COFFEE BOOK
on
NEURODEGENERATIVE DISORDERS

Neuroscience Research Lab
Department of Neurology, PGIMER Chandigarh, India

Foreword

Honestly, it is an extreme privilege to get a chance to write the foreword for a Coffee book where my (read it, our) research investments will be up for many future discussions over a cup of Chai or Coffee from the famous Tuck shop at PGI. First, to say it loud, the NRL's contribution has been enormous in shaping up my career in the field of neuroscience and I will highly regard that throughout my life.

My association with NRL was scripted on a bright morning during August 2007 when I met Dr. Akshay at the other side of a conference table for an interview. I came from Bangalore with my couple of years of industry expreience after my Masters and wanted to make a mark in a hardcore research laboratory with an absolute aim for the coveted degree, Ph.D. My journey at NRL statred as a Senior Research Fellow on 15th of September 2007 and I left NRL for my post-doctoral training on 8th October 2015. As you guessed it, in these eight years, I earned my Ph.D. working on the pathophysiology of Alzheimer's disease (AD) and evaluating the role of umbilical cord blood (UCB) derived stem cells as a potential therapy in AD like mice. This study, for the first time, showed that a primitive population of lineage negative (lin-ve) stem cells from human UCB could rescue learning and memory in rodents in dose dependent manner (PMID: 25989508). Glad to mention, this was one of the studies which paved forward to future studies at NRL in extrapolating the underlying molecular mechanism to established the link of BDNF mediated rescue behind this outcome.

In the process, there were many publications where I actively or passively contributed and therefore, you will find my name in many articles even after relieving from NRL. We, at NRL, looked into many pathophysiological aspects of different neurodegenerative disorders (NDD), their *in-vitro* and rodent models and avenues for ptential therapeutic targets. This vision has led to many interesting findings and related pubilcations in last decade. To highlight, we could come up with a consensus review article collaborating with international authors from the field of Alzheimer's disease (AD) to explain how pre-clinical studies can be translated into successful clinical trials for AD. Also, there were critical reviews on the current perspectives of several other NDDs such as Parkinson's disease, Amyotrophic Lateral Sclerosis, Muscular Dystrophy, Spinal Cord injuries and Age-related Macular Degeneration in the context of Indian scenario and beyond. It is a great idea to come up with this Coffee book where such articles will find a place for your attention and reference. I am sure, one will be enriched with so much of knowledge from the content of this compilation that it will certainly trigger new directions of research in near future. Hope this Coffee book serves you the best of your scientific appetite.

Avijit Banik, Ph.D.
Alumni, Neuroscience Research Lab.

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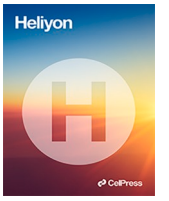

ALS plasma reduces the viability of NSC34 cells via altering mRNA expression of VEGF: A short report

Radhika Khosla [a], Hemant Bhagat [b], Parth Lal [c], Akshay Anand [a,*]

[a] Neuroscience Research Lab, PGIMER, Chandigarh, India
[b] Department of Anesthesia and Intensive Care, PGIMER, Chandigarh, India
[c] Advanced Paediatric Centre, PGIMER, Chandigarh, India

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ABSTRACT

Introduction: Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder that progressively leads to motor neuron degeneration at the neuromuscular junctions, resulting in paralysis in the patients. The clinical diagnosis of ALS is time taking and further delays the therapeutics that can be helpful if the disease is diagnosed at an early stage. Changes in plasma composition can be reflected upon CSF composition and hence, can be used to study the diagnosis and prognosis markers for the disease.
Aim: To develop a simple model system using motor neuron like cell line after plasma induction.
Method: Neuroblastoma × Spinal Cord hybridoma cell line (NSC34) was cultured under appropriate conditions. 10% ALS patients' plasma was added to the media, and cells were conditioned for 12 h. Cell survival analysis and differential gene expression of a panel of molecules (published previously, VEGF, VEGFR2, ANG, OPTN, TDP43, and MCP-1) were done.
Results: ALS patients' plasma impacted the life of the cells and reduced survival to nearly 50% after induction. VEGF was found to be significantly down-regulated in the cells, which can be explained as a reason for reduced cell survival.
Conclusion: ALS plasma altered the expression of an essential neuroprotective and growth factor VEGF in NSC34 cells leading to reduced viability.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a degenerative disorder that includes neuromuscular interactions. The neurons degenerate, and the muscles get atrophied, causing paralysis. The disease grows rapidly in some cases and relatively very slowly in others. ALS is sporadic in approximately 90% of the cases and familial in rest of the 10%, where C9orf72 and SOD1 are the most studied genes for the familial origin of the disease [1]. Mutations in various other genes have been studied for sporadic ALS. However, no single molecule has been assigned to the pathology of the disease to date, making the prognosis more difficult.

Proteomic studies on the biofluids from ALS patients have shown altered levels of various physiologically important proteins [2–4]. Our previous studies have focused on a specific panel of the molecules studied in relation to ALS [5–7]. The levels of these molecules were analysed in the CSF and plasma of ALS patients. The specificities of the molecules in the panel have been explained well in our

* Corresponding author. Neuroscience Research Lab, Department of Neurology, Post graduate Institute of Medical Education and Research, Chandigarh, India, 160012.
E-mail address: akshay1anand@rediffmail.com (A. Anand).

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previous studies. The panel contains Vascular Endothelial growth factor (VEGF) and its receptor VEGFR2, Angiogenin (ANG), Optineruin (OPTN), Transactive Response DNA Binding Protein 43 (TDP43), and Chemokine Ligand 2 (CCL2).

The first three proteins in the panel are involved in vascularisation and also have been found to play neuroprotective roles in CNS [8,9]. VEGF is a growth factor and acts as a trophic factor for the survival and proliferation of the surrounding cells [9]. OPTN and TDP43 proteins are involved in protein inclusions formed inside the dying neurons. TDP43 is an important transcription factor, and its mislocalisation affects cell survival [10]. OPTN is another molecule that is involved in regulating autophagy [11] and is found to be involved in the process of neuroinflammation along with CCL2, an important marker for neuroinflammation.

Neuroblastoma × spinal cord hybrid (NSC34) cell line is a model for studying cellular-level ALS pathology as the cell line has characteristic features of motor neurons. Hence, ALS pathology can be created in the cell line, and different prognosis and therapeutic approaches can be studied with its help (details in method section). Although some previous studies have shown the toxicity of ALS patients' CSF in the *in-vitro* [12] and *in-vivo* [13] system, no previous studies have tested the effect of plasma from patients *in-vitro*. However, some early studies have studied the serum cytotoxicity *in-vitro* and *in-vivo* [14,15]. The CSF of the ALS patients reduced the cell viability of the NSC34 cells and also caused ALS-like symptoms in mice. Some studies have shown that ALS CSF-induced neurodegeneration in the NSC34 cells can be reversed after the administration of neuroprotective molecules such as VEGF, BDNF [16–19].

Altered biochemical changes in the systemic circulation are representative of the altered composition of CSF and can be used for biomarker discovery for neurodegenerative diseases. Studies combining CSF and plasma analysis can be more helpful in studying brain pathology [20,21]. Plasma can be collected non-invasively and may be used to create the ALS pathology, as shown by the current study. The plasma of ALS subjects has been shown to have varying protein configurations than normal healthy individuals [7]. To see how the composition of the plasma affects the cells, the cell viability was analysed using MTT assay. Further, the gene expression of the proteins proposed in the panel was analysed in the cells. However, blood-brain barrier has an important role to play in this interaction of plasma with neurons; hence *in-vivo* studies for plasma are warranted along with CSF.

2. Methods

2.1. Cell line

Neuroblastoma × Spinal Cord Hybridoma cell line is commonly abbreviated and mentioned as NSC34 cells. The cell line is a hybrid of spinal cord cells taken from 12 to 14 days old embryonic mice and the mice neuroblastoma N18TG2 cells. The cell line developed by Cashman et al. in 1992 is an appropriate model for the studies concerning motor neurons as the cells mimic the properties of motor neurons [22]. The cell line is a mixture of large motor neurons and small neurons. The cell line was provided to us by Dr. Vegasna Radha and Dr. Archana from CCMB, Hyderabad. The cells were cultured using the DMEM Glutamax (Dulbecco's Minimum Essential Medium with Glutamax, 10569-010, Gibco, Grand Island, US) supplemented with 10% Fetal Bovine Serum (FBS, 10270106, Gibco, Thermo, Brazil) and 1% Penicillin-Streptomycin (Pen-Strep, (10,000 U/mL),15140122, Gibco, Thermo, Grand Island, US) at 37°c with 5% CO_2 in the incubator.

2.2. Subjects

The plasma of nine ALS patients was used for inducing the cultured cells in various combinations of plasma samples. The study was approved by the Institutional Ethics Committee (Ethical reference no. NK/5365/PhD/382). Mean ALS FRS R score and mean disease duration (in months) of ALS patients were 31.9 ± 10.46 and 21.87 ± 13.74, respectively. The disease progression rate (ΔFS) for the group of ALS patients was 0.736 [23]. Healthy individuals' plasma was used as a control for the induction experiment of cells (eight). Also the non-induced or unstimulated cells were used as an additional experimental control. The blood samples were collected from the participants after they consented to participate. The plasma was isolated from the whole blood by centrifugation at 1500 RPM for 30 min.

2.3. Induction of cells

The cultured cells were induced with media containing the plasma of patients and controls for 12 h. 10% plasma media was used for the induction of cells. The complete media (DMEM with 10% FBS) was further supplemented with 10% plasma from the subjects. The plasma samples were pooled for three subjects in each category for all the experimental setups in different combinations. Before induction, the cells were subjected to serum starvation for 12 h by culturing in DMEM only (without FBS supplementation). Serum starvation was done to bring all the cells on the same cell cycle stage, i.e. G0, to avoid any effect of different cell cycle stages on MTT results [24]. After serum starvation, the cells were cultured for 12 h with plasma media. Further assessments were done after this induction.

2.4. MTT assay

To analyse the effect of plasma from human subjects on the cell line or to test the toxicity of the plasma of ALS patients, the cell viability was analysed using MTT assay. MTT assay helps to assess the percentage cell survival . MTT is a colorimetric assay, and results are based on the color intensity of the end products. Cells were seeded in a 96-well plate at a density 10,000 cells/well. The cells were allowed to be confluent. Thereafter, the cells were serum starved for 12 h and then treated with plasma for 12 h before adding MTT

substrate. MTT substrate i.e. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl Blue Tetrazolium Bromide, M2128-500 MG, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.5 mg/ml working solution with 10% DMEM-FBS media, when added to the culture media, it got reduced by live cells and got converted from yellow solution to formazan crystals, which were then dissolved with the help of DMSO and color intensity (Optical density, OD) was measured at 595 nm with a microplate reader (Bio-Rad Laboratories, California, USA). Succinate dehydrogenase in the mitochondria of live cells causes the reduction of MTT reagent. Untreated cells were used as the experimental control. The percentage cell viability for MTT assay can be calculated using the below formula:

$$\text{Percentage cell viability} = \frac{OD\ of\ treated\ cells - OD\ of\ blank\ wells}{\text{OD of untreated cells – OD of blank wells}} \times 100$$

2.5. mRNA expression

After analyzing the cell viability, mRNA expression was analysed for genes referred to in the introduction section. RT-qPCR was done for mRNA expression. The cells were seeded in T-25 flasks till they became confluent. The cells were treated in the same manner as for MTT assay. After the treatment was complete, the images of cells were studied for morphological alterations, after which the media was discarded. Untreated cells were used as experimental controls. Cells were washed with PBS and trypsinised using Trypsin-EDTA (0.25%, 1×, 25200056, Gibco, Thermo, Canada). Immediately RNA isolation was done using the standard kit protocol for RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was done thereafter immediately using the Verso cDNA synthesis kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA).

Primer sequence and details have been reproduced in Table 1. 10 μl of the reaction mixture was prepared by adding cDNA samples, Power up SYBR Green Master Mix reagent (Applied Biosystems, Foster City, California, USA), primers (forward-reverse, Eurofins Genomics India Pvt. Ltd.) and RNAase free water. The PCR was done using the Step one Real-Time PCR System (Applied Biosystems, Foster City, California, USA). 50 ng of cDNA concentration was used for the reaction. The PCR was done in triplicates. β-actin was used for the normalization of the gene expression, and relative fold change was calculated in the gene expression. The PCR program setup is shown in Fig. 1; the default program of the ABI step one software for calculating ΔΔCt with SYBR green reagent has been used for the gene expression.

2.6. Statistical analysis

The data was tested for distribution using the one-sample K–S test. It was found to be parametric, and hence, ANOVA was used to compare the cell viability followed by post-hoc analysis (Tukey HSD test), and Independent *t*-test was applied to compare the fold change for genes in the ALS plasma-treated cells and control plasma-treated cells using the ΔΔCt values. The data from minimum three experiments was considered for the analysis. Each experiment was done in triplicates.

3. Results

3.1. ALS patient plasma is toxic for the NSC34 cells

Decreased cell viability was seen in the cells treated with ALS patients' plasma in comparison to healthy individuals' plasma and untreated cells (Fig. 2). Plasma has been found to be toxic to motor neuron-like cells. Considering this loss in the cell viability of the cells treated with ALS plasma ($p = 0.04$), gene expression was analysed in the cells.

Table 1
Primer sequences of the genes quantified using RTq-PCR. The genes tested are Vascular Endothelial Growth Factor (VEGF), VEGF receptor 2 (VEGFR2), Angiogenin (ANG), Optineurin (OPTN), Trans-active Response DNA Binding Protein 43 (TDP43) and Chemokine Ligand 2 (CCL2).

Gene		Primer Sequence
VEGF	F	5′-CGATTGAGACCCTGGTGGA-3′
	R	5′-GTCTTTCTTTGGTCTGCATTCAC-3′
VEGFR2	F	5′-TCATAATAGAAGGTGCCCAGGA-3′
	R	5′-CGTAGGACAATGACAAGAAGGA-3′
ANG	F	5′-AACCTCACCCTGCAAAGATG-3′
	R	5′-GTGGACAGGCAAACCATTCT-3′
OPTN	F	5′-TGTTTCAAAGAGGAGCCGAG-3′
	R	5′-ATCACATGGATCTGAAGCGT-3′
TDP43	F	5′-CAACTCTAAGCAAAGCCCAG-3′
	R	5′-ATCTACCACTTCTCCATACTGAC-3′
MCP1	F	5′-GCCAACTCTCACTGAAGCC-3′
	R	5′-CGTTAACTGCATCTGGCTGAG-3′
β-actin	F	5′-AGCCATGTACGTAGCCATCC-3′
	R	5′-CTCTCAGCTGTGGTGGTGAA-3′

F-Forward, R-Reverse.

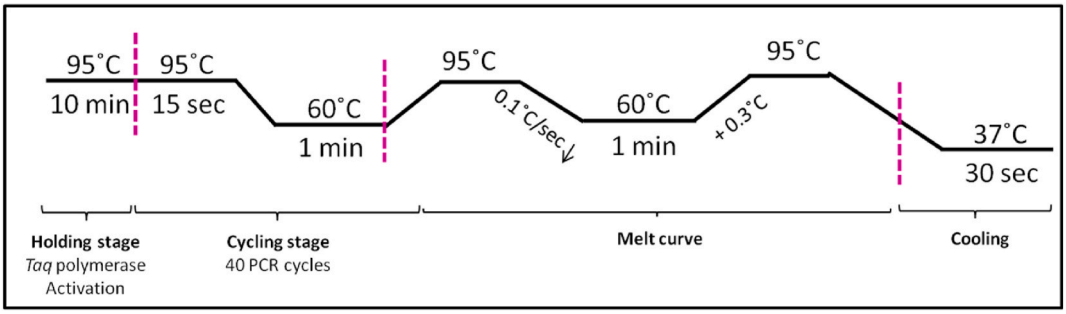

Fig. 1. PCR program used for the quantification using ΔΔCt method with SYBR Green dye. First is the holding stage for the activation of reagents, then the cycling stage with 40 PCR cycles, each having denaturation, extension, and annealing. Melt Curve was also tested to analyse the integrity of the amplifications.

3.2. ALS plasma treatment altered the morphology of the NSC34 cells

The characteristic morphology of the NSC34 cells is neuron-like with dendrites, and axons. This cell line is an adherent cell line and the cells are found adhered to the culture dishes and attain their characteristic neuron-like shape. However, the ALS plasma treatment resulted in an alteration of the morphology of the cells. Instead of having the characteristic shape, the cells lost their adherent property and started floating as circular cells suspended in the media. Also, the cell density was reduced in the culture dish treated with ALS plasma (Fig. 3).

3.3. Treatment of cells with ALS plasma significantly reduced the VEGF mRNA expression in the cells

The relative fold change in the mRNA expression was calculated using the SYBR method. Fold change was analysed for the following genes: VEGF, VEGFR2, ANG, OPTN, TDP43, and CCL2 (Fig. 4). The fold change for all the genes in the untreated cells was considered as one, and the fold change for the cells treated with plasma was normalized to that. VEGF was significantly downregulated in the ALS plasma-treated cells in comparison to cells treated with control plasma (Fig. 4b). A decrease in the VEGF expression was noted, mimicking the reduced VEGF levels in the ALS plasma as shown in our previous study [5]. The difference in fold change for the other genes was not significant (Fig. 4a, c-f).

4. Discussion

ALS was first defined by Charcot as a Neuromuscular disorder in which neuronal degeneration leads to muscle wasting and ultimately paralysis. Within three to five years of the onset of the disease, the patients got choked to death because of respiratory failure. Various proteins till now have been explored for the biomarker potential for studying prognosis and therapeutic studies. CSF from ALS

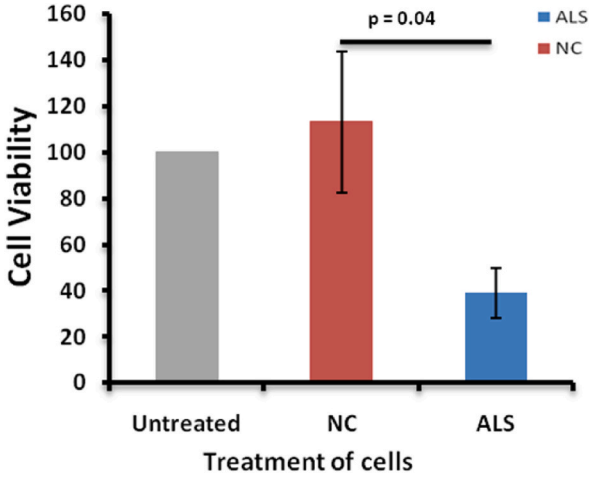

Fig. 2. The cultured NSC 34 cells were serum starved for 12 h and then kept under treatment for 12 h. MTT assay was done to estimate cell viability. There was a reduction in the percentage of live cells in the ALS plasma-treated cells in comparison to untreated and normal control treated plasma cells. n = 5 (five experiments were done in triplicates). Untreated cells have been used as an experimental negative control. Abbreviations; NC, Normal control (NSC34 cells treated with healthy individuals plasma), ALS, Amyotrophic Lateral Sclerosis (Cells treated with ALS patients plasma).

4

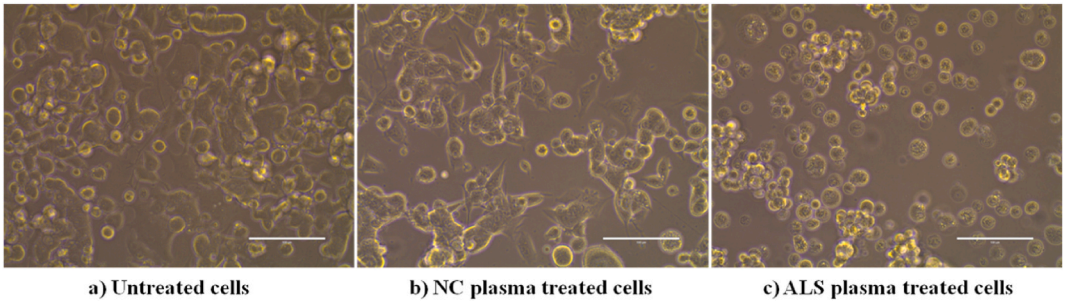

Fig. 3. Altered morphology of the cells treated with ALS patients' plasma. (a) The characteristic morphology of the untreated NSC34 cells. The cells are rounded but they are adherent cells and are in the diving stage. (b) The cells after the control plasma treatment have acquired the characteristic neuronal shape. (c) The cells are more rounded in structure and are floating as a suspension in the media after treatment with the ALS patients' plasma. Abbreviations, NC, Normal control, ALS, Amyotrophic Lateral Sclerosis.

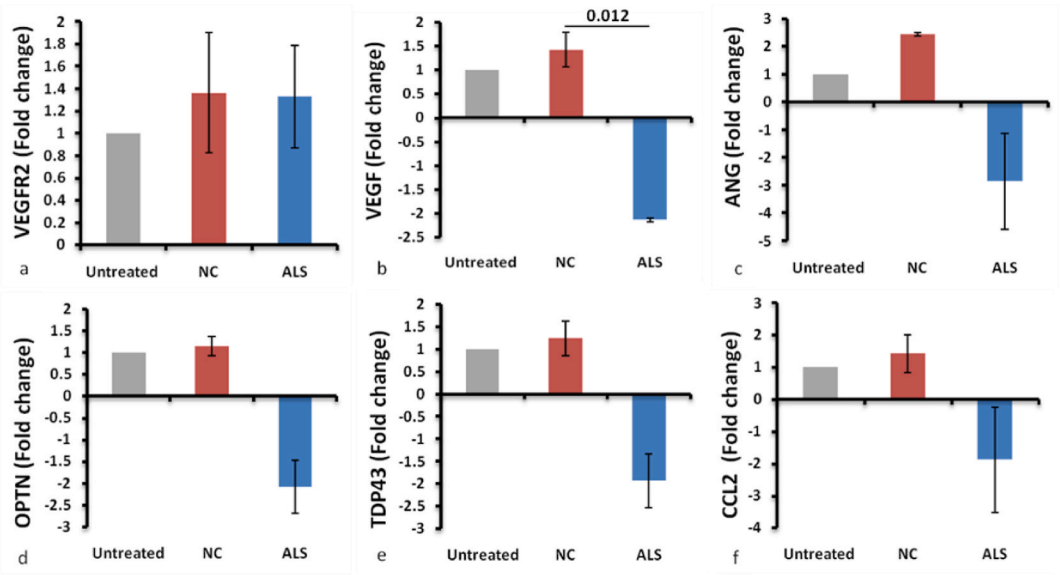

Fig. 4. Fold change in the mRNA expression of VEGFR2 (a), VEGF (b), ANG (c), OPTN (d), TDP43 (e), and CCL2 (f). (a) VEGFR2 has not shown much change in the expression. (b) VEGF has been significantly downregulated in ALS patients' plasma-treated cells. p-0.012. (c–f) Other genes (ANG, OPTN, TDP43 and CCL2) have shown downregulation though not significant. n = 3 (Three experiments done in triplicates). Abbreviations, VEGF, Vascular Endothelial Growth Factor, VEGFR2, VEGF receptor 2, ANG, Angiogenin, OPTN, Optineurin, TDP43 Transactive Response DNA Binding Protein 43, CCL2, Chemokine Ligand 2.

patients has shown to be toxic in the *in-vitro* and *in-vivo* systems. Also, the toxicity caused by CSF has shown to be rescued after adding the neurotrophic factors, VEGF [12] and Brain Derived Neurotrophic Factor (BDNF) [18] to these systems.

However, in our case, no CSF toxicity was observed even when tested at higher dosages (Supplementary Fig. 1). This observation might be explained by the lower progression rate of disease in our ALS patients in comparison to previous studies where CSF toxicity was shown [17,23]. The disease pattern in India has been described as a slow progressing disease in comparison to western counterparts in various studies [25,26]. Galán et al. observed that CSF toxicity was patient-specific and not all patients' CSF was toxic. They had also shown that there is no correlation between CSF toxicity and the survival of ALS patients [27]. Since the changes in the CSF were presumably reflected in the plasma [20,21], the potential of ALS plasma could also be explored for creating such a model system. Some earlier studies have shown the serum of ALS patients to be toxic for *in vitro* systems [28,29].

Our previous studies have analysed a panel of proteins in the ALS plasma (completely) and CSF (partially) of the same cohort. In the study, a similar decreasing trend for VEGF in both fluids was reported [5,7]. A predictive logistic regression model developed using the demographics and the protein levels in both fluids of the cohort showed the combined role of all three markers, demographics, protein levels in the plasma and CSF, in the prediction of the disease [6]. Hence, the analysis of plasma from ALS patients in the *in-vitro* system is warranted. The current study has shown that the ALS plasma is toxic for the NSC34 cells and it decreases the cell viability to almost half of the untreated cells. Along with the decrease in cell viability, the cells also lose their characteristic shape as well as the adherent property, as shown by the cells in suspension (Fig. 2).

5

Since the panel of the molecules we have studied till now play an important role in the pathology besides providing neuroprotection to the cells, the same panel has been analysed in the NSC34 cells in the present study. VEGF is found to be significantly downregulated in cells treated with ALS plasma in comparison to cells treated with control plasma. However, protein level estimation in the NSC34 cells or in the supernatant, after stimulation with plasma could not be done in the present study. Absence of protein level correlation with mRNA expression is a limitation of the current study.

The downregulation of VEGF is in concert with the decrease in VEGF levels in the plasma of ALS patients of our cohort as reported in our previous study [5]. Different studies have shown that VEGF administration could reverse the degeneration in the NSC34 cells although VEGF mRNA expression was not observed in the NSC34 in these studies [17,19]. The downregulation of VEGF in our study is consistent with this role of VEGF. However, there are contrasting reports regarding the VEGF levels in the biofluids of ALS patients. Gao et al. reported elevated VEGF levels in the ALS CSF and serum. They could observe an inverse correlation between the VEGF levels and disease progression rate. VEGF is a neuroprotective molecule and the downregulation of which can be attributed to the reduced viability of the NSC34 cells in our study.

Another reason for studying the plasma *in-vitro* system for ALS is to explore the possibility of ALS progression to be a dying back phenomenon [30–32]. Since dying back theory of ALS progression suggests that the degeneration starts at the muscular or the neuromuscular junction level. Fischer et al. in a case report of a sporadic ALS patients' autopsy, has shown that there were indications of denervation and re-innervation of muscles, but the motor neurons did not indicate any abnormality [33]. It may be speculated that there is some altered composition of plasma or reduction in certain growth factors in the plasma that might trigger the degeneration in ALS patients. Also, there might be a possibility that degenerating muscles release some degenerative factors in the plasma and at the neuromuscular junction, which leads to the degeneration of motor neurons [34]. The degeneration first starts at the muscular level and then moves toward neurons. This warrants the need to target plasma composition as a biomarker for ALS and also this warrants more studies targeting the therapeutic approaches for muscles. This can further be supported by the fact that only motor neurons get affected in ALS and not the other neurons.

5. Conclusion

ALS plasma altered the expression of an essential neuroprotective and growth factor VEGF in NSC34 cells leading to reduced viability.

Author contribution statement

Radhika Khosla: Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Hemant Bhagat: Parth Lal: Contributed reagents, materials, analysis tools or data.

Akshay Anand: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18287.

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REVIEW ARTICLE

Ayurvedic Herbal Therapies: A Review of Treatment and Management of Dementia

Vinod Srivastava[1], Deepali Mathur[2], Soumyashree Rout[2], Bikash Kumar Mishra[2], Viraaj Pannu[3], and Akshay Anand[4,*]

[1]*College of Health and Behavioral Sciences, Fort Hays State University, Hays, Kansas 67601, USA;* [2]*Department of Neurology, Apollo Hospitals, Bhubaneswar, Odisha, India;* [3]*Department of Internal Medicine, Jersey Shore University Medical Center, Neptune, New Jersey, USA;* [4]*Research Lab, Department of Neurology, PGIMER, Chandigarh, India*

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Abstract: Dementia has been characterized by atypical neurological syndromes and several cognitive deficits, such as extended memory loss, strange behavior, unusual thinking, impaired judgment, impotence, and difficulty with daily living activities. Dementia is not a disease, but it is caused by several neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Lewy's bodies. Several drugs and remedies are indicated for alleviating unusual cognitive decline, but no effective pharmacological treatment regimens are available without side effects. Herbal drugs or traditional medicines like Ayurveda have been known for facilitating and corroborating the balance between mind, brain, body, and environment. Ayurvedic therapy comprises 600 herbal formulas, 250 single plant remedies, and natural and holistic health-giving treatments that relieve dementia in patients and increase vitality. Ayurvedic Rasayana herbs [rejuvenating elements] strengthen the brain cells, enhance memory, and decrease stress. The current medicine scenario in the treatment of dementia has prompted the shift in exploring the efficacy of ayurvedic medicine, its safety, and its efficiency. This review presents the literature on several herbal treatments for improving dementia symptomatology and patients' quality of life.

Keywords: Ayurveda, dementia, ashwagandha, turmeric, brahmi, Shankhapushpi.

1. INTRODUCTION

Dementia, characterized by the ongoing decline of brain functioning, such as reasoning, thinking, and memory, has become the most significant global challenge [1]. Dementia is a syndrome [a group of symptoms], and the normal aging process does not necessarily cause dementia [2]. Instead, it is caused by the damage to brain cells by several factors and diseases, such as Alzheimer's and stroke [1]. Almost 60% - 70% of dementia cases are Alzheimer-induced dementia, followed by 20%-25% of vascular dementia and 5% of Lewy Body dementia [2, 3]. Age is one of the significant risk factors in people with dementia; however, other differential factors have also been discerned, and younger people in their 30s, 40s, and 50s [below 65] are also at risk, constituting 9% of dementia cases [1, 4]. Although the causes of Alzheimer's disease have not been fully understood, the age-related changes in the human brain, genetics, and environmental factors, including lifestyle, are associated with this progressive brain disease [3, 5]. The late-onset of Alzheimer's disease is the most common type appearing in the mid-60s compared to early-onset [between the 30s and 60s] [6]. Alzheimer's is the leading cause of dementia and is highly correlated with age, raising the incidence of dementia later in people's lives [7]. However, several studies project the primary etiology of early dementia is Alzheimer's disease, followed by Vascular Dementia and Frontotemporal Lobar Degeneration, highlighting the significant role of environmental factors leading to epigenesis, but those perturbations do not get manifested until later life [8-10].

The prevalence of dementia may double every 5-6 years after 60-65 years of age until 90, and approximately 30% of individuals over 85 years may also be at risk of developing dementia [2, 3, 7]. Some of the common risk factors besides neurogenetics that have been found associated with dementia are diabetes mellitus, hypertension, obesity, unbalanced diet, social isolation, alcohol use, and tobacco consumption [11-14]. According to the WHO report, around 50 million people have dementia worldwide, and approximately 9.9 million new dementia cases are found every year [1]. The increment in the neurological disorder will multiply and may turn 82 million by 2030 and 152 million by 2050, according to the WHO report [5]. Comparatively, low and middle-income countries have reported a higher incidence of dementia than

*Address correspondence to this author at the Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India; E-mail: akshay1anand@rediffmail.com

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developed countries [15]. Dementia has been recognized as the second highest disability syndrome and constitutes the seventh primary cause of death of individuals above 70 years [5, 16]. WHO estimates that 50% of subjects experiencing dementia remain undiagnosed, and almost one-third of patients discharge from the hospital without much improvement in functional capabilities [17].

There has been a lot of debate regarding the pathogenesis of Alzheimer's disease, in particular the difference in pathogenesis between early and late-onset Alzheimer's and dementia. A consensus among researchers has been noticed that the pathogenetic cause of early-onset Alzheimer's, which can have familial roots, is the premature excessive deposition of amyloid beta plaques due to various mutations, such as PSEN1, PSEN2, or APP mutations [18, 19, 20], whereas the pathogenesis of sporadic Alzheimer's disease is mainly undetermined [10, 21]. Late-onset Alzheimer's was thought to be associated with amyloid beta; however, unlike its early-onset counterpart, amyloid beta does not seem to have an etiological relationship [21, 22]. Schneider *et al.* reported mixed brain pathologies for most dementia cases in older adults [21-23]. Gorelick *et al.* suggested the importance of vascular contributions to cognitive impairment and dementia in later life and reported that the neuropathology of cognitive impairment could be a complex interplay of Alzheimer's disease and microvascular brain damage in later life [23]. Furthermore, global cerebral hypoperfusion has been noticed to be associated with the increased progression of late-onset Alzheimer's disease [24], and the study by Moon *et al.* showed a correlation between Carotid intima-media thickness [CIMT] and progression of dementia after adjustment for various baseline risk factors for cognitive impairment [25]. Atherosclerosis and vascular phenomenon have been indicated to be the significant co-contributors to cognitive impairment and dementia, and any intervention that can reduce the burden of atherosclerosis can, in turn, reduce the burden of late-onset Alzheimer's progression [7].

Over the last five decades, numerous pharmacological and non-pharmacological approaches and interventions have been used to treat, stop progression, and manage dementia effectively; however, there are no effective ways to cure dementia. Pharmacological treatments could be expensive and potentially hepatotoxic, requiring patients to undergo regular liver checkups and appropriate dose modifications [26, 27]. The absence of specific medical treatment to manage dementia patients' neurological, behavior, and neurodegenerative diseases has been challenging for clinicians and caregivers. Providing adequate care to dementia patients requires a collaborative approach between healthcare professionals and family members, which could be expensive, challenging, and emotionally taxing for all stakeholders [28]. Almost 20% of healthcare professionals providing care to dementia patients may be at risk of experiencing mental health problems [17, 29]. These challenges and the growing number of dementia cases have motivated healthcare workers to emphasize early diagnosis and find evidence of alternative treatments for dementia patients with comorbid medical and/or mental health conditions, which may also be preventive in nature. Additionally, rigorous scientific research is required for any alternative treatments to be included in evidence-based best practices to optimize people's physical, emotional, psychological, and cognitive well-being. Ayurvedic medicine's use globally has surged, and research to quantify evidence has also been growing [8]. As a result, this study has attempted to present an overview of the Ayurvedic medicine system and approach and evaluate the recent evidence found in the literature to treat and/or manage dementia. The current review has excluded herbs that do not come under Ayurvedic medicine purview. However, clinical trials and the use of herbal medicine in clinical treatments of diseases have several challenges.

Although plants are the source of almost a quarter of medicines globally, a few countries [like India, ancient Greece, Egypt, and China] where alternate herbal medicine originated still practice traditional herbal medicine [30]. Ayurvedic medicine is well known in India, but due to a lack of established funding, effective government policies, and Ayurvedic operational, clinical, and quality management standards, the clinical trials and standardized Ayurvedic medicine manufacturing could not take off until recently [30, 31]. Ayurvedic medicinal plants have also been labeled as dietary supplements, and the lack of awareness among people regarding the safe and unsafe use of herbal medicine and its interactions with allopathic [modern] medicine has created practical barriers to demand and supply and clinical trials [30]. Lately, with shifting focus and policy changes in India, evidence of clinical trials of Ayurvedic medicine is burgeoning. As a result, it is important to provide people, researchers, and clinicians with adequate updated information and an overview of the Ayurvedic medicine system to bring changes in their lives and help deal with the growing challenges of dementia, which is the focus of the current review.

2. AYURVEDIC MEDICINE

Among several medicinal sciences, Ayurveda is the oldest medical system that originated in India from one of the Vedas called Atharvaveda and has discussed dementia and age-related problems. Ayurveda is medical knowledge system of holistic healing, emphasizing natural plant-based medicine and living in balance with nature for the health and well-being of human beings [32, 33]. Ayurveda has discussed various classifications of diseases, treatment modalities [including behavioral interventions], surgeries [mentioned in Sushruta Samhita by Nagarjuna], and lifestyle changes to improve ailments, health, and general well-being [33]. Ayurveda also elaborates on the knowledge of energy points on the body [marma] to be used as diagnostic and treatment tools of diseases through therapeutic massages [33, 34].

The mechanics of the Ayurvedic approaches in the therapeutic application of Ayurvedic herbs is integrative, harmonizing biological, neurological, and behavioral diseases and disorders and spiritual aspects of human life. Ayurveda [Ayur Vidya] is called the Knowledge or Science of Life, which emphasizes integrating mind, body, social, psychological, and spiritual aspects of human existence while maintaining balance with nature [35]. Ayurveda believes that life consists of five basic *panchamahabhutas* [five elements of life; linguistically translated as air, fire, water, earth, ether] and spirit, the consciousness, which is the reflection of the pure consciousness [36]. Fire holds an

essential place in Ayurvedic medicine and corresponds to metabolism and metabolic stress generated by the interaction of various body parts/organs, such as brain electromagnetic waves and the digestive system [37]. Air denotes gaseous movements and motion, such as heart beating, breathing, and neural transmissions; water represents liquid in the body, such as blood, digestive, tongue, and genital secretions; earth denotes anatomical and mechanical structures in the body; ether corresponds to space, such as pores in the body and spiritual energy [37]. These are the foundational blocks of understanding Ayurvedic medicine, as their disbalance cause *doshas* [biohumors].

Ayurveda takes a functional approach to human anatomy, which is the interplay of different organs corresponding to different sensory inputs [38]. Ayurveda views disease as the imbalance of metabolic processes consisting of five basic *panchamahabhutas* [five elements of life], and imbalances distress physical, psychological, spiritual, and environmental systems of human life [32, 33, 36]. To attain the highest level of health and well-being, an individual must take responsibility [follow the doctrine of karma] and understand themselves as part of the macrocosm to harmonize one's existence with the surrounding environment and nature. Ayurveda holds that salvation [Moksa] is the epistemological transformation toward self-actualization, transcending mundane life toward higher consciousness [39]. This journey is identified as spiritual, epistemological, intellectual, social, and psychological evolution towards self-actualization, promoting happiness [decreasing stress and anxiety], love, compassion, physical and mental health, and harmony with the environment and nature [39]. It emphasizes the importance of valid knowledge achieved through analytical decision-making processes of perception, inference, comparison, testimony, and intuition and recommends using valid knowledge for diagnostic analysis and understanding of general 'truth' [32, 33, 36, 40].

Furthermore, yoga, which is the oldest recorded behavioral treatment modality, recommends cognitive and behavioral interventions for various medical and mental health problems, emphasizing mental modification and perfecting the human body [39]. Yoga views the mind as an aggregate of biopsychosocial experience known through introspection, creating self-awareness. Self-awareness can promote a healthy lifestyle, spiritual well-being, and balance with the immediate environment and nature, which are holistic and promotive approaches to well-being (Fig. **1**). Righteous actions and behaviors conjoined with a mindful and balanced diet, nutrition, and food digestion can have therapeutic preventive and restorative effects on disease (Fig. **1**). Lastly, identification of treatment and management of ailments, such as dementia and cognitive problems, may need remedial approaches and can also have compensatory effects (Fig. **1**). Thus, the Ayurvedic wellness framework is grounded in the person-in-environment perspective. In Ayurveda, patients' non-diseased states are separated from diseased states, and it emphasizes balancing the life forces to decrease mind and body distress and increase well-being (Fig. **2**). Furthermore, Ayurveda distinguishes patients by diseased states of patients' phenotypical constitutional specificity based on three *doshas* [biohumors - fluid/semi-fluid excitant secretions] - *Vata, Pitta,* and *Kapha*, representing all movements in the body and mind, all metabolic processes in the body impacting at physical and mental levels, and all stability functions in the body respectively; these three factors constitute the state/inclination of mind and body [38].

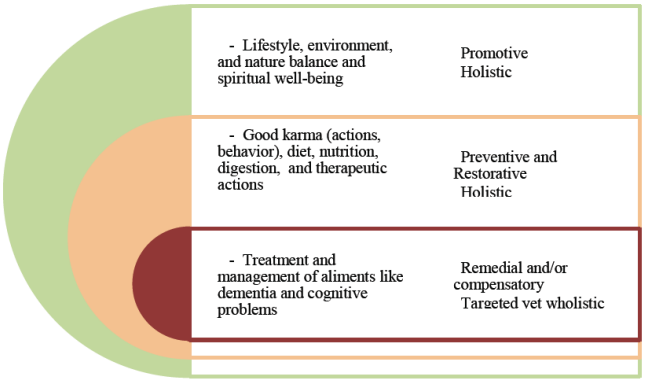

Fig. (1). Ayurvedic diagnostic and treatment approaches. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

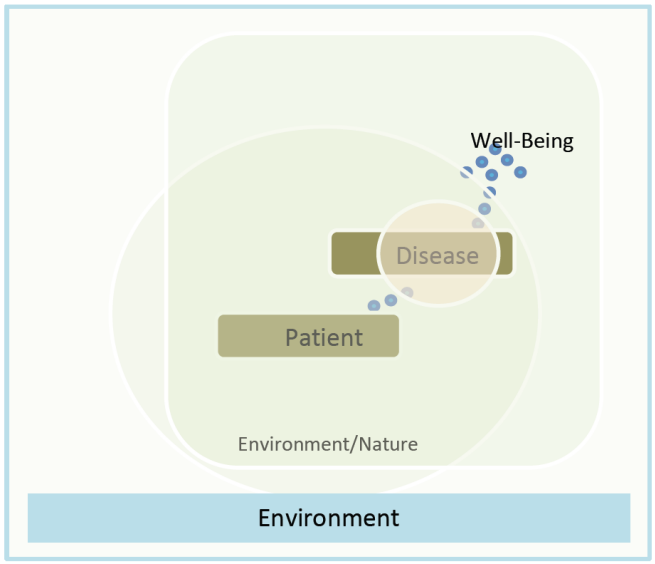

Fig. (2). Ayurvedic framework. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Ayurvedic approaches are similar to what Evidence-Based-Practice [EBP] modern science claims. Ayurveda advocates basing the clinical decision-making process on analytical reasoning and evidence in testimonies [recorded practice wisdom as evidence], understanding of the client's unique problems and preferences [including connection with social environment and nature], and the utilization of the clinician's expertise. Ayurveda maintains that therapeutic approaches to treating diseases and helping patients maintain or achieve optimal wellbeing are possible by harmonizing mind, body, and environment processes, which resembles the integrative health approaches.

3. DEMENTIA AND AYURVEDA

According to Ayurveda, dementia is caused by the imbalance of three doshas [biohumors] – Vata, Pitta, and Kapha [functions of motion, digestions, and cumulation, respectively] [41]. Imbalanced Vata components of the body are predominantly associated with dementia [42, 43]. Dietary factors have a critical role in the pathogenesis of dementia [40], and constipation has a higher incidence rate in dementia patients and has been observed to accelerate the progression of neurodegenerative pathology [44]. Different lifestyles, sleeping, and eating preferences/patterns have also been associated with the age-related issues of the predominance of Vata dosha, and Vata dosha dominance is the prime factor associated with constipation [Krura Kostha] and neuropsychological disturbances in dementia patients [40]. Sleep disorders and sleep disturbances due to napping during the day and waking up at night increase Kapha and Vata dosha, respectively [40]. Kapha sustains body mass, structure, and versatility [45-47], especially for the Vata imbalance [35]. Ayurvedic medicine has explained the different usage and qualities of 700 herbs and 6,000 formulations for curing various disease sufferers [34]. It illustrates approximately 5,000 signs and symptoms of several disorders and has several ayurvedic medicines for treating dementia. Herbal medicine may be free from chemicals, additives, and preservatives, with low side effects and beneficial outcomes. This review attempts to describe different ayurvedic processes and remedies for the management of dementia.

4. AYURVEDA TREATMENT: MEDICINAL PLANTS USED FOR DEMENTIA TREATMENT

Ayurveda has been using herbal plants since 5,000 B.C. as medicinal resources and considered them health promoters, restorators of dosha imbalances, and curators of ailments [48]. Two of the Vedas, Rigveda [4500 to 1600 B.C.] and Atharvaveda, have demonstrated different medicinal plants, their uses, and their effects on the body. Ayurvedic herbalism targets to improve the neuro-endocrine-immune system by increasing anti-inflammatory and antioxidant derivatives [49, 50]. Traditional remedies also stimulate patients' memory and cognitive function in an enhanced state [51, 52]. Some of the herbal remedies have been mentioned for the treatment of dementia. Adjunct therapies, social and spiritual dimensions, diets/nutrition, and yoga have been excluded from this review.

5. ASHWAGANDHA [WITHANIA SOMNIFERA]

Ashwagandha is a predominant analeptic ayurvedic medication that belongs to the Solanaceae family and has been used for thousands of years [53, 54]. Alkaloids like alanine, withananine, somnine, and steroidal lactones are the most important compounds of *Withania Somnifera* compositions; its roots have frequently been used for medicines [53, 55]. Research indicates that the ashwagandha root influences nerve functions, antioxidant activities, free radicals, and inflammatory ventures to support defense mechanisms, enhance sexual performance, and reduce stress reactivity [55, 56].

As a *rasayan* [medicine], its overall alkaloid extraction from the root portion helps different mammalian families' central nervous systems to remain calm [55]. Additionally, roots prohibit NF-kB dynamization and obstruct mass-production of β- amyloid, which lessens apoptotic cell death and revitalizes synaptic functions [57]. Furthermore, its WL-A compound [steroid lactone] strengthens antioxidant activities by transferring Nrf2 to the nucleus [57-59]. WL-A also promotes the regeneration of nerves by reducing semaphorin 3A.

In a recent study, Ashwagandha was also found to facilitate the level of cholinergic like acetylcholinesterase and dopaminergic activities in the brain, and the memory and cognitive activities of dementia patients were improved [60, 61]. Another study has demonstrated that applying methanolic extracts of Ashwagandha to human neuroblastoma cells with a proper dose and time resulted in nerve growth, developed synapse patterns, and caused regeneration of axons and dendrite [53, 57, 62, 63]. According to Elhadidy, oxidative stress in rats due to aluminum trichloride [$AlCl_3$] in the cortex, hippocampus, and striatum can be prevented by the daily consumption of ashwagandha extract. This medication also prevents lipid peroxidation and the production of NO [64]. In addition, Ashwagandha prevents mitochondrial disruption, rejuvenates energy levels, and decreases brain inflammation.

6. CHEMICAL STRUCTURE OF ASHWAGANDHA

Ashwagandha is a 2 m tall and 1 m wide woody perineal shrub with tomentose branches whose structure is radially expanded from the central stem and produces green bell-shaped flowers. The brownish stem of this shrub also consists of small, green elliptic leaves located at the opposite side of flowering shoots and generates orange-red fruits, which shape like spherical berries with multiple pale brownish seeds, encircled by inflated calyx [5-8mm in diameter]. When the ashwagandha fruit reaches the ripe stage, it becomes red in color. Generally, the calyx [5mm long] of this shrub is found to be shorter than the corolla [5-8 mm long] [65].

Ashwagandha is composed of numerous chemicals with major characteristics for use in the pharmacotherapeutics medicinal department. The most important chemical components are steroidal lactones formed by "withanolides, withaferin A, 27-deoxywithaferin A, withanolide-D, withanosides, and withasomniferols A-C. It also contains countless alkaloids like anaferine, anahygrine, cushohygrine, dl-isopelletierine, 3-tropyltigloate, *etc*. The different parts of this shrub consist of various chemicals; for example, in the stem, Withasomnilide, withasomniferanolide, somniferanolide, somniferawithanolide, somniwithanolide; leaf part: 24,25-Dihydrowithanolide A, withanolide A, withanone, withaferin A, 27-hydroxy withanone, and 17-hydroxy withaferin A, 27-deoxy-16-en-withaferin A, 2, 3-dihydro-3*β*-hydroxywithanone, *etc*.; root segment: Withanolide E, withanolide F, withanolide G, withanolide H, withanolide I, withanolide J, withanolide K, withanolide L, withanolide M, and even fruits contain withanamide F,withanamide G, withanamide H, withanamide I, and many more [66, 67].

7. TURMERIC [CURCUMA LONGA]

Turmeric, an herbaceous medicinal plant, belongs to the Zingiberaceae family and possesses antioxidant, anti-septic, and anti-inflammatory preventive properties. As a derivative of curcuma longa, turmeric is a rhizome and root. It carries powerful biological properties that decrease oxidative stress and reactivity and enhances cognitive activities associated with aging procedures [55, 57]. In addition to curcumin, additional turmeric ingredients, including odorous turmerones like α - turmerones, β- turmerones, α - santalene, *etc.,* have significant anti-inflammatory and antioxidant characteristics to be used as a medicinal remedy for different purposes [68].

By activating the Nrf2- keap1 pathway, turmeric exerts antioxidant effects to decrease genomic variability. Curcumin interacts with keap1 [ECH-associated protein1] and liberates Nrf2 [which is primarily found in the cytoplasm] to move towards the nucleus, where it binds with antioxidant components of DNA to facilitate gene expression. Genes governed *via* Nrf2 comprising antioxidant enzymes, DNA revivify enzymes, and anti-inflammatory proteins strengthen the cell's capacity to repair the damage caused by repression of pro-inflammatory cytokines like ROS, IL-8, and TNF-α [57, 69, 70, 71]. In addition to this, curcumin decreases inflammatory activities by suppressing PLA2 [phospholipaseA2] and COX-2 [cycloxygenase-2] enzymes, which metabolize neural phospholipids and prostaglandins. Furthermore, through the Aβ-induced rat model of AD, it has been shown that curcumin improves memory by decreasing GFAP and COX-2 manifestation [57, 72], but the effectiveness of amyloid treatments in humans needs to be established.

Whenever turmeric [Curcumin] was given to AD mice, they displayed reduced plaque deposition and decreased oxidative and inflammatory activities [55, 57, 73, 74]. According to Brondino, curcumin defends PC12 and endothelial cells against Aβ toxicity and tau hyperphosphorylation in AD transgenic mice, decreasing Aβ oligomer and fibril formation [73,75]. In addition to this, curcumin decreases the rate of oxygen species like ROS, which blocks APP cleavage and revitalizes synaptic flexibility in AD mice [75, 76, 77, 78]. Furthermore, turmeric can decalcify the liver, normalize cholesterol levels, suppress allergies, and promote the digestive and immunity systems [55, 79, 80].

8. CHEMICAL STRUCTURE OF TURMERIC

The height of turmeric plant is 1 mm tall with highly branched but has a short stem and long leaves. The leaves of this ayurvedic plant are alternatively arranged in two rows and produce pale yellowish flowers. Again, the leaves are separate to form a leaf sheath, petiole, leaf blade, and the false stem also developed from the leaf sheath. The petiole length is 50-115 cm, whereas the leaf blade is 75-115 cm long. The most important part is the yellowish-orange colored rhizome, whose upper part is rough and segmented skin, approximately 2.5-7.0 cm long, and diameter measures about 2.5 cm [1 inch] [5, 6].

The major ingredient of turmeric is carbohydrate, i.e., 69.9%, remaining like 6.3% protein, 3.5% minerals, 5.1% fat, and 13.1% moisture are present [81]. Like Ashwagandha, the rhizome of turmeric contains different chemicals like curcuminoids and possesses demethoxycurcumin, curcumin, bisdemethoxycurcumin, and sesquiterpenoids including germacrone, bisacumol ar-turmerone, curlone, curcumene, curcuminol, β-bsabolene, α and β termerones, zingiberene, *etc*. [81, 82]. Another constituent of the rhizome is a volatile oil that can be acquired by a known process called steam distillation but also incorporated by borneol, d-sabinene, d-α-phellandrene, cineol, sesquiterpenes, and zingiberene [82, 83]. These chemicals are available in turmeric, but it also contains L-beta-curcumene, limonene, manganese, niacin, nickel, norbixin, pcymene, phosphorous copper/zinc, potassium, calcium, *etc*. [84].

9. BRAHMI [BACOPA MONNIERI]

Another ayurvedic herb related to the Scrophulariaceae family is primarily found in swampy and marshy regions of Southeast Asia, tropical Asia, sub-tropical United States, tropical Africa, and Australia [57,85]. Bacopa Monnieri is used to improve memory and intelligence and decrease stress and anxiety. It has also been used for the treatment of various diseases like epilepsy, asthma, and insomnia. Additionally, Brahmi possesses antioxidant, anti-inflammatory, anti-diabetic, and anti-arthritis properties. It also has gastrointestinal and muscle tranquilizer effects. Furthermore, it has been used as a fever reducer, pain reliever, lowering hypertension agent, and displays neuronal and liver-protecting properties. The main chemical components of Brahmi are saponins and triterpenoid saponins, including bacosides A and B, bacosaponins A, B, and C, and alkaloids containing nicotine, herpestine, and brahmine. Other primary constituents of this nootropic herb are betulinic acid, aspartic acid, glutamic acid, serine, stigmasterol, stigmastanol, β- sitosterol, and saponin glycosides like pseudo-jujubogenin glycosides [85, 86].

Brahmi's components exhibit antioxidant properties [especially bacoside A and B] and defend the brain from oxidative stress and various age-related cognitive decline [87]. Recently, it has been proven that bacosides expanded expressions of antioxidant molecules like SOD, GSH, and HSP70 and acted as a free radical scavenger by preventing lipoxygenase activities in the brain [42, 57, 87, 88]. Additionally, this advancing mode of various antioxidant enzymes was found in the prefrontal cortex, hippocampus, and striatum of the rat brain when treated with Brahmi for 21 days [85]. In AD dementia cases, these bacosides also provide neuroprotection to specific regions, such as the prefrontal cortex, hippocampus, and striatum of the brain, from cytotoxicity and DNA damage [85, 89]. The extracts of Brahmi [*i.e.,* bacosides] also possess a higher potential for inhibiting lipid peroxidation, chelating irons, and other divalent metals, which lead to reduced oxidative stress associated with β-amyloid [85, 90]. Due to bacosides and glutathione peroxidase, iron chelation increases in the brain [57, 85, 91], which improves cerebral vasodilation and enhances memory and learning ability [57, 85]. Due to the vasodilator property of Brahmi, which is mediated by nitric oxide, it controls systolic and diastolic pressure and ca+ fluctuations without influencing cardiac rhythm [85, 92].

Even bacosides can appear as regulators for membrane phosphorylation and dephosphorylation processes [57, 93] due to enhancing protein and RNA turnover activities in different brain areas like the hippocampus [94]. A combination of bacosides A and B protects the brain from smoking-induced neurological damage [57, 95]. Brahmi acts as an herbal neuroprotective agent that reduces the inflammatory levels in the brain by decreasing microglia-induced interleukin, TNF-α, and caspase-10 levels [42, 96-98]. Brahmi's betulinic acid also acts as a triterpenoid, which decreases COX-2 expression and production of prostaglandins due to minimizing the rate of inflammation in the brain cells [42]. As a therapeutic herb, Brahmi interacts with neurotransmitters, improves memory [99, 100], and enhances brain plasticity by increasing BDNF by 1.3 times and Arc by 2 times the expression of the brain cells [85, 101]. Research shows that Brahmi's anticholinergic effects in the AD of rat model increased cognitive functions [55, 102]. It has also been shown that Brahmi treatment inhibited acetylcholine and choline acetyltransferase activities in hippocampal and frontal cortexes muscarinic cholinergic receptors [55, 89]. Another study showed that neurons are protected from beta-amyloid-induced cell apoptosis by suppressing cellular acetylcholinesterase activities using Brahmi extracts [55]. Bacosides of Brahmi reduce hypobaric-hypoxia-induced cognitive dysfunction and other associated neurological disorders [88, 103].

Brahmi also plays a critical curative role in treating amnesia in dementia, Alzheimer's, and schizophrenia patients in various ways. For example, Brahmi can reverse amnesia and function like benzodiazepines, scopolamine, quinoline derivatives, and phenytoin, for which disruption of long-term potentiation [LTP] occurs. It has been shown by different tests that Brahmi inhibited the increased levels of mitogen-activated protein kinase [MAP kinase], phosphorylated CREB [pCREB], and inducible nitric oxide synthase [iNOS] in patients with amnesia induced by diazepam, while other proteins like cAMP [cyclic adenosine monophosphate], total CREB, total nitrate, and nitrite PDE were unaffected or normalized [104]. By utilizing Brahmi extracts, amnesia patients showed that their nitrite levels were normal. Additionally, Brahmi reversed amnesic effects caused by L-NNA-induced anterograde and retrograde amnesia but did not reverse amnesia in rats induced with MK-1 [104]. Brahmi may perform as a neuroprotective medicinal herb for Parkinson's, stroke, and epilepsy as it has been observed to enhance serotonin levels and activate CREB and 5-HT3A receptors in the hippocampus in postpartum rats, promoting learning abilities [100, 104, 105].

10. CHEMICAL STRUCTURE OF BRAHMI

Brahmi is a perineal herb that is 60-90 cm long, highly branched, and extended up to 5-35cm. This non-aromatic herb has well-expanded yellow-colored roots and has a 1 mm thick, greenish stem with nodes and internodes, but the taste is somehow unsweetened. Brahmi leaves are 8-15 mm long, 4 mm broad, and oblong-shaped. Its leaf blade is attached to the stem, which is called sessile; the lower surface is covered with dots and produces different colored five-petal flowers like white, pink, purple, *etc*. Bracteoles are broader than pedicles and 6-30mm long [85, 106].

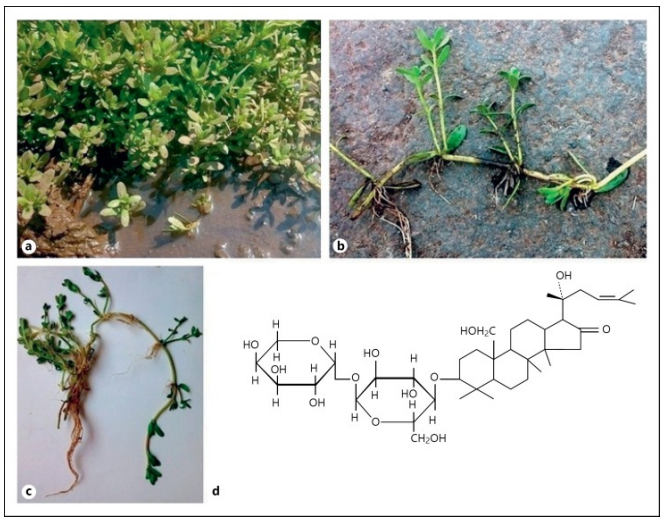

Fig. (3). Plant botanical morphology and Bacosides chemical composition [85]. Reprinted from Annals of Neurosciences, 24(2), Chaudhari KS, Tiwari NR, Tiwari RR, Sharma RS., Neurocognitive effect of nootropic drug Brahmi (Bacopa Monnieri) in Alzheimer's Disease, 111-22, 2017, with permission from S. Karger AG, Basel." *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

11. SHANKHAPUSHPI [CONVOLVULACEAE PLURIC-AULIS]

Shankhapushpi, an ayurvedic medicinal herb, belongs to the family of Convolvulaceae, which enhances learning, memory, and intelligence [57, 107]. Every part of Sankhapushi plants bears medicinal properties of strengthening memory acquisition, retention, and retrieval [107]. Generally, it is found in India and contains different secondary metabolites, such as triterpenoids, flavonol, glycosides, and steroids, which help in alleviating various nervous disorders like stress, anxiety, mental fatigue, and insomnia. Sankhapushpi also facilitates diverse neuropharmacological processes [55, 57, 108], and it has soothing effects on the body due to its effectiveness in regulating stress hormones like cortisol and adrenaline [57, 109, 110]. The presence of different chemicals, such as glucose, sucrose, starch, coumarins, sitosterol, convolvine, and convolidine, minimizes various ulcers and pain, controls neurotoxicity levels, enhances bone marrow quality, and increases nerve tissues [108, 111].

Ethanolic extract of CP decreases cholesterol, LDL cholesterol, triglycerides, and phospholipid levels in serum and provides antioxidant effects on the body [57, 109, 112, 113]. It has also been proven that ethanolic extraction of Shankhapushpi enhances brain nourishment by increasing acquisition and raising brain protein contents [109, 114, 115]. Additionally, CP acts as a muscle relaxant by decreasing the ethyl acetate portion [109] and acting as an antidepressant through interacting adrenergic, dopaminergic, and serotonergic systems utilizing ethanolic extrication [108, 116, 117]. It is also found that ethanolic extraction of CP enhances acetylcholine activities in the hippocampal CA1 and CA3 regions, dendritic intersections, and branching point numbers to support brain development, plasticity, and cognitive activities [57, 118-120]. Furthermore, methanolic extracts of CP, especially from stems and leaf callus, enable the body to defend against

tonic convulsion and show anti-convulsant activities [108, 121, 122]. CP also provides nutrition to every single layer of skin and is used in beauty products [109].

There are three different varieties of Shankhapusphi, such as Canscora Decussata (CD), Clitoria Ternatea (CT), and Evolvulus Alsinoides (EA). Sethiya [2018] verified the four traditional herbs' neuropharmacological activities *in vitro* to understand the antioxidant potential, AchE inhibition, 5-LOX enzyme inhibition, β- amyloid-induced neurotoxicity on neuro-2A, and *in vivo* assays like scopolamine-induced memory retrieval. After testing various parameters, he found different pharmacological potency in these four botanical herbs, and the order of activity was found as EA> CD>CP>CT [123].

12. CHEMICAL STRUCTURE OF SHANKHAPUSHPI

This medicinal herb consists of several branches, usually expanding widely on the ground and extending up to 30cm. Branched roots are cylindrical, and their color is also converted from brown to light brown. Even stems are found to be cylindrical, having nodes and internodes, while the length of the light green leaves is found to be 10.5-2cm and 0.1- 0.5 cm broad. Shankhapushpi flowers are primarily white and purple in color [124].

The whole Shankhapushpi plant is enriched in several medicinal properties as its different part contains several types of chemical constituents like kaempferol, taraxerone, taraxerol, N-hexacosanol, delphinide, *etc*. Another chemical group of alkaloids is also found in this plant, including sankhapusine, convosine, convolidine, confoline, phyallbine, convolamine, subhirsine, *etc.* This plant also consists of other groups like flavonoids, glycosides, phenolic compounds, steroids, *etc*. [125]. It also contains carbohydrates consisting of maltose, sucrose, rhamnose, *etc.,* besides myristic acid [39.9%], palmitic acid [66.8%], and linoleic acid [2.3%] [126].

13. GOTU KOLA [CENTELLA ASIATICA]

Gotu Kola [Centella Asiatica] is a herbaceous perennial plant that grows mostly in temperate and swampy areas in several regions of the globe [57, 127]. CA has a long and dense stem with smooth leaves of green to reddish-green color. Stems are interconnected and belong to the family Apiaceae [55, 57, 127]. In addition to scentellin, asiaticin, and centillicin, GK consists of pentacyclic triterpenoids, such as Asiatic acid (AA), madecassoside acid (MA), asiaticoside, and madecassic acid, which purify the blood, enhance memory, boost learning abilities, promote longevity, and decrease high blood pressure [57, 128-130].

AD and dementia patients have lower levels of phosphorylated CREB, which is an essential factor in the progression of memory and cognitive functions. Yanan [2008] observed that water extract of GK improves CREB phosphorylation and increases BDNF levels, facilitating axon regeneration and neuronal dendritic arborization, indicating neuroprotection in rats. He also identified the increasing molecular mechanism of memory and cognitive activities caused by the enhancement of CREB phosphorylation. Using GK treatment, Yanan [2008] specified some definite pathways that stimulate CREB phosphorylation, such as PKA [protein kinase A], nitric oxide signaling, and MAPK/

Fig. (4). Shankhapushpi. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

ERK/RSK pathways [129]. In another study, it has also been found that AA of GK decreases the glutamate-induced cognitive deficits and controls the lipid peroxidation, GSH, and superoxide dismutase levels in the hippocampus and cortex of the brain [130-132]. Additionally, it was found that AA of GK protects the body from glutamate-induced dementia and increases memory retrieval [132,133].

Furthermore, GK derivatives, such as AA and asiaticoside, can minimize hydrogen peroxide-induced cell death and lessens the number of free radicals. These proficient derivatives were also able to inhibit AD type of dementia and Aβ toxicity, which causes neural death *in vitro* [134-136]. Gray [2015] noticed that the GK treatment protects the body from Aβ-induced mitochondrial dysfunction and oxidative stress by using MC65 and SH-SY5Y neuroblastoma cells but also shows higher ATP production and the stimulation of antioxidant response genes, such as NFE2L2, GCLC, and NQO1 [136]. Furthermore, Gray [2017] found that using GK extraction treatment, both brains of the aged Tg2576 and WT mice possessed enhanced synaptic plasticity, increased dendritic arborization, and spine density [137]. Justin [2018] reported the GK's CWA neuroprotection capability against aluminum toxicity. He claimed that AA of GK decreases Al 3+ induced intracellular ROS production and mitochondrial membrane depolarization and prevents apoptosis activities and disruption of DNA in *in vitro* models of AD disease [138]. The use of GK extract also shows a substantial impact on patients with epilepsy disorder [57].

14. CHEMICAL STRUCTURE OF GOTU KOLA

Gotu kola is not only a perineal but also identified as a creeper herb growing 15 cm long. The stem of this medicinal herb starts at the rooting points, morphologically thin, striated, and greenish in color. Generally, the kidney-shaped leaves are covered with crenate margins and possess lengths of 1-5 cm and 2-6 cm broad. Small 3-4 white or purple flowers are found in the umbels [139].

There are different chemical groups present in this medicinal herb. Gotu kola consists of multiple types of triterpenic acids, including Madasiatic acid, Madecassic acid, Thankunic acid, Indocentoic acid, Euscaphic acid, Terminolic Isothankunic acid, Asiatica acid, *etc.* Different chemicals that constitute triterpenic sugar esters are available, such as Asiaticoside [A, B, C, D, E, F], Braminoside, Brahmoside, Brahminoside, Thankuniside, Centellasaponin A, Centellasapogenol, *etc.* Even steroid groups of triterpenoids are found in which stigmasterol and sitosterol are included. By analyzing Gotu kola, other chemical groups are also identified like flavonoids, such as kaempferol, astragalin, catechin, Rutin, *etc.,* vitamins: nicotinic acid, ascorbic acid, β -carotene, minerals, calcium, phosphorus, iron, potassium, magnesium, manganese, zinc, sodium, and copper. In addition to this, different essential oil, chemicals, and amino acids are also found [139, 140].

15. GUGGULU [COMMIPHORA MUKUL]

Guggulu is an exotic plant and belongs to the Burseraceae family. It is widely distributed from northern Africa to central Asia. It is a herb with an average height of about 3m and thin and peppery bark, and it grows mostly in arid and semi-arid climates [141]. Its juice [oleo gum resin] is extracted from cracks and fissures of the bark of different plant species [Commiphora Mukul, C. Molmol, C. Abyssinica, C. Burceraceae, and C. Whighitti] [55]. Aromatic oleogum resin is a pale yellow-brown color substance with a sharp, bitter taste [57, 142]. The oleoresin of Guggulu has been found to contain complex mixture compounds, such as water-soluble gum [30% to 60%], alcohol soluble resins [20% to 40%], and volatile oils [8%], which harbor numerous pharmacological activities [55, 57]. The major constituents of water-soluble gum are mucilage, sugar, and proteins, whereas alcohol-soluble gum consists of comiphorinic acids and heerabomyrrhols. Volatile oils are composed of terpenes, sesquiterpenoids, cuminic aldehyde, eugenol, ketone steroids Z- and Eguggulsterone, and guggulsterols I, II, and III [143, 144]. Additionally, Guggulu includes ferulic acids, phenols, and other non-phenolic aromatic acids that possess antioxidant activities against hydroxyl radicals and are used to treat different neurodegenerative and oxidative stress-related disorders [145, 146].

Fig. (5). Morphology of Gotu kola. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Additionally, Guggulusterone is a potent antagonist of the nuclear hormone receptor involved in cholesterol metabolism to lower total cholesterol levels. The administration of guggulipid [Z- Guggulsterone] has been found to decrease LDL cholesterol and triglyceride levels in serum of both animal and human models [147, 148]. These biological activities can also explain the hypolipidemic effects of Guggulu extraction [149]. The efficacy of Guggulu in hypolipidemic activities has been found, and it acts as an efficacious antagonist ligand for the farnesoid X receptor, a nuclear receptor that is activated through bile acids [147, 150]. A number of findings suggest a strong association between APP [amyloid precursor protein] processing, cholesterol, and AD [18, 151]. During *in vitro* and *in vivo* experimentation, it was observed that different cholesterol pools within the plasma membrane bilayer were affected by modulating membrane cholesterol levels and these cholesterol pools were differen-

tially sensitive to Aβ disrupting effect [152]. The evidence in the literature indicates an association between the utilization of cholesterol-lowering drugs and decreased prevalence of dementia [18,55,153]. Gugulipid acts as a substantial cholesterol-lowering, antioxidant, and anti-acetylcholine esterase antagonist against the streptozotocin-induced memory deficit model of dementia in rats [55, 154].

16. CHEMICAL STRUCTURE OF GUGGULU

The height of this medicinal tree ranges between1.2 to 1.8 m, with several branches producing brownish-red flowers but possessing shorter pedicles. This dwarf plant has palmately compound leaflet and bears drupe-type fruits containing one seed. In the ripe stage, fruits become red [155].

Like other herbs, this therapeutic tree also contains multiple varieties of fatty acids, myristic acid, palmitic acid, steric acid, arachidic acid, and oleic acid [156, 157].

17. MUSKROOT/SPIKENARD [NARDOSTACHYS JATAMANSI]

Nardostachys Jatamansi [NJ] is a flowering plant and belongs to the valerian family. It grows in Nepal, India, and Bhutan and is also known as muskroot or spikenard. Its rhizomes and roots are used for ayurvedic therapies. Different phytochemical components produced by muskroot include Acaclin, Ursolic acid, Octacosanol, Nardosinonediol, Oleanolic acid, and β- Sitosterol. It also contains other terpenoids, such as spirojatamol, nardostachysin, jatamols A and B, and calarenol. This medicinal plant is also used for preparing perfumes due to its intense aromatic essential oils.

NJ herbal extract has antioxidant properties. It decreases chronic fatigue syndrome [CFS], lipid peroxidation, nitrite and superoxide dismutase levels and increases low catalase levels. Furthermore, NJ alcoholic extract improves memory and learning capacity and reverses the amnesia induced by diazepam [1 mg/kg] and scopolamine [0.4mg/kg] in young and aged rats. NJ was reported to reverse the aging-induced amnesia in mice. It has established itself as a powerful and useful memory enhancer in both older individuals and patients experiencing age-associated dementia [55, 157, 158].

18. CHEMICAL STRUCTURE OF MUSKROOT

Muskroot is a perennial plant that is hairy and grows up to 10-60 cm long. Generally, this medicinal herb's roots [2.5 to 7.5 cm] are used for therapy and pharmaceutical applications. Narrow, long leaves of muskroot are rosy and slightly pink in color. Bilateral flowers not only possess bisexual characteristics but also develop the capacity to form clusters. The rhizomes of this herbal plant are 2.5 to 7.5 cm long and covered with reddish-brown tufted fibers. The upper surface of 4 mm lengthy muskroot fruits is hairy.

This herbal medicinal plant contains volatile oil, like sesquiterpenes, which also comprise other chemicals, such as nardostachone, jatamol, nardosinone, jatamansic acid, pyrnocoumarin A and B. Through chemical analysis, it has been

Fig. (6). Guggulu plants and rasins of Guggulu. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Fig. (7). Rhizomes of muskroot. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Table 1. Drugs with clinical trials models and their benefits.

Drugs Name	Disease	Model	Benefit	Refs.
Ashwagandha	Chronic stress	Human	Decreases stress and anxiety	[160]
	Cognitive dysfunction	Human	Reduces cognitive impairment level	[161]
	Neurodegeneration, Cognitive disability	Rat	Re-establishing neurite growth, defend neurons from apoptosis	[162]
	Inflammatory mechanism	Rat	Reduces inflammation for long term	[163, 164]
	Memory disorder		Enhances memory	[164, 165]
Turmeric	Dementia	Rat	Enhances cognition, improve memory	[166]
	Memory disorder	Non-demented adults	Increases memory and attention	[167]
	Alzheimer's	Human	Enhances cognition, depression agitation	[168]
Brahmi	Dementia	Human	Improves cognition	[169]
	Dementia	Human	Reduces stress, improves memory and cognition	[170]
Shankhapushpi	Cognitive dysfunction	Rat	Cognition level	[171]
	Poor Memory	Children	Increases alertness of mind, attention, and memory level	[172]
	Neurotoxicity, memory disorder	Rat	Decreases neurotoxicity level, strengthen memory	[123]
Gotukola	Alzheimer's	Rat	Improves behavior	[173]
		Healthy people	Increases working memory, cognition function	[174]
Guggulu	Hypercholesterolemia	Human	Decreases total cholesterol level	[149]
	Neuroinflammation	Rat	Decreases behavior abnormality, inflammation level	[175]

Table 2. Drugs mechanism in clinical trials.

Drugs	Disease	Mechanisms Observed in the Clinical Trial	Refs.
Brahmi	Memory disorder	Increases memory level	[176]
		Improves cognition level	[177, 178]
Gotukola		Increases working memory, cognition function	[124, 179, 180]
Centella Asiatica		Improves cognitive and mood disability and enhance working memory	[175]
Ginko Biloba	Alzheimer's Vascular dementia	Increases cognition function and reduces high blood pressure, headache, and dizziness.	[181]
Withania somnifera	Cognitive dysfunction	Increases level of functionality, attention, cognition, and even reaction time also changes	[161]
Muskroot	Cognitive disorder, depression, memory disturbance	Regulates nervous functions, increase learning, memory, reduce depression and express normal cognitive effects	[182]
Turmeric	Dementia, memory impairment	Improves memory and cognition level	[183]

confirmed that sesquiterpenes also include other chemicals like sitosterol, angelicin, elemol, and calarene. Muskroot also contains unstable oils, gum, sugar, starch, ketone, lupeol, propionate, and cyclohexanol ester [159].

The following two tables summarize clinical trials and drugs efficacy in different clinical models (Tables **1** and **2**).

CONCLUSION

The evidence in the literature illustrates that ayurvedic medicinal plants, such as Ashwagandha, Gotu Kola, Guggulu, Turmeric, and Brahmi, have valuable therapeutic properties in the treatment of dementia. Using these medicinal plants based on the evidence in the literature to provide person-specific treatment regimens can significantly be helpful in addressing dementia symptomatology and improving quality of life. Furthermore, these herbal remedies have a low toxicity threshold compared to other pharmacological drugs. Dementia involves neurodegeneration and can be addressed using herbal medicines; however, Ayurveda recommends Rasayana [herbal remedies], adjunct therapies [like panchakarma therapy], and lifestyle changes for managing dementia effectively. Yoga and meditation may also help rejuvenate the brain cells and improve memory and confidence. With the growing acceptance and use of herbal medicine worldwide, Ayurveda offers natural, cost-effective, well-tolerated, and holistic treatment regimens to manage dementia. However, there are many challenges to overcome in accessing Ayurvedic treatment. Several issues that need attention are manufacturing pharmaceutical-grade herbal medicine, conducting new RCTs to generate evidence, and evaluating the efficacy of ayurvedic medicine administration along with many available pharmacological drugs, quality control, and safety.

LIST OF ABBREVIATIONS

PSEN1 = Presenilin-1

PSEN2 = Presenilin-2

APP = Amyloid Protein Precursor

NF-kB = Nuclear Factor Kappa B

Nrf2 = Nuclear Factor Erythoid 2 Related Factor 2

ROS = Reactive Oxygen Species

IL-8 = Interleukin-8

TNF-alpha = Tumor – Necrosis Factor -Alpha

GFAP = Glial Fibrillary Acidic Protein

APP = Amyloid Protein Precursor

SOD = Superoxide Dismutase

HSP70 = Heat Shock Protein 70

BDNF = Brain Derived Neurotrophic Factor

CP = Convulvulus Pluricaulis

GK = Gotukola

NJ = Nardostachys Jatamansi

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ORIGINAL RESEARCH

Genotyping of Clinical Parameters in Age-Related Macular Degeneration

Priya Battu[1,*], Kaushal Sharma[1,*], Rajarathna Thangavel[2], Ramandeep Singh[3], Suresh Sharma[4], Vinod Srivastava[5], Akshay Anand[1]

[1]Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India; [2]Department of Ophthalmology, Vasan Eye Care, Chennai, India; [3]Advanced Eye Centre, Post Graduate Institute of Medical Education and Research, Chandigarh, India; [4]Department of Statistics, Panjab University, Chandigarh, India; [5]College of Health and Behavioral Sciences, Fort Hays State University, Hays, KS, USA

*These authors contributed equally to this work

Correspondence: Akshay Anand, Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, 160012, India, Tel +911722756094, Email akshay1anand@rediffmail.com

Background: Optical coherence tomography (OCT) parameters like subretinal fluid (SRF), intra retinal fluid (IRF) and retinal detachment (RPED) etc are routinely accessed by ophthalmologists in patients with retinal complaints. Correlation of OCT findings with genotype and phenotype of AMD patients is relatively unexplored. Here, we have investigated the association of OCT parameters' with genetic variants along with protein expressions and examined their clinical relevance with AREDS (Age-Related Eye Disease Study) criteria in AMD patients.

Methods: For this study, samples were recruited from Advanced Eye Centre, PGIMER, Chandigarh, India. Case-only analysis of anonymous imaging data (OCT/Fundus) acquired during the routine clinical evaluation of patients was done to examine the OCT findings in the AMD patients. TaqMan genotyping assays were used to analyze the single nucleotide polymorphisms in these patients. ELISA (enzyme linked immunosorbent assay) was used to estimate the protein levels of these genes in serum. Information pertaining to lifestyle/habits was also collected by administering a standard questionnaire at the time of recruitment of the patients.

Results: Intra-retinal fluid (IRF) was associated significantly with the LIPC genotype (p=0.04). Similarly, smoking status and early AMD were also associated with the APOE genotype (p=0.03). Additionally, variants of IER-3 and SLC16A8 were also found to be associated with co-morbidities (p=0.02) and males (p=0.02), respectively. RPED has shown a significant association with AREDS criteria, which demonstrated an area under AUROC around 72%.

Conclusion: Results of genotype–phenotype association can give a precise impression of AMD severity and can be beneficial for the early diagnosis of AMD cases.

Keywords: age-related macular degeneration, RPE detachment, OCT parameters, TIMP-3, HTRA1, IPC, APOE, anti-VEGF therapy, AREDS

Introduction

Age-related macular degeneration (AMD) is a retinal degenerative disorder that develops in late life, generally after 50 years of age. It is a painless condition but results in irreversible central vision loss.[1] It is the third most common cause of blindness in the world. It has been estimated that, by 2020, 196 million people will be suffering from AMD. This number is predicted to increase to 240 million by 2088.[2] AMD can occur due to impaired functioning of choroidal blood vessels, retinal pigment epithelial cells, Bruch's membrane (BM), and the photoreceptor layer.[3] AMD is broadly categorized into dry and wet forms. The advent of high-resolution OCT imaging (optical coherence tomography) brought new insights into the evaluation and monitoring of phenotypic variations of retinal layers in AMD patients.[4,5] Ophthalmologists

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frequently use it for qualitative phenotypic manifestations assessment, such as cystoid spaces, retinal pigment epithelium detachments (PEDs), sub-retinal fluid (SRF), and/or vitreomacular pathologies.[6]

The characteristic feature of dry AMD, such as drusen (deposition of lipofuscin between the RPE and its underlying basement membrane – Bruch's membrane), appears as the distinct elevation of the RPE with varying reflectivity on an OCT image. These are seen as hyper-reflective stacks below the RPE layers on an OCT image, causing RPE irregularity. Moreover, the sub-retinal fluid appears as an opaque space between the neuro-sensory retina's rear end and the RPE/choroid-capillaries complex reflection. The RPE detachment gives slightly more reflection on OCT. This increased reflectivity of the RPE severely overshadows the noise from the underlying choroid. In SD-OCT, geographic atrophy appears as a central thinning of the retina over a degenerated zone of RPE. Pigment epithelial detachment is usually responsible for impaired vision, usually indicated with choroidal neovascularization (CNV). Therefore, the identification of pigment epithelial defects (PEDs) by OCT analysis carries a prognostic value.[7,8] These morphological changes are being used to determine the course of diagnosis and treatment of such patients to enhance AMD management.

AMD is a multifactorial disease. Inflammation, drusen formation (lipofuscin genesis), and neovascularization contribute to AMD's pathophysiology.[9] Many factors have been linked with AMD occurrence and progression, but age being the most important factor. Rudnicka et al have found that the risk of AMD incidence increases four times with an increase in age by 10 years. Additionally, family history, smoking habits and previous cataract surgery have been identified as risk factors for AMD.[10] Cholesterol, diabetes and menopausal age have also been associated with AMD.[11] Results have also found that higher circulating levels of white blood cells that the gut microbiome has been observed to be different among AMD cases and controls[12] which can be used as biomarkers for AMD.[13] In addition to environmental factors, genetics also play a crucial role in the manifestation of AMD.[14]

Many studies have examined the association of AMD with various genetic factors. Genetic variants of complement factors (C2, CFI, CFH, CCL2), angiogenesis (VEGFs, VEGFRs, etc), pro-angiogenic genes (TIMP3, ADAMTS9), and metabolizing genes (LIPC, APOE) genes have been reported to be associated with the risk of AMD[15–20] genetic polymorphisms in different genes have been linked to AMD like *CFHY402H* (rs1061170), *ARMS2* (rs10490924), *C2* (rs547154), *ABCA1* (rs1883025), *VEGFA* (rs4711751) are associated with advanced AMD, ie, neovascular form of AMD,[21] genetic polymorphisms in TLR are associated with AMD in Indian population.[19] Some studies have shown a negative association of AMD with genetic variants of rs2075650 ofApoE,[22] rs10468017 of hepatic lipase (LIPC),[23] and allele T of variant rs493258of hepatic lipase.[24] Hence, the exact role of these genes can be defined by examining their expression profile in serum of AMD patients. For example, lipid metabolizing proteins (LIPC and APOE), monocarboxylic acid transporter protein SLC16A8, TIMP-3,[20] angiogenic VEGF[16] and HTRA1,[25] ARMS2, COL8A1[26] levels were found to be increased in serum of AMD patients in comparison to controls. Daily life activities like sleeping patterns are also known to modulate the protein expression of the genes.[15] We have also identified that genetic variants and subsequent protein alterations especially lipid metabolising proteins (APOE and LIPC) can modulate the anti-VEGF response in wet AMD patients.[53] But, the translation of such studies to diagnostic and therapeutic advancement remains neglected because most of the studies lack the approach to consider the clinical findings, genotype, protein expression and socio-demographic variables as an integral entity to dissect the AMD complexity. Hence, we have conducted a pilot study where an association between genotype, phenotype, protein levels and demographic variables has been investigated and attempted to investigate the association of genetic differences with OCT findings of AMD patients.

Materials and Methods

Study Design

The present study is a case-only analysis of 53 AMD patients attending the retina clinic of Advanced Eye Centre, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh during the period 2014–2018. Patients were recruited after obtaining written consent at the time of enrollment. The ethical approval for the study was obtained from the Institutional Ethical Committee, PGIMER, Chandigarh. Retrospective case-only analysis of anonymous imaging data was acquired during the routine clinical evaluation of patients diagnosed with AMD. This study received ethical

approval from the PGIMER Ethical Committee (No: PGI/IEC/2005-06; dated: 23.07.2013), PGIMER, Chandigarh, India, and followed the provisions of the ethical approval. Study was conducted in accordance with the declaration of Helsinki. Participants were informed about purpose and nature of the study before recruiting them.

Recruitment of Patients

The patients included in this study were recruited from the co-author's clinic. The inclusion criteria of research subjects included a diagnosis of AMD following AREDS criteria during a dilated Fundus examination by this study's co-author, a retina specialist.

Only patients aged 50 or above were recruited as research subjects of this study. Group 1: Each eye had no drusen or non-extensive small drusen (AMD category 1); Group 2 (Intermediate Drusen): At least one eye had one or more intermediate drusen, extensive small drusen, or pigment abnormalities associated with AMD (AMD category 2); Group 3 (Large Drusen): At least one eye had one or more large drusen or extensive intermediate drusen; Group 4 (Geographic Atrophy): At least one eye had geographic atrophy. Group 5 (Neovascular): Choroidal neovascularization or RPE detachment in one eye (nondrusenoid RPE detachment, serous sensory or hemorrhagic retinal detachment, subretinal hemorrhage, subretinal pigment epithelial hemorrhage, subretinal fibrosis, or evidence of confluent photocoagulation for neovascular AMD.[27]

Patients below the age of 50 and having AMD-like clinical features but associated with some other pathological conditions, such as diabetic retinopathy, uveitis, and near-sightedness were excluded from the study.

OCT Findings

Macular OCT image of AMD patients was acquired using Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, CA) with a super-luminescent diode (840 nm). It can obtain 27,000 optical coherence A-scans per second. Macular cube and radial scans were performed in the eyes of AMD patients. Retinal layer thickness (μm) was estimated by analyzing the macular cube (512 × 128) protocol covering a 10.4° radius foveal area. Additionally, 6 mm radial lines consisting of 128 A-scans per line and cross-hair protocol including two 6 mm lines (6–12 to 9–3 o'clock) at 512 scan resolution were also carried out in these eyes.

Distance between anterior inner limiting membrane (ILM) and posterior RPE was measured to denote the retinal thickness (μn). Tabular output mode was used to analyze foveal thickness from the OCT image. Morphological deformities in the retinal layer were analyzed based on OCT images, which have been reported in previous literature, including subretinal fluid (SRF), intra-retinal fluid (IRF), pigment epithelial detachment (RPED), RPE irregularity (RPE Irr), and fibrosis. Clinical parameters obtained from OCT images like retinal thickness, presence, and location of intra-retinal cysts, RPED, SRF were also observed in clinical trials for CNV AMD patients to show morphological integrity of retinal layers.[28]

SRF was identified as a non-reflective space between the posterior RPE layer and the neuro-sensory retina above. The intra-retinal fluid was determined by the presence of cysts that were defined as round, minimally reflective spaces within the neuro-sensory retina. PED was described as a focal elevation of the reflective retinal pigment epithelium (RPE) band over an optically clear or moderately reflective space.

Table 1 Sociodemogaphic details of AMD patients recruited in the study

	Gender		Smoking Status		Co-Morbidities	
	Males	**Females**	**Yes**	**No**	**Yes**	**No**
AMD (n=53)	29	24	14	39	39	14

Collection of Socio-Demographic Data

The demographic information like age, gender, smoking habits, food habits and co-morbidities (eg hypertension, cardiovascular diseases, diabetes etc) of the patients was collected, like by administering a standard questionnaire. Socio-demographic details of the study population are described in Table 1.

Isolation of Serum

2–4 mL blood was taken in the vacutainers containing clot activators (BD Biosciences, USA). Centrifugation was carried out at 2500 rpm (high knob) at room temperature for 30 minutes. The upper clear layer of serum was collected in centrifuge tubes and stored at −80◦C till further uses, after proper labeling and coding.

ELISA Estimation

The serum levels of LIPC (Hepatic Lipase C), TIMP-3 (Tissue inhibitor of metalloproteinases-3), B3GALTL (Beta 3-Glucosyltransferase), IER-3 (Immediate Early Response −3), SLC16A8, (Solute Carrier Family 16 Member 8), ADAMTS9 (ADAM Metallopeptidase With Thrombospondin Type 1 Motif 9), HTRA1 (High-Temperature Requirement A Serine Peptidase 1), and APOE (Apolipoprotein E) proteins were estimated using commercially available kits (Qayee-Bio, China). The experiment was performed as per the manufacturer's protocol for the estimation of proteins in serum. Experiments were conducted after standardization. Standards were run in duplicates and samples were run in random duplicates. The absorbance reading was taken at 450 nm on an ELISA reader (Biorad, USA). Total protein estimation was performed with 400 times diluted serum samples to normalize the ELISA values.

Estimation of Total Protein

Total protein was estimated using Bradford's method. Bovine serum albumin was used as standard in these experiments. Bradford reagent (Sigma, USA) and autoclaved water was used in 1:4 dilution and the absorbance was measured at 595nm using ELISA reader (Biorad). Normalization of ELISA counts were carried out by using values obtained from total protein estimation.

PBMC Isolation and DNA Extraction

4 mL blood sample was taken in EDTA vacutainer (BD Biosciences, USA) and RBCs were allowed to settle for 2 hours at room temperature. Upper layer was collected and carefully layered on equal volume of histopaque previously taken in a separate tube. It was subjected to centrifugation at 1500 rpm (REMI, India) for 30 minutes. Three layers were obtained after this procedure. The middle buffy layer was aspirated out and taken in the centrifuge tube. Two washes of 1X PBS were given at 5000rpm for 5 minutes at 4◦C. The PBMC pellet was suspended and stored at −80 0 C for further use. Genomic DNA was extracted using commercially available kits (Qiagen, Germany). UV spectrophotometer (Beckman Coulter) was used to estimate concentration and integrity of the isolated DNA by measuring absorbance at 260nm, after labeling and coding, DNA was stored at -20^{0}C till further use.

Analysis of Single Nucleotide Polymorphisms

Single nucleotide polymorphisms were analyzed by TaqMan genotyping assay (ABI, USA). Real-time PCR was carried out to analyze SNPs for eight genes, namely *LIPC* (rs920915), TIMP-3 (rs5749482), *B3GALTL* (rs9621532), *IER*-3 (rs3130783), *SLC16A8* (rs8135665), *ADAMTS9* (rs6795735), *HTRA1* (rs11200638) and *APOE* (rs4420638). Briefly, PCR conditions included a denaturation step at 95◦C for approximately 10 minutes, an extension step at 95◦C for 15 seconds, and 60◦C for 1 minute. The process was repeated for 40 cycles. The reaction mixture's total volume was 10 µL, with 20ng as the total concentration of genomic DNA in the TaqMan assay reaction.

Statistical Analysis

Frequencies for studied genotypes have been measured to distribute clinical parameters obtained from OCT image analysis, and their statistical significance has been calculated through the *chi*-square test. Pearson's correlation was used to find a relationship between OCT biomarkers and genotypes. Independent Student's *t*-test was used to analyze statistical

significance of differential expressions between the genetic variants. To establish the correlation between existing AREDS criteria (used for AMD classification) and OCT parameters, we used Pearson's correlation. Diagnostic efficacy and specificity of the OCT parameters to identify AMD patients were calculated through ROC curve and area under ROC (AUROC) curve. All the values were reported with a 95% confidence interval, and p-values ≤ 0.05 were taken to be statistically significant. Statistical analysis was done by using SPSS 22.0 (SPSS, USA). The power analysis was conducted, and its values varied from 0.78 to 0.99 for this study for varying sample sizes from 41 to 53; values were found to be at 95% CI, and p values were statistically significant ($p < 0.05$).

Results

Relationship Between Genotypic Frequency and Clinical Parameters

For all the eight variants, we analyzed the association of genotype with clinical parameters obtained from OCT. The socio-demographic details of the recruited AMD patients are mentioned in Table 1. For the ADAMTS9 variant, the homozygous T/T genotype association was seen with all the clinical findings. The association of the homozygous C/C genotype was lowest with all the clinical parameters. The number of patients with homozygous A/A genotype in the APOE gene was highest for all clinical findings. For the LIPC variant, homozygous G/G genotype patients had the highest incidence of all clinical findings than homozygous C/C variant and heterozygous C/G variant. LIPC genotypes were found to be significantly associated with intra-retinal fluid, among the other clinical findings (Table 2). For Intra-retinal fluid, the highest number of individuals was homozygous G/G, followed by heterozygous C/G and homozygous C/C. In the case of the HTRA1 variant, patients with the homozygous G/G genotype had the highest incidence of SRF, IRF, and RPE irregularity. Patients with homozygous A/A genotype had the highest incidence of subretinal fibrosis. The incidence of RPED was found to be equal in patients with homozygous A/A and heterozygous A/G genotypes (Table 2). Non-significant variants are shown in the supplementary information (Table S1) Results are graphically represented in the supplementary text. (Figures S1–S8).

Association of Genetic Variants with Socio-Demographic Variables

Out of 43 patients, 74.1% of the subject had co-morbidities, and among those with co-morbidities, 59.37%, 25%, and 15.62% had homozygous T/T, heterozygous C/T, and homozygous C/C genotype, respectively, for ADAMTS9 variant. For ApoE variants, out of 49 patients, 75.5% had co-morbidities. Among those with co-morbidities, 45.03% had homozygous A/A genotype, and 3.97% had heterozygous A/G genotype, respectively. Genotype was significantly associated with smoking status (P=0.03) (Table 3). Among variants of B3GALTL, out of 46 individuals, 78.26% had co-morbidities among those with co-morbidities, 68.75%, 34.37%, and 9.37% had homozygous T/T, heterozygous C/T, and homozygous C/C genotypes, respectively. For IER-3- variant, out of 41 patients, 62.79% had co-morbidities among those with co-morbidities, and 88.8% had heterozygous A/G genotype, and 11.11% had homozygous A/A genotype. Co-morbidities were also found to be significantly associated with the IER-3 genotype (P= 0.02). We also looked at the association of SLC16A8 with socio-demographic variables. We found that out of 44 patients, 77.27% had co-morbidities among those with co-morbidities, 55.88%, 38.23%, and 5.88% had homozygous C/C, heterozygous C/T and HomozygousT/T genotypes, respectively. Also, gender was significantly associated with the SLC16A8 genotype (P=0.02). For the HTRA1 variant, out of 46 patients, 78.26% had co-morbidities among those with co-morbidities, 50%, 33%, and 16.66% had homozygous G/G, heterozygous A/G, and homozygous A/A variants, respectively (Table 3). For variants of LIPC, out of 53 patients, 73.58% had co-morbidities; among those with co-morbidities, 51.28%, 43.58%, and 5.1% had homozygous G/G, heterozygous C/G and homozygous C/C variants, respectively. Similarly, for TIMP-3 variants, out of 51 patients, 70.5% had co-morbidities, and among those with co-morbidities, 86.0% had homozygous C/C, and 13.8% had heterozygous G/C genotypes. The association of genotypes and socio-demographic variables is graphically represented in the supplementary text (Figures S9–S16). Additionally, the association of genotype of the variants studied with early, intermediate and advanced AMD is represented graphically in Figures S17 and S18.

Table 2 Association of genotypes of studied SNPs with clinical findings of AMD patients. LIPC *(Lipase C)* was found to be significantly associated with IRF (intra retinal fluid)

Parameters	ADAMTS9 (N=43)				APOE (N=49)				HTRA1 (N=47)				LIPC (N=53)			
OCT Findings	**TT (N=25)**	**CC (N=5)**	**CT (N=13)**	**p**	**AA (N=45)**	**AG (N=4)**	**GG (N=0)**	**P**	**GG (N=7)**	**AA (N=22)**	**AG (N=18)**	**P**	**GG (N=27)**	**CC (N=4)**	**CG (N=22)**	**P**
SRF	7	2	4	0.86	10	2	0	0.21	2	6	7	0.72	4	1	8	0.21
IRF	13	1	5	0.37	21	0	0	0.07	5	6	10	0.06	12	4	7	0.04
RPED	12	0	3	0.06	17	0	0	0.13	1	8	8	0.37	7	2	7	0.60
RPE Irr	22	3	12	0.18	36	3	0	0.81	6	16	17	0.42	19	4	20	0.12
Fibrosis	8	1	3	0.77	12	0	0	0.23	2	6	3	0.68	9	2	2	0.07

Table 3 Association of genotype of studied genetic loci with gender, smoking status, co-morbidities and diagnosis based on AREDS criteria: There was a significant association between genotype and some studied variables. ADAMSTS9 genotype aas associated with AREDS criteria. IER-3 genotype was associated with the co-morbidities, and SLC16A8 genotype was associated with gender in AMD patients

Parameters		ADAMTS9				APOE				B3GALTI				IER-3			
		TT	CC	CT	P	AA	AG	GG	P	TT	CT	CC	P	AA	AG	GG	P
Gender	Male	15	3	7	0.93	25	3		0.45	12	3	10	0.08	4	18		0.53
	Female	10	2	6		20	1			16	0	5		5	14		
Smoking	Smoker	7	3	3	0.29	11	3		0.03	6	1	5	0.66	2	9		0.72
	Non-smoker	18	2	10		34	1			0	0	0		7	23		
Co-morbidity	Absent	6	0	5	0.24	11	1		0.98	6	0	4		6	8		0.02
	Present	19	5	8		34	3			22	3	11		3	24		
AREDS	Early	0	1	0	0.08	0	1		0.002	1	0	0	0.59	0	0		
	Intermediate	4	1	3		8	0			6	1	3		2	8		
	Advanced	21	3	10		37	3			21	2	12		7	24		
Parameters		**SLC16A8**				**HTRA1**				**LIPC**				**TIMP-3**			
Gender	Male	CC	TT	CT	P	GG	AA	AG	P	GG	CC	CG	P	CC	GG	GC	P
	Female	19	1	5	0.02	4	12	11	0.91	18	3	8	0.07	25	0	5	0.52
Smoking	Smoker	7	1	11		3	10	7		9	1	14		16	0	5	
	Non-smoker	5	1	4	0.584	3	3	6	0.19	6	3	5	0.07	8	0	4	0.17
Co-morbidty	Absent	21	1	12		4	19	12		21	1	17		33	0	6	
	Present	7	0	3	0.60	1	4	5	0.612	7	2	5	0.52	10	0	5	0.11
AREDS	Early	19	2	13		6	18	12		20	2	17		31	0	5	
	Intermediate	0	0	0	0.47	1	0	0	0.08	1	0	0	0.47	1	0	0	0.697
	Advanced	4	1	3		1	5	1		7	1	2		8	0	3	
		22	1	13		5	17	17		19	3	20		32	0	7	

Abbreviations: AREDS, age-related eye disease study; ADAMTS9, a disintegrin and metalloproteinase with thrombospondin motifs 9; APOE, apolipoprotein E, B3GALTL, Beta-1,3-glucosyltransferase; IER-3, immediate early response 3, SLC16A8, solute carrier family 16 member 8; HTRA1, high-temperature requirement A serine peptidase 1; LIPC, lipase C, hepatic type; TIMP-3, tissue inhibitor of metalloproteinases 3.

Table 4 Association of clinical variants obtained from OCT images and their association with existing AREDS score designated with Pearson's *Chi*-square values (p-value). RPED was found to be associated with AREDS score

Count		AREDS			
		AREDS 3	AREDS 4	AREDS 5	p-value
SRF	Absent	8	6	16	
	Present	1	3	9	
	Total	9	9	25	0.369
IRF	Absent	6	5	13	
	Present	3	4	12	
	Total	9	9	25	0.749
RPED	Absent	8	8	12	
	Present	1	1	13	0.021
	Total	9	9	25	
RPE Irr	Absent	2	2	2	
	Present	7	7	23	
	Total	9	9	25	0.414
FIbrosis	Absent	8	5	18	
	Present	1	4	7	0.289
	Total	9	9	25	

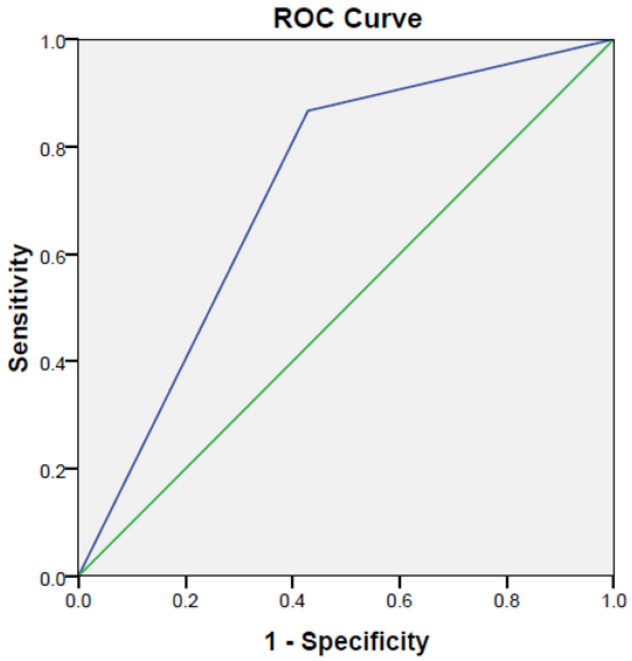
Abbreviations: AMD, age-related macular degeneration; SRF, subretinal fluid; IRF, intraretinal fluid; RPED, retinal pigment epithelium detachment; RPE Irr, retinal pigment epithelium irregularity.

Protein Expression versus Genotype

We have also investigated the association of protein levels with the difference in genotype. We observed that the protein level was similar among the different genotypes, with a slight difference between protein expression in HTRA1 and LIPC genotypes. Although, the differences were not statistically significant, as seen in Table S2.

Association of Clinical Parameters with AREDS Criteria

Additionally, we have attempted to associate previously mentioned clinical parameters, including SRF, IRF, RPED, RPE irregularity, and fibrosis with prevailing AREDS criteria for AMD patients. The *chi*-square result (Pearson's *chi*-square p= 0.021) has demonstrated a significant association of RPED with AREDS criteria (Table 4), suggesting that the new approach is crucial for AMD patients' diagnosis. However, the association with other non-significant clinical parameters with AREDS may be due to the study's inadequate sample size. Additionally, results of area under the receiver operating curve (AUROC), which found to be around 72% (p=0.019) to determine the sensitivity and specificity of the model (Figure 1) with minimum standard error 0.08 and with a close range of 95% confidence intervals (CI 0.562–0.876). Results are suggesting RPED could be a leading parameter for diagnosing AMD cases from the population (Table 4).



Area Under the Curve				
Test Result Variable(s):AREDS				
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.719	.080	**0.019**	.562	.876

a. *Under the nonparametric assumption*
b. *Null hypothesis: true area = 0.5*

Figure 1 Area under ROC (AUROC) to predict AMD cases from the normal population based on the criteria of association between RPED and AREDS.

Discussion

Many studies on neurodegenerative disorders by our lab has found that various genes are implicated in neurodegenerative conditions like Parkinson's, ALS and AMD in the Indian population.[29–36] Also, we have been exploring various therapeutic strategies for these degenerative conditions.[37–39] This investigation is also an attempt to find a missing link between clinical practice and lab findings. The present study aimed to demonstrate morphological deformities (as reflected by high-resolution OCT images) and see if these clinical findings can be associated with AMD's genetic variants. Genome-wide association studies have identified specific genetic loci that are associated with AMD.[12,13,40] A GWA study has identified genes, HTRA1, and CFH as significant contributors to AMD's risk.[41] Another study has found that genetic variants (frequency < 0.1%) of complement factor H (CFH), complement factor I (CFI), and tissue inhibitor of metalloproteinases(TIMPs), including a splice variant in SLC16A8 suggest causal roles for these genes, in AMD. The difference in ethnic backgrounds may also be one factor responsible for AMD pathology. In a study on the Italian population, SNPs in LIPC (Hepatic Lipase), SLC16A8 (Solute carrier family 16 members 8), and TIMP-3 (Tissue inhibitor of metalloproteinases) were recognized as susceptibility factors responsible for causing AMD.[30] Seddon et al have reported that the TT genotype of the LIPC variant was linked to a lower risk of AMD independent of socio-demographic variables like smoking, BMI (Body Mass Index), and a diet rich in lutein.[42]

OCT imaging has paved the way for better management of AMD and emerged as the gold standard for diagnosing wet AMD besides assessing anti-VEGF treatment responses and evaluating disease progression.[43,44] Hence, the present study has been carried out to understand the association of genetic variants and protein expressions with OCT parameters to exhibit the genotype-phenotypic alterations to strengthen further AMD's diagnostic protocol in clinical setup (no controls included). Karacorlu et al have studied the morphology of Bruch's membrane by using SD-OCT (Spectral Domain Optical Coherence Tomography) of CNV patients in association with anti-VEGF treatment.[45] We considered

five phenotypic changes: Intra-retinal fluid, Subretinal fluid, RPE irregularity, RPE detachment, and retinal fibrosis and found a significant association of LIPC genotypic variants with intra-retinal fluid ($p<0.04$).

In addition to genetics, socio-demographic factors like age, sex, weight, occupation, education, food habits, physical activity, night sleep hours, exposure to sunlight, water intake, and co-morbidities, microbiota status etc are believed to be the major risk factors for AMD. As AMD is a degenerative disease associated with ageing, age remains the most important risk factor for AMD incidence. Interestingly, women have also been reported to be at higher risk of developing AMD. Many studies have shown that smoking status confers the risk of development and progression of AMD and other diseases.[42–52] We also found the association of smoking status and the number of individuals in different genotype groups of the APOE gene ($p<0.03$).[14] Several studies have shown a positive correlation between smoking and AMD.[14,16–19] Myers et al have found that smoking is positively associated with a high risk of converting the early form to moderate form of AMD.[47] Another study reported smoking to be associated with the occurrence of AMD.[48] For example, Rim et al found that the risk of developing advanced AMD is related to smoking's current or past status.[49] In addition to lifestyle, the co-existence of a diseased state increases the probability of AMD occurrence. Hypertension, cardiovascular abnormalities, and diabetes have also been reported in AMD as a comorbid condition. In the current study, we have found that co-morbidities were significantly associated with genotypic variants of IER-3 ($p<0.02$). A previous study carried out by Vassilev et al also found an association between AMD, Diabetes, history of eye diseases, and cardiovascular disorders,[50] highlighting the importance of environment and history of illness on the pathogenesis of AMD. Environment plays a vital role in people's adaptation and self-regulation.[10] Rohrer et al have shown an association of complement factor products, SNPs smoking, and BMI with AMD in the population of South Carolinians. The study results have also demonstrated that AMD was more common in people of European descent than Americans.[51] Additionally, Europeans were found to have a higher risk of developing AMD with more copy numbers of rs3766404 (CFH) and a lower chance with more copy numbers of rs1536304 (*VEGFA)*.

AREDS criteria are used routinely to diagnose AMD, but this study examined the potential association of genotype with AREDS. Interestingly, we have found a significant association between the early stage of AMD and genotypes of APOE ($p<0.002$). Additionally, we have also demonstrated that the genotypic variation of SLC16A8 ($p<0.02$) was significantly associated with male AMD patients in the Indian population.

The discovery of biomarkers that postulate AMD's association with genetic variants may facilitate early AMD diagnostics and therapeutics. Therefore, our analysis of the protein expressions in the AMD patients' serum has shown a varying degree of expression among genotypes of all studied genes. We did not find a significant correlation between genotype and expression levels for genes, indicating that there may be other genetic factors exerting their pathologic effects. The initiation of disease and its progression could be influenced by a varied degree of genetic penetrance of different gene loci. One of the limitations of this study was the small sample size, but this pilot study has provided the initial data for a large cohort study to strengthen AMD's better management. Lee et al have shown that previously collected OCT images may be used to generate retinal flow maps from structure images of patients.[52] Conclusively, the present study results have suggested that expression of lipid metabolizing proteins (LIPC and APOE), pro-angiogenic protein (ADAMTS9), and Serine protease HTRA1 may be considered to reflect the alterations in OCT image parameters.

Additionally, OCT image parameter SRF has demonstrated the association with APOE, LIPC, and HTRA1genotypes. Similarly, RPED has also been found to be associated with ADAMTS9 levels. But these findings need to be validated on larger sample size by considering population-based genotype susceptibility to redefine the diagnostic criteria of AMD pathology.

Conclusion

Intra-retinal fluid (IRF) was associated significantly with the LIPC genotype ($p=0.04$). Similarly, smoking status and early AMD were also associated with the APOE genotype ($p=0.03$). Additionally, IER-3 variants and SLC16A8 genotypes were also found to be associated with co-morbidities ($p=0.02$) and males ($p=0.02$), respectively. RPED has shown a significant association with AREDS criteria, which demonstrated an area under AUROC around 72%. In

addition to genetic association findings with gender, smoking status and co-morbidities, this pilot study highlights the association of OCT biomarkers with genetic polymorphisms and diagnostic criteria like AREDS in AMD patients. We propose that further Analysis of GWAS using various OCT parameters of AMD patients may help us in identifying disease-modifying genes and aid in developing personalized therapies.

Data Sharing Statement

All the relevant data of the manuscript is available with the corresponding author of the manuscript and accessible whenever asked.

Ethical Approval and Consent to Participate

This study received ethical approval from the PGIMER Ethical Committee (No: PGI/IEC/2005-06; dated: 23.07.2013), PGIMER, Chandigarh, India, and followed the provisions of the ethical approval. Participants were recruited only after taking written consent from them.

Consent to Publication

All the authors give consent to publish this manuscript.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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Modulated anti-VEGF therapy under the influence of lipid metabolizing proteins in Age related macular degeneration: a pilot study

Kaushal Sharma[1,2], Priya Battu[1], Ramandeep Singh[3]✉, Suresh Kumar Sharma[4] & Akshay Anand[1]✉

Age-related macular degeneration (AMD) is a devastating retinal disease that results in irreversible vision loss in the aged population. The complex genetic nature and degree of genetic penetrance require a redefinition of the current therapeutic strategy for AMD. We aimed to investigate the role of modifiers for current anti-VEGF therapy especially for non-responder AMD patients. We recruited 78 wet AMD cases (out of 278 AMD patients) with their socio-demographic and treatment regimen. Serum protein levels were estimated by ELISA in AMD patients. Data pertaining to the number of anti-VEGF injections given (in 1 year) along with clinical images (FFA and OCT) of AMD patients were also included. Visual acuity data (logMAR) for 46 wet AMD cases out of a total of 78 patients were also retrieved to examine the response of anti-VEGF injections in wet AMD cases. Lipid metabolizing genes (LIPC and APOE) have been identified as chief biomarkers for anti-VEGF response in AMD patients. Both genotypes 'CC' and 'GC' of LIPC have found to be associated with a number of anti-VEGF injections in AMD patients which could influence the expression of B3GALTL,HTRA1, IER3, LIPC and SLC16A8 proteins in patients bearing both genotypes as compared to reference genotype. Elevated levels of APOE were also observed in group 2 wet AMD patients as compared to group 1 suggesting the significance of APOE levels in anti-VEGF response. The genotype of B3GALTL has also been shown to have a significant association with the number of anti-VEGF injections. Moreover, visual acuity of group 1 (≤ 4 anti-VEGF injections/year) AMD patients was found significantly improved after 3 doses of anti-VEGF injections and maintained longitudinally as compared to groups 2 and 3. Lipid metabolising genes may impact the outcome of anti-VEGF AMD treatment.

Degenerative changes of macular photoreceptors (rod and cones) can lead to irreversible vision loss in aged population. Age related macular degeneration has been associated with 52 independent genetic variants and various environmental factors like smoking, age, food habits, comorbidities[1,2]. Recently, our data has also indicated that association of sleeping pattern and activities of daily living with AMD which can stimulate the pathological changes by modulating protein expression[3]. Despite growing knowledge of AMD genetics, not much advancement in treatment of AMD has been noted in the field. Currently, anti-VEGF injection is prescribed for wet AMD patients in order to offer symptomatic relief to increasing visual acuity[4]. However, current therapies for both dry (vitamin supplementations) wet AMD (anti-VEGF injection) have been reported to retard the photoreceptor degeneration. Short term safety of intravitreal bevacizumab with an average of 2–3 injections per 3 months with a maximum of 4 injections was also investigated[5]. This has shown significant improvement in retinal thickness, analyzed by OCT along for visual acuity[6]. Withdrawal of bevacizumab therapy has been found to enhance the chance of recurrence of wet AMD by 10% every successive year[7]. Dose Optimization and frequency of

[1]Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India. [2]Advanced Pediatrics Centre, Department of Pediatrics, Post Graduate Institute of Medical Education and Research, Chandigarh, India. [3]Advanced Eye Centre, Department of Ophthalmology, Post Graduate Institute of Medical Education and Research, Chandigarh, India. [4]Department of Statistics, Panjab University, Chandigarh, India. ✉email: mankoo95@yahoo.com; akshay1anand@rediffmail.com

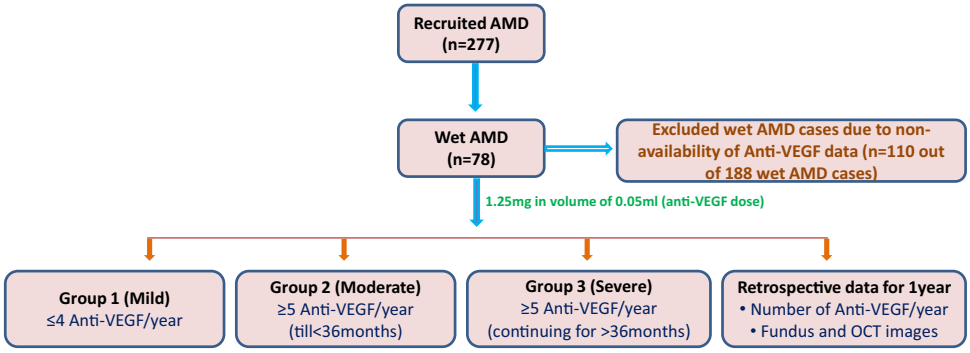


Figure 1. Schematic representation of groups categorised in study.

Anti-VEGF injection can be influenced by genetic variants and the interactions between them. Genetic variant of CCT3 gene rs12138564 has been coupled to improved outcome of anti-VEGF treatment. On the contrary, the results from same study have also revealed a decreasing anti-VEGF response under the influence of rare genetic variants of *C10orf88* and *UNC93B1* genes in wet AMD patients[8]. Our previous genetic investigation on genetics AMD on Indian patients has defined the biological significance of systemic inflammation[9–11], impaired angiogenic mechanism[12–14], oxidative stress[15] which showed TLR3 independent[16] aggravation of AMD pathology along with the substantial contribution of environmental factors. Exploring the genetic penetrance of rare and common genetic variants and their pathological implication under the influence of confounders can determine the genetic complexity and susceptibility of AMD[17] which can influence the disease phenotype and treatment outcome. This is suggestive of possible association of genetic variation and the influence of environmental factors (with or without interactions) which may modulate the outcome and number of anti-VEGF treatment in AMD patients which can contribute in AMD management. This study also describes the genetic susceptibility towards the response of Anti-VEGF treatment in Indian AMD patients.

Methodology

Recruitments of participants. The study population comprised of 277 patients with AMD recruited from Advanced Eye Centre, PGIMER, Chandigarh, India. Analysis of Anti-VEGF response was carried out on 78 cases of active wet AMD. Although the patients were recruited prospectively, the data of 11 patients was retrieved (from same recruited patients) retrospectively to examine the number of anti-VEGF injections given in a year. Moreover, the data of visual acuity was retrieved for 46 AMD cases out of a total of 78 wet AMD patients recruited in the study. The written informed consent was obtained from all the participants after explaining the nature of study. The experimental protocols were approved by Institute Ethical Committee (IEC) (No: PGI/IEC/2005-06; dated: 23.07.2013), PGIMER, Chandigarh, India. The study adhered to the study protocol and conducted as per the ethical guidelines laid down by Institute Ethical Committee, PGIMER, Chandigarh, India. The participants were also asked about the history of prescribed medication for any ailment along with AMD pathology. The socio-demographic (SD) details including smoking, alcohol consumption, and food habits (prior or current) etc. were also noted.

Treatment regimen of Anti-VEGF therapy. The details of a total number of anti-VEGF injections and an estimated duration of AMD pathology was obtained individually for each patient. Intravitreal Bevacizumab (1.25 mg/0.05 ml) was given to wet AMD patients. We categorised the wet AMD patients based on number of anti-VEGF injections given as described in Fig. 1. We administered three monthly doses of Bevacizumab followed by *pro re nata* (PRN) treatment. However, strict PRN could not be followed up in many patients owing to financial, and other logistic reasons in our part of the world.

Clinical details. Clinical severity and categorization of AMD was done by a retina specialist by recording the fluorescein fundus angiography (FFA) and optical coherence tomography (OCT) images. AREDS criteria were adopted to classify the AMD pathology in the population. Snellen's best corrected visual acuity (VA; US feet 20/20) data of 46 wet AMD patients out of the total of 78 AMD cases was collected at three time points including first (baseline), third and final visit of AMD patients along with the total visit (in months) made to the Department of Ophthalmology, PGIMER, Chandigarh. VA values were converted to logMAR scale and were considered for final data analysis. We did not take into the account the type of CNV (Classic or Occult) in our wet AMD patients. This is the limitation of our study.

Serum extraction. Blood sample of patients was collected in Sodium citrate vacutainer and kept at room temperature for 1–2 h. Samples were centrifuged at 1800 rpm for 20–30 min at room temperature. Upper layer sample (serum) were collected and stored in − 80 °C for further experimental uses.

Genomic DNA extraction. Genomic DNA from peripheral blood mononuclear cells (PBMCs) was extracted using commercially available kit (Qiagen, USA) to perform the SNP analysis. DNA was stored at − 20 °C till conducting the experiments.

Total protein estimation. Bradford's method was adopted to estimate the total protein levels in the patient's serum. Briefly, diluted serum (600 times) was mixed with diluted Bradford's reagent (1:4 ratio). Absorbance of the reaction was taken at 595 nm using ELISA reader (BioRad, USA).

Retrospective analysis. In order to understand the response of anti-VEGF injections in different AMD phenotypes, we retrieved the clinical data of AMD patients (n = 11) including the number of anti-VEGF shots and clinical images (both FFA and OCT) in 1 year of duration.

SNP analysis. Single nucleotide polymorphism (SNP) analysis was carried out for lipid metabolizing genes like LIPC (rs920915) and APOE (rs769449), pro-angiogenic genes including ADAMTS9 (rs6795735) and TIMP3 (rs5749482), regulatory genes *e.g.* B3GALTL (rs9542236), IER3 (rs3130783), HTRA1 (rs11200638) and SLC16A8 (rs8135665, monocarboxylic transporter protein). SNP analysis was carried out on StepOne real time PCR (Applied Biosysystems Inc., Foster city, CA) by using Taq Man assay (ThermoFisher, USA) as per the manufacturer's instruction. Briefly, genomic DNA (20 ng) and 5ul of Taqman master mix was taken in the 10 µl of total volume of reaction setup. FAM and VIC tagged probes, to discriminate the allelic variation in genome at particular site, was added to the reaction. Reaction without genomic DNA was considered as negative control. Analysis of raw data to demonstrate the allelic condition (homozygous dominant/recessive and heterozygous) was performed using *Genotyper* and *StepOne V2.0* softwares (Applied Biosysystems Inc., Foster city, CA).

ELISA. Serum levels of lipid metabolizing (APOE and LIPC), pro-angiogenic (TIMP-3 and ADAMTS9), regulatory (HTRA1, IER3 and B3GALTL) and monocarboxylic acid transporter (SLC16A8) proteins were estimated by commercially available ELISA kits (Qayee Biological Technology Co. Ltd., Shanghai, China). Serum samples were diluted before performing the experiments. The protocol was followed as per the manufacturer's instructions. Briefly, diluted serum samples were incubated with primary and secondary antibodies in dark at 37 °C for one hour. Washing was carried out 5 times, using 1X diluted washing buffer before adding the substrates to the reaction. Reaction was terminated by adding stop solution followed by estimation of absorbance at 450 nm in ELISA reader (BioRad, USA). The values were further neutralized with total protein levels for respective patients.

Statistical analysis. Comparative analysis of protein expression between various groups was estimated using One-way ANOVA, independent *T*-and Mann–Whitney tests. Pearson's chi square analysis was applied to reveal the association between number of anti-VEGF treatment and genotype frequency of various SNPs along with SD parameters. Logistic regression analysis was carried out to study the association of number of anti-VEGF shots and protein expression. Moreover, changes in protein expression with respect to single nucleotide polymorphism (for respective gene) were also analysed using contrast analysis with or without controlling anti-VEGF numbers. Wilcoxon sign-ranked test was employed to compare the changes in visual acuity of AMD patients throughout treatment regimen. Multivariate model analysis was performed to understand the effect of genotype interactions on anti-VEGF response (number of anti-VEGF injections per year). Survival curve was also generated for current data set in order to show direct relationship between number of anti-VEGF and progression of AMD pathology. Z-proportions test was applied to compare minor allele frequency (MAF) derived from GAW studies (INDEX-DB and IndiGenomes) conducted on Asian population with current study.

Results

Association of anti-VEGF injections with socio-demographic details. Results of *chi-square* suggest that alcohol addiction could be a modulator for anti-VEGF response in Indian AMD patients. Similarly, AMD patients with history of cataract surgery (single or both eyes cataract surgery) can also significantly alter the anti-VEGF response. Both results indicate the complex nature of AMD pathology where activities of daily living and associated ailment could act as a modifier for anti-VEGF response in AMD (Table 1).

Genotype influences anti-VEGF response in AMD pathology. Chi-square analysis has revealed a significant association of B3GALTL and LIPC variants with anti-VEGF response in Indian AMD patients. Results demonstrate that the frequency of homozygous 'CC' and heterozygous 'CT' of B3GALTL are more frequent in AMD patients, being moderate and non-responsive towards anti-VEGF response with context to number of injections given to the patients. Similarly, both homozygous 'CC' and heterozygous 'GC' genotypes of LIPC are also associated with number of injections given to AMD patients (Table 2). A complex nature of AMD pathology due to its heterogeneity and genetic interaction along with equal contribution of environmental factors has been widely investigated which has also been supported by our data. However, we did not find significant association of remaining genotypes with the number of anti-VEGF injections given to the wet AMD patients (Table S1).

Comparison of minor allele frequency derived from Asian GWAS studies. We have compared the minor allele frequencies (MAF) of studied genes with GWA studies conducted especially on Asian (INDEX-DB) and Indian (IndiGenomes) population by considering the fact of small sample size for final analysis in current

	Status	Avastin response			Total	P-value
		Mild	Moderate	Non-responsive		
Alcohol habit	Never	37	9	5	51	**0.024**
	Past	5	0	2	7	
	Current	8	8	1	17	
Total		50	17	8	75	
Cataract surgery	No surgery	29	8	3	40	**0.018**
	One eye surgery	22	8	3	33	
	Both eyes surgery	0	1	2	3	
Total		51	17	8	78	

Table 1. Association of anti-VEGF response (based on number of anti-VEGF injections given during the course of disease) with daily living habits (Socio-demographic details) of AMD patients including alcohol consumption and cataract surgery in AMD patients. Mild- < 4 Avastin/year; Moderate- ≥ 5 Avastin/year; Non-responsive- ≥ 5 Avastin/year and continuous for > 36 months.

	Genotypes	Anti-VEGF response			Total	P-value
		Mild	Moderate	Non-responsive		
B3GALTL Genotype (rs9542236)	Homozygous TT	32	6	2	40	0.033
	Homozygous CC	1	0	1	2	
	Heterozygous CT	13	9	2	24	
Total		46	15	5	66	
LIPC genotype (rs920915)	Homozygous GG	18	8	1	27	0.013
	Homozygous CC	0	2	2	4	
	Heterozygous GC	25	5	4	34	
Total		43	15	7	65	

Table 2. Association of genotypes of (Pearson's *Chi*-square) B3GALTL (rs9542236) and LIPC (rs920915) with number of anti-VEGF injections given to AMD patients to demonstrate the genetic susceptibility of both genes towards response of anti-VEGF treatment in AMD pathology. Mild- < 4 Avastin/year; Moderate- ≥ 5 Avastin/year; Non-responsive- ≥ 5 Avastin/year and continuous for > 36 months.

Genotype	Allele	MAF frequency current study	MAF from IndiGenome	MAF from INDEX-DB	P-value
B3GALTL (rs9542236)	C	28 (0.21)	0.18	NA	0.41
LIPC (rs920915)	G	88 (0.67)	0.73	NA	0.38
ADAMTS9 (rs6795735)	T	95 (0.73)	0.77	NA	0.49
APOE (rs769449)	A	9 (0.07)	0.08	0.083 (GnomAD)	0.71*
HTRA1 (rs11200638)	A	86 (0.67)	0.34	NA	< 0.001
TIMP3 (rs5749482)	C	12 (0.08)	0.15	NA	0.15
IER-3 (rs3130783)	A	111 (0.91)	0.91	NA	0.99
SLC16A8 (rs8135665)	T	34 (0.27)	0.19	NA	0.13

Table 3. Comparison of minor allele frequency derived from IndiGenome and INDEX-DB GWAS with current study. MAF: Minor allele frequency; *p-value based on comparison between IndiGenome and current study.

study. Results of Z-test proportions did not show significant alteration of MAF between IndiGenomes and current study except *HTRA1* (Table 3). Our study has indicated that response of anti-VEGF injections was found to be varied based on *LIPC* genotype and the level of APOE. We did not find frequencies of minor alleles of the studies genes in INDEX-DB except *APOE* gene which was found to be similar as frequency shown in IndiGenomes. However, references genomes from both studies haven't assessed the effect of different genotypes on anti-VEGF response or any kind of treatment strategies.

LIPC genotype influences protein expression. Associated genotypes of LIPC with anti-VEGF numbers have also been found to influence the majority of protein expression analysed in the study. We have demonstrated that homozygous 'CC' genotype of LIPC variant show enhanced expression of regulatory (HTRA1, B3GALTL and IER3), monocarboxylic transporter protein SLC16A8, and levels of LIPC itself. Moreover, sig-

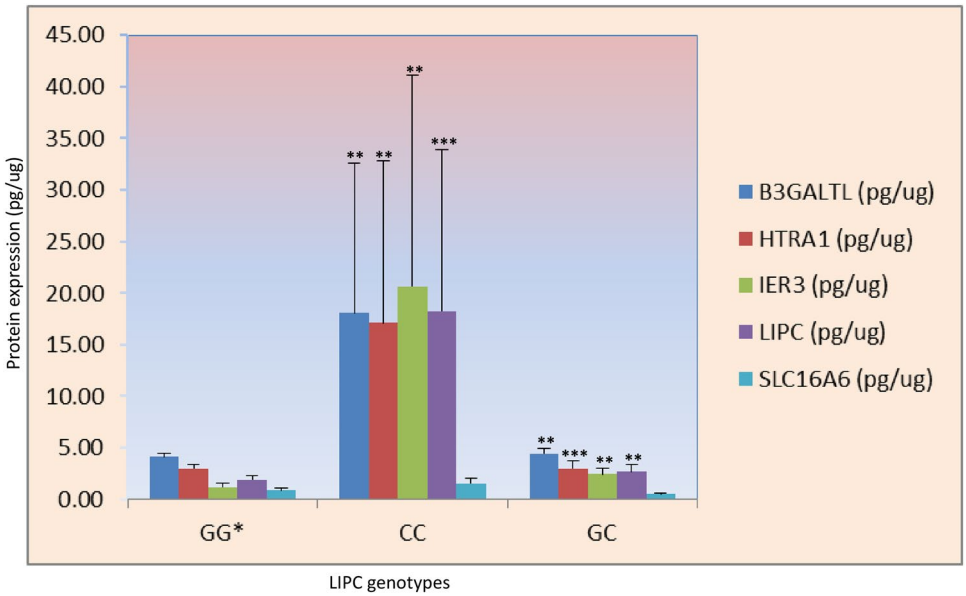


Figure 2. Impact of LIPC genotype on protein expression. Significant elevated expressions of B3GALTL, HTRA1, IER3 and LIPC were seen in 'CC' genotype of LIPC genetic variant (rs920915) as compared to both reference 'GG*' and heterozygous 'GC' alleles. *GG** Reference allele. Bar is representing SEM; $P < 0.05$.

Genotype	Genotypes	Significant genotypes+			After controlling Anti-VEGF numbers				
		Contrast estimate	SE	p-value	B	SE	t-value	p- value	95% CI
ADAMTS9 (pg/ug)	CC vs. TT*	−.358	4.585	.938	.030	.139	.213	0.83	−0.249–0.309
	CT vs. TT*	2.321	2.471	.352					
APOE(pg/ug)	AA vs. GG*	.001	.002	.732	0.00002	0.00006	.364	0.72	−0.0001–0.00015
B3GALTL (pg/ug)	CC vs. TT*	−4.770	7.311	.517	.062	.124	.499	0.62	−0.186–0.309
	CT vs. TT*	−2.260	1.910	.242					
HTRA1 (pg/ug)	AA vs. GG*	.512	2.168	.814	−.003	.098	−.030	0.98	−0.199– 0.193
	AG vs.GG*	−0.689	2.253	.786					
LIPC (pg/ug)	CC vs. GG*	17.578	3.972	< 0.0001	−.131	0.100	−1.314	0.19	−0.332–0.070
	CG vs. GG*	0.827	1.801	.648					
TIMP3 (pg/ug)	CC vs.GG*	0.011	0.011	.327	−0.0002	0.00048	−.320	0.75	−0.001–0.001
	GC vs. GG*								
IER-3 (pg/ug)	GG vs. AA*	2.045	3.834	.596	0.032	.153	.209	0.83	−0.277–0.341
	AG vs. AA*								
SLC16A8(pg/ug)	TT vs. CC*	−1.020	.638	.116	0.004	0.015	.285	0.77	−.025–0.034
	TC vs. CC*	−.410	.247	.103					

Table 4. Contrast estimate to see the impact of genotype and response of anti-VEGF in AMD. Contrast estimate indicates the significant of per unit change in genotype (nucleotide/polymorphism) from 'GG' (reference genotype) to 'CC' in LIPC genetic variant (rs920915) by alteration the LIPC levels (17.58 pg/unit changes). Alteration in expression levels with reference by changing in nucleotides ('GG' to 'CC') didn't show any alterations indicating the indirect implication of anti-VEGF injections in AMD pathology (by considering the anti-VEGF numbers as covariate).

nificant alteration of protein expression, including HTRA1, IER-3 and LIPC, has also been examined in heterozygous 'GC' genotype of LIPC variants (Fig. 2). However, we did not find significant alteration of proteins among B3GALTL genotypes which has also showed the association with number of anti-VEGF injection in AMD patients (Table 2). Similarly, the expression of studied proteins were not found to be significantly altered with reference to other genotypes except the SLC16A8 expression between 'AA' and 'GA' genotypes of HTRA1 (Table S2).

Additionally, contrast estimate indicated significant changes in LIPC levels by 17.578 pg/ug with alteration of genotype i.e. from 'GG (reference genotype)' to 'CC' genotype ($p = < 0.0001$) which is consistent with our previous results[15] (Table 4). Interestingly, we did not find any significant alteration for any other protein levels against the changes in genotypes (of studied variants) while considering anti-VEGF number as covariate. Results

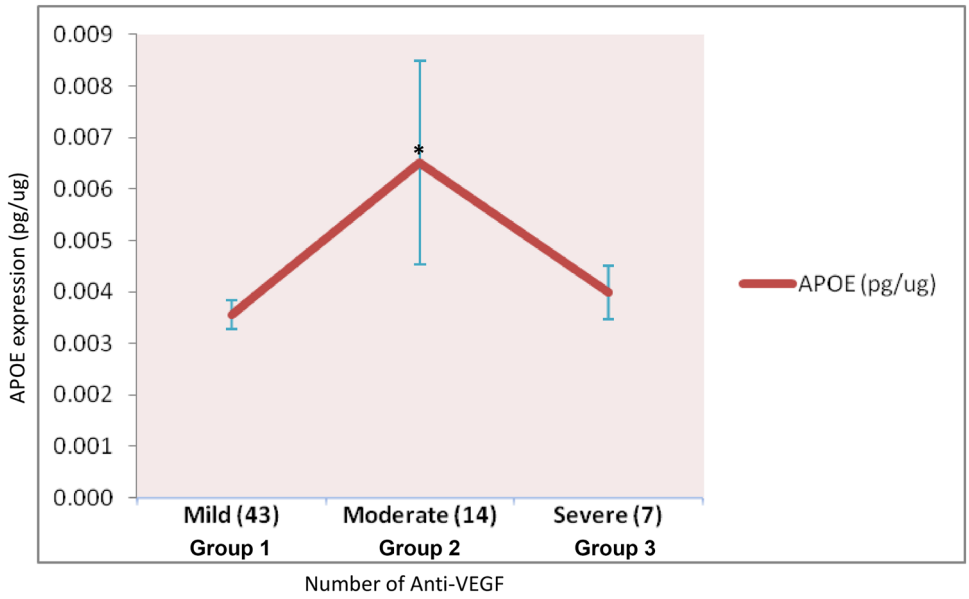


Figure 3. APOE expression in mild, moderate and severe groups of anti-VEGF response is based on the number of injections in wet AMD patients. Significantly higher levels of APOE were seen in moderate group as compared to mild group. Bar is representing SEM; $P < 0.05$.

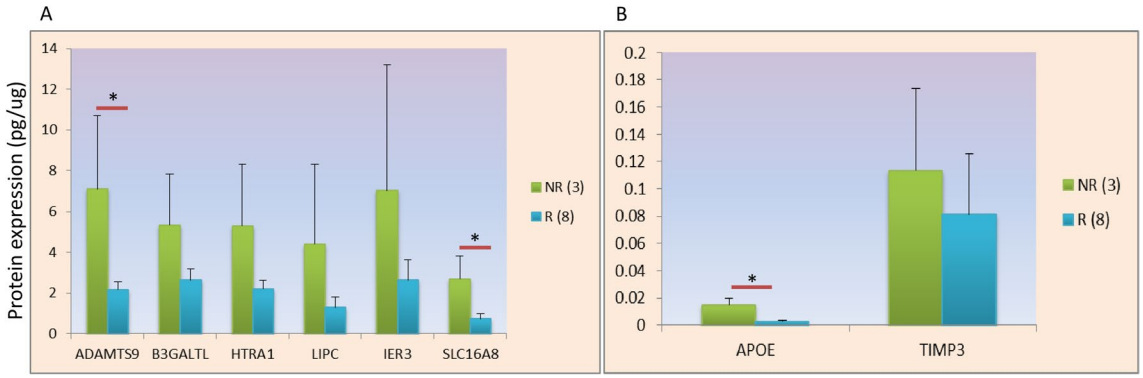


Figure 4. Differential expression of proteins in retrospectively group (Group 4). (**A**) Significant higher expression of ADAMTS9 and SLC16A8 in anti-VEGF non-responder (≥ 5 anti-VEGF injections/year), as compared to responders (≤ 4 anti-VEGF injections/year) in wet AMD patients. (**B**) APOE expression significantly higher in non-responder (≥ 5 anti-VEGF injections/year) for anti-VEGF AMD patients in comparison to responders (≤ 4 anti-VEGF injections per year). NR: non-responsive wet AMD for anti-VEGF treatment; R: responsive wet AMD for anti-VEGF treatment. Bar is representing SEM; $P < 0.05$.

show an indirect role of lipid metabolism by regulating the action of associated proteins (LIPC) in controlling the anti-VEG response. Results also signify the biological significance of particular genotype (of variants), genetic and allelic interactions under the influence of confounders which may influence the various protein expressions thereby modulating the AMD treatment outcome after anti-VEGF.

APOE mediated anti-VEGF response in AMD. Enhanced APOE levels with successive anti-VEGF injections (≥ 5 of per year) in AMD patients have suggested the APOE dependent anti-VEGF response in Indian AMD (Fig. 3). Significantly elevated expression of APOE has been observed in moderate group (group 2; ≥ 5 anti-VEGF injections/year and continuing for < 36 months) as compared to mild group (group 1; ≤ 4 anti-VEGF injections/year). Similarly, APOE levels were also found to be higher in severe group (group 1; ≥ 5 anti-VEGF/year and continuing for > 36 months) as compared to mild group of AMD, though it was not statistically significant. Results suggested that lipid metabolizing genes (especially APOE and LIPC) may modulate the action of anti-VEGF in AMD pathology.

To further validate the results suggesting the role of lipid metabolizing genes in anti-VEGF response, we assessed the scale of anti-VEGF injections given to AMD patients (for 11 AMD patients, Fig. 4). Pearson's correlation analysis has revealed the positive correlation between anti-VEGF treatment and expression of ADAMTS9 (PCC = 0.629; P = 0.020), APOE (PCC = 0.872; P = < 0.0001) and SLC16A8 (PCC = 0.656; P = 0.014). Response

Coefficients[a]							
	Unstandardized coefficients		Standardized coefficients			95.0% confidence interval for B	
Model	B	Std. error	Beta	t	P-value	Lower bound	Upper bound
Constant	.514	.423		1.215	0.255	− .443	1.470
APOE	251.530	47.041	.872	5.347	< 0.0001	145.116	357.945

Table 5. Logistic regression analysis to show the association of number of anti-VEGF injection on APOE expression in AMD pathology in retrospectively analyzed AMD patients. [a]Dependent variable: anti-VEGF number.

Group	Mean ± SD logMAR						P-Value						Average follow up (months)
	Baseline VA		VA after 3 injections		Final VA		Left 1st Vs 3rd	Right 1st Vs 3rd	Left 1st Vs final	Right 1st Vs final	Left third vs final	Right third vs final	
	Left eye	Right eye	Left eye	Right eye	Left eye	Right eye							
Group 1 (n = 35)	0.95 ± 0.60	0.97 ± 0.50	0.75 ± 0.58	0.82 ± 0.61	1.07 ± 0.68	1.0 ± 0.63	0.003	0.007	0.334	0.807	0.025	0.225	65
Group 2 (n = 7)	0.92 ± 0.53	0.56 ± 0.24	0.59 ± 0.30	0.75 ± 0.26	1.49 ± .52	1.49 ± 0.64	0.109	0.665	0.357	0.180	0.144	0.180	75
Group 3 (n = 4)	0.33 ± 0.23	0.63 ± 0.17	0.28 ± 0.31	0.39 ± 0.26	1.25 ± 0.46	1.82 ± 0.13	0.317	0.109	0.109	0.066	0.109	0.068	103

Table 6. Response of anti-VEGF treatment on visual acuity (logMAR) among different anti-VEGF groups of AMD patients (*i.e.* group 1, 2 and 3) and total follow-up (in months) during the course of disease.

of anti-VEGF treatment on AMD pathology in modulating the protein expression was further analysed and modelled by regression analysis to support the Pearson's correlation results. Adjusted *Cox* and *Snell's* R^2 values as 0.734 and 0.761, respectively were observed for logistic model. Regression analysis has demonstrated that APOE is significantly associated with anti-VEGF injections in a period of time (in one year) in Indian AMD pathology (Fig. 4 & Table 5). Results suggest that APOE and LIPC may act as chief modulator for anti-VEGF treatment in AMD patients.

When we compared the visual acuity data among studied groups, significant improvement of visual acuity from baseline was observed in group 1 AMD cases after three doses of anti-VEGF treatment as compared to group 2 and group 3. However, visual acuity was also improved in case of group 2 and 3 AMD cases after 3 doses of anti-VEGF treatment but it was non-significant. Number of anti—VEGF injections were further correlated with visual acuity (VA) of group-wise AMD patients along with their total follow up. Results have also shown that while comparing final visual acuity of group 2 and 3, AMD cases within group 1 worsened. Longitudinal follow-up of patients revealed more consistent results of visual acuity examined in group 1 AMD patients as compared to group 2 and group 3 (Table 6). This may require more anti-VEGF injections to stabilize the visual acuity as in case of group 2 and 3 in our results.

Influence of genetic interaction on anti-VEGF response. Our results have shown the role of lipid metabolizing genes in modulating anti-VEGF response in AMD pathology. Hence, we further attempted to assess the impact of genetic interaction on anti-VEGF response in AMD. The analysis of data revealed a significant genotype interaction among ADAMTS9-TIMP3 genes in AMD pathology. However, we did not find direct influence of genotype interaction on response of anti-VEGF treatment (in terms of number of injections given) and association with disease progression (Table 7). Results also suggest that studied SNP variants and their genetic interactions, especially among pro-angiogenic genotypes (ADAMTS9-TIPM3), may exacerbate the AMD pathology suggesting an indirect implication of the same on anti-VEGF response.

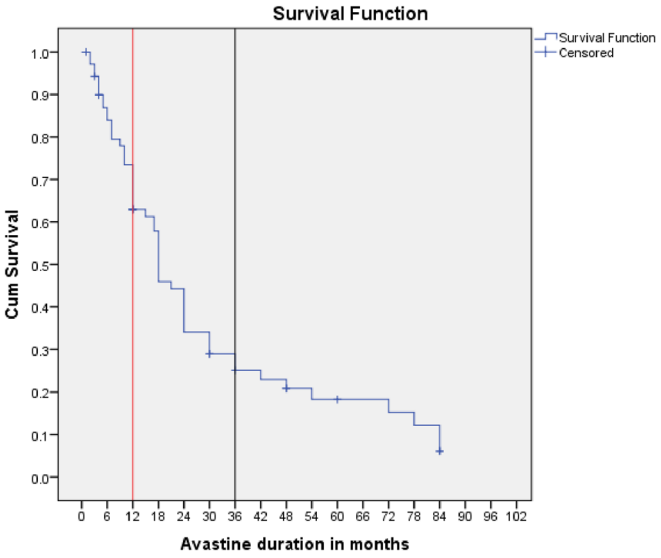
We wanted to examine the progress of disease in patients as with the duration of disease (in months), such as the effect of anti-VEGF treatment, until the occurrence of the AMD pathology. For this purpose, survival analysis was performed and Kaplan–Meier survival curve revealed that at 12 months anti-VEGF treatment can provide 64% symptomatic recovery from AMD, while at 36 months, it was only 25% (Fig. 5). Subsequently, symptomatic relief from AMD by anti-VEGF treatment waned in patients receiving the successive anti-VEGF treatment with gradual increase in number of injections (anti-VEGF). This may be due to uncontrolled activity of lipid metabolizing proteins under the influence of confounders along with the genetic complexity of an individual[15]. Moreover, we have also determined the median survival time by locating the (time 'in months'), at which the cumulative survival proportion is 0.5. In our study, median survival rate due to the effect of anti-VEGF treatment is 18 months with standard error of 1.849 and confidence intervals (14. 38–21.63) (Fig. 5).

Multivariate tests							
Genotype interactions	**Effect**	**Test**	**Value**	**F**	**Hypothesis df**	**Error df**	**P-value**
B3GALTL (rs9542236) * LIPC (rs920915)	Intercept	Pillai's Trace	.342	9.875	2	38	< 0.0001
	Anti-VEGF number	Pillai's Trace	.056	1.137	2	38	.331
	B3GALTL genotype	Pillai's Trace	.011	.103	4	78	.981
	LIPC genotype	Pillai's Trace	.475	6.078	4	78	< 0.0001
	B3GALTL * LIPC genotype	Pillai's Trace	.051	1.014	2	38	.372
APOE (rs769449) * HTRA1 (rs11200638)	Intercept	Wilks' Lambda	.260	58.273	2	41	< 0.0001
	Anti-VEGF number	Wilks' Lambda	.943	1.246	2	41	.298
	APOE genotype	Wilks' Lambda	.810	4.795	2	41	.013
	HTRA1 genotype	Wilks' Lambda	.781	2.695	4	82	.036
	APOE * HTRA1	Wilks' Lambda	.835	1.938	4	82	.112
Pro-angiogenic genotype interaction ADAMTS9 (rs6795735) * TIMP3 (rs5749482)	Intercept	Pillai's Trace	.698	46.138	2	40	< 0.0001
	Anti-VEGF number	Pillai's Trace	.006	.127	2	40	.881
	ADAMTS9 Genotype	Pillai's Trace	.408	5.260	4	82	.001
	TIMP3 genotype	Pillai's Trace	.370	11.751	2	40	< 0.0001
	ADAMTS9 * TIMP3 genotype	Pillai's Trace	.480	6.466	4	82	< 0.0001
Regulatory genotype interaction HTRA1 (rs11200638) * IER3 (rs3130783)	Intercept	Pillai's Trace	.189	4.090	2	35	.025
	Anti-VEGF number	Pillai's Trace	.035	.640	2	35	.533
	HTRA1 genotype	Pillai's Trace	.071	.666	4	72	.618
	IER3 genotype	Pillai's Trace	.002	.028	2	35	.972
	HTRA1 * IER3 genotype	Pillai's Trace	.033	.596	2	35	.557
Cellular function SLC16A8 (rs8135665) * B3GALTL (rs9542236)	Intercept	Pillai's Trace	.100	2.271	2	41	.116
	Anti-VEGF number	Pillai's Trace	.008	.175	2	41	.840
	SLC16A8 genotype	Pillai's Trace	.091	.998	4	84	.413
	B3GALTL	Pillai's Trace	.086	.941	4	84	.445
	SLC16A8 * B3GALTL	Pillai's Trace	.007	.146	2	41	.864
Lipid metabolizing APOE (rs769449) * LIPC (rs920915)	Intercept	Pillai's Trace	.324	8.871	2	37	.001
	Anti-VEGF number	Pillai's Trace	.013	.249	2	37	.781
	APOE genotype	Pillai's Trace	.006	.112	2	37	.895
	LIPC genotype	Pillai's Trace	.057	.553	4	76	.697
	APOE * LIPC	Pillai's Trace	.078	1.575	2	37	.221

Table 7. Multivariate analysis to demonstrate genotype interaction of studied SNPs (based on their cellular functions) and influence of anti-VEGF treatment on AMD pathology. Results showed significant genotype interaction of pro-angiogenic genes including ADAMTS9 (rs6795735) and TIMP3 (rs5749482), but didn't show direct influence of genotype interactions on number of anti-VEGF injections in Indian AMD patients.

Discussion

The need for personalized medicine cannot be emphasised unless the genetics and nature of interactions with genetic variants and environmental factors well understood which acts as a roadblock towards translational approach in AMD genetics[18]. This study has attempted to understand the unique outcome of anti-VEGF treatment under the influence of confounders and genetic variants. We have shown the outcome of anti-VEGF (in context to number of injections given during the disease course) associated with both environmental (alcohol consumption and cataract history) and genetic factors (genetic variants of B3GALTL and LIPC). Poor response of Aflibercept has also been observed with higher BMI and geographic atrophy AMD patients[19]. Aqueous humor levels of angiogenic and pro-angiogenic proteins including VEGF-A, VEGF-C, interleukin 8, endothelin 1, HGF (Hepatocyte growth factor), HB-EGF (Heparin-binding epidermal growth factor-like growth factor), follistatin, and angiopoietin 2 were also found to be elevated after intravitreal injection of bevacizumab[20]. ATG haplotype of rs699947 (− 2578 C/A), rs2010963 (+ 405 C/G) and rs3025039 (+ 936 C/T) SNPs has been earlier shown to be associated with 'poor' responder of intravitreal bevacizumab in Tunisian AMD Patients[21]. Our results suggest that VEGF could be a potential identifier for anti-VEGF response by considering the lipid metabolizing genes as a modifier (especially APOE and LIPC) which is consistent with our previous report in the field[12]. Recently, TT genotype of CFH genetic variant (Y402H) was shown to increase the function and response of intravitreal ranibizumab in AMD patients[22]. Interestingly, a significant alteration in LIPC (lipid metabolizing), TIMP-3 (angiogenic) and SLC16A8 (monocarboxylic transporter) was observed in CFH negative AMD cases[23]. Our results have also revealed the association of genetic variants of B3GALTL and LIPC with the number of anti-VEGF injections in Indian AMD patients. Moreover, we also found a significant differential expression of B3GALTL, HTRA1, IER3 and LIPC proteins among subgroups of LIPC genotype. Genetic interaction of various genotypes can also influence the outcome of anti-VEGF treatment in AMD pathology. We have demonstrated



Means and Medians for Survival Time							
Mean[a]				Median			
		95% Confidence Interval				95% Confidence Interval	
Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
29.606	3.526	22.695	36.516	18.000	1.849	14.376	21.624

[a]Estimation is limited to the largest survival time if it is censored.

Figure 5. Survival curve to demonstrate the symptomatic recovery in wet AMD patients after treating with anti-VEGF injections during the course of disease.

a significant interaction between pro-angiogenic ADAMTS9-TIMP3 genotypes. However, we did not find significant association between number of anti-VEGF injections and such genetic interaction studied in our population. This indicate a complex nature of AMD pathology and associated response of anti-VEGF treatment which can be dependent on the nature of genetic interaction along with contribution of confounders[24]. Moreover, our results have also showed that both APOE and LIPC may act as biomarkers to differentiate degree of anti-VEGF response in wet AMD cases with respect to number of anti-VEGF injection given to the patients. The treatment strategy for lipid metabolism (by targeting APOE and/or LIPC) along with anti-VEGF may be a crucial step for effective management of AMD. Results of visual acuity and changes VA after anti-VEGF treatment have suggested the group 1 as a responder in comparison to group 2 and 3 where anti-VEGF treatment did not lead to significant changes in VA (especially after 3 doses of anti-VEGF injections). Out results of visual acuity and number of anti-VEGF injections have further supported the hypothesis of current study which indicates subsequent changes in number of anti-VEGF injections (or response) and visual acuity outcome based on genetic susceptibility of AMD patient.

Conclusively, results indicate the prominent biological significance of lipid metabolizing molecules (including APOE and LIPC) which may influence the anti-VEGF outcome in AMD patients. Impact of genetic variants and their interaction cannot be ignored in modulating the anti-VEGF response which must be considered for redefining the management of AMD pathology. However, conclusion of this study was drawn on limited number of samples along with number of anti-VEGF injections. Visual acuity of anti-VEGF treated groups has also suggested that group 1 AMD patients (≤ 4 anti-VEGF injections/year) respond to anti-VEGF treatment and showed more persistent visual acuity as compared to group 2 (≥ 5 anti-VEGF injections/year till < 36 months) and 3 (≥ 5 anti-VEGF injections/year for > 36 months). Final visual acuity of group 2 and 3 have further deteriorated than group 1 AMD cases indicating the longitudinal implication of genetic susceptibility (especially through LIPC and APOE) and response towards anti-VGEF treatment (also the number of anti-VEGF injections). This study could serve as substrate to design larger study on geographically diverse range of population based on their genetic susceptibility, genetic interactions, penetrance and influence of environmental factors.

Data availability

Whole data can be provided by first and corresponding authors of the manuscript without any restriction whenever required.

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Author contributions

K.S.: Execution of experiments, data acquisition, co-conceptualization, analysis and writing of manuscript; P.B.: Writing of the manuscript, retrieving of clinical data; R.S.: Clinical analysis and investigation of patients, and editing of manuscript; S.K.S.: Data analysis and editing of the manuscript; A.A.: PI, acquired funding, conceptualization and editing of the manuscript.

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Competing interests

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Correspondence and requests for materials should be addressed to R.S. or A.A.

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RESEARCH ARTICLE

Serum Levels of ARMS2, COL8A1, RAD51B, and VEGF and their Correlations in Age-related Macular Degeneration

Priya Battu[1], Kaushal Sharma[1], Manjari Rain[1], Ramandeep Singh[2] and Akshay Anand[1,*]

[1]*Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India;* [2]*Advanced Eye Centre, Post Graduate Institute of Medical Education and Research, Chandigarh, India*

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Abstract: ***Background*:** Many factors including genetic and environmental are responsible for the incidence of Age-related Macular Degeneration (AMD). However, its pathogenesis has not been clearly elucidated yet.

***Objective*:** This study aimed to estimate the Age-Related Maculopathy Susceptibility 2 (ARMS2), Collagen type VIII Alpha 1 chain (COL8A1), Rad 51 paralog(RAD51B), and Vascular Endothelial Growth Factor (VEGF) protein levels in serum of AMD and control participants and to further investigate their correlation to understand AMD pathogenesis.

***Methods*:** For this case-control study, 31 healthy control and 57 AMD patients were recruited from Advanced Eye Centre, Post Graduate Institute of Medical Education and Research, Chandigarh, India. A blood sample was taken and serum was isolated from it. ELISA (enzyme-linked immunosorbent assay) was used for the estimation of proteins in the serum of patients.

***Results*:** ARMS2 and COL8A1 levels were significantly elevated in the AMD group than in the control group. The highest levels of ARMS2, COL8A1, and VEGF proteins were recorded for the wet AMD sub-group. The study results endorsed significant positive correlation between these following molecules; ARMS2 and COL8A1 ($r = 0.933$, $p < 0.0001$), ARMS2 and RAD51B ($r = 0.704$, $p < 0.0001$), ARMS2 and VEGF ($r = 0.925$, $p < 0.0001$), COL8A1 and RAD51B ($r = 0.736$, $p < 0.0001$), COL8A1 and VEGF ($r = 0.879$, $p < 0.0001$), and RAD51B and VEGF ($r = 0.691$, $p < 0.0001$).

***Conclusion*:** The ARMS2 and COL8A1 levels were significantly higher and RAD51B was significantly lower in the AMD group than controls. Also, a significant statistical correlation was detected between these molecules, indicating that their interaction may be involved in the pathogenesis of AMD.

Keywords: Age-related macular degeneration, ARMS2, COL8A1, RAD51B, VEGF, ELISA.

1. INTRODUCTION

Age-Related Macular Degeneration (AMD) is a degenerative disorder of the central retina, leading to the loss of photoreceptors and decreased visual acuity. AMD is a multifactorial disease influenced by environmental and genetic factors [1]; however, its pathophysiology has not been understood clearly [2]. It has been categorized phenotypically into dry and wet forms. In dry form, the mounds of lipoprotein along with complement factors and oxidized pigments accumulate in sub-retinal spaces called drusen, leading to Retinal Pigment Epithelium (RPE) cell death. In the wet form, Choroidal Neovascularization (CNV) advances, and the new fragile blood vessels arise from underlying choroid which infiltrates and leaks their contents into the sub-retinal spaces. Such actions are followed by photoreceptor cell death and disturbed integrity of the RPE monolayer. The advanced form of AMD causes vision loss in the elderly [3]. Many studies have linked genetic variants of biomolecules Age-Related Maculopathy Susceptibility 2 (ARMS2), Collagen VIII(COL8A1), Rad 51 paralog (RAD51B), Vascular Endothelial Growth Factor (VEGF), and others with AMD susceptibility [4]. The serum levels of these proteins could be associated with AMD incidence [5, 6]. The change in serum levels further supports genetically regulated biomolecule involvement and suggests their physiological significance in AMD pathogenesis. ARMS2 is a protein of the extracellular matrix of the choroid. The variants in the corresponding locus are associated with AMD but its function has not been explored yet [7, 8]. Deficiency of ARMS2 due to insertion-deletion variant might lead to accumulation of drusen by inhibiting clearance of cellular debris mediated by complement system [9]. Similarly, COL8A1 is another extracellular protein, which is a part of the Descemet membrane and is required

*Address correspondence to this author at the Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India; Tel: +911722756094;
E-mail: akshay1anand@rediffmail.com

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for normal anterior eye development [10]. In contrast, RAD51B is a protein necessary for DNA repair and maintenance of chromosomal integrity [11]. Its role is suggested in recombinational related repair [12]. Variants in COL8A1 and RAD51B are associated with the risk of neovascular AMD development [13]. Another protein VEGF has also been widely explored in AMD and has been implicated in vasculogenesis and angiogenesis, which have been associated with many cancers and ocular diseases [14]. Intravitreal anti-VEGF is generally given as a treatment of a wet form of AMD [15, 16]. It has been demonstrated that VEGF inhibition decreases local complement factor H (CFH)and other complement proteins in the eye *via* reduced VEGFR2/PKC-α/CREB signaling [17]. The studies mentioned earlier show the involvement of these four proteins in AMD; however, the synergistic role of ARMS2, COL8A1, and RAD51B under the influence of VEGF has not been explored yet, to understand the pathological role in AMD. In this study, we have attempted to investigate the expression of mentioned proteins in AMD. This could be beneficial to target AMD pathology more precisely by modulating the treatment strategy accordingly and also examined if there is any correlation between the levels of these proteins in AMD patients and whether they constitute a pathway.

Table 1. Age and gender distribution in the study groups and subgroups.

Group	Age (Years) *Mean (SD)*	P-value	P-value *(Among the Three Sub-groups of AMD)*	Gender *(Male and Females)*	P -value`
Control	57 (9.61)	Reference	-	M=22 F=9	Reference
AMD	68 (8.77)	<0.0001		M=36 F=21	0.460
Wet	70(8.48)	<0.0001	0.192	M=10 F=8	0.274
Dry	64 (8.10)	0.011		M=11 F=4	1.000
Dry/Wet	69 (9.08)	<0.0001		M=11 F=7	0.478

Age is represented as mean ± standard deviation. Age differences were analyzed by an independent sample T-test between AMD *vs.* control group and AMD *vs.* control subgroups. Gender differences were tested by SISA statistics two by two tables. Pearson's p-values were reported for sample size more than 5 and Fischer t-test values were taken for sample size less than 5. ANOVA was applied to evaluate the age difference between the subgroups. The significance was observed at $p \leq 0.05$. Here, for 6 AMD patients, subgroup analysis could not be done as for them subgroup diagnosis was not apparent.
Abbreviations: AMD, Age-related macular degeneration; F, females; M, males.

2. MATERIALS AND METHODS

2.1. Subject Recruitment

This is a case-control study conducted by recruiting 88 participants consisting of 31 healthy controls (age ≥ 50 years) and 57 AMD patients(age ≥ 50 years). They were recruited from January 2018 to May 2019 at the Advanced Eye Center, Post Graduate Institute of Medical Education and Research after obtaining Institutional Ethical Committee approval. All the participants identified themselves as North Indians. Research subjects signed the informed consents and voluntarily agreed to participate in the study. Following the study's exclusion criteria, patients with diabetic retinopathy, uveitis, myopia, and conditions resembling AMD features such as Adult Vitelliform Macular Dystrophy were excluded from the study. A detailed proforma was filled for collecting socio-demographic details of the patients.

Fluorescein angiography and spectral-domain Optical Computed Tomography (OCT) were used to diagnose and classify AMD. Patients were divided into three categories based on phenotypical characteristics such as dry AMD(unilateral/bilateral), wet AMD (unilateral/bilateral), and dry/wet AMD defined by dry in one eye and wet in another.

The age and gender details of the study groups are summarized in Table **1**.

2.2. Serum Isolation

For isolation of serum, 4 ml of blood was collected in clot activator vacutainer (BD, USA) and kept at room temperature for 30 min. The clotted blood was centrifuged at 3000 rpm for 30 min (REMI, India) to separate serum as the top layer. Serum was transferred to microcentrifuge tubes and stored at -80°C until further use.

2.3. Assessment of ARMS2, COL8A1, RAD51B, and VEGF Levels in Serum of AMD Patients

The serum levels of ARMS2, COL8A1, RAD51B, and VEGF were estimated using commercially available kits (Qayee-Bio, China). The experiments were performed as per the manufacturer's instructions for the estimation of proteins in serum. Standardization for sample dilution was carried out before conducting the experiment. Standards were done in duplicates and random duplicates were put for samples. The absorbance reading was taken at 450 nm on the ELISA reader (Bio-Rad Laboratories, USA). The values obtained by ELISA were normalized later to the respective total protein concentration. Each sample's ELISA value was divided by the total protein value for the respective sample for the four biomolecules.

2.4. Total Protein Estimation

Bradford's method was used for total protein estimation. Standard concentrations were prepared from Bovine serum albumin with 2X dilutions ranging from 6.25 to 1000 µg/ml. 10 µl of standard and serum samples were loaded into the 96 well glass plate followed by the addition of 200 µl of Bradford reagent (Sigma, USA) pre-diluted with autoclaved water in 1:4 dilution. Samples were mixed thoroughly by tapping and incubated at room temperature for 10 minutes. Absorbance was measured at 595 nm using the ELISA reader

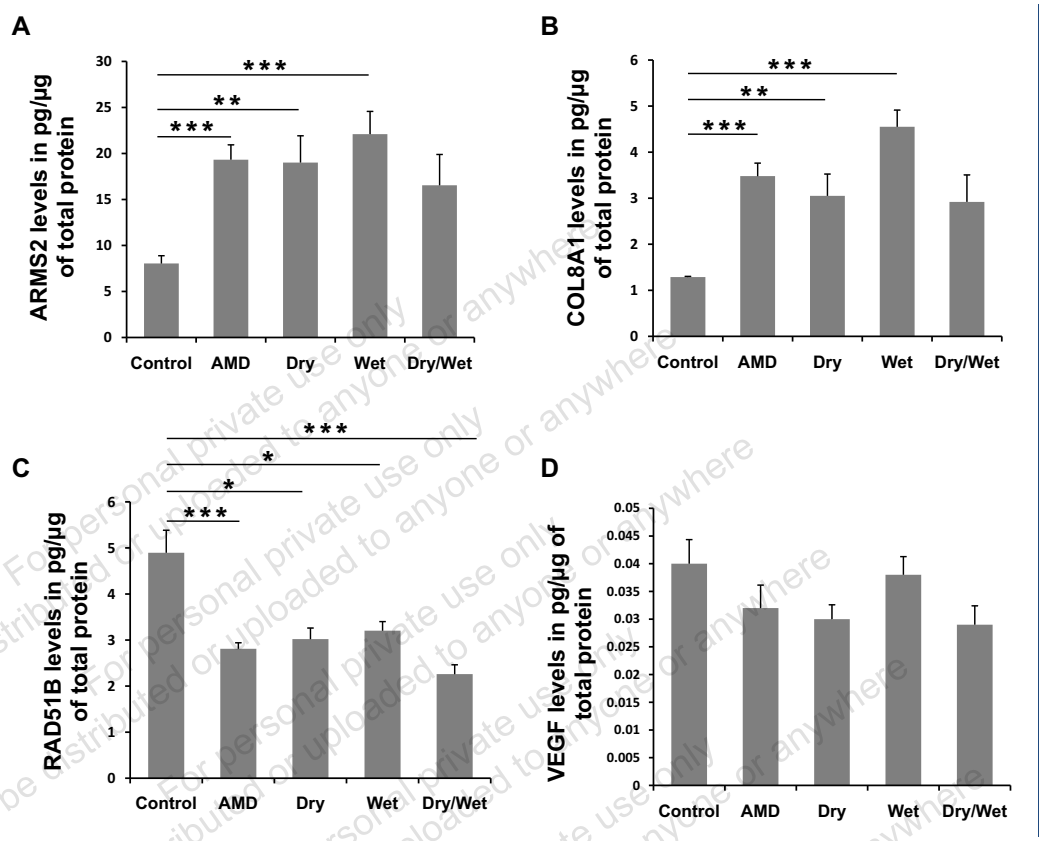

Fig. (1). Estimation of protein concentration by ELISA. (**A**) ARMS2 levels were significantly higher in the AMD group than in the control group. Also, its level was significantly higher in wet and dry subgroups than in the control group. (**B**) COL8A1 levels were also higher in the AMD group than in the control group. The levels were higher in wet and dry subgroups than control group (**C**). RAD51B levels were significantly lower in AMD and AMD sub-groups than control group (**D**). VEGF levels were lower in AMD patients but it was non-significant. The mean values were reported for the proteins after normalization with total protein counts. Normality was checked by the Shapiro-Wilk test. Mann-Whitney U test was used to estimate the difference of protein concentrations between the groups. The significance was observed at $p \leq 0.05$. The * represents $p \leq 0.01$-0.05, ** represents $p \leq 0.001$-0.01, and *** represents $p \leq 0.001$. The levels of ARMS2 and COL8A1 were significantly higher, and RAD51B was significantly lower in the AMD group as compared to controls, suggesting the role of these proteins in AMD which should be further investigated. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Bio-Rad Laboratories, USA). Serum samples were assayed in triplicates and the average value was taken into consideration for normalization of ELISA counts.

2.5. Statistical Analysis

Statistical analysis was performed on SPSS 21.0 (SPSS Inc., USA) and SISA (https://www.quantitativeskills.com/sisa/). Normality was checked by the Shapiro-Wilk test. Mann-Whitney U test was used to estimate the difference of protein concentrations between the groups. Age differences were analyzed by independent sample T-test between AMD *vs.* control group and AMD sub-groups *vs.* control group. Gender differences were tested by SISA statistics two by two tables. Pearson's p-values were reported for the sample size of more than 5 and Fischer t-test values were reported for sample size less than 5. The significance was observed at $p \leq 0.05$. Spearman's rho coefficient was used to check the correlation between the biomarkers, as applicable. Correlation data were significant at $p \leq 0.01$ after Bonferroni's correction ($p = 0.05/6$). ANOVA was used to evaluate the age difference between the subgroups. STRING 11.0 was used to predict the relationship among the proteins.

3. RESULTS

ARMS2 levels were significantly higher in the AMD group (19.32 ± 12.16 pg/ µg) as compared to the control group (8.04 ± 4.62 pg/µg). The ARMS2 levels were significantly higher in dry (19.01 ± 11.25 pg/µg) and wet subgroup (22.10 ± 10.45 pg/µg) as compared to controls. Hence, the highest levels of ARMS2 were observed in the wet group (Fig. **1A**, Table **2**).

COL8A1 levels were found to be significantly higher in AMD (3.48 ± 2.10 pg/µg) as compared to the control group (1.28 ± 0.80 pg/µg). The COL8A1 levels were significantly higher in two AMD sub-groups, dry (3.05 ± 1.84 pg/µg) and wet (4.55 ± 1.46 pg/µg), as compared to controls, with the highest being in wet AMD (Fig. **1B**, Table **2**).

Table 2. Serum protein concentrations of biomarkers in two study groups and three subgroups.

Protein	Control	AMD	Wet	Dry	Dry/wet
ARMS2, pg/ µg	8.04 ± 4.62	19.32 ± 12.16	22.10 ± 10.45	19.01 ± 11.25	16.54 ± 14.20
COL8A1, pg/ µg	1.28 ± 0.80	3.48 ± 2.10	4.55 ± 1.46	3.05 ± 1.84	2.92 ± 2.48
RAD51B, pg/ µg	4.89 ± 2.67	2.810 ± 0.98	3.20 ± 0.84	3.02 ± 0.94	2.26 ± 0.86
VEGF, pg/ µg	0.040 ± 0.023	0.032 ± 0.031	0.038 ± 0.014	0.030 ± 0.01	0.029 ± 0.015

Data are represented as mean ± standard deviation. ELISA was used to estimate the concentration of proteins in serum. The mean values were reported for the proteins after normalization with total protein counts.

Abbreviations: AMD, Age-related macular degeneration; ARMS2, Age-Related Maculopathy Susceptibility 2, COL8A1 Collagen type VIII Alpha 1 chain; RAD51B, Rad 51 paralog; VEGF, Vascular Endothelial Growth Factor.

RAD51B levels were significantly decreased in the AMD group (2.81 ± .98 pg/µg) as compared to the control group (4.89 ± 2.67 pg/µg). Similarly, the levels were significantly less in AMD sub-groups *i.e.* dry(3.02 ± 0.94 pg/µg), dry/wet (2.26 ± .86 pg/µg), and wet (3.20 ± .84 pg/ µg) subgroup when compared to control (Fig. **1C**, Table **2**).

No significant difference was observed in VEGF levels in AMD group (0.032 ± 0.031 pg/µg) and sub-groups *i.e.* dry (0.030 ± 0.01 pg/µg), wet (0.038 ± 0.014 pg/µg) and dry/wet (0.029 ± 0.015 pg/µg), and were compared with control (0.040 ± 0.023 pg/µg). However, the highest level of VEGF was recorded for the wet sub-group amongst the subgroups (Fig. **1D**, Table **2**).

We also found significant correlations between these proteins in AMD group (Fig. **2A**-**F**). Positive correlation was found between ARMS2 and COL8A1 (r = 0.933, p<0.0001), ARMS2 and RAD51B (r = 0.704, p<0.0001), ARMS2 and VEGF (r = 0.925, p<0.0001), COL8A1 and RAD51B (r = 0.736, p<0.0001), COL8A1 and VEGF (r = 0.879, p<0.0001), and RAD51B and VEGF (r = 0.691, p<0.0001), and these proteins were also found to be positively correlated in AMD subgroup (Tables **3**-**5**).

We also observed that these proteins were positively correlated to each other in the control group (Table **6**), including ARMS2 and COL8A1 (r = 0.707, p<0.0001), ARMS2 and RAD51B (r = 0.907, p<0.0001), ARMS2 and VEGF (r = 0.972, p<0.0001), COL8A1 and RAD51B (r = 0.710, p<0.0001), COL8A1 and VEGF(r = 0.683, p<0.0001), and RAD51B and VEGF (r = 0.872, p<0.0001), The results indicate that these proteins are positively correlated in both controls and AMD patients and are associated with the pathophysiology of AMD.

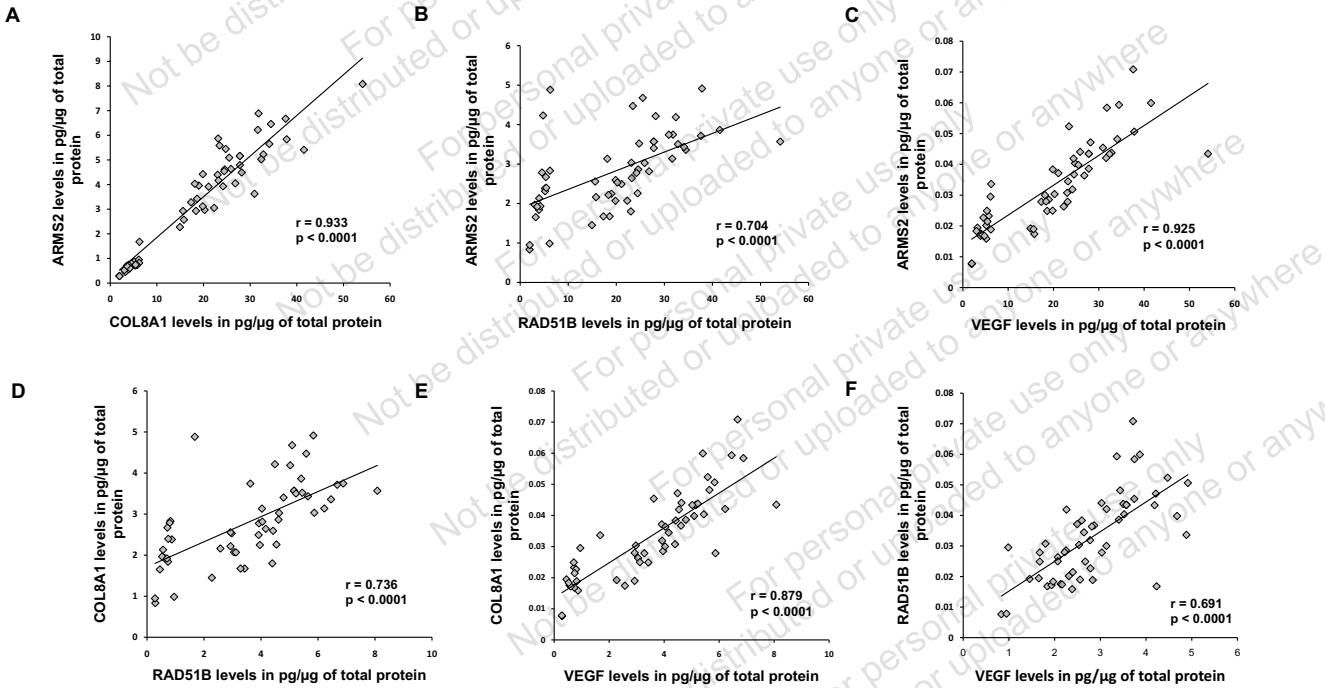

Fig. (2). Correlation of serum protein concentration of ARMS2, COL8A1, RAD51B, and VEGF in AMD group. (**A**) ARMS2 and COL8A1, (**B**) ARMS 2 and RAD51B, (**C**) ARMS2 and VEGF, (**D**) COL8A1 andRAD51B, (**E**) COL8A1 and VEGF, and (**F**) RAD51B and VEGF. Pearson's correlation was performed for RAD51 B and VEGF, and for the rest of the correlations, Spearman's correlation was used. Correlation data were significant at p≤0.01 after Bonferroni's correction (p=0.05/6). All the molecules were significantly correlated with each other in the AMD group, suggesting the involvement of these proteins in the pathophysiology of AMD. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 3. Correlations among biomarkers in wet AMD.

-	ARMS2	COL8A1	RAD51B	VEGF
ARMS2	r = 1	-	-	-
COL8A1	r = 0.703* p = 0.002	r = 1	-	-
RAD51B	r = 0.088 p = 0.727	r = 0.543* p = 0.030	r = 1	-
VEGF	r = 0.850 p<0.0001	r = 0.661* p = 0.005	r = 0.391 p = 0.109	r = 1

Spearman's correlation was performed for correlations marked with '*', whereas for the rest of the tests, Pearson's correlation was performed. Correlation data were significant at p≤0.01 after Bonferroni's correction (p=0.05/6). A strong positive correlation was observed for COL8A1 and ARMS2, ARMS2 and VEGF, RAD51B and COL8A1, and COL8A1 and VEGF.Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; p, p-value; r, Spearman's/Pearson's correlation coefficient; RAD51B, Rad 51 paralog; VEGF, Vascular Endothelial Growth Factor.

Table 4. Correlations among biomarkers in dry AMD.

-	ARMS2	COL8A1	RAD51B	VEGF
ARMS2	r = 1	-	-	-
COL8A1	r = 0.896* p<0.0001	r = 1	-	-
RAD51B	r = 0.785 p<0.001	r = 0.864* p<0.0001	r = 1	-
VEGF	r = 0.965 p<0.0001	r = 0.882* p<0.0001	r = 0.878 p<0.0001	r = 1

Spearman's correlation was performed for correlations marked with '*', whereas for the rest of the tests, Pearson's correlation was performed. Correlation data were significant at p≤0.01 after Bonferroni's correction (p=0.05/6). A strong positive correlation was observed for all the molecules. Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; p, p-value; r, Spearman's/Pearson's correlation coefficient; RAD51B, Rad 51 paralog; VEGF, Vascular Endothelial Growth Factor.

Table 5. Correlations among biomarkers in dry/ wet AMD.

-	ARMS2	COL8A1	RAD51B	VEGF
ARMS2	r = 1	-	-	-
COL8A1	r = 0.988* p<0.0001	r = 1	-	-
RAD51B	r = 0.781* p<0.0001	r = 0 .779* p<0.0001	r = 1	-
VEGF	r = 0.890* p=<0.0001	r = 0.868* p=<0.0001	r = 0.796 p=<0.0001	r = 1

Spearman's correlation was performed for correlations marked with '*', whereas for the rest of the tests, Pearson's correlation was performed. Correlation data were significant at p≤0.01 after Bonferroni's correction (p=0.05/6). A strong positive correlation was observed for all the molecules. Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; p, p-value; r, Spearman's/Pearson's correlation coefficient; RAD51B, Rad 51 paralog; VEGF, Vascular Endothelial Growth Factor.

Table 6. Correlation among the proteins in the control group.

-	ARMS2	COL8A1	RAD51B	VEGF
ARMS2	r = 1	-	-	-
COL8A1	r = 0.707* p = <0.0001	r = 1	-	-
RAD51B	r = 0.907* p = <0.0001	r = 0.710* p = <0.0001	r = 1	-
VEGF	r = 0.972* p = <0.0001	r = 0.683* p = <0.0001	r = 0.872 p = <0.0001	r = 1

Spearman's correlation was performed for correlations marked with '*', whereas for the rest of the tests, Pearson's correlation was performed. Correlation data were significant at p≤0.01 after Bonferroni's correction (p=0.05/6). A strong positive correlation was observed for all the molecules. Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; p, p-value; r, Spearman's/Pearson's correlation coefficient RAD51B, Rad 51 paralog; VEGF, Vascular Endothelial Growth Factor.

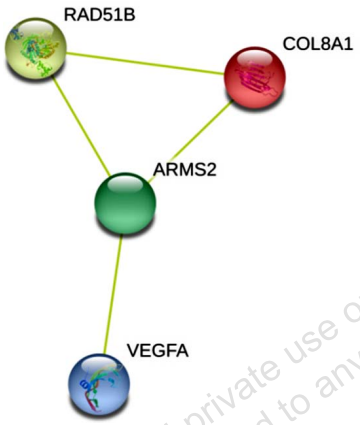

Fig. (3). Interaction among the four proteins shows 'text mining, represented by green interconnections. Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; RAD51B, Rad 51 paralog; VEGFA/VEGF, Vascular Endothelial Growth Factor. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

An inter-relation was also predicted among these proteins by STRING 11.0 at the level of text mining (Fig. **3**).

4. DISCUSSION

The present study reports the serum expression of ARMS2, COL8A1, RAD51B, and VEGF and their correlation in AMD. We found that expressions of ARMS2 and COL8A1 were up-regulated in AMD patients with the highest expression in wet AMD as compared to healthy controls. Although RAD51B was significantly lower in AMD than healthy controls, an increasing trend was observed in AMD when moving from dry to wet AMD. However, the data could not be adjusted for the difference in age and gender as the Mann-Whitney U test was used to evaluate the difference. All the four proteins were positively correlated in controls and AMD irrespective of the type of AMD, suggesting that these proteins are physiologically interrelated. Hence, their levels in AMD are dependent on the expression of each other or any common third factor, indicating a common pathway involved in AMD pathogenesis. STRING 11.0 could predict the relationship of these proteins from text mining, but it is not evident whether these proteins are co-expressed, co-existent, or possess homology.

VEGF, which is involved in neovascularization, is a well-known therapeutic target to treat CNV. Cumulative neutralization of VEGF and angiopoietin-2(ANG-2) has been shown to decrease CNV leakage, inflammation, and retinal cell damage, suggesting a non-redundant role of increased VEGF in neovascularization [18]. We observed that VEGF levels were significantly and positively correlated with COL8A1, RAD51B, and ARMS2. An increase in VEGF leads to CNV which may occur due to damage to the extracellular matrix. However, the response to anti-VEGF treatment is dependent on the genetic makeup of an individual [19]. The complex architecture of AMD has been demonstrated through various reports, which indicate an equal contribution of both genetic and environmental factors.

Since AMD involves photoreceptors (RPE) and Bruch's membrane, a complex pathway in its pathogenesis is expected. COL8A1 and ARMS2 are involved in the structural maintenance of the extracellular matrix. COL8A1 is involved in the integrity of Bruch's membrane and may contribute to drusen accumulation in AMD [20]. It also supports cell proliferation and invasion in cancer [21]; thus, increased COL8A1 might be implicated in the formation of CNV. Furthermore, ARMS2 interacts with several proteins present in the extracellular matrix, one of which is COL1A1 (Collagen type 1 alpha Chain), a significant component of Bruch's membrane. The AMD pathology might involve an increase in ARMS2 and COL1A1 in an interactive manner, thereby up-regulating angiogenic genes, including VEGF, *via* COL1A1 [22].

Cell survival is of importance in AMD as degeneration and death of photoreceptors and RPE are involved in geographic atrophy, associated with dry AMD. Moreover, the DNA damage response pathway has also been linked with stress due to ageing [23]. In this context, RAD51B gains importance by maintaining chromosomal integrity through DNA repair by homologous recombination repair [24]. We observed lower RAD51B in dry AMD and dry/wet AMD, where degeneration is prominent, and higher in wet AMD.

RAD51B brings about its function by forming a protein complex, called the BRCA1-associated genome surveillance complex with BRCA1 (breast cancer type 1 susceptibility protein), involved in genome stability and tumor suppression [25, 26]. BRCA1 also regulates endothelial function by suppressing VEGF [27-29]. We hypothesize that RAD51B is insufficient in AMD to form a protein complex with BRCA1 to bring about DNA repair. Thus, BRCA1 is available to interact with estrogen receptors, suppressing VEGF expression. Hence, it will be interesting to study BRCA1 association with RAD51B and VEGF in AMD pathophysiology.

CONCLUSION

The involvement of multiple pathways in AMD poses considerable obstruction in designing therapeutics for it [30]. The necessity of understanding AMD pathophysiology and uncovering new therapeutic targets had led to the present study. The limitations of this study are differences in the mean age of AMD and sample size. We have tried to diminish the effect of age difference by including participants above 50 years of age in both the groups and adjusting p-values for the age, wherever applicable. Studies with larger sample sizes and age-matched groups would further strengthen our findings. Moreover, including biomolecules such as BRCA1, COL1A1, and others in addition to ARMS2, COL8A1, RAD51B, and VEGF would provide useful leads to unravel the pathophysiology of AMD.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The approval was provided by the Institutional Ethical Committee of the Post Graduate Institute of Medical Education and Research (PGIMER), India (No: PGI/IEC/2005-06; dated: 23.07.2013) and INT/IEC/2019/000524.

HUMAN AND ANIMAL RIGHTS

No animals were used in the studies that are the basis of this research. All the human procedures were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

All the participants signed the informed consent and voluntarily agreed to participate in the study.

STANDARDS OF REPORTING

The study conforms to the STROBE guidelines.

AVAILABILITY OF DATA AND MATERIALS

The dataset that supports the results and findings of this research is available from the correspondence author, [AA], on reasonable request.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Role of Microglia and Astrocytes in Spinal Cord Injury Induced Neuropathic Pain

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Gurwattan S. Miranpuri[1], Parul Bali[2], Justyn Nguyen[1], Jason J. Kim[1], Shweta Modgil[3], Priya Mehra[3,4], Seah Buttar[1], Greta Brown[1], Noemi Yutuc[1], Harpreet Singh[1], Aleksandar Wood[1], Jagtar Singh[4], and Akshay Anand[3,5,6]

Abstract

Background: Spinal cord injuries incite varying degrees of symptoms in patients, ranging from weakness and incoordination to paralysis. Common amongst spinal cord injury (SCI) patients, neuropathic pain (NP) is a debilitating medical condition. Unfortunately, there remain many clinical impediments in treating NP because there is a lack of understanding regarding the mechanisms behind SCI-induced NP (SCINP). Given that more than 450,000 people in the United States alone suffer from SCI, it is unsatisfactory that current treatments yield poor results in alleviating and treating NP.

Summary: In this review, we briefly discussed the models of SCINP along with the mechanisms of NP progression. Further, current treatment modalities are herein explored for SCINP involving pharmacological interventions targeting glia cells and astrocytes.

Key message: The studies presented in this review provide insight for new directions regarding SCINP alleviation. Given the severity and incapacitating effects of SCINP, it is imperative to study the pathways involved and find new therapeutic targets in coordination with stem cell research, and to develop a new gold-standard in SCINP treatment.

Keywords

Spinal cord injury, Neuropathic pain, Microglia, Astrocytes, Matrix metalloproteinases, JNK, P2 receptors

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Introduction

The nature and extent of a spinal cord injury (SCI) is diverse and complicated. There are many symptoms, including, but not limited to, paralysis, myelopathy, and damage to white matter and grey matter. The complexity of injury is increased manifold as nerve fiber damage compromises sensation and motor signal transmittance to and from the brain, while grey matter damage results in segmental losses of interneurons. The utilization of corticosteroid (methylprednisolone sodium succinate), surgical interventions, and physiotherapy are the only methods for treatment in current health care, and these methods display limited success.[1] Yet, recent advances in the fields of stem cell biology have revolutionized neuroprotective and regenerative interventions.

Neuropathic pain (NP), because of its relatively unexplored molecular mechanism and widespread clinical morbidity, is extremely debilitating for SCI patients. In addition, NP is extremely resistant to treatment with current analgesic drugs, solidifying the necessity to find efficacious treatment options. Acknowledged in the current research are the emerging role of WNK1; cation-dependent chloride transporters (NKCC1) activation and inhibition by bumetanide[2,3]; cannabinoid receptor (CB2) and anti-hyperalgesia effect of WIN

[1] Department of Neurological Surgery, University of Wisconsin, School of Medicine and Public Health, Madison, Wisconsin, United States
[2] Department of Biological Sciences, Indian Institute of Science Education & Research Mohali, India
[3] Neuroscience research lab, Department of Neurology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India
[4] Department of Biotechnology, Panjab University, Chandigarh, India
[5] CCRYN- Collaborative Centre for Mind Body Intervention through Yoga
[6] Centre of Phenomenology and Cognitive Sciences, Panjab University, Chandigarh, India

Corresponding
Gurwattan S. Miranpuri, Department of Neurological Surgery, University of Wisconsin, School of Medicine and Public Health, Madison, Wisconsin WI 53792, United States.
E-mail: miranpuri@neurosurgery.wisc.edu

55,212-2[4]; bradykinin (B1) and vanilloid-1 (TRPV1) receptor antagonists[5,6]; and PPAR-gamma agonists in preventing neuropathic pain.[7] Time-specific changes in expression of matrix metalloproteinase-2 (MMP-2) in SCI-induced NP (SCINP)[8] and improved functional recovery with folic acid therapy has been found.[9] Likewise, bone marrow stromal cells (BMSCs) following lumbar puncture have shown some promising results in alleviating NP, including allodynia and hyperalgesia in chronic constriction injury (CCI) and spared nerve injury mice models.[10]

Delving further into NP research, glia-mediated inflammatory reactions have been found to play a pivotal role in the introduction and development of NP. Microglia plays a fundamental role in proliferation, differentiation, and synaptic hemichannel growth in neurons. They are also known to be involved in the regulation of infection in brain tissue through innate and adaptive immune responses and maintaining homeostasis, respectively. Because of its major role in the neuroinflammatory process for neurodegenerative diseases, the study and utilization of microglia awakened from its relative dormancy.[11] A noteworthy discussion on microglial cells history[12] reveals their origin, differentiation, homeostasis, and implication in health and disease. For example, microglia have been recognized to have a critical role in Alzheimer's, Parkinson's, and Adrenoleukodystrophy.[13]

Another glial cell type involved in providing neuroprotection is spinal cord astrocytes that release astrocytic mediators, for example, cytokines, chemokines, and growth factors for this purpose.[14] Unfortunately, the mechanism behind how astrocytes release astrocytic mediators is unclear, due in part to the lack of research on astrocytes because of their complexity in differentiation and seeding. Although astrocytic connexin-43 is implicated in gap junctions and communication of cytosolic contents via glial syncytia and to the extracellular space, the mechanism for this contribution remains unclear. Despite this, many studies have implicated astrocytes in facilitating or maintaining NP.

Studying molecular mechanisms,[15] discovered in the murine nerve injury model, MMPs activate and sustain NP. MMP-9 induces NP through interleukin-1beta cleavage and microglia activation at the acute stage. Similarly, latent stage MMP-2 maintained NP through the continuation of interleukin-1beta cleavage, though instead activating astrocytes.[16] Tissue inhibitors of MMPs (TIMPs) inhibit the activity of MMPs by regulating tissue proteolysis. As discussed, earlier, microglia and astrocytes help in tissue repair and breakdown in CNS. Therefore, studying the role of these glial cells in relation to NP may provide new insights into NP treatments.[17]

Role of Glial Cells in Neuropathic Pain

Earlier pain induced after SCI was thought to be a result of anatomical, neurochemical, and excitotoxic alterations with changes in ion channels.[18–23] However, the current treatment modalities targeting neuronal activity by modifying the ion channels proved to be inadequate.[24] The focus was then shifted to neuron dysfunction, which until recently was thought to arise from neuroimmune modulation. Recent studies have made it evident that NP development involves complex mechanisms involving not only neuronal cells but also glial cells. These modulations are mainly contributed by the resident glial cells of the spinal cord. Astroglia and microglia are main cells attributing to inflammatory response and have been implicated in pain induction after injury.[25] SCI is followed by extensive neuroinflammation because of activation of these glial cells releasing chemokines and cytokines.[26] Thus, targeting neuroinflammation may open new treatment avenues for management of NP.[27]

Implication of Microglia in Spinal Cord Injury-Induced Neuropathic Pain

Microglia play an important role in the maintenance and health of the CNS and are known to aide in neuronal differentiation and in the production of synaptic bonds. Microglia develop early in the embryonic yolk sac, contribute to brain development, and continue their work much into adulthood. The role of microglia in peripheral pain is well studied, and activation of these cells after partial sciatic nerve ligation,[28] spinal nerve ligation,[29] and sciatic nerve inflammation[30] have been found. Fractalkine, a microglial activator, induces allodynia and hyperalgesia shown by behavioral parameters.[31] Like peripheral injury, microglial activation has been reported in SCI [32–35,23] and showed that the microglia shift from resting to an activated state in rats undergone T9 spinal cord contusion injury. These activated microglia contribute to chronic pain induction and maintenance after SCI. Microglial response at different timepoints determined that activation plateaued between two and four weeks after injury. In spinal nerve injury models, hyperactive microglia were found to increase the levels of P2X4 receptors. The expression was specific to microglia cells, while neuron and astrocytes remained unaffected.[36] P2X4 are purinergic receptors, and ATP is a known mediator of NP; therefore, it was suggested that upregulation of these receptors is linked to NP induction. However, the mechanism was not fully understood. Later, it was demonstrated that these receptors on stimulation lead to Brain-derived Neurotropic Factor (BDNF) secretion from activated microglia.[37] It was further found to affect NMDA receptors' NR1 subunit in spinal cord dorsal horn neurons, which results in pain. This was supported by the studies in P2X4-deficient mice that, after peripheral nerve injury induction, display impaired BDNF signaling and lack hyperalgesia.[38] Another molecule, CCL21, was shown to rapidly express in injured sensory neurons. Further investigations in CCL21-deficit mice established the link between CCL21 and microglial P2X4. It was found that these deficit mice lack allodynia and reported with lower P2X4 expression.[39] Later it was

demonstrated that a wide range of purinergic receptors were activated by ATP in response to nerve injury that led to microglial activation and subsequently NP (Multiple PY2). Molecular mechanism, such as those involving MAPK, ERK, and p38, have been implicated in signal transduction from microglial activation. For example, ROCK activation in spinal microglia has been shown with p38 MAPK activation and induction of NP.[40] Thus, it can be suggested that spinal microglia play a role in NP induction and may be explored for their use as potential targets for chronic pain treatment.

Long-term pain sensation is maintained by neuronal-glia interactions. Early phase and chronic phase are separately maintained by different mechanisms. Mice with nerve injury had persistent microglia activation for more than three months in the spinal cord. It was seen that microglia involvement is far beyond the cytokine and chemokine signaling that last for a limited period. Chronic inflammation by microglia, when targeted with immunotoxin Mac1-saporin, helps in pain reversal.[41] Evidently, microglia are strongly tied to inducing NP through MMP-9 activation in the spinal cord. The activation and deactivation of microglia through MMP-9 injection and inhibition has been demonstrated.[15] As an example, NAC attenuated remifentanil-induced postoperative hyperalgesia via inhibiting the cleavage of IL-1β, a substrate of MMP-9 in DRG, significantly inhibiting glial activation and neuron excitability in the spinal dorsal horn.[42]

Implication of Astrocytes in Spinal Cord Injury-Induced Neuropathic Pain

Astrocytes are cells lining the neurons and are involved in neuroinflammation by activating astrogliosis. Like microglia, long-lasting changes in astrocytes have been observed in in-vivo models of SCI. Astrogliosis is associated with development and NP persistence. Various mechanisms have been proposed through which astroglia contribute in NP. Astrocyte-related markers such as glial fibrillary acidic protein (GFAP) and aquaporin 4 are elevated in SCI rats.[34] Upregulated levels of GFAP and -p38 MAPK were also reported below and at level of injury, further supporting astrogliosis role in pain (Carlton et al. 2009).[43] Elevation of Connexin-43 (CX-43), a gap junction protein, points toward increased connectivity between adjacent astrocytes.[44] Wang and Xu[45] discussed the role and association of Cx-43 and pannexin channels in SCINP, and they concluded that CX-43 is an emerging therapeutic target for NP. Studies have also shown that these effects are a result of upregulation of Tropomyosin-related kinase B.T1 (TrkB.T1)-driven astrocytes (Figure1). TrkB.T1 is an isoform of TrkB receptors that are expressed on astrocytes and an increased number of TrkB.T1^{+} cells after injury were reported that sustained through eight weeks.[46] In concordance with this, TrkB.T1 deletion in astrocytes led to reduced astrocyte proliferation in thoracic contusion SCI and improved hind limb withdrawal threshold.[47] Role of chemokines in activation of astrocytes has also been implicated in establishment of NP. Animals with spinal nerve ligation (SNL) have elevated levels of chemokines. CXCL13 has been shown to activate spinal astrocyte via another chemokine CXCR5. CXCR5-/-mice demonstrated lack of NP following SNL as CXCR5 was essential for activation of glial cells. Thus, neuron-astrocyte interaction lead to CXCL13 production by neuron cells that further activates astrocytes through CXCR5, inducing NP.[48] c-Jun N-terminal kinase (JNK)/monocyte chemoattractant protein-1 (MCP-1) pathway in astrocytes is also found to involved in NP development.[49] A separate study[50] bridges the role of both JNK1/2 and MMPs in NP where inhibition of astrocyte activation in the spinal cord by tetramethylpyrazine (TMP) prevented CCI-induced neuroinflammation. These findings demonstrate implication of JNK-MMP-2/9 in attenuating NP. In a mouse CPIP model, phosphorylated c-jun N-terminal kinase 1/2 (pJNK1/2) is downstream of spinal MMP-2. The MMP-2 inhibitor reversed the increase of glial fibrillary acidic protein (GFAP), the astrocyte biomarker, and pJNK1/2 on day three post injury.[51] The findings in this study include (a) increase in GFAP, but no significant effect on ionized calcium binding adaptor molecule 1, IBA1, a reactive microglial biomarker; (b) inhibition of astrocytes with fluorocitrate, but no inhibition of microglia with minocycline results in attenuation of allodynia in injured mice correlated with enhanced spinal levels pJNK1/2; (c) pJNK1/2 inhibitor, SP600125, showed decline in allodynia in injured mice; (d) increased expression levels of spinal MMP2, mainly NeuN a neuron biomarker; and (e) intrathecal administration of MMP-2 inhibitor, APR 100, resulting in delayed allodynia and decreased spinal levels of GFAP and pJNK1/2. Recent studies have also indicated that neuroinflammation plays a vital role in the occurrence and promotion of NP and that anti-inflammatory therapy has the potential to relieve the pain.[14] Thus, both microglia and astrocytes are linked to NP induction. In the next section, we will review studies that targeted these cells to ameliorate NP.

Glial Cells as Therapeutic Targets

Microglial Targets for Alleviating NP

Regulating microglia activity is thought to be a possible approach in impeding chronic pain progression. As discussed, activated microglia through production of various immuno-inflammatory molecules contributes to a state of chronic pain. These molecules lead to the activation of intracellular cascades in microglia cells generating and sustaining chronic pain.[11] Earlier studies with the goal of managing pain have explored the neuropathic roles of microglia and pharmacological interventions targeting activation of these cells.

Inhibiting p38 MAPK

Decades earlier, the glia cell-modifying functions of drugs like fluorocitrate and propentofylline had been shown to

reduce pain sensitivity.[52,53] These drugs however did not distinguish glial cell types and which cells are responsible for pain sensitivity. Mounting evidence demonstrates the activation of microglia cells as exhibited by elevated levels of markers such as CD 11b and Iba 1. Phosphorylated p38 MAP kinase expression was also another characteristic feature of SCI and is believed to be another key regulator of NP. It was reported that p38MAPK was activated only in microglia after SCI. With the activation of p38, microglia produce proNGF via the p38MAPK-mediated pathway. When Minocycline was administered, it showed significant reduction in proNGF levels. The reduction was mediated by the inhibition of the p38 MAPK phosphorylation by the drug. Maintaining its levels using inhibitors has been shown to ameliorate NP.[54] Inhibitors of p38 have shown remarkable efficacy in reducing pain. SB203580, p38 inhibitor, has shown promising results in SNL-induced allodynia[29,36] while FR167653 or CNI-1493 has been reported to prevent NP in different neuropathy modes.[55,31] FR167653, another inhibitor of p38, reverses allodynia in SNL model for almost 6 h after single injection of 50 mg/kg, i.p. (Intra-peritoneal). Multiple injections of FR167653 maintained pain reception for two days after the last injection. Propentofylline given in nerve injury showed NP reversal by microglial response inhibition.[56] Likewise, efficacy of p38 inhibitor, SB203580 given intrathecally was seen only when provided before seven days of injury.[57]

Altering Expression of Microglia Through Purinergic Receptors

ATP modulates microglial activity and is a ligand of the P2-purinoceptor family. Various P2 receptor subtypes such as P2X4, P2X7, and P2Y12 are expressed on microglia and are known to play a role in NP development. It has been shown that $P2Y_{12,}$ metabotropic purinergic receptor is linked to activated microglia and decreased expression of $P2Y_{12}$, decreasing progression of NP.[58] Administration of P2X1-4 receptor antagonist, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) reverses tactile allodynia, while pyridoxal phosphate6-azophenyl-2′,4′-disulphonoic acid (PPAD), which inhibits P2X1–3, 5, 7 receptors without affecting P2X4 receptors, does not respond to tactile allodynia. This suggests that P2X4 receptor activation is required in pain stimulation. Antisense oligonucleotide against P2X4 receptors showed attenuated pain in nerve injury (Tsuda et al., 2003). Similar results were seen in genetic models with deleted P2rx4 gene.[38] These receptors are believed to stimulate pain induction through calcium influx and BDNF release[59], further supported by experiments on P2X4 receptor-deficient mice showing impaired microglial BDNF expression.[38] Furthermore, mice deficient in P2X4 receptor that had undergone peripheral nerve injury showed no sign of mechanical allodynia strengthening BDNF and P2X4 association with NP.[60–62]

Pain sensitivity reduction in P2X7 receptor-deficient mice indicates a role of these receptors in NP.[63] Pharmacological blockade using N-(adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl) methyl]-2-chloro-benzamide[64] or A-839977[65] has shown promising results. Likewise, Perez-Medrano et al., 2009 found positive effects of various cyanoguanidine antagonists of receptor for pain reduction. P2X7 receptor effects are mediated through the convergence to p38 MAPK signaling. However, while in P2X4 receptor-signaling, BDNF is a key molecule, P2X7 receptor signaling is regulated by the release of interleukin-1β and cathepsin S from microglia.[66] Apart from P2X receptors, microglia are known to have a wide range of P2Y receptors such as P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12.[67–70] P2Y12 receptor is involved in eliciting pain sensitization.[57] Administration of 2Me-SADP, P2Y12 receptor agonist, mimics similar pain behavior in rats as nerve-injured animals.[58] Moreover, genetic manipulation of P2ry12 gene or suppression of expression through antisense oligonucleotides prevents mechanical allodynia.[58,71] Thus, it can be implicated that purinergic receptor mediated pain induction is an upstream process that converges to p38 MAPK signaling cascade.

Inhibition of Expression of Matrix Metalloproteinase/Induction of TIMPs Through Microglia

In peripheral nerve injury, Chattopadhyay[72] reported a dramatic increase of MMP-9. It was found that this upregulation of MMP-9 was linked with proinflammatory cytokines: TNF-α and IL-1β. Deletion of MMP-9 gene leads to significant reduction in NP, which further suggests the role of MMP in pain induction.[72] This opens a new avenue for NP management by MMP targeting. Mice with chronic constrictive injury (CCI)-induced NP, when treated with N-acetyl-cysteine (NAC), showed reduction in NP via a mechanism of MMP inhibition.[16] Both in vitro and in vivo experiments showed suppression of the activity of MMP9 and MMP2. NAC blocked the maturation of interleukin-1β, a substrate of MMPs, inhibiting the phosphorylation of protein kinase Cγ, NMDAR1, and mitogen-activated protein kinases. In this mechanism, NAC inhibited microglia activation but with no effect on astrocytes, thus demonstrating a safe and effective approach via strong inhibition of MMPS. TIMPs are endogenous inhibitors of MMPs[73] comprising of four inhibitors: TIMP1, TIMP2, TIMP3, and TIMP4.[74] Of these, TIMP1 and TIMP2 have specifically reported to alleviate the pain behavior by inhibiting MMP 2 and MMP 9.[15,75] Evidence of other small molecule inhibitors of MMPs that are employed to reduce the NP are reviewed.[76]

Other Microglial Targets in Alleviation of NP

Although proliferation of microglia is correlated with NP, candidate molecules for this activation of spinal microglia remain elusive. A recent study reported that peripheral nerve injury induces microglial proliferation and pain through de novo expression of colony-stimulating factor 1 (CSF1) sensory neurons.[77] It was discovered that CSF1 binds to CSF1

receptors in microglia, which then activates and increases proliferation of microglia. Furthermore, they found that microglial membrane adapter protein, DAP12, is downstream of CSF1, which induces pain.[76] Likewise, recombinant Macrophage-CSF injection in rats induced microglial proliferation and development of mechanical allodynia, suggesting the role of M-CSF as a candidate molecule for induction of microglia proliferation.[78] Furthermore, Platelet-activating factor (PAF)/PAFr signaling has been implicated in peripheral nerve injury in spinal cord signaling. Autocrine or paracrine effects of PAF among the activated microglia and neurons have been shown in NP induction.[79] A recent study demonstrated that partial sciatic nerve ligation-induced pain can be attenuated by deficiency of lysophosphatidyl choline acyl transferase 2 (LPCAT2), a PAF biosynthetic enzyme.[80] Explored mechanisms exhibited LPCAT2 in wild-type spinal cord microglia, with no expression of LPCAT2 in LPCAT2-KO mice reduced spinal PAF expression. Also, pretreatment with PAF receptor antagonist ABT-491 showed a decline in ATP-stimulated PAF biosynthesis in macrophages designated as PAF–pain loop, thus demonstrating a novel therapeutic target that may lead to alleviation of NP.[80]

Astrocytes Targets for Alleviating NP

Microglia have long been implicated in NP, unlike astrocyte involvement, which is still a new concept. Astrocyte activation is dependent on interaction between these cells and neurons, as well as release of factors from both cell types**.** In CPIP animal model, fluorocitrate inhibited activation of astrocyte in mice and attenuated the development of allodynia in them, while minocycline (microglia inhibitor) could not show the same effects. Thus, drugs targeting astrocytes are being explored for NP regulation rather than microglial pathway.[51]

Astrocytic Glutamine-Signaling

Glutamate transporters are involved in pathological pain induction, and spinal astrocyte glutamate transporters are downregulated in pain. This aspect is also explored for possible therapy in chronic pain. In vivo imaging in rodent models has reported the role of metabotropic glutamate receptor five (mGluR5) signaling in S1 astroglia (Figure 1). Activation of this pathway induces allodynia, which is reversed by blocking the signaling.[81] Adenoviral infusion of GLT1 gene results in overexpression in astrocytes. Spinal GLT-1 gene transfer prevented the induction of partial sciatic nerve ligation–induced allodynia.[69] Consistent with these findings, riluzole inhibited NP by reducing extracellular glutamate by EAATs.[82] Moreover, ceftriaxone also prevented allodynia by selectively upregulating GLT-1 expression.[83] Taken together, it can be implicated that decreased astrocytic excitatory amino acid transporter in the spinal cord led to activation of glutamatergic synaptic pathway related to pathological pain. Propentofylline increases GLT-1 and GLAST expression and suppresses pain symptoms.[84] The roles of GLT-1 and GLAST are further supported by the use of amitriptyline, a first-line drug for the NP treatment, which is shown to reverse the GLT-1 and GLAST downregulation.[85]

Blocking JNK Signaling in Astrocytes:

The studies related to translocator protein (TSPO) linked the activation of JNK signaling with chronic pain. Mouse model of spinal nerve ligation showed that Ro5-4864, TSPO agonist, reported reduced NP and that this was attributed to inhibition of astrocyte and p-JNK1 activation. Along with this, p-ERK was also reduced, suggesting the involvement of both JNK and ERK signaling.[86] Another JNK inhibitor, D-JNKI-1, ameliorated NP in SNL model of induced allodynia.[87] Later, MCP-1 was identified as main downstream molecule in JNK induced pain sensitization.[88] The association of JNK/MCP pathway was evident with the usage of drugs such as Tanshinone IIA (TIIAS) that targets this signaling and showed protection against NPin SNL animal models. Animals treated with TIIAS had elevated paw withdrawal threshold and reduction in astrocytic activation. Pathway analysis reported reduced JNK phosphorylation and MCP1 release in treated mice.[49] Fluorocitrate, an astrocyte inhibitor, provided protection in development of allodynia in CPIP-injured mice. This effect was suggested to be achieved through increased spinal levels of p-JNK.[51] SP600125 (JNK inhibitor) prevents the development of allodynia in the same mice model, and double immuno- staining showed colocalization of pJNK1/2 with GFAP, suggesting astrocytic involvement. Further studies revealed the involvement of matrix metalloproteinase-2 (MMP2) as they were upregulated. Intrathecal APR 100 (MMP-2 inhibitor) showed delayed development of allodynia with decreased levels of GFAP and pJNK1/2. This suggests the crosstalk between MMP2/JNK1/2 and MCP in astrocyte activation and pathogenesis of pain hypersensitivity.[51] Effectiveness of SP600125 in prevention of SNI and ddC-induced nociceptive behavior was also shown in another study where amitriptyline alone could not attenuate pain; however, when SP600125 was co-administered with amitriptyline, an antinociceptive effect was reported.[89] It was found that p-JNK was upregulated in SNI and ddC-exposed mice and that amitriptyline treatment further increased its expression. Additionally, it promoted astrocyte activation reported by elevated glial fibrillary acidic protein (GFAP) levels. Both JNK and glial activation was attenuated by co-administration of JNK inhibitor. Thus, it can be suggested that inhibiting astrocyte JNK activation exacerbates the amitriptyline analgesic response.[89] Modulating spinal astrocytes by targeting the JNK/MCP1 pathway can be an efficient approach against NP.

Other Astrocytes Targets for NP

Besides JNK and glutamate inhibitors, various other compounds have been tested for regulating astrogliosis in NP. One such compound is a well-known antioxidant, lycopene. Analgesic effects of lycopene were exhibited through prevention of Cx-43 protein downregulation.[90]

Lycopene treatment increased the expression of Cx-43 protein in spinal astrocyte cultures in a TNF-dependent manner, which was found to be reduced in the NP model. Similarly, when mice with a partial sciatic nerve ligation were treated with repeated doses of lycopene, it resulted in inhibition of down regulation of Cx-43 expression in the spinal cord dorsal horn (SCDH) along with reduction in mechanical hypersensitivity.[90] Pioglitazone, a PPAR**γ** agonist, was shown to impart analgesic effects by reducing astrocyte activation, while administration of PPAR**γ** antagonist GW9662 abolished these effects, suggesting involvement of PPAR**γ** mechanisms.[91] Similarly, the analgesic activity of docosahexaenoic acid (DHA, 22:6 n-3) was confirmed in a rat model of a chronic constriction injury (CCI) where treatment with DHA showed reduced GFAP-positive astrocyte in the SCDH and ameliorated NP.[92]

Future Directions

Although contemporary methods do not yield many results in the treatment of SCINP, glial cell-based therapies in SCI bring hope for the alleviation of symptoms and the development of a cure for NP. Because of the expansive research conducted for SCINP, there are many pathways that are available for targeting by current FDA-approved drugs and by other drugs that may still be in development. In a review,[93] it has been discussed that MMPs and TIMPs should be the primary focus of clinical studies because of their extensive studies and high implication of their function in NP. In addition to MMP-2/9, JNK-1/2, astrocytes, and microglia have been found to have a critical role in NP. Many studies have shown the specific roles these tissue types play in NP, and thus it is paramount to create a treatment of NP through targeting astrocytes and microglia. Studies have shown the effects of targeting each cell type independently, but there were no studies that demonstrated the treatment of both types of cells simultaneously. This raises the possibility of a dual treatment resulting in an additive effect, thus increasing the effectiveness and efficiency of the elimination of NP. Further studies should assess the effectiveness of a dual approach of treatment of astrocytes and microglia. Coupled with that, there are efficient methods to produce specific cell types such as astrocytes and microglia from stem cells to be utilized in both study and treatment. Thus, it may be time for this method to be utilized in a clinical setting as relevant human in-vitro studies and animal model have demonstrated exceptional promise. Furthermore, because of the lack of clinical relevance of translational animal models, it is advised that labs should consider the exploration of other models, such as the WMS™ model–relevant as a model for human spinal cord pathology and practical use as a platform for developing therapeutic delivery technologies.[94]

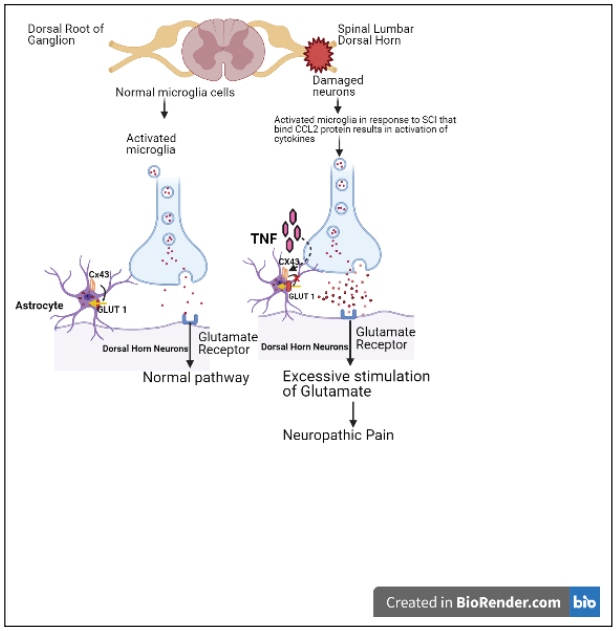

Figure 1. Schematic Representation of Spinal Cord Injury Causes Neuropathic Pain Induces by Elevation of Inflammatory Marker and Cytokines.

Source: Author [Priya Mehra; The schematic was designed using biorender software].

Abbreviations

CX43, connexin; TNF, Tumor Necrosis Factor; GLUT1, Glutamate receptor 1.
NAC, N-Acetyl-Cysteine;
DRG, Dorsal Root Ganglia;
FDA, Food and Drug Administrations;
CXCL, C-X-C Motif Chemokine 13;
CXCR, C-X-C Chemokine Receptor;
NMDAR1, N-Methyl-D-Aspartate Receptors 1;
CPIP, Chronic Post-Ischemia Pain;
GLAST, Glutamate–Aspartate Transporter;
CNS, Central Nervous System;
ATP, Adenosine Triphosphate
NMDA, N-methyl D-aspartate;
NR, Hetero-Oligomeric Proteins Receptor Subunits;
CCL, Chemokine (C-C Motif) Ligand;
MAPK, Mitogen-Activated Protein Kinases;
ERK, Extracellular-Signal-Regulated Kinase;
ROCK, Rho-Associated Protein Kinase

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Authors' Contribution

All the authors contributed to concepts, definition of intellectual content, literature search, manuscript editing, and review.

Statement of Ethics

The paper reflects the authors' analysis in a truthful and complete manner. The paper properly credits the meaningful contributions of co-authors and co-researchers. This material is the authors' own original work. The paper has not been previously published. All sources used are properly credited.

Declaration of Conflicting Interests

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ORCID iDs

Parul Bali https://orcid.org/0000-0003-0849-938X

Akshay Anand https://orcid.org/0000-0001-7947-5209

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Effect of Retinal Injury Induced by Laser Photocoagulation on Visuospatial Memory in Mouse Model

Priya Mehra[1,2] Parul Bali[3] Jagtar Singh[1] Pradip Kumar Saha[4] Akshay Anand[2]

[1]Department of Biotechnology, Panjab University, Chandigarh, India
[2]Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[3]Department of Biological Science, IISER - Indian Institute of Science Education and Research, Mohali, India
[4]Department of Obstetrics and Gynecology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Address for correspondence Parul Bali, PhD, Department of Biological Science, IISER - Indian Institute of Science Education and Research, Mohali 140306, Punjab, India (e-mail: parul28bali@gmail.com);

Akshay Anand, PhD, Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India (e-mail: akshay1anand@rediffmail.com).

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Abstract

Visual pathway reveals the connection between neuronal activity of the brain and eye. The neural networks of brain amplify the retinal signals resulting in the formation of visual image. The laser injury in the retina may affect the visual pathway and may lead to disruption of neuronal signals/activity. Therefore, we aimed to study the effect of retinal injury induced by laser on cognitive abilities in laser-induced mouse model. We have established laser model to understand the relation between retina and brain by disrupting retinal pigment epithelial (RPE) layer and evaluate the effect of laser-induced retinal injury on visuospatial memory. Age- and sex-matched C57BL/6J male mice were taken for conducting the experiments. The laser model was established by using laser photocoagulator to disrupt the RPE layer of the retina. After defined irradiation of laser onto mouse retina, the fundus fluorescein angiography was performed to confirm the laser spots. The visuospatial and short-term memory was performed using neurobehavioral test, that is, Morris water maze (MWM), and passive avoidance, respectively. In MWM experiment, results showed that escape latency time, which was taken by healthy and laser-injured mice was comparable. This was further validated by another neurobehavioral analysis, that is, passive avoidance that showed nonsignificant difference between these two groups using independent *t*-test. Visuospatial memory may not be affected by retinal injury induced by laser photocoagulation. It may depend on the power of the laser and duration of the laser. The severe injury in the retina such as optic nerve damage may cause dysfunctioning of visual pathway.

Keywords
- visual impairment
- laser photocoagulation
- retinal degeneration
- cognition
- memory

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Introduction

Several eye disorders such as age-related macular degeneration (AMD), diabetic retinopathy, glaucoma, retinal vein occlusion, retinal ischemia, and retinal pigmentosa have retinal degeneration as a main symptom. It is considered as one of the major causes of blindness.

Visual impairment affects retina, optic nerve, and different parts of brain such as thalamus, optic chiasma, optic radiation, occipital lobes, and visual cortex. According to the World Health Organization, globally 285 million people are visual impaired, out of which 39 million are blind and majority of people are above 50 years of age or more,[1] whereas 123.7 million people are with refractive errors, 6.9 million with glaucoma, 65.2 million with cataract, 3 million with diabetic retinopathy, and 11 million with AMD, and it is expected to double in next 10 years.[2] In developing countries, leading causes of visual impairment are AMD, glaucoma, and diabetic retinopathy.

However, the prevalence of visual impairment is expected to increase twofold by 2050.[3,4] Moreover, there is a close relationship between visual impairment and cognitive decline as both are neurodegenerative disorders and are related to aging.[5,6] The study showed the relation between macular degeneration and Alzheimer's disease (AD) in older age group resulting in poor reading ability, distorted blurred faces due to lack of sensory stimulus, and brain stimulus activity.[7] It was studied that visual performance has significant effect on memory deterioration.[8] A study reported correlation of memory and learning with vision in 1,000 cataract-operated patients and found significant improvement in memory task and learning.[9] Therefore, such results show that memory and learning are significantly associated with vision.

Various research have shown the association of visual performance with cognition function.[6,7] The dysregulations of blood retinal barriers has implicated age-related cognitive decline.[10] In neurological disorders, disruption of retinal blood barrier leads to cognitive decline as signaling get disrupted.[11] Laser model has been used to study the visuospatial memory by either disrupting retinal layers or optic nerve that transmits signal from retina to brain where visual image formation occur known as visual perception.[12] The primary site of injury using laser photocoagulation is retina. There are two methods by which retina get disrupted by laser, that is, one by targeting the Bruch's membrane that is an innermost layer of the choroid below retinal pigment epithelial (RPE) layer. The proliferation of choroidal vessels takes place that penetrate RPE through Bruch's membrane and leakage of lipids and blood from these new fragile blood vessels under the retina and sometimes between the retinal layers also occurs. This process is known as choroidal neovascularization.[13] Second method is by directly targeting the RPE that absorb laser beam, which gets converted into thermal energy leading to denaturation of cellular proteins.[14] The retinal injury model can be validated by visualizing damaged retina through fundus fluorescein angiography (FFA), optical coherence tomography, and electroretinogram. In our study, we have established the laser model and tried to investigate that whether the retinal injury affects visuospatial memory.

Some studies have shown that visual impairment leads to visual cortex damage. It has shown that neuronal cells in the retina as well as its thickness also get affected in AD.[15,16] The retina shares various functions with central nervous system, transmission of signal leads to visual perception, cognition, image formation, and spatial memory. Therefore, keeping in mind this doubted or unconfirmed relation between visual problems and visuospatial memory or cognitive learning, this laser-induced retinal injury model was established. This model was used to study the effects of laser on visuospatial memory or cognitive abilities via retinal degeneration using standardized parameters such as 100-µm size of the laser.

Methods

All the experiments were performed under good laboratory practice guidelines. All the protocols were approved by the study director and quality assurance personnel.

Study Design

In this study, 6 to 8 weeks C57BL/6J male mice were used. Animals were fed on standard diet and RO drinking water was available *ad libitum*. Animals were maintained in a 12-hour light/dark cycle. Utmost care was taken while performing experiments. All the experiments were conducted in accordance with Institutional Animal Ethical Committee (105/IAEC/720).

Laser Photocoagulation

Mice were anesthetized with the mixture of ketamine and xylazine in the ratio 1:10 intraperitoneally in to mice and their pupil was dilated with 1% tropicamide and phenylephrine. A slit lamp connected with laser photocoagulator (IRIDEX) with 532 nm argon green laser wavelength was used. Eight laser shots were given to both eyes. The laser shots were given around optic disc in each eye with coverslips as a contact lens. The size of laser spots was 100 µm; duration of exposure to laser pulse was 100 milliseconds; power of the laser was 200 mW. These all parameters were already standardized in our laboratory.[17] The mice whiskers were trimmed and eyes were kept moist and clean with sterile saline. After that, mice were placed on slit lamp laser system. The fundus was observed using coverslip with one drop of hydroxypropyl methylcellulose. Once the site got visualized, eight laser shots were given to each eye in circular fashion around the optic disc.

Fundus Fluorescein Angiography

To validate the laser model, FFA was done after 24 hours of laser injury. The animals were anesthetized and pupil was dilated. The mice received tail vein injection of 0.2 µL of fluorescein dye diluted in 1 mL normal saline. Immediately

after injecting, fundus was focused. The vascular leakage and size of laser lesion was determined by using Heidelberg Engineering software. The laser-induced leakage validated the establishment of laser model.

Neurobehavioral Analysis

Rotarod

The rotarod consists of circular rod rotating at constant speed to measure muscular strength, motor coordination of mice. After 1 month of laser injury, neurobehavioral assessment was done. The mice were kept on rotating rod at constant speed of 15 rpm for 180 seconds. The mice were allowed to rotate on rotarod for 3 minutes to maintain the balance and to avoid falling. The time taken by the mice to fall from rotating rod was recorded. The trial was done for three times daily followed by Morris water maze (MWM).

Morris Water Maze

MWM was performed to measure the spatial memory of the mice. After laser injury, the mice underwent neurobehavioral analysis. It was 7 days protocol, first 6 days were acquisition days and the seventh day was retrieval day. Before MWM, mice were screened for swimming ability by rotarod experimentation. MWM consists of circular tank filled with water with equally divided four quadrants named Q1, Q2, Q3, and Q4. A circular white platform was submerged 1 cm below the water surface kept in Q1 quadrant. The visual cues were placed on the walls and pool side. The background of water was colored white to identify and track the darker mice against bright background in ANY-maze software. The complete protocol was recorded and tracked by using ANY-maze video software connected with webcam. The water temperature was maintained between 21 and 24°C. Four trials were done every day and each trial was for 120 seconds. ANY-maze software measures various parameters such as escape latency time of the mice, mean speed from platform to different quadrant, time spent by mice in each quadrant, distance traveled by mice from each quadrant to platform, and mean distance from the platform.

Passive Avoidance

Passive avoidance is the learning task used to measure short-term memory. The instrument divided into two equal parts named light chamber and dark chamber with a gate between two. This protocol was performed for 3 days for 10 minutes. On first day, mice were allowed to explore both chambers freely. On second day (acquisition day) mice were kept in the light chamber and gate was opened after 30 seconds and then time was recorded when mice entered the dark chamber. In dark chamber, the mice received mild foot shock of 20 mW and after that mice were removed immediately from the box. On third day (test day), the time spent by mice in light chamber was recorded to avoid foot shock in dark.

Enucleation of Eye

Mice were euthanized by overdose of ketamine: xylazine combination. The eyes were enucleated and stored at 80°C for further experiments.

Results

Retinal Assessment

FFA: The fundus imaging was done after 24 hours of laser injury. The vascular leakage at the injury site showing laser lesions in eight laser group. The images were captured by Heidelberg Engineering software as shown in ►**Fig. 1**.

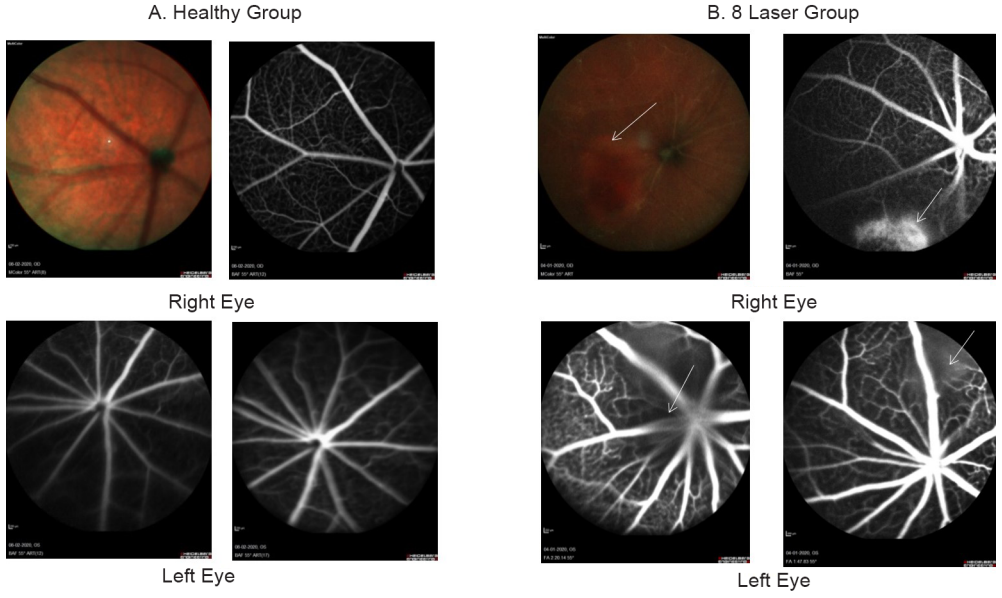

Fig. 1 Fundus fluorescein angiography shown fundus image of the eye. (**A**) The healthy control group shows no leakage of fluorescein dye in left and right eyes. (**B**) Although in eight laser group, the white arrows show leakage at injury site in both right and left eyes of laser group after 24 hours of laser injury.

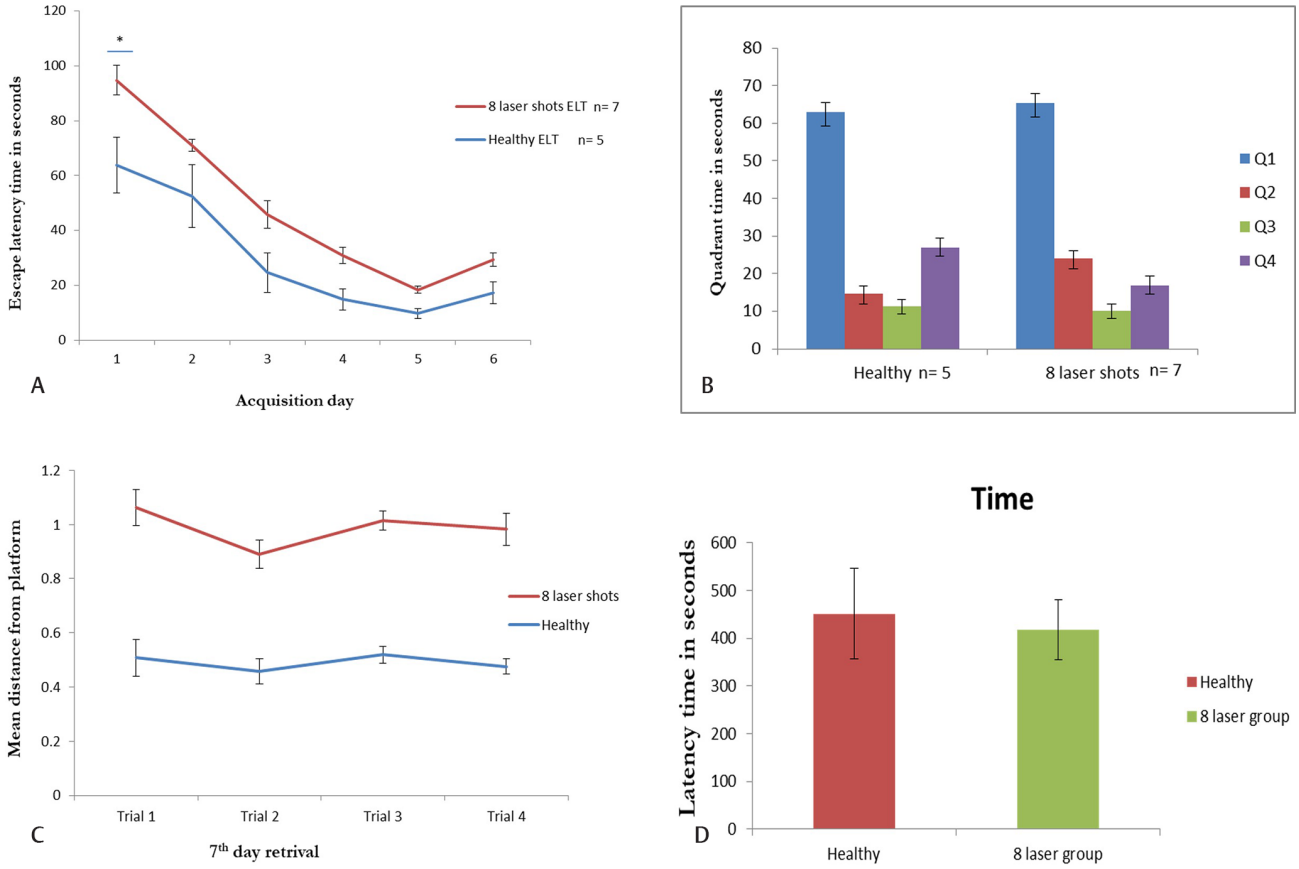

Fig. 2 The MWM analysis showing learning in mice. (**A**) The day-wise escape latency time during acquisition days of mice in healthy control (*N* = 5) and eight laser injury (*N* = 7). The ELT was found to be more in mice with eight laser group at day 1 as compared with the healthy control group. (**B**) *Quadrant time*: The time spent by mice in MWM quadrants Q1, Q2, Q3, and Q4 on retrieval day (seventh day). (**C**) The search error measured as mean distance from hidden platform traveled was less in healthy group as compared with laser group but nonsignificant. (**D**) Passive avoidance found more latency in eight laser group as compared with healthy group. The trial lasts for maximum 10 minutes. ELT, escape latency time; MWM, Morris water maze.

Neurobehavioral Assessment

Morris water maze: Before MWM, the mice were undergone rotarod experiment to determine the swimming ability. All mice were found to be swim well, and their motor coordination and muscular strength was strong. After three trials of 3 minutes, mice had undergone MWM experiment. ANY-maze software was attached to webcam to track the mice. It was found significant reduction in the swimming path on day 1 in healthy group as compared with laser group (►**Fig. 2A**). However, all mice were learned to reach the platform but were nonsignificantly. ►**Fig. 2B** depicts that the mice spent time mice in each quadrant at seventh day of the experiment. It was found that mice spent more time in Q1 quadrant where the hidden platform was placed but nonsignificantly, whereas, mice spent maximum time in Q2 and Q4 quadrants significantly (►**Fig. 2B**). The mean distance from platform was found to be nonsignificant in both groups (►**Fig. 2C**).

Passive Avoidance

After MWM, to confirm the learning and memory ability, the mice were subjected to passive avoidance in which time spent by mice in the light chamber to avoid shock was noted. We found that mice had learned and remain in the light chamber maximum time for 10 minutes on day 3 in both healthy control and laser group (►**Fig. 2D**).

Statistical Analysis

IBM SPSS version 22 was used for the statistical analysis, whereas baseline values were checked for normalcy and baseline group comparisons were done by using independent samples *t*-test.

Discussion

Visual system is the process in which signals get transmitted, processed, and interpreted by axonal retinal ganglion cells and primary cortex in brain to form visual perception. The neuronal cells in the retina extend axons from retinal ganglion cells to optic nerve to brain mainly in primary cortex to form image. Brain is able to remember entire series of images formed by retina when we move eye results in spatial memory. Since the image produced by eye is directly correlated with the brain[18] as the electrical signal get transmitted from retinal photoreceptors cells to brain through optic nerve which is responsible for visual perception, thus impairment

in the visual function will affect the brain function and understanding directly. Understanding the mechanism of visual and cognitive function will provide evidence to understand the disease with another important perspective. This invokes the need to study the effects of visual impairment on brain function and to study the effect of the treatment used for vision repair on the brain function.

In retinal degenerations such as retinitis pigmentosa and AMD, diabetic retinopathy begins with loss of photoreceptor, impaired macula and later on results in loss of inner retinal neurons, but significant numbers of bipolar and ganglion cells remain for many years that can be reversed by electrical stimulation of the retina or the optic nerve.[19]

In our study, we observed that laser-induced retinal injury do not affects the learning and memory. In spatial memory test, laser-injured mice showed learning and memorizing at the retrieval day when the hidden platform was removed. This test was validated by fear-motivated test in which laser-injured as well as healthy mice spent maximum time in light chamber to avoid foot shock. Some of the studies have shown that learning and visual memory may not be correlated. Yancopoulos (2016) used anti-VEGF treatment to abolish clinical neovascularization; however, investigations to examine its association with cognitive improvement have been lacking. While they found significant results in the treatment of angiogenic eye disorders, they did not report the impact on neurobehavioral.[13,20] Prusky et al examined the role of visual acuity in animal model and found that the mice with reduced visual acuity were found to be deficit in place of learning and do not differ their learning ability in MWM.[21] Similarly, Storchi et al demonstrated that innate behavior has various advantages in vision test mainly by determining the visual capabilities with intact visual function in mice model and in retinal degeneration.[22]

Hence, we studied the association between laser-induced retinal injury and memory in mice model. Many studies have shown that the neuronal and retinal degeneration are correlated with each other. Neuronal cell death results in cognitive and visual impairment. Also, neurodegeneration affects retina. In neurological disorders such as AD and Parkinson's disease, retinal thickness reduced in mild cognition impairment as compared with healthy control group.[23,24] Although various studies from our group have independently analyzed the role of risk and genetic factors in onset of neurodegeneration, we had not examined the association between two clinical situations, that is, vision loss and cognitive deficit.[25-43] However, there are studies that have shown the correlation between visual impairment and cognition in the population and found significant association between them.[44-46]

Oktem et al found significant correlation between retinal thickness and mild cognition in AD patients as compared with control group.[47] However, our results have shown that visuospatial memory is independent of retinal injury induced by laser photocoagulation.

Limitations of the Study

The molecular analysis such as reverse transcription polymerase chain reaction, immunohistochemical staining to check the expression of different neuronal marker and apoptotic marker is yet to be done. The samples were stored at –80°C for further experimentation.

Conclusion

Visuospatial memory may not affect by retinal injury induced by laser photocoagulation. It may depend on the power of the laser and duration of the laser. The severe injury in the retina such as optic nerve damage may cause dysfunctioning of visual pathway.

Ethical Approval

The ethical approval was taken by Institute Animal Ethical committee. The ethical approval number is 105/IAEC/720.

Funding

None.

Conflict of Interest

None declared.

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RESEARCH ARTICLE

Sleeping pattern and activities of daily living modulate protein expression in AMD

Kaushal Sharma[1,2], Ramandeep Singh[3], Suresh Kumar Sharma[4], Akshay Anand[1]*

1 Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India, **2** Advanced Pediatrics Centre, Department of Pediatrics, Post Graduate Institute of Medical Education and Research, Chandigarh, India, **3** Department of Ophthalmology, Post Graduate Institute of Medical Education and Research, Chandigarh, India, **4** Department of Statistics, Panjab University, Chandigarh, India

* akshay1anand@rediffmail.com

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Abstract

Degeneration of macular photoreceptors is a prominent characteristic of age-related macular degeneration (AMD) which leads to devastating and irreversible vision loss in the elderly population. In this exploratory study, the contribution of environmental factors on the progression of AMD pathology by probing the expression of candidate proteins was analyzed. Four hundred and sixty four participants were recruited in the study comprising of AMD (n = 277) and controls (n = 187). Genetics related data was analyzed to demonstrate the activities of daily living (ADL) by using regression analysis and statistical modeling, including contrast estimate, multinomial regression analysis in AMD progression. Regression analysis revealed contribution of smoking, alcohol, and sleeping hours on AMD by altered expression of *IER-3*, *HTRA1*, *B3GALTL*, *LIPC* and *TIMP3* as compared to normal levels. Contrast estimate supports the gender polarization phenomenon in AMD by significant decreased expression of *SLC16A8* and *LIPC* in control population which was found to be unaltered in AMD patients. The smoking, food habits and duration of night sleeping hours also contributed in AMD progression as evident from multinomial regression analysis. Predicted model (prediction estimate = 86.7%) also indicated the crucial role of night sleeping hours along with the decreased expression of TIMP-3, IER3 and SLC16A8. Results revealed an unambiguous role of environmental factors in AMD progression mediated by various regulatory proteins which might result in intermittent AMD phenotypes and possibly influence the outcome of anti-VEGF treatment.

1. Introduction

Most of the degenerative diseases (*e.g.* AMD and Alzheimer's disease) have shown complex phenotypes based on both gene-environment interactions which have propensity to alter the cellular functions by gene expression changes [1, 2]. AMD is characterized by degenerative changes in macular photoreceptors and vision impairment in elderly. It is associated with various environmental factors and 52 independent genetic loci [3]. However, most of reported AMD alleles have not been probed for interaction with environmental factors rendering the genetic studies of AMD an incomplete and unimpactful analysis.

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AMD literature is replete with evidence in support of the contribution of both genetic and environmental factors in the progression of AMD, but fails to define the architecture of this complex phenotype. However, smoking has been much investigated with context to AMD and found to exhibit its effect through generation of oxidative stress [4] and induce angiogenic cascade [5, 6] in order to promote angiogenesis of choroidal blood vessels. Moreover, smoking exposure has been shown to exert the pathological changes akin to AMD by blocking alternative complement pathways and by lipid dysregulation in RPE cells [7]. Studies have also shown that the combined effect of both alcohol consumption and smoking might further exacerbate the AMD pathology by influencing the activity of SOD (superoxide dismutase) and glutathione peroxidase activity [8]. Our previous reports have also defined the pathological role of oxidative stress [9], impaired angiogenesis [10, 11] and inflammatory cascade (mediated through CCL2 and CCR3) [12–14]. Similar pathological hallmarks have also been exhibited by other degenerative diseases including AD, ALS etc. [15–18]. Recently, we have also identified genetic variants of TIMP3, APOE and HTRA1 genes to contribute towards the complexity of Indian AMD [19]. The exact mechanism of action of the associated environmental factors to modulate the function wide genetic architecture in AMD is not adequately investigated although it is generally accepted to play a key role in AMD pathology. Exposure of environmental factors is possibly to bring the epigenetic modifications at the gene/genome (methylation of CpG Island) as well as on histone protein (*acetylation*, *phosphorylation*, *methylation*, *citrullination*, *ubiquitylation*, *ribosylation*, and *sumoylation*) levels which could modulate the expression of proteins and their mediated cellular mechanism [1]. Temporal nature of smoking and dietary induced AMD pathology by altering the protein expression indicates the epigenetic regulation of disease progression [20]. Revealing the understanding of rare and common genetic variants, copy number variations along with mitochondrial genetics, and their contributions in the AMD pathology under the influence of environmental factors, enable us to redefine the diagnosis and propose a new therapeutics regimen [21–23].

We report that there is an alteration in expressions of HtrA Serine Peptidase 1 (*HTRA1*), Tissue inhibitor of metalloproteinase-3 (*TIMP*-3) and Immediate Early Response 3 (*IER*-3) in sleep deprived individuals or AMD patients with increase in sleep duration, prompting further research [24, 25]. This has implication for superior diagnosis and management of patients affected by AMD. We wanted to examine the nature and extent of the role of environmental factors in exerting its influence on genetic components and whether these are governed by epigenetic or epistatic interactions.

2. Materials and methods

2.1. Recruitment of participants

We have recruited around 464 participants in present study which comprised with both AMD (n = 277) and controls (n = 187). Participants were recruited as per the inclusion-exclusion criteria mentioned in the study along with their informed written consent. The study has started the recruitment of participants from 2010 and finished the same in 2017. The recruitment of participants and clinical examinations were performed from the Department of Ophthalmology, PGIMER, Chandigarh, India and experiments were conducted in Neuroscience Research Lab, PGIMER, Chandigarh, India. The study was conducted as per the approval of Institute Ethical Committee of both PGIMER (No: PGI/IEC/2005-06; dated: 23.07.2013) and Panjab University (IEC No. 131A-1, dated: 29.10.2014). All methods pertaining to study were performed in accordance with the relevant guidelines and regulations laid down by IECs. The study could be considered as a representative to replicate the same in large cohort.

2.2. Clinical investigations

Clinical evaluation of AMD phenotypes was carried out by retina specialists which included fluorescein fundus angiography (FFA) of dilated retina of the patients. Patients were clinically classified based on the drusen deposits and leaky vessels captured as fundus images. Moreover, the extent of macular photoreceptors degeneration and thickness of retinal layers were also examined by Optical Coherence Tomography (OCT) of patients. AMD patients were classified based on the clinical features observed and stratified by the AREDS criteria. Based on the presence of clinical parameters including drusen, neovascular lesions and atrophy of photoreceptors, AREDS stratified the AMD patients into 5 categories. Briefly, AMD patients with A few small drusen (<63 microns in diameter) fall in the AREDS 1. AREDS2 was characterized as multiple small drusen, a few intermediate drusen (63 to 124 microns in diameter), and/or RPE abnormalities. Many intermediate drusen with at least one large druse (≥125 microns in diameter) classified as AREDS3. Atrophy of foveal photoreceptors was characterized as greographic atrophy (AREDS4) and finally, patients with any leakage between retinal layers or neovascular features were classified as AREDS5.

2.3. Activities for daily living (ADL) details

A well-defined questionnaire was introduced to collect the socio-demographic details of the studied participants. ADL details prominently included the daily living activities (food habits, smoking, alcohol), education and profession, any medication, physical activities and/or yogic practices, sleeping pattern, etc which mostly associated with person's life style. Food habit was categorized *i.e*. vegetarian, prior history of non-vegetarian and/or non-vegetarian, based on the consumption of food for at least six months or more since the date of recruitment. Non-vegetarian participant was defined based on consumption of chicken, fish and/or mutton or any one of them. Smokers were also categorized (non-smoker, prior/past-smoker, current smoker) based on the current and/or past-history of smoking, if any, of the participants who must be smoking for minimum six months (in case of prior or current smoker) at the time of recruitment. Similarly, participants were also classified (non-alcoholic, prior/past-alcoholic and current alcoholic) based on the alcohol consumption (past or current) with minimum 6 months of alcohol consumption history. To see the impact of sleep hours of the participants, we have classified participants in to three categories namely as sleep deprived (<6 hours sleep), 6–7 hours' sleep and rise before 6AM (6–7 hours' sleep) and >6–7 hours' sleep and late sleep or late rise (after 6AM). Moreover, we also have asked participants whether they have been instructed to take medication for any ailment including cardiovascular, hypertension, diabetes, migraine and stroke history by a physician in addition to AMD.

2.4. Serum extraction

3ml of blood sample was withdrawn from participants and were subjected to centrifugation at 2500rpm for 30minutes. Upper supernatant layer was collected and stored at -80˚C for further experimentation.

2.5. Total protein estimation

Total protein in the serum of participants was estimated by Bradford's method. Samples were diluted (ranges from 200–600 times) with distilled water before performing the assay. Bradford's reagent was added in 1:4 dilutions in the experiment. Absorbance of samples was taken at 595nm by ELISA reader (BioRad, USA).

2.6. ELISA

Serum levels of proteins involved in pro-angiogenesis (*e.g*. *ADAMTS9*, *TIMP*-3), cellular regulatory proteins (like *IER*-3, *B3GALTL*, *HTRA*1), monocarboxylic acid (pyruvic acid or lactate) transporter (SLC16A8) and lipid metabolizing proteins [hepatic lipase (*LIPC)* and apolipoprotein E (*APOE*)] were estimated using commercially available ELISA kit. Protocol was followed as per available recommendations with the kit and absorbance was recorded at 450nm. Values of ELISA were normalized with total protein of the respective sample. Levels of protein were compared with control populations.

2.7. Genotype analysis

Genotype analysis was also carried out for same set of genes involved in various cellular functions *e.g*. lipid metabolizing proteins [*LIPC* (rs920915) and *APOE* (*rs769449*)], pro-angiogenesis [*e.g*. *ADAMTS9* (rs6795735), *TIMP*-3 (rs5749482)], cellular regulatory proteins [like *IER*-3 (rs3130783), *B3GALTL* (rs9542236), *HTRA*1 (rs11200638)] and monocarboxylic acid transporter [e.g. SLC16A8 (rs8135665)] to associate with ADL.

2.8. Statistics

Data was assessed for normal distribution in the population using normal quantile plot (O-Q plot) and Kolmogorov-Smirnos (K-S) tests. Differential protein expression in various groups stratified on the basis of socio-demographic details, was analyzed by ANOVA. Logistic regression analysis was utilized to analyse the effect of exposure of environmental factors (like smoking, food habit, alcohol consumption *etc*) by creating variables for prior and current status of activities of daily living (ADL). To examine the differential protein expression due to gender polarization effect in AMD patients, contrast analysis was carried out. Predictive modeling based on clinical severity and associated expression changes were analyzed by regression analysis. Multinomial regression analysis was done to analyze the contribution of ADL in AMD severity. Moreover, the prediction model based on ADL and expression level of proteins was put forwarded to diagnose AMD cases more precisely.

3. Results

3. 1. Association of socio-demographic factors

Chi-square analysis of the data revealed a significant association of various factors with AMD patients including profession, accident, consumption of anti-inflammatory drugs of participants. There is a significant difference between mean age of AMD and Control ($p<0.001$). Results reveal marginal association of physical activity and education of an individual with AMD pathology (Table 1).

Activities of daily living (ADL) of the participants were also analyzed to examine if association existed between AMD and these variables. Association of AMD patients with BMI, smoking habits (both prior and current habit) and abnormal sleeping pattern was noted. Moreover, it was higher in AMD patients as compared to control (Table 2).

Frequencies of clinical features of AMD patients were also calculated as presented in Table 3. Recruited AMD patients showed advanced form of AMD clinical features (AREDS 5) involving bilateral phenotype. Further dissection of bilateral phenotypes of AMD patients revealed the numbers as 28, 34 and 82 with bilateral dry, bilateral wet and dry-wet bilateral phenotypes, respectively. Approximately, 42% of AMD patients were also diagnosed with and cataract and underwent the surgery to treat the same.

Table 1. Comparative demographic characteristics of AMD and controls.

Features	AMD(n)	Controls(n)	p-value
Gender			
1. Male	171 (61.73%)	99 (53.51%)	0.833
2. Female	106 (38.27)	86 (46.87%)	
Age (Mean ± SD)	68.30 ± 9.086	56.94 ± 11.266	<0.0001***
Anti-Inflammatory drugs¥			<0.0001***
1. No Inflammatory	144 (53.33%)	139 (81.76%)	
2. Anti-Inflammatory drugs	126 (46.67%)	31 (18.24%)	
Occupation¥			<0.0001***
1. Professional	62 (22.63%)	8 (5.19%)	
2. Semi professional	48 (17.52%)	6 (3.90%)	
3. Clerical	41 (14.96%)	37 (24.03%)	
4. Skilled	07 (2.56%)	13 (8.44%)	
5. Semi-skilled	12 (4.38%)	28 (18.18%)	
6. Unskilled	103 (37.59%)	62 (40.26%)	
7. Unemployed	01 (0.36%)	0	
Education¥			0.063
1. Professional or honor	61 (22.18%)	46 (28.57%)	
2. Graduate or Post Graduate	21 (7.64%)	20 (12.42%)	
3. Intermediate	23 (8.36%)	18 (11.18%)	
4. High school	74 (26.91%)	35 (21.74%)	
5. Middle school	19 (6.91%)	15 (9.32%)	
6. Primary school	57 (20.73%)	19 (11.80%)	
7. Illiterate	20 (7.27%)	08 (4.97%)	
Physical activity¥			0.052
1. Physically active	111 (40.81%)	78 (49.37%)	
2. Inactivity	161 (59.19%)	80 (50.63%)	
Accident history¥			0.029*
1. Accident history	55 (19.93%)	18 (11.69%)	
2. No accident history	221 (80.07%)	136 (88.31%)	

¥ Some missing values.

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3.2. Activities of daily living influence protein expression

We also attempted to study the gross impact of various ADL on protein expressions in AMD patients. Our results revealed a significantly enhanced LIPC levels in AMD patients who smoke and have non-vegetarian food habits (prior) suggesting an impaired lipid metabolism (IDL to LDL formation) due to malfunction of LIPC in AMD pathology (Fig 1A & 1E). Interestingly, the sleeping pattern of AMD patients [6-7hrs sleep, waking time before 6AM in morning (normal sleep) versus >7-8hrs sleep, late sleep or late wakefulness] was found to display a significant effect on HTRA1 levels. Documentation of consequently altered HTRA1 levels suggests the role of impaired circadian rhythm on AMD patients and the biological significance of HTRA1 being amenable to such regulation. However, more research is required (Fig 1G). We did not find significant alteration in protein levels under the influence of smoking, participant's food habits and disturbed sleeping pattern (Fig 1B–1D, 1F and 1H).

The *beta* coefficient (B) of logistic regression analysis revealed that significantly decreased expression of IER-3 (-0.288), B3GALTL (-0.214), LIPC (-0.172), TIMP-3 (-63.696) along with increased levels of HTRA1 (0.696) were observed in Indian AMD, without adjusting the ADL.

Table 2. Comparative frequencies of activities of daily livings (like BMI, smoking, alcohol consumption, food habit and sleeping pattern) of AMD and control participants.

Features	AMD (n)	Controls (n)	p-value
BMI¥			0.003*
1. **Under weight**	10 (3.75%)	07 (4.46%)	
2. **Normal**	175 (65.54%)	87 (55.41%)	
3. **Over Weight**	50 (18.73%)	53 (33.76%)	
4. **Obese**	32 (11.98%)	10 (6.37%)	
Smoking habit¥			0.010*
1. Never smoker	185 (67.52%)	128 (81.01%)	
2. Prior smoker	54 (19.71%)	17 (10.76%)	
3. Current smoker	35 (12.77%)	13 (8.23%)	
Alcohol consumption			0.650
1. Never Alcohol	186 (67.15%)	112 (71.34%)	
2. Prior Alcohol	30 (10.83%)	14 (8.92%)	
3. Current Alcohol	61 (22.02%)	31 (19.74%)	
Food habit ¥			0.163
1. **Vegetarian**	147 (53.26%)	78 (50%)	
2. **Non-vegetarian**	86 (31.16%)	61 (39.10%)	
3. **Prior nonveg**	43 (15.58%)	17 (10.90%)	
Night sleeping hours¥ 269			0.006*
1. **6–7 hrs sleep, rise before 6AM**	157 (58.36%)	81 (54.36%)	
2. **Sleep deprived (<6hrs sleep)**	29 (10.78%)	05 (3.35%)	
3. **>7–8 sleep, late sleep or late rise (after 6AM)**	83 (30.86%)	63 (42.29%)	

¥ Some missing values.

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Logistic regression analysis estimated the individual effect of either prior or current status of ADL on protein expressions in AMD pathology (Table 4). Similar results were noted by adjusting smoking and alcohol habits. Past history of alcohol consumption was also found to significantly decrease IER3, B3GALTL, LIPC, TIMP3 expressions and increase HTRA1 levels. Additionally, prior history of alcohol consumption has potential to modulate the AMD pathology by -0.641 unit as compared to those who consume vegetarian diet (95% CI = 0.278–0.998; P = 0.049). Prior non-vegetarian history revealed marginal association with AMD by

Table 3. Clinical characteristics of North-West Indian AMD recruited in the study.

AMD features	Phenotypes	Number	Percent (%)
AMD phenotypes	Dry AMD	42	15.2
	Wet AMD	91	32.9
	Bilateral AMD	144	52.0
Cataract¥	No cataract	157	57.30
	Unilateral Cataract	53	19.34
	Bilateral cataract	64	23.36
Eye surgery¥	No eye surgery	160	64.78
	Unilateral surgery	96	35.03
	Bilateral surgery	18	06.57

¥ Some missing values.

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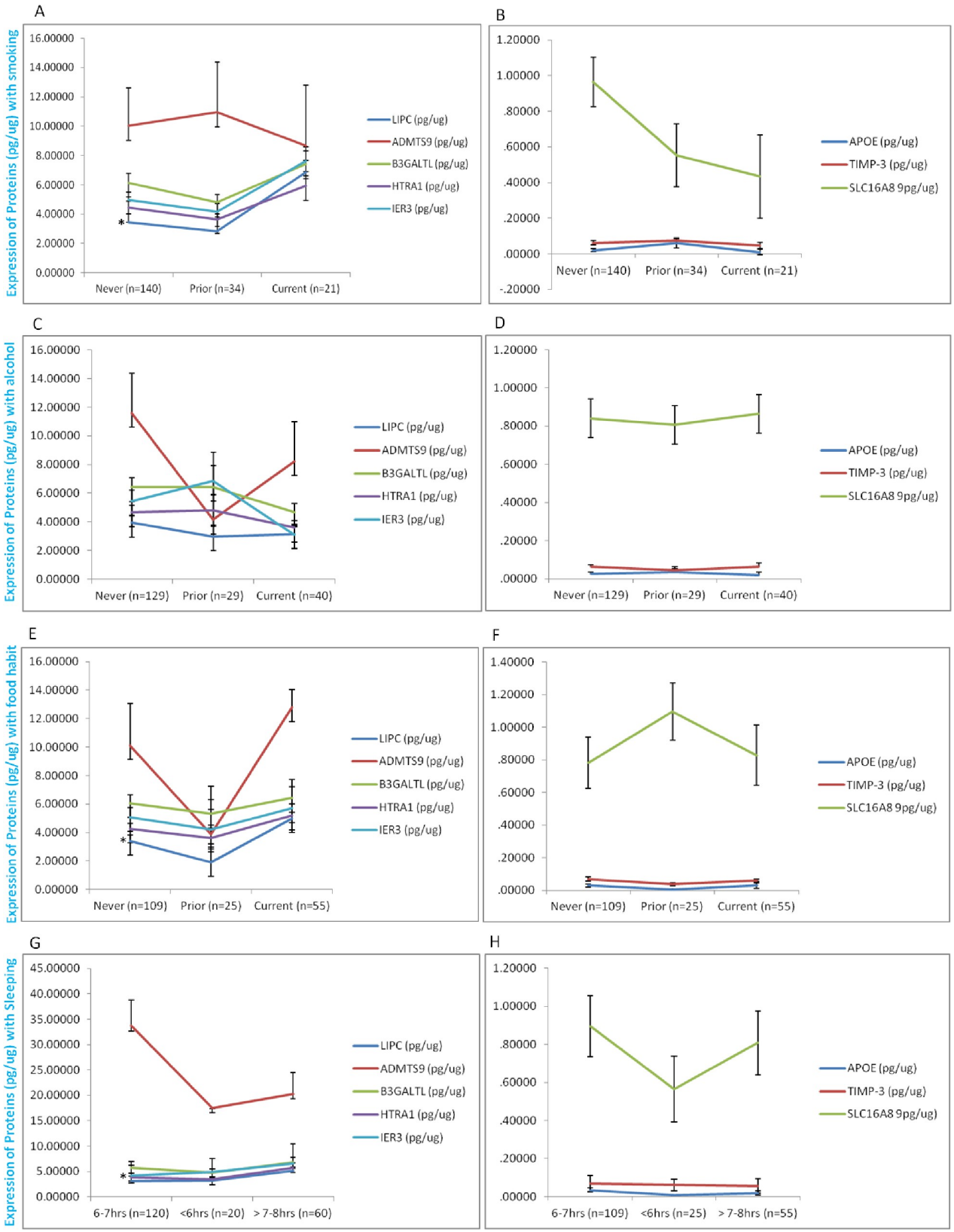

Fig 1. Expression of protein under ADL. Expression of lipid metabolizing (LIPC and APOE), proagniogenic (TIMP3 and ADAMTS9), regulatory (HTRA1, B3GALTL, and IER3) and momocarboxylic acid transporter (SLC16A8) proteins under the influence of ADL. LIPC (pg/ug) levels were

significantly elevated in 'current smoker' AMD patients (A & E). Altered sleeping patter can be associated with HTRA1 levels in AMD pathology indicating the crucial role of circadian rhythm in degenerative diseases like AMD (G).

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modulating the expression of HTRA, B3GALTL, IER3, LIPC and TIMP3 with changing it -0.691 (B = beta coefficient) unit with reference to never smoker (95% CI = 0.233–1.076; P = 0.076). Interestingly, by adjusting the sleeping pattern of AMD patients, it decreased the expression of IER3 (B = -.351; 95% CI = .605-.819; P = <0.0001) and TIMP3 (B = -44.128; P = <0.0001) significantly. Moreover, altered sleeping pattern (person who slept late or woke up after sun rise) revealed the changes in the expression of IER3 and TIMP3 by 0.757 unit (B coefficient) as compared to normal sleep (95% CI = 1.2–3.785; P = 0.01). Significant changes in IER3 (B = -.314; 95% CI = .637-.838; P = <0.0001), TIMP3 (B = -41.969; P = <0.0001) and SLC16A8 (B = -0.184; 95% CI = 1.022–1.415; P = 0.027) expression were observed while adjusting the physical activity of AMD patients (Table 4). Our results support the previous findings which indicate the crucial contribution of environmental factors including smoking, food habits, physical activity and alcohol consumption in AMD pathology by regulating the proteins expression. The association of sleeping pattern with AMD shows the biological importance of HTRA1, IER3 and TIMP3 which may have roles in modulating age-related changes in retinal layers, representative of AMD pathology.

3.3. Gender polarization effects of SLC16A8 and LIPC expressions in AMD

Females are considered to be more susceptible for AMD pathology, though we did not find any significant difference in frequency of AMD male and female. We also attempted to examine the gender effect on protein expression in Indian AMD patients. Contrast estimate was done using univariate analysis of variance to analyze the difference in protein expressions among male and female of studied population (Table 5). Contrast estimate (CE) for SLC16A8 [CE = -0.768; F = 5.451; 95% CI = -1.418- (-)-0.119; P = 0.021] and LIPC [CE = -0.644; F = 7.357; 95% CI = -1.112- (-)-0.175; P = 0.007] was found to be significantly decreased between male and female control population. Such differential expression of both proteins was not observed among AMD male and female. It may be argued that differential expression of both SLC16A8 and LIPC is required to regulate various mechanisms under the influence of a set of hormones and may confer the protective mechanism for age-related changes.

3.4. ADL contribution in advancement of AMD severity

To assess the independent contribution of ADL (including smoking, food habits, physical activity, sleeping hours and alcohol consumption) on AMD severity (AREDS criteria), we subjected the data to multinomial logistic regression. The model demonstrated a highly significant association of both past (B = -1.286; P = <0.0001) and current non-vegetarian food habit (B = -0.667; P = 0.001) in the advancement of AMD pathology (Table 6). Results showed that current non-vegetarian and past history of non-vegetarian history can contribute to AMD by B values of -0.667 and -1.286 units as compared to reference category (vegetarian diet). However, the prediction of model was not satisfactory.

Similarly, past and current status of smoking has also showed a significant association in progression of AMD pathology. Contribution of past (B = -1.275; P = <0.0001) and current smoking (B = -2.435; P = <0.0001) was observed in exacerbating the AMD pathology with prediction probability of around 68.4% (Table 6). Results for alcohol consumption in progression of AMD pathology has shown a comparable trend highlighting the contribution of past (B = -1.803; P = <0.0001) and current status of alcohol consumption (B = -1.077; P = <0.0001) as

Table 4. Logistic regression analysis to estimate the changes in protein expression under the influence of ADL.

Variables in the Equation									
		B	S.E.	Wald	Df	Sig.	Unadjusted	95% C.I.	
								Lower	Upper
Step 5e	IER3 levels	-.288	.066	19.371	1	<**0.0001**	.749	.659	.852
	B3GALTL levels	-.214	.065	11.037	1	**0.001**	.807	.711	.916
	HTRA1 levels	.696	.149	21.744	1	<**0.0001**	2.006	1.497	2.687
	LIPC levels	-.172	.081	4.539	1	**0.033**	.842	.719	.986
	TIMP3 levels	-63.696	8.666	54.027	1	<**0.0001**	.000	.000	.000
	Constant	1.484	.220	45.353	1	0.000	4.412		
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Adjusted for smoking**	**95% C.I.**	
								Lower	**Upper**
Step 1a	IER3 levels	-.287	.066	18.980	1	<**0.0001**	.751	.660	.854
	B3GALTL levels	-.214	.064	11.335	1	**0.001**	.807	.713	.914
	HTRA1 levels	.690	.147	21.984	1	<**0.0001**	1.994	1.494	2.660
	LIPC levels	-.171	.082	4.345	1	**0.037**	.843	.717	.990
	TIMP3 levels	-62.852	8.660	52.672	1	<**0.0001**	.000	.000	.000
	Smoking code			1.649	2	0.438			
	Smoking code(1)	-.507	.395	1.648	1	0.199	.602	.278	1.306
	Smoking code(2)	-.049	.494	.010	1	0.920	.952	.361	2.508
	Constant	1.538	.226	46.431	1	0.000	4.657		
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Adjusted for Alcohol**	**95% C.I.**	
								Lower	**Upper**
Step 1a	IER3 levels	-.291	.068	18.513	1	<**0.0001**	.748	.655	.854
	B3GALTL levels	-.222	.065	11.756	1	**0.001**	.801	.705	.909
	HTRA1 levels	.707	.151	21.784	1	<**0.0001**	2.028	1.507	2.728
	LIPC levels	-.185	.083	4.983	1	**0.026**	.831	.706	.978
	TIMP3 levels	-63.702	8.623	54.572	1	<**0.0001**	.000	.000	.000
	Alc code			5.397	2	0.067			
	Alc code(1)	-.679	.441	2.374	1	0.123	.507	.214	1.203
	Alc code(2)	-.641	.326	3.870	1	**0.049**	.527	.278	.998
	Constant	1.705	.246	47.879	1	0.000	5.502		
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Adjusted for Food habit**	**95% C.I.**	
								Lower	**Upper**
Step 1a	IER3 levels	-.286	.067	18.201	1	<**0.0001**	.751	.659	.857
	B3GALTL levels	-.209	.065	10.368	1	**.001**	.811	.714	.921
	HTRA1 levels	.674	.149	20.503	1	**.000**	1.963	1.466	2.628
	LIPC levels	-.163	.081	4.017	1	**.045**	.850	.724	.996
	TIMP3 levels	-63.781	8.722	53.475	1	<**0.0001**	.000	.000	.000
	Food Habit code			3.219	2	.200			
	Food Habit code(1)	-.058	.286	.040	1	.841	.944	.539	1.655
	FoodHabit_code(2)	-.691	.390	3.140	1	.076	.501	.233	1.076
	Constant	1.613	.255	40.018	1	.000	5.018		
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Adjusted for sleeping**	**95% C.I.**	
								Lower	**Upper**
Step 1a	IER3 levels	-.351	.077	20.720	1	< .0001	.704	.605	.819
	TIMP3 levels	-44.128	7.184	37.735	1	< .0001	0.000	.000	.000
	Night Slp code			8.606	2	.014			
	Night Slp code(1)	-.568	.616	.851	1	.356	.567	.169	1.895

(*Continued*)

Table 4. (Continued)

Variables in the Equation									
	Night Slp code(2)	.757	.293	6.666	1	**.010**	2.131	1.200	3.785
	Constant	1.367	.239	32.617	1	.000	3.923		
		B	**S.E.**	**Wald**	**df**	**Sig.**	**Adjusted for Physi activity**	**95% C.I.**	
								Lower	**Upper**
Step 1a	IER3 levels	-.314	.070	20.140	1	< **.0001**	.730	.637	.838
	TIMP3 levels	-41.969	6.908	36.913	1	< **.0001**	.000	.000	.000
	SLC16A8 levels	.184	.083	4.913	1	**.027**	1.202	1.022	1.415
	Physi Activ code(1)	-.039	.263	.022	1	.883	.962	.574	1.611
	Constant	1.350	.276	23.919	1	.000	3.859		

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compared to reference category (Table 6). The prediction probability of the model was about 65%. Interestingly, sleep deprived (<6hours sleep) and >7-8hrs sleep, late sleep or late rise subjects have also shown the significant impact on progression of AMD severity. Results have shown that sleep deprived (B = -1.885; P = <0.0001) and >7-8hrs sleep, late sleep or late rise (B = -.681; P = <0.0001) patterns contribute to the progression of AMD severity with a prediction probability of about 60% (Table 6). Pearson and deviance values of Goodness-of-fit model were found to be non-significant for the analysis. Results are suggested an independent role of ADL (environmental factors), especially sleep, in the progression of AMD pathology-which has never been analyzed previously.

3.5. Altered sleeping pattern and expression of IER3, TIMP3 and SLC16A8 confer the AMD

Association of sleep pattern and AMD pathology has not been adequately investigated. We have attempted to further dissect the impact of sleeping pattern in AMD patients. Regression analysis shows that night sleeping hours (B = 0.449; Exp(B) = 1.567; 95% CI = 1.1–2.23; P = 0.013) along with the expression of IER3 (B = -.444; Exp(B) = 0.641; 95% CI = 0.512–0.804; P = <0.0001) and TIMP3 (B = -23.54; Exp(B) = <0.0001; 95% CI = 0.000–0.004; P = 0.010) are significantly associated with AMD pathology. However, the marginal association of SLC16A8 expression (B = -.332; Exp(B) = .717; 95% CI = 0.506–1.017; P = 0.062) was also observed (Table 7). Results suggest the imperative role of sleeping pattern of an individual which may activate the various age-related mechanisms by influencing pertaining protein expressions. Our results indicate the biological significance of IER3, TIMP-3 and SLC16A8 expression to be influenced by alter sleeping hours of an individual. Classification table also strengthens our hypothesis with 86.7% validity of this regression model to predict the AMD pathology.

Table 5. Contrast estimate using univariate analysis of variance to differentiate the expression pattern on basis of gender for control population.

Variables	F-value	Contrast estimate (CE)	p-value	95% CI	
				Lower	Higher
SLC16A8	5.451	-0.768	0.021	-1.418	-0.119
LIPC	7.357	-0.644	0.007	-1.112	-0.175

[Female (n) = 86; male (n) = 99].

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Table 6. Multinomial logistic regression to examine the contribution of ADL in AMD severity.

Parameter estimates						
	Parameters	**B**	**S.E.**	**Wald**	**df**	**p-value**
Food habit	Non-vegetarian	-0.667	.198	11.388	1	**0.001**
	Prior Non-vegetarian	-1.286	.247	27.020	1	**<0.0001**
Smoking	Past smoker	-1.275	.288	19.572	1	**<0.0001**
	Current smoker	-2.435	.390	39.054	1	**<0.0001**
Night Sleep hours	Sleep deprived (<6hrs sleep)	-1.885	.311	36.716	1	**<0.0001**
	>7-8hrs sleep, late sleep or late rise	-.681	.195	12.206	1	**<0.0001**
Alcohol	Past Alcohol	-1.803	.280	41.547	1	**<0.0001**
	Current Alcohol	-1.077	.209	26.619	1	**<0.0001**

Reference category: [a]Vegetarian habit; [a]non-smoker habit; [a]6-7hours sleep or wake up before 6AM; [a]Never alcoholic.

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4. Discussion

Disease pathology of AMD is known to be influenced by both genetic and environmental factors evident by our quantitative outcome of protein expression under the influence of environmental factors [1]. In general, the ambiguity in the nature and extent of interaction between environmental and genetic factors has significantly hampered the pace of clinical translation in the field of AMD genetics [2]. Current AMD genetics warrants comprehensive analysis in the manner it can illustrate the contribution and interactions of contributory factors along with their degree of penetrance in disease progression. Majority of ageing diseases progress by cumulative genetic changes under temporal exposure of ADL consequently result in cellular and molecular alterations including protein homeostasis, metabolic dysfunction and aberrant signaling processes. The altered cellular and molecular crosstalk may confer complexity to the age related diseases thereby confounding an effective and precise diagnosis and treatment regime for complex disorders like AMD [26]. Therefore, a careful consideration of environmental and genetic components and their nature of interactions (and/or extent of interaction) may likely provide a precise AMD phenotype and personalized management strategies. The treatment strategy which can deal with the synergistic and/or cumulative action of contributory factors could provide a better outcome to therapies for AMD [26, 27]. Our ANOVA

Table 7. Regression analysis to predict the AMD pathology under the influence of ADL.

Variables in the Equation							
	B	**S.E.**	**Wald**	**Df**	**p-value**	**Exp(B)**	**95% CI**
Night sleep pattern	.449	.180	6.205	1	**0.013**	1.567	1.1–2.23
TIMP3 levels	-23.54	9.194	6.555	1	**0.010**	.000	0.000–0.004
IER3 levels	-.444	.115	14.907	1	**<0.0001**	.641	0.512–0.804
SLC16A8 levels	-.332	.178	3.487	1	**0.062**	.717	0.506–1.017
Constant	.192	.445	.187	1	0.666	1.212	
Classification table							
		Predicted					
		Group code				**Percentage corrected**	
		AMD		**Controls**			
Group	AMD	111		17		**86.7**	
	Control	22		50		69.4	
Overall percentage						80.5	

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results demonstrate that smoking and non-vegetarian food habit can effectively alter the LIPC expression that may exacerbate the AMD pathology. Interestingly, altered expression of HTRA1 under the influence of altered sleep cycle, can accelerate the AMD pathology thereby providing opportunity to correct the dysregulated circadian rhythm.

Various studies have been carried out to illustrate the significance of environmental factors on genetic components. Our results show that smoking, gender, age, diet *etc* as contributing confounders and have been significantly associated with complement factors, CFH variant, other variants of other genetic loci including *ARMS2*, *IL-8*, *TIMP3*, *SLC16A8*, *RAD51B*, *VEGFA etc* [28–31]. In our earlier report, smoking was found to be associated with TC genotype of CFH variant (Y402H) along with marginal association of AG genotype of TLR3 (rs3775291) with non-vegetarian food habit which also exhibited confounding effect on CFH expression and modulated TLR3 mediated functions in AMD [32, 33]. Interestingly, we also have found the pathological role of TIMP-3, SLC16A8, IER3 and LIPC in CFH independent manner in Indian AMD [34]. Moreover, eotaxin-2 was also significantly altered when smoker and non-smoker AMD cases were compared [35]. These results point out that the interaction between genetic and environmental factors which often lead to complex phenotype of disease [36].

Logistic regression analysis, by creating the dummy variables, enabled us to identify the effect of prior and current status of ADL like smoking and food habits *etc*. The results unambiguously reveal that prior or current history of non-vegetarian diet, smoking and alcohol can significantly alter the expression of IER-3, TIMP-3, B3GALTL, LIPC and HTRA1, suggesting the involvement of prior exposure of these habits as responsible for changes that may activate the age related molecular and cellular mechanisms. However, not many studies have revealed the association and biological significance of sleeping hours on AMD pathology. Khurana *et al* (2016) reported the high chance of geographic atrophy with increase in sleeping hours [24]. Similarly, short sleep has also been reported to be associated with increased susceptibility of AMD [25]. Similarly, our results from regression anlaysis indicate the pathological implication of altered sleeping hours of AMD. This illustrates the mechanistic importance of HTRA1, IER-3 and TIMP-3 in regulation of circadian rhythms. A marginal association was also reported for SLC16A8. Multinomial regression analysis showed a significant contribution of sleeping hours in AMD progression along with existing factors like smoking, alcohol, food habit *etc*. Temporal protein expressions in differential environmental exposure indicate the plausible role of epigenetics in AMD which has been evident by the 48% higher activity of DNA methyltransferases (DNMTs) in addition to enhanced DNMT1 and DNMT3B levels in AMD as compare to controls. Results also showed the higher methylation of LINE1 in AMD patients [37]. Methylation analysis has demonstrated the epigenetic regulation of *SKI*, *GTF2H4*, *TNXB and IL17*RC genes and their mediated functions in AMD pathology [38, 39].

Gender has additionally been found associated with AMD showing higher susceptibility for females ([40]. However, we did not find any significant difference in frequency between AMD females and males. Surprisingly, contrast estimate results showed differential expression of SLC16A8 and LIPC between control male and female (was not seen among AMD male and female) which may support the sex susceptibility and gender polarization hypothesis in the context of ADL. However, hormonal difference between both genders, their different cellular and molecular action, along with association with SLC16A8 and LIPC, has not been investigated in this report.

5. Conclusion

Conclusively, consideration of environmental factor, sleeping patterns and genetics of an individual must be profiled in order to provide the precise diagnostic and therapeutic benefit to

AMD patients. Genetic interaction, gene-protein interaction and gene-environmental interaction, along with nature of interactions and investigation of epigenetic pattern, can enable us to understand the penetrance of each component while facilitating personalized medicine hypothesis. Moreover, exploratory studies to examine the multiple genetic variations (especially in heterogenic disease like AMD), the degree of genetic penetrance of 'hot spots' or other genetic variants (mutation penetrance) may develop various genetic recombinant phenotypes (with varied genetic interactions) for disease pathology under the influence of environmental factors [41, 42]. Hence, complete mapping of genetic interactions, their genetic penetrance, epigenetics status and grading of epistatic interactions under the influence of confounder will provide precise disease phenotype. This could be dealt by modulating the therapeutics. Instead of cellular therapies, herbal or natural therapies could provide benefit in environmental induced age related changes or diseases by regulating the cellular and molecular pathways [43–47]. However, this requires an ADL framework for optimal treatment outcome.

6. Strengths and limitations

Study has first time demonstrated the biological significance sleeping pattern, in addition to already existing confounders (*e.g.* smoking, food habits, alcohol consumption) in AMD pathology by examining the altered expression of prominent biomarkers. Sleeping pattern could regulate the angiogenesis and survival of photoreceptors in AMD pathology as indicated by results described in Table 4. Moreover, interesting involvement of SLC16A8 and LIPC (Table 5) in protection mechanism has also provided the pilot data for further investigation in field of AMD which suggest further diversification and complexity of AMD to strengthen the diagnostics and therapeutic outcome accordingly [48]. This led hamper the clinical translation in neurodegenerative diseases including Alzheimers disease and AMD [18, 49]. However, further validation and replication of the results must be reconfirmed in larger cohort (by including Asian and Caucasian population) with precise mechanism of AMD pathogenesis.

Supporting information

S1 File. Sleeping pattern and activities of daily living modulate protein expression in AMD (PONE-D-20-29337R1).
(DOCX)

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Author Contributions

Conceptualization: Kaushal Sharma, Akshay Anand.

Formal analysis: Kaushal Sharma, Suresh Kumar Sharma.

Funding acquisition: Akshay Anand.

Investigation: Kaushal Sharma, Ramandeep Singh.

Methodology: Kaushal Sharma, Ramandeep Singh.

Project administration: Akshay Anand.

Software: Kaushal Sharma.

Supervision: Suresh Kumar Sharma.

Writing – original draft: Kaushal Sharma.

Writing – review & editing: Ramandeep Singh, Suresh Kumar Sharma, Akshay Anand.

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RESEARCH ARTICLE

Amyotrophic Lateral Sclerosis (ALS) prediction model derived from plasma and CSF biomarkers

Radhika Khosla[1], Manjari Rain[1], Suresh Sharma[2], Akshay Anand[1]*

1 Department of Neurology, Neuroscience Research Lab, Post Graduate Institute of Medical Education and Research, Chandigarh, India, **2** Department of Statistics, Panjab University, Chandigarh, India

* akshay1anand@rediffmail.com

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a degenerative disorder of motor neurons which leads to complete loss of movement in patients. The only FDA approved drug Riluzole provides only symptomatic relief to patients. Early Diagnosis of the disease warrants the importance of diagnostic and prognostic models for predicting disease and disease progression respectively. In the present study we represent the predictive statistical model for ALS using plasma and CSF biomarkers. Forward stepwise (Binary likelihood) Logistic regression model is developed for prediction of ALS. The model has been shown to have excellent validity (94%) with good sensitivity (98%) and specificity (93%). The area under the ROC curve is 99.3%. Along with age and BMI, VEGF (Vascular Endothelial Growth Factor), VEGFR2 (Vascular Endothelial Growth Factor Receptor 2) and TDP43 (TAR DNA Binding Protein 43) in CSF and VEGFR2 and OPTN (Optineurin) in plasma are good predictors of ALS.

Introduction

Amyotrophic Lateral Sclerosis (ALS), a multi-system neurodegenerative disorder, is a rare motor neuron disease. The symptoms involve the degeneration of upper and lower motor neurons along with weak muscular strength, lost ability of movement and speech leading to total or partial paralysis. Talbott et al have reported the global prevalence of disease to be 6/100,000 individuals [1] with an approximate male: female ratio of disease incidence to be 1:3 [2]. Riluzole is the only known Food and Drug Administration (FDA) approved drug for ALS which gives only symptomatic relief to patients [3].

Diagnosis and prognosis of ALS is dependent upon clinical investigations. Various models have been proposed to predict the survival and prognosis of the disease [4–8]. These can also help in analysing the course of disease progression during clinical trials. Diagnosing ALS using clinical investigations can take a long time that leads to certain delay in starting the treatment of patients. Hence, diagnosing ALS at the earlier stages of the disease is immensely important.

Abbreviations: ALS FRS R, Revised ALS Functional Rating Score; ALS, Amyotrophic Lateral Sclerosis; ANG, Angiogenin; BMI, Body Mass Index; CCL2, Chemokine Ligand 2; CSF, Cerebrospinal Fluid; ELISA, Enzyme Linked Immunosorbent Assay; FDA, Food and Drug Administration; OPTN, Optnieurin; PGIMER, Post Graduate Institute of Medical Education and Research; ROC, Receiver Operating Characteristics; SPSS, Statistical Product and service Solutions; TDP43, Transactive response DNA Binding Protein 43; VEGF, Vascular Endothelial Growth Factor; VEGFR2, VEGF Receptor 2.

Biomarkers are the measures that can provide significant information about the disease prediction or progression. In our previous study, out of a panel of six biomolecules including Vascular Endothelial Growth Factor (VEGF), VEGF receptor 2 (VEGFR2), Angiogenin (ANG), Optineurin (OPTN), Transactive response DNA binding protein 43 (TDP43) and Chemokine Ligand 2 (CCL2), five biomolecules were found to be significantly altered in plasma [9]. In another study, Cerebrospinal Fluid (CSF) from the same cohort (approx. half of the patients) was analysed for the same six molecules [10]. Three of the molecules, involved in the angiogenic and neuroprotective pathway, were found to be significantly altered. In the absence of a single biomarker for the disease diagnosis, analysing a panel of molecules in various biofluids simultaneously can have predictive value for ALS. The previous logistic regression model was proposed based on VEGF and CCL2 mRNA levels, serum levels of CCL2 and consumption of smoking and alcohol data with high sensitivity and specificity [11]. However, the model included fewer numbers of patients and only three biomolecules i.e VEGF, CCL2 and lipid hydroperoxides were studied.

We aimed to develop a predictive statistical model based on new panel of six bio-molecules analysed in Plasma and CSF of patients along with their socio-demographic characteristics of patient population. The forward stepwise (binary likelihood) logistic regression model proposed in the present study can predict ALS with high sensitivity and specificity.

Methods

Participants

Total 239 participants (107 ALS and132 controls) were recruited. All the participants provided informed consents. The study approval was provided by the Institutional ethical committee (IEC approval number PGI/IEC/2014/2249) of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. ALS patients were recruited from the Neurology outpatient Department. Among all participants, biomarkers were estimated in 187 unhaemolysed plasma and 86 CSF samples. Socio-demographic data was also collected for variables such as gender, age, BMI, smoking, alcohol, diet, ALSFRS-R, disease onset and duration of the disease. The criterion for including samples in statistical analysis was that there is no missing value for any of the 21 variables. The logistic regression for developing the model was developed considering 23 ALS patients and 14 controls. The patients were diagnosed clinically and recruited on the basis of revised El Escorial criteria [11–13]. All the patients were found to be sporadic on the basis of family history. According to el Escorial criteria, the patients were categorised as definite/possible/probable ALS.

Statistical analysis and modelling

All the statistical tests were done using Statistical Product and service Solutions (SPSS v 23.0 SPSS Inc., Chicago, USA). Descriptive statistics was applied to analyse the distribution of data for various parameters. Binary Logistic regression model was applied for predicting risk of ALS based on the quantitative and qualitative data collected. Total 21 variables were tested including the proteins levels such as VEGF, VEGFR2, ANG, OPTN, TDP43 and CCL2 in plasma and CSF and socio-demographic details such as gender, age, BMI, smoking, alcohol, diet, ALSFRS-R, disease onset and duration of the disease. A forward stepwise (likelihood ratio) method was used for applying the model.

Results

Development of ALS predicting logistic regression model

Forward stepwise (likelihood ratio) binary logistic regression analysis was performed to compute the predicted risk (P) of ALS with the help of the following equation

$$P = \frac{1}{1 + e^{-Y}}$$

Where, Y is model score.

Before calculating Y, Hosmer–Lemeshow goodness of fit statistic was applied to test whether the given data fits to the logistic model. Null hypothesis (H_0) indicating that the given data fits well to the logistic model was tested and chi square (χ^2) = 0.468, degree of freedom (df) = 8 and p = 0.994 suggests that the logistic model is adequately in agreement with the null hypothesis and fits the data.

Omnibus test of model coefficients also confirmed that forward stepwise (likelihood) binary logistic regression is highly appropriate analysis for generating predictive equation. Omnibus test yielded $\chi^2 = 255.58$, df = 7 and $p<0.001$.

The Wald test showed that out of the 21 predictors only 7 predictors were significant and can predict the risk of ALS. Following equation was obtained from the Beta values obtained by Wald test and are presented in Table 1.

Model Score (Y) = -57.04 + 0.151 Age—0.243 BMI -2501.477 VEGFR2 plasma level + 93.109 OPTN plasma level—0.244 VEGF CSF level + 3.184 VEGFR2 CSF level—0.130 TDP43 CSF level.

Adequacy of the logistic regression model was supported by -2 log-likelihood method with $\chi^2 = 51.73$. Coefficient of determination (R^2) was computed using Cox and Snell's, and Nagelkerke's R^2, to check the association of variables in current logistic regression model. R^2 close to 1 suggests strong association of selected independent variables with dependent variables. The present logistic regression model has Cox and Snell's $R^2 = 0.684$ and Nagelkerke's $R^2 = 0.912$.

Validity of logistic regression model

The Correct classification using logistic regression model of ALS was 94%. Sensitivity and specificity of the logistic regression model was 98% and 93%, respectively. Receiver operating characteristic (ROC) curve with 7 predictive variables revealed that the model for predicting ALS risk is an excellent model, as the area under the curve was 99.3% (Fig 1). As expected the ROC curve has low standard error of 0.003 with 95% confidence interval as 0.986–0.999 (Table 2).

Discussion

ALS is a motor neuron disease caused by degenerative changes in the motor neurons of spinal cord and cortical regions in brain. The degenerated neurons lead to impaired synaptic connections with muscles leading to paralysis in patients. In severe cases this may lead to respiratory failure causing fatality [14]. 10% of the cases have family history of ALS and are known as familial ALS cases. However, 90% of the cases are sporadic and occur because of mutations in varied number of genes. Most commonly associated cases are of C9ORF72 and SOD1 genes [15]. The variability in the pathophysiology of disease makes it a multi system degenerative disease or a multivariate disease. This multi system degeneration obscures the diagnosis, prognosis and treatment strategies even more. However, early prediction of the disease and predictive

Table 1. Significant independent variables revealed by maximum likelihood method for logistic regression equation.

Variables	Beta (β)	Standard error	Wald	Degree of freedom	p-value
Age	0.151	0.040	14.601	1	<0.001
BMI	-0.243	0.106	5.296	1	0.021
VEGFR2 plasma level	-2501.477	668.648	13.996	1	<0.001
OPTN plasma level	93.109	33.889	7.548	1	0.006
VEGF CSF level	-0.244	0.071	11.777	1	0.001
VEGFR2 CSF level	3.184	0.639	24.841	1	<0.001
TDP43 CSF level	-0.130	0.038	11.378	1	0.001
Constant	-57.040	13.953	16.713	1	<0.001

Abbreviations: BMI Body Mass Index, VEGFR2 Vascular Endothelial Growth Factor Receptor 2, OPTN Optineurin, VEGF Vascular Endothelial Growth Factor, CSF Cerebrospinal Fluid, TDP 43 Transactive Response DNA Binding Protein 43.

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prognostic patterns for individualised or cohorts of patients can help in finding effective treatment strategies for ALS and delaying ALS-related adversity and mortality.

Failing to find a single molecule or factor for diagnosis, opting for a panel of markers or some statistical models or equations can help in prediction of ALS. Such models can also help in analysing the prognosis of disease in patients. We have analysed such a panel of markers that are involved in pathways of pathology of disease. VEGF [16–18], VEGFR2 [19, 20] and ANG [21, 22] have been studied in respect to angiogenic pathways as well as in neuroprotective pathways. The dysregulated levels of angiogenic molecules can cause oxidative stress. Oxidative stress has been linked to neuronal degeneration in various studies [23, 24]. The soluble counterpart of another VEGF receptor (sVEGFR1) has been associated with ALS in previous lab studies. These molecules have been shown to be neuroprotective in various studies. Other

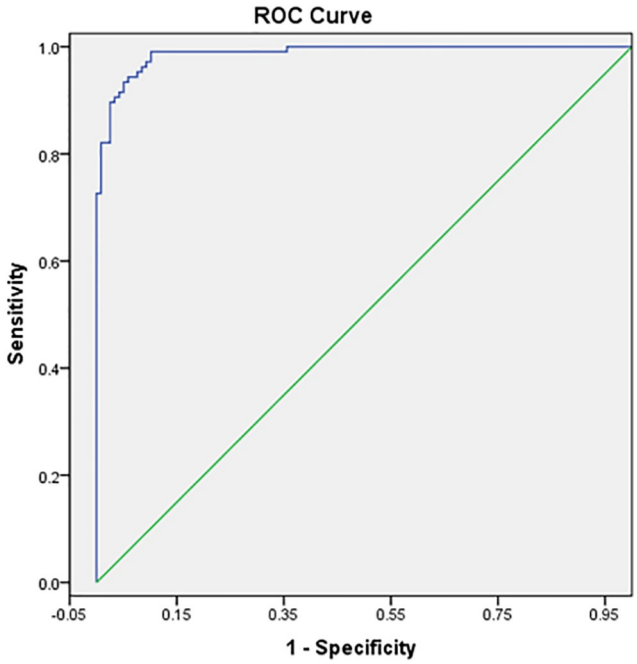

Fig 1. Receiver operating characteristic (ROC) for the forward stepwise binary logistic regression model for predicting ALS risk.

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Table 2. Validity of logistic regression model.

Area Under the Curve				
Test Result Variable(s): Predicted probability				
Area	**Standard Error[a]**	**p value[b]**	**Asymptotic 95% Confidence Interval**	
			Lower Bound	**Upper Bound**
0.993	0.003	<0.001	0.986	0.999

[a]. Under the nonparametric assumption

[b]. Null hypothesis: true area = 0.5

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two molecules OPTN [25] and TDP43 [26] are associated with proteinopathy, which is a characteristic of ALS. Both molecules have been found to be accumulated in protein inclusions in the cytoplasm of neurons. Also TDP43 levels have been measured in CSF and serum of ALS patients as it is a major content of motor neuron inclusions. CCL2 is the main molecule of neuroinflammation pathway, which is also a characteristic feature of ALS [18, 27–29]. VEGF and CCL2 have also been shown to contribute significantly to the regression model developed by Gupta et al [11]. In this study, the molecules have been studied in plasma and CSF, both. Since there are theories that CSF is the fluid that may carry the pathogenic markers responsible for degeneration of motor neurons, measurement of proteins in CSF is important.

In combination with molecular markers simple socio-demographic factors such as age and BMI can also contribute significantly as predicting factors in logistic regression model. Also the protein levels in serum [30, 31] and SNPs [29, 32–34] can be seen in various candidate molecules and then the biomarker potential of these molecules can be explored using such regression models. Analysing the panel of markers in plasma and CSF both along with socio-demographic and clinical details can add more value to the model developed and improve the sensitivity and specificity of the model. The model has the potential of prediction of ALS even though other prognostic and survival prediction models have also been developed in the past years.

The model should be tested on larger cohorts to study the validity and predictability. Also, the markers should be analysed in CSF to blood and at cellular level (in the form of gene expression) to add to the validity of models.

Conclusion

The proposed forward stepwise (binary likelihood) logistic regression model has shown high sensitivity and specificity. Also, the 99.3% area under the curve is indicative of the excellence of the model in predicting the risk of ALS. However, lesser sample size can be shortcoming of the predictive model. Developing such risk predicting models or combined models that can predict the risk of disease as well as survival using bigger cohorts of participants can be helpful in understanding the aetiology of ALS.

Supporting information

S1 Data.
(XLSX)

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Author Contributions

Conceptualization: Akshay Anand.

Data curation: Radhika Khosla.

Formal analysis: Radhika Khosla, Suresh Sharma.

Funding acquisition: Akshay Anand.

Investigation: Radhika Khosla.

Methodology: Radhika Khosla.

Project administration: Akshay Anand.

Supervision: Akshay Anand.

Validation: Suresh Sharma, Akshay Anand.

Visualization: Akshay Anand.

Writing – original draft: Radhika Khosla, Manjari Rain.

Writing – review & editing: Radhika Khosla, Manjari Rain, Akshay Anand.

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Working Memory Alterations Plays an Essential Role in Developing Global Neuropsychological Impairment in Duchenne Muscular Dystrophy

Rahul Tyagi[1], Harshita Arvind[1], Manoj Goyal[2], Akshay Anand[1] and Manju Mohanty[3]**

[1] Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, [2] Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India, [3] Department of Neurosurgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Background: Neuropsychological profile of Indian Duchenne muscular dystrophy (DMD) subjects remains unidentified and needs to be evaluated.

Methods: A total of 69 DMD and 66 controls were subjected to detailed intelligence and neuropsychological assessment. The factor indexes were derived from various components of Malin's Intelligence Scale for Indian Children (MISIC) and Rey Auditory Verbal Learning Test (RAVLT).

Results: Poor verbal and visual memory profiles were demonstrated by DMDs, which include RAVLT-immediate recall (IR) (p = 0.042), RAVLT-delayed recall (DR) (p = 0.009), Rey–Osterrieth complex figure test (RCFT)-IR (p = 0.001), and RCFT-DR (p = 0.001). RAVLT-memory efficiency index demonstrated poor verbal memory efficiency (p = 0.008). Significant differences in the functioning of working memory axis [RAVLT T1 (p = 0.015), recency T1 (p = 0.004), Digit Span Backward (p = 0.103)] were observed along with reduced performance in visuomotor coordination, visuospatial, and visual recognition abilities. Block designing efficiency index and attention fraction showed a normal performance in DMD kids.

Conclusion: Working memory deficits were found to be the crucial element of cognitive functioning in DMD cases. Working memory interventions may be beneficial to improve the neuropsychological profile in DMD.

Keywords: neuropsychology, DMD, Duchenne muscular dystrophy, intelligence, working memory

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Daniela Smirni,
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Reviewed by:
Umbertina Conti Reed,
University of São Paulo, Brazil
Jennifer Andrews,
University of Arizona, United States

****Correspondence:***
Manju Mohanty
manjumohanty2011@gmail.com
Akshay Anand
akshay1anand@rediffmail.com

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a rare X-linked inherited progressive neuromuscular dysfunction caused by pathogenic variations in the *DMD* gene encoding a rod-shaped protein called dystrophin, which provides an anchor between the cytoskeleton and extracellular matrix resulting in proper muscular integrity and strength (Koenig et al., 1987; Kunkel et al., 1987). The absence of dystrophin results in loss of structural integrity leading to progressive muscular weakness

ultimately resulting in loss of ambulation and early death in the twenties due to respiratory and cardiac dysfunction (Moser, 1984; Mercuri and Muntoni, 2013).

Though *Duchenne de Boulogne* reported intellectual disability in his descriptions (Duchenne, 1868), the cause of impairment of cognitive domains remain undetermined. Several initial reports indicated a moderate reduction in the intelligence quotient (IQ) in approximately one-third of patients with DMD with more severe affection of verbal compared to performance IQ (Cohen et al., 1968; Cotton et al., 2001, 2005). These studies also suggested the crucial role of dystrophin protein in the development of the intellectual trajectory. A comparison of DMD to patients with similar conditions like spinal muscular atrophy provided evidence of the cognitive deficits not due to motor dysfunction (Billard et al., 1992). The presence of non-progressive mental retardation is a prominent clinical feature in DMD (Bresolin et al., 1994). We have also reported non-progressive intellectual and neuropsychological deficits in our DMD cohort (Tyagi et al., 2019b).

Subsequent research reported variable degrees of impairment in specific cognitive domains involving immediate verbal memory, visual memory, and attention (Anderson et al., 1988). Donders and Taneja (2009) reported significant deficits in the delayed verbal recall without any difference in immediate memory tasks in DMD patients compared to their unaffected siblings (Donders and Taneja, 2009; Anand et al., 2015). Recent studies have also reported deficits in the executive functioning including planning and inhibition among DMD patients (Mento et al., 2011) when compared to controls (motor impairment: SMA, typically developing children: rheumatoid arthritis, and unaffected sibling). The brain is also an important site of dystrophin expression, where deficit may cause severe mechanical alterations. Along with full-length dystrophin protein (Dp427), short dystrophin isoforms including Dp260, Dp140, Dp116 Dp71, and Dp40 are also expressed from the internal promoters, which are named according to their length in kilodaltons (Muntoni et al., 1995; Daoud et al., 2009; Naidoo and Anthony, 2020). Full-length dystrophin is expressed in a tissue-specific manner through the proximal promoters in the brain, whereas short dystrophin isoforms are expressed through distant upstream promoters (Boyce et al., 1991). In mouse, dystrophin was reported to be localized in the neuronal postsynaptic specializations of cerebellar Purkinje's cells and in cerebral cortical pyramidal cells (Lidov et al., 1990). Moreover, human DMD brain study also reported deficiency of dystrophin in the postsynaptic densities (PSD) of the brain (Kim et al., 1995). Dp140, Dp71, and, an alternatively spliced shortest isoform, Dp40 are reported to be expressed in the various brain regions including the cerebral cortex, cerebellum, and hippocampal dentate gyrus (Greenberg et al., 1996; Lidov and Kunkel, 1997; Daoud et al., 2009; Tozawa et al., 2012; Naidoo and Anthony, 2020). The expression of dystrophin and short isoforms in the crucial brain regions indicates their crucial role for higher-order cognitive functioning (Doorenweerd, 2020). Dystrophin's localization and interaction in the brain regions for performing higher-order functions necessitate the domain-wise investigation according to the population dynamics (Wicksell et al., 2004; Doorenweerd, 2020; Tyagi et al., 2020).

Similar genotype–phenotype correlation studies have previously associated the neurological and intellectual outcomes in various neurological disorders in which genetic screenings indicated specific pathologies (Anand et al., 2007, 2012a,b, 2015; Sharma et al., 2012; Goyal et al., 2014). However, in Indian children with DMD, studies elucidating the cognitive and neuropsychological functioning are scarce. A South Indian study reported significantly lower IQ in DMD subjects compared to the normative data (Perumal et al., 2015). However, the small sample size, absence of a matched control group, and less coverage of neuropsychological domains were major limitations of this study. Hence, we studied the cognitive function of DMD children by assessing various cognitive and neuropsychological domains and compared these results with a matched control group.

MATERIALS AND METHODS

Participants

The current study included 69 children with DMD. Sixty-six age-, sex-, and education-matched children and adolescents in the age group of 6–16 years served as a control group. The inclusion criteria included a diagnosis of DMD based on clinical features and genetic tests. The exclusion criteria included children having psychiatric co-morbidities including autism spectrum disorders, attention deficit hyperactivity disorder (ADHD), and epilepsy, etc. The study was approved by the Institutional Ethical Committee (IEC). Written informed consent was obtained from the parents or legal guardians (as children were minor) before inclusion in the study. The study was conducted in the Neuroscience Research Lab of Postgraduate Institute of Medical Education and Research, Chandigarh, India. The pathogenic variants in the *DMD* gene were obtained by multiplex ligation-dependent probe amplification as described earlier in our cohort (Tyagi et al., 2019a).

Assessment of Cognitive Functions

Intelligence

Malin's Intelligence Scale for Indian Children (MISIC) [an Indian adaptation of Wechsler Intelligence Scale for Children (WISC)] was used to assess intelligence (Malin, 1970). It measures both verbal and performance IQ. *Verbal subsets* included tests of information, comprehension, arithmetic, analogies, and similarities; vocabulary; and Digit Span (DS) test, while *performance subsets* included the picture completion, block designing, coding, maze, and object assembly. Raw scores for each case were converted into age-adjusted test quotients using the normative data. Verbal IQ (VIQ) and performance IQ (PIQ) were obtained by averaging the tests of verbal subsets and performance subsets, respectively. The average score of VIQ and PIQ was used to obtain the global intelligence quotient (IQ).

Assessment of Specific Cognitive Domains

Subjects with IQ > 69 were carried forward for a detailed assessment of specific cognitive domains. Details of test batteries and corresponding cognitive and neural correlates are provided in **Supplementary Table 1**.

Rey Auditory Verbal Learning Test

Rey Auditory Verbal Learning Test (RAVLT) was used to measure learning, working, short- and long-term memory, susceptibility to interference, serial positioning effect, recognition memory, and verbal memory efficiency index. In the present study, the adapted version for the Indian population was used (Kar et al., 2004). A list of 15 nouns (list A) was read aloud during five consecutive trials. In each trial, an interval of 1 s was maintained between presentations of two nouns. A subsequent list (list B) of 15 words were presented after list A (trials 1–5) as an interference. The subject was asked to recall the list A immediately after the list B task. After 20 min, the subject was then instructed to recall list A again to access the long-term verbal memory. To assess the recognition, 30 words (15—list A and 15 other words) were presented and the subject was asked to recognize list A out of 30 words. Omissions (from list A) and commissions (non-list A words) were recorded. The number of words correctly recalled in each RAVLT trial as well as in immediate (after list B) and delayed recall (after 20 min) tasks were used as scores. Learning capacity was assessed by summing the total list A words recalled over five trials. The subject's susceptibility to interference was obtained through proactive interference (PI) and retroactive interference (RI). PI reveals the negative effect of previously learned material in the acquisition or recall of new information (Vakil et al., 2010). Similarly, RI reveals the negative effect of new learning in recalling previously learned information. Serial positioning effect was assessed by obtaining primacy (first one-third of 15 words), middle (middle one-third of 15 words), and recency (last one-third of 15 words) scores of list A trials 1–5 (Boone et al., 2005). RAVLT Memory Efficiency Index (MEI) was obtained by using all RAVLT components based on a previous study (Ricci et al., 2012). Calculations to obtain various factor indexes from RAVLT data have been depicted in **Supplementary Table 2**.

Rey–Osterrieth Complex Figure Test

It was administered to measure visual learning, short- and long-term visual memory, and visuo-constructive ability of the child. This figure consists of a complex design with multiple subcomponents, which was placed in front of the subject. For visuo-constructive ability, the subject was instructed to draw the figure on the paper with freehand. For visual memory, the complex figure was recalled twice, an immediate recall (IR) immediately after copying task and delayed recall (DR) after 30 min. Scoring was done based on the accuracy and placement of the components of a complex figure based on a previous study (Duley et al., 1993).

Stroop Color and Word Test

Stroop test, a measure of executive functioning, was used to record response inhibition, selective attention, and cognitive flexibility (Golden, 1975). The Stroop Color and Word Test (SCWT) consists of a 5-by-20 matrix of words representing three colors (red, blue, and green) each in three sheets to record neutral, congruent, and incongruent tasks. The first sheet consisted of 100 (5 × 20) names of three colors (red, blue, and green) printed only in black color to record the neutral task. The second sheet consisted of 100 (5 × 20) XXXX symbols printed in three colors (red, blue, and green) to record congruency. The third sheet consisted of 100 (5 × 20) color names, which were printed in another color (red/blue/green), e.g., red-written word printed in blue/green color as an incongruent task. Subjects were instructed to name the color instead of reading the written words down the column. Forty-five seconds were provided to finish each task. The number of words read in each sheet was considered the score of a participant. The last task has an interference component because it requires the participant to override or inhibit a reading response. This test measures the ease with which a person can shift his or her perceptual set to conform to changing demands and inhibit the usual response from interfering with the unusual one. The interference component (also called Stroop effect) was calculated based on the following formula: Stroop effect = SCWT color (raw) − SCWT color − word (raw) (Jensen and Rohwer, 1966).

Children's Color Trail Test A & B

Children's Color Trail Test (CCTT) is a measure of sustained attention and is found to be very sensitive to brain damage (Llorente et al., 2003). It has two parts, part A and part B. In trail A, circles were numbered 1 to 15 in two colors: yellow and pink. The subject was required to connect the numbers 1 to 15. Trail B consisted of the 1 to 15 numbered circles in two colors: pink and yellow, and the subject was required to link the numbered circles with alternative colors. The time taken to complete the task and interference index was considered an outcome measure. The interference component was calculated based on the following calculation: CCTT interference index = (CCTT2 time raw score − CCTT1 time raw score)/CCTT1 time raw score.

Color Cancellation Test

This test is a measure of focused attention, accurate visual scanning, and activation or inhibition of rapid response (Llorente et al., 2003). A sheet consisting of 150 circles in five different colors (red, yellow, blue, black, and gray) was presented. The subject was required to cancel only the red and yellow circles as fast as possible. The time taken to complete the task was used as scores.

Controlled Oral Word Association Tests

It measures phonemic verbal fluency. The original Controlled Oral Word Association Tests (COWA) used alphabets starting with FAS to generate words (Ruff et al., 1996). It has been adapted for the Indian population (Kar et al., 2004). Subjects were asked

to generate words beginning with Ka, Pa, and Ma as many as possible in 1 min. Scoring was done according to acceptable new word formation over three trials.

Animal Naming Test

The animal naming test is a measure of the category verbal fluency. The animal naming test requires the subject to generate the names of animals as many as possible in 1 min. Scoring was done according to the generation of newly formed words (Regard et al., 1982).

Visual Recognition Test

This test has been selected from the NIMHANS battery for children manual (Kar et al., 2004). It is a measure of visual agnosia and the capacity of the subject to recognize subjects visually. The test consists of a card with 10 pictured objects that were required to be recognized and named by the subject.

Statistical Analysis

SPSS (ver. 21.0) was used to perform statistical analysis. Kolmogorov–Smirnov (KS) test was performed to check the normality of data variables. A comparison between two independent normally distributed data was carried out by independent Student's *t*-test with equal or unequal variance (Welch's correction). For non-normally distributed data, a non-parametric Mann–Whitney *U* test was performed. A *p*-value $\leq$ 0.05 was considered to be significant for testing the hypothesis. The study seeks to show that working memory underlies cognitive deficits in this population. However, this cannot be demonstrated by simply showing that working memory tasks are correlated with all the other tasks. Hence, regression analyses and domain factor scores were derived to demonstrate dissociation by showing that other domains do not perform as consistently as working memory in predicting other skills. Linear regression was performed and scales of working memory functioning were considered dependent variables (RAVLT1, Digit Span Backward, RAVLT MEI, WMI, and Stroop task color word scale). The forward selection method was chosen for analysis. Principal component analysis was performed followed by varimax rotation with Kaiser normalization. Eigenvalue > 1 was considered. The factor structure of DMD cases and controls was analyzed. An absolute value below 0.3 was considered a coefficient display.

TABLE 1 | Demographic details of study participants.

Demographic parameter	DMD (*n* = 69)	Control (*n* = 66)	*p*-value
Age, mean (SD)	10.78 (2.65)	10.37 (2.1)	0.371
Education level, mean (SD)	4.22 (2.3)	4.21 (2.05)	0.971
Income per month (INR)	34,755 (48621)	40,131 (52555)	0.626
Age of onset (years), mean (SD)	4.16 (2.10)	NA	NA
Disease duration	6.36 (2.7)	NA	NA

RESULTS

Demographic Variables

Demographic details of DMD and control subjects have been provided in **Table 1**.

Intellectual Functioning in DMD

General intellectual abilities of DMD subjects were categorized based on ICD-10 guidelines. Out of 69 DMD patients, 48 (69.56%) had adequate intelligence (IQ > 84) and 16 (23.18%) demonstrated borderline intelligence (IQ 70–84). Only 5 (7.24%) subjects demonstrated intellectual disability (IQ < 70), among which 1(1.44%) had moderate and 4 (5.79%) had mild intellectual disability. Similarly, verbal IQ was found to be impaired in 8.69% DMD cases whereas 5.79% demonstrated impairment in performance IQ. In the control group, all had adequate intelligence.

Duchenne muscular dystrophy subjects revealed a mean IQ of 90 (SD = 14.54; range = 42–123), VIQ of 87 (SD = 14.71), and PIQ of 93 (SD = 16.29) shown in **Table 2**. Verbal discrepancy (VIQ-PIQ) of −6.50 (SD = 10.80) was observed compared to −2.07

TABLE 2 | Comparison of general intelligence among DMD and control population.

General intelligence	DMD Mean (SD)	Control Mean (SD)	*t*-value	*p*-value
Verbal intelligence quotient	87 (14.71)	108 (14.30)	−8.559	<0.001
Performance intelligence quotient	93 (16.29)	110 (13.00)	−6.695	<0.001
Intelligence quotient	90 (14.54)	109 (11.33)	−8.571	<0.001
VIQ-PIQ	−6.50 (10.80)	−2.07 (15.51)	−1.918	0.058

TABLE 3 | Comparison of subsets of general intelligence between DMD and control groups.

MISICsubtests	DMD Mean (SD)	Control Mean (SD)	*t*-value	*p*-value
Verbal subtests				
Information	12.08 (4.57)	15.58 (4.96)	−4.179	<0.001
Comprehension	9.86 (4.73)	15.20 (5.76)	−5.782	<0.001
Arithmetic	7.80 (2.75)	10.58 (2.55)	−5.959	<0.001
Digit span	7.83 (2.46)	9.03 (2.37)	−2.830	0.005
Similarity	10.27 (4.96)	15.11 (5.12)	−5.163	<0.001
Performance subtests				
Picture completion	6.94 (2.73)	9.75 (2.66)	−5.895	<0.001
Block design	16.02 (12.84)	21.69 (11.75)	−2.522	0.013
Coding	27.91 (15.82)	38.85 (13.03)	−4.109	<0.001
Maze	14.98 (10.12)	17.68 (1.88)	−2.013	0.048
Object assembly	7.750 (7.19)	11.914 (5.52)	−2.692	0.009
Factor indexes				
VCI	255 (57.14)	338 (50.78)	−8.685	<0.001
WMI	172(27.41)	201 (29.37)	−5.907	<0.001
PRI	165 (54.16)	205 (47.26)	−4.498	<0.001
FOD	172 (27.41)	201 (29.80)	−5.795	<0.001

(SD = 15.51) of control with significant difference ($t = -1.918$, $p = 0.05$). Seventy-eight percent of DMD participants exhibited less VIQ than the PIQ.

Assessment of Specific Cognitive Abilities

Specific cognitive abilities were assessed only in DMD cases ($n = 64$) with IQ ≥ 70 and the performance was compared with healthy controls. Both groups were matched on age and education.

Intelligence

The mean IQ of DMD and control groups were 92 (SD = 11.48) and 109 (SD = 11.33), respectively, and the differences were significant concerning VIQ and PIQ. Factor index analysis in the DMD subjects revealed poor performance in the Verbal Comprehension Index ($p < 0.001$), Working Memory Index ($p < 0.001$), and Perceptual Reasoning Index ($p < 0.001$) in comparison to the control. Scores have been depicted in **Table 3**.

Sustained and Focused Attention

We used CCTT and CCT to measure sustained and focused attention. The mean CCTT completion time score between DMD and control groups was obtained. DMD group took more time ($p < 0.001$) in completing CCTT-A, a measure of psychomotor sequencing, visual tracking, processing speed, and graphomotor skills, indicating poor performance in the attention domain. Similarly, CCTT-B, representing divided attention, set-switching, inhibition, and working memory/sequencing, was also poorly performed by the DMD group ($p < 0.001$). However, the CCTT interference score was found to have significant ($p = 0.05$) differences in comparison to the control group. CCT, a measure of selective attention and visual scanning, was poorly performed by DMD subjects ($p < 0.001$). Errors made in the CCT task were comparable to the control group.

Executive Functioning

Executive functioning was measured by COWA, Animal Naming Test (ANT), and SCWT task performance. COWA performance indicates the subject's phonemic knowledge and language fluency. The mean retrieval in COWA was 4.29 (SD = 3.00) in comparison to the control group 6.16 (SD = 2.75) with significant differences ($t = -3.675$, $p < 0.001$). In the semantic fluency assessment, DMDs demonstrated a raw score of 8.95 (SD = 3.49) whereas the raw score of controls was 11.48 (SD = 4.08) with significant differences ($t = -3.804$, $p < 0.001$) as depicted in **Table 4**.

Performance on SCWT

Scores of SCWT revealed a poor DMD performance on all measures of SCWT as represented in **Table 4**. Neutral word task score indicating poor personal tempo and speed showed significant poor performance among DMD subjects ($t = -3.361$; $p = 0.001$). The congruent task, a measure of color perception, has also been found to be affected. The incongruent task, a measure of response inhibition and negative priming, was also affected. Stroop effect was calculated and DMD subjects were found to be susceptible to interferences ($p = 0.036$) in comparison to the control group. It indicated poor cognitive control and selective attention of participants.

We used the Visual Recognition Task as a measure of parietal lobe functioning. DMD subjects recognized a reduced

TABLE 4 | Comparison of neuropsychological variables between DMD and control groups.

	Control		DMD		*t*-value/*z*-value	*p*-value	
NP	**Mean**	**SD**	**Mean**	**SD**			**Cohen's *D***
COWA-total	18.52	8.27	12.88	9.01	−3.690	<0.001***	0.65
COWA avg	6.16	2.75	4.29	3.00	−3.675	<0.001***	0.65
ANT	11.48	4.02	8.95	3.49	−3.804	<0.001***	0.67
RCFT-copy	33.01	3.78	28.55	8.77	−3.174	0.002**	0.66
RCFT-IR	25.38	6.86	19.50	9.17	−3.606	0.001**	0.73
RCFT-DR	24.71	6.37	19.21	9.01	−3.460	0.001**	0.70
Stroop-W	58.12	12.75	48.02	17.54	−3.361	0.001**	0.66
Stroop-C	45.83	9.84	35.16	13.46	−4.617	<0.001***	0.91
Stroop-CW	28.03	8.53	20.90	7.75	−4.551	<0.001***	0.87
Stroop effect	17.50	7.58	13.98	9.44	−2.126	0.036*	0.41
CCTT1	34.13	16.05	62.00	42.61	4.212	<0.001***	−0.87
CCTT2	67.28	24.85	100.78	50.41	4.138	<0.001***	−0.84
CCTT interference	1.15	0.78	0.80	0.56	−1.951*	0.051	0.52
CCT	86.75	33.28	127.72	53.75	4.584	<0.001***	−0.92
VRT	9.44	0.73	8.22	1.21	−6.474	<0.001***	1.22

*Number of control (n = 64) and DMD subjects assessed in various scales: COWA and ANT (n = 64), RCFT (n = 44), Stroop test (n = 49), CCTT and CCT (n = 46), and VRT (n = 55). Level of significance *P < 0.05; **P < 0.01; ***P < 0.001.*

number of pictured objects in comparison to the control group (**Table 4**). Block designing (BD) task was used to measure visuo-constructive abilities in DMD. Though the test quotient revealed poor visual-motor coordination ($t = -2.522$, $p = 0.013$), we formed an efficiency index by using test parameters to obtain a motor component-free analysis. There was no difference between DMD and control cases on BD efficiency (0.183). The time taken to complete the BD was comparable to the control subjects ($p = 0.551$). However, the level of complexity of block designing tasks that also require working memory manipulation of resources was found to be affected ($p = 0.011$). Maze scores were analyzed to measure visuospatial ability, a crucial element of working memory. DMD subjects were found to be poor in comparison to the control group. The test quotient ($p < 0.001$) and the time taken in completing the maze task was also significantly different ($p = 0.024$). Similarly, errors made in the maze performance was also significantly higher in the DMD group ($p = 0.009$).

We used RAVLT, DST, and Rey–Osterrieth complex figure test (RCFT) to measure verbal and visual memory changes in DMD and control groups. Comparison of RAVLT learning trials between DMD and control groups showed significant differences in trial 1, $U = 1{,}570$, $p = 0.015$ with a mean (SD) value of 6.24 (2.33) for the DMD group compared to 7.15 (1.92) for the control group, checking these units properly. In trial 1, 27% of DMD subjects showed impairment. The learning trend of DMD subjects was similar to the control group as evidenced by similar performance in each learning trial after trial 1.

Rey Auditory Verbal Learning Test immediate and delayed recall scores were observed to be significantly different in DMD subjects (IR: $U = 1{,}651$, $p = 0.042$; DR: $U = 1{,}525$, $p = 0.009$), indicating poor short- and long-term verbal memory. Moreover, DMD subjects also demonstrated a significant reduction in the long-term percent retention (LTPR) ($U = 1{,}537$; $p = 0.010$) as depicted in **Figure 1**.

No differences in the learning capacity were observed in the DMD subjects ($p = 0.071$). An analysis of serial positioning effect revealed poor recency in DMD cases. This measure of immediate memory significantly differed in comparison to the control group ($U = 1{,}478$, $p = 0.004$).

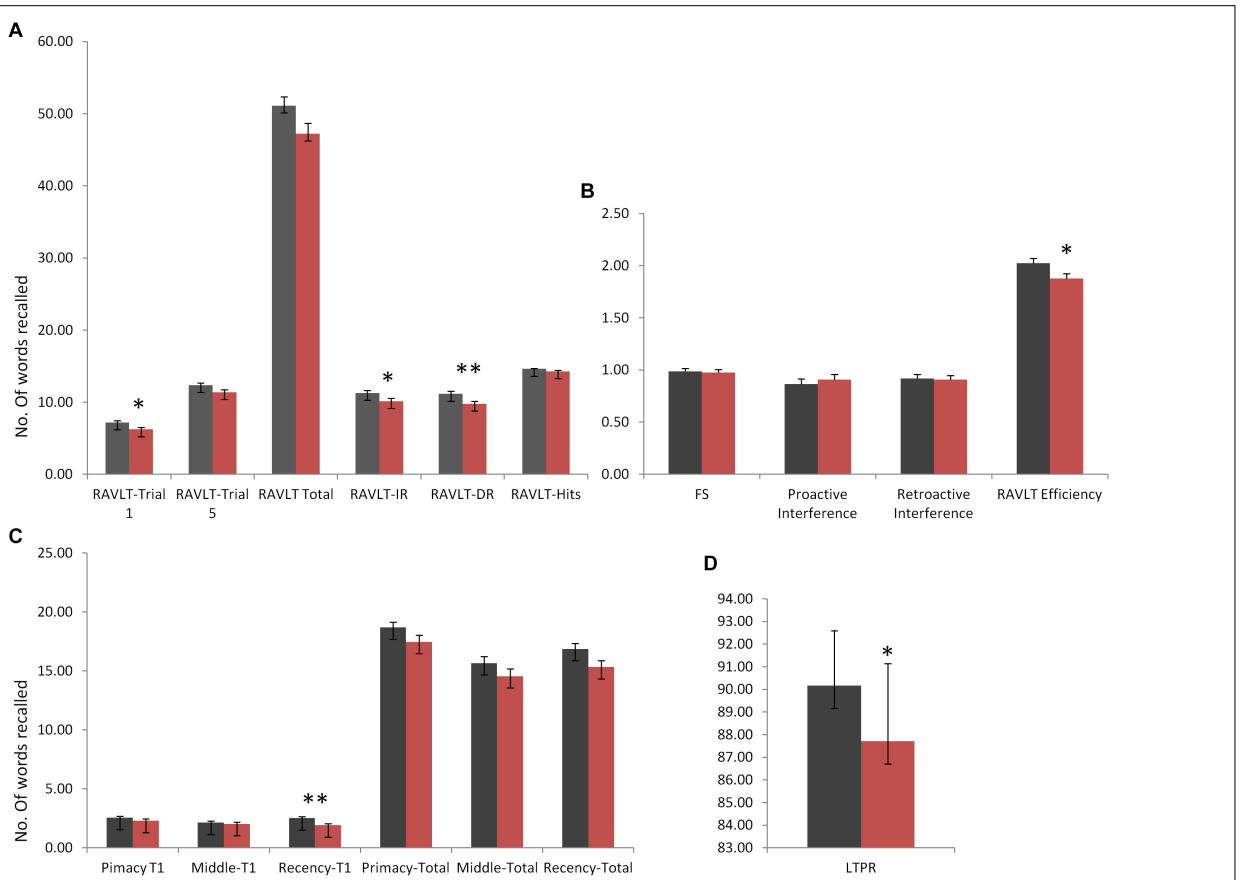

FIGURE 1 | Rey Auditory Verbal Learning Test (RAVLT) data represents trends of verbal memory impairment in Duchenne muscular dystrophy (DMD) subjects. **(A)** A significant difference in trial 1, immediate and delayed verbal memory. **(B)** No difference was observed in the susceptibility to interferences through proactive interference (PI) and retroactive interference (RI) scores. RAVLT-Memory Efficiency Index (MEI) was found to have cut-off values close to the patients of behavioral variant frontotemporal dementia (bvFTD). **(C)** Among serial positioning factors, recency T1 score was found to be reduced significantly. **(D)** Long-term percent retention of DMD subjects was also affected. All results expressed as mean ± standard error of mean (SEM). Data was statistically analyzed using SPSS 16.0 by independent *t*-test/non-parametric test (Mann–Whitney test) as applicable. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TABLE 5 | Comparison of RAVLT variables between DMD (n = 63) and control group (n = 66).

Cognitive domain and neuro psychological battery	Neuro psychological battery variables	DMD Mean ± SD	DMD Mean Rank	Control Mean ± SD	Control Mean rank	*Z*-value	Mann–Whitney *U*	*p*-value
RAVLT	RAVLT-trial 1	6.24 (2.33)	56.91	7.15 (1.92)	72.72	−2.424	1,570	0.015
•Verbal learning	RAVLT-trial 5	11.38 (2.89)	58.89	12.33 (2.36)	70.83	−1.832	1,694	0.067
•Short-term verbal memory	RAVLT-learning capacity	47.24 (11.52)	58.92	50.97 (9.90)	70.80	−1.805	1,696	0.071
•Long-term verbal memory	RAVLT-IR	10.16 (3.23)	58.20	11.29 (2.93)	71.49	−2.031	1,651	0.042
	RAVLT-DR	9.78 (3.04)	56.20	11.14 (3.11)	73.40	−2.626	1,525	0.009
	RAVLT-hits	14.27 (1.18)	58.88	14.61 (0.76)	67.70	−1.648	1,703	0.099
	Omission	0.75 (1.19)	68.33	0.38 (0.72)	59.11	−1.722	1,690	0.085
	Commission	0.87 (2.10)	66.80	0.52 (1.07)	60.50	−1.147	1,782	0.251
	LTPR	87.71 (27.25)	56.40	90.23 (20.01)	73.21	−2.561	1,537	0.010
RAVLT	Primacy-T1	2.30 (1.29)	61.56	2.52 (1.08)	68.29	−1.061	1,862	0.289
•Serial positioning effect	Middle-T1	2.03 (1.22)	63.06	2.12 (1.13)	66.85	−0.593	1,957	0.553
	Recency-T1	1.92 (1.15)	55.46	2.53 (1.15)	74.11	−2.916	1,478	0.004
	Primacy-total	17.46 (4.50)	60.92	18.61 (3.71)	68.89	−1.215	1,822	0.224
	Middle-total	14.56 (5.04)	61.51	15.59 (4.68)	68.33	−1.039	1,859	0.299
	Recency-total	15.33 (4.35)	58.94	16.85 (3.87)	70.79	−1.806	1,697	0.071
RAVLT	Proactive interference	0.91 (0.41)	65.68	0.86 (0.28)	64.35	−0.203	2,036	0.839
•Susceptibility to interferences	Retroactive interference	0.91 (0.31)	61.66	0.92 (0.17)	68.19	−1.008	1,869	0.313
	Forgetting speed	0.98 (0.22)	60.57	0.99 (0.18)	68.19	−1.171	1,803	0.242
	RAVLT efficiency	1.8 (0.36)	56.06	2.0 (0.27)	73.54	−2.655	1,516	0.008

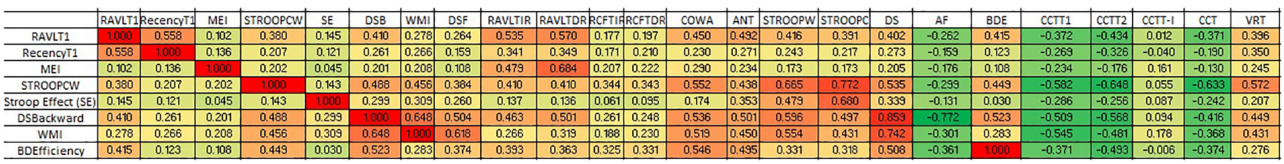

FIGURE 2 | Correlation heat map showing association of working memory with other cognitive domains.

However, primacy and middle words recalling were intact and comparable to the control group. Factor scores including proactive interference, retroactive interference, and forgetting speed from the RAVLT data are compiled in **Table 5**, which showed no difference when compared to control subjects. RAVLT Memory Efficiency Index (RAVLT-MEI) was calculated by incorporating various RAVLT components as shown in **Supplementary Table 2**. An RAVLT memory efficiency index of 1.8 was significantly lower among DMD subjects than the control group with RAVLT-MEI of 2.0 (U = 1,516, P = 0.008). Impairment in the RAVLT trials has been depicted in **Table 5**.

DST

The Digit Span subtest of MISIC provides an opportunity to understand the attentional loop of working memory. The DMD group's Digit Span forward performance was statistically comparable to the control group (p = 0.159), indicating a normal auditory short-term memory and simple verbal expression. However, Digit Span Backward task performance revealed an inefficient working memory domain in comparison to the control group (p = 0.013). Differences between the DSF and DSB tasks have been suggested to tease out attention from the working memory (Hale et al., 2002). The fraction of attention was obtained by the pooled Digit Span score and it was found to be comparable to the control group (U = 1,663, p = 0.067). It also indicated the crucial role of working memory in the DMD cognitive functioning.

Performance on RCFT

Compared to control group, DMD patients demonstrated a significantly lowered score on the RCFT copy (t = −3.174, p = 0.002), immediate recall (t = −3.606, p = 0.001), and delayed recall (t = −3.460, p = 0.001) as depicted in **Table 4**.

TABLE 6 | Linear regression of DMD neuropsychology data.

Dependentvariable	Model	Unstandardized coefficients		Standardized coefficients	*t*-score	*P*-value
		B	Std. error	Beta		
RAVLT1*	(Constant)	2.104	0.810		2.597	0.011*
	RAVLTDR	0.437	0.071	0.545	6.166	<0.001***
	(Constant)	1.103	0.867		1.272	0.207
	RAVLTDR	0.356	0.075	0.444	4.760	<0.001***
	STROOPW	0.035	0.013	0.251	2.693	0.008**
DSTB	(Constant)	0.578	0.536		1.079	0.290
	STROOPW	0.058	0.010	0.762	6.112	<0.001***
	(Constant)	2.877	0.922		3.120	0.004**
	STROOPW	0.040	0.010	0.527	3.850	0.001**
	CCTT2	−0.016	0.006	−0.398	−2.907	0.007**
RAVLT1	(Constant)	4.755	0.740		6.424	<0.001***
	COWAAVG	0.379	0.108	0.561	3.519	0.002**
	(Constant)	11.628	3.112		3.736	0.001**
	COWAAVG	0.465	0.107	0.687	4.334	<0.001
	MISICVIQ	−0.079	0.035	−0.359	−2.264	0.032*
	(Constant)	10.986	2.907		3.778	0.001**
	COWAAVG	0.408	0.103	0.604	3.970	0.001**
	MISICVIQ	−0.104	0.034	−0.473	−3.035	0.006**
	RAVLTIR	0.284	0.126	0.351	2.255	0.033*

*Level of Significance *P < 0.05; **P < 0.01; ***P < 0.001.*

Estimating the Effect Size

Cohen's *D* values were calculated for each parameter. Among the RAVLT parameters, recency T1 (effect size, ES = 0.53) and RAVLT efficiency index (ES = 0.63) showed a medium effect size. RCFT data showed medium effect in the RCFT copy (ES = 0.66), RCFT-IR (ES = 0.73), and RCFT-DR (ES = 0.70). Executive functioning domains including Stroop congruent (ES = 0.91) and incongruent task (ES = 0.87), CCTT-A (ES = −0.87), CCTT-B (ES = −0.84), and CCT (ES = −0.92) tasks revealed large ES. The effect size for Visual Recognition Test (VRT) was found to be very large (ES = 1.22).

Association of Working Memory Components With Various Cognitive Domains

Correlation between various cognitive domains showed that working memory components have been highly correlated with the executive functioning domains. It also revealed the flow of domain dysfunctions primarily due to working memory alterations. The short-term verbal memory revealed a significant positive correlation with working memory components especially RAVLT-T1 (Spearman rho = 0.535; $p < 0.001$). However, verbal long-term memory correlated strongly with the RAVLT memory efficiency index (Spearman rho = 0.684; $p < 0.001$). The data indicate that learning in RAVLT requires efficient working memory. However, long-term memory formation is an effect of various components including working memory, retention, and recognition. However, in comparison to verbal memory, visual memory correlated less with the verbal working memory. Moreover, the more complex cognitive domains such as executive functioning have been strongly associated with the working memory components as depicted in **Figure 2**.

TABLE 7 | Rotated component matrix[a].

	Component				
	1	2	3	4	5
STROOPC	0.813				
STROOPW	0.801				
STROOPCW	0.749				
DSBackward	0.730				
DSForward	0.711				
COWATOTAL	0.595				
ANT	0.560				
RCFTIR		0.934			
RCFTDR		0.928			
RCFTCOPY		0.738			
RecencyT1			0.805		
RAVLTIR			0.770		
RAVLTDR			0.761		
MiddleT1				0.926	
RAVLT1				0.637	
PrimacyT1					0.923

Extraction method: principal component analysis. Rotation method: Varimax with Kaiser Normalization[a].
[a]Rotation converged in five iterations.

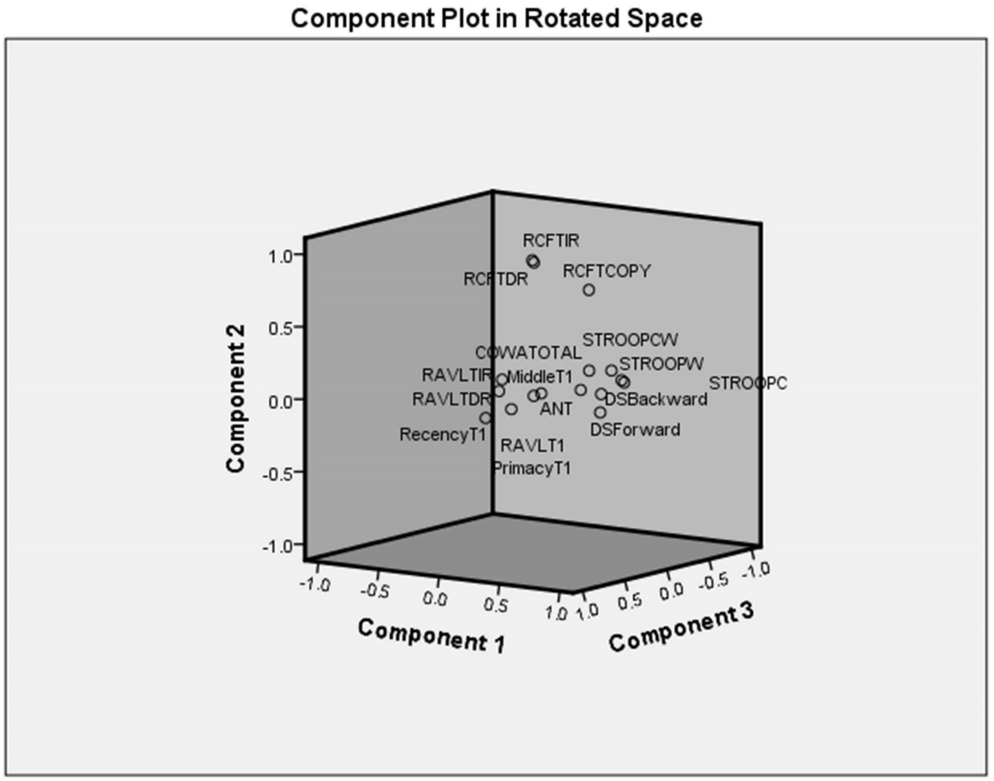

FIGURE 3 | Component plot in rotated space.

Linear regression analysis was performed considering RAVLT1 and Digit Span Backward task as dependent variables. The model with RAVLT delayed recall, and Stroop neutral task revealed a *B* value of 1.103. A higher *B* value of 2.104 was observed in a model with RAVLT delayed recall alone. All of the predictors in both models revealed statistical significance. Adjusted *R* square was 0.335 for the model as presented in **Table 6**.

Principal Component Analysis

Principal component analysis extracted five components (eigenvalues > 1) that explained 75% variance. **Table 7** shows the factor structure in five components with higher eigenvalues. The component plot is provided in **Figure 3**.

DISCUSSION

Inadequate intelligence in approximately 30% DMDs (Cotton et al., 2001) and poor functioning of various neuropsychological domains necessitate immediate rehabilitation strategies. In contrast to previous reports, approximately 7% of our DMD cohort revealed intellectual disability (IQ < 70). DMD subjects had a mean IQ of 90 (14.54) indicating average intelligence. There are inconsistent findings in previous studies that reported different intelligence quotients in different ethnic populations. A recent study conducted in South Indian DMD patients reported low average intelligence (mean IQ = 88.5 ± 13.18), which also indicates population-based investigation and intervention regime (Perumal et al., 2015). Verbal components remained vulnerable in ours as well as the South Indian cohort as VIQ was found to be affected more than the performance IQ score.

Assessment of verbal memory through RAVLT revealed deficits in immediate and delayed verbal memory in our cases. The poor performance on RAVLT trial 1 revealed deficits in the short span memory process, which may lead to difficulties in the maintenance and manipulations of verbal information. However, we found an adequate trend of learning after trial 1 till trial 5 indicating a normal learning trend in the DMD subjects. Thus, multiple exposures (rehearsal) of information may help DMD subjects to perform complex cognitive performance. RAVLT memory efficiency index (RAVLT-MEI) (Ricci et al., 2012), which combined measures of encoding and retention, revealed a marked reduction of scores. Ricci et al. (2012) reported the cut-off range of 1.2 and 1.9 to differentiate the patients with Alzheimer's disease/behavioral variant frontotemporal dementia (bvFTD) and bvFTD/normal controls to evaluate the prognostic impact of this factor index. Similarly, in our study, RAVLT-MEI value for DMD was found to be 1.8, suggesting lower levels of verbal retention and encoding, similar to the patients with frontotemporal dementia, which emphasizes the role of frontal and temporal lobes in DMD subjects (**Figure 1**). RAVLT-MEI score may also indicate the working memory resources of the DMD brain. The DMD group showed a normal trend in the acquisition of the list of words learned initially (primacy) and middle in order. However, recently, learned information

was inadequate, indicating poor working memory as also evidenced by RAVLT trial 1 performance and MEI (**Figure 1**). The arithmetic subtest and Digit Span Backward task further indicated deficits in the attention loop and working memory axis.

Along with the poor performance of verbal working memory, DMD subjects also demonstrated poor visual memory, visuo-constructive, and visuospatial abilities. However, the role of motor restrictions in these tasks necessitated the investigation of motor-free efficiency indexes negating the muscular effects. Therefore, block designing efficiency scores were introduced in this study, which demonstrated a normal visuo-constructive ability in DMD. Also, DMD subjects were able to normally switch between the neural circuits in the presence of the appropriate stimulus. Previous studies have observed a less consistent poor visual memory (Billard et al., 1992). In contrast, our study subjects demonstrated poor visual immediate and long-term memory. A likely explanation could be the variable mutation location and affected dystrophin isoforms in our cohort (Tyagi et al., 2019a). Furthermore, linear regression analysis of working memory alterations through RAVLT1 was associated with the long-term verbal memory. It suggested that working memory loss also affects verbal long-term memory. We previously associated and validated the working memory alterations with Dp140 isoform through computational cognitive modeling (Tyagi et al., 2020). Recent radiological investigation reported extensive abnormalities in white matter of DMD cases affecting Dp140 isoform (Preethish-Kumar et al., 2020). White matter microstructure has previously been associated with working memory (Tokariev et al., 2019). The contribution of altered working memory in affecting other neuropsychological domains was underexplored in DMD.

An assessment of visual working memory could have shed more light on the poor visual immediate and long-term memory in DMD. Previously, Cowan et al. (2010) demonstrated that visual working memory capacity highly correlates with intelligence in children. Underperformance in the CCTT task indicated difficulty in the alternating and sustained visual attention, perceptual tracking, simple sequencing, and psychomotor speed. The DMD group took a longer time in finishing CCTT-A and CCTT-B. Semantic (ANT) and phonemic (COWA) verbal fluency scores were not similar to the scores obtained in the control group, indicating poor verbal ability and executive control. Poor Stroop Color and Word Task (SCWT)-Word (W) performance of the DMD group corresponds to personal tempo, speech motor problems, and learning disabilities. SCWT color and color word performance was found to be altered in the DMD group, which reflected poor health of the primary visual cortex since it is responsible for the spatial selectiveness and color perception (Johnson et al., 2001; Solomon and Lennie, 2007). Factor indexes such as RAVLT-memory efficiency index, primacy and recency factors, proactive and retroactive interferences, LTPR, block designing efficiency, and attention fractions provide evidence of alterations as well as strengths of DMD brain processes especially in disorders demonstrating motor restrictions.

Working memory is strongly associated to the attentional control as a part of central executive. Luria (1973) provided three model components of a working brain in his book, which consisted of memory system, attention system, and activation system. Processing of attention requires effective processing of four sub-components, which include working memory as a central component along with competitive selection, top-down sensitivity control, and automatic bottom-up filtering for salient stimuli (Knudsen, 2007). Information obtained from the environment is filtered before its access to the working memory based on appropriate stimulus. This phenomenon is referred as bottom-up filtering for salient stimuli. However, based on the strength and quality of the signals, information is selected for getting access of working memory. This phenomenon is referred as competitive selection. These two processes are crucial before processing of appropriate information before further encoding. Limited working memory capacity plays an essential role of presenting and manipulating the information in conjunction to the activation of brain areas to support the attentional control (Knudsen, 2007). Thus, working memory may contribute to the formation of short-term memory as well as it is inextricably inter-related to attentional processing. Our study results propose a deeper investigation to study the molecular aspects of understanding dystrophin isoform-associated mechanism of working memory regulations. Moreover, interventions at the level of working memory axis (working memory, attention, and visuospatial functioning) may probably improve the neuropsychological impairments in DMD.

CONCLUSION

Duchenne muscular dystrophy subjects showed poor general intellectual abilities as compared to the normal population. Among the cognitive domains analyzed, working memory functioning seems to affect DMD neuropsychological profile. It can be used as a potential target for rehabilitation strategies. Novel factor indexes including RAVLT-MEI demonstrated poor verbal memory efficiency. The DMD group also underperformed on attention, executive functioning, visual memory, verbal fluency, and sustained and focused attention tasks.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AA was responsible for the conceptualization and editing and serves as the grant PI. RT was responsible for co-conceptualization under supervision, genetic and neuropsychological data acquisition, experiments and analysis, statistical analysis, drafting, and editing the manuscript. HA was responsible for neuropsychological data acquisition. MG is the co-supervisor of the first author. MM was responsible for supervision of neuropsychological assessment, analysis, and validation of data and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2020.613242/full#supplementary-material

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What We Fail to See in Neuro-Genetic Diseases: A Bird's Eye View from the Developing World

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Navami Samaranayake[1], Pulasthi Dissanayaka[1], Isuru Gunarathna[1], Lakmal Gonawala[1], Nalaka Wijekoon[1], Pyara Rathnayake[2], Darshana Sirisena[3], Harsha Gunasekara[4], Athula Dissanayake[5], Sunethra Senanayake[3], Akshay Anand[6], Kapaettu Satyamoorthy[7], Ashwin Dalal[8], and K. Ranil D. de Silva[1]

Abstract

Background: Progressive neurological genetic diseases are not rare. They cause psychosocial damages to its victims. This article focuses on common psychosocial issues faced by those from the developing world.
Methods: A multicentre observational survey of 246 patients from teaching hospitals in Sri Lanka. Participants were clinically and genetically confirmed by neurologists and the Interdisciplinary Centre for Innovation in Biotechnology and Neuroscience (ICIBN) respectively from 2014 to 2018. Convenience sample with random geographical distribution. Factors were equally weighted. ANOVA, Student's t-test and chi-square analysis were used. Statistical Software R Statistics—version 3.5 and one-sample t-test with CI = 95% was used. This study meets the ethical guidelines of the local institutional review boards which are in compliance with the Helsinki Declaration.
Results: Sample included 184 males and 62 females of 3–76 years with either Duchenne muscular dystrophy (n=121), spinocerebellar ataxia (n = 87) or Huntington disease (n = 38). Mean income of the affected is lower than the standard average monthly income ($P \leq .001$). Consultation visits depend on the monthly income (CI 20421.074–34709.361; $P \leq .001$).
Conclusion: Poverty is inversely proportionate to the patients' living conditions. As developing countries are financially challenged, it is a societal challenge to rebuild our values to enhance their living status.

Keywords

Poverty, developing country, neurological disorder, Sri Lanka

Introduction

> Being unwanted, unloved, uncared for, forgotten by everybody, I think that is a much greater hunger, a much greater poverty than the person who has nothing to eat.
>
> —Mother Teresa

> Poverty is the worst form of violence.
>
> —Mahatma Gandhi

Poverty is a major cause of ill health and a barrier to accessing healthcare when needed. Ill health, in turn, is a major cause of poverty. This is partly due to the costs of seeking healthcare, which include not only out-of-pocket spending on care but also transportation costs and any informal payments to providers. It is also due to the considerable loss of income associated with illness in developing countries, both of the breadwinner and family members who may be obliged to stop working or attending school to take care of an ill relative. In addition, poor families coping with illness might be forced to sell assets to cover medical expenses, borrow at high interest rates or become indebted to the community.[1]

[1] Interdisciplinary Centre for Innovation in Biotechnology and Neuroscience, University of Sri Jayewardenepura, Nugegoda, Sri Lanka
[2] Lady Ridgeway Hospital for Children, Colombo, Sri Lanka
[3] National Hospital of Sri Lanka, Colombo, Sri Lanka
[4] Sri Jayewardenepura General Hospital, Colombo, Sri Lanka
[5] Teaching Hospital Karapitiya, Galle, Sri Lanka
[6] Neuroscience Research Lab, Institute of Medical Education and Research (PGIMER), Chandigarh
[7] Manipal School of Life Sciences, Manipal Academy of Higher Education, Manipal, India
[8] Centre for DNA Fingerprinting and Diagnostics, Hyderabad, Telangana, India

Corresponding author:
K. Ranil D. de Silva, Interdisciplinary Centre for Innovation in Biotechnology and Neuroscience, University of Sri Jayewardenepura, Nugegoda, Sri Lanka.
E-mail: ranil@sjp.ac.lk

Genetic disorders can create a whole package of problems socially, economically for an affected family and to the society that will consume 5%–10% of the total health budget of a country for treatments. In Sri Lanka and India common monogenic and complex disorders including neurodegenerative disorders such as Duchenne muscular dystrophy, Huntington's disease, spinocerebellar ataxias[2,3,13-19] and congenital disorders remain high with rising concern towards the necessity for a genetic testing service. Genetic testing in Sri Lanka is almost non-existent in government hospitals and only confined to a few centres in the private sector. The corresponding author has been successful in establishing the one and only free genetic testing service for selected neurodegenerative and neuromuscular disorders. To date, this service is able to provide genetic testing reports worth of few millions of Sri Lankan rupees.

Case 01

A wheelchair-bound teacher with a family history of spinocerebellar ataxia type 1 shared her story of a failed marriage. The disease onset was seen during her second pregnancy, upon which her husband had demanded for an abortion as he did not want any more 'sick' individuals in his family. After refusing to have abortion, she was harassed physically and mentally by the husband and the mother-in-law. Abortions are considered illegal in Sri Lanka. Due to depression she had attempted suicide by ingesting a toxic substance. Although she and the foetus had survived the incident, her husband forcefully took the guardianship of the first born. Hence, she had left the family to join her mother. Her mother was having multiple comorbidities, although she remains the only support system for the patient. Currently, all three live under a barely constructed house with a single room. Even if her first child does not even recognize her anymore and has no contacts with her, she is determined to give a better education to her second child and take him to the epitome of success.

Case 02

Two wheelchair-bound teenage boys presented with Duchenne muscular dystrophy. Their mother being a housewife and the father being a labourer hardly earn a living. Even with disabilities, the older sibling had won a gold medal from an all-island art competition. The parents yearn to fulfil their children's needs to the best of their abilities even though that demands cutting down their fundamental needs including the money they spend on meals. Even with such effort, they only save enough to take their children to the leading children's hospital annually, because the cost it takes to travel to the hospital with their two wheelchair bound children is greater than their monthly income.

Case 03

One family visit revealed a dependent woman in her 60s with Huntington disease, confined to bed in a secluded room without ventilation. This patient with urine incontinency was kept on a mattress that was soaked in urine, under which crawled maggots. Her adult son was waiting till his mother dies because the family no longer wanted to support her needs. Although financially stable, she was unable to live the final stage of her life with tender loving care.

Methodology

A multi-center observational survey was conducted on patients identified after through neurological examination at teaching hospitals in Sri Lanka from 2014 to 2018. Initially, 292 patients who were clinically diagnosed by consultant neurologists were referred for the genetic testing at ICIBN, University of Sri Jayewardenepura, Sri Lanka. This survey included 246 participants (males n = 184, females n = 62) of 3–76 years, who were genetically confirmed for Duchenne muscular dystrophy (n = 121, age range 2–18, mean age: 9 years), spinocerebellar ataxia (n = 87, age range 21–73, mean age: 44 years) and Huntington disease (n = 38, age range 25–59, mean age: 45 years). Written informed consents were obtained from every participant where applicable. For incompetent patients, surrogate consent was taken. Clinical data and sociodemographic information were collected using standard questionnaire and clinical batteries. This study meets the ethical guidelines of the local institutional review boards which are in compliance with the Helsinki Declaration. All factors were equally weighted. ANOVA, Student's t-test and chi-square analysis techniques were used as the statistical tests. Statistical Software R Statistics—version 3.5 was used with extension packages. Analysis was done using one-sample t-test with confidence interval of 95%.

Results

Around 60% of the affected have a monthly income that ranges between 28,000 LKR (156 USD) and 65,000 LKR (362 USD) (Table 1, Table S1, Figure 1). The mean household income of an affected family is 30,531 LKR (170 USD) and

Table 1. Monthly Income Distribution of the Cohort

Income Category/LKR	Income Category in US$	Percentage (%)
<8000	<44.55	2.45
8001–12000	44.55–66.81	3.67
12001–16000	66.81–89.08	7.35
16001–20000	89.08–111.35	15.10
20001–28000	111.35–155.89	7.76
28001–35000	155.89–194.86	18.78
35001–45000	194.87–250.54	19.18
45001–65000	250.54–361.88	20.82
>65000	>361.88	4.90

Source: Authors own.

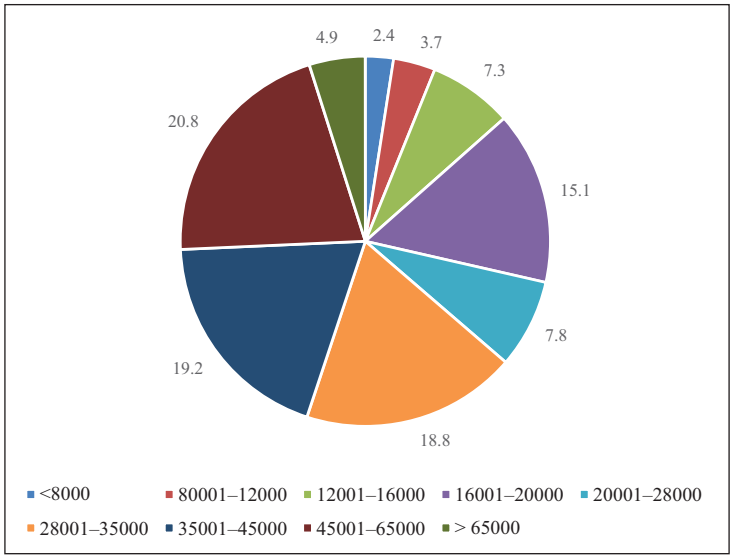

Figure 1. Monthly Income (LKR) Percentage of the Cohort

Source: Authors own.

Table 2. Statistical Analysis Summary Table

Analysis	Used Statistical Method	Confidence Intervals/Degrees of Freedom	*P* Value
Economic status and affected number of family members	ANOVA	95%	.245
District vs frequency of consultations	Chi-square	95 (df)	.6143
Monthly income vs frequency of clinic visits	ANOVA	95%	7.98×10^{-7}
Salary vs district	ANOVA	95%	.783
Dependency between wheelchair bound patients and source of water for daily bathing	Chi-square	8 (df)	2.2×10^{-16}
Dependency between wheelchair bound patients and type of toilet	Chi-square	4 (df)	2.2×10^{-16}
Dependency between wheelchair bound patients and frequency of clinic visits	Chi-square	10 (df)	4.318×10^{-7}
Type of toilet vs economic score	Kruskal–Wallis	2 (df)	8.989×10^{-12}
Living area vs frequency of clinic visits	Chi-square	5 (df)	.3776

Source: Authors own.

34,494 LKR (192 USD) in rural and urban areas, respectively. Table 2 illustrates the significant correlations found in the patient cohort.

In the analysed population, 61% of patients use squatting type toilets (Figure 2a), 63% of patients use pipelines as the water source (Figure 2b), 92% receive neurology consultations (Figure 2c), while 64% of the cohort consult a neurologist once in every 6 months (Figure 2d).

Discussion

Although progressive neurological genetic diseases are not rare in the world, including developing countries, they cause grave psychosocial damages to its victims.[4–6] Throughout the world 7.6 million children are born annually with a severe genetic disorder or birth defect, out of which nearly 95% of them are from the developing countries. The prevalence and burden of genetic disorders, birth defects and common complex diseases are generally higher in developing countries.[7,8] The aforementioned cases illustrate the common psychosocial issues displayed in this cohort.

At a time where the inherited disease community remains as a highly neglected cluster within Sri Lanka, ICIBN-USJ being the only centre in the state sector provides free neurogenetic testing service facilitating the vision emphasized by the World Health Organization in terms of 'harnessing

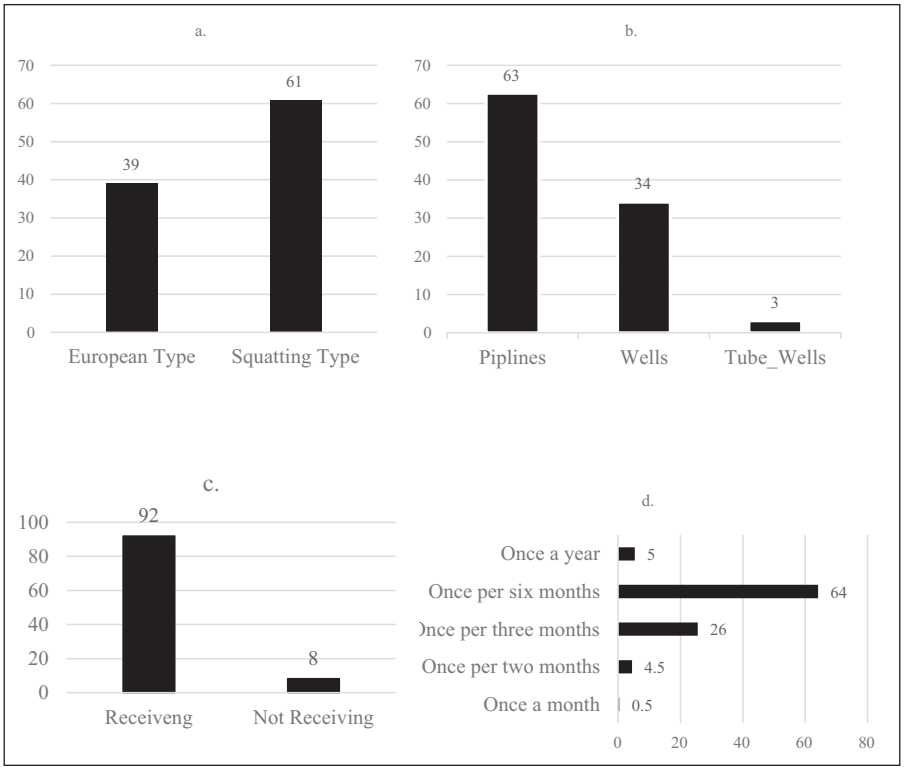

Figure 2. Distribution of psychosocial factors: (a) Types of toilets. (b) Type of water source. (c) Percentage of receiving consult of neurologist. (d) Percentages frequencies of neuroconsultations.

Source: Authors own.

the genomic knowledge and have it contribute to health equity, especially among developing nations'. However, 5,000– 10,000 LKR (27–54 USD) is spent on chemicals and consumables per test, where the cost is mainly covered by funding obtained by the principal investigator through international and local funding agencies. Thus, the authors request from the philanthropists and nongovernmental organizations to facilitate free molecular diagnostic services in developing countries.

The ongoing research and free molecular diagnostic service at ICIBN-USJ has resulted in the establishment of a unique national biobank. Our goal is to foster the research and educational aspirations of the inherited disease community through international collaboration in research leading to double doctoral degrees in human resource development, clinical trials and patient registries, advancing the search for new/modifier genes, founder effects, admixtures and pharmacogenetic effects utilizing this national biobank with the following assets as at May 2020:

1. DNA bank with associated sociodemographic and clinical data from
   a. Over 2,000 patients, with the following breakdown: Stroke—500, Parkinson's disease (PD)—370, and controls—500.
   b. Genetically diagnosed/genetically negative for common mutation patients in the following numbers: Duchenne muscular dystrophy—178/27, spinal muscular atrophy—24/11, limb-girdle muscular dystrophy—1/15, Huntington's disease—39/38, spinocerebellar ataxia—69/127, myotonic dystrophy—4/2.
2. 'Brain Bank' with 76 autopsy brain samples from ageing individuals, with immunohistochemical stains for neuropathological markers associated with dementia-related disorders and genotyping data on candidate genes for stroke.
3. Serum and urine samples from patients with DMD, SCA and HD.

Poverty and Heath

As these diseases are often not recognized, prevention through proper guidance is not anticipated leading to a high cost to the affected individual, their families and the healthcare sector.[7,8] Typically, nearly 50% of medical expenses in low-income nations are derived from out-of-pocket payments, compared to 30% in middle-income nations and 14% in high-income nations.[9]

According to the data collected through the survey it was evident that the frequency of neurological consultations depends on the monthly income of the affected family ($P \leq .001$). It should be noted that even among our cohort, 16% (DMD, n =12; SCA, n = 19; HD, n = 08) was already wheelchair-bound. The minimum mean distance the patients have to travel to reach the closest regional hospital ranged between 40 km and 50 km. However, being a Third World country the public transport system in Sri Lanka is poor. With disease progression, patients find it cumbersome to use ordinary infrastructure because most public places, even the transport system or ordinary schools, lack facilities for the disabled. This issue is not given priority due to financial constraints. Consequently, patients find it difficult to reach out for medical care. This scenario is highlighted in Case 02 where the cost it takes to travel to the hospital with wheelchair-bound children is greater than their monthly income.

There was a significant association between wheelchair-bound patients and the frequency of clinic visits ($P \leq .000$). Monthly income and the frequency of visits as well as wheelchair-bound patients and the frequency of their clinic visits are significantly associated with each other (Table 2).

According to Sri Lankan census in 2016, the average household income in rural and urban areas are 58,137 LKR (323.67 USD) and 88,692 LKR (493.79 USD), respectively.[10] The mean household income of an affected family is 30,531 LKR (169.98 USD) and 34,494 LKR (192.04 USD) in rural and urban areas, respectively ($P \leq .000$). According to Table 1 around 60% of the affected have a monthly income that ranges between 28,000 LKR (155.89 USD) and 65,000 LKR (361.88 USD) (Table 1). One reason for this is when one member is suffering from a progressive disease, another adult in the family has to invest all of that individual's time caring for the patient.

Caregivers tend to go from one doctor to another and from one treatment modality to another because of poor understanding about the disease and its management. Upon failure of this 'doctor-shopping' phenomenon, caregivers tend to give up on the patients resulting in confinement of the patient as shown in Case 03.

Although these diseases do not have a permanent cure, 55% of the considered sample leaned towards traditional medicine. Only 7% of those who received Ayurveda treatment had experienced improvement in the signs and symptoms of disease, while 47% of those who received traditional medicine reported satisfaction. Therefore, it is important to explore these medical modalities. If these positive methods are supported through evidence, a holistic approach can be implemented in handling these patients which would prevent patients from withholding treatment entirely.

Dependency between wheelchair-bound patients and source of water appears to be significant. These patients mostly use pipelines as the water source for bathing according to chi-square test results ($P \leq .001$; CI: 5.50731). Type of toilets and the economic score and wheelchair-bound patients and the type of toilet used are significantly associated with each other (Table 2). They use European type of toilets according to chi-square test ($P \leq .001$; CI: 9.48779). The Kruskal—Wallis test for the economic score and the type of toilets showed a significant association ($P \leq .001$; chi-squared = 29.267; df = 4). The greater the patient is affected economically, the lesser the standard of the toilets. Most wheelchair-bound patients have squatting type toilets. Though most basic needs are covered in majority of the study sample despite their economic status, the attention towards utilizing European type toilets is limited (Table 2). Enlightening caregivers about improving the quality of life of patients even with small changes in the household is crucial.

Since the free health system is maintained in Sri Lanka, the medical insurance services are not well-established with low- and middle-income families to which most of our patients with neurogenetic disorders belong. Moreover, the established medical insurance policies in Sri Lanka hardly cover the expenses incurred at Out Patient Department tests, thus promoting indoor insurance covers.

Mobile clinics have been conducted by ICIBN-USJ around the country based on selected government hospitals for better availability, including the Northern Province (Jaffna Teaching Hospital), which was abandoned for nearly 30 years due to a civil war. Jaffna in the Northern Province is home to many consanguineous families. With the recent testing conducted there, new clusters of patients with clinical heterogeneity were identified. However, post-trauma stress of war that may have influenced these diseases was not systematically studied in these war-torn areas.

Tradition and Health

Before colonization, Sri Lankan tradition revolved around the concept of 'religion and village'. The bond the villagers had for one another was exquisite. The superhuman qualities such as compassion, kindness and humanity were seen in the locals. The religious leaders acted as mediators between conflict groups and as a source of comfort in addressing mental turmoil. However, with globalization these qualities have dissolved, building high parapet 'emotional' walls in the community.

Although Sri Lanka is a multi-religious country, where many gloriously claim to be fully devoted, the core qualities of each religion have evaporated with time. Religious centres rarely function as a resource centre for the needy because of the busy lifestyle of the community. We believe it is the obligation of those centres to address the concerns of the villagers in a more fruitful manner so that everyone gets the due benefit. Not only can they function as a place that adds tranquillity to the minds of the affected, but they can also work at the initial level where fundamental human

needs are addressed. We believe that if this support had been there, patients would seldom attempt suicide or deliberate self-harm.

According to Sri Lankan penal code, therapeutic abortions are only allowed when the mother's life is at risk.[11] As noted in Case 01, abortions are desired and performed illegally and silently in the country. Victims tend to hide these progressive neuro-degenerative genetic diseases due to stigma and once known, the patient is segregated from the society. Hence, instead of due to these reasons patients remain underdiagnosed.

Parents suffer when a child is diagnosed with a genetic disease. Mothers show constant self-blame and experience pressure from their in-laws because they are branded as the root cause for the disease. Parents express fear of having more children. Due to lack of communication with the medical practitioners, they fail to find out about the alternative ways such as adaptation, surrogating and donor sampling methods. However, lack of availability and affordability also play a key role in limiting these options even when known.

Enhancing the qualities of humankind should be addressed from the primary education level where the children are taught to accept differences, share with the needed and be non-judgmental. Even though Sri Lanka has a total adult literacy rate of 91.2%,[12] these psychological aspects of the community remain unaddressed. This is indirectly the failure of emotional intelligence of the country as well.

Conclusion

Poverty is invariably increased in the affected people. As a developing country, addressing the fundamental needs of the affected is financially inconvenient. Instead of waiting till the governing bodies implement the needful, there are major changes we can adopt as a society to help those in need and reduce the stigma. It is a societal challenge to rebuild our deep-rooted traditional values to form a better community.

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Authors Contributions

N. S., N. W., L. G., P. D. are Research Assistants at the Interdisciplinary Centre for Innovation in Biotechnology and Neuroscience, University of Sri Jayewardenepura, Nugegoda, Sri Lanka and have co-written the final manuscript. I. G. is the statistician who contributed in co-writing the statistical analysis. Acquisition, analysis, or interpretation of data were done by I. G., L. G., and N. W.

P. R., D. S., H. G., A. D., S. S. are Consultant Neurologists and contributed in clinical aspect of the patients. A. A., K. S., A. D. provide academic guidance for the PhD students in Molecular Diagnostics. R. D. S. is the principal investigator and has developed the manuscript from its inception to the final version.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Statement

The study was approved by the Ethical Committee of Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka which is in compliance with the Helsinki Declaration (Ethics no 34/14 and 449/09).

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ORCID iD

K. Ranil D. de Silva https://orcid.org/0000-0002-8738-9412

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Transplantation Efficacy of Human Ciliary Epithelium Cells from Fetal Eye and Lin-ve Stem Cells from Umbilical Cord Blood in the Murine Retinal Degeneration Model of Laser Injury

Sridhar Bammidi[1,*], Parul Bali[1,2,*], Jaswinder Kalra[3], and Akshay Anand[1]

Abstract

A number of degenerative conditions affecting the neural retina including age-related macular degeneration have no successful treatment, resulting in partial or complete vision loss. There are a number of stem cell replacement strategies for recovery of retinal damage using cells from variable sources. However, literature is still deficit in the comparison of efficacy of types of stem cells. The purpose of the study was to compare the therapeutic efficacy of undifferentiated cells, i.e., lineage negative stem cells (Lin-ve SC) with differentiated neurosphere derived from ciliary epithelium (CE) cells on retinal markers associated with laser-induced retinal injury. Laser-induced photocoagulation was carried out to disrupt Bruch's membrane and retinal pigmented epithelium in C57BL/6 mouse model. Lineage negative cells were isolated from human umbilical cord blood, whereas neurospheres were derived from CE of post-aborted human eyeballs. The cells were then transplanted into subretinal space to study their effect on injury. Markers of neurotropic factors, retina, apoptosis, and proliferation were analyzed after injury and transplantation. mRNA expression was also analyzed by real-time polymerase chain reaction at 1 week, and 3-month immunohistochemistry was evaluated at 1-week time point. CE cell transplantation showed enhanced differentiation of rods and retinal glial cells. However, Lin-ve cells exerted paracrine-dependent modulation of neurotrophic factors, which is possibly mediated by antiapoptotic and proliferative effects. In conclusion, CE transplantation showed superior regenerative outcome in comparison to Lin-ve SC for rescue of artificially injured rodent retinal cells. It is imperative that this source for transplantation may be extensively studied in various doses and additional retinal degeneration models for prospective clinical applications.

Keywords

subretinal, laser injury, lineage negative stem cells, ciliary epithelium cells, umbilical cord blood, retinal degeneration

Introduction

Degenerative conditions of the retina, viz. retinitis pigmentosa, age-related macular degeneration, diabetic retinopathy, glaucoma, etc., do not have any successful treatment to reverse the vision loss. Their widespread use in industrial, medical, and military fields has caused severe vision loss in a number of individuals[1]. Laser injuries are also a part of severe retinal damage, as a result of occupational eye injuries[2].

Cell-based therapies may provide treatment avenues for injuries as well as degenerative disorders by using

[1] Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[2] Department of Biophysics, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[3] Department of Obstetrics and Gynaecology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
* Both the authors contributed equally and are co-first authors of this article.

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Corresponding Author:
Akshay Anand, PhD, Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India.
Email: akshay1anand@rediffmail.com

reproducible laser injury models. Current ongoing stem cell work findings have also raised hopes of individuals suffering from such untreatable disorders[3–5]. Stem cells from different sources such as neural stem cells, retinal progenitors, hematopoietic, mesenchymal, embryonic, and induced pluripotent stem cells (iPSCs) have been studied for their differentiation potential into neuronal, retinal as well as glial cell lineages[3,6,7]. Stem cell therapies for targeting such chronic diseases may provide effective solutions. However, the field suffers from lack of comparative analysis of the various types of stem cells even though there is overemphasis on iPSCs[8] and embryonic stem cells[9] without any comparative studies between the two and/or other types of stem cells.

Recent studies show that stem cell transplantation at the site of neural injury or neuronal niche may facilitate differentiation into neurons, resulting in functional improvements in animal models[10,11]. Although the use of stem cells derived from umbilical cord blood (UCB) has been employed with several injury/disease models involving the central nervous system, it is limited as compared to the burgeoning number of cord banks being set up in the Asian countries[12]. The study tested the effect of UCB-derived stem cells in laser-induced injury in rabbit trabecular meshwork[13]. Similarly, the efficacy of transplantation of ciliary epithelium (CE) cells has not been adequately investigated let alone compared with other cell types despite repeated failure to regenerate the damaged or diseased retina. However, several groups have identified and continued with studies of transplantation of CE stem cells in retinal injury models[14,15].

In our previous study, we characterized human umbilical cord blood (hUCB)-derived lineage negative stem cells (Lin-ve SC) on the basis of morphology and cell surface marker expression[16]. We identified these cells under scanning electron microscopy and reported homogenous morphology, i.e., size, shape, and structure as compared to lineage positive and mononucleated cells. Lin-ve SC showed significantly increased expression of hematopoietic stem cells expression, i.e., CD117 and CD34, which ameliorated amyloid-induced memory loss upon transplantation in mouse model. The presence of CD34+ and CD117+ in Lin-ve SC showed 99% positive expression of CD45 marker[17]. These cells exerted neurotrophic factors mediated paracrine effects causing anti-apoptotic activity and amyloid clearance by the activation of astrocytes[18,19].

As lasers have been used for creating the models of retinal degeneration in fish, rodents, and primates[20,21], we established a modified animal model, injuring the Bruch's membrane and the surrounding retinal pigment epithelium (without causing choroidal neovascularization). CE cell–derived neurospheres were cultured under in vitro conditions using recombinant fibroblast growth factor (rhFGF) and recombinant epidermal growth factor (rhEGF), which showed increased size and number on sixth day of culture[22]. These neurospheres were dissociated and transplanted in the subretinal space of mice after laser injury. This resulted in marked increase in neurotrophic marker expression.

This study describes the comparative efficacy of differentiated human fetal CE-derived neurospheres and undifferentiated Lin-ve hUCB-derived stem cells, in the subretinal space of laser-induced retinal injury mouse model. We wanted to understand which source provides effective outcomes. Our results showed that the transplantation of CE cells presumably differentiated it into rods and retinal ganglion cells (RGCs) after migration from subretinal space. However, Lin-ve SC rescued the retinal damage by inducing neurotrophic factors, which may exert antiapoptotic and proliferation activity. Hence, we conclude that differentiated (CE cells) cells can be a superior source for cell transplantation therapy in comparison to undifferentiated (Lin-ve SC) cells after laser injury.

Materials and Methods

Animals

C57BL/6 J syngeneic mice ($N = 3$ per group) of 6- to 8-week-old male were used in the study after the approval from Institutional Animal Ethics Committee (IAEC), Post Graduate Institute of Medical Education and Research, Chandigarh, India [71(69)/IAEC/423]. Animals were kept in animal house facility in a 12-h light/dark cycle (LD 12:12). Mice were fed with standard chow diet and access them freely to clean drinking water.

Laser-Induced Retinal Injury in Mouse Model

The laser-induced retinal injury was established using Argon green laser (532 nm, Iris Medical, USA). The C57 mouse was anesthetized with xylazine hydrochloride (10 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) and ketamine hydrochloride (100 mg/kg) (Nirlife, Sachana, Gujarat, India) in 1:10 ratio. Mice were kept over heating pad to prevent cold cataract. The local anesthesia, i.e., lignocaine solution, was given to the cornea and 1% tropicamide solution (Akums Drugs and Pharmaceuticals Ltd, Haridwar, Uttarakhand, India) applied for pupil dilation. The fundus shots were obtained through slit lamp by placing the anesthetized mice in front of laser photo-coagulator (IRIDEX, Mountain View, CA, USA). Eight even laser spots in circular fashion were subjected around optic disk in both the eyes. The standardized parameters used in laser photocoagulation were spot size of 100 μm, power of 200 mW, and duration of pulse of 100 ms. Sham control group was operated for subretinal injection without injecting any liquid/vehicle.

Isolation of Lin-ve Stem Cells from hUCB for Subretinal Transplantation

Human UCB samples were obtained after the approval from Institutional Committee for Stem Cell Research and Therapy (IC-SCRT: Approval No. PGI-ICSCRT-53-2014/1469), Post Graduate Institute of Medical Education and Research, Chandigarh-India. Pregnant women of age 20–35 years and

≥28 weeks of gestation period were included in the study. UCB was taken from the umbilical cords of newborns after filling proper informed consent, in the presence of an independent witness. The collected UCB was layered over histopaque solution (Sigma-Aldrich) for enrichment of MNCs using density gradient centrifugation. These MNCs were then subjected to magnetic associated cell sorter (MACS) (Miltenyi Biotech, Bergisch Gladbach, Germany) for the isolation of Lin-ve SC using human Lin-ve isolation kit (Miltenyi Biotech). The kit works on the basis of biological affinity of biotin and streptavidin. The primary antibody cocktail consisted of biotinylated monoclonal antibodies, which include CD123, CD235a (Glycophorin A), CD56, CD19, CD16, CD15, CD14, CD11b, CD3, and CD2.

MNCs were suspended in MACS-BSA buffer and 10 µl of biotin antibody cocktail was added per 107 cells. These were incubated for 15–20 min at 4–8°C and tapped/gently agitated regularly. The cells were then incubated with the second solution, i.e., streptavidin coated magnetic microbeads. Twenty microliters of these microbeads per 107 cells was added and incubated again with gentle tapping for 20–30 min at 4–8°C. The cells were then diluted with 1–2 ml of the buffer, mixed well, and washed twice at 1,500 rpm for 5 min at 4°C. Supernatant was completely pipetted out. Up to 10^8 cells were suspended in 500 µl of freshly made PBS/MACS-BSA buffer. The cells were then subjected to magnetic separation. The magnetic separation unit along with magnetic separation column was kept in a laminar hood and the column was washed with appropriate amount of buffer solution twice. This was shifted to a fresh collection tube, and the cell suspension was passed through the column at a very slow and steady rate, taking care that there are no air spaces/bubbles inside the column. One milliliter of the buffer was then passed through the column twice to completely elute the cells of interest. Turbid white suspension was obtained as flow through.

Ciliary Epithelial Isolation and Culture

Human fetal eye globes were obtained from abortus after legal termination of pregnancy up to 20 weeks of gestation in accordance to ethical guidelines approved by IC-SCRT (Approval no. PGI-ICSCRT-53-2014/1469). The samples were collected from the terminations at the MTP-OT, SLR, and the Emergency-OT, PGIMER, Chandigarh.

Appropriate informed consents were obtained for every donor, on a prescribed consent form, and all the detailed procedures and objectives related to sample collection were explained to them. All the donors were screened and the following conditions were noted before collection of eye samples:

Inclusion Criteria. The samples were obtained of mid-trimester and up to 20 weeks of fetal abortions. This was on suggestion from the experts at both Department of Obstetrics and Gynecology and the Institutional Committee on Stem Cell Research (IC-SCR), PGIMER, Chandigarh.

Exclusion Criteria.

- Hepatitis virus B and C
- Human immunodeficiency virus
- Samples bearing congenital abnormalities
- Any malformation of fetus effecting head
- Evidence of chorioamnionitis (fever, foul smell liquor)
- Intrauterine fetal death

The fetal eyes were carefully enucleated immediately after abortion and transported in an ice cold sterile Hanks' Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY, USA). The surgical procedure was performed under stereo-zoom microscope (Leica EZ4, Leica Microsystems, Wetzlar, Germany) in order to isolate CE. Eye was held with forceps and a cut at the anterior edge of pars plana was made in order to carefully get the strip of ocular tissue containing CE. Ciliary rings were isolated and washed with sterile HBSS without taking nonpigmented epithelium, retinal pigmented epithelium (RPE), iris as well as retina. CE was dissociated first mechanically using blade and trypsinized using 4–5 ml of 0.25% trypsin (Gibco, Life Technologies, Burlington, ONT, Canada) with ethylenediaminetetraacetic acid at 37°C for 20–30 min. The action of trypsin was further neutralized with advanced Dulbecco's modified Eagle's medium/F12 (Gibco). The unwanted debris was removed by 0.70 µm cell strainer (BD Biosciences, Discovery Labware, Durham, NC, USA) and then centrifuged at 800G for 10 min. The number of pigmented cells was estimated with hemocytometer (Hausser Scientific, Horsham, PA, USA) and also with automatic cell counter (Millipore, Darmstadt, Germany). Finally, 3,000 cells/well were cultured in 96-well plate using retinal culture medium containing advanced DMEM/F12 (Gibco), 2 mM L-glutamine (Gibco, Paisley, Scotland, UK), N2 supplement (Gibco, Life Technologies), 100 U penicillin–streptomycin (Gibco), and fungizone (Gibco, Paisley, Scotland, UK). The cells were provided with proliferative growth factors, i.e., rhEGF (20 ng/ml; R&D Systems, Minneapolis, MN, USA) and rhFGF basic (20 ng/ml; R&D Systems). The size, shape, and structure of culture neurospheres were estimated using Image J software. The sixth day CE cultured neurospheres were harvested for subretinal transplantation in laser injured retina of mouse.

Transplantation of hCE-Derived Neurospheres and hUCB Lin-ve Stem Cells

We labeled our transplanted cells with fluorescent dye–carboxyfluorescein succinimidyl ester (CFDA-SE) (Sigma-Aldrich) in order to track the Lin-ve SC population and the CE-derived neurospheres after transplantation. For CFDA

Table 1. List of Antibodies Used in Immunohistochemical Experiments Real-Time PCR.

Antibody		Make	Dilution
Primary antibodies			
1.	Rhodopsin	Santa Cruz Biotechnology, USA	1:100
2.	Thy1	Santa Cruz Biotechnology, USA	1:100
3.	BDNF	Santa Cruz Biotechnology, USA	1:100
4.	CNTF	Santa Cruz Biotechnology, USA	1:100
Secondary antibodies			
1.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200
2.	Cy3 conjugated Donkey α Mouse	Jackson Immunoresearch, USA	1:200
3.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200
4.	Cy3 conjugated Donkey α Rabbit	Jackson Immunoresearch, USA	1:200

BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; PCR: polymerase chain reaction.

staining, cells were counted and 50,000 were suspended in 1 µl of PBS, then transplanted after 24 h of injury through transcorneal subretinal route. The endpoint analysis was carried out at 1 week and 3 months after transplantation. The subretinal injection in mice was performed under surgical microscope after giving anesthesia. The prick was made at the cornea–scleral junction with beveled 31G insulin needle and pressure was released without injuring the cornea. A microsyringe (EXMIRE, Fuji, Shizouka, Japan) of 33G was introduced through the pricked area and moved behind the lens and moved till it touched the retina without exerting pressure on it. One milliliter suspension of 50,000 cells was injected carefully and bleb formation was observed. This served as a marker for successful subretinal delivery.

Immunohistochemistry

The eye balls were enucleated after sacrifice and frozen at −80°C and cryosectioned (Leica Cryostat, Buffalo Grove, IL, USA). Immunohistochemistry was performed to analyze the protein expression changes in the retinal layers viz. rhodopsin (Santa Cruz Biotechnology, Dallas, TX, USA), Thy1 (Santa Cruz Biotechnology), brain-derived neurotrophic factor (BDNF; Santa Cruz Biotechnology), and ciliary neurotrophic factor (CNTF; Santa Cruz Biotechnology). The sections were fixed using HistoChoice (Sigma-Aldrich) and then incubated with primary antibody (1:100 dilution) at 4°C overnight. Next day, the sections were kept in Cy3 labeled secondary antibody solution (1:200 dilution) (Jackson Immunoresearch, West Grove, PA, USA) for half an hour and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) (1:1,000 dilution) (Table 1). The sections were subsequently imaged under a confocal microscope to analyze the protein expression.

Real-Time Polymerase Chain Reaction

Relative fold change in the expression of the mRNA levels was detected using real-time polymerase chain reaction (PCR). The RNA was isolated from the dissected retinae of the enucleated eye balls, and converted to cDNA libraries, using standard kit protocols (Qiagen, Velno, The Netherlands and Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed using primers for rhodopsin (Eurofins, Bangalore, India), Thy1 (Eurofins), BDNF (Sigma-Aldrich, New Delhi, India), CNTF (Sigma-Aldrich, New Delhi, India), BCl2 (Eurofins), and Ki67 (Eurofins). β-Actin (Sigma-Aldrich, New Delhi, India) was used for an endogenous housekeeping control (Table 2). Expression levels were quantified and analyzed using StepOne, Applied Biosystems real-time PCR software (Thermo Fisher Scientific).

Statistical Analysis

Data were analyzed by calculating mean ± standard error of the mean, and normality of data was analyzed using 1-KS sampling test. The data were analyzed by one-way analysis of variance (ANOVA) followed by LSD and Scheffe and Dunnett's test for post hoc analysis. Statistical analysis of results was analyzed using 16.0 version of SPSS. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ were considered as statistically significant.

Results

CE Cell Transplantation Enhances the Formation of Rod Cells

We used laser-induced retinal injury mouse model without causing CNV. For this, we targeted eight shots in a circular pattern around the optic disc. These shots were focused at the Bruch's membrane, which resulted in the damage to RPE and caused the breach of blood retinal barrier. We validated this model using fundus fluorescein angiography showing leakage in the retinal blood vessels as well as electroretinogram that showed changes in the wave pattern[22]. RPE plays crucial role in providing nutrients to the retina and phagocytosis of photoreceptor's outer segment for its renewal[23]. Therefore, we wanted to analyze the rhodopsin expression, a pigment that makes up rod photoreceptors. The immunohistochemistry for its expression showed significant reduction

Table 2. List of Genes Analyzed and Their Primer Sequences Used for Real-Time PCR.

Sl no.	Gene	Primer sequence	
1.	Rho (mouse)	Forward	5′-CAGTACTCGGAATGCAGCAA-3′
		Reverse	5′-CAGTCTTCAGGGGCTCTGTC-3′
2.	Thy1 (mouse)	Forward	5′-ATTCAGGCCTGCCGGGGTAC-3′
		Reverse	5′-AGTTCTTTCGTGAGCATGGA-3′
3.	BDNF (mouse)	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′
4.	CNTF (mouse)	Forward	5′-GCGAGCGAGTCGAGTGGTTGTCTG-3′
		Reverse	5′-TTAGCTTTCGGCCACCAGAGTGGAGAATTC-3′
5.	Ki67 (mouse)	Forward	5′-CAGTACTCGGAATGCAGCAA-3′
		Reverse	5′-CAGTCTTCAGGGGCTCTGTC-3′
6.	Bcl-2 (mouse)	Forward	5′-GCCCTTCGGAGTTTAATCAG-3′
		Reverse	5′-TACACTTGCACACACACGCT-3′

BDNF: brain-derived neurotrophic factor; CNTF: ciliary neurotrophic factor; PCR: polymerase chain reaction.

in rhodopsin levels in mice injured with laser, in comparison to the control mice. We then transplanted two types of cells, i.e., sixth day dissociated neurospheres differentiated from CE cells or undifferentiated Lin-ve SC derived from human UCB. The protein expression of rhodopsin at 1 week was found to be significantly higher in the retina transplanted with CE differentiated cells in comparison to the group transplanted with Lin-ve SC (Fig. 1A, B). The mRNA expression of rhodopsin was also estimated at 1 week and 3 months after transplantation. We found increased expression of rhodopsin in CE cells transplanted group as compared to Lin-ve SC transplanted group (Fig. 1C).

Differentiated CE Neurospheres Increase the Retinal Ganglion Cells (RGCs) of Retina

RGCs are a type of neurons present on the inner layer of retina. They transmit the visual stimulus at photoreceptor layer by engaging two types of neurons, i.e., bipolar and amacrine cells[24]. As rhodopsin was found to be significantly altered by laser-induced injury therefore, we wanted to analyze the effect of injury as well as transplantation of Lin-ve SC on RGCs. We found reduction of Thy1 protein expression after laser-induced retinal injury. However, transplantation of CE cells induced the upregulation of Thy1 expression (in comparison to control and laser injured mice) evident from quantitative analysis of immunohistochemical staining carried out at 1 week after transplantation (Fig. 2A, B). The mRNA expression of Thy1 showed significant increase after Lin-ve SC transplantation at 1 week, but this expression was abolished significantly when analyzed at 3 months after transplantation (Fig. 2C).

Neurotrophic Modulation by Paracrine Effects Exerted by Undifferentiated hUCB Lin-ve SC Transplantation

The existing literature of stem cell transplantation suggests that these cells either differentiate or aid integration in the host tissues, while others argue that stem cells act by providing neuroprotection mediated by the release of paracrine factors[25]. In the light of paracrine effects, we analyzed the expression levels of BDNF as well as CNTF in all the groups. We report that BDNF levels were upregulated more by transplantation of undifferentiated Lin-ve SC as compared to differentiated CE cells, when analyzed at 1 week (Fig. 3A, B). The mRNA expression of BDNF was concomitantly found increased after Lin-ve SC transplantation (in comparison to laser injured mice at 1 week). However, this effect was again diminished when analyzed at a longer time duration, i.e., 3 months (Fig. 3C).

CNTF is a ciliary neurotrophic factor and regarded as a survival factor for various neuronal types[26]. We estimated CNTF by immunohistochemistry, using confocal microscopy (Fig. 4A). By quantitative analysis (by Image J software), we found significant CNTF expression in laser injured mice. CNTF protein expression was also found upregulated in mice transplanted with CE cells in comparison to Lin-ve SC transplanted mice (Fig. 4B). The mRNA expression of CNTF, analyzed by real-time PCR, showed significant increase in Lin-ve SC transplanted mice in comparison to all other groups at 1-week after transplantation. However, this expression was diminished after 3 months of transplantation (Fig. 4C).

hUCB Lin-ve SC Transplantation Enhances Antiapoptotic and Proliferative Activity in Laser Injured Retina

The stem cells are reported to exert paracrine effects by releasing neurotrophic factors upon transplantation. These neurotrophic factors act as a ligand and initiate cell signaling pathways. BDNF acts as a ligand for TrkB receptor and activates CREB transcriptional factor, further promoting cell survival and maintenance of neuronal cells[27]. Likewise, CNTF is known to initiate Jak-STAT pathway and provides antiapoptotic and proliferative effect[28]. Bcl2, a well-known antiapoptotic marker, acts as a downstream molecule of CNTF initiated pathway. We found significant upregulation

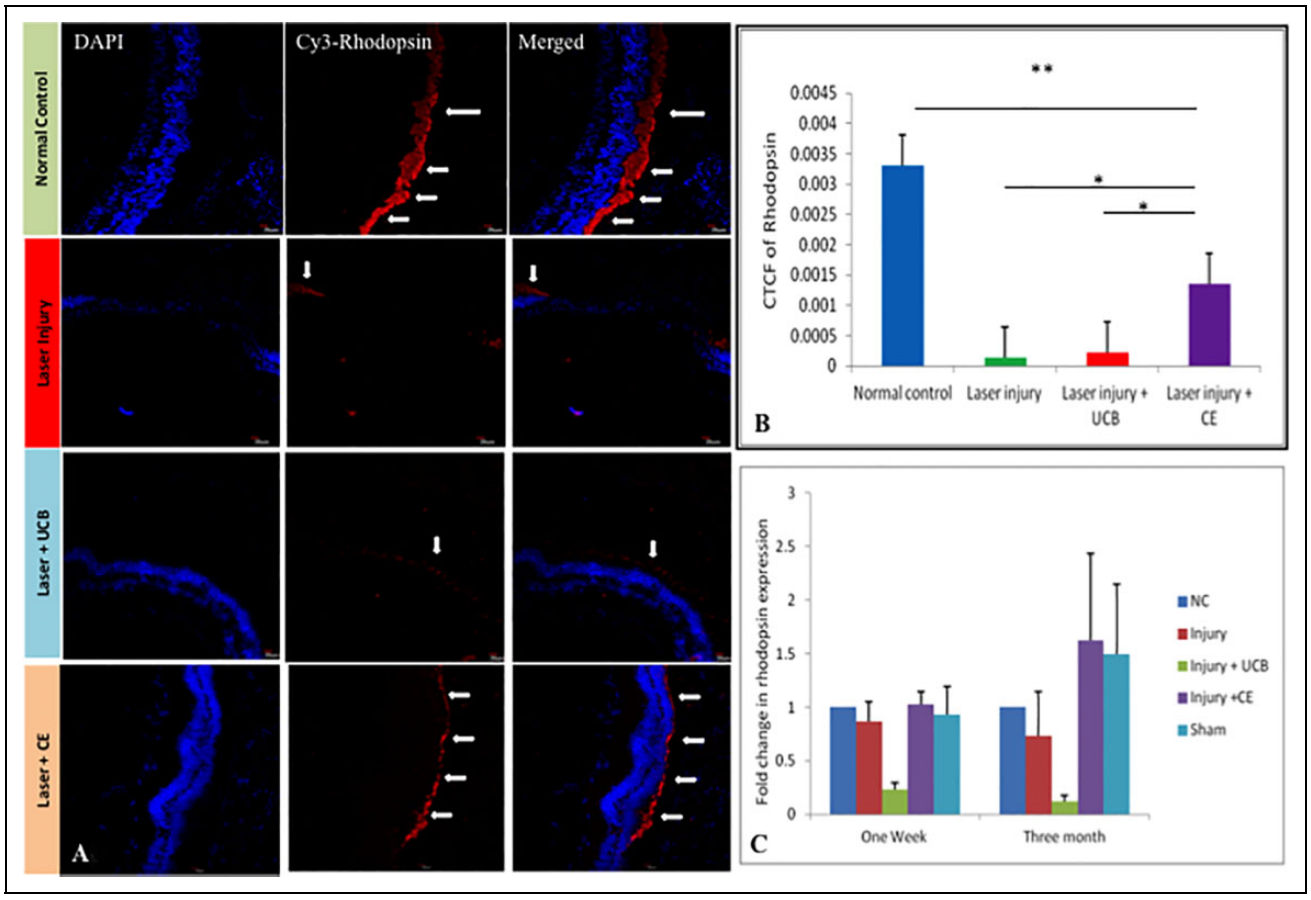

Figure 1. Differentiated CE cells transplantation enhances rod cells expression. (A) Immunohistochemistry of rhodopsin showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured retina mice, laser injured transplanted with Lin-ve SC mice, and laser injured transplanted with CE cells mice. (B) Quantitative protein expression of rhodopsin at 1-week time point measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of rhodopsin in all four groups was analyzed using real-time PCR at 1 week and 3 months after transplantation. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post-hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$ and **$P \leq 0.01$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

of Bcl2 mRNA expression after Lin-ve SC transplantation in laser injured mice in comparison to all the groups of 1 week and 3 months after transplantation. However, Bcl2 expression remained unaffected at 3 months after cells transplantation (Fig. 5A).

As proliferation markers are routinely analyzed in stem cell therapies, Ki67, a nuclear antigen of proliferation[29], was found highly upregulated upon transplantation of hUCB-derived Lin-ve SC (as compared to other groups at 1 week after transplantation). However, Ki67 expression was unaltered in all the groups at 3 months after transplantation (Fig. 5B).

Discussion

The series of degenerative changes in retina are due to diseased state with certain injuries resulting in irreversible damage. The currently used drugs have potential for symptomatic relief without halting the disease progression. Our data provide the comparative outcomes of transplantation of differentiated versus undifferentiated cells by utilizing a reproducible model of laser injured mouse retina. We wanted to examine the role of candidate markers of retinal repair and proliferation by transplantation of stem cells isolated from different origins. For that purpose, human eye from abortus fetuses was used to harvest the CE sourced stem cells. Second, we purified the Lin-ve SC from hUCB, a richly harvested source of undifferentiated stem cells.

Retina is an accessible and well-studied part of central nervous system. The retinal cellular structures have been studied in detail[30,31]. The existing studies provide evidence of the existence of stem cells in rodent retina. CE is one of the sources of stem cells shown as a rich source of cells with tremendous differentiation potential[15]. It has also been shown to possess the capacity to differentiate into specific retinal cell lineages for better

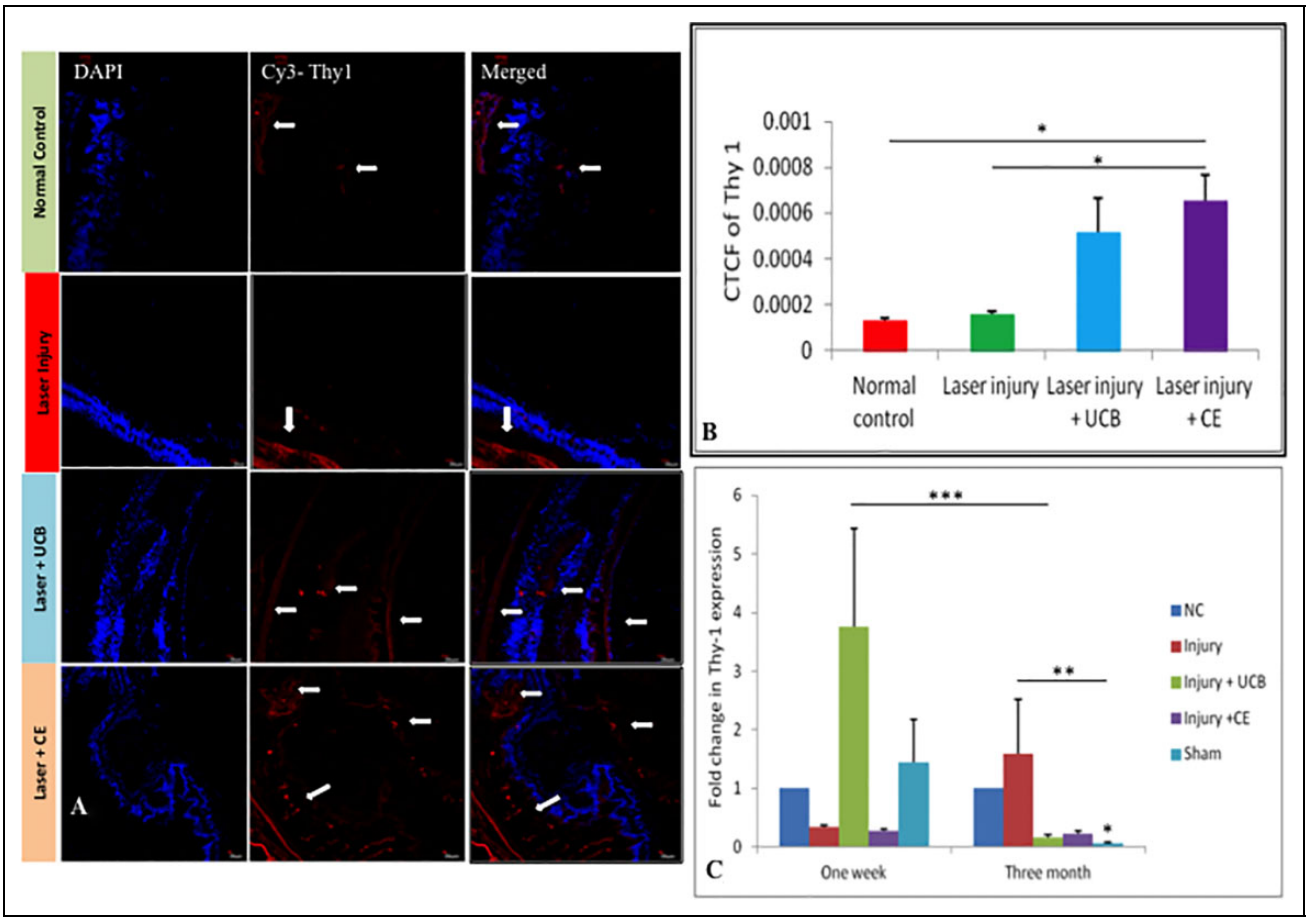

Figure 2. Differentiated CE cell transplantation enhances expression of retinal ganglion cells. (A) Immunohistochemistry of Thy1 showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control mice, laser injured retina mice, laser injured transplanted with Lin-ve SC mice, and laser injured transplanted with CE cells mice. (B) Quantitative protein expression of Thy1 at 1 week after transplantation was measured by corrected total cell fluorescence of immunohistochemistry images using Image J software (C). mRNA expression of Thy1 in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

integration into host tissue because of a similar niche. The pigmented CE has been shown to result in BrdU incorporation when provided with mitogens in vivo along with increase in cyclin D1 and Ki67 expression[32]. These cells, when stimulated with growth factors, rhEGF and bFGF, have been shown to form self-renewing colonies expressing retinal progenitor marker, i.e., Chx10[33]. Therefore, we used neurospheres that were differentiated from CE cells from human fetal eyes to examine how the stem cell markers are altered in laser-induced retinal injury in comparison to other cell types. We subretinally transplanted sixth day dissociated neurospheres into the laser-induced injury in retina of the mouse model. A significant expression of rhodopsin and Thy1 was reported in the mice retina after 1 week of transplantation (Figs. 1, 2). These data suggest that CE cells are involved in the rescue of retinal injury by activating the progenitor cells residing in the retina, which aid their differentiation into rods and RGCs. In comparison, CE cells are better positioned in the repair by activating the retinal markers and exerting neurotrophic effects superior to Lin-ve SC.

It has been shown that mesenchymal stem cells derived from hUCB exert neuroprotective effects by secreting several trophic factors including TGFbeta-1, NT-3, BDNF, and CNTF. These stem cells showed repair and regeneration of damaged neurons when transplanted in rat optic tract model[34]. Further, studies have shown transplantation of these cells intravenously in a neonatal hypoxic ischemia induced injury rat model could recover motor abilities mediated by neurotrophic factors, i.e., GDNF, BDNF, and NGF[35]. Endogenous cells secrete these neurotrophic factors mediated by paracrine effectors of transplanted cells. These

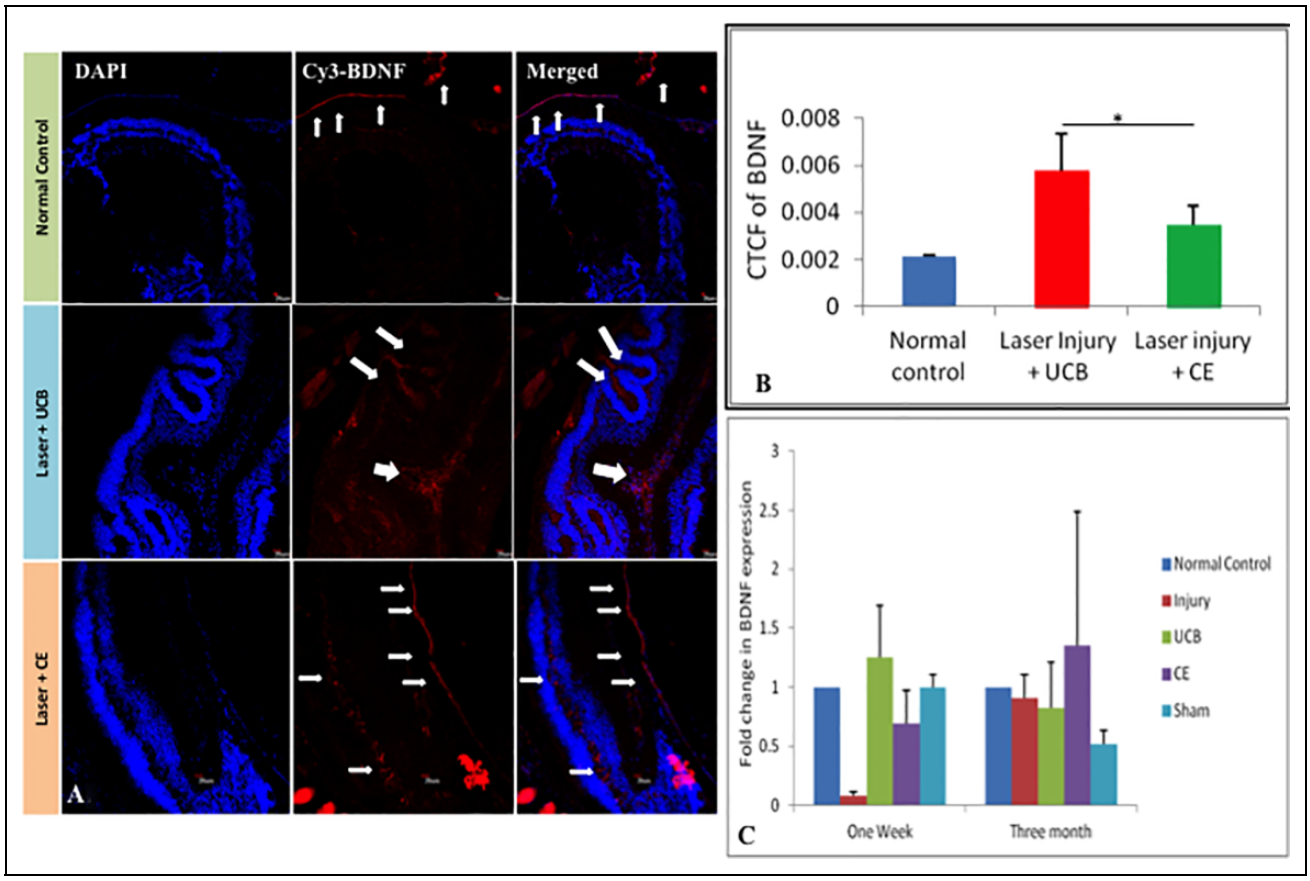

Figure 3. Neurotrophic factor modulation by the Lin-ve SC transplantation. (A) Immunohistochemistry of BDNF showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured mice transplanted with Lin-ve SC, and laser injured mice transplanted with CE cells. (B) Quantitative protein expression of BDNF at 1-week time point measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of BDNF in all four groups was analyzed using real-time PCR at 1-week as well as 3-month time point. Statistical analysis was performed using one-way ANOVA test for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$ was regarded as statistically significant. ANOVA: analysis of variance; BDNF: brain-derived neurotrophic factor; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

trophic factors generally play an active role in neuronal development and survival by promoting proliferation, regeneration, and maturation of neurons[32]. The administration of trophic factors, e.g., CNTF, BDNF, and NT-4 has resulted in prevention of ON-injury-induced RGC death[36]. Other studies have described the effect of trophic factor administration (CNTF, BDNF, or PEDF) via intravitreal route on prevention of phototoxic-induced degeneration of photoreceptors cells[37]. Hence, such studies provide the necessary basis of studying the levels of these paracrine factors in transplantation studies. Our results that there is upregulation of BDNF after Lin-ve SC transplantation are consistent with other studies (Fig. 3).

Our results showed that there was significant enhancement in the expression of CNTF in the Lin-ve SC transplanted retina (when compared to the laser injured and CE cells transplanted group). CNTF is known to regulate via JAK/STAT pathway[28]. Bcl2 comprises the downstream effector molecule[38] and hence the upregulation of Bcl2 and CNTF mRNA (Figs. 4C, 5A). However, immunohistochemistry shows the shoot in the level of neurotropic factors in injured group, which are maintained sustainably by the CE injected group while there is not the similar sustenance in the UCB injected group. The dichotomy of CNTF mRNA and protein expression can be ascribed to post-translational differences in both the experimental and intervention groups. This can be further examined by using specific inhibitors of CNTF.

Ki67 is the universal molecule for investigating proliferation. It was found to have increased expression upon Lin-ve SC transplantation (Fig. 5B). The activity of the enhanced endogenous proliferation needs further elaboration. Therefore, our results unanimously reveal that hUCB-derived Lin-ve SC, when transplanted at the site of retinal injury, induce expression of neurotrophic factors, which may exert neuroprotective effect mediated by the antiapoptotic and

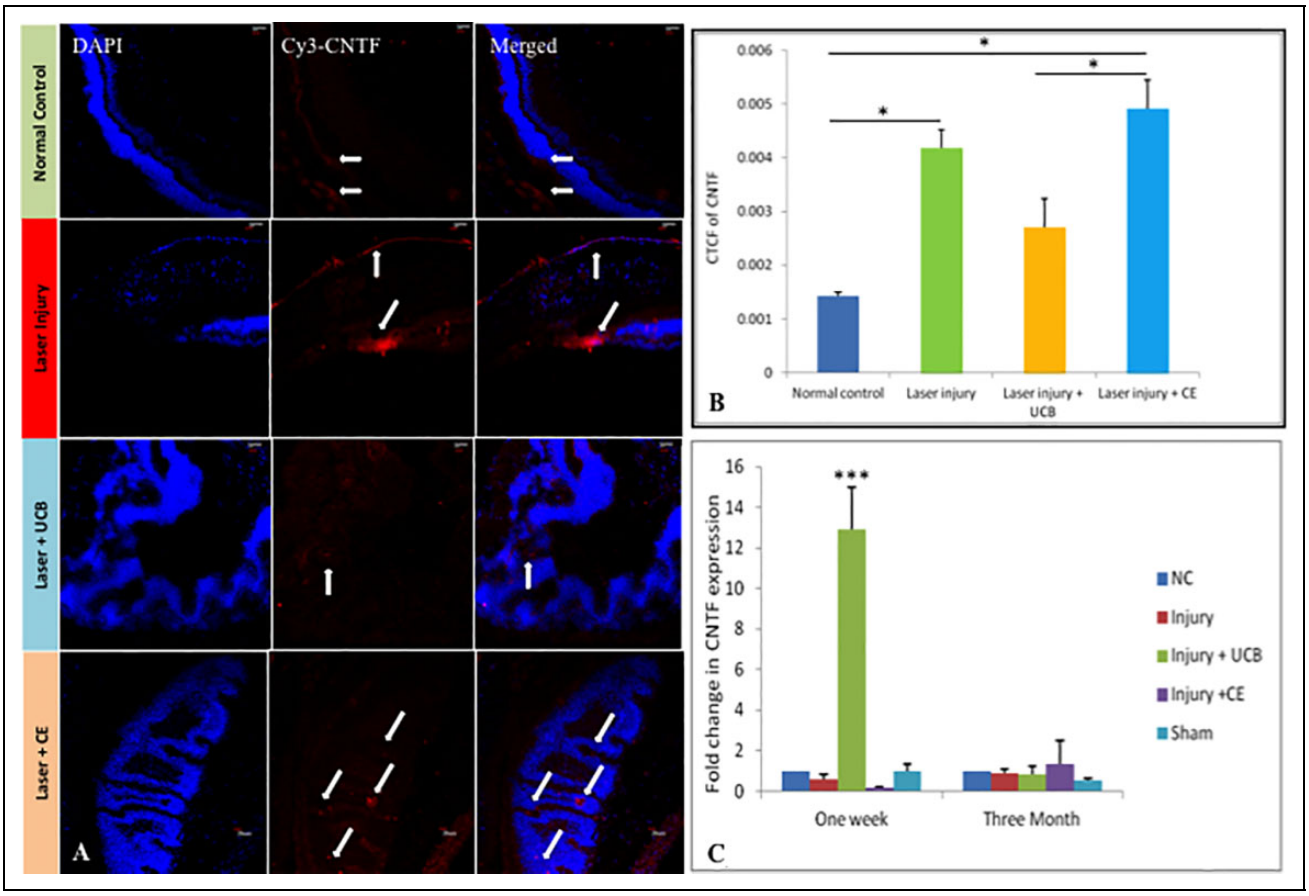

Figure 4. CE cell transplantation upregulated ciliary neurotrophic factor in the laser injured retina. (A) Immunohistochemistry of CNTF showing Cy3 (red fluorescence marked by arrows) expression bound to primary antibody at 20× visualized under confocal microscope in normal control, laser injured mice, laser injured mice transplanted with Lin-ve SC, and laser injured mice transplanted with CE cells. (B) Quantitative protein expression of CNTF at 1-week time after transplantation measured by corrected total cell fluorescence of immunohistochemistry images using Image J software. (C) mRNA expression of CNTF in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA for immunohistochemistry and real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; CNTF: ciliary neurotrophic factor; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

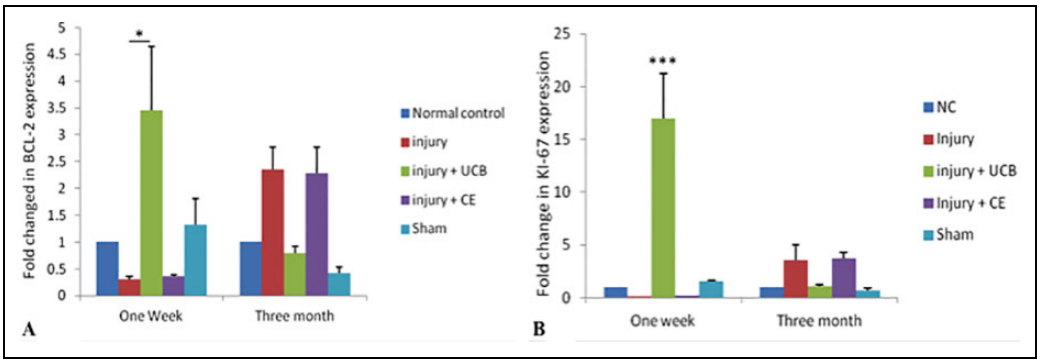

Figure 5. Antiapoptotic and proliferative activity enhanced by Lin-ve SC transplantation in laser injured retina. (A, B) mRNA expression of antiapoptotic marker Bcl2 and proliferative marker, i.e., Ki67 in all four groups was analyzed using real-time PCR at 1 week as well as 3 months after transplantation. Statistical analysis was performed using one-way ANOVA for real-time PCR results. This was followed by post hoc analysis using LSD, Scheffe, and Dunnett's test. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ were regarded as statistically significant. ANOVA: analysis of variance; CE: ciliary epithelium; Lin-ve SC: lineage negative stem cells; PCR: polymerase chain reaction.

proliferative mechanisms. Importantly, our study indicates the need for the multiple doses of Lin-ve SC transplantation instead of single dose as the paracrine effects noted in the study did not last for longer duration investigated.

Briefly, we explain the superior outcomes from transplantation of CE-related stem cells by ascribing it to same niche, i.e., retina providing conducive niche for rescue when compared to undifferentiated Lin-ve SC. In the context of noted paracrine effects, Lin-ve SC provide comparable outcomes but mediated by antiapoptotic and proliferative mechanisms. However, as Lin-ve SC effects lasted for a few weeks, it is suggested to examine the associated pathway at extended time frame using multiple doses of Lin-ve SC. Such studies will be helpful in utilizing the cord blood stored in the banks and for developing therapies for untreatable disorders of the retina. The main limitation of the study is that we were not able to perform immunohistochemistry of BDNF due to limited availability of eye samples.

Conclusion

The transplantation of CE cells showed superior outcomes than the undifferentiated Line-ve UCB-derived SC when tested in retinal injury mouse model. Based on the retinal marker analysis, we hypothesize that while the CE cells aid the formation of rods and ganglion cell layer, the Lin-ve SC transplantation promotes antiapoptotic and proliferative activity by neurotrophic modulation. We could not test the therapeutic outcome on the neuroprotective or paracrine effects from transplantation of multiple doses of Lin-ve SC or those at extended time points as the study was limited for a few weeks. Also, we have neither tested the per se effect of the transplantation of prominent markers on retinal repair nor evaluated the physiological changes. Hence, this study provides the rationale for more comprehensive analysis of the different sources of stem cells and their transplantation effects for extended duration. It is pertinent to note that clinical trial recently used RPE cells differentiated from autologous iPSCs and resulted in successful transplantation[39,40]. Our study thus provides preliminary data for future clinical application of the stem cell therapy for treating several retinal injuries.

Data Availability

All raw data are available with the first author of this manuscript.

Ethical Approval

Ethical approval for animals was obtained from Institutional Animal Ethics Committee (IAEC), Post Graduate Institute of Medical Education and Research, Chandigarh, India [71(69)/IAEC/423]. Human UCB samples were obtained after the approval from Institutional Committee for Stem Cell Research and Therapy, Post Graduate Institute of Medical Education and Research, Chandigarh, India (IC-SCRT: Approval No. PGI-ICSCRT-53-2014/1469).

Statement of Human and Animal Rights

All the experimental procedures were conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSEA) guidelines.

Statement of Informed Consent

The samples were obtained after filling the proper informed consent forms by participants after explaining the complete protocol.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD


Akshay Anand https://orcid.org/0000-0001-9003-3532

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Gene networks determine predisposition to AMD

Kaushal Sharma[a,b], Neel Kamal Sharma[c], Ramandeep Singh[d], Suresh Kumar Sharma[b,e], Akshay Anand[a,*]

[a] Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[b] Centre for Systems Biology and Bioinformatics, Panjab University, Chandigarh, India
[c] Scientific Research Department, Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Bethesda, MD 20889, United States of America
[d] Department of Ophthalmology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[e] Department of Statistics, Panjab University, Chandigarh, India

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ABSTRACT

Purpose: AMD genetic studies have revealed various genetic loci as causal to AMD pathology. We have described the genetic complexity of Indian AMD by describing the interaction of genotypes and subsequent changes in protein expression under the influence of environmental factors. This can be utilized to enhance the diagnostic and therapeutic efficacy in AMD patients.
Design: Genotype association was studied in 464 participants (AMD =277 & controls = 187) for eight genetic variants and their corresponding protein expression
Methods: SNP analysis and protein expression analysis was carried out in AMD and controls in tandem with longitudinal assessment of protein levels during the course of AMD pathology. ANCOVA and contrast analysis were used to examine the genotypic interactions and corresponding alterations in protein levels. In order to identify the important genetic variants Logistic Regression (LR) modeling was carried out and to authenticate the model Area under the Receiver Operating Characteristic curve (AUROC) were also computed.
Results: We have found genetic variants of rs5749482 (TIMP-3), rs11200638 (HTRA1), rs769449 (APOE) and rs6795735 (ADAMTS9) to be associated with AMD, concomitant with significant alterations of studied proteins levels. Analysis also revealed that the genetic interaction between APOE-HTRA1 genotypes and changes in LIPC levels (> 6 pg/ug) by one unit change in SNP, play a crucial role in AMD. LR model suggested that the seven factors (including both genetic and environmental) can be utilized to predict the AMD cases with 88% efficacy and 95.6% AUROC.
Conclusion: Results suggest that diagnostic and therapeutic strategy for Indian AMD must include estimation of genetic interaction and concomitant changes in expression levels of proteins under influence of environmental factors.

1. Introduction

Age related macular degeneration (AMD) is characterized as multifactorial heterogenous disease. AMD is characterized with deposition of drusen (constituted of lipoproteins, complement factors, oxidized lipids and pigment) between RPE (retinal pigment epithelium) and choroid in early ageing stage. Neovascular characteristics can be seen in advanced stage of AMD which can leak fluid in between retinal layers (wet AMD) and can further lead to atrophy of foveal photoreceptors (geographic atrophy) [1]. Both environmental and genetic factors have been associated with AMD pathology. Results from the recent study performed on Caucasian population have found various genetic variants to be significantly associated with AMD pathogenesis [2]. Despite the growing knowledge in field of AMD genetics, there is no advancement in diagnostic and treatment strategies to combat AMD. However, a potential tool for early diagnosis may be developed with the help of genetic studies. Such a tool could include analysis of the genetic interactions and complexity between of SNP variants along with their association with disease progression. Moreover, interaction between genetic variants, especially intronic SNPs with associated changes in expression pattern of protein, under the influence of environmental factors, can provide better insight of gene-phenotype correlation and may pave way towards the development of precise diagnostic tool for personalized medicine. This approach is lacking in most studies.

* Corresponding author.
E-mail address: akshay1anand@rediffmail.com (A. Anand).

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Association of socio-demographic data with genotype, and genotype-expression along with correlation analysis are key components in the study of complex diseases like AMD. It is postulated that such analysis may help in translation of basic research for societal need both from diagnostic and treatment point of view. To this end, it is important to ascertain the imperative role of candidate gene whether it is exerting its deleterious effect independently or by interacting with other genetic loci of an individual [3].

The present study was planned to map causal role of genetic and allelic variants among candidate genes and examine their correlation through protein expression profiles, AMD phenotypes and disease progression. We have also examined the association of socio-demographic parameters with the risk modifying genetic factors with variants in single ethnic North-West Indian AMD population. ANCOVA (analysis of covariance) and contrast analysis revealed the impact of single nucleotide changes, especially intronic variants, on alteration in LIPC (hepatic lipase) levels by estimating SNP's interactions in AMD patients thereby suggesting the impact of intronic variants in AMD diagnosis and treatment modules. Additionally, logistic regression has revealed precise diagnosis of Indian AMD with 88% classification efficacy (95.6% AUROC) by considering patient's age, food habits, diabetes and serum levels of IER3 (Immediate Early Response 3), HTRA1 (HtrA Serine Peptidase 1), and TIMP3 (tissue inhibitor of metalloproteinase 3) which may also be beneficial for management of AMD patients.

2. Methodology

2.1. Study design and recruitment of participants

We had recruited AMD patients from Advanced Eye Centre, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. 277 AMD participants were recruited in the study. Age matched controls (n = 187) were also recruited from Joshi Foundation camps held in Chandigarh region. The participants were included in the study as per the inclusion-exclusion criteria approved by the Institute ethical committee after obtaining informed written consent (Fig. S1). 18 AMD participants were followed up. The study adhered to the Institute Ethical Committee guidelines as per approval No: PGI/IEC/2005–06; dated: 23.07.2013 with matching approvals from Ethical Committee, Panjab University, Chandigarh (IEC No. 131A-1, dated: 29.10.2014). Study protocols adhered to guidelines of Helsinki declaration. The characteristics of recruited subjects have been presented in Table 1.

2.2. Clinical investigation and AREDS scoring of AMD patients

Comprehensive ocular examination and AREDS scoring was performed by retina specialist, Advanced Eye Centre, PGIMER, Chandigarh. Visual acuity, intra ocular pressure and fundus assessment of dilated eye were noted during clinical examination. AREDS scoring of AMD patients was carried out as per the AREDS criteria (Age-Related Eye Disease Studies) [4]. All AMD patients were screened through fluorescein fundus angiography in order to assess the morphology and number of drusen besides mapping the leakage in the retinal layers. Diagnosis of disease pathology was based on clinical findings of fluorescein angiographic and ophthalmoscopy.

2.3. Socio-demographic details of the participants

Socio-demographic details of participants were collected through a standardized questionnaire comprising of various activities of daily living (ADL) like food habits, smoking, alcohol consumption, habitat and comorbidities (supplementary file) *etc* along with clinical details of the participants.

Table 1
Tabular representation of characteristics of Indian AMD patients. ¥missing values in the studied population.

AMD features	Phenotypes	Number	Percent (%)
Gender	AMD Male	171	61.73
	AMD Female	106	38.27
	Control Male	99	52.94
	Control Female	88	47.06
Age (average ± SD)	AMD	68.30 ± 9.086	
	Control	56.94 ± 11.266	
AMD phenotypes	Dry AMD	42	15.2
	Wet AMD	91	32.9
	Bilateral AMD	144	52.0
Uni-bilateral AMD phenotypes distribution	Unilateral dry	42	15.16
	Unilateral wet	91	32.86
	Bilateral dry	28	10.10
	Bilateral wet	34	12.27
	Dry-wet bilateral	82	29.61
AMD phenotypes as per AREDS	AREDS 3	56	20.2
	AREDS 4	33	11.9
	AREDS 5	188	67.9
Avastin treatment¥	No Avastin	100	37.03
	Avastin	170	62.97
Anti-AMD drug (vitamin's supplement) ¥	No AMD drugs	80	29.53
	Anti-AMD drugs	191	70.47

2.4. Genetic analysis and protein expression

We undertook genetic analysis and expression profile of eight genes and their variants (Table S1). TaqMan assay was employed to identify the genetic variations in the population as per the manufacturer's instructions. Complete details are available as supplementary files.

Similarly, in order to assess the expression of these genes, ELISA of the respective proteins was carried out in the serum of AMD patients and further compared with controls. The procedure was followed as per the guideline provided by the manufacturers. The levels of candidate proteins were normalized to the total protein concentration of the respective samples. The complete procedure for both ELISA and total protein estimation is available in supplementary file.

2.5. Statistical analysis

Normality of current data was assessed by Normal Quantile plot (Q-Q plot) and Kolmogorov- Smirnov (K—S) tests. Univariate and multivariate logistic regression analysis were used for association of genetic frequency with disease pathology in the population. Further, genotype frequency (both homozygous recessive and heterozygous) of studied SNPs was compared with socio-demographic data including smoking, food habits, sleeping hours *etc* using univariate and multivariate logistic regression (adjusted for sex, age, smoking, food habit) by calculating Odd's ratio (OR) with 95% confidence interval (CI). Interaction analysis (gene-gene interaction) was performed to establish the genotypes interactions of significant SNPs found in the study. Moreover, fold changes in protein expression along with genotype interaction was also computed by contrast parameter analysis. To estimate the diagnostic efficacy and specificity of the logistic regression model to identify AMD cases from population was established through ROC curve and area under ROC (AUROC) curve. Independent t-test was employed to evaluate the differences in expression of proteins between two groups i.e. case (AMD) and control. Differential expression of protein with their respective SNP variant was also evaluated by one-way Analysis of variance (ANOVA). Spearman's correlation test was employed to find the correlation between analysed proteins in serum of the participants. p-value ≤ 0.05 was considered significant. All the statistical analysis

was performed using IBM SPSS Statistics version 20.0, Chicago, Illinois, USA software.

3. Results

3.1. Genotype association with Indian AMD

We performed the genetic association of eight SNP variants with AMD pathology as mentioned in Table S1. Univariate and multivariate analysis of SNPs showed significant association of TIMP-3 (rs5749482), HTRA-1(rs11200638), APOE (rs769449; Apolipoprotein E) and ADAMTS9 (rs6795735; ADAM Metallopeptidase With Thrombospondin Type 1 Motif 9) genotypes with Indian AMD patients. Heterozygous 'GC' genotype of SNP rs5749482 (TIMP-3) showed significant association with AMD pathology (95% CI = 1.132–3.358; OR = 1.949; p = 0.016), examined through univariate regression analysis. Results also revealed the higher frequency of homozygous 'CC' of same SNP in AREDS 3 and 4 grade AMD patients as compared to AREDS 5, computed by univariate (95% CI = 0.011–0.926; OR = 0.101; p = 0.043) as well as multivariate (CI = 0.008–0.803; OR = 0.082; p = 0.032) logistic regression. Moreover, rs11200638 SNP (HTRA-1) also showed a strong association with AMD pathology. Genotyping results further showed a significantly higher frequency of homozygous 'AA' genotype (rs11200638) in AMD patients in comparison to controls analysed through both univariate (95% CI = 0.148–0.493; OR = 0.270; $p \leq 0.0001$) and multivariate (95% CI = 0. 161–0.668; OR = 0.327; p = 0.002) logistic regression. Similarly, homozygous 'AA' genotype of rs11200638 SNP was also found to be significantly associated with AREDS 5 grade AMD as compared to AREDS 3–4. Genotype analysis of SNP rs769449 (APOE) with AMD pathology showed 'AG' genotype and its association with Indian AMD patients as compared to controls analysed by both univariate (95% CI = 1.265–4.173; OR = 2.298; p = 0.006) and multivariate (95% CI = 1.509–7.462; OR = 3.355; p = 0.003) logistic regression. However, we did not find any significant difference in genotype frequencies of rs769449 among different AMD subtypes. Moreover, rs6795735 SNP (ADAMTS9) also showed marginal difference in homozygous 'CC' genotype frequency in AMD and controls participants, analysed by unadjusted logistic regression (95% CI =0.086–1.052; OR = 0.300, p = 0.060). While adjusting for age, smoking, sex, food habits and alcohol, homozygous 'CC' genotype (of rs6795735 SNP) was found to be associated with AMD pathology through logistic regression analysis (95% CI =0.004–0.535; OR = 0.049, p = 0.013) (Table 2). Association analysis did not show any significant difference between genotype frequencies among AMD subtypes for rs6795735. We did not find any association of other SNPs including rs920915 (LIPC), rs9542236 (B3GALTL), rs3130783 (IER3) and rs8135665 (SLC16A8 or MCT3; Monocarboxylate transporter 3) with AMD pathology (Table S2).

Receiver operating curve (ROC) curve was plotted (1-specificity along x-axis and sensitivity along y-axis) and AUROC was computed to assess the authenticity of logistic regression model performed on significant genetic variants (APOE, TIMP-3 and HTRA1) and non-significant SNPs to diagnose the AMD cases from normal controls. ROC curve was also plotted to compare genetic interaction between significant (AUROC 60.2%, p = 0.082) and non-significant (AUROC 57.3%, p = 0.211) intronic variant to predict Indian AMD. Results suggested that it is imperative to consider the genetic interaction analysis to the studied cumulative impact on disease phenotype. This may play crucial role in diagnostic efficacy of AMD patients and so on with treatment paradigm (Fig. S2).

3.2. Association of allele frequencies with AMD

Analysis showed that the allele frequencies of ADAMTS9, TIMP-3, HTRA1, APOE and LIPC SNPs are linked to AMD pathology (Table 2). Allele 'C' of both pro-angiogenic SNPs including rs5749482 (TIMP-3) (95% CI = 1.258–3.073; OR = 1.966; p = 0.003) and rs6795735 (ADAMTS9) (95% CI = 0.393–0.883; OR = 0.011; p = 0.011) showed higher frequencies in AMD as compared to controls, signifying the crucial role of neovascularization in AMD pathology. Results indicate significantly higher frequency of 'C' allele of TIMP-3 rs5749482 in dry phenotype (AREDS 3 & 4) of AMD while comparing with wet (AREDS 5) (95% CI = 0.235–0.786; OR = 0.430; p = 0.005). Univariate logistic regression has also revealed the significant association of AMD with both 'C' (95% CI = 1.084–2.233; OR = 1.556; p = 0.016) and 'A' (95% CI = 1.210–3.760; OR = 2.135; p = 0.008) alleles of lipid metabolizing genes (SNPs) involving rs920915 (LIPC) and rs769449 (APOE), respectively. Allele 'A' frequency was also significantly much higher in dry (AREDS3-4) as compared to wet subtype (95% CI = 0.061–0.214; OR = 0.114; $p \leq 0.0001$). Moreover, genetic variant of HTRA-1 rs11200638 analysis also demonstrated the higher number of 'A' allele in AMD cases in comparison to controls using univariate logistic regression (95% CI = 0.307–0.595; OR = 0.427; $p \leq 0.0001$). Results suggest the 'A' as risk allele for disease progression due to higher number in wet AMD as compared to dry AMD phenotype (95% CI = 1.570–3.615; OR = 2.382; p ≤0.0001) (Table 2). Allele frequencies which were not found to be associated with North-West Indian AMD are mentioned in Table S3.

3.3. Association with socio-demographic factors

By considering AMD as multifactorial disease known to be associated with various genetic and environmental factors, we estimated the genetic association of SNPs with various socio-demographic parameters for their possible contribution to AMD pathology. Logistic regression analysis revealed that the sleeping pattern of AMD participant was found to be associated with 'GC' and 'CC' genotypes of TIMP3 rs5749482 (95% CI = 0.163–0.876; OR = 0.378; p = 0.023) and LIPC rs920915 (95% CI = 1.551–10.558; OR = 4.047; p = 0.004), respectively (Table 3). However, marginal association of sleeping pattern and 'CC' genotype of ADAMTS9 rs6795735 was also observed in AMD pathology using multivariate logistic analysis (95% CI = 0.097–1.043; OR = 0.318; p = 0.059). Food habits of the AMD participants has also shown the association with 'AG' genotype of IER3 rs3130783 (95% CI = 0.236–0.995; OR = 0.485; p = 0.048). 'CC' genotype of LIPC rs920915 has shown the marginal association with non-vegetarian diet by AMD patients while comparing with vegetarian participants (95% CI = 0.990–6.087; OR = 2.455; p = 0.053). Moreover, smoking habits and alcohol consumption were also found to be associated with genotypes of HTRA1 rs11200638, APOE rs769449 and LIPC rs920915 (Table 3).

Logistic regression analysis results indicated that sleeping pattern, food habit, smoking and alcohol consumptions can play crucial role in AMD pathology by showing their significant association with various genotype.

3.4. Protein expression in AMD

Expression level of above mentioned genes showed significant alterations between AMD and controls. Results revealed the significant enhancement of pro-angiogenic proteins (ADAMTS9 and TIMP-3), lipid metabolizing proteins (including LIPC and APOE), regulatory proteins (e.g. HTRA-1, IER-3 and B3GALTL) and monocarboxylic protein transporter (e.g. SLC16A8) in AMD cases, in comparison to controls. On the contrary, expression of SLC16A8 proteins was significantly decreased in AMD patients as compared to control participants (Table 4). Comparing the levels between dry and wet AMD phenotypes, the results have shown the significantly higher levels of HTRA1 (p = 0.048) and LIPC (0.043) proteins in wet AMD as compared to dry AMD pathology in North Indian Population. Dry AMD subtype has revealed significantly enhanced expression of SLC16A8 in comparison to wet AMD (Fig. S3). Moreover, while analyzing AREDS subtypes, SLC16A8 was also

Table 2
Logistic regression and allele frequency distribution of genetic variants in Indian AMD. Logistic regression analysis to associate the genotype frequencies of various genetic variants with North-West Indian AMD using univariate and multivariate method. Association of allele frequencies of various genetic variants with North-West Indian AMD by univariate logistic regression.

genotypes		Groups				Unadjusted p-value			Multivariate adjusted for age, sex, food habit, smoking, alcohol			AIC	BIC
		AMD		Controls		p-value	OR	95% CI	p-value	OR	95% CI		
rs6795735	TT	114	52.5%	57	61.3%	Ref							
(ADAMTS9)	CC	20	9.2%	3	3.2%	0.060	0.300	0.086–1.052	0.013	0.049	0.004–0.535	231.4	264.1
	CT	83	38.2%	33	35.5%	0.382	0.795	0.476–1.329	0.785	0.911	0.465–1.783		
	T	311	71.7%	167	81.1%	Ref							
	C	123	28.3%	39	18.9%	0.011	0.011	0.393–0.883					
		AREDS 3 & 4 (dry)		**AREDS 5 (wet)**									
rs6795735	TT	36	50.0%	78	53.8%	Ref							
(ADAMTS9)	CC	9	12.5%	11	7.6%	0.245	0.564	0.215–1.481	0.185	0.497	0.176–1.398		
	CT	27	37.5%	56	38.6%	0.888	0.957	0.522–1.754	0.851	0.941	0.499–1.774		
	T	99	68.8%	212	73.1%	Ref							
	C	45	31.3%	78	26.9%	0.343	**0.**809	0.523–1.254					
		AMD		**Controls**									
rs5749482 (TIMP-3)	GG	184	81.1%	77	67.5%	Ref							
	CC	5	2.2%	6	5.3%	0.090	2.868	0.850–9.677	0.397	2.178	0.360–13.195	288.8	322.4
	GC	38	16.7%	31	27.2%	0.016	1.949	1.132–3.358	0.146	1.657	0.839–3.274		
	G	406	89.4%	185	81.1%	Ref							
	C	48	10.6%	43	18.9%	0.003	1.966	1.258–3.073					
		AREDS 3 & 4 (dry)		**AREDS 5 (wet)**									
rs5749482 (TIMP-3)	GG	53	72.6%	131	85.1%	ref							
	CC	4	5.5%	1	0.6%	0.043	0.101	0.011–0.926	0.032	0.082	0.008–0.803		
	GC	16	21.9%	22	14.3%	0.110	0.556	0.271–1.141	0.176	0.598	0.284–1.259		
	G	122	83.6%	284	92.2%	Ref							
	C	24	16.4%	24	7.8%	0.005	0.430	0.235–0.786					
		AMD		**Controls**									
rs11200638 (HTRA1)	GG	42	20.7%	42	36.5%	Ref							
	AA	100	49.3%	27	23.5%	< 0.0001	0.270	0.148–0.493	0.002	0.327	0.161–0.668	291.1	324
	GA	61	30.0%	46	40.0%	0.335	0.754	0.425–1.339	0.945	0.976	0.490–1.944		
	G	145	35.7%	130	56.5%	Ref							
	A	261	64.3%	100	43.5%	< 0.0001	0.427	0.307–0.595					
		AREDS 3 & 4 (dry)		**AREDS 5 (wet)**									
rs11200638 (HTRA1)	GG	20	29.9%	22	16.2%	Ref							
	AA	24	35.8%	76	55.9%	0.006	2.879	1.347–6.154	0.006	3.074	1.376–6.867		
	GA	23	34.3%	38	27.9%	0.317	1.502	0.677–3.332	0.583	1.272	0.539–3.00		
	G	73	50.7%	82	30.1%	Ref							
	A	71	49.3%	190	69.9%	< 0.0001	2.382	1.570–3.615					
		AMD		**Controls**									
rs769449 (APOE)	GG	200	88.9%	94	77.7%	Ref							
	AA	0	0.0%	0	0.0%								
	AG	25	11.1%	27	22.3%	0.006	2.298	1.265–4.173	0.003	3.355	1.509–7.462	286	315.9
	G	425	94.4%	215	88.8%	Ref							
	A	25	5.6%	27	11.2%	0.008	2.135	1.210–3.760					
		AREDS 3 & 4 (dry)		**AREDS 5 (wet)**									
rs769449 (APOE)	GG	63	86.3%	137	90.1%	Ref							
	AA	0	0.0%	0	0.0%								
	AG	10	13.7%	15	9.9%	0.394	0.690	0.294–1.620	0.421	0.697	0.290–1.677		
	G	99	68.8%	289	95.1%	Ref							
	A	45	31.3%	15	4.9%	< 0.0001	0.114	0.061–0.214					
		AMD		**Controls**									
rs920915 (LIPC)	G	287	67.7%	101	57.4%	Ref							
	C	137	32.3%	75	42.6%	0.016	1.556	1.084–2.233					
		AREDS 3 & 4 (dry)		**AREDS 5 (wet)**									
	G	99	68.8%	289	95.1%	Ref							
	A	45	31.3%	15	4.9%	< 0.0001	0.114	0.061–0.214					

significantly increased in AREDS 4 as compared to AREDS 5 AMD patients ($p = 0.017$). Marginal alterations of B3GALTL levels ($p = 0.073$) have also been observed between the dry and wet AMD phenotypes (Fig. S3). Similarly, TIMP-3 expression between AREDS subtypes have also exhibited alteration between the AREDS subtypes (higher in AREDS 3 versus AREDS4) which was marginally significant ($p = 0.057$) (Fig. S3).

Importantly, estimation of protein expression longitudinally can provide better insights into disease progression and involvement of particular cellular mechanism(s) based on ongoing treatment of AMD patients. We have thus analysed the expression of ADAMTS9, APOE, and IER-3 longitudinally with a minimum interval of one year. Results have shown significantly decreased serum IER-3 levels in AMD during the course of disease and the prescribed treatment paradigm indicating the mechanistic role of IER 3 in pathophysiology of Indian AMD (Table S5).

Table 3
Association of covariates with genotype frequencies in Indian AMD patients using logistic regression analysis.

Genotype	Number (frequency)				Unadjusted *p* value			Multivariate analysis adjusted for age, sex		
					P-value	OR	95% CI	P-value	OR	95% CI
ADAMTS9 rs6795735										
	AMD disturbed sleep		**AMD normal sleep**							
TT	47	56.0%	62	49.2%	Ref					
CC	4	4.8%	15	11.9%	0.079	0.352	0.110–1.129	0.059	0.318	0.097–1.043
CT	33	39.3%	49	38.9%	0.690	0.888	0.497–1.590	0.676	0.882	0.490–1.589
TIMP3 rs5749482										
	AMD disturbed sleep		**AMD normal sleep**							
GG	77	90.6%	102	75.6%	Ref					
CC	0	0.0%	5	3.7%	0.999	0.000	0.000	0.999	0.000	0.000
GC	8	9.4%	28	20.7%	0.023	0.378	0.163–0.876	0.012	0.334	0.141–0.789
HTRA1 rs11200638										
	AMD Alcohol		**AMD Never alcoholic**							
GG	10	15.4%	32	23.2%	Ref					
AA	31	47.7%	69	50.0%	0.390	1.438	0.629–3.287	0.193	1.892	0.724–4.944
GA	24	36.9%	37	26.8%	0.102	2.076	0.864–4.986	0.040	3.029	1.052–8.718
IER3 rs3130783										
	Vegetarian AMD		**Nonveg AMD**							
AA	94	75.2%	90	87.4%	Ref					
GG	3	2.4%	0	0.0%	0.999	0.000	0.000	0.999	0.000	0.000
AG	28	22.4%	13	12.6%	0.048	0.485	0.236–0.995	0.057	0.491	0.237–1.020
APOE rs769449										
	AMD Smoker		**AMD non smoker**							
GG	61	81.3%	137	92.6%	Ref					
AA	0	0.0%	0	0.0%						
AG	14	18.7%	11	7.4%	0.015	1.691	1.108–2.580	0.031	2.907	1.103–7.664
LIPC rs920915										
	Vegetarian AMD		**Nonveg AMD**							
GG	58	50.9%	42	43.3%	Ref					
CC	9	7.9%	16	16.5%	0.053	2.455	0.990–6.087	0.063	2.407	0.954–6.070
GC	47	41.2%	39	40.2%	0.646	1.146	0.641–2.049	0.661	1.143	0.630–2.071
	AMD Smoker		**AMD non smoker**							
GG	37	54.4%	62	43.7%	Ref					
CC	10	14.7%	15	10.6%	0.809	1.117	0.455–2.742	0.989	0.993	0.373–2.643
GC	21	30.9%	65	45.8%	0.060	0.541	0.286–1.025	0.050	0.500	0.250–1.001
	AMD disturbed sleep		**AMD normal sleep**							
GG	35	42.7%	60	48.8%	Ref					
CC	17	20.7%	8	6.5%	0.007	3.643	1.426–9.307	0.004	4.047	1.551–10.558
GC	30	36.6%	55	44.7%	0.829	0.935	0.508–1.720	0.817	0.929	0.498–1.734

Table 4
Differential expression of proteins in serum of AMD and their comparisons with controls.

Proteins	Group	N	Mean	F-value	t-value	P-value
ADAMTS9 (pg/ug)	AMD	206	10.065	34.122	3.105	0.00206
	Controls	142	2.629			
APOE (pg/ug)	AMD	206	0.026	18.619	2.526	0.011939
	Controls	155	0.0036			
B3GALTL (pg/ug)	AMD	190	6.054	10.534	3.763	0.000201
	Controls	125	3.393			
HTRA1 (pg/ug)	AMD	193	4.45	13.295	3.823	0.000158
	Controls	130	2.618			
LIPC (pg/ug)	AMD	193	3.675	45.747	4.401	< 0.0001
	Controls	149	1.619			
TIMP3 (pg/ug)	AMD	187	0.061	18.008	4.239	0.000029
	Controls	146	0.021			
IER3 (pg/ug)	AMD	186	5.5	41.988	5.561	< 0.0001
	Controls	145	1.519			
SLC16A8 (pg/ug)	AMD	187	0.841	24.551	−2.307	0.021696
	Controls	131	1.378			

Interestingly, HTRA1 (F = 3.901; p = 0.022) and LIPC (F = 7.295; p = 0.001) levels were also found to be significantly altered between the genotypes of SNPs of respective genes using ANOVA analysis. Post hoc results for HTRA1 showed marginally significant alterations in levels between homozygous ‘AA' versus heterozygous ‘GA' with mean difference (MD) of 1.68 pg/ug units (95% CI = −0.0271-3.38; p = 0.055). Similarly, homozygous ‘GG' versus homozygous ‘CC' (MD = −3.65 units; 95% CI = −6.09-1.22; p = 0.001) and heterozygous ‘GC' versus homozygous ‘CC' (MD = 3.48 units; 955 CI = 1.06–5.90; p = 0.002) have shown significant alteration in LIPC levels between them. Hence, results indicate that a defined genotype of HTRA1 and LIPC in Indian AMD (intra-group) can alter respective protein levels. This may be beneficial to for development of treatment strategy with or without existing Anti-VEGF therapy (Table S6).

3.5. Protein expression in uni-bilateral AMD

Results have shown no significant changes in the ADAMTS9, APOE, B3GALTL, HTRA1 and IER3 levels in serum of AMD patients with respect to AMD phenotypes in both eyes. However, ANOVA analysis revealed significant changes in expression in LIPC and SLC16A8 levels and showed marginal alteration in TIMP3 levels in Indian AMD patients. Intra-group assessment (post hoc) revealed a significant changes in LIPC levels between unilateral wet and unilateral dry AMD cases (p = 0.014). Similarly, alteration in SLC16A8 levels was observed between bilateral dry and unilateral wet (p = 0.013) and also marginally significant differences between bilateral dry and wet-dry bilateral AMD phenotype (p = 0.058) (Fig. 1A). TIMP-3 expressions also differed marginally between the groups (p = 0.056). However, we did not find significant alterations in protein levels while comparing unilateral and

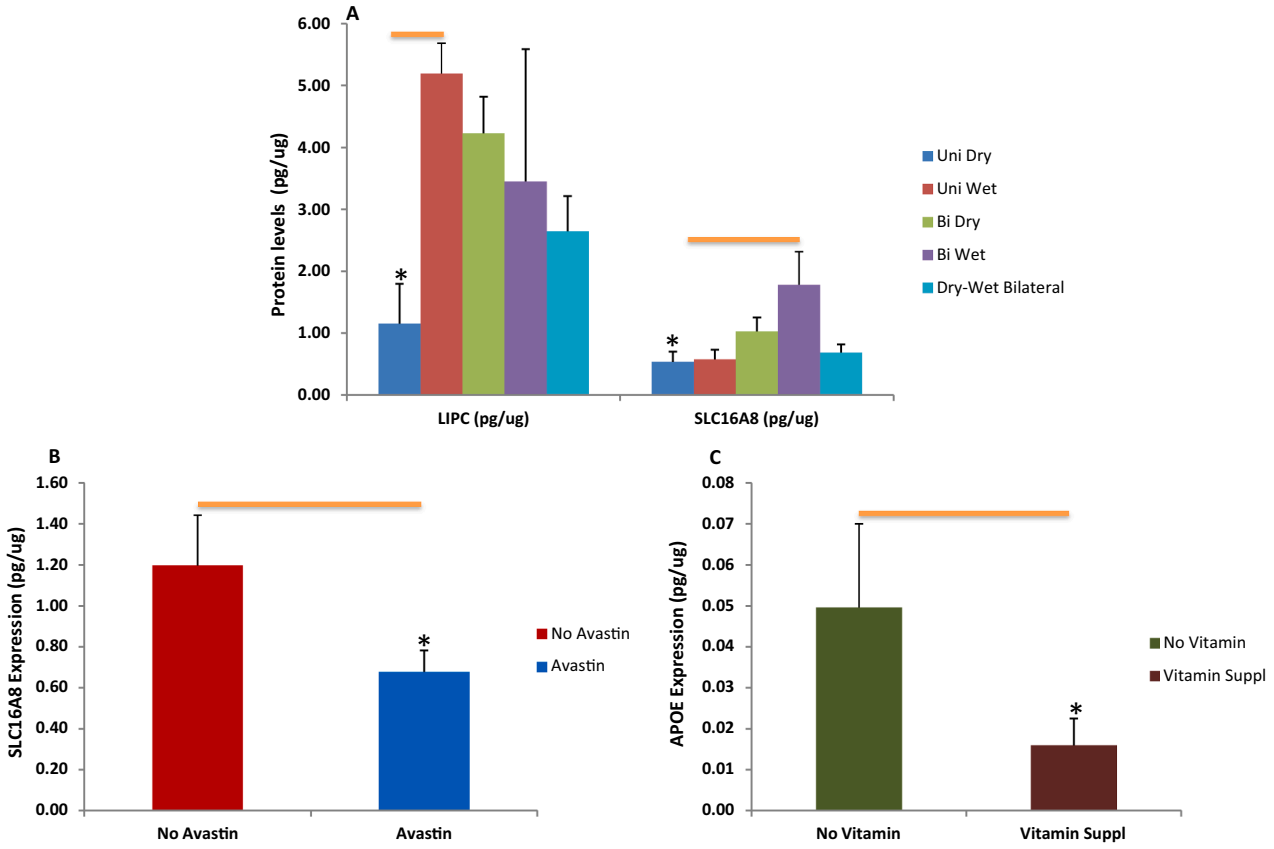


Fig. 1. (1A) Expression levels of LIPC and SLC16A8 along with error bars in unilateral-bilateral AMD pathology. (n) uni wet (63); uni dry (20); Bi wet (29); Bi dry (25); dry-wet (50). (1B–1C) Effect of treatment (including both anti- VEGF and vitamin supplementations) on the protein expressions (B) Alteration in SLC16A8 levels in Avastin and non-Avastin treated AMD patients, (C) Effect of vitamins supplementation on APOE levels. pg: pictogram; ug: microgram; error bar representing SE (*$p > 0.05$).

bilateral disease phenotypes for both dry as well as wet AMD.

Results suggest the protein expression of LIPC, SLC16A8 and TIPM-3 can be modulated based on clinical phenotypes and/or vice versa which can be correlated with clinical and treatment outcome of AMD patients.

3.6. Genetic interactions influence protein expression

To understand the heterogenic complexity in North-West AMD patients, we performed analysis of covariance (ANCOVA) between significant genetic variants i.e. rs11200638 (*HTRA*-1), rs5749482 (*TIMP*-3) and rs769449 (*APOE*) and dissected the statistical interactions between them. Genotypes of both rs769449 (*APOE*) and rs11200638 (*HTRA*-1) showed significant interaction between them (B = 0.782; Wald = 4.963; 95% CI; 1.099–4.349; OR = 2.186; $p = 0.026$). Interestingly, results signify the imperative role of gene-gene interactions and their cumulative outcome in heterogenic complex disease like AMD (Table 5). It has been shown that there is genetic interaction between HTRA1and APOE (Table 5).

Furthermore, we employed the contrast parameter estimate in order to unveil the impact of genetic composition on proteins expression. Results have demonstrated that one unit change in rs920915 LIPC genotype i.e. from homozygous 'CC' to homozygous 'GG' can alter the LIPC expression by > 6.0 pg/ug folds when controlling both significant (rs769449, rs5749482 and rs11200638) (SE = 1.747; $p = 0.0001$) and non-significant (rs920915, rs9542236, rs8135665, rs3130783, and rs6795735) genetic variants (SE = 2.137; $p = 0.002$) (Table 6). Hence, it can be inferred from results that heterogenic complexity in North-West Indian AMD is not only confined at the genetic level but can also be regulated by various cellular mechanisms.

Genetic interactions (gene-gene interaction) could indicate the precise heterogenic complexity of disease conditions like AMD. Genetic complexity arising from gene-gene, gene-protein and gene-

Table 5
Statistical genetic interactions between significant SNPs (including HTRA1, TIMP-3 and APOE) using ANCOVA analysis.

Variables in the Equation							
	B	S.E.	Wald	df	p-value	OR	95% CI
APOE genotype	0.102	0.235	0.188	1	0.664	1.107	0.699–1.754
HTRA1 genotype	0.143	0.218	0.429	1	0.512	1.154	0.752–1.770
TIMP3 genotype	0.266	0.197	1.830	1	0.176	1.305	0.887–1.918
APOE-HTRA1	**0.782**	**0.351**	**4.963**	**1**	**0.026***	**2.186**	**1.099–4.349**
APOE-TIMP-3	−0.285	0.339	0.708	1	0.400	0.752	0.387–1.461
HTRA1-TIMP-3	0.478	0.378	1.599	1	0.206	1.612	0.769–3.380
Constant	−1.687	0.397	18.006	1	0.000	0.185	

Table 6
Representation of gene-gene interaction and impact of protein expression using contrast estimation analysis.

Contrast Results: After controlling for significant and non-significant genetic variants							
Genotype		Significant Genotypes[+#]			Non-significant genotypes[#]		
		Contrast Estimate	Std. Error	p-value	Contrast Estimate	Std. Error	p-value
ADAMTS9 (pg/ug)	Level CC vs. Level TT*	−3.091	10.033	0.759	−17.123	12.833	0.185
	Level CT vs. Level TT*	−2.641	6.480	0.684	−11.090	7.846	0.161
APOE(pg/ug)	Level AA vs. Level GG*	0.049	0.032	0.122	0.064	0.047	0.172
B3GALTL (pg/ug)	Level CC vs. Level TT*	1.101	2.067	0.595	0.494	2.668	0.853
	Level CT vs. Level TT*	−1.387	1.415	0.330	0.375	1.761	0.832
HTRA1 (pg/ug)	Level AA vs. Level GG*	1.144	1.181	0.334	1.282	1.677	0.447
	Level AG vs. Level GG*	−0.201	1.337	0.881	−1.562	1.978	0.432
LIPC (pg/ug)	Level CC vs. Level GG*	6.659	1.747	**0.0001****	6.914	2.137	**0.002****
	Level CG vs. Level GG*	−0.326	1.129	0.773	−0.956	1.289	0.460
TIMP3 (pg/ug)	Level CC vs. Level GG*	−0.030	0.121	0.802	0.046	0.038	0.234
	Level GC vs. Level GG*	0.033	0.026	0.202			
IER-3 (pg/ug)	Level GG vs. Level AA*	0.366	2.047	0.858	−6.171	9.771	0.529
	Level AG vs. Level AA*				−1.227	2.433	0.615
SLC16A8 (pg/ug)	Level TT vs. Level CC*	−0.933	0.514	0.072	−0.548	0.754	0.469
	Level TC vs. Level CC*	0.118	0.298	0.694	0.113	0.396	0.775

[+] By controlling significant genotype: APOE, HTRA1 & TIMP3.
[#] By controlling non-significant genotype: ADAMTS9, B3GALTL, LIPC, IER3 & SLC16A8.
* Showing reference genotype.

sociodemographic interactions can influence disease phenotype and its progression which may further alter the therapeutic outcome of existing treatment in AMD patients. Results of the Spearman's correlation have indicated that *IER*-3, *B3GALTL*, *TIMP*-3 and *HTRA*-1 are more prominent proteins found to be correlated with the expression of most of studied proteins in Indian AMD patients. Moreover, levels of ADAMTS9, LIPC, APOE and SLC16A8 show the correlation with altered expressions of HTRA-1 and B3GALTL (Table S4). Conclusively, results suggest the cross-talk between proteins and their mediated functions which may regulate various cellular and molecular processes in Indian AMD pathology.

3.7. Effect of AMD treatment on protein levels

The effectiveness of the treatment is governed by genetic composition and susceptibility of an individual. The effect of treatment (Avastin and vitamin supplements) on protein levels were also analysed in North-West Indian AMD patients. Results of the study revealed a significant alteration in SLC16A8 and APOE levels with the treatment of Avastin (Anti-VEGF) ($p < 0.05$) and vitamin supplementations drugs ($p < 0.05$), respectively (Fig. 1B-1C). No significant difference in other protein levels were observed among mentioned subgroups. However, we did not find any significant alteration in CFH levels in Avastin treated AMD as compared to non-Avastin AMD group [5]. No study has revealed the direct evidence between anti-VEGF and/or vitamin supplementations and alterations of both proteins SLC16A8 and APOE levels in AMD patients. This may provide the needed information to predict the treatment outcome when with Anti-VEGF therapy is used.

3.8. Logistic regression model

We also attempted to establish the statistical equation to predict the AMD cases in early life which can be useful to prognostication and diagnosis of AMD. In this study, forward stepwise (likelihood) binary logistic regression (BLR) analysis has shown seven variables which found to be best fit in equation out of 24 variables. Seven variables which was significantly associated with prediction of AMD cases viz age, food habits, comorbidity, diabetes and serum levels of IER3, HTRA1, TIMP3 as depicted in equation (Table 7).

$$f(y) = \frac{1}{1 + e^{-y}}.$$

Table 7
Best fit for maximum likelihood significance of independent variables analysed by logistic regression equation. Moreover, classification of North-West Indian AMD cases by maximum likelihood computed by logistic regression equation.

Variables in equation						
Variables	B	S.E.	Wald	df	p-value	Exp(B)
Age	−0.132	0.020	42.504	1	< 0.0001	0.876
Food Habit	−0.463	0.238	3.793	1	0.051	0.629
Co-morbidity	−1.633	0.427	14.617	1	< 0.0001	0.195
Diabetes	2.275	0.448	25.752	1	< 0.0001	9.729
IER3 levels	−0.409	0.094	19.036	1	< 0.0001	0.664
HTRA1 levels	0.688	0.155	19.729	1	< 0.0001	1.991
TIMP3 levels	−108.604	14.924	52.954	1	< 0.0001	0.000
Constant	10.512	1.626	41.792	1	0.000	36,747.561
Classification table						
		Predicted				
Group code		Group code				Percentage
		AMD	Control			corrected
	AMD	241	24			90.9
	Control	24	131			84.5
Overall percentage						88.6

Forward stepwise BLR model is used to compute the probability. Let

$$Y = \beta_0 + \beta_1 X_1 + \ldots + \beta_k X_k \quad (1)$$

and define

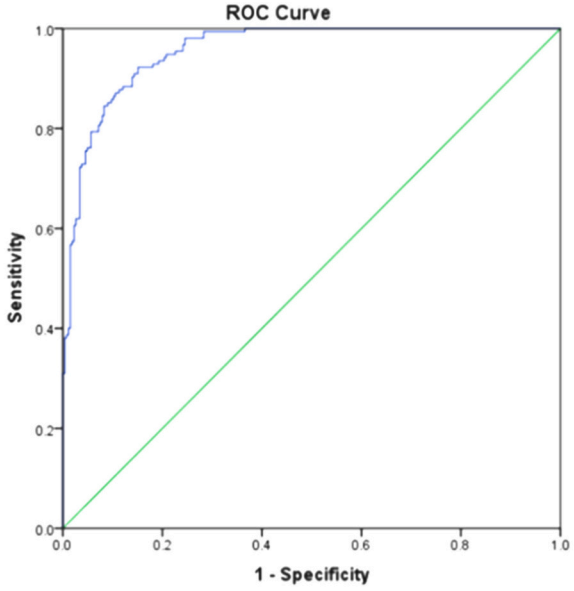
$$P(X) = \frac{1}{1 + e^{-\left(\beta_0 + \sum_{j=1}^{k} \beta_j X_j\right)}}$$

Therefore

$$\text{logit } P(X) = \log \frac{P(X)}{1 - P(X)} = \beta_0 + \sum_{j=1}^{k} \beta_j X_j \quad (2)$$

To estimate the likelihood model on the given data, null hypothesis was considered to be best fit to predict the AMD cases more precisely from the population.

Model predictability for best fit of AMD cases was determined by Hosmer–Lemeshow goodness which shows the chi square (χ^2) = 4.217, degree of freedom (df) = 8, and $p = 0.837$. Hence, logistic model is satisfactorily justify the null hypothesis that the data fits well to the



Area Under the Curve				
Test Result Variable(s):	Predicted probability			
			Asymptotic 95% CI	
Area	Std. Error[a]	Asymptotic Sig.[b]	Lower Bound	Upper Bound
.956	.009	.000	.939	.973

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

Fig. 2. ROC of logistic regression model to put forward a probable statistical best fit equation to predict AMD cases from the population with 95% AUROC.

logistic regression. Moreover, coefficient of determination (R2) analysed by both Cox-Snaell and Nagelkerke's tests were observed around R2 = 0.553 and R2 = 0.755, respectively which suggesting the strong association of independent variables with dependent variables in current logistic regression model. The predicted equation is:

$$Y = 10.512 - 0.132\,(\text{age}) - 0.463\,(\text{food habit}) - 1.633\,(\text{comorbidity}) + 2.275\,(\text{diabetes}) - 0.409\,(\text{IER3 levels}) + 0.688\,(\text{HTRA1 levels}) - 108.604\,(\text{TIMP3 levels})$$

3.9. Authenticity of the model

Current model equation obtained by using forward stepwise logistic regression analysis has showed 88.6% predictability of AMD cases classification in the studied population (Table 7). Moreover, the sensitivity and specificity of the model sing stepwise logistic regression analysis has derived the best fit model to predict the AMD cases from the population with around 95.6% AUROC (Fig. 2).

4. Discussion

Global AMD genetics has revealed various independent genetic loci to be associated with Caucasian AMD [2,6]. Not many studies have been carried out to identify the genetic complexity of AMD pathology which describe the diagnostic and therapeutic efficacy. Current investigations have explored the genetic association of rs5749482 (TIMP-3), rs11200638 (HTRA1), rs769449 (APOE) and rs6795735 (ADAMTS9) loci with North-West Indian AMD patients who has unique diet and geographical distribution. Majority of this population doesn't smoke due to religious diktats. Results have also exhibited the genotype association with other covariates like smoking, food habit, sleeping pattern *etc* in AMD patients. Moreover, allele distribution of genetic loci including rs5749482 (TIMP-3), rs11200638 (HTRA1), rs920915 (LIPC), rs769449 (APOE), and rs6795735 (ADAMTS9) were also found to be significantly different between AMD and control population which indicates various risk and protective alleles in Indian AMD pathology. We also examined the protein expression of the same set of genes in order to examine the causal impact of SNP changes, especially intronic variants. This is lacking in most of GWAS and Caucasian AMD genetic reports [7]. Additionally, our results have extensively estimated the protein levels of candidate genes between AMD and controls. This showed significant alteration between two groups. HTRA1 and LIPC levels have exhibited a significant difference between two different disease phenotypes of AMD i.e. wet and dry subtypes. Similarly, SLC16A8 and TIMP3 levels were also found to be altered with the progression of disease i.e. differential expressions in AREDS subtypes. Expression results of LIPC and SLC16A8 levels have also exhibited differential expressions in uni and bilateral Indian AMD patients. Our prior study has showed the changes in eotaxin-2 levels with their disease phenotypes in uni- and bilateral AMD condition of patients [8]. However, the longitudinal follow up of the AMD participant has revealed the changes in only IER-3 levels during the course of disease and prescribed treatment strategy. Our previous study has also demonstrated the altered expression of *LIPC, TIMP-3, IER3* and *SCL16A8* in *CFH* negative AMD cases and indicated that AMD pathology is independent of CFH which may be govern through these genes [9]. Hence, our results conclusively are suggesting various aspects in advancement of and diagnostic and treatment paradigm which can assist existing Anti-VEGF therapy, can be prescribed in non-responsive AMD patients and/or changes in treatment strategy based on the genetic makeup (genotype based differential expression), genetic interaction and per nucleotide changes in protein expression.

Surprisingly, ANOVA and ANCOVA both analyses have indicated that importance of genetic makeup of the participants which can influence the expression pattern of proteins regardless of genomic location and biological significance. Our results of contrast analysis have shown significant alteration of LIPC levels with changing the genetic

makeup by one unit, indicating the genetic interaction through intra and intergenic variants which may impact the pertaining cellular functions. Intragenic and epistatic interactions, especially in multigenic disease pathologies, cannot be ignored. Such investigations have showed the significant genetic association between genotypes of HTRA1 and APOE. Therefore, contrast analysis demonstrates the fold changes in LIPC levels with respect of their reference genotype. Both results indicate AMD as genetic complex disease (based on geographical distribution) and may be dealt diagnostically and therapeutically by considering the degree of complexity and fold changes in protein levels with reference to single nucleotide changes which can be beneficial to set the amount of dose with or without existing treatments (Anti-VEGF or Vitamin supplements) or provide novel treatment based on patients genetic complexity. Lately, gene-gene interaction strategy has demonstrated the synergistic effect on AMD pathology i.e. by epistatis interaction [10] which can further modulate the drug response in patients [11]. Moreover, correlation results have also implicated the alteration of protein expression which is dependent on each other suggesting co-expression of proteins in Indian AMD which could further indicate the crosstalk between various cellular downstream signaling and regulatory processes.

Interestingly, our studies reveal the significant decrease in levels of SLC16A8 and APOE in patients being treated with Anti-VEGF and vitamin supplementations, respectively. Though, how AMD treatment (Avastin and vitamin supplementations) leads to changes in SLC16A8 and APOE levels could be a matter of investigation as most of AMD treatment based on anti-VEGF therapy [10,11]. Our results on AMD patients specify the responsiveness and effectiveness of AMD treatment which can be determined by genetic interaction and complexity of patients based on their genetic and expression data. This can provide insights into personalized medicine for AMD.

Our attempt to understand the complexity of Indian AMD, results of logistic regression model has also supported the genetic interaction along with environmental factors and comorbidity prevail in participant. Hence, comprehensive genetic analysis by investigating gene-gene, gene-protein and gene-environmental interactions can provide the precise genetic network (epistatic interaction) and their associated phenotypic outcome which could be beneficial to map the treatment strategy and development in personalized medicine [12,13]. In our previous study, we had demonstrated the gene-gene interaction with two different SNPs of CCL2 variants to predict the AMD pathology independently [14]. Importantly, LR model has also suggested that AMD diagnostic efficacy can be enhanced by considering by patient's age, food habits, diabetes and serum levels of *IER3, HTRA1, TIMP-3* and could also be useful to specify the treatment paradigm based on such results. Estimation of same set of proteins in vitreous fluid can directly reflect the pathological alterations at microenvironment in AMD pathology which is a limitation of this study.

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Contribution

KS: Data acquisition, co-conceptualization, analysis and writing of manuscript; NKS: Manuscript editing and sample collection; RS: Clinical investigations of participants; SKS: Co-conceptualization, data analysis and statistical modeling, editing of the manuscript; AA: PI, acquired funding, conceptualization and editing of the manuscript.

Author's statement

None of the authors have showed competing financial interests with this manuscript and have read the manuscript and agreed to authorship as it is presented.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2020.09.044.

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Association of Plasma biomarkers for Angiogenesis and Proteinopathy in Indian Amyotrophic Lateral Sclerosis Patients

Shweta Modgil[1] Radhika Khosla[1] Abha Tiwari[1] Kaushal Sharma[1] Akshay Anand[1]

[1]Department of Neurology, Neuroscience Research Lab, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Address for correspondence Akshay Anand, PhD, Department of Neurology, Neuroscience Research Lab, Post graduate Institute of Medical Education and Research, Chandigarh, India 160012 (e-mail: akshay1anand@rediffmail.com).

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Abstract

Background Amyotrophic lateral sclerosis (ALS) is a rare motor neuron disease with progressive degeneration of motor neurons. Various molecules have been explored to provide the early diagnostic/prognostic tool for ALS without getting much success in the field and miscellaneous reports studied in various population.

Objective The study was aimed to see the differential expression of proteins involved in angiogenesis (angiogenin [ANG], vascular endothelial growth factor [VEGF], vascular endothelial growth factor receptor 2 [VEGFR2], etc), proteinopathy (transactive response DNA binding protein-43 [TDP-43] and optineurin [OPTN]), and neuroinflammation (monocyte chemoattractant protein-1[MCP-1]) based on the characteristics of ALS pathology. Though, suitable panel based on protein expression profile can be designed to robust the ALS identification by enhancing the prognostic and diagnostic efficacy for ALS.

Methods A total of 89 ALS patients and 98 nonneurological controls were analyzed for the protein expression. Expression of angiogenic (VEGF, VEGFR2, and ANG), neuroinflammation (MCP-1), and proteinopathy (TDP-43 and OPTN) markers were estimated in plasma of the participants. Proteins were normalized with respective value of total protein before employing statistical analysis.

Results Analysis has exhibited significantly reduced expression of angiogenic, proteinopathy, and neuroinflammation biomarkers in ALS patients in comparison to controls. Spearman's correlation analysis has showed the positive correlation to each protein.

Conclusion Altered expression of these proteins is indicating the prominent function in ALS pathology which may be interdependent and may have a synergistic role. Hence, a panel of expression can be proposed to diagnose ALS patient which may also suggest the modulation of therapeutic strategy according to expression profile of patient.

Keywords
- amyotrophic lateral sclerosis
- angiogenic markers (VEGF, VEGFR2, angiogenin)
- TDP-43
- optineurin
- MCP-1/CCL-2

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Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a devastating neurodegenerative disease characterized by progressive degeneration of neurons and muscles.[1] The disease is believed to share common genetic link; however, only 10% of the diagnosed cases have family history (mainly associated with C9ORF72 mutations [40% of cases], superoxide dismutase-1 [SOD1] mutations [10-20% of cases] and TAR DNA binding protein-43 [TDP-43, 4% of cases][2] and remaining 90% of the cases are sporadic. The incidence of ALS has been reported to be 1.5 to 2.5 per 100,000 individuals per year[3] with worldwide prevalence of 6 in 100,000 individuals.[4] In India, approximately 5 of 100,000 individuals get affected from ALS[5] with higher prevalence in males than females.[6] Upper and lower motor neurons in the cerebral cortex, brainstem, and spinal cord degenerate because of which movement is affected.[7] As the disease progresses, all voluntary muscles are affected and daily activities like walking, talking, eating are severely compromised. This is followed by adverse effect on involuntary muscles including respiratory, as well as cardiac muscles, proving to be life-threatening. Patients suffering from ALS die within 1 to 5 years of the detection of the disease because of respiratory or cardiac failure[8] with a few patients surviving up to 10 years.

In spite of decades of research, the prognosis of the disease remains elusive with limited treatment strategies. Riluzole is the only Food and Drug Administration (FDA)-approved drug for ALS and can only provide symptomatic relief.[9] Earlier diagnosis of the disease is challenging but critical for management of the ALS patients. Neuroinflammation is prominently correlated with ALS disease onset and is found to be associated with monocyte chemoattractant protein-1 (MCP-1), and other inflammatory cytokines (and receptors like CCR2), fibronectin, interleukins, etc.[10] A marked variation in expression profile of these molecules has been described. In most of the familial ALS patients, at least one of these genes has been found to be affected. However, SOD1 is mutated in 20% of patients while TDP-43 mutation has been linked with 3 to 4% familial ALS cases.[11] It has been shown that TDP-43 gets accumulated in the neurons of ALS patients, also termed as TDP-43 proteinopathy. Increased level of TDP-43 has been reported in the cerebrospinal fluid (CSF) and plasma of patients as compared with controls.[12] Along with VEGF (vascular endothelial growth factor), VEGFR2 (VEGF receptor 2), and ANG (angiogenin; hypoxia responsive gene responsible for vascularization) are also believed to be associated with disease. Similarly, OPTN (optineurin) is known to be deposited as inclusion bodies but its levels in the plasma or serum not analyzed yet.

Protein expression analysis of various circulating proteins (in biofluid) has potential for biomarker discovery and can aid in the early diagnosis/prognosis and advancement in treatment strategies in ALS. For this reason, the expression of proteins known to be involved in this disease are routinely being examined in biological fluids and correlated with disease severity and progression. However, results from various studies lack consistency making their potential as a biomarker for disease prediction unreliable. For example, VEGF levels were found to be unaltered in ALS patients' CSF and spinal cord sections,[13,14] while in the ALS serum, VEGF was reported to be elevated.[15,16] We have earlier shown in a study on North Indian population that VEGF-A is increased in ALS patients' serum, as well as CSF.[16] Similarly, studies related to other associated molecules in the ALS pathology, like ANG,[17] TDP-43,[18] MCP-1, have also showed diverse reports. Conclusively, ALS diagnostic efficacy can be enhanced by proposing a panel of protein expression chip to precisely identify the ALS with increasing efficacy.

Present study has attempted to examine the expression of these molecules including VEGF, ANG, TDP-43, OPTN, VEGFR2, and MCP-1 in ALS patient's plasma to propose the probable diagnostic panel for early diagnose ALS panel.

Materials and Methods

Patient Recruitment and Sample Collection

A total of 89 ALS patients and 98 genetically unrelated healthy controls were recruited for the study as per the informed consents, duly approved by the institutional ethical committee. Patients visiting outpatient department (OPD), who were clinically diagnosed to have ALS, were included. All the patients were found to have sporadic onset of disease without any family history. Mean ALS functional rating scale (FRS) score was found to be 34.59. Patients were categorized according to the ALS FRS-R scoring into minimal, mild, and moderate-to-severe categories[19] in accordance with increasing severity of the disease. Four criteria come under the ALS FRS-R covering functional assessment of trunk, cervical, lumbosacral region, and respiratory functions. Each section has three questions with answers ranging from 0–4.

Characteristics of the Patients Recruited: Sociodemographic Analysis

Out of total 89 patients recruited, 67 participants were males and 22 were females, that is, nearly 75.28% of participants were males and 24.72% were females suggesting higher prevalence of the disease in males than females. The average age of all the ALS participants was 48.43 years and 41.98 years in case of controls. After measuring height and weight of the participants, the body mass index (BMI) was calculated as BMI = weight(kg)/height(m^2). No significant difference was found between the BMI of patients (average, 22.7 kg/m^2) and control (average, 24.10 kg/m^2).

Amyotrophic Lateral Sclerosis Diagnostic Classification

The severity of ALS disease in Indian patients was based on ALS FRS R scoring. The ALS patients were classified into three categories as per the score obtained which includes minimal, mild, and moderate-to-severe. ALS FRS is the functional

rating scale designed to assess the progression of the disease in ALS patients. The scale includes factors related to physical health and health of motor functions, as well as respiratory functions. Patients are categorized based on the scoring.[20]

El Escorial criteria is a diagnostic criteria for ALS patients. Patient group can be divided into the following three categories according to the criteria: definite, probable and possible.[21]

Plasma Isolation

Blood sample was taken in ethylene diamine tetra-acetic acid (EDTA) coated vials (BD vacutainers) and mixed thoroughly to avoid blood coating. The samples were layered on equal volume of Histopaque (HiSep LSM 1077, HiMedia Laboratories, Mumbai, Maharashtra, India) and then centrifuged at 1,500 rpm for 30.0 minutes at room temperature, kept at room temperature. Plasma was collected from upper layer which appeared as transparent fluid.

Enzyme-Linked Immuno-sorbent Assay

Sandwich enzyme-linked immunosorbent assay (ELISA) was used for the estimation of various molecules. Commercially available ELISA kits were used to estimate the protein levels for ANG, VEGF, VEGFR2, OPTN, TDP-43 (Qayee Bio-Technology Co., Ltd., Shanghai, China), and MCP-1 (Diaclone SAS, Besancon, France) in plasma of participants as per the standard protocol described by manufacturer. Briefly, 50 µL of standard and diluted samples (range: 2–10 times of dilution) were added to the wells, after which HRP conjugated antibody was added. The plate was set for incubation at 37°C for 1 hour. This allowed the antigen to bind with antibody precoated in the wells of ELISA plate. After washing the plate, 50 µL of chromogen solution A and B were added in dark and the plate was incubated for 10 minutes at 37°C in dark. The stop solution was added and estimation was done using ELISA plate reader (iMARK reader, BioRad) at 450 nm. OD values were noted.

Total Protein Estimation

Total protein was estimated by Bradford's method using Bovine serum albumin (BSA) as standard. Coomassie brilliant blue reagent (Bio-Rad Protein Assay Dye Reagent Concentrate, 450 mL no.: 5000006, Bio-Rad Laboratories, Hercules, California, United States) was used for total protein estimation and absorbance was taken at 595 nm. ELISA values were normalized with total protein present in the sample. The mean and standard deviation was calculated for each protein.

Statistical Analysis

Data were analyzed by using SPSS version 21. Data normality was analyzed using Kolmogorrov–Smirnov test and Shapiro–Wilk test depending upon the sample size. Mann–Whitney *U*-test was applied to compare ALS and normal control groups. Kruskal–Wallis test was employed to analyze the comparative protein levels in ALS subtypes including minimal, mild, and moderate-to-severe to see the protein variation with the disease severity of ALS, Spearman's analysis was done to see correlation between studied proteins and to see the probable mechanistic crosstalk in ALS pathology. Protein levels were also correlated with age, BMI, and duration of disease (in months) using Spearman's correlation analysis. The association of variations in the protein levels with various sociodemographic parameters was analyzed using parametric and nonparametric tests. The sociodemographic parameters included El Escorial criteria, gender, smoking habits, alcohol consumption, feeding habits, onset of the disease (early or late onset), and duration of the disease (short duration or long duration).

Results

Protein Expressions in Indian Amyotrophic Lateral Sclerosis Patients

Differential Total Protein in Amyotrophic Lateral Sclerosis Patients

Total protein estimation for the plasma samples of the patients and controls indicated increased level of the total protein concentration in the patient group as compared with controls (►**Fig. 1**). These total protein values were used for normalization of the target protein concentrations estimated using ELISA.

Target Protein Concentration

ELISA results indicated lower levels of all the above-mentioned six proteins in ALS patients' plasma as compared with normal controls. OPTN levels were significantly reduced in patients (►**Fig. 2A**, $p = 6.9 \times 10^{-5}$). When categorized among minimum, mild, and moderate-to-severe, on the basis of disease progression, the protein levels were found to be marginally decreased in severe category compared with other subtypes. The decrease was, however insignificant (►**Fig. 3A**, $p = 0.443$). Plasma from ALS patients was found to have significantly lower levels of TDP-43 (►**Fig. 2B**, $p = 1.4 \times 10^{-3}$). Reduced expression both OPTN and TDP-43 are suggesting the proteinopathy in Indian ALS.

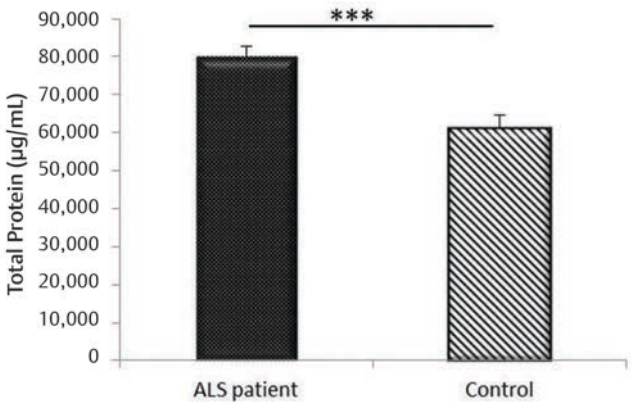

Fig. 1 Total protein expression and comparison of ALS patients and normal controls. ALS, amyotrophic lateral sclerosis.

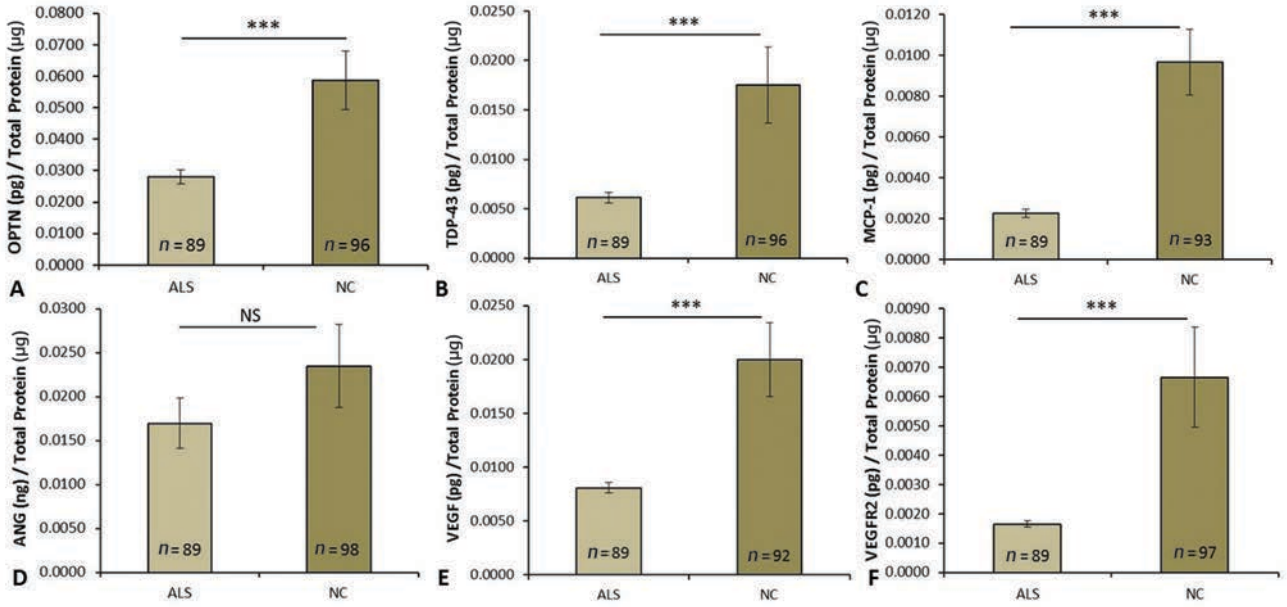

Fig. 2 Plasma protein levels of (**A**) optineurin (OPTN), (**B**) TDP-43 (TAR DNA binding protein), (**C**) MCP-1 (monocyte chemoattractant protein-1), (**D**) Angiogenin (ANG), (**E**) VEGF (vascular endothelial growth factor) and (**F**) VEGFR2 (VEGF receptor 2) estimated by ELISA in ALS patients and controls. All data are expressed as mean ± SEM. Significance was considered at $p \leq 0.05$, $^{***}p$ value ≤0.001. ALS, amyotrophic lateral sclerosis; NC, normal control; NS, nonsignificant.

Moreover, significant decrease in level of MCP-1 was also reported in ALS patients compared with control (►**Fig. 2C**, $p < 10^{-6}$). Likewise, categorization according to ALSFRS score showed nonsignificant alterations in MCP-1 level with disease severity (►**Fig. 3B**, $p = 0.435$) suggesting that a minute changes in MCP-1 may ubiquitously stimulate ALS pathology.

When we analyzed the expression of angiogenic markers, for example, VEGF, VEGFR2, and ANG, we did not find any significant changes in ANG levels between ALS and controls (►**Fig. 2D**, **p** = 0.262). Though, angiogenic proteins including VEGF (►**Fig. 2E**, $p < 10$) and VEGFR2 expressions (►**Fig. 2F**, $p < 3.4 \times 10$) were also significantly decreased in ALS patients in comparison to controls. However, similar to other proteins, the comparison of protein levels among the subcategories of ALS patients did not reveal any marked difference. Though downward trend was observed for VEGF (►**Fig. 3C**, $p = 0.335$), ANG (►**Fig. 3D**, $p = 0.703$), TDP-43 (►**Fig. 3E**, $p = 0.638$), and VEGFR2 (►**Fig. 3F**, $p = 0.808$) with increased disease severity yet the difference was insignificant.

Protein–Protein Correlation

ELISA results have showed the decreased expression of studied proteins. However, biological interactions between them to show pathological significance in ALS have not been established yet. We have analyzed the multiple correlation using Spearman's test to investigate the protein–protein interactions, as well as correlation of protein levels, with age, BMI, and duration of disease in Indian ALS patients. Results have showed strong positive correlation between all studied proteins. These proteins may be interdependent (►**Table 1**). The pathological characteristics has also suggested the neuroinflammatory-, angiogenic-, and proteinopathy-associated changes in ALS patients. Results implicate the prospective interactions and cross-talk between these proteins in the progression of ALS pathology in Indian population. No significant correlation was found between protein levels and parameters such as age, BMI, and duration of disease (►**Supplementary Table 1**; available online only). Interestingly, results have revealed significant alter levels of ANG in definite, probable, and possible ALS patients based on EI Escorial scoring (►**Supplementary Table 2**; available online only)

Discussion

Analysis of biomarkers can be useful in the early diagnosis of diseases. Especially having panel of molecules can help better in diagnosis of disease, instead of analyzing a single molecule in plasma. Having chip-based tools that can analyze panel of interacting biomarkers for a disease can prove helpful in diagnosis, as well as prognosis of disease. Plasma proteomics is being used increasingly for the analysis of concentrations of various biomarkers in the blood, although it is very expensive. Plasma is considered as an important biofluid for assessing diffusion of proteins from several tissues. Current research is directed in strengthening early diagnosis of ALS or at least analyzing the prognosis of disease via varying protein levels. VEGF is a major angiogenic molecule that is responsible for vascularization at the time of development, as well as later in life. Various studies have assessed the concentration of VEGF in serum, plasma, and CSF of ALS patients at various stages of the disease. *VEGF* levels in serum and CSF have been found to be increased in some of the studies. For example, Gao et al also measured VEGF concentration in patients at

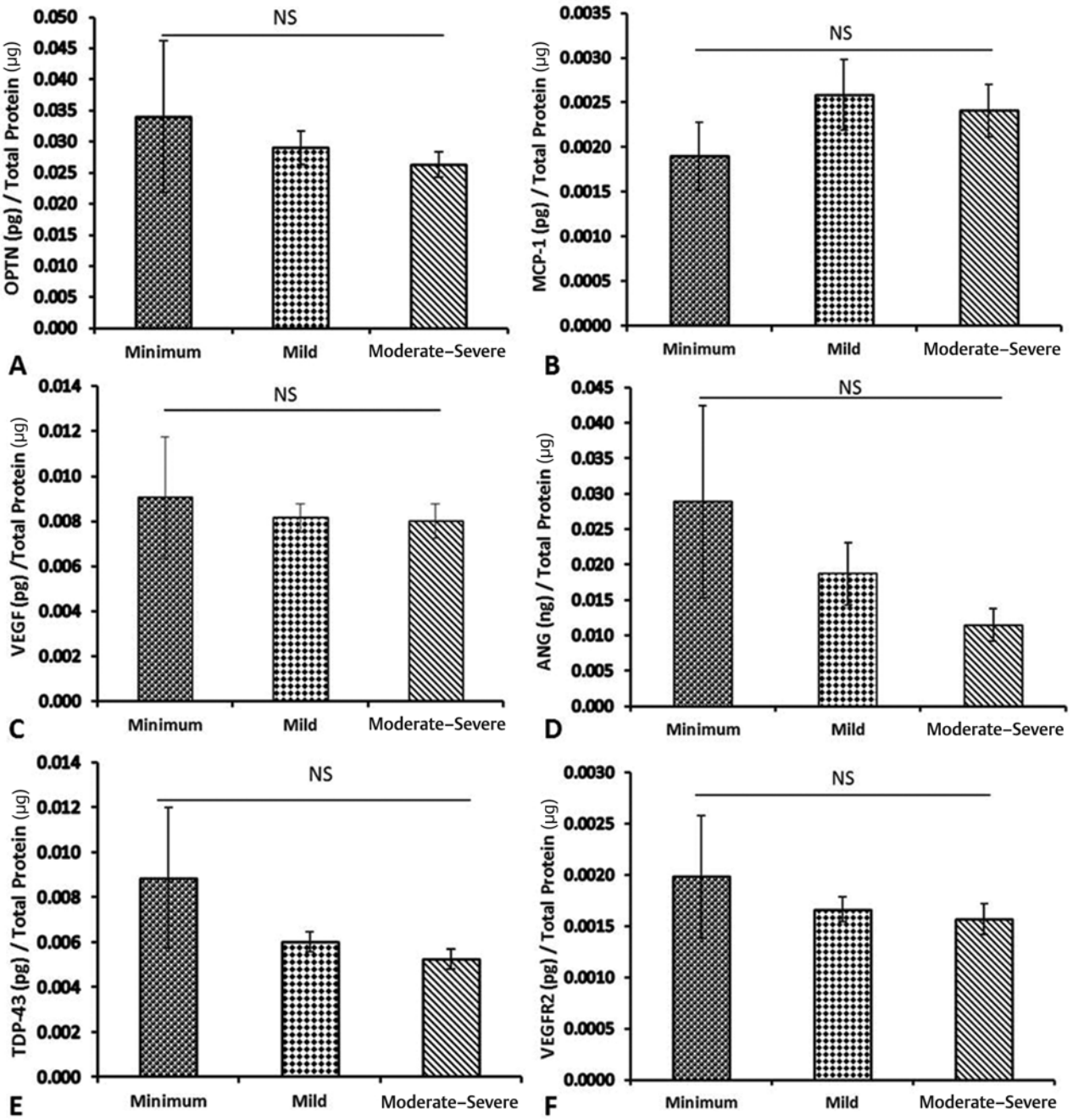

Fig. 3 Association of severity of ALS (minimum, mild, and moderate–severe) based on ALS FRS-R score with (**A**) optineurin (OPTN), (**B**) MCP-1 (monocyte chemoattractant protein-1) (**C**) VEGF (vascular endothelial growth factor), (**D**) angiogenin (ANG), (**E**) TDP-43 (TAR DNA binding protein) and (**F**) VEGFR2 (VEGF receptor 2) in ALS patients. Data are represented as mean with standard error as error bar. Significance was calculated by Kruskal–Wallis test and considered at $p \leq 0.05$. ALS, amyotrophic lateral sclerosis; FRS, functional ration scale; NS, nonsignificant.

different time intervals after the disease onset. According to this study, the upregulation of VEGF level was found to be more in patients with the progression of the disease rather than during 12 months.[15] They argued that VEGF levels get upregulated in patients as the disease progresses.[16] However, Nygren et al showed that CSF concentration of VEGF is not increased in patients.[14] When the postmortem spinal cord sections were analyzed, it was similarly found that there was no increase in VEGF levels. But in our study, we found opposite trend in Indian patients. *VEGF* level was decreased significantly in patients as compared with controls, although we did not carry out the autopsy studies. More research in this direction can clarify this variation depending upon family history and demographic analysis. As VEGF is required for angiogenesis, decreased VEGF concentration may exacerbate degeneration of motor neurons because of hypoxia created by reduced vascularization.[22,23] This can also explain the increase in ANG levels of patients. VEGF levels reported

Table 1 Spearman's correlation analysis between studied proteins in Indian ALS pathology

MCP-1	$r = 1$					
VEGF	$r = 0.599$ $p < 10^{-6}$	$r = 1$				
VEGFR2	$r = 0.591$ $p < 10^{-6}$	$r = 0.596$ $p < 10^{-6}$	$r = 1$			
ANG	$r = 0.639$ $p < 10^{-6}$	$r = 0.411$ $p = 6.4 \times 10^{-5}$	$r = 0.772$ $p < 10^{-6}$	$r = 1$		
TDP-43	$r = 0.583$ $p < 10^{-6}$	$r = 0.446$ $p = 1.2 \times 10^{-5}$	$r = 0.759$ $p < 10^{-6}$	$r = 0.833$ $p < 10^{-6}$	$r = 1$	
OPTN	$r = 0.723$ $p < 10^{-6}$	$r = 0.888$ $p < 10^{-6}$	$r = 0.792$ $p < 10^{-6}$	$r = 0.662$ $p < 10^{-6}$	$r = 0.620$ $p < 10^{-6}$	$r = 1$
Spearman's correlations	**MCP-1**	**VEGF**	**VEGFR2**	**ANG**	**TDP43**	**OPTN**

Abbreviations: ANG, angiogenin; ALS, amyotrophic lateral sclerosis; MCP-1, monocyte chemoattractant protein-1; OPTN, optineurin; TDP, TAR DNA binding protein; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

in various studies have shown mixed results.[17] In our study, VEGF was found decreased. It can be hypothesized that decreased VEGF levels might lead to hypoxia in response to which hypoxia inducing factor (HIF-α) getting activated that may further induce ANG expression, either as a compensatory response to hypoxia associated with low-VEGF levels or exerting neurogenic effects. Although, our study didn't show any significant difference of ANG in patients as compared with controls, Cronin et al reported increased ANG expression. In this context, it is pertinent to state that Cronin et al did not report any correlation between VEGF and ANG levels.[17] But in our study, we found significant correlation between both the molecules.

In our study,TDP-43 levels were found decreased significantly in ALS patients when analyzed by ELISA.TDP-43 proteinopathy is an important characteristic of ALS and frontotemporal dementia (FTD).[24,25] Granules of TDP-43 are found to get aggregated in the cytoplasm of neurons[26] due to which neurons begin to degenerate. Expectedly, TDP-43 concentration in plasma and serum has been reported to be increased in several studies,[12,27] albeit no such study from India has so far analyzed this. TDP-43 is involved in the regulation of angiogenic genes.[28] TDP-43 regulates progranulin in tandem with VEGF. Briefly, it acts as an inducer of angiogenic genes which can be studied in tandem while analyzing TDP-43 role in ALS. Further, TDP-43 proteins have a nuclear localization signal that allows it to enter the nucleus and act as inducer. However, in the various cases of ALS, disruption of nuclear localization signal (NLS) causes formation of TDP-35 and TDP-25 fragments which start accumulating in cells and form protein aggregates. These protein aggregates further entrap TDP-43 molecules and form protein inclusions.[29] Therefore, it is attractive to hypothesize that hypoxia, being an important risk factor in ALS, coupled with importance of angiogenesis in the neuroprotection, TDP-43 levels might influence the angiogenic pathway in severe forms of ALS. However, this needs to be examined in larger sample size.

In developing an understanding of angiogenesis–hypoxia cross talk in ALS, VEGFR2'srole in ALS cannot be underestimated, as it acts as a receptor of VEGF. In our study, its levels showed decreasing trends. It is pertinent to point out that there is decrease in VEGFR2/VEGFR1 as reported in various studies.[30,31] Decrease in VEGFR2 can be ascribed to feedback loop moderated by VEGF expression. The discussion of angiogenesis–hypoxia axis in the pathogenesis of ALS is incomplete without reviewing the cross talk of OPTN with TDP-43. OPTN has nuclear factor-κB (NF-κB) suppressive activity and inhibits the tumor necrosis factor (TNF)-α–mediated NF-κB activation. Mutations in the OPTN activate TNF-αand the caspase pathway,[32,33] disrupting the nuclear localization signal of TDP-43. Consequently, it is found increased and accumulated as protein inclusions in the motor neurons of ALS patients as shown by IHC studies on post mortem spinal cord sections.[34] For decrease in TDP43 values, as found in our study, it can be hypothesized that excessive accumulation of TDP43 in cells can be a cause of decreased plasma levels of TDP-43. As TDP-43 is unable to target the nucleus, it cannot induce *VEGF*- (angiogenic genes) causing hypoxia. In this study, we found a decrease in OPTN in plasma of ALS patients, possibly hampering the regulation of TDP-43, as postulated above.

MCP-1 was also found to be significantly decreased in ALS patients. MCP-1 levels are also known to be elevated in ALS patients due to associated neuroinflammation in the progression of ALS.[16] As the severity of the disease progresses, the alterations are expected to be enhanced. OPTN, VEGF, ANG, TDP-43, and VEGFR2 show decreasing trend as the severity of the disease progresses (in accordance with ALSFRS scoring) but the difference was not significant. Along with discussing differential levels of various proteins, we tried to hypothesize the possible interaction pattern between these proteins and that such panels of interacting molecules can be studied for analyzing their diagnostic or prognostic potential. Possible association and interaction between these molecules have been presented in ►**Fig. 4** .

Conclusion

The candidate biomarkers analyzed in this study showed fluctuating trends in the plasma of ALS patients. VEGF, VEGFR2, OPTN, TDP-43, and MCP-1 were downregulated and were

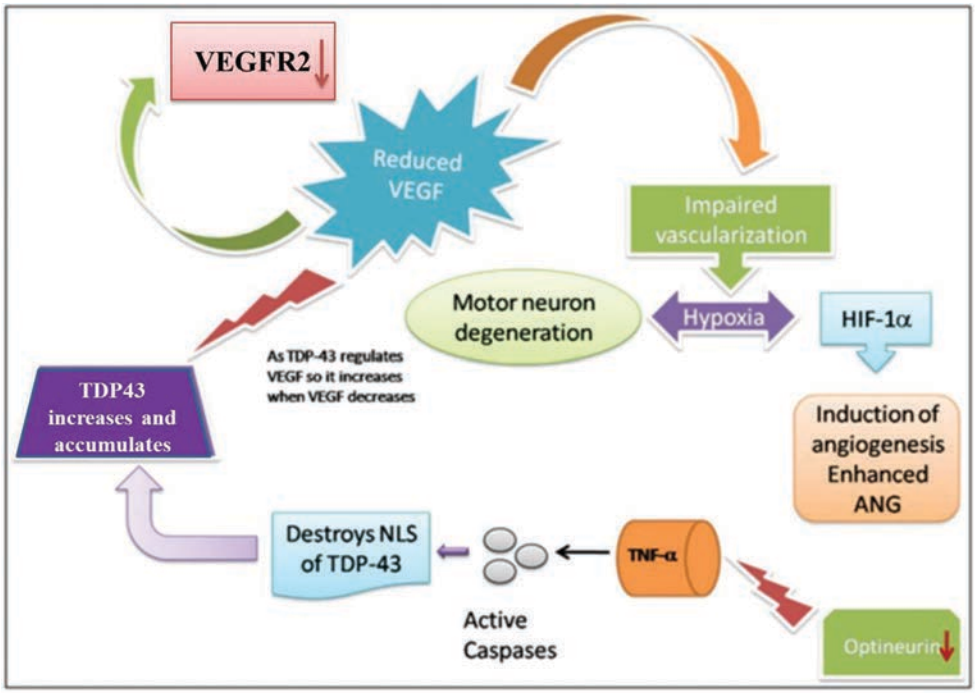

Fig. 4 Proposed schematic showing the role of six major proteins in causing ALS-reduced VEGF may be responsible for the hypoxia in brain because of which HIF-1α (hypoxia inducing factor-1α) gets activated consequently inducing ANG activation to compensate hypoxia. Because VEGF is reduced, its receptor soluble VEGFR2 may also be downregulated. Decreased VEGF may also lead to enhanced TDP-43 which may compensate for reduced VEGF by increasing its expression. Decreased OPTN also leads to disruption of NLS (nuclear localization signal) of TDP-43 due to which it gets accumulated in the cytoplasm. ALS, amyotrophic lateral sclerosis; ANG, angiogenin; OPTN, optineurin; TDP, TAR DNA binding protein; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

positively correlated to each other, suggesting a cross-talk exists among these five biomarkers. A comprehensive study is required to analyze the effect of these biomarkers on the disease progression to understand the role in the disease progression or for early diagnosis of the disease. Further research in this direction is required.

Note

Each demographic factor is divided into categories and the mean protein levels of markers were compared for the respective categories. No significant difference was found between protein levels with respect to various categories of these factors

Authors' Contributions

S.M.: planning and execution of experiments, data generation and analysis, writing, and editing of manuscript; R.K.: experimentation, data generation and analysis, writing, and editing of manuscript; A.T.: experimentation and data generation. K.S.: data analysis and editing of manuscript; A.A.: corresponding author and editing of manuscript.

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Conflict of Interest

None declared.

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CLINICAL RESEARCH ARTICLE

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Identifying putative cerebrospinal fluid biomarkers of amyotrophic lateral sclerosis in a north Indian population

Radhika Khosla MSc[1] | Manjari Rain PhD[1] | Shreyas Chawathey MBBS[2] | Shweta Modgil PhD[1] | Rahul Tyagi PhD[1] | Keshav Thakur MSc[1] | Viraaj Pannu MBBS student[3] | Suresh Kumar Sharma PhD[4] | Akshay Anand PhD[1]

[1]Neuroscience Research Lab, PGIMER, Chandigarh, India

[2]D. Y. Patil University School of Medicine, Navi Mumbai, India

[3]Government Medical College and Hospital, Chandigarh, India

[4]Department of Statistics, Panjab University, Chandigarh, India

Correspondence
Akshay Anand, Neuroscience Research Lab, Department of Neurology, Post graduate Institute of Medical Education and Research, Chandigarh, India-160012,
Email: akshay1anand@rediffmail.com, Phone: +91 9914209090.

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Abstract

Introduction: Evidence-based information about cerebrospinal fluid (CSF) levels of biomarkers in patients with amyotrophic lateral sclerosis (ALS) is limited.

Methods: Vascular endothelial growth factor (VEGF) and its receptor vascular endothelial growth factor receptor 2 (VEGFR2), optineurin (OPTN), monocyte chemoattractant protein-1 (MCP-1), angiogenin (ANG), and TAR DNA-binding protein (TDP-43) were quantified by enzyme-linked immunoassay in the CSF of 54 patients with sporadic ALS and 32 controls in a case-control study design.

Results: CSF levels of VEGF (P = .014) and ANG (P = .009) were decreased, whereas VEGFR2 was higher (P = .002) in patients with ALS than in controls. TDP-43 positively correlated with MCP-1 (P = .003), VEGF (P < .001), and VEGFR2 (P < .001) in patients with ALS.

Discussion: Our findings suggest possible utility of VEGF, VEGFR2, and ANG as biomarkers for use in ALS treatment trials.

KEYWORDS

amyotrophic lateral sclerosis, angiogenesis, cerebrospinal fluid, muscle wasting, neuromuscular disorder

1 | INTRODUCTION

A growing body of evidence points to involvement of growth factors, such as vascular endothelial growth factor (VEGF)[1-3] and its receptor vascular endothelial growth factor receptor 2 (VEGFR2), angiogenin (ANG),[4,5] optineurin (OPTN),[6,7] TAR DNA-binding protein (TARDBP or TDP-43),[5,8-11] and monocyte chemoattractant protein-1 (MCP-1),[12,13] as well as many other biomolecules in the pathogenesis of amyotrophic lateral sclerosis (ALS). These molecules and/or their downstream targets may serve as potential biomarkers, and thus they are targets for directed therapy.

Along with angiogenic effects, VEGF exerts neuroprotective effects.[2,14] VEGF double-knockout mice have been found to show ALS-like pathology.[15] VEGF exerts its angiogenic and neuroprotective role with the help of its main receptor, VEGFR2, via the PI3K/Akt pathway.[16,17] ANG, a ribonuclease (RNase) family protein, is another major molecule involved in vascularization and angiogenesis. Mutations in *ANG* and altered expression of *ANG* have been linked to various cases of both sporadic ALS (SALS) as well as familial ALS (FALS).[18] ANG is also required for the VEGF–VEGFR2 cell survival pathway.[19]

Proteinopathy is a hallmark of ALS, with TDP-43 as the main protein found in the protein inclusions of motor neurons. TDP-43 acts as a transcription factor that shuttles between the cytoplasm and

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised; ANG, angiogenin; BMI, body mass index; CCR2, chemokine receptor 2; CNS, central nervous system; CSF, cerebrospinal fluid; FALS, familial amyotrophic lateral sclerosis; M:F, male:female; MCP-1, monocyte chemoattractant protein 1; OPTN, optineurin; NF-κB, nuclear factor-kappaB; PGIMER, Post Graduate Institute of Medical Education and Research; SALS, sporadic amyotrophic lateral sclerosis; TDP-43, TAR DNA-binding protein; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

nucleus. Impaired functioning of TDP-43 leads to formation of TDP inclusions.[20]

Another molecule of importance found in the protein inclusions of ALS patients is OPTN. Mice with depleted OPTN have shown axonopathy because of necroptosis. It has also been shown to play an active role in nuclear factor-kappaB (NF-κB)–mediated regulation of neuroinflammation,[21] which is another hallmark of ALS. Several chemokines and interleukins have been associated with the disease, of which MCP-1 is well studied. MCP-1 is a member of the C-C chemokine family of proteins and regulates infiltration of monocytes and T cells that cause inflammation.

There is an unmet need to further study these molecules and delineate their roles in diagnosis, prognosis, and treatment of ALS. Due to the multiple postulated mechanisms giving rise to ALS, several researchers have proposed the use of a molecular panel to detect the disease signature. Thus, in this study, we measured levels of six putative biomarkers (VEGF, VEGFR2, ANG, OPTN, MCP-1, and TDP-43) in the cerebrospinal fluid (CSF) of patients with SALS and explored whether there are correlations between the levels of these molecules and the severity and duration of ALS. The CSF level of these six biomolecules may have interdependent roles in the pathogenetic mechanisms of ALS, which may relate to hypoxia, proteinopathy, and/or neuroinflammation.

2 | METHODS

2.1 | Subjects

Our study received approval from the institutional ethics committee of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. All study participants gave written informed consent before enrollment. CSF samples were collected from ALS patients in the Neurology Inpatient Department of PGIMER and stored at −80°C for more than 2 years. Inclusion criteria were a diagnosis of definite/probable/possible ALS according to revised El Escorial criteria (as diagnosed by experienced neurologists based on clinical assessment)[22] and age between 20 to 65 years. ALS was defined based on family history.

Disease duration was defined as the time between symptom onset and diagnosis. We used the ALS Functional Rating Scale—Revised (ALSFRS-R) to evaluate the function of ALS patients. ALS patients were graded as minimum (ALSFRS-R score >40), mild to moderate (ALSFRS-R score 30-39), moderate to severe (ALSFRS-R score 20-29), or advanced disease (ALSFRS-R score <20).[22]

Exclusion criteria were hepatic, renal, gastroenterological, respiratory, cardiovascular, endocrinological, neurological, immunological, or hematological diseases; hypothyroidism or hyperthyroidism; cognitive impairment with insignificant decisionmaking capacity; major depression; schizophrenia or dementia, including Alzheimer disease; and frontotemporal degeneration (as determined empirically by the study neurologists).

Controls samples were collected from our Institute's Trauma Center. Inclusion criteria allowed for otherwise healthy subjects with limb injury who were 20 to 65 years of age. In addition to the exclusion criteria for patients, additional exclusion criteria were head/brain or spinal cord injury, pre-existing muscular weakness, chronic or acute muscular or neurological disorder, infectious or inflammatory diseases, and vaccinations or treatments with immunoglobulins within 3 months preceding sample collection, along with the exclusion criteria of patients.

3 | ENZYME-LINKED IMMUNOASSAY

Human VEGF, VEGFR2, OPTN, TDP-43, ANG, and MCP-1 levels were obtained using sandwich enzyme-linked immunoassay (ELISA) kits. The kits for VEGF, VEGFR2, OPTN, TDP-43, and ANG were procured from Qayee Biological Technology (Shanghai, China) and for MCP-1 from Diaclone SAS (Besancon, France). The immunoassays were carried out as described by the supplier's instructions. CSF samples were not diluted for ELISA. Briefly, after adding samples and horseradish peroxidase conjugate, the plate was incubated. The plate was then washed and substrate reagent was added. The stop solution was then added and the plate was assayed within 20 minutes of addition of stop solution. Absorbance was taken at 450 nm with the ELISA reader (Bio-Rad Laboratories, Hercules, California). A standard curve was plotted for each experiment and values of respective proteins for all the samples were calculated. $R^2 \geq 0.97$ was considered appropriate for the test.

3.1 | Statistical analysis

Statistical analyses were performed using SPSS version 23 (IBM Corp, Armonk, New York). The Kolmogorov-Smirnov test was initially

TABLE 1 Demographic and clinical features of patients with sporadic ALS and healthy controls

Demographic and clinical features	ALS (n = 54)	Controls (n = 32)	*P* value
Age (years)	48.01 ± 12.24 (n = 52)	38.12 ± 16.43 (n = 31)	.003
BMI (kg/m^2)	22.09 ± 4.13 (n = 49)	24.25 ± 5.40 (n = 28)	.082
Sex (male/female)	43/10 (n = 53)	28/4 (n = 32)	.554
Duration (months)	19.34 ± 15.13 (n = 46)	—	—
ALSFRS-R score	34.37 ± 6.17 (n = 48)	—	—

Note: Data expressed as mean ± standard deviation, except sex, which is expressed as number of samples.
Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised; BMI, body mass index.

carried out to assess the normality of data pertaining to the CSF levels for each molecule. The non-normal data sets were analyzed using the Mann-Whitney *U* test. Independent *t* tests were used for analysis of data that were normally distributed. Analysis of coviariance was applied to control *P* values for age by using general linear model. Significance was considered at $P \leq .05$. Pearson or Spearman rho test statistics were used to correlate the data sets, based on the applicability. There were some patients whose sociodemographic and clinical data were unavailable, and these were excluded from analysis of correlation between clinical severity (ALSFRS-R score) and duration of illness against the CSF levels of various biomarkers. The Bonferroni correction test for multiple comparisons was applied to obtain significant correlations. The cut-off *P* value after Bonferroni correction for correlation between biomarkers and clinical severity or duration of illness was $P \leq .004$ and among the biomarkers was $P \leq .003$.

TABLE 2 Cerebrospinal fluid levels of VEGF, VEGFR2, TDP-43, and OPTN in ALS patients and healthy controls

Biomolecules	ALS	Controls	*t* value	*P* value (95% CI)	Age-adjusted *P* value (95% CI)
VEGF (pg/mL)	161.28 ± 15.44 (n = 54)	169.20 ± 10.30 (n = 27)	−2.405	.019 (1.36 to 14.47)	.014 (1.83 to 15.80)
VEGFR2 (pg/mL)	37.35 ± 2.19 (n = 48)	35.46 ± 2.29 (n = 24)	3.390	.001 (0.78 to 3.00)	.002 (0.71 to 3.10)
TDP-43 (pg/mL)	105.70 ± 24.22 (n = 48)	112.07 ± 13.88 (n = 30)	−1.313	.193 (−16.06 to 3.30)	.230 (−16.63 to 4.06)
OPTN (pg/mL)	619.92 ± 45.82 (n = 46)	606.20 ± 27.77 (n = 31)	1.491	.140 (−4.61 to 32.05)	.083 (−2.28 to 36.73)

Note: Data expressed as mean ± standard deviation.
Abbreviations: ALS, amyotrophic lateral sclerosis; CI, confidence interval; OPTN, optineurin; TDP-43, TAR DNA-binding protein; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

TABLE 3 Cerebrospinal fluid levels of ANG, MCP-1, and MCP-1/VEGF ratio in ALS patients and healthy controls

	ANG (ng/mL)		MCP-1 (pg/mL)		MCP-1/VEGF	
Statistical measures	ALS (n = 47)	Control (n = 31)	ALS (n = 54)	Control (n = 27)	ALS (n = 54)	Control (n = 26)
Median	151.53	155.85	486.76	599.86	3.18	3.31
Range	54.59	34.09	1517.06	1637.96	10.59	10.74
Minimum	141.85	134.30	287.86	354.16	1.51	1.92
Maximum	196.44	168.39	1804.93	1992.12	12.10	12.67
25th percentile	148.01	150.50	391.21	449.71	2.50	2.75
75th percentile	153.62	160.77	749.03	783.15	3.89	4.82
Z value	−2.594		−1.788		−1.027	
P value	.009		.074		.304	

Note: Data expressed as median and range.
Abbreviations: ALS, amyotrophic lateral sclerosis; ANG, angiogenin; MCP-1, monocyte chemoattractant protein 1; VEGF, vascular endothelial growth factor.

TABLE 4 Correlation analyses for biomolecules in ALS patients

MCP-1	$r = 1$					
VEGF	$r = -0.006$; $P = 0.963$	$r = 1$				
VEGFR2	$r = 0.268$; $P = .065$	$r = 0.325$; $P = .024$[a]	$r = 1$			
ANG	$r = 0.062$; $P = .676$	$r = 0.230$; $P = .120$	$r = 0.077$; $P = .609$	$r = 1$		
TDP-43	$r = 0.425$; $P = .003$	$r = 0.719$; $P < .001$[a]	$r = 0.545$; $P < .001$[a]	$r = 0.332$; $P = .024$	$r = 1$	
OPTN	$r = 0.188$; $P = .211$	$r = 0.021$; $P = .887$[a]	$r = 0.060$; $P = .693$[a]	$r = 0.218$; $P = .151$	$r = 0.148$; $P = .328$[a]	$r = 1$
Biomolecules	MCP-1	VEGF	VEGFR2	ANG	TDP-43	OPTN

Note: The Spearman rho correlation was used to determine *P* values, unless noted otherwise; *P* values are significant at $P \leq .05 / 15 = .003$ after Bonferroni correction.
Abbreviations: ALS, amyotrophic lateral sclerosis; ANG, angiogenin; MCP-1, monocyte chemoattractant protein 1; OPTN, optineurin; TDP-43, TAR DNA-binding protein; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.
[a]The Pearson correlation used to determine *P* values.

4 | RESULTS

4.1 | Clinical and demographic characteristics

No FALS patients were enrolled. Fifty-four SALS patients and 32 control subjects were enrolled in our study. The sociodemographic features of participants are summarized in Table 1.

4.2 | Quantitative protein expression

ELISA revealed changes in various biomarkers associated with ALS pathology. The data for VEGF, VEGFR2, TDP-43, and OPTN were normally distributed, whereas those for ANG, MLP-1, and MCP-1/VEGF were not normally distributed. VEGF and ANG were reduced; VEGFR2 was elevated significantly among the SALS patients compared with the control group (Tables 2 and 3).

4.3 | Correlation analyses

No significant correlations were observed between the biomolecules and ALSFRS-R criteria or duration of illness (see Table S1 online). Correlations between biomarkers revealed that TDP-43 was positively correlated with MCP-1, VEGF, and VEGFR2 (Table 4).

5 | DISCUSSION

In this study we showed that levels of CSF VEGF in patients with ALS decreased compared with controls; VEGFR2 was significantly elevated and ANG was decreased in the CSF of ALS patients. Despite significant *P* values, there was substantial overlap between the values of the control and ALS groups. We have previously reported the reduced expression of chemokine receptor 2 (CCR2), receptor of MCP-1,[23] and upregulation of MCP-1[3] and VEGF[3] in north Indians. Several researchers have reported that the MCP-1/VEGF ratio in ALS significantly differs from healthy and neurological controls and may be used as a diagnostic marker for ALS.[12] However, we did not find any significant difference in MCP-1/VEGF ratio between ALS patients and controls.

A number of studies have shown that VEGF is elevated in the CSF and serum of ALS patients. However, some studies have also shown CSF levels of VEGF to be lower in the diseased group, similar to our observation, although this has not been ascribed to disease duration.[24-26]

Reduced expression of VEGF has been found to result in a neurodegenerative condition in mice, similar to human ALS, and therapeutic interventions with either *VEGF* or VEGF protein have yielded benefit in those studies.[27] Our study is consistent with the earlier findings, as we reported lower levels of VEGF in the ALS group along with a concomitant rise in CSF levels of VEGFR2. Studies involving knockout mice showed that low VEGF levels in mice with $Vegf^{\delta/\delta}$ could result in motor neuron degeneration owing to two possible pathways. First, due to peripheral arteriolar dysregulation, there is a decline in vascular supply and prolonged chronic ischemia. Endothelial cell dysfunction may also impair central nervous system (CNS) homeostasis due to disintegration of blood-brain barrier.[28] Decreased VEGF levels may lead to these outcomes, as VEGF is needed at a particular "maintenance" level for appropriate function and survival of endothelial cells. However, its temporal analysis may shed more light on this.[29] Second, VEGF acts as a neurotrophic factor, and thus a fall in its levels implies impaired neuroprotection, leading to reduced survival of neurons.

The effects of VEGF are exerted by VEGFR2 activation and downstream activation of the PI3K-Akt signaling pathway, inhibiting p38 MAP kinase phosphorylation and, consequently, preventing Bcl-2 downregulation, inhibiting apoptosis.[30,31] Further, VEGF reduces neuronal susceptibility to glutamate-induced excitotoxicity by causing induction of AMPA receptor GluR2 subunit expression.[32] VEGF and its cognate receptors, VEGFR1 and VEGFR2, are expressed in the spinal motor neurons of mice as well as humans,[28] and overexpression of VEGFR2 causes a delay in onset of disease and improves the duration of survival in mutant SOD1-G93A mice.[33]

Some authors have argued that dysregulation of the hypoxia response, coupled with changes in mediators of the VEGF pathway, VEGF promoter polymorphisms, and certain variant genotypes, result in low VEGF levels in the CSF. One such example is the -2578AA genotype that is associated with ALS in some male patients, linking lower VEGF concentrations and pathogenesis of ALS.[1,25,34] However, further studies are needed to clarify these findings. Future studies may help to further examine the current evidence of utility of VEGF as a biomarker in the management of ALS. Some studies reported upregulation of VEGFR2 as well as VEGF (in the CSF of ALS) due to autocrine and paracrine functions of VEGF on motor neurons, apparently to protect them from injury due to various derangements that induce apoptosis.[35] Further, a positive correlation of VEGF with VEGFR2 was observed in this study, supporting reduced neuroprotection and vascularization via VEGFR2.

In addition, another growth factor, ANG, essential for angiogenesis and cell survival was decreased in the CSF of ALS patients. Reduced ANG is associated with reduced ribosome biogenesis and cell proliferation.[19] ANG is also involved in regulatory function crucial for cell growth.[36,37] Because ANG activates the NF-κB–mediated cell survival pathway and Bcl-2–mediated antiapoptotic pathway, it is crucial for neuronal survival in ALS.[38,39] Further, ANG is also implicated in neuroprotection in ALS.[40] Thus, a deficiency of ANG may lead to decreased cell survival, a characteristic of ALS.

Our study reveals a positive correlation of TDP-43 with the angiogenesis markers VEGF, VEGFR2, and ANG. TDP-43 binds to VEGF mRNA and controls its stability, ultimately regulating progranulin levels involved in cell growth.[41] Survival of motor neurons is decreased with loss of TDP-43 as there is reduced non-homologous end-joining and accumulation of double-strand breaks in DNA.[42] Interestingly, the MCP-1 levels correlated positively with TDP-43,

suggesting that a reduced immune response, as indicated by decreased MCP-1, may lead to loss of TDP-43 function.

Our study has certain limitations. The total number of eligible ALS/control subjects, number of ALS/control subjects who declined participation, and their reasons for nonparticipation were not recorded. Thus, the patient population included in the study may not represent the overall ALS population. The numbers of samples obtained from patients and controls were small and some of the demographic data of controls were unavailable to us. The smaller sample size may be one of the reasons that we were unable to obtain significant correlations between clinical severity and duration of disease with CSF biomolecules. Although we tried to include matched ALS and control subjects with similar medical histories (other than ALS), other unknown differences may exist between ALS patients and controls that could affect biomarker outcomes. Further, there were differences in age between ALS patients and controls that could not be controlled for; thus, adjusted and nonadjusted *P* values have been presented. Another limitation is that cognitive impairment and frontotemporal degeneration were diagnosed by neurologists using empirical means, rather than specific instruments.

In conclusion, our findings add to the evidence of utility of VEGF, VEGFR2, and ANG for use as biomarkers in prognosis and therapeutic applications for management of ALS. Further studies are needed to understand roles of a number of putative biomarkers of this rare neurological disease.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

ORCID

Viraaj Pannu https://orcid.org/0000-0001-6315-0186
Akshay Anand https://orcid.org/0000-0001-9003-3532

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Can Cheiromancy Predict Mean Survival or Fatality of a Patient with Amyotrophic Lateral Sclerosis?

Akshay Anand[1,*] Sudesh Prabhakar[1] Keshav Thakur[1,*] Rahul Tyagi[1] Vinod Shastri[2] Devi Prasad Tripathi[3] Suresh Sharma[4]

[1]Department of Neurology, Neuroscience Research Lab, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[2]Faculty of Arts, Jyotirvigyan Kendra, Rajasthan University, Jaipur, India
[3]Department of vastushastra, Lal Bahadur Shastri Rashtriya Sanskrit Vidyapeetha, New Delhi, India
[4]Department of Statistics, Panjab University, Chandigarh, India

Address for correspondence Akshay Anand, PhD, Department of Neurology, Neuroscience Research Lab, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India (e-mail: akshay1anand@rediffmail.com).

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Abstract

Background The past three decades have seen palmistry as an interface to human health. There have been no previously organized attempts in utilizing this knowledge to predict the state of disease.

Objective Due to unavailability of any biological marker for diagnosing amyotrophic lateral sclerosis (ALS) till date, we attempt to examine whether palmistry could be used for detecting the onset and survival of patient suffering from ALS.

Methods Patients suffering from ALS attending the neurology outpatient department at Postgraduate Institute of Medical Education and Research, India were selected for study. Palm photographs were obtained from all patients including controls after their consent. Patients suffering from other comorbidities such as diabetes, hypertension, migraine, as well as smokers and nonsmokers were included in the study. Twenty-six ALS patients, 30 neurological controls, and 34 healthy age matched controls were recruited in the study. Retrospective analysis of the palm pictures based on blinding method was performed by academically qualified palmists.

Results The results demonstrated the need for further studies in the subject even though the observations made were independent by both the palmists.

Conclusion This study opens new vistas for cheiromancy to be further explored for analysis in larger samples.

Keywords
- ALS
- cheiro
- palmistry
- survival time

Introduction

Palmistry is generally considered as an occult science. Believed to have originated in India, it spread from Asia and the whole world with time. William John Warner, also known as Cheiro, was an Irish astrologer who learned palmistry in India and later spread it to Europe. A term cheiromancy was coined after William John Warner. Although not much literature exists consisting of studies using palmistry as a diagnostic tool for disease onset, however, certain studies have used palmistry or dermatoglyphic as one of the tools for predicting the disease onset.[1,2] Palmistry employs the study of line to predict disease mind.[3,4] Bhargava and Sathawane in 2012 suggested that dermatoglyphics can find its application in medical diagnosis of various diseases through invention of new or unusual patterns.[5,6] This makes description of certain crease, curves and lines defined as heart line, head line, life line, simian crease, Sydney crease, etc.[2-4] hold a significant value and based on this, the palmists or any cheirologist may suggest the current position of an individual. However, one should

*Akshay Anand and Keshav Thakur contributed equally to this study.

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not confuse between the palmistry and dermatoglyphic as the latter is considered as more scientific method of analyzing and correlating the crease and palm patterns with the genetics of disease. The patterns obtained from fingerprints are related to different disorders uniquely. In context to dermatoglyphics, several theories have been proposed to investigate the hand patterns in diseased patients. First theory came up in 1924 by Bonnevie, discussing the formation of patterns and crease on palms.[7] Later Cummins in mid-1950s tried to find the relation between palm patterns as well as geometry of palm.[8] Cummins' theory is still considered for analysis of hands and foot today.[9] Recently, in 2013, Kücken and Champod have tried to explore the relation between stress factor's impact and geometry of feet and hands.[10] In current study, we have attempted to examine the relation of palm morphology with survival in amyotrophic lateral sclerosis(ALS) patients. It had been for the first time that science of palmistry is being tested for predicting the disease condition and the survival of patient to verify whether it can be used as a tool to diagnose the onset and time the patient will live after onset of disease.

Materials and Methods

Data analysis was performed in Neuroscience Research Lab, Postgraduate Institute of Medical Education and Research, India.

Study Design

Patients recruitment: ALS patients were recruited from outpatient department of neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. Age and sex matched controls were also obtained from the hospital. A total of 90 subjects were selected out of which 26 ALS, 30 neurological controls, and 34 age matched healthy controls were recruited.

Analysis: A retrospective analysis was performed on the palm pictures captured from all the above-mentioned subjects after obtaining informed consent. These palm pictures were stored electronically after proper coding. Study was ethically approved by institutional ethical committee.

Blinding: Blinding was done for palm pictures as per laboratory's established protocol and was provided to the palm analysts for further interpretation. Palmists were not aware of the status of patients as to which disease are they suffering from. No prior information about status of palm pictures was provided to palmists (thus reducing the potential bias for ALS in palmistry). These images were analyzed independently by the palmists (►**Fig. 1**).

Palm Analysis

Palm analysis was performed by two academically qualified (PhD) palmists. Both were given same set of data and were asked to analyze the images (►**Table 1**).

Physical signs and symptoms on palm decide about disease and death of an individual.[11] Palm is of different shapes such as square, rectangular, circular, rukshakriti (bear shape), and cow's mouth shape.[11-14]

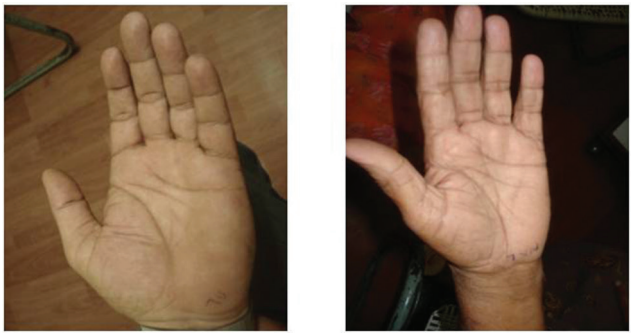


Fig. 1 Representative palm pictures of subjects that were provided to palm analyst for predicting their health condition and their survival tenure.

An individual with square palm and nails with a bit of pale blue color indicates that person's lifespan can be extended with drugs only. Normally, person with square palm stick to their own work but due to branching of heart line on the mount of Venus indications for brain disease especially brain hemorrhage may be the cause of person's death.

A rectangular palm of stiff or rough appearance indicates lung infection may become reason for death.

If the life line of dying person's palm is intersected by islands or diagonal lines, then heart problem brain disease or any kind of accident becomes a reason for death.

Rough skinned palm with blue color nail characters results in heart problems and brain hemorrhage due to uninvited tensions. However, if nails are curved outward with redness than that person is predicted to have long life, but if sun line is intersected by a line from the mount of Venus going toward heart line is an indicator of high cholesterol levels in body and signals paralysis and vision loss of that individual (►**Table 2**).

Interpretation of lines as per the palmists are as follows:[11,12]

1. If heart line goes till mount of Saturn and divides into two parts by getting into a shape of an island before reaching the mount of Saturn, then it indicates that patient might die of lung infection during surgical procedure.
2. If head line is being intersected by Saturn line and later head line is branched into three parts, then it indicates the death of person due to delayed diagnosis or due to some surgical error.

Later, the information from palmists was matched with the data which were obtained from ALS patients. These data were not shown to palmists; neither the nature of subject nor age, sex, etc. was disclosed. Comorbidities were also taken into account to understand if they have any impact on subject's survival (►**Table 3**).

Patients were later contacted by telephone to know the status of their survival by team members of Neuroscience Research Lab.

Statistical Analysis

Statistical analysis was performed using nonparametric tests (Kaplan–Meier and Mann–Whitney) to compare the analysis done by palmists. Apart from this analysis was performed to check whether how much the information provided by

Table 1 Names and description of creases present on palm along with their position in the image

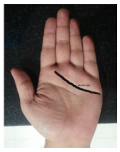
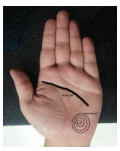
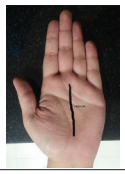
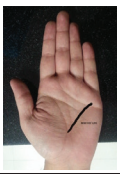
S. No.	Name of lines	Description	Position of line in palm
1.	Hridayrekha (heart line)	Heart line originates from small finger up to index finger	
2.	Mstishkrekha (head line)	Head line although below heart line originates at the center of index finger and thumb and moves toward moon mount on the palm	
3.	Life line	Life line originates from the center of index finger and thumb and extends through the middle of palm making a semicircle till the base of thumb	
4.	Suryrekha	Sun line originates at the base of ring finger till the base of sun mount just above the heart line	
5.	Fate line	Fate line originates from the base of thumb till the base of middle finger	
6.	Mercury (business) line	Mercury line originates from the base of thumb and extends up to small finger	

Table 2 Different color of palm indicates impact on health condition of an individual

S. No.	Palm color	Indication	Time span
1.	Blue	Death due to infectious disease	Not defined
2.	Black tone	Death	15 d
3.	Light yellow colored	Heart/brain disease	30 d–3 y
4.	Redness	Recovery from heart disease	Not defined
5.	White/light red palm	Immediate death or dead person	Not defined
6.	Bright blue palm	Fast recovery from surgical procedure	Not defined

Note: This table describes the color difference along with their indication and possible time limit in which the individual may get affected.

palmist match with phenotype of ALS in patients recruited for study.

Results

Analysis of results showed results which need to be further validated in larger sample. The observations were made independently by two palmists. The data from first palmist showed 15.4% prediction of fatality for ALS subjects with 3.85% similarity to ALS phenotype, as compared with 40% accurate prediction of ALS phenotype and only 24% of fatality in ALS patients as per the second palmist (►**Table 4**).

When we performed the survival analysis of the ALS patients, significant difference (p = 0.047) was observed between median survival times by the palmists as compared with actual lifespan of ALS patients, calculated by patient follow-up (►**Fig. 2**).

Table 3 List of comorbidities taken into account while analyzing the palm pics

Comorbidity	
1. Heart disease	12. Obese/overweight
2. Diabetes/autoimmune disease	13. No comorbidity
3. Hb/other blood disease	14. Migraine/headache
4. Liver disease	15. Underweight
5. Frontal stress/brain related problems/depression/ paralysis	16. Sleep problem/mental trauma
6. Kidney disease	17. Abnormal blood pressure
7. Coma	18. Eye disease
8. Age-related problems	19. Memory deficits
9. Terminal Illness	20. Breathing trouble/ respiratory issues
10. Intense pain resulting death	21. Arthritis/joint diseases
11. Thyroid problem	

Discussion

Palm reading or palmistry, also called as cheiromancy (meaning hand prediction) in older times, is an analysis of a person's hand to foretell the future aspects of life including health outcomes. Health-related predictions such as diseases and ailments have also been studied since long time, and there are various studies that indicate the relation between palms and physical well-being[11]; however, most of such studies are not designed scientifically. Mount of Venus and heart line are considered as a representative of warnings for diseases related to heart, kidney, diabetes, urinal tract, and mental depression, while various other diseases such as familial deafness, leprosy, and rheumatoid arthritis have been believed to be related to the simian line.[12,13] As such, in current study, astrologers have taken into consideration the line and physical appearance of palm for deducing an observation over a patient. They have highlighted the importance of lines and mounts and also defined how analyzing the lines can help in predicting the condition of a patient. It is believed that analyzing the palm pictures the person's nature and lifestyle can be predicted.[14] The palm lines are also used to predict human life's bad or good events as well as various diseases and accidents. Currently available literature about palmistry and its scientific application are a result of scientific analysis performed on the palm contours of diseased patients.[14-16] In 2011, a study published in *International Journal of Morphology* associated the chromosomal aberration with pattern of simian and Sydney lines or life line and head line.[4] In support, similar observation with context to palm creases has been made in current study where palmists reported that palms having branching of heart line on the mount of Venus indicate brain disease or brain hemorrhage. Karnick in one of his article cited the work of Dobson et al where they have tried to examine the correlation between the palmar keratoses and any form of cancer. They found an association with 46% palms of men and 28% women, as compared with only 12% of normal men and 5% of normal women. Another study was reported in 1990 where these palmar keratoses were studied in association with bladder cancer.[18] Newrick et al found a strong relationship between length of life line and age. They evaluated 100 autopsies and found that length of right hand life line and age at death were statistically correlated even though according to Karnick, person's longevity is influenced by the heart line.[1,19] Various other studies have associated dermatoglyphic patterns with dental caries,[20] gynecological cancers,[21] autism,[22] nonsyndromic cleft lip,[23]

Table 4 Data obtained from two palmists on the health and phenotype of ALS patient

	Correct prediction of diseased organ for ALS	Correct prediction of fatality for ALS	Correct prediction of diseased organ for neurological controls	Correct prediction of diseased organ for normal controls
Observer 1	1/26 = 3.85%	4/26 = 15.4%	5/27 = 18.5%	1/34 = 2.94%
Observer 2	10/25 = 40%	6/25 = 24%	4/30 = 13.3%	1/34 = 2.94%

Abbreviation: ALS, amyotrophic lateral sclerosis.

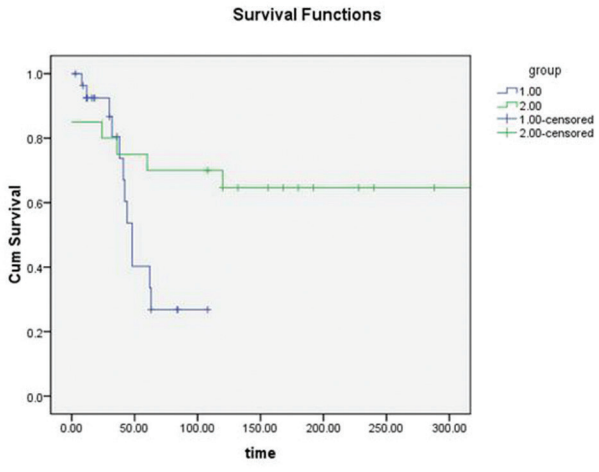


Fig. 2 Survival analysis curve of ALS patients. Blue line indicates actual lifespan and green line indicates lifespan of ALS patients according to the palmists. ALS, amyotrophic lateral sclerosis.

Actual lifespan was confirmed telephonically by the neuroscience research team. The cumulative survival curves (Kaplan–Meier) show that, after 5 years, the actual survival was only 25%, while that predicted by palmist was 70%.

and breast cancer.[24,25] In current study, the palmist took into consideration the color pattern, tone of palm, as well as skin pattern of palm in predicting the survival and condition of patient. As a result, initially both observers were able to give 40% correct prediction of diseased organ with palms of ALS patients (palm pictures were blinded to the observer). The fatality prediction for ALS patients of both the observers did not match the actual data. As per observer's record, 15.4% for observer 1 and 24% of patient were predicted to die soon due to some brain-related disorder. On performing the statistical analysis, we observed the significant difference of $p = 0.047$. In Kaplan–Meier's survival analysis, palmist observations reported the survival of up to 70% among ALS patients, whereas the actual survival was only 25%. The current study suffers from a limitation. The palm pictures were available in JPEG format and no real-time observations or interactions were allowed. Such analysis can be extended to other fatal diseases and its role in predicting the disease condition in an individual can be similarly ruled out before concluding that palmistry does not hold significance in health care.

Authors' Contributions

A.A. contributed to study design and editing, S.P. screened the ALS patients, S.S. was involved in statistical analysis and interpretation of data. V.S., K.T., and R.T. acquired and compiled the data and K.T. prepared the preliminary draft of the manuscript.

Conflict of Interest

A.A. reports grants from ICMR, during the conduct of the study.

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Angiogenesis-Centered Molecular Cross-Talk in Amyotrophic Lateral Sclerosis Survival: Mechanistic Insights

Keshav Thakur,[a,b] Abha Tiwari,[a] Kaushal Sharma,[a,c] Shweta Modgil,[a] Radhika Khosla,[a] & Akshay Anand[a,*]

[a]Neuroscience Research Lab, Post Graduate Institute of Medical Education and Research, Chandigarh, India; [b]Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India; [c]Centre for Systems Biology and Bioinformatics, Panjab University, Chandigarh, India

*Address all correspondence to: Akshay Anand, PhD, Professor, Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India-160012; Tel.: +91 9914209090, E-mail: akshay1anand@rediffmail.com

ABSTRACT: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized with progressive muscle atrophy. We have attempted to establish the link between angiogenesis and cellular survival in the pathogenesis of ALS by compiling evidence described in various scientific reports. The phenotypes of human ALS have earlier been captured in the mutant SOD1 mice as well as by targeted deletion of the hypoxia response element (HRE) from the promoter of the mouse gene for vascular endothelial growth factor (VEGF). Indirect evidence shows that angiogenesis can help prevent oxidative stress, and hence, enhance cell survival. VEGF and angiogenin chiefly regulate the process of angiogenesis. Transactive response DNA-binding protein 43 (TDP-43) is usually found inside the nucleus, but in large number of cases of ALS, it accumulates in the cytoplasm (TDP-43 proteinopathy). Interestingly, TDP-43 proteinopathy is found to be aggravated in the presence of the *OPTN* mutation, which is the genetic factor that is responsible for such accumulation. Interaction of TDP-43 with progranulin can further affect the angiogenesis in ALS patients by regulating activity of VEGF receptors, but conclusive evidence is needed to establish its role in pathogenesis of ALS. Certain mutations in *UBQLN2* and *UBQLN4* indicate that ubiquitination has a role in ALS pathobiology, but its link to angiogenesis has not been adequately studied. Recent studies have shown that several mutations in RNA-binding proteins (RBPs) can also cause ALS. Conclusively, in this review, we have attempted to argue the role of angiogenesis in enhanced ALS survival rate is probably regulated with the activation of NF-κβ. Additionally, interaction between OPTN and TDP-43 can also impact the transcription of various angiogenic molecules. Whether targeting angiogenic substances or TDP-43 can provide clues about extending ALS survival rate, in combination with current treatments, can only be evaluated after additional studies.

KEY WORDS: optineurin, vascularization, VEGF, angiogenin, TDP-43

I. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurological disorder characterized by degeneration of motor neurons and progressive atrophy of muscles. ALS was first studied in 1848 when it was described as progressive muscular atrophy.[1] Then in 1873, Charcot described the disease and distinguished it from spinal muscular atrophy, based on heredity. The disease came to be known as Charcot's disease later on.[2] ALS is also known as Lou Gehrig's disease, after the famous baseball player Lou Gehrig. The disease is more prevalent in athletes and soldiers.[3,4]

ALS is a catastrophic neurodegenerative disease characterized by the loss of motor neurons in adults, which disrupts coordination between voluntary muscles and the brain. Because it is difficult to define the complete pathogenesis of this lethal disease, additional studies are required. Although the disease can be hereditary background, very few cases are familial. Only 10% of ALS cases are found to be familial (fALS), and 90% of the cases are sporadic (sALS). Mutations in four genes are associated with familial cases of ALS: *SOD1*, *C9orf72*, *TDP43*, and *FUS*. However, Andersen and Al-Chalabi[5] have shown that there may be more prevalence of the familial cases. Out of the 10% of familial cases, 20% are attributable to mutation in superoxide dismutasae (SOD1).[6] Apart from SOD1 studies, lipid peroxidation has also been examined to estimate the

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oxidative burden in ALS patients, because it is one of the pathological hallmarks in disease progression. Simpson et al.[7] confirmed the role of oxidative stress in ALS by measuring the level of lipid peroxidation product 4-hydroxy-2,3-nonenal (HNE) in serum and cerebrospinal fluid (CSF) of patients with ALS. They also assessed the level of monocyte chemotactic protein-1 (MCP-1) along with HNE and confirmed the role of increased oxidative stress and immune system activation in ALS. Vascular abnormalities may have a causal association with neurodegenerative diseases such as Alzheimer's disease and ALS because they occur before symptoms do. A direct consequence of such abnormalities is the accumulation of neurotoxic and vasculotoxic molecules in the interstitial fluid (ISF) because of hampered clearance.[8] The resulting hypoxic conditions pave the way for neuronal degeneration, which needs to be investigated comprehensively with both *in vitro* and *in vivo* tools.

ALS is accepted as a multigene disorder worldwide. The last two decades of ALS research has focused on identifying various genetic variants that increase the risk or progression of disease. Genome-wide association studies carried out in this regard have revealed involvement of different genes that were previously considered unimportant. However, literature on ALS is also replete with conflicting and unverifiable reports of putative diagnostic and prognostic biomarkers, thus hampering knowledge translation into clinical application.

II. ANGIOGENESIS AND CELLULAR SURVIVABILITY IN ALS PATHOLOGY

The process of angiogenesis is a major aspect in various pathological phenotypes, such as ALS, age-related macular degeneration (ARMD), and cancer. Studies from Indian patients have revealed significantly elevated levels of vascular endothelial growth factor-A (VEGF-A) in biofluids (serum and CSF) of ALS patients. These elevated VEGF-A levels are thought to contribute to the enhanced mean survival time after disease onset in North Indian patients with ALS. Chemokine ligand-2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1) were also found to be increased in the CSF of these ALS patients[9] and upregulated in their peripheral blood mononuclear cell (PBMC).[10] These interesting investigations also revealed a reduction in C-C chemokine receptor type 2 (CCR2) in PBMCs of ALS patients.[11] These molecules have been recently shown to be involved in the cell survival pathways through angiogenesis and are thus speculated to play a critical role in neurodegeneration. An important area of investigation is whether the enhanced mean survival time resulting from elevation of VEGF is sustainable across other stages of disease and whether it occurs by participation of additional molecules in determining ALS outcome. The review thus seeks to advance our knowledge by discussing the role of angiogenesis in cellular survival mediated through VEGF/sFLT-1, transactive response DNA-binding protein 43 (TDP-43), angiogenin, optineurin, and other such molecules implicated in ALS pathology.

III. MAINTENANCE OF NEUROVASCULATURE IN ALS

Various studies have shown the link between VEGF expression and motor neuron degeneration. Altered VEGF expression leads to impaired vascularization.[12] During development, VEGF regulates the vascularization pattern and also the establishment of the nerve network with the blood vessels, along with various other molecules.[13] Neuropilin is the common receptor for VEGF and semaphorin A.[14] Interaction and cross-talk between these molecules at the time of development guides axons and leads the axon terminals to their synaptic connections. Along with VEGF, VEGF receptors VEGFR1 (flt-1) and VEGFR2 (flk-1), and fibroblast growth factor 1 (FGF1) also aid development of the neurovasculature.[15] A recent study has shown that there could be a direct relationship between the deficit of VEGF and motor neuron degeneration, which is considered to be the main characteristic of ALS pathology. VEGF can protect motor neurons from cell death and can even delay processes like neurodegeneration in animal models of ALS.[16] Angiogenin is another molecule linked to the vascularization process. Angiogenin is a small protein of the ribonuclease family that regulates angiogenesis. It is a hypoxia responsive gene.[17] Angiogenin can alter the action

of VEGF, and absence of angiogenin can lead to impaired vascularization, despite the presence of VEGF, because ANG is a downstream molecule of VEGF cell survival pathway.[18] Mutation in ANG was first found to be associated with ALS in 2006 by Greenway et al.[19] The patients were of Irish or Scottish descent. After this study, reports emerged from German,[20] Dutch, North American, French,[21] and Italian subjects about the role of mutations in *ANG* in ALS cases,[22–24] but studies done in Asia have not adequately addressed this important area of investigation. This is even more critical because Indian patients are known to survive far longer than Caucasian patients with ALS. Angiogenin is translocated to the nucleus to exert its effect through RNA (ribonucleic acid), which is essential for VEGF activity. SNPs and/or mutations in *ANG* reported in ALS usually lead to loss of function, thus hampering its nuclear translocation and ribonucleolytic activity. This renders it incompetent to induce angiogenesis.[25] Along with vascularization, VEGF has also been considered to be neuroprotective.[26] ANG plays an important role in angiogenesis induced by other growth factors, such as VEGF and FGF.[27]

Genes induced by hypoxia were first associated with ALS by Oosthuyse et al.[28] They found ALS-like symptoms in mice upon deletion of the hypoxia response element (HRE) in the promoter of VEGF.

The neuroprotective nature of ANG has also been demonstrated by Subramanian et al.[29] with pluripotent P19 embryonal carcinoma (EC) cells. This cell line was used as a model of neuroectodermal differentiation. Different variants of human ANG (hANG) were used for cell line stimulation. hANG-ALS variants were not found to have a neuroprotective effect as compared to hANG.[29] Thus, the establishment of a link between hypoxia and ALS pathogenesis has placed angiogenic factors at the focal point for ALS investigations.

A. Receptors Involved in Neurovasculazation and Their Cross-Talk with Various Angiogenic Molecules

VEGF mediates its action with the help of tyrosine kinase receptors. VEGFR1 (Flt-1) and VEGFR2 (Flk-1) are the membrane bound receptors of VEGF. They are agonists of VEGF and regulate both angiogenesis and cell survival via activation of PI3k/Akt and MEK/ERK pathways. Other receptors for VEGF are the neuropilins, which also act as common receptors for semaphorins. Soluble forms of VEGFR1 (sFlt-1) act as antagonists to the action of VEGF. It binds to VEGF outside cells and inhibits the effect of VEGF. The biological responses to VEGF are believed to be mainly mediated through kinase insert domain receptor (KDR), whereas phosphatidylinositol glycan anchor biosynthesis class F (PIGF) shows high affinity for Flt-1.[30–32]

sFlt-1 regulates the expression of VEGF in the tissue fluid via a negative feedback mechanism. Ahmad et al.[33] found enhanced expression of sVEGFR1 on increasing the level of VEGFA in the culture medium.

In the first study, Anand et al.[34] showed sVEGFR1 to be unexpectedly downregulated in the serum of ALS patients relative to controls with proportionate reduction in its levels with increasing severity of ALS. The binding of angiogenin to α-actin is an essential feature of angiogenesis, and it promotes Akt-1 activation, thus supporting cell survival.[27,35] VEGF also activates the same pathway,[36] and therefore, its sequestration by sVEGFR1/sFLT1 depletes the cell of its antiapoptotic activity. The Akt pathway targets NF-κB which inhibits apoptotic pathways.[37]

Although, VEGF and its receptors along with angiogenin mainly regulate the angiogenic mechanism, we speculate that other molecules, such as sFLT1, TDP-43, and optineurin, play a nonredundant role in angiogenic mechanisms and cross-talk with the angiogenic pathway. Therefore, the synergistic effect of these molecules in angiogenic and cell survival mechanisms is discussed here.

B. TDP-43 Proteinopathy and Angiogenesis

Inclusion bodies are considered to be major hallmark of the disease. Mainly, two kinds of inclusion bodies are found: ubiquitin-positive skein-like inclusions and ubiquitin-negative Bunina bodies.

The composition of these ubiquitinated inclusions (UBIs) was not clear until very recently when Arai et al.[38] and then Neumann et al.[39] recognized TDP-43 as a major component of UBIs in ALS and frontotemporal lobar degeneration (FTLD). TDP-43 is a protein that plays important role in alternative

splicing. TDP-43 also acts as a transcription factor. It possesses a nuclear localization signal (NLS) as well as a nuclear export signal. Mutations in the NLS lead to accumulation of TDP-43 in the cytoplasm in the form of inclusion bodies and protein aggregates.[40] Truncations in the C-terminus of TDP-43 have been associated with protein mislocalization.[41] TDP-43 mutations are thus implicated in several cases of ALS. In patients with fALS, dominantly inherited TDP-43 mutations were found.[42–46] A study carried out on transgenic mice expressing TDP-43 that has the A315T mutation showed that TDP-43 is involved.[47] In either direct or indirect alteration of protein degradation pathways may lead to ubiquitinated protein accumulation and subsequent neuronal degradation.[48]

TDP-43 is the most common protein involved in the pathogenesis in ALS and accounts for almost 97% of familial ALS conditions. In normal conditions, TDP-43 is localized to the nucleus, whereas in the disease state, pathological TDP-43 is found in the cytoplasm[49] of motor neurons and spinal cord. The protein has abnormal structure and function. Even though silencing TDP-43 itself is not appropriate because of its RNA binding, it is crucial for cellular functions.[50] Recruitment of p62, ubiquilin-2, and optineurin is closely associated with aggregation of TDP-43. Hence, it is required to reduce aggregation, which could be processed by acetylation of TDP-43. This would render it prone to phosphorylation, leading to ubiquitination and causing dysfunctional mitochondria.[49]

Downregulated NF-κB was found in ALS patients that might contribute to loss of neuronal protection.[51] Furthermore, caspase activation by tumor necrosis factor alpha (TNF-α) leads to induction of proteolytic cascades. Truncations in the C-terminal of intact protein leads to accumulation of protein aggregates in the cytoplasm. Routing of the fragments of TDP-43 protein (TDP-25 and TDP-35) towards autophagy can reduce this protein aggregations.[52] Loss of function ANG K17I mutation is detected with TDP-43 accumulation in the cytoplasm,[53] indicating that angiogenin has a role in tRNA cleavage and disruption of protein translation.[54] These results also support the hypothesis that angiogenesis and protein aggregation due to translation failure are interlinked pathways in ALS. In the absence of NLS, the truncated TDP-43 is likely to form cytoplasmic aggregates, which induces toxic stress within the cell.[25]

Recent studies have shown that the presence of TDP-43 largely affects the expression of progranulin,[55] which is associated with progressive ALS with active degeneration in motor tracts and glial cells.[56] Eguchi et al. have reported the angiogenic role of progranulin in tumors.[57] It regulates the angiogenesis process in VEGF-independent manner. In ALS patients with decreased VEGF levels, progranulin-dependent angiogenesis presumably counteracts the stress reduction pathway. As expected, progranulin-associated loss of function mutation in FTD patients has shown TDP-43 proteinopathy and hampered autophagy.[58] Because of common features of TDP stress granule accumulation in both FTD and ALS, progranulin can be studied as a novel candidate gene for ALS.[59] It has been shown that progranulin is directly involved in stimulation of VEGF.[60] Colombrita et al.[55] showed the alteration of TDP-43 and progranulin levels in cultures of the NSC-34 cell line. Although there was not much change in the expression of VEGF in those cells after silencing the *TDP-43* gene, there was a significant change in expression of progranulin ($p < 0.05$). The authors also analyzed the effect of overexpression of TDP-43, which enhanced the expression of VEGF and growth factor progranulin (GRN) GRN genes, leading to decreased levels of progranulin (up to 70% to 75%).[55] Mutations in multifunctional GRN also causes FTLD with TDP-43 protein accumulation. Various studies on animal models of GRN showed negative regulation of TNF-α signaling.[61] A study on mice with PGRN (progranulin) deficiency resulted in autophagy impairment. The pathological forms of TDP-43 cleared by autophagy accumulate rapidly in PGRN deficient mice.[58] These results provide insights into interconnections between angiogenic events and proteinopathy in ALS, highlighting the need to investigate proportional expression of TDP-43/VEGF-PGRN as biomarkers of disease pathogenesis.

Chds are ATP-dependent helicases containing DNA-binding proteins. Their role as differentiating markers for hematopoietic stem cells and their

cross-talk with TDP-43 marks them as a potential candidates in the group of molecules that affect the diseased state of ALS by cross-talk with vascularization processes. Chd4 is a member of same family. This gene is commonly shared by both neurons and hematopoietic stem cells for their differentiation, making this a primary target for studying MNDs in the context of vascularization.[62] As mentioned earlier, Chd genes are ATP dependent. Thus it is possible that this affects mitochondrial functions. TDP-43 also suppresses Chd1, a chromatin remodulator responsible for protection of cells from stress condition. Upregulation of TDP-43 greatly reduces Chd1 expression, thus increasing the levels of stress granules in cell.[63] A study in 2016 by Gomez-Del Arco et al.[64] showed that chromatin remodeling genes regulate the mitochondrial function in heart and skeletal muscles. Chd4/NuRD complex is established as a transcriptional repressor in cell differentiation processes. Studies reveal that the Chd4/NuRD complex binds to the promoter region of mitochondria-regulating genes, likely Pgc1, thus controlling its expression. Cells deficient in Chd4 were unable to produce enough ATP, and the loss of Chd4 impacted the expression of Pgc.[65] These studies have advanced our understanding of the role of mitochondrial dysfunction, angiogenesis, and ALS (Fig. 1).

C. TDP-43–OPTN Proteinopathy and Angiogenesis

Mutations in *TDP-43* and *FUS* optineurin (*OPTN*) (a neuroprotective agent in the optic nerve) have been reported in both familial and sporadic cases of ALS.[66] Maruyama et al.[66] were the first to report three types of mutations for optineurin (located on chromosome 10) in ALS, two of which are homozygous. One of these homozygous mutations was the deletion of exon 5, observed in familial ALS, and the other was a nonsense mutation in *Q398X*, which is found in both familial as well as sporadic cases of ALS. A loss of function mutation in the optineurin gene plays an important role in ALS disease generation. Optineurin works as an adaptor protein for ubiquitin binding, which regulates

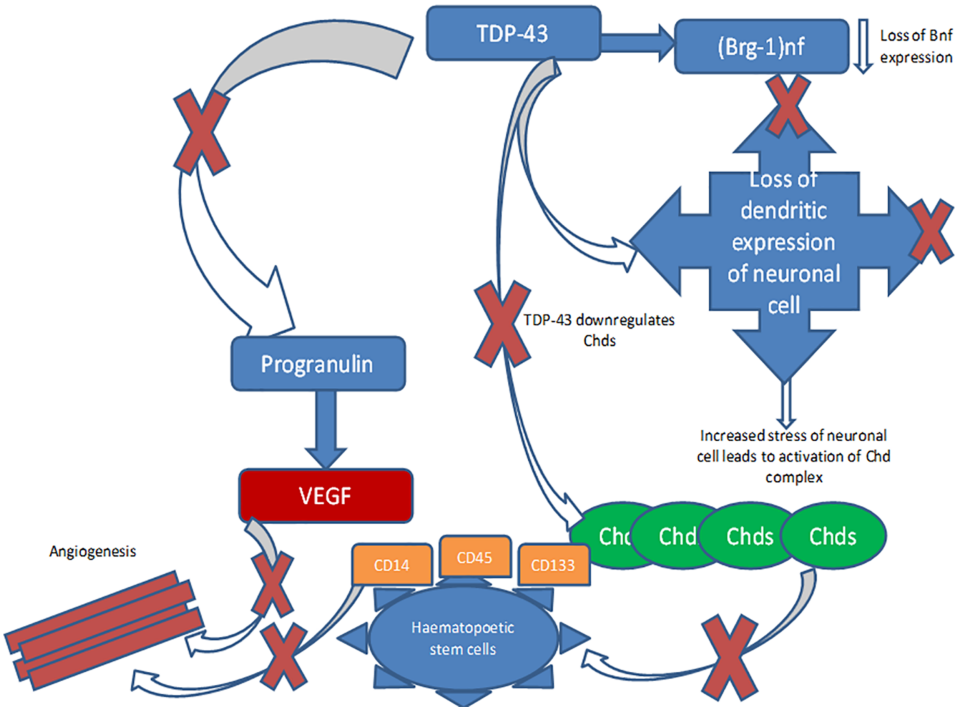

FIG. 1: Epigenetic dysregulation affecting angiogenesis in ALS

the interconnected pathways leading to cell death via autophagy or necroptosis. Mutations in proteins that interact with optineurin, such as TBK1 and p62, suggest a common pathogenic pathway for cell death. With the limited presence of optineurin, many associated factors impact the neuroprotective pathways in which optineurin plays a pivotal role. Hence, the presence of optineurin is directly correlated with the degeneration of ALS. It has been reported that the missense mutation in the *OPTN* gene described the cytoplasmic distribution different from that of the wild-type form of immunoreactive cytoplasmic inclusions. A heterozygous missense mutation E478G was observed in familial ALS.[66] The results indicated the localization of optineurin to distinctive skein-like inclusions of anterior horn neurons and their neurites in spinal cords of sALS and some fALS cases, but not in the cases linked to SOD1 or in the ALS transgenic mouse models overexpressing ALS-linked mutant SOD1. This clearly indicates that OPTN, just like TDP-43 and FUS, influences the pathology of ALS in a manner apart from the SOD1-linked pathway.[67]

The cell survival mechanisms generally converge through certain common pathways, which are speculated to be shared by *angiogenin*, *sVEGFR1/sFLT1*, *TDP-43* and *optineurin*, driven by the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and the serine threonine kinase Akt (Fig. 2). The NF-κB pathway may also be induced by various stimuli that impact the final outcome of the pathway in various cases. One of the inducing molecules for NF-κB pathway is TNF-α which recruits caspases resulting in cell death. Optineurin is believed to be a part of the TNF-α (tumor necrosis factor alpha) signaling pathway influencing it in a manner that regulates cell death.[68] *Optineurin*, which is known to regulate NF-κB pathway, has been shown to

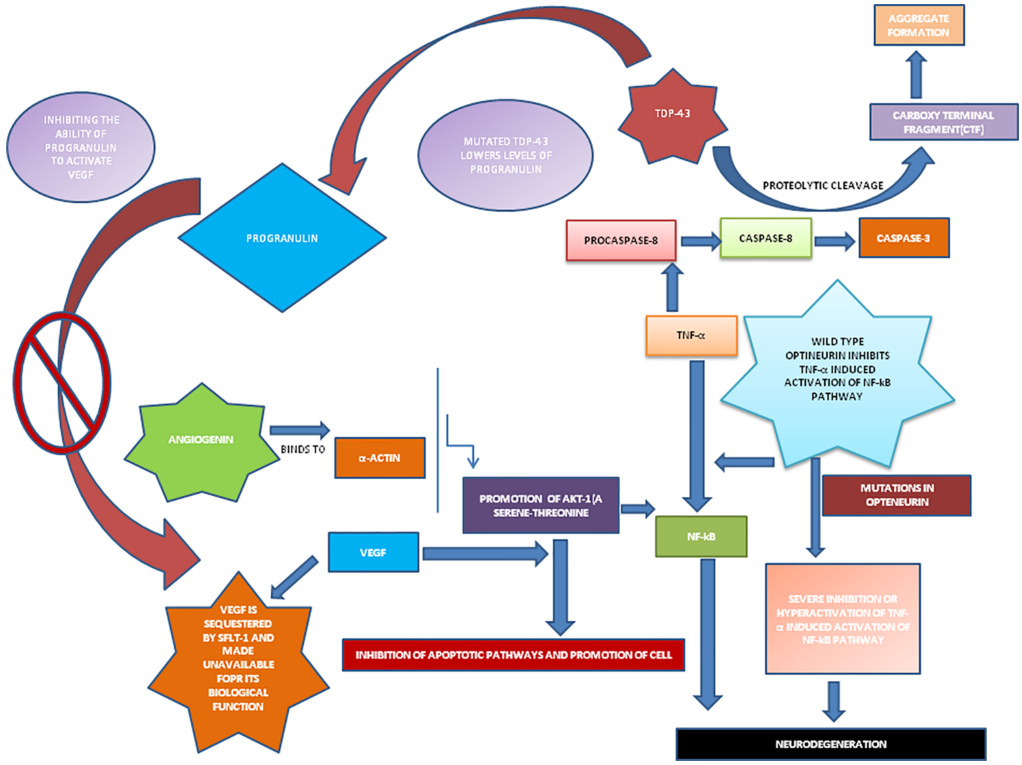

FIG. 2: Schematic representation of the cross-talk between candidate molecules and known pathways like Akt-1, caspases, and neurodegeneration

colocalize with TDP-43 inclusions. The mutations that lead to functional changes in *optineurin* may severely inhibit or hyperactivate TNF-α induced activation of NF-κB pathway leading to neurodegeneration.[69] Uncontrolled proteolytic cleavage of TDP-43 have been provoked by hyperactive TNF-α, consequently leading to formation of aggregates (TDP-43 proteinopathy) inside cells and ECM mediated by NF-κB pathway, which can further stimulate the caspase cascades.[70] In ALS patients with optineurin mutations, the NF-κB expression pattern is altered. Sako et al.[51] also studied the role of NF-κB in ALS. Immunohistochemical studies were carried out on the spinal anterior horn of patients with sALS, an ALS patient with a mutant optineurin (OPTN-ALS), and three controls.[71] Exome sequencing in ALS patients identified loss-of-function (LoF) mutations in *TBK1*. TBK 1 interacts with optineurin through its C-terminal TBK1 coiled-coil domain (CCD2). The mutant allele results in loss of interaction and mitigation of protective effects of optineurin.[72] Loss of optineurin hampers damaged mitochondrial clearance by autophagosomes.[73] TBK1 mediates phosphorylation of OPTN and strengthens the retention of OPTN/TBK1 on ubiquitinated mitochondria.[74] Quantitative proteomics has reported TBK1 association with various other adaptors of autophagy p62/SQSTM1, which has been associated with ALS risk[75] and is another receptor molecule involved in autophagy,[76] is also found to be regulated by TBK1 activation.[74] SQSTM1 recognizes the LC3B site in phagosomes, and the L341V mutation of SQSTM1 has a defective recognition site for the LC3 region that reduces the binding affinity.[77] A positive relationship was established between TBK-1 and VEGF expression in a hypoxia model,[74,78] suggesting that angiogenic factors are recruited in response to overexpressing TBK1. TBK1-mediated gene induction of VEGF, FGF1, and FGF2 has been seen in solid tumors.[79] Extending the situation to ALS pathology, haploinsufficiency of TBK1 in disease may negatively affect the expression of angiogenic markers, such as VEGF and angiogenin, as reported in many studies.[80–82] The exact cascade of the mechanism is still unknown. However, recent studies suggest a strong linkage between the newly identified genes and earlier well-characterized genes involved in ALS pathology.

Studies mentioned previously suggest an imperative role for *OPTN* in cellular survival in ALS pathology and can further our understanding of the phenomenon, which is hampered in ALS pathology (see Fig. 2).

Therefore, we can also hypothesize that *optineurin* and *TDP-43* interaction can lead to enhanced survival rate (as seen in Indian patients), mediated by a neovascular/angiogenic mechanism synergistically contributing to cell survival pathways and involving motor neuron degeneration.

IV. PERSISTENT INFLAMMATION AND ANGIOGENESIS

As earlier studies pertaining to ALS have uncovered the role of VEGF-A and chemokine ligand-2 (CCL-2) in the pathogenesis of ALS,[83] correlating these changes in proteins and progression of disease in a larger cohort of ALS patients, on a longitudinal analysis, will give credible evidence useful for developing new treatments. There is a decrease in microglia[84] population in the spinal cord with disease progression.[50] In a mouse model, prior to disease onset, splenic monocytes expressed a differentiated macrophage phenotype, which included increased levels of chemokine receptor CCR2. Next, expression of the microglial level of CCL2 and other chemoattractants increased, which probably recruited monocytes to the CNS via spinal cord–derived microglia. In the case of human ALS, similar monocytes undergo an ALS-specific microRNA inflammatory response similar to that observed in the ALS mouse model,[85] establishing a link between the animal model and the human disease. VEGF has been found to induce activation of the phosphatidylinositol 3-kinase (PI3-K)/Akt antiapoptotic pathway and is thus a target molecule to reduce neuronal cell death associated with ALS.[36] Therapeutic measures against ALS, involving VEGF-A gene therapy are, therefore, being increasingly investigated.

Another molecule involved with inflammatory stress is granulin, whose deficiency upregulates CCl2.[86] Furthermore, granulin mutations and stress

stimuli induce changes in TDP-43.[87] The majority of therapeutic strategies for ALS are being sought based on the control of neuroinflammation much like other neurodegenerative disorders.[88] It is for the same reason that inhibition of the CCl2 pathway is frequently suggested as the therapeutic approach, to delay glial activation and promote neuronal survival.[89]

Moreover, spinal cord tissue analysis of ALS patients has revealed elevated transcripts of dendritic cell markers (e.g., CD83) and monocytic/macrophage/microglial transcripts,[90] increased expression of cyclooxygenase-2 (COX-2),[91] connective tissue growth factor (CTGF),[92] CCl2,[90] and VEGF receptor (VEGFR)-1[92] enhanced activity of glutamate dehydrogenase (GDH) accompanied by reduced levels of glutamate and aspartate.[93] The increase in CTGF expression is explained by the fact that CTGF plays an important role in astrogliosis, which is often seen as the consequence of hypoxic conditions and is, therefore, a pathological hallmark of ALS.[92] Gliosis is also related to the enhanced GDH activity as reported by Malessa et al.[93] The study has also suggested disruption in cholinergic transmission in the spinal cord of ALS patients, thus contributing to the reduced amino acid levels.

V. OXIDATIVE STRESS AND ANGIOGENESIS

SOD1 is the first gene discovered to be involved in ALS pathology. Mutant SOD1 forms aggregates in motor neurons and enhances production of reactive oxygen species (ROS), which is a well-known pathophysiology of SOD gain-of-function mutation in ALS patients. However, another link of SOD1 mutation has been reported where it was suggested that mutant SOD1 binds to the 3′ UTR region of VEGF mRNA, and after interaction with HuR and TIAR, ribonucleoprotein forms a complex that negatively affects expression of VEGF. The study pointed out that post-transcriptional regulation of VEGF expression by mutant SOD1 is impaired by interaction with key regulatory proteins.[94]

Pretreatment of the cells with VEGF has been shown to protect the culture against oxidative stress-induced motor-neuron-like cell death via the activation of PI3-K and/or MAPK signaling pathways.[36] Based on similar lines, Lunn et al.[95] demonstrated a decrease in VEGF and VEGFR2 levels in the spinal cord of G93A-SOD1 ALS mice and further emphasized the role of VEGF mediated PI3K/Akt signaling in neuroprotection.

In the earlier context, it is important to introduce the role of CHCHD10 protein, which is located in the intermembrane space, and its missense mutation in FTD-ALS patients has been reported. The protein is essential for mitochondrial ultrastructure because the mutant allele causes abnormalities mainly in cristae.[96] It is part of a mitochondrial contact site and cristae organizing system (MICOS) complex.[97] In mitochondria, CHCHD10 has been found to work as the HRE that interacts with cytochrome oxidase (COX) and helps in oxygen consumption. However, when localized in the nucleus, it acts as a transcriptional repressor for genes that harbor OREs. Because of its hypoxia sensitivity, it plays a pivotal role in the regulatory network that responds to altered oxygen levels. In ALS patients with mitochondrial dysfunction, CHCHD10 mutations contribute to the disease progression by reduced oxygen sensitivity and altered angiogenesis.[97]

More recently, in addition to the molecular network associated with the ALS condition, analysis of whole genome sequencing for detection of associated loci has shown constructive loss of function mutation in gene NEK1, which could be responsible for inherent ALS.[98] Both NEK1 and c21orf2 are involved in the DNA damage response, and studies have demonstrated interaction between these two proteins during DNA repair.[99] Both gene variants have been identified in ALS exome studies. The NEK1 variant with loss-of-function mutation and its association with ALS has been recently defined.[100] The gene has multiple functions, including cilia formation, microtubule stability, neuronal morphology, and polarity.[98] NEK1 has been shown to affect the stability of von Hippel-Lindau tumor suppressor (pVHL) by phosphorylation in *in vitro* and *in vivo* studies.[101] In the study, VHL phosphorylation did not affect expression of HIF. However, pVHL has been demonstrated to regulate HIF expression in various other studies,[102,103] so a plausible role of NEK1 in hypoxia, oxidative stress, and angiogenic processes cannot be discounted.

VI. OTHER RISK ALLELES IN ALS

The mapping of the human genome has led to characterization of the *C21orf2*, *MOBP*, and *SCFD1*[104] genes, which have been newly connected to increased risk of ALS. The SNP-based heritability is approximately 8.5%, having a distinct and significant role in identifying low-frequency variants with the frequency of 1% to 10%.[104] Other genes have also been found to be associated with ALS. Recently, mutations in *ubiquilin 2* (*UBQLN2*) were associated with dominant inheritance of ALS along with frontotemporal dementia (ALS–FTD).[22,38,39,105] Neuropathological analysis of the mice with end-stage disease has revealed the accumulation of ubiquitinated inclusions in the brain and spinal cord, astrocytosis, fewer hippocampal neurons, and reduced staining of TDP-43 in the nucleus, with concomitant formation of ubiquitin^{+} inclusions in the cytoplasm of spinal motor neurons.[22,38,39,105] Missense mutations in *ubiquilin 2* (*UBQLN2*) identified as the cause of X-linked dominant ALS–FTD has revealed the accumulation of ubiquitinated inclusions present in brain and spinal cord.[106,107] The *UBQLN4* gene variant has also been found to be associated with ALS. The UBQLN4^{D90A} mutation impairs the ubiquitin-proteasome system, which interferes with the β-catenin signaling pathway, disrupting the breakdown of β-catenin and resulting in accumulation of β-catenin leading to structural defects in motor neurons. Edens et al.[108] studied the effect of mutated UBQLN4 in Zebra fish and mouse models. The mutation in these models caused a change in the shape of motor neurons in the spinal cord. Most cases of ALS have mutated RNA-binding proteins. RBPs have been found to be associated with familial ALS. Bakkar et al.[109] studied the published literature with IBM Watson, comparing the data to look for semantic similarities and any new connections between entities involved. They found five new RBPs that were associated with ALS. These have been previously associated in some of the studies. Five RBPs, hnRNPU, Syncrip, RBMS3, Caprin-1, and NUPL2, showed significant alterations in ALS relative to controls.[109] Additionally, Münch et al.[110] showed the importance of point mutations of the p150 subunit of *dynactin* (*DCTN1*) in ALS. Despite the large number of gene loci found to be associated with ALS, their cross-talk with angiogenic molecules remains enigmatic.

VII. CONCLUSION

The review of various studies on ALS suggests the importance of neovascularization and its regulatory processes in the enhanced survival of motor neurons in certain patients living with ALS for long periods. Studies also signify the imperative role of angiogenic processes chiefly governed by VEGF (associated receptors). This can regulate angiogenesis by interacting with *ANG*, thereby enhancing cell survival. We also described the molecular interaction between TDP-34 and OPTN, which may influence the outcome of angiogenic pathways by affecting VEGF and its associated molecules. Additionally, this review has provided mechanistic insights into molecular interactions between different molecules involved in pathological changes associated with ALS. Identifying the molecular interactions that influence angiogenic processes and mediate cell survival can lead to a paradigm shift in diagnostic and treatment strategies in ALS research.

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Original Article

The Role of *Dystrophin* Gene Mutations in Neuropsychological Domains of DMD Boys: A Longitudinal Study

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Rahul Tyagi[1], Vivek Podder[2], Harshita Arvind[1], Manju Mohanty[3], and Akshay Anand[1]

Abstract

Background: Duchenne Muscular Dystrophy (DMD) is a fatal muscular dystrophy of pediatric population coupled with other secondary comorbidities including mental retardation and neuropsychological impairments. Mutation location in the dystrophin gene, have been associated with neuropsychological functioning in DMD.
Purpose: We investigated temporal changes in the neuropsychological functioning of DMD subjects, hitherto understudied.
Methods: Subjects with suspected DMD were enrolled according to the ethical guidelines. Genetic confirmation by Multiplex Ligation Dependent Probe Amplification was carried out to identify pathogenic deletion or duplication in dystrophin gene. Intellectual and neuropsychological functioning was assessed by using standardized batteries. Investigated neuropsychological domains included visual, verbal and working memory, selective and sustained attention, executive functioning, verbal fluency, and visuo-constructive and visuo-spatial abilities. The assessments were carried out at baseline and followed for one time point in 30 cases.
Result: The follow-up assessment revealed that neuropsychological functioning did not worsen with time. Improvements were seen in block designing task (p = 0.050), serial positioning primacy effect (p = 0.002), Stroop incongruent task (p = 0.006), visual long-term memory (p = 0.003) and attention (p = 0.001). DMD cases with mutation location affecting short dystrophin isoform (Dp140) also showed improvement in these domains.
Conclusion: No temporal alterations were found in DMD subjects, though improvements in few domains were observed. Neuropsychological rehabilitation may be useful in improving the quality of life in DMD subjects.

Keywords

DMD, neuropsychology, cognition, longitudinal, follow-up, dystrophin

Introduction

Duchenne muscular dystrophy (DMD) is a fatal X-linked genetic neuromuscular disorder, characterized clinically by rapidly progressive and disabling muscle weakness, present from birth and exclusively occurring in males. DMD is caused by an X-linked recessive frameshift mutation in the *dystrophin* gene that ensues absent or non-functional muscle dystrophin protein and resultant muscle fibre degeneration, leading to chronic peripheral inflammation.[1] Dystrophin functions as a direct signalling molecule and connects the extracellular matrix to the cytoskeleton. It is a part of the dystrophin-associated glycoprotein complex.[2,3] It is the most common childhood muscular dystrophy with an estimated incidence of 200 per million male live births.[4] By the age of 3, patients with DMD exhibit motor inabilities in such as walking, running, climbing, jumping, waddling gait, difficulty in standing, followed by upper limb weakness and pseudohypertrophy by the age of 5. This is followed by progressive worsening of the symptoms and with death due to respiratory failure or cardiac arrhythmia before the third decade of life.[5]

In addition to skeletal muscle pathology and loss of physical strength, a subset of children with DMD is characterized by global cognitive impairment. Previous works suggest that in DMD patients, intelligence quotient (IQ) distribution is downshifted one standard deviation with a lower verbal IQ than performance IQ. It is reported that DMD patients might also have specific neuropsychological deficits

[1] Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
[2] Department of General Medicine, Kamineni Institute of Medical Sciences, Narketpally, Nalgonda, Telangana, India.
[3] Department of Neurosurgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
Corresponding author:
Manju Mohanty, Department of Neurosurgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
E-mail: manjumohanty2011@gmail.com
Co-corresponding author:
Akshay Anand, Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.
E-mail: akshay1anand@rediffmail.com

including poor performance in working memory, executive function, attention deficits, and impaired reading and language acquisition skills.[1,6] Previous studies have led to hypothesis that these specific neuropsychological deficits resonate with cerebellar lesions due to similarity in cognitive impairments.[7] Even though dystrophin is often characterized in muscles, it is also found in various other tissues including the brain. Multiple studies have shown the association between the loss of dystrophin and cognitive impairments. Multiple studies from both clinical and animal models attribute the lack of dystrophin expression in the brain to the development of the cognitive and behavioural alterations in DMD.[8–10] Some patients with DMD also have a higher incidence of neurobehavioral disorders including attention-deficit/hyperactivity disorder (ADHD), anxiety disorder, autism spectrum disorders (ASD), epilepsy and obsessive-compulsive disorder. Experimental studies have found that dystrophin is expressed in neurons within specific brain regions including the cortex, cerebellar Purkinje cells, Cornu Ammonis (CA) region of the hippocampus, retina and the peripheral nerve. These might be responsible for some of the neuropsychological deficits.[11]

It is important to note that myelination is critical in the central nervous system (CNS) for complex brain processing and therefore the disorders affecting the neuronal myelination, by a process regulated by oligodendrocytes in the CNS, may produce neurological deficits.[12] In a recent study, researchers have found that for proper maturation of oligodendrocytes and effective myelination during postnatal brain development, normal expression of dystrophin isoforms is required. Oligodendrocytes express three different forms of dystrophin, Dp427, Dp140 and Dp71, and loss of oligodendroglial dystrophin, particularly Dp427, was found to be contributory to neurodevelopmental deficits in their experimental mdx mouse model of DMD. In this study, in mice without functional Dp427 dystrophin protein had late development of myelination with significantly affecting the cerebral cortex.[13] A past review identified lack of Dp427 to be associated with progressive muscle weakness in all DMD patients, likely responsible for both muscle degeneration and brain dysfunction.[14]

Despite involvement of common gene isoforms, Wingeier et al. in their study found no correlation of declining cognitive function with the progression of muscular deterioration.[7] Another study reported that cognitive impairment in DMD is non-progressive and unrelated to the severity of muscle disease. Additionally, varying phenotypic expressions of specific neuropsychological impairments is also notable in DMD patients.[15] The reason for this divergence is inconclusive, but this might be associated with the timing and localization of human dystrophin isoforms expression.[1] In contrast, previous studies reported that intellectual functioning in DMD patients deteriorates as the disease progresses with progressive reduction in all IQ scores.[15] As previously noted, varying neuropsychological deficits affect overall cognitive performance of the boys with DMD. For example, boys with DMD often have problems in short-term verbal working memory and increased risk of learning disability resulting from poor phonological awareness/processing. They often encounter problems with reading as discussed in a study, whereby 40% of boys with DMD have been shown to have reading problems. It is also found that they have lower academic achievement scores than expected of their level of cognitive functioning.[16] In addition to academic performance, they also face poor health-related global quality of life potentially posing them at risk of depression, anxiety and stress.[17,18] A successful care of DMD patients thus requires comprehensive, multidisciplinary plan including psychosocial care, in addition to a pharmacological approach.

In order to plan clinical trials to establish efficacy of interventions targeting different neuropsychological impairments, longitudinal studies in DMD patients are required. This will help to explore how, over the course of time, neuropsychological function changes with progression of DMD. Additionally, this can help with risk stratification and screening and offering specific neuropsychological rehabilitation. Future studies could include acquisition of longitudinal data in order to examine which cognitive and neuropsychological functions in DMD are non-progressive or progressive. This is important in counselling and future planning. Previous studies suggested that more research is needed about characterizing the features of neuropsychological profile in determining the use and effectiveness of cognitive rehabilitation and retraining for children with DMD.[5] In-depth review of the literature has revealed that there are no longitudinal studies that have investigated whether the cognitive and neuropsychological impairment in DMD is progressive. To the best of our knowledge, this is the global first longitudinal study which has described the neuropsychological function in DMD patients. The aim of this longitudinal study was to use a battery of intelligence, learning and memory tests to characterize the neuropsychological profile in boys with DMD by following them up for long-term changes in various domains.

Methods

Subjects: A total of 30 DMD subjects were recruited according to the guidelines of Institutional Ethics Committee (IEC) of Postgraduate Institute of Medical Education and Research, Chandigarh, India. Informed assent and written informed consent was obtained from the participants before enrolment. The study was approved by IEC vide no. INT/IEC/2015/732 dated 19 November 2015. The recruitment guidelines adhered to the Helsinki Declaration. The DMD patients were enrolled with the help of Indian Association of Muscular Dystrophy (IAMD). Cases were also recruited retrospectively with the help of patient support groups. The prevalence-based sample size was derived, that is, 1/3500 males for DMD. For inclusion in the study, cases with characteristic clinical features of the Duchenne phenotype were identified. The cases with BMD or intermediate phenotypes and other myopathies

were not considered for inclusion. The entire study was conducted according to the quality assurance protocols of the Neuroscience Research Lab. Genetic diagnosis was carried out by Multiplex Ligation Dependent Probe Amplification (MLPA) as described previously.[19,20]

IQ: Malin's intelligence scale for Indian Children (MISIC), an adaptation to Wechsler intelligence scale for children (WISC), was employed to assess the IQ. Briefly, verbal- and performance-based IQs (VIQ and PIQ) were derived to finally form the IQ. VIQ was derived by six subtests, that is, information, comprehension, arithmetic, digit span, vocabulary and similarity. PIQ was derived from four subtests, that is, picture completion, block designing (BD), coding and maze. The detailed description is provided in the supplementary material**.**

Neuropsychological Assessments: Neuropsychological assessments were carried out in 30 DMD cases. Memory (visual and verbal), attention (selective and sustained), executive functioning (cognitive flexibility, cognitive control, response inhibition, interference), verbal fluency (semantic and category) and visuo-constructive ability were assessed using standard test batteries including Rey Auditory Verbal Learning Test (RAVLT), Rey–Osterrieth Complex Figure Test (RCFT), Stroop Colour and Word Test (SCWT), Colour Cancellation Test (CCT), Children's Colour Trail Test (CCTT), Visual Recognition test (VRT), Controlled Oral Word Association (COWA), Animal Naming Test (ANT). Follow-up assessments were carried out at single time point. The detailed description is provided in the supplementary material.

Statistical Analysis

We used SPSS version 21 to analyse the neuropsychological data. Normal distribution was analysed by Kolmogorov–Smirnoff statistics. Normally distributed data was further analysed by paired *t* test. Level of significance was analysed at $p < 0.05$.

Results

Participants: A total of 30 cases diagnosed with DMD were enrolled. Participant demographic details have been provided in Table 1. Genetic investigations were carried out in all DMD cases. Representative electropherogram is provided in Figure 1.

Table 1. Details of Participants

Variables	Mean (SD)
Cases	*n* = 30
Gender	All males
Age	11.54 (2.71)
Education	4.93 (2.87)
Age of onset	3.54 (1.41)
Disease duration	8.31 (3.17)
Follow-up duration	10 months
Dp140 isoform alteration	*n* = 20

Source: Authors' own data.

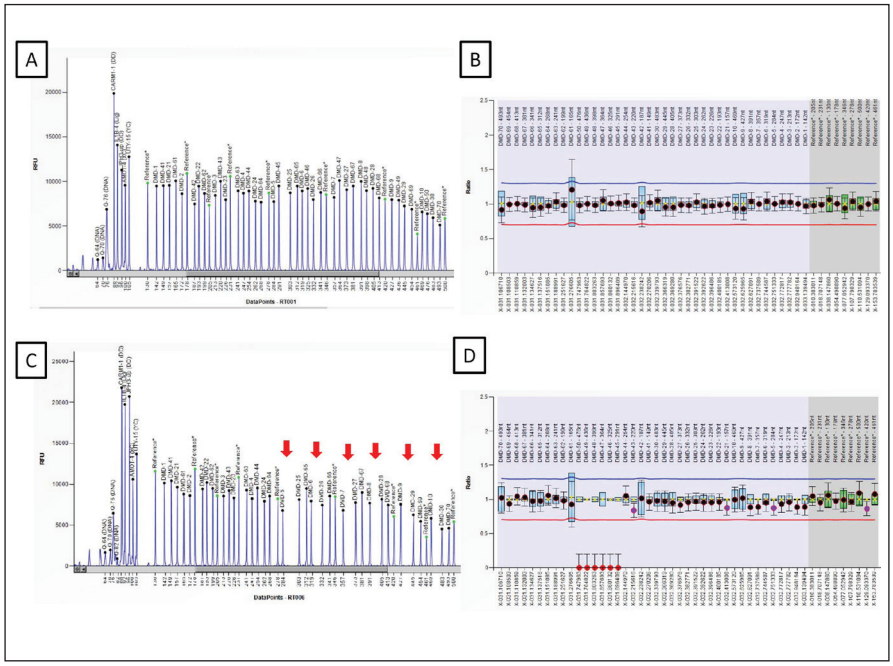

Figure 1. Electropherogram Obtained after Multiplex Ligation Dependent Probe Amplification (MLPA) PCR Followed by Capillary Electrophoresis of the Amplified Products. (A & B) Electropherogram and Ratio Chart Representing Profile of a Normal Control Sample. (C & D) Electropherogram and Ratio Chart Representing Deletions Between Exon 45–50 (see arrow) in the Patients Clinically Diagnosed for DMD. Ratio Between 0.70 and 1.30 is Considered in the Normal Range While a Ratio of 0.00 is Considered as Deletion (Depicted in Red Dots).

Source: Authors' own.

Longitudinal Analysis of Cognitive and Neuropsychological Profile in DMD Subjects

Follow up of 30 DMD subjects was carried out to assess the progression of impairment in the general and specific cognitive domains. The mean follow-up duration was 10 months. Among the MISIC subsets, the DMD group showed marginally significant improvement in the block designing task (t = –2.074, p = 0.050). Moreover, the mean levels achieved in the block designing task was improved to two levels with significant improvement in the block designing efficiency (t = –2.706, p = 0.014). However, the mean time in completing the block designing task was significantly increased in the follow-up (t = –2.741, p = 0.013). An improved serial positioning effect of primacy component in trial 1 showed a statistically significant improvement (t = –3.422, p = 0.002). DMD subjects also performed better and took less time in the colour cancellation task in the follow-up (t = 3.929, p = 0.001). Remaining variables were comparable to the pre-follow-up status (Tables 2–5).

Table 2. Comparison of General Intelligence on Pre and Post Follow-Up in DMD Subjects (n = 30) Using Paired t Test

Cognitive Domain and Neuropsychological Battery	Neuropsychological Battery Variables	DMD-Pre Mean ± SD	DMD-F Mean ± SD	t Value	P Value
• Verbal intelligence • Performance intelligence • General intelligence	Information	93 ± 14.63	94 ± 11.99	–0.576	0.570
	Comprehension	84 ± 21.71	88 ± 12.43	–1.192	0.245
	Arithmetic	85 ± 14.28	86 ± 11.60	–0.438	0.665
	Digit span	88 ± 14.43	86 ± 14.97	0.974	0.340
	Vocabulary	78 ± 11.57	75 ± 14.17	1.022	0.334
	Similarity	94 ± 35.14	102 ± 33.26	–1.063	0.303
	VIQ	89 ± 11.87	92 ± 13.36	–1.464	0.154
	Picture completion	79 ± 21.14	83 ± 12.62	–1.078	0.293
	Block designing	93 ± 30.23	103 ± 19.97	–2.074	**0.050**
	Coding	84 ± 35.94	95 ± 23.60	–1.569	0.132
	Maze	107 ± 39.00	110 ± 12.53	–0.414	0.683
	PIQ	66 ± 10.80	63 ± 6.78	0.559	0.591
	IQ	97 ± 14.00	100 ± 15.62	–1.419	0.167

Source: Authors' own data.

Note: Bold values represent significant p values.

Table 3. Comparison of Neuropsychological Variables in DMD Subjects on Follow-Up (n = 30) Using Paired t Test for RAVLT Variables

Cognitive Domain and Neuropsychological Battery	Neuropsychological Battery Variables	DMD-Pre Mean (SD)	DMD-F Mean (SD)	t Value	P Value
RAVLT • Verbal learning • Working memory • Short-term verbal memory • Long-term verbal memory	RAVLT-trial 1	6.68 (2.58)	7.50 (3.27)	–1.856	0.074
	RAVLT-trial 5	12.00 (3.09)	12.61 (2.45)	–1.030	0.312
	RAVLT-learning capacity	50.11 (12.44)	52.39 (12.86)	–1.156	0.258
	RAVLT-IR	11.04 (3.12)	11.71 (3.02)	–1.565	0.129
	RAVLT-DR	10.61 (3.00)	11.36 (3.65)	–1.446	0.160
	LTPR	90.02 (23.39)	90.50 (30.53)	–0.066	0.948
RAVLT • Serial positioning effect • Working memory	**Primacy T1**	**2.53 (1.23)**	**3.39 (1.34)**	**–3.422**	**0.002**
	Middle-T1	1.96 (1.07)	2.17 (1.33)	–0.691	0.495
	Recency-T1	1.86 (1.09)	1.93 (1.65)	–0.232	0.818
	Primacy-total	18.60 (4.05)	19.92 (4.31)	–1.655	0.110
	Middle-total	15.53 (4.24)	16.35 (4.89)	–1.107	0.278
	Recency-total	15.10 (4.66)	16.60 (5.3)	–1.499	0.145
RAVLT • Susceptibility to interferences	Proactive interference	0.93 (0.34)	0.94 (0.68)	–0.113	0.911
	Retroactive interference	0.93 (0.19)	0.94 (0.26)	–0.258	0.799
	Forgetting speed	0.97 (0.18)	0.90 (0.29)	1.164	0.254
	RAVLT efficiency	1.96 (0.28)	2.03 (0.34)	–1.287	0.209

Source: Authors' own data.

Note: Bold values represent significant p values.

Table 4. Comparison of Neuropsychological Variables in DMD Subjects on Follow-Up (n = 30) Using Paired t Test

Cognitive Domain and Neuropsychological Battery	Neuropsychological Battery Variables	DMD-Pre Mean (SD)	DMD-F Mean (SD)	*t* Value	*P* Value
COWA and ANT • Executive Functioning Semantic Fluency • Category Fluency	COWA-K	6.04 (3.65)	6.07 (3.31)	–0.082	0.935
	COWA-M	5.18 (3.76)	5.57 (3.26)	–0.763	0.452
	COWA-P	4.57 (3.61)	5.03 (3.15)	–1.045	0.305
	COWA-Avg	5.18 (3.48)	5.45 (3.03)	–0.813	0.423
	ANT	9.60 (3.67)	8.80 (3.26)	1.046	0.304
• Executive Functioning Cognitive Flexibility • Cognitive Control • Response Inhibition • Interference	Stroop-w	52.14 (20.11)	60.91 (20.70)	–2.523	0.020
	Stroop-C	**38.05 (13.78)**	**47.50 (15.87)**	**–3.059**	**0.006**
	Stroop-CW	23.79 (9.14)	26.58 (14.98)	–1.138	0.267
	Stroop effect 1	14.18 (9.70)	18.50 (11.20)	–1.617	0.121
	Stroop effect 2	0.48 (0.16)	0.51 (0.26)	–0.650	0.522
	Stroop effect 3	0.65 (0.20)	0.61 (0.18)	0.876	0.391
RCFT • Visuo-constructive ability • Visual short and long-term memory	RCFT-Copy	31.05 (6.70)	32.52 (3.53)	–1.068	0.298
	RCFT-IR	21.39 (8.87)	23.98 (8.84)	–1.661	0.111
	RCFT-DR	**21.05 (8.25)**	**25.09 (6.11)**	**–3.417**	**0.003**

Source: Authors' own data.

Note: Bold values represent significant p values.

Table 5. Comparison of Neuropsychological Variables in DMD Subjects on Follow-Up (n = 30) Using Paired t Test

Cognitive Domain and Neuropsychological Battery	Neuropsychological Battery Variables	DMD-Pre Mean ± SD	DMD-F Mean± SD	*t* Value	*P* Value
DIGIT span test • Short term memory • Working memory	DSF	5.17 (1.03)	5.30 (1.06)	–0.680	0.503
	DSB	3.17 (1.70)	3.04 (1.55)	0.680	0.503
Maze • Visuo-spatial planning	MAZE-TT	158.87 (93.58)	165.73 (81.84)	–0.272	0.790
	MAZE-E	6.69 (8.31)	6.13 (8.66)	0.872	0.397
Block design test	BD-TT	147.19 (104.72)	215.86 (105.21)	–2.741	0.013
	BD-levels	4.95 (2.73)	6.33 (2.73)	–3.512	0.002
	BD-EFFIC	0.22 (0.24)	0.35 (0.26)	–2.706	0.014
CCTT and CCT attention • Divided attention • Focused attention • Interference	CCTT1	46.06 (21.76)	47.17 (24.67)	–0.190	0.851
	CCTT2	83.00 (39.65)	75.17 (34.82)	1.677	0.112
	CCT	**137.15 (60.20)**	**93.45 (40.47)**	**3.929**	**0.001**
	CCTT interference	0.89 (0.53)	0.79 (0.80)	0.466	0.647
	CCT error	1.22 (2.02)	2.28 (2.08)	–1.679	0.111
VRT • Visual agnosia	VRT	8.05 (1.50)	8.48 (1.36)	–1.441	0.165

Source: Authors' own data.

Note: Bold values represent significant p values.

Table 6. Representing Temporal Changes in Neuropychological Functioning Due to *DMD* Gene Mutation Affecting Dp140 Isoform

Scale	Variable	Pre (SD)	Post (SD)	*t* Value	*P* Value
RAVLT	Primacy effect	2.50 (1.46)	3.22 (1.43)	–2.060	0.050
SCWT	SCWT-colour	37.46 (13.96)	46.00 (17.43)	–2.504	0.028
RCFT	RCFT-delayed recall	20.17 (9.21)	25.32 (6.15)	–3.457	0.004
CCT	Colour cancellation	155.86 (60.01)	105.21 (42.38)	3.317	0.006

Source: Authors' own data.

Effect of Mutation Location on Temporal Change in Neuropsychological Functioning

We analysed the trends of neuropsychological functioning in cases with distal mutation location affecting Dp140 isoform. Among 30 DMD subjects, 20 had mutations in the *DMD* gene affecting Dp140 isoform, that is, exon 44 or upstream. No changes in the cognitive and neuropsychological functioning were observed over time in majority of parameters except primacy, Stroop colour and word task-colour component, and RCFT-delayed recall, which showed improvement from baseline assessment as shown in Table 6.

Discussion

We provide a comprehensive longitudinal analysis of cognitive and neuropsychological profile in DMD subjects. The detailed analysis of neuropsychological domains and their progressive nature in boys with DMD provide better understanding of the use and effectiveness of specific rehabilitation regime required for retraining these patients. Additionally, this will enable future interventional studies targeting specifically impaired neuropsychological function.

When investigating cognitive process, analysing different aspects of the function is critical. In the present study, 30 boys with DMD were assessed for the progression of impairment in the general and specific cognitive domains over a mean follow-up duration of 10 months. The findings of this study showed that after a mean follow-up of 10 months, boys with DMD had no change in their general, verbal and performance intelligence. Data regarding non-progressive nature of intelligence was consistent with previous findings. DMDs have lower verbal IQ score than performance IQ score, and all IQ scores progressively reduce as the disease progresses.[15, 21] The risk of cognitive deficit is determined by the location of mutation in the *DMD* gene that ensues specific functional dystrophin isoforms as described earlier. For example, patients who get lower IQ score were found to have a mutation in the distal region of the gene, whereas those with full-length mutation had highest scores.[22] However, our study confirmed superior cognitive performance on block design task, designed to assess visuospatial ability, with significant improvement in the designing efficiency.

The study also undertook the neuropsychological assessment of boys with DMD for the RAVLT. We found a significant improvement in serial positioning effect of primacy component. In this effect, the person is assessed for the tendency to better recall the first items in a list than those in the middle or last. The finding that DMD patients had improvement in primacy component reflects their ability to improve the long-term memory after repeated exposures. However, there is a paucity of evidence that showed this effect in DMD patients. A previous study investigating serial positioning memory of boys with DMD found their inability to sustain attention to the task; however, temporal changes were not investigated.[23]

Furthermore, executive function and information processing speed were assessed with Stroop Colour Test (SCT), Stroop Colour and Word Test (SCWT), COWA test. Stroop test is used to measure cognitive flexibility and selective attention.[24] Examination was performed at baseline and during follow-up rounds. Our study found significant improvement in the SCT during follow-up, suggesting improvement in the executive function of this population. The improved performance on tests assessing executive functions such as cognitive flexibility is in contrast to a past study which showed poor performance on tests for executive function among DMD patients.[25] Chamova et al. reported poor performance on all neuropsychological tests (general cognitive abilities, verbal memory, attention and executive functions) in patients with non-functional Dp140 isoforms.[9] Remmelink et al. examined the effect of an absent full-length dystrophins (Dp427) on behavioural consequences in DMD patients and found a deficit in cognitive flexibility.[26]

In our study, all other neuropsychological functions remained unchanged over the period. However, improvement in colour cancellation task, block design task, visual long-term memory and primacy effect indicate possibilities of improvement in cognitive domains. The domains that remained unchanged can be further analysed in future studies, by profiling the expression of dystrophin isoforms in post-mortem brain samples of the DMD patients. This will help elucidate underlying genetic basis for the observed variable phenotypic changes in the specific neuropsychological function. Additionally, interventional studies can enhance characterization of clinical and genetic variability and develop newer interventions specific to neuropsychological deficits. This may also serve to explore genotype-phenotype relationship in subsets of DMD patients with other coexisting neurodevelopmental disorders such as ADHD and ASD.

The significant improvement of executive functions in our study suggests that genetic prediction models can be developed to facilitate risk assessment, early detection and targeted treatment in such patient populations. Bailey et al. have recently developed a bioinformatics tool, called DMD Open access Variant Explorer (DOVE), to facilitate effective analysis of pathologic *DMD* gene variants, resulting in scope of precision medicine treatment for DMD.[27]

The functional improvement observed during the follow-up period shows that boys with DMD may be more amenable to neurocognitive rehabilitation. The substantial economic burden of physical and neuro-developmental disability makes DMD patients vulnerable. Several studies have shown such economic burden of DMD on patients and their family.[28, 29] Since the advent and progress in multidisciplinary management for DMD, the functional outcome, quality of life and longevity of the patients have significantly been improved.

Conclusion

The neuropsychological profiling of *DMD* patients provides a well-recognized pattern of cognitive strengths and weaknesses among *DMD* patients. This opens new vistas to explore other comorbid neurodevelopmental and neuropsychiatric disorders. The variation in phenotypic manifestation of neuropsychological deficits was found to vary with location of the *DMD* gene and effect of the mutation on CNS-expressed isoforms. Further research with larger sample size and multi time point analysis will be required to understand the involvement of various domains. The neuropsychological domains that remained unchanged need to be explored in future interventional studies with increased sample size in order to explore the changes on such domains and develop newer targeted neurocognitive interventions. Additionally, improved executive function in our study population reflects their receptibility to neurocognitive interventions. Future longitudinal studies with increased sample size and long-term follow-up are imperative.

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Author Contributions

Akshay Anand: Conceptualization, management of the study, editing and final approval the manuscript.

Rahul Tyagi: Co-conceptualization under supervision, genetic and neuropsychological data acquisition, experiments and analysis, statistical analysis, drafting and editing the manuscript.

Vivek Podder: Drafting the manuscript.

Harshia Arvind: Neuropsychological data acquisition.

Manju Mohanty: Supervision in neuropsychological assessment, analysis and validation of data.

Ethical Statement

The study was approved by Institute Ethics Committee of PGIMER, Chandigarh vide no. INT/IEC/2015/732 dated 19 November 2015.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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RESEARCH ARTICLE

Repurposing Pathogenic Variants of *DMD* Gene and its Isoforms for DMD Exon Skipping Intervention

Rahul Tyagi[1], Sumit Kumar[1], Ashwin Dalal[2], Faruq Mohammed[3], Manju Mohanty[4], Paramvir Kaur[1] and Akshay Anand[1,*]

[1]*Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India;* [2]*Diagnostics Division, Center for DNA fingerprinting and Diagnostics, Hyderabad, India;* [3]*Genomics and Molecular Medicine Unit, Institute of Genomics and Integrative Biology, New Delhi, India;* [4]*Department of Neurosurgery, Postgraduate Institute of Medical Education and Research, Chandigarh, India*

Abstract: ***Background*:** Duchenne Muscular Dystrophy (DMD) is a progressive, fatal neuromuscular disorder caused by mutations in *the DMD* gene. Emerging antisense oligomer based exon skipping therapy provides hope for the restoration of the reading frame.

***Objectives*:** Population-based *DMD* mutation database may enable exon skipping to be used for the benefit of patients. Hence, we planned this study to identify *DMD* gene variants in North Indian DMD cases.

***Methods*:** A total of 100 DMD cases were recruited and Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to obtain the deletion and duplication profile.

***Results*:** Copy number variations (deletion/duplication) were found in 80.85% of unrelated DMD cases. Sixty-eight percent of cases were found to have variations in the distal hotspot region (Exon 45-55) of the *DMD* gene. Exon 44/45 variations were found to be the most prominent among single exon variations, whereas exon 49/50 was found to be the most frequently mutated locations in single/multiple exon variations. As per Leiden databases, 86.84% cases harboured out-of-frame mutations. Domain wise investigation revealed that 68% of mutations were localized in the region of spectrin repeats. Dp140 isoform was predicted to be absent in 62/76 (81.57%) cases. A total of 45/80 (56.25 %) and 23/80 (28.70%) DMD subjects were predicted to be amenable to exon 51 and exon 45 skipping trials, respectively.

***Conclusion*:** A major proportion of DMD subjects (80%) could be diagnosed by the MLPA technique. The data generated from our study may be beneficial for strengthening of mutation database in the North Indian population.

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1. INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a rare and incurable disorder caused due to mutations in the *DMD* gene located on Xp21 loci. *DMD* gene mutations are the primary cause for pathology and progression of this neuromuscular disorder resulting in the atrophy of muscles [1]. The presence of a single copy of X-chromosome in males increases their susceptibility to the disorder. Most of the mutations found in the *DMD* gene are deletions/duplications and are non-randomly distributed. Due to deficiency of dystrophin protein, there is a progressive muscular weakness, which causes a reduction in the sarcolemmal elasticity and results in death due to respiratory and cardiac failure, usually in their twenties [2]. Other co-morbidities associated with DMD include scoliosis (curvature of spine), variable degrees of cognitive and neuropsychological alterations [3]. The expression of *DMD* gene is regulated by a number of internal promoters, which result in different shorter isoforms of dystrophin protein with varying sizes. Clinical heterogeneity in DMD is attributed to differential expression of full length and shorter dystrophin isoforms in various tissues. Full-length dystrophin (Dp427) was reported to be localized in the muscle (Dp427m), cortical and hippocampal neurons (Dp427c) [4] and purkinje isoforms (Dp427p) [5]. The muscle-specific full-length dystrophin protein primarily functions by stabilizing dystrophin-associated protein complex (DAPC). The proximal promoters express Dp260 and Dp116 in the retina and peripheral nerves, respectively [6, 7]. The *DMD* gene translation through distal promoters results in

*Address correspondence to this author at the Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India; Cell: +919914209090 / +919815968102;
E-mail: akshay1anand@rediffmail.com

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Dp140, Dp71 and DP40 isoforms, believed to be expressed in the central nervous system (CNS). The regulatory site for Dp140 isoform lies in exon 44/45 and the mutations in this region may result in the loss of Dp140 isoform, reported to be crucial for cognitive function in DMD [8]. Localization of different dystrophin isoforms in crucial body organs suggests its role in the regulation of complex neuromuscular mechanisms. Various domains are implicated in the functional role of dystrophin protein, including actin-binding domain (ABD), central rod like spectrin like repeats (SpR), cystein rich domain (CRD) and C-terminal region (CTR). ABD interacts with actin cytoskeleton, SpRs provides flexibility and stretching to the protein, CRD establishes the interactions with β-dystroglycans; and CTRs interact with other protein components of DAPC [9].

Multiplex polymerase chain reaction (mPCR) techniques developed by Chamberlain [10, 11], Beggs [12] and Kunkel [13] target gene of interest by using traditional PCR-reaction and dominate the genetic testing in many developing countries. However, mPCR was used extensively to screen 18-32 exons of *DMD* gene [11-13]. Since detection of duplications and other mutations are not possible by the use of mPCR [14-16], multiplex ligation dependent probe amplification (MLPA) has emerged as a useful and robust technique for *DMD* gene screening [14, 17, 18]. Most of the genetic epidemiology studies of *DMD* in various regions of Indian subcontinent are carried out by mPCR [19], which limits the detection of most prominent mutations. Globally, majority of the DMD patients were reported to harbor deletions (~68%) followed by small mutations (~20%) and duplications (~ 11%) of one or >1 exons [20]. Asian database shows 72% of mutations as large deletions out of the 1819 subjects submitted to their inventory [21].

With gene therapy trials still awaiting success, steroids are the only means to manage the initial course of the devastating disease. Antisense Oligomer (AOs) based exon-skipping gene therapy is by far the most promising and fast emerging approach to partially restore the reading frame, but highly expensive and unaffordable for the cases with low economic status. The resulting pseudo-expression of functional dystrophin protein through splicing events may render severe symptoms of DMD into a milder phenotype akin to Becker Muscular Dystrophy (BMD). The appropriate strategy for exon skipping requires detailed information about the mutation location for the excision of a minimum number of exons for correcting the reading frame.

By the time exon skipping therapy becomes cost-effective and more technologies emerge with time, there may be an impending requirement to generate a population-specific *DMD* mutation database. Majority of the DMD mutation data has been reported from the South Indian population [22] which differs from the North Indian population with respect to ethnic background. Moreover, North Indian studies have been dominated by mPCR and thus may underrepresent the mutation spectrum of *DMD* gene. This study is therefore an attempt to not only study the *DMD* mutations but also to identify the loss of corresponding dystrophin isoforms.

2. MATERIALS AND METHODS

2.1. Participants

A total of 100 male DMD patients were recruited between 2012-2017 with the help of the Indian Association of Muscular Dystrophy (IAMD) after obtaining informed consent as per Institute Ethics Committee guidelines. No control group was enrolled for genetic investigations. The sample size has been estimated by utilizing significance and power values attributed to the study. The sample size was calculated according to the prevalence of DMD, *i.e.* 1/3500 males. To achieve the power of 80%, a sample size of ~70 DMD cases was required. Cases with characteristic clinical features of the Duchenne phenotype with early age of onset were included in the study which was followed by genetic confirmation. Cases were also recruited retrospectively with the help of patient support groups. Informed assent and written informed consent were mandatory for inclusion. The cases with BMD or intermediate phenotypes were not considered for inclusion. Moreover, cases with other myopathies were also excluded. The entire study was conducted according to the quality assurance protocols of the Neuroscience Research Lab, acknowledged by the Quality Council of India.

2.2. Isolation of Genomic DNA

Five ml of blood was collected and Peripheral Blood Mononuclear Cells (PBMCs) were isolated through Ficoll density centrifugation. *QIAamp DNA Blood Mini kit* was used to isolate DNA from the PBMCs or whole blood sample according to the manufacturer's protocol. DNA was quantified using a UV spectrophotometer *(DU730, UV/VIS spectrophotometer, Beckman Coulter)*. DNA samples with yield ranged from 50-150 ng/µl were used for further analysis. Qualitative analysis of DNA was performed in 0.8% agarose. The DNA samples were then coded and stored at -20ºC.

2.3. Multiplex Ligation Dependent Probe Amplification (MLPA)

2.3.1. Amplification of the Probes

Probe sets P034 and P035 (*MRC-Holland, Amsterdam, the Netherlands*) were used for detecting mutation in the target region spanning 1-79 exons of *DMD* gene. To rule out the possibility of disorders with similar phenotypes, the genes namely, *SMN1, LMNA, DYSF, MYOT, CAV3, APP and PSEN* were also screened (Supplementary Table **1**). MLPA procedure was carried out as per established protocol [14]. Briefly, 50 ng/µl of DNA samples (along with three reference samples) were denatured at 95°C for 1 min, followed by incubation at 60°C for 16-20 h for hybridization reaction. Hybridized probes were ligated by using ligase buffers and DNA ligase enzymes (Fig. **1**).

Amplification was performed using FAM modified primer mix and SALSA DNA polymerase. Fragment separation was carried out in the ABI platform through capillary electrophoresis. Coffalyser.NET software (MRC Holland, *Amsterdam, the Netherlands*) was used to obtain electropherograms. Alternately, GeneMarker software (*Softgenetics*) was also used to analyze the .fsa data to obtain the ratios.

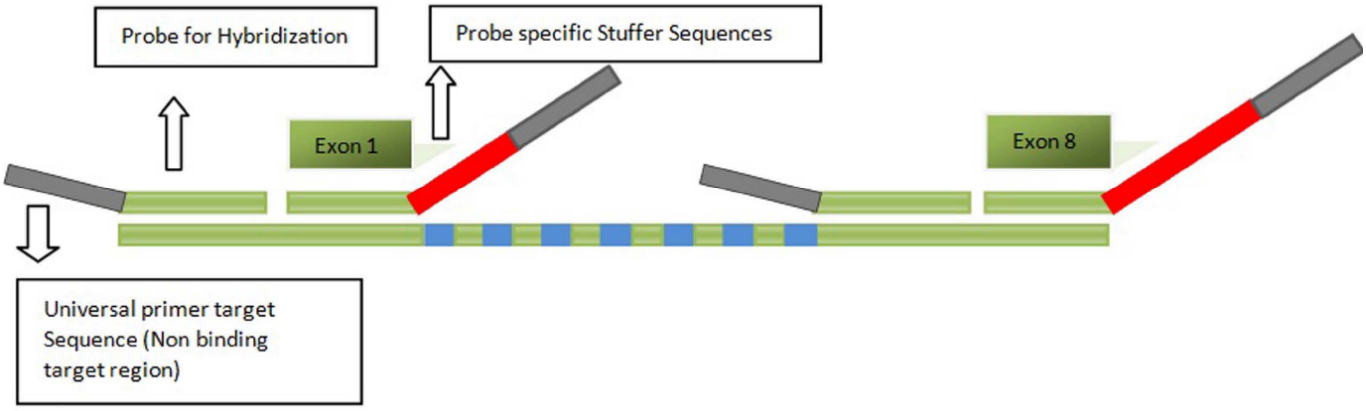

Fig. (1). Schematic representation of MLPA probe which consists of oligonucleotides for the target region, universal primer binding region and stuffer sequences for unique amplicon length. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Dosage Quotient (DQ) ratio was generated by comparing the electropherogram of DNA samples of DMD cases with that of reference samples. Affected domains were predicted based on mutation location at the corresponding domain. Dp140 expression was predicted based on mutation upstream and downstream of exon 44, as previously described. The proximal hotspot region of exon 2-20 and distal hotspot region of exon 44-55 were considered. Reading frame concordance was obtained from Leiden databases.

3. RESULTS

3.1. Patient Demography

The demography profile has been provided in Table **1**. A total of 100 DMD cases were recruited from various geographical locations of Chandigarh, Delhi, Punjab, Haryana, Himachal Pradesh, Rajasthan and Uttar Pradesh. The majority of them (66%) belonged to the urban habitat. Out of 100 cases, 80% were Hindus and 17% were Sikhs. Six families had two affected children and both the siblings were included in the study. Among these, 3 siblings had deletion, 1 had duplication and 2 could not be detected through MLPA. Hence, there were 94 unrelated DMD cases.

3.2. Distribution of Exonic Mutations

Among the 94 unrelated DMD cases, MLPA detected deletion or duplication in 76/94 (80.85%) cases and no mutation in 18/94 (19.14%) cases. Representative electropherograms and ratio charts have been provided in Fig. (**2**).

Among these 94 unrelated families, 69/94 (73%) DMD subjects revealed deletions, whereas, in 7/94 (7%), duplications were observed. Among the deletions, single exon deletions (SEDel) were observed only in 16/69 (23.18%) cases. The exon 45 deletion was the most common SEDel; however, the most frequently deleted either as single or multiple exon deletions were exon 49/50. Out-of-frame and in-frame mutations were found in 66/76 (86.84%) and 10/76 (13.15%), respectively. We found exon 45-55 to be the most commonly affected region in 68% of the cases followed by exon 1-20 in 13%. Domain wise analysis revealed that 68% cases harboured mutations in the SpR and 6% in the ABD. In the remaining 6% cases, mutation lesion ranged from the ABD and SpR domains. The spectrum of deletion and duplication profile of *DMD* gene has been described in Fig. (**3**) and Table **2**.

Table 1. Demographic characteristics of DMD cases (n=100).

Variables	DMD Group (Cases)
Sample Size	100
Gender	Male (100%)
Age	11.18 ± 3.71
Phenotype	DMD
Sibling pairs	6 Pairs
Religion	
Hindu	80 (80 %)
Sikh	17 (17 %)
Others	3 (3 %)
Habitat	
Rural	34 (34%)
Urban	66 (66%)
Geographical Distribution	
Chandigarh	10 (10%)
Punjab	20 (20%)
Delhi	29 (29%)
Himachal Pradesh	16 (16%)
Haryana	12 (12%)
UP	11 (11%)
Rajasthan	2 (2%)

The detailed mutation profile representing mutation type as per Human Genome Variation Society (HGVS) nomenclature and the predicted dystrophin protein expression has been provided in Table **3**. DMD cases who did not exhibit any alterations in the copy number status indicated the probability of point mutation in the dystrophin gene. We could not perform further experiments in MLPA negative cases. Furthermore, screening of *SMN1, LMNA, DYSF, MYOT, CAV3, APP* and *PSEN* genes did not reveal del/dup in cases with no del/dup in *DMD* gene (Supplementary Fig. **1**).

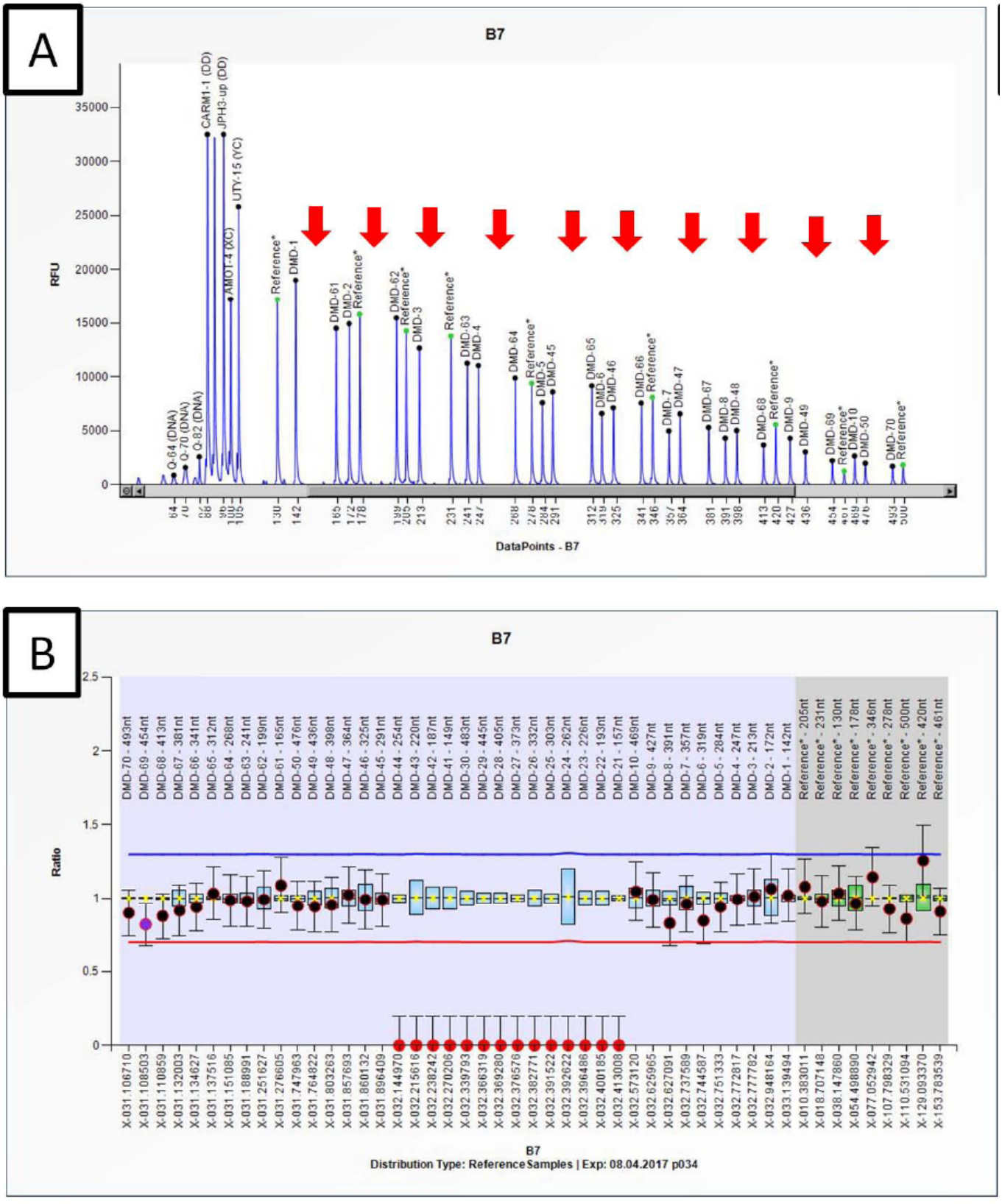
A
B7
RFU
DataPoints - B7
B
B7
Ratio
B7
Distribution Type: Reference Samples | Exp: 08.04.2017 p034

Fig. (2). contd….

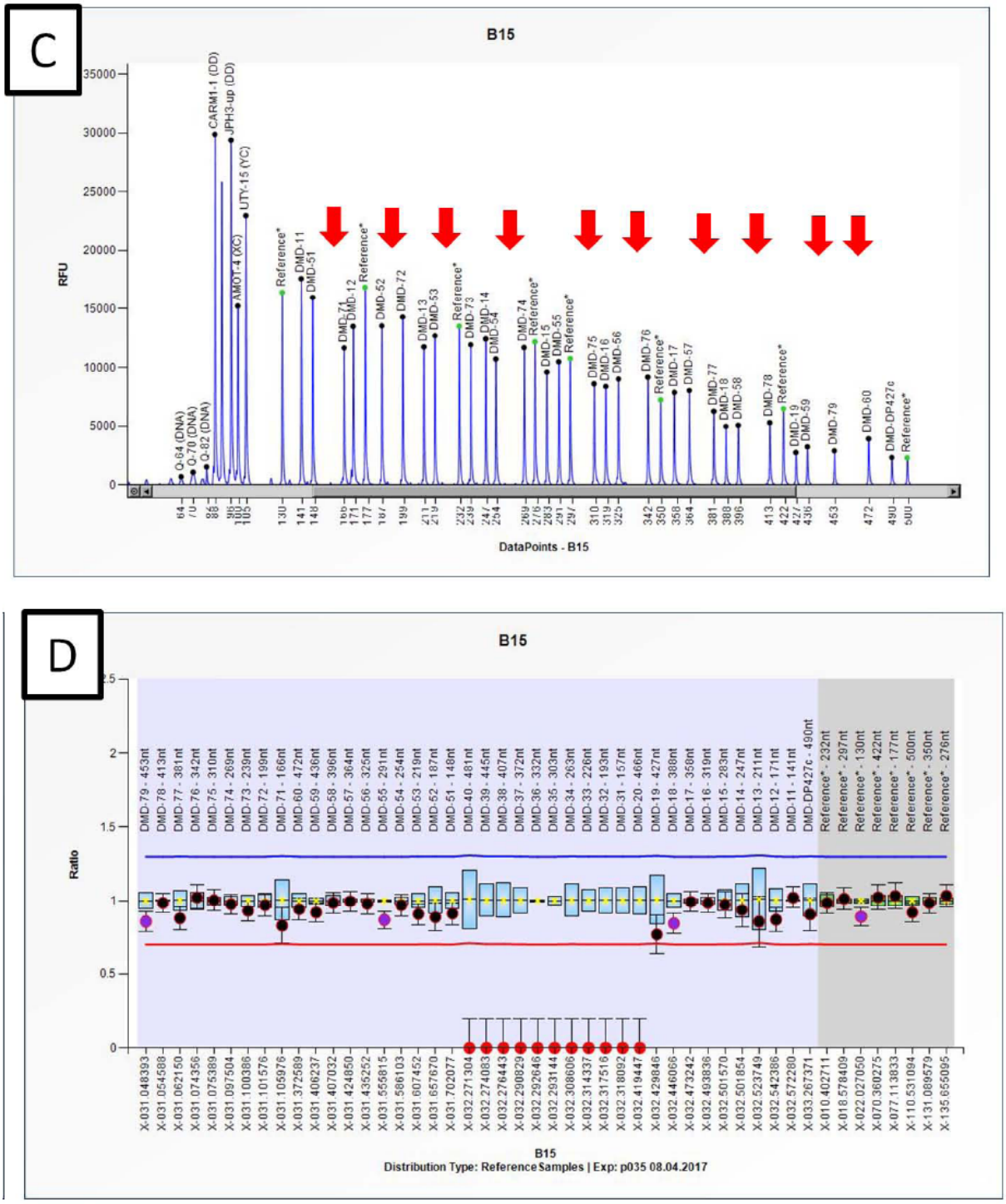

Fig. (2). Electropherogram and ratio chart obtained through coffalyser.NET showing profile of DMD subject with long stretch deletion between Exon 20 to 44. (**A-B**) Electropherogram and ratio chart showing deletion from Exon 31 to 40 and Exon 20 covered by P035 probemix. (**C-D**) Electropherogram and ratio chart showing deletion from Exon 41 to 44 and Exon 21 to 30 covered by P034 probemix. Ratio between 0.70-1.30 is considered in the normal range while ratio of 0.00 was considered as deletion (depicted in red dots and arrows). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3. Predicted Loss of Short Dystrophin Isoforms and Target Range for Exon Skipping

Mutation profile analysis indicated a loss of short dystrophin isoforms in DMD. Out of 76 cases, 14% cases were predicted to have affected full-length dystrophin protein. Retina specific Dp260 isoform was predicted to be affected in 66% cases. CNS specific Dp140 isoform was predicted to be absent in 64% DMD cases. Moreover, in 3% cases, Dp116 was also affected. Mutation data revealed that exon 51 skipping would be effective in 56.25% DMD cases. However, based on the frequency of exonic mutations 28.75% DMD cases could be amenable to Exon 45 skipping (Table **4**).

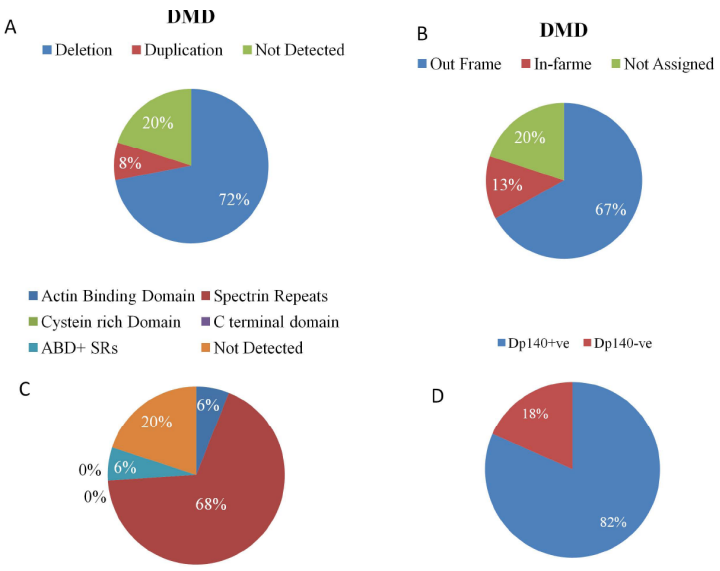

Fig. (3). Distribution of mutations in the cases with DMD. Pie diagram showing **A)** Mutation rate **B)** Predicted proportion of cases with out-of-frame or in-frame mutations according to Leiden Databases. **C)** Domain wise distribution of mutations **D)** Proportion of presence or absence of Dp140 Isoform in DMD cases. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Distribution and spectrum of *DMD* gene variations in 94 unrelated DMD cases.

Variables	N (%)
Total DMD cases	**100**
MLPA +ve Cases	80/100 (80%)
MLPA -ve Cases	20/100 (20%)
Sibling pairs	6
Unrelated DMD cases	94
Copy number variations (CNVs)	76/94 (80.85%)
CNV-ve	18/94 (19.14%)
Proximal Hotspot (exon 2-20)	11/76 (14.47%)
Distal Hotspot (exon 45-55)	62/76 (81.57%)
Dp140+ve	14/76 (18.42 %)
Dp140-ve	62/76 (81.57%)
Out of frame	66/76 (86.84 %)
In-frame	10/76 (13.15 %)
Deletions	69/94 (73%)
Deletions among CNVs	69/76 (90.78%)
Single exon deletion (SEDel)	16/69 (23.18%)
Most common SEDel	Exon 45: 5/69 (7.24)
Multiple exon deletion patterns	53/69 (76.81%)
Proximal Deletions	10/69 (14.49%)
Proximal-Distal Deletions	4/69 (5.79%)
Distal Deletions	57/69 (82.60 %)
Out-of-frame Deletions	62/69 (89.85 %)
In-frame Deletions	7/69 (10.14 %)
Most Prominent Exon Deletion	Exon 49/50
Duplications	7/94 (7%)
Duplications among CNVs	7/76 (9.21%)
Single exon Duplications (SEDup)	1/7 (14.28 %)
Multiple exon Duplications	6/7 (86%)
Proximal Duplications	6/7 (86%)
Distal Duplications	1/7 (14.28%)
Out-of-frame Duplications	4/7 (57.14%)
In-frame Duplications	3/7 (4.85 %)

4. DISCUSSION

The analysis of mutation spectrum has revealed mutations in 80.85% DMD cases with 73% deletions and 7% duplications in unrelated families. Previous Indian studies have reported 75% [16] and 86.6% [22] mutation detection rate by MLPA. Spectrin repeat domain was found to be affected in the majority of cases. Based on the mutation location, Dp140 was found to be prominently affected in our study group, which may explain cognitive and behavioral abnormalities in DMD. Similarly, alterations in other dystrophin isoforms including Dp116 and Dp260 are crucial for understanding the disease spectrum because the absence of Dp260 isoform in the retina is believed to affect rod pathway signaling in electroretinogram studies [23]. Analysis of other genes involved in myopathies and cognitive impairment revealed the monogenic effect of *DMD* gene mutation in the development of Duchenne phenotype in our cohort.

In our study, 81.57% DMD subjects revealed mutations in the 45-55 hotspot regions (Table **2**); however, exon 2-20 mutations were detected only in 14.47% DMD cases. Various studies have reported exon 2-20 and 45-55 regions to be the hotspot regions for *DMD* gene mutation falling under the rod domain (SpR) while exon 56-79 mutations are rare [24-26]. The mutation frequencies and hotspot regions in our study cohort were found to be similar to the global spectrum of dystrophin gene mutations; however, we did not examine the junctional breakpoints. Breakpoints need to be confirmed by sanger/next-generation sequencing approaches for confirming the eligibility of DMD cases. Our study suggests the hotspot region of Exon 45-55 and Exon 2-20 as a prominent target site for multi-exon targeting of Phosphorodiamidate morpholino oligomers (PMOs).

With overall 23.18% cases with single exon deletions, our study reports exon 44/45 to be the equally prominent single-exon copy number rearrangements. This location was reported to be varied in Asian populations. In this context, Exon 50 has been reported to be the most prominent SEDel

Table 3. Mutation profile of indian duchenne muscular dystrophy cases (n=100).

Patient	Age	HGVS Nomenclature	Genetic Mutation	Dystrophin Protein and Isoform	ORF Prediction	Domain Dp140-ve	CNS Dp140 Isoform
P-1	9	c.6291-?_6438+?del	Del Exon 44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-2	9	c.7661-?_8027+?del	Del Exon 53-54	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-3	8	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-4	4	c.94-?_3603+?del	Del Exon 03-26	Dp427(M/L/C/P)	IF	ABD, SPR	Dp140+ve
P-5	7	c.6615-?_7309+?del	Del Exon 46-50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-6	9	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-7	8	c.6439-?_6614+?del	Del Exon 45	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-8	13	c.265-?_649+?del	Del Exon 05-07	Dp427(M/L/C/P)	OF	ABD	Dp140+ve
P-9	12	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-10	9	c.6439-?_6912+?del	Del Exon 45-47	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve
P-11[@]	13	c.94-?_264+?dup	Dup 3-4	Dp427(M/L/C/P)	IF	ABD	Dp140+ve
P-12[@]	12	c.94-?_264+?dup	Dup 3-4	Dp427(M/L/C/P)	IF	ABD	Dp140+ve
P-13	11	c.6291-?_6438+?dup	Dup 44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-14	10	c.6913-?_7660+?del	Del Exon 48-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-15	6	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-16	11	c.650-?_960+?del	Del Exon 08-09	Dp427(M/L/C/P)	OF	ABD, SPR	Dp140+ve
P-17	12	c.6615-?_6912+?del	Del Exon 46-47	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-18	10	c.6913-?_7660+?del	Del Exon 48-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-19	10	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-20	15	c.6615-?_7200+?del	Del Exon 46-49	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-21	9	c.6615-?_7200+?del	Del Exon 46-49	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-22	8	c.2804-?_3603+?dup	Dup Exon 22-26	Dp427(M/L/C/P)	OF	SPR	Dp140+ve
P-23	8	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-24	14	c.6439-?_7200+?del	Del Exon 45-49	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve
P-25	8	c.7310-?_7542+?del	Del Exon 51	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-26	8	c.2169-?_5922+?dup	Dup Exon 18-41	Dp427(M/L/C/P), Dp260	OF	SPR	Dp140+ve
P-27	8	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-28	11	c.7201-?_7309+?del	Del Exon 50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-29	6	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-30	10	c.6291-?_6438+?del	Del EXON 44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-31[#]	8	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-32[#]	13	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-33	16	c.6913-?_7309+?del	Del Exon 48-50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-34	14	c.1150-?_7200+?del	Del Exon 11-49	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve

(Table 3) contd….

Patient	Age	HGVS Nomenclature	Genetic Mutation	Dystrophin Protein and Isoform	ORF Prediction	Domain Dp140-ve	CNS Dp140 Isoform
P-35	11	c.6439-?_6614+?del	Del Exon 45	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-36	9	c.6118-?_6290+?del	Del Exon 43	Dp427(M/L/C/P), Dp260	OF	SPR	Dp140+ve
P-37	14	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-38[$]	9	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-39	11	c.1150-?_7200+?del	Del Exon 11-49	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve
P-40	9	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-41	10	c.7201-?_7309+?del	Del Exon 50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-42	8	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-43	7	c.6615-?_7542+?del	Del Exon 46-51	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-44	13	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-45	10	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-46	5	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-47	9	c.6291-?_6438+?del	Del Exon 44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-48	12	c.6439-?_6614+?del	Del Exon 45	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-49[$]	14	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-50	13	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-51	15	c.6615-?_8217+?del	Del Exon 46-55	Dp427(M/L/C/P), Dp260, Dp140, Dp116	OF	SPR	Dp140-ve
P-52	13	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-53	16	c.1150-?_7098+?del	Del Exon 11-48	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve
P-54	19	c.6291-?_8217+?dup	Dup 44-55	Dp427(M/L/C/P), Dp260, Dp140, Dp116	OF	SPR	Dp140-ve
P-55	17	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-56	18	c.6439-?_6614+?del	Del Exon 45	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-57	16	c.94-?_1482+?dup	Dup Exon 3-12	Dp427(M/L/C/P)	IF	ABD,SPR	Dp140+ve
P-58	19	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-59	10	c.1332-?_2380+?del	Del Exon 12- 19	Dp427(M/L/C/P)	OF	SPR	Dp140+ve
P-60	10	c.7099-?_7309+?del	Del Exon 49-50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-61	11	c.6913-?_7660+?del	Del Exon 48-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-62	12	c.265-?_649+?dup	Dup Exon 5-7	Dp427(M/L/C/P)	OF	ABD	Dp140+ve
P-63	14	c.6439-?_6614+?del	Del Exon 45	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-64	8	c.6913-?_7660+?del	Del Exon 48-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-65	15	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-66	17	c.6615-?_7309+?del	Del Exon 46-50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-67	19	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-68	3	c.7310-?_7542+?del	Del of Exon 51	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-69	19	c.6913-?_7309+?del	Del Exon 48-50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve

(Table 3) contd….

Patient	Age	HGVS Nomenclature	Genetic Mutation	Dystrophin Protein and Isoform	ORF Prediction	Domain Dp140-ve	CNS Dp140 Isoform
P-70	9	c.6615-?_7310+?del	Del Exon 46-51	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-71	15	c.94-?_2949+?del	Del Exon 03-22	Dp427(M/L/C/P)	IF	ABD,SPR	Dp140+ve
P-72	14	c.6439-?_7660+?del	Del Exon 45-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-73	15	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-74	11	c.2381-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-75[&]	13	c.7099-?_6438+?del	Del Exon 20-44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-76[&]	9	c.7099-?_6438+?del	Del Exon 20-44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-77	7	c.6615-?_7310+?del	Del Exon 46-51	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-78	7	c.7310-?_8217+?del	Del Exon 51-55	Dp427(M/L/C/P), Dp260, Dp140, Dp116	OF	SPR	Dp140-ve
P-79	12	c.6615-?_8027+?del	Del Exon 46-54	Dp427(M/L/C/P), Dp260, Dp140	IF	SPR	Dp140-ve
P-80	6	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-81	9	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-82	12	c.650-?_3603+?del	Del Exon 8-26	Dp427(M/L/C/P)	OF	ABD, SPR	Dp140+ve
P-83	11	c.650-?_1812+?del	Del Exon 08-15	Dp427(M/L/C/P)	OF	ABD, SPR	Dp140+ve
P-84	4	c.7201-?_7660+?del	Del Exon 50-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-85	11	c.7543-?_8027+?del	Del Exon 52-54	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-86	6	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-87	17	c.6291-?_6438+?del	Del Exon 44	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-88	7	c.7543-?_7660+?del	Del Exon 52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-89^	14	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-90^	10	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-91	10	c.7201-?_8027+?del	Del Exon 50-54	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	NA
P-92*	14	c.94-?_264+?del	Del Exon 3-4	Dp427(M/L/C/P)	IF	ABD	Dp140+ve
P-93	8	c.6439-?_8027+?del	Del Exon 45-54	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-94	6	c.7099-?_7660+?del	Del Exon 49-52	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-95*	19	c.94-?_264+?del	Del Exon 3-4	Dp427(M/L/C/P)	IF	ABD	Dp140+ve
P-96	19	c.6615-?_6912+?del	Del Exon 46-47	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-97	9	c.7201-?_7309+?del	Del Exon 50	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve
P-98	11	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-99	10	Not Assigned	No Del/Dup	Not Assigned	NA	NA	NA
P-100	11	c.7310-?_7872+?del	Del Exon 51-53	Dp427(M/L/C/P), Dp260, Dp140	OF	SPR	Dp140-ve

Abbreviations: Dp: Dystrophin Protein, Dp260, Dp140, Dp116, Dp71: Short Dystrophin isoforms (260, 140, 116 and 71 in kiloDaltons), M/L/C/P: Muscle/ lymphoblastoid cells/Cortex/Purkinje Cells, Del: Deletion, Dup: Duplication, ORF: Open reading frame, OF: Out of frame, IF: In-frame, ABD: Actin binding domain, SPR: Spectrin Repeats. [@,#,$,&,^, *]: Sibling groups.

found in Singapore, exon 49/50 in Japanese and exon 51 in Vietnamese populations [27], which pertains to Dp140 isoform. In a study conducted in 112 DMD cases of the South Indian region, deletions were detected in 73% of the cases. SEDels were detected in 20.4% (23.18% in our study) and in contrast to our study, the most common SEDel was exon 50 with 38.5% cases (exon 44/45 in our study) [28]. Though the most prominently mutated single exons differed in both studies, yet both locations are pertinent to Dp140 isoform. SEdels need to be confirmed by alternate methods like PCR

Table 4. Amenability of exon skipping in Indian DMD subjects.

Amenability to Exon Skipping	DMD Cases	Dystrophin Isoform Restored	Target
Exon 51	45/80	Dp160, Dp71, 40	Muscle and CNS
Exon 45	23/80	Dp71, 40	Muscle and CNS
Exon 43	14/80	Dp140, 71, 40	Muscle, CNS, Kidney
Exon 28	14/80	Dp260, Dp140, 71, 40	Muscle and CNS, Kidney and retina

and sequencing. However, we could not validate the SEdel/dup by sequencing which may have revealed potential polymorphism or point mutation in the probe binding site. In a study conducted in the East Indian region, although the deletion rate of 65.7% was reported, deletion in the distal hot spot exonic region was noted in 82.61% of cases and that in the proximal hotspot, region was 10.87% [29]. In a South Indian MLPA based study, Vengalil *et al.* reported deletions and duplications in 91% and 9% cases, respectively from a cohort of 279 DMD subjects [30] and reported exon 50 deletion to be the most common mutation.

Most prominently mutated exons in our study, *i.e.* exon 44/45, are also considered to have a breakpoint in the dystrophin gene [31, 32]. It is important to discuss the evolutionary significance of this exonic location. An admixture of dystrophin exon 44 regions between the Neanderthal genome and expanding Homo sapiens nearly between 80 and 50 thousand years ago, was reported [33]. In addition, the prevalence of DMD in the African black population (1/250,000) being less than the UK (1/40,000) suggests the role of admixture in the instability of the loci [34].

Bhattacharya *et al.* reported ABD and CRD as a hub of mutational events [9]; however, our cohort mainly represented SpR domains. In view of frequently encountered mutation of exon 49 and 50, Exon 51 skipping approach might benefit a large proportion [45/80 (56.25%)] of our study group. Before exon-skipping therapeutics took shape, Wilton in 1999 showed using *mdx* mouse model, that AONs can remove the mutation in the exon resulting in increased dystrophin production by its repetitive administration [35].

Antisense oligonucleaotide (AO) based exon skipping therapies are the most promising approach to treat DMD. PMOs are important AOs with the ability to skip multiple exons. Eteplirsen (exondys51) became the first FDA approved exon skipping therapy for DMD in the United States [36]. PMO based Exondys 51 or Eteplirsen is the first FDA approved therapy applicable for 13% of DMD based on a <1% increase in pseudo-expression of dystrophin. Despite a limited improvement in dystrophin expression, multi-exon skipping potentially increases the amenability to 80-90% of DMDs [37]. For exon-skipping therapeutics to be successfully applied, it is important to populate the genetic database with information about dystrophin isoforms and define its distribution pattern corresponding to various organ systems. German human genome database, which shows 2982 mutations occurring in *DMD* gene [38], is an example of such databases. Similarly, in France, more than 13,500 registrations were made from 31 different countries, by generating a mutation database in 2015.

CONCLUSION

This study updates the existing *DMD* gene mutation spectrum in the Indian population. Besides strengthening the mutation databases, amenability to exon skipping trials and impaired cognitive functions associated with Dp140 isoform could be predicted through the type and location of mutation. Since, early institution of treatment benefits in terms of enhanced life expectancy and reduced morbidities, a newborn screening program for DMD will be of paramount importance in countries like India with a high prevalence of the disorder. Genetic counseling, prenatal and carrier screening are crucial for the prevention and management of DMD. However, awareness in the medical fraternity and general population; and empowering patient support groups may be beneficial to reduce the disease burden.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Ethics Committee of PGIMER, Chandigarh, India vide approval Int/IEC/ 2015/732.

HUMAN AND ANIMAL RIGHTS

No animals were used for the study that are the basis of this research. All experimental protocols on patients were followed according to the guidelines of Institutional Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India.

CONSENT FOR PUBLICATION

Written informed consent was obtained before the recruitment of study subjects.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [A.A.], upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

AUTHORS' CONTRIBUTIONS

AA: Conceptualization, management of the study, editing & Final approval the manuscript, RT: Co-conceptualization under supervision, Data Acquisition and analysis as PhD student, Experiments, writing the manuscript: SK: Experiment Assistance PK: Assistance in Data Acquisition MM: Co-investigator in grant application FM: Experiments related to Capillary sequencing: AD: Inter-laboratory validation of the data.

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Altered Expression of Heat Shock Protein-27 and Monocyte Chemoattractant Protein-1 after Acute Spinal Cord Injury: A Pilot Study

Vidyasagar Boraiah[1] Shweta Modgil[2,3] Kaushal Sharma[2,4] Vivek Podder[5] Madhava Sai Sivapuram[6] Gurwattan S. Miranpuri[7] Akshay Anand[2] Vijay Goni[1]

[1]Department of Orthopaedics, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[2]Department of Neurology, Neuroscience Research Laboratory, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[3]Department of Zoology, Panjab University, Chandigarh, India
[4]Centre for System Biology and Bioinformatics, Panjab University, Chandigarh, India
[5]Department of General Medicine, Kamineni Institute of Medical Sciences, Narketpally, Telangana, India
[6]Department of General Medicine, Dr. Pinnamaneni Siddhartha Institute of Medical Sciences and Research Foundation, Chinna Avutapalli, Andhra Pradesh, India
[7]Department of Neurological Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States

Address for correspondence Akshay Anand, PhD, Department of Neurology, Neuroscience Research Laboratory, Post Graduate Institute of Medical Education and Research, Chandigarh, India (e-mail: akshay1anand@redifmail.com).

Vijay Goni, MS, Department of Neurology, Neuroscience Research Laboratory, Post Graduate Institute of Medical Education and Research, Chandigarh, India (e-mail: vijaygoni@gmail.com).

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Abstract

Background Spinal cord injury (SCI) leads to serious complications involving primary trauma and progressive loss due to inflammation, local ischemia, or infection. Despite a worldwide annual incidence of 15 to 40 cases per million, methylprednisolone is the only treatment available to alleviate neurologic dysfunction; therefore, research is currently focused on identifying novel targets by biochemical and molecular studies.

Purpose Here, we investigated the expression of various molecular markers at the messenger ribonucleic acid (mRNA) and protein level at day 0 and day 30 post-SCI.

Methods Enzyme-linked immunosorbent assay (ELISA) was performed to determine the expression of CASPASE-3 and heat shock protein-27 (HSP-27) in serum samples. Real-time polymerase chain reaction (RT-PCR) was performed to determine the level of mRNA expression of vascular endothelial growth factor receptor-1 (VEGFR-1), VEGFR-2, HSP-27, monocyte chemoattractant protein-1 (MCP-1), and CASPASE-3.

Results HSP-27 expression at day 30, as compared with day 0, showed significant downregulation. In contrast, there was elevated expression of MCP-1. ELISA analysis showed no significant change in the expression of CASPASE-3 or HSP-27.

Conclusion There may be possible opposing role of HSP-27 and MCP-1 governing SCI. Their association can be studied by designing in vitro studies.

Keywords
- angiogenesis
- CASPASE-3
- heat shock protein-27
- monocyte chemoattractant protein-1
- inflammation
- molecular markers
- spinal cord injury
- vascular endothelial growth factor
- vascular endothelial growth factor receptor-1
- vascular endothelial growth factor receptor-2

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Introduction

Injury to the spinal cord less than 3 weeks of age is considered acute spinal cord injury (SCI). SCI results in devastating complications, and the reversal of resulting deficits is a challenge for medical research. Despite extensive research to understand the pathophysiology of SCI, there exists no effective treatment that can reverse the deficits or interrupt the ongoing damage to the spinal cord following SCI.[1] Most SCIs are reported from high-velocity road traffic accidents, falls, crimes, and recreational activities with the incidence of injuries on the rise in geriatric populations.[2] Cervical spine is commonly affected as it is the most flexible region.[3]

A biphasic phenomenon best describes the pathophysiology of SCI. This includes a primary phase and secondary phase of injury.[4] The primary injury is caused by initial trauma, ischemia, demyelination, or infection. Further damage to the spinal cord continues in the secondary phase of injury characterized by tissue edema, electrolyte imbalance, cell death, free radical formation, excitotoxicity, chemotaxis, and immune cells infiltration.[5] Once initiated, all these mechanisms perpetuate a self-propagating cycle leading to deleterious consequences.

To stabilize the spinal column and prevent further damage, urgent intervention is required soon after surgery. Current treatments use a combination of medical, surgical, and rehabilitation therapy[6] although advantages from this combined intervention are not usually curative. Inflammation proceeds different phases. The phagocytic phase involves removal of debris from the site of injury followed by a proliferative phase characterized by revascularization aided by angiogenesis and extracellular matrix deposition, and finally, a modeling phase where wound retraction and tissue homeostasis are achieved.[7,8] It has been suggested that secondary mechanisms may exacerbate complications, and therefore, controlling the secondary phase is also important for modifying the deficits.[9] The identification of signal molecules is important to develop an understanding of the repair mechanisms. Current research is, therefore, focused on discovering newer molecular targets on which treatment modalities for acute SCI can be tested.

Many molecules are involved in injury mechanisms. Vascular endothelial growth factor (VEGF) has been studied in the pathogenesis of SCI and known to have dual neurotropic effects: by directly acting on the neurons to promote neurite extension and by activating glial cells that produce various growth factors promoting neuronal growth,[10]making it an attractive target for investigation in SCI.[11] Similarly, the heat shock proteins (HSPs) are primarily released because of acute stress, and levels of expression of these highly conserved proteins are increased following SCI to preserve neuronal cells and repress chronic inflammation.[12-14] Conversely, monocyte chemoattractant protein-1 (MCP-1) recruits cells to the site of injury that includes memory T cells, monocytes, and dendritic cells.[15] In this respect, it is not clear whether recruited immune cells exacerbate tissue damage or promote repair[16] but likely depend on the type of cells involved. A delicate balance between the two can be deciphered by sampling cerebrospinal fluid (CSF) at various time intervals.[17] Furthermore, SCI and its long-term neurological deficits involve apoptosis of neurons and oligodendroglia in regions unaffected by the initial injury. This regulated apoptosis is executed through the caspase family of cysteine proteases.[18]

The aforementioned molecules are interrelated through various pathways and are involved in the pathogenesis of SCI. We, therefore, examined the role of these molecules in neuronal protection in acute SCI with the hope that this will result in the emergence of newer treatment targets for developing treatment modalities or predicting injury outcome.

Methods

Recruitment of Participants

All patients with acute traumatic SCI who presented to ATC emergency of the Post Graduate Institute of Medical Education and Research (PGIMER) trauma center in Chandigarh, India, between January 1, 2016, and February 26, 2017, were considered. Patients with sustained acute traumatic SCI with neurological deficits within a wide age group representing injury from all vertebrae levels were included in the study. Patients with any other comorbidities, injury to other organs, and without neurological deficits were excluded from the study. A total of 42 patients had met the inclusion criteria. All 42 patients were examined clinically and advised the requisite investigations with noncontrast computed tomography. The first samples for all 42 patients were taken in the emergency room and followed up after 30 days. Fourteen patients were lost in the follow-up and therefore excluded from the study. The remaining 28 patients were included in the study (►**Fig. 1**).

Treatments Given

All patients underwent posterior decompression surgery with pedicle screw fixation for posterior spinal fusion. Surgery was done within 10 days from the date of injury. After surgery, all patients were additionally treated with aceclofenac 75 mg for 2 weeks, hydrocortisone 100 mg thrice daily for 5 days, and antibiotics (cephalosporin and amikacin) for 5 days.

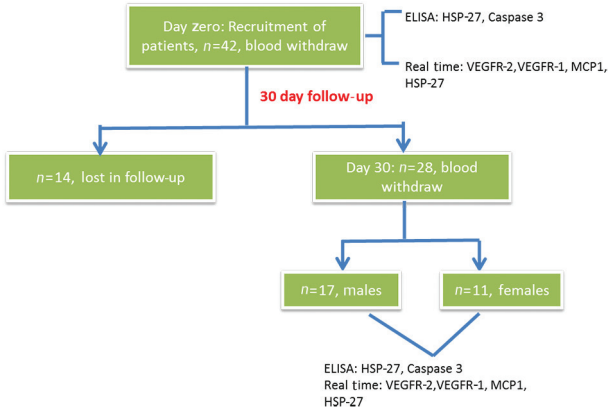

Fig. 1 The work flow of the study conducted to estimate the expression levels of different genes. ELISA, enzyme-linked immunosorbent assay; HSP-27, heat shock protein-27; MCP-1, monocyte chemoattractant protein-1; VEGFR-1, vascular endothelial growth factor receptor-1.

Follow-Up Period

Each patient was followed up after 30 days, and blood samples were taken on the 1st day and 30th day. The first day was considered as the date of the presentation with injury in the hospital. At the 30th day, no neurological improvements were observed in any of the patients.

Ethical Committee Approval

The ethical approval for the recruitment of the participants and to conduct the study was taken from the Institutional Ethical Committee, PGIMER, Chandigarh, vide letter number NK/558/Res, dated February 4, 2014.

Sample Collection and Isolation of Peripheral Blood Monocytes, Plasma, and Serum

Five milliliters of blood was collected in a serum separator tube from SCI patients at day 1 (before intervention) and day 30. It was subjected to centrifugation for 15 minutes at 3000 rpm, and serum was collected. Blood was also collected in an ethylenediaminetetraacetate tube and kept at room temperature for ~2 to 3 hours to settle. The upper yellowish portion was collected and layered on an equal volume of Histopaque and centrifuged at 1800 rpm for 30 minutes. Finally, from the interphase of plasma and Histopaque, a buffy coat of peripheral blood mononuclear cells (PBMCs) was collected and stored in ribonucleic acid (RNA) later (Sigma Aldrich, United States), while plasma was collected in a separate vial and stored in–80°C ultrafreezer.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the protein expression in serum samples at both time points for CASPASE-3 and HSP-27. Analysis was made using commercially available ELISA kits (Genxbio). Briefly, serum samples were plated on a precoated antibody ELISA plates and incubated for 2 hours at 37°C. The washing with buffer was followed by secondary antibody incubation at room temperature for 1 hour. Absorbance was taken at 450 nm using ELISA reader as described by the manufacturer. Total protein concentration of samples was estimated using the Bradford method. A standard curve using bovine serum albumin was used as a protein standard, and ELISA concentrations were further normalized by their respective total protein concentrations.

RNA Isolation and cDNA Synthesis

PBMCs stored in RNA later were used for RNA isolation. Cells were washed using 1X PBS to remove RNA later, and then RNA isolation was performed using a commercially available kit (Qiagen, United States). RNA was used as a template to synthesize complementary deoxyribonucleic acid (cDNA) as per kit protocol (Thermo Scientific, United States). The expression of different genes was determined by subjecting cDNA to real-time polymerase chain reaction (RT-PCR) analysis (Applied Biosystems) using specific primers (Sigma, United States and Eurofins, Genomics) (►**Fig. 1**).

Reverse Transcriptase Polymerase Chain Reaction

Marker gene expression was analyzed by RT-PCR. As angiogenic, inflammatory, and stress-related markers may change following SCI, the mRNA expression of VEGFR-1, VEGFR-2, HSP-27, and MCP-1 was normalized to B-actin housekeeping gene and subsequently quantified. The quantitative PCR data were analyzed using the method of Livak and Schmittgen[19]. The primer annealing temperature was optimized using gradient PCR validated by agarose gel electrophoresis. The samples were subjected to PCR analysis using specific primers. The relative fold change was determined for each sample. The primer details are shown in ►**Table 1**.

Statistical analysis

All the results were expressed as mean ± standard error of mean. The data were statistically analyzed using SPSS version 16.0. Data normality was analyzed using 1-KS sampling. The statistical significance of data was computed using the Mann–Whitney *U* test, and $p < 0.05$ was considered as statistically significant.

Results

Our study included 17 male patients (60.7%) and 11 female patients (39.3%) (►**Fig. 2A**), suggesting higher incidence of SCI in male patients in accordance with the present trend.[20] Minimum age of the patient was 17 years and the maximum was 65 years, with the mean age being 41.07 ± 13.711 years (►**Fig. 2B**). Of 28 patients, 14 patients sustained injury to thoracic spine, 10 patients to lumbar spine, and 4 patients to cervical spine (►**Fig. 2C**).

Table 1 The sequence of the primers and annealing temperature used for polymerase chain reaction

Gene name	Forward primer sequence	Reverse primer sequence	Annealing temperature
VEGFR-1	GCTGTGCGCGCTGCTT	AACTCAGTTCAGGACCTTTTAATTTTGA	63°C
VEGFR-2	TGATACTGGAGCCTACAAGTGCTT	CCTGTAATCTTGAACGTAGACATAAATGA	58.9°C
HSP-27	CGTGGTGGAGATCACTGGCAAGC	CGGGCCTCGAAAGTGACCGG	63°C
MCP-1	5′-AGCAGCAAGTGTCCCAAAGA-3′	5′-TTGGGTTTGCTTGTCCAGGT-3′	64.2°C

Abbreviations: HSP-27, heat shock protein-27; MCP-1, monocyte chemoattractant protein-1; VEGFR-1, vascular endothelial growth factor receptor-1.

Protein Estimation

ELISA was performed to estimate change in protein levels of HSP-27 and CASPASE-3 after 1 month of follow-up. Protein estimation using ELISA showed no significant difference in the level of HSP-27 (p = 0.423) and CASPASE-3 (p = 0.979) between day 1 and day 30 (►**Fig. 3**). We further analyzed the data and found that there was no significant change in both CASPASE-3 and HSP-27 in relation to age, gender, severity, and level of vertebrae involved (data not shown).

Gene Expression

VEGFR-2 expression was found to be elevated after 30 days of post-trauma to spinal cord; however, the increase was not statistically significant (p = 0.867). Similarly, expression of VEGFR-1 showed no significant change in the follow-up group (►**Figs. 4A**, **B**). The relative expression of HSP-27 on day 1 was compared with day 30 (►**Fig. 5**), and we found a significant decrease in the expression of HSP-27 (p = 0.001) at 30th day posttrauma. Expression of MCP-1 showed a significant elevation at 30th day posttrauma (►**Fig. 6**).

Discussion

SCI is a devastating condition with serious consequences. Proinflammatory and anti-inflammatory mechanisms participating in the secondary phase play a decisive role

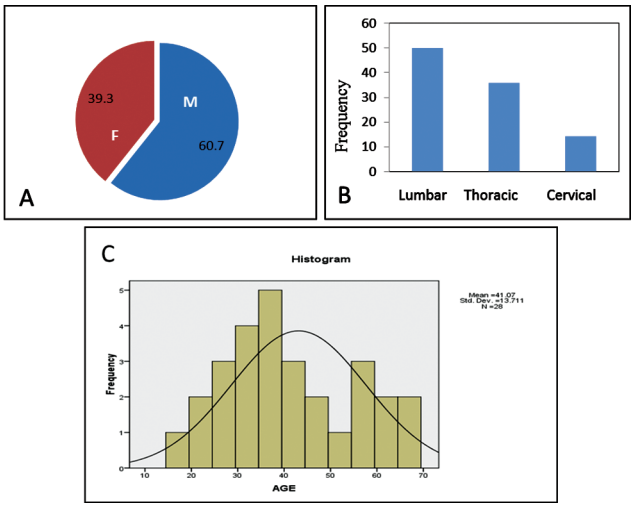

Fig. 2 (**A**) Pie chart showing gender distribution in the study. (**B**) Histogram showing age distribution. (**C**) Bar chart showing percentage-wise distribution of levels of vertebral injury.

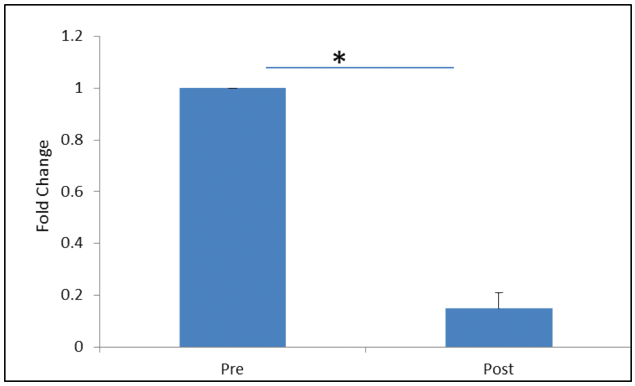

Fig. 5 Fold change in gene expression levels of heat shock protein-27 at day 0 and day 30 post–spinal cord injury. *p > 0.05.

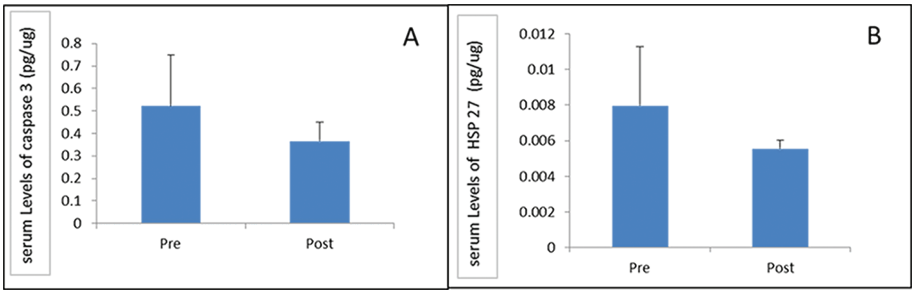

Fig. 3 Comparing (**A**) CASPASE-3 and (**B**) heat shock protein-27 expression in serum at day 0 and day 30 post–spinal cord injury. HSP-27, heat shock protein-27.

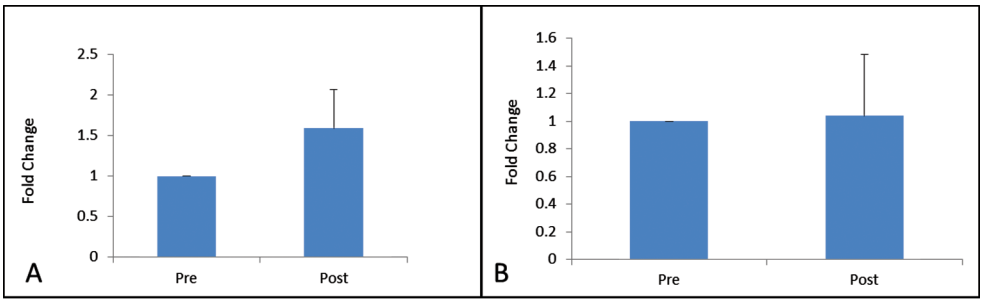

Fig. 4 Fold change in gene expression levels of vascular endothelial growth factor receptor-1 (**A**) and vascular endothelial growth factor receptor-2 (**B**) at day 0 and day 30 post–spinal cord injury.

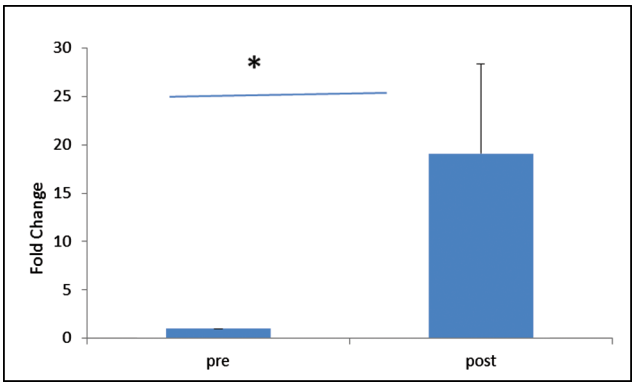

Fig. 6 Fold change in gene expression levels of monocyte chemoattractant protein-1 at day 0 and day 30 post–spinal cord injury. $^{*}p > 0.05$

in the outcome.[21] Different biomarker genes are expressed in a complex manner during secondary phase, but only a few of them are analyzed. The protective mechanisms include upregulation of regeneration-associated genes and neurotrophic factors.[22] The present study was performed to probe any possible pattern in the expression of various angiogenetic biomarkers such as VEGFR-1, VEGFR-2, HSP-27, MCP-1, and CASPASE-3 in traumatic SCI so that the understanding of aforementioned secondary mechanisms could serve as a basis for devising new strategies for pharmacological interventions. Because of limited funds, ELISA could be performed only for CASPASE-3 and HSP-27.

VEGF promotes cell survival by reducing apoptosis and repairing blood vessel damage.[23] Quantification of CASPASE-3 protein by ELISA post-SCI did not show any significant change in the posttraumatic period, which may suggest that CASPASE-3 is not altered by VEGFR, suggesting its redundant role in angiogenesis and neurogenesis post-SCI. These studies are in contrast to those performed by Voss et al,[24] where VEGF has shown to have a nonredundant role. As discussed earlier, VEGF helps in restoration of blood vessels' damage by promoting angiogenesis and thereby inhibits the apoptotic machinery. VEGF exerts its angiogenic effects through its two major tyrosine kinases receptors, VEGFR-1 and VEGFR-2. Whereas VEGFR-1 recruits hematopoietic precursor cells and helps in the migration of monocytes and macrophages, VEGFR-2 plays a major role in the regulation of vascular endothelial cells.[25] Quam et al[26] reported the stimulation of VEGFR following ischemia resulting in the formation of newer vasculature and it has positive effects in promoting neurogenesis. Following neurological stress or trauma or any kind of injury, VEGFR is shown to be released by microglial, astrocytes, and monocytes as a compensatory response for inflammatory reactions and to protect neurons. Our study analyzed the PBMCs for any possible change in the expressions of both VEGFR-1 and VEGFR-2. As reported, the increase in expressions of both VEGFR-1 and VEGF-2, even though not significant, may exert neuronal protection and regeneration by promoting effects through angiogenesis. Interestingly, their increase can be antagonized by the inflammatory mechanisms following posttrauma, thus indicating the fine molecular balance that directs the cellular homeostasis. Insignificant change in the VEGF-1 and -2 can be due to delayed recovery, which can be investigated further with a long-term follow-up with sampling at intermittent intervals. Furthermore, antagonistic mechanisms to neuronal recovery like deposition of chondroitin sulfate at neuronal ends that occur in secondary phase after spine trauma can be contributory.[27] A study done in rats found that the expression of VEGFR mRNA and protein levels get upregulated immediately following SCI; however, the levels get normalized after 14 days of SCI.[28] Another study reported a significant decrease in VEGF levels after 1 day of surgery and it was maintained after 1 month post-SCI.[29] HSP-27, being a stress protein and released from living cells on exposure to stress that occurs usually in chronic diseases, trauma, and infections, has been reported to be elevated after SCI and suggesting that it plays a key role in modulating secondary phase of spine injury by acting as a molecular chaperone and repairing the partially damaged neurons.[14] Consistent with this study, a pre- and posttreatment study on rats with peroxisome proliferator-activated receptor inhibitors showed the protective neurological response that is due to elevation of HSP.[30,31] There are additional studies with HSP-27 that shows its angiogenic and neurogenic potential. A few studies have also shown that the elevation of this protein following any kind of stress or tissue injury is associated with angiogenesis.[32] In contrast to this, mutations in HSP 27 result in decreased expression of HSP and binding of heat shock factor to heat shock element resulting in impaired neuroprotection.[33,34] We did not analyze any single nucleotide polymorphisms (SNPs) in HSP-27. In this context, a significant decrease in the levels of HSP-27 in the posttraumatic period indicates a subdued compensatory response. The cause of delayed activation could not be analyzed although HSP-27 and CASPASE-3 expression are often with apoptotic and angiogenic activity.

Similarly, the expression of MCP-1 in the acute phase of posttraumatic SCI was elevated within 30 days from the time of injury. This can be an intrinsic response to the inflammation caused after secondary SCI since MCP1 is released by monocytes, macrophages and dendritic cells in response to inflammatory reaction. A study in a rat model reported increased expression of MCP-1 in secondary SCI, due to inflammatory cytokines—interleukin-1β and tumor necrosis factor α. The same study reported that after anti-MCP-1 gene therapy, levels of MCP-1 expression, monocytes, and macrophage infiltration were reduced, further supporting inflammatory role of MCP-1. The reduction of MCP-1 expression is, therefore, protective during secondary SCI as it inhibits apoptotic process and reduces neuronal and astrocyte injury.[35]

It has been reported that the angiogenic role of MCP-1 through the p44/42 MAPK (Erk1/2) pathway upregulates hypoxia-inducible factor (e.g., VEGF) in the aortic endothelial cells.[36] Upregulating the levels of VEGF-A also results in angiogenesis, pointing out a possible compensatory role by VEGF in SCI.[37] It is possible that after SCI, there is activation of chemotactic activity resulting in an increase in the number

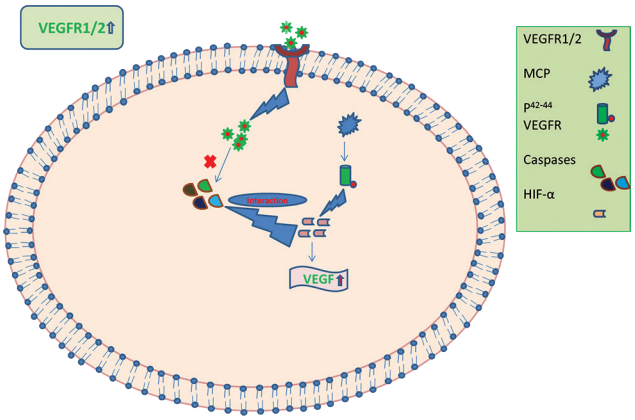

Fig. 7 Plausible mechanism of interaction of various biomarker genes in the secondary phase of spinal cord injury. HIF, hypoxia inducible factor; MCP, monocyte chemoattractant protein; VEGFR-1, vascular endothelial growth factor receptor-1.

of various chemotactic factors such as MCP-1, which further activates VEGFR (►**Fig. 7**). We postulate that VEGFR-1 and VEGFR-2 and MCP-1 are important biomarkers released after SCI trauma and this is supported by other studies.[31,38] They may exert their angiogenic influence to protect neurons from senescence and activate neuronal regeneration; however, their interaction with HSP-27 at other time points remains to be determined.

Limitations

The study consisted of a small sample size, and study participants were not studied with long-term follow-up. This study did not consider patients with any other comorbid conditions. The study was only limited to the group of patients who had undergone decompression surgery

Conclusion

The decreased mRNA HSP-27 expression may indicate a subdued compensatory response or delayed activation, which needs further investigation at other time points. On the other hand, increased MCP-1 expression can constitute an intrinsic response to the inflammation caused after secondary SCI, suggesting a possible inverse association of HSP-27 and MCP-1 with SCI. These may be investigated as potential biomarkers in larger studies, where CSF samples can also be analyzed.

Funding

None.

Conflict of Interest

None declared.

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REVIEW ARTICLE

Is Brain-Derived Neurotrophic Factor: A Common Link Between Neurodegenerative Disorders and Cancer?

Radhika Khosla[a #] Avijit Banik[b], Sushant Kaushal[a], Priya Battu[a #], Deepti Gupta[c] and Akshay Anand[a,*]

[a]*Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, Chandigarh, India;* [b]*Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA;* [c]*Department of English and Cultural Studies, Panjab University, Chandigarh, India*

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Abstract: ***Background*:** Cancer is a common disease caused by the excessive proliferation of cells, and neurodegenerative diseases are the disorders caused due to the degeneration of neurons. Both can be considered as diseases caused by the dysregulation of cell cycle events. A recent data suggests that there is a strong inverse association between cancer and neurodegenerative disorders. There is indirect evidence to postulate Brain-derived Neurotrophic Factor (BDNF) as a potential molecular link in this association.

***Discussion*:** The BDNF levels are found to be downregulated in many neurodegenerative disorders and are found to be upregulated in various kinds of cancers. The lower level of BDNF in Alzheimer's and Parkinson's disease has been found to be related to cognitive and other neuropsychological impairments, whereas, its higher levels are associated with the tumour growth and metastasis and poor survival rate in the cancer patients.

***Conclusion*:** In this review, we propose that variance in BDNF levels is critical in determining the course of cellular pathophysiology and the development of cancer or neurodegenerative disorder. We further propose that an alternative therapeutic strategy that can modulate BDNF expression, can rescue or prevent above said pathophysiological course. Larger studies that examine this link through animal studies are imperative to understand the putative biochemical and molecular link to wellness and disease.

Keywords: BDNF, homeostasis, neurodegenerative disorders, BDNF/TrkB cascade, alternative therapy, Alzheimer's disease.

1. INTRODUCTION

During the development of the nervous system, various neuronal growth factors play an important role in influencing the survival and growth of the developing neurons [1]. These growth factors are categorized as Neurotrophins, Neuregulins and GDNF family growth factors [2, 3]. The Brain-Derived Neurotrophic Factor (BDNF) belongs to the family of neurotrophin and shows various therapeutic actions associated with cognitive impairment in various neurodegenerative diseases [4]. It has been observed that in various neurodegenerative diseases, such as ALS and Alzheimer's disease (AD), the level of BDNF is found reduced, significantly adding to the disease pathophysiology [5]. The lower BDNF level in the plasma is responsible for cognitive impairment, stress, fatigue and depression [6].

In contrast, in several types of cancers, the plasma level of BDNF is found to be raised and plays a critical role in the metastasis [7]. Through various intrinsic cellular and molecular pathways, BDNF may exert an oncogenic influence to the cancerous cells thus accelerating tumour growth and metastasis. It is hypothesised on the basis of several studies that there must be an appropriate or homeostatic level of BDNF in plasma that ensures the prevention of both cognitive impairment and cancer [8]. Neurodegenerative diseases and cancers can, therefore, be examined in the context of such opposite molecular influence because neurodegenerative disorders involve cell death and cancer is characterised by unregulated cell proliferation [9]. A current GWAS study investigating the genetic link between cancer and AD cases has revealed that a large sample size ranging from 9931 to 54,162 has both positive and negative association on their Single Nucleotide Polymorphisms (SNPs) with a range of cancers such as breast, colon, lung, ovarian and prostate cancer. This suggests a common genetic etiology between cancer and AD, indicating the need to examine these associations [10]. Thus, BDNF could be a critical factor in regulating homeostasis, and possibly any deviation in its expression may trigger either the process of tumorigenesis or neurodegeneration. The focus of this review article is to evaluate the homeostatic role of BDNF in the pathophysiology of several

*Address correspondence to this author at the Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India; Tel: +919815968102;
E-mail: akshay1anand@rediffmail.com
[#]*These authors contribute equally*

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kinds of cancers and neurodegenerative diseases so that, we could postulate appropriate interventions based on matching the desirable effects [11]. Further, we have discussed how a holistic approach as such the practice of Yoga, mind-body techniques can sustain cellular homeostasis. As Yoga and exercise increase the BDNF levels, it is worthwhile to design studies to probe its opposing role in two forms of the disease [12, 13]. This article may provide insights into our understanding of the pathophysiology of neurodegenerative disorders and cancer from the perspective of BDNF and propose a comprehensive intervention for effective management of these disorders (Fig. **1**).

2. CANCER AND NEURODEGENERATIVE DISORDERS: AN INVERSE ASSOCIATION

2.1. Exploring the Relationship Between Cancer and Alzheimer's Disease (AD)

Driver *et al*. in Framingham Heart study [14] described the inverse association between cancer and AD by a community based prospective cohort analysis. The study included 1278 participants with or without the history of cancer and followed it for 10 years and estimated the risk of developing AD. In a case-control analysis, when normalized to their age, sex and smoking habits, the cancer survivors were found to be at a lesser risk of probable AD and *vice versa*. Another prospective cohort study [15] showed an inverse association between prevalent dementia and future cancer risk in more than 3000 people, aged at 65 years and above. A follow up was carried out for 5.4 years for dementia and 8.3 years for cancer. It was found that patients with Dementia showed lower risks of cancer and *vice versa*. (Table **1**) with the increasing evidence of such inverse association, it is hypothesized that there could be dynamic molecular interactions governing cellular regeneration and degeneration processes in both disease conditions [16].

2.2. Exploring the Relationship Between Cancer and Parkinson's Disease (PD)

The emerging reports point out the inverse association between Cancer and the prevalence of PD. Driver *et al* conducted a case-controlled nested study in a cohort of 22,071 male physicians from the US and followed them up for 22 years determining that cancer survivors had a decreased risk of PD diagnosis. The incidence of any cancer was found to be reduced in PD cases (13.1%) as compared to their age-matched controls (14.8%) [17]. Another meta-analysis study by Xie *et al.* suggested that PD patients from the cohort of western countries were found to be significantly associated with reduced risk of developing colorectal cancer. They analysed 13 studies from the region including 343,226 PD patients and showed that irrespective of the variations in study designs, gender and location of tumor, this inverse association was constant in the population [18]. Although the above studies confirm the inverse association between cancer and PD, no such studies have been carried out in larger Asian populations.

Several studies have recognised the importance of the link between cancer and neurodegenerative diseases and their inverse association but molecular mechanisms involved in the association are unknown. Nixon *et al* studied this inverse relationship in association with obesity. As obesity is the common risk factor associated with both diseases, they have studied the association of leptin and adiponectin with both diseases. Both molecules have opposing effects. They

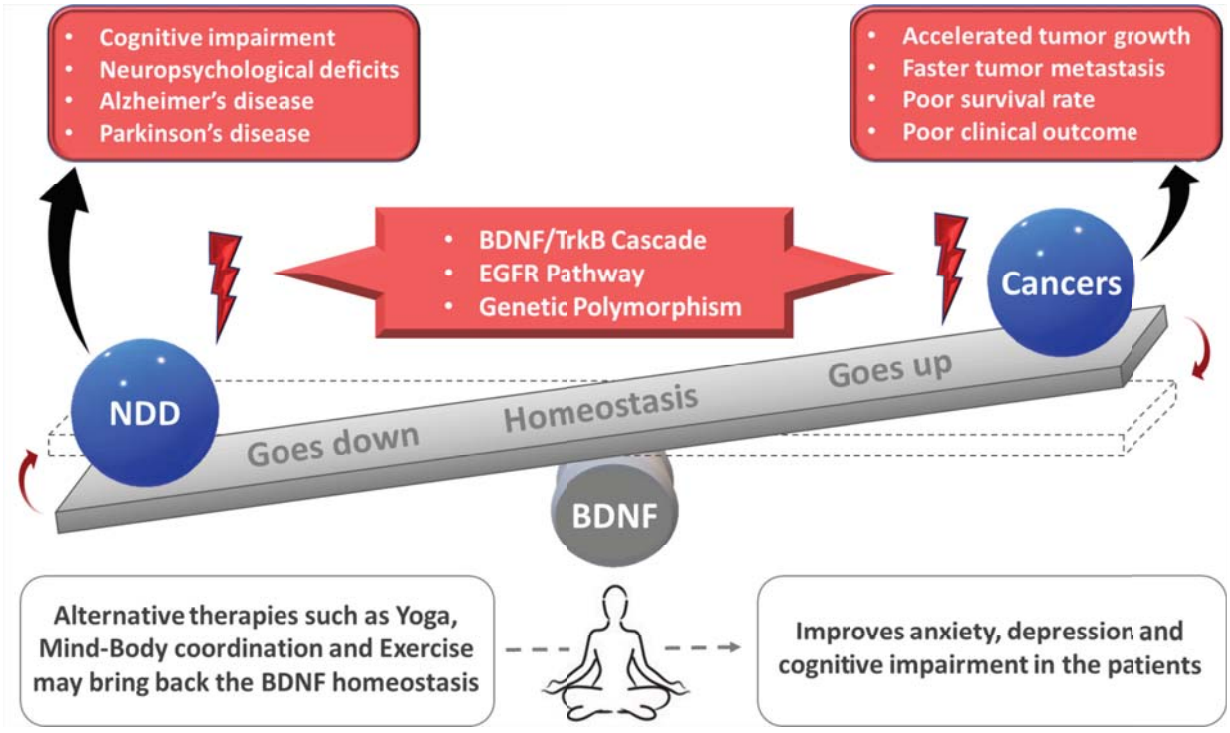

Fig. (1). Schematic representation shows the association between cancer, neurodegenerative disorders and BDNF induced pathology that how an alternative therapeutic approach may manage these diseases/disorders by maintaining the BDNF homeostasis. NDD: neurodegenerative disorders.

Table 1. Table showing various studies that shows reduced BDNF in the neurodegenerative diseases.

Aims	Outcome
Investigating the association between serum BDNF concentration and mild cognitive impairment (that may further lead to AD).	The serum BDNF concentration was observed to be decreased and reduction in BDNF can be associated with cognitive impairment. Serum BDNF concentration was significantly lower in aMCI patients [20].
To investigate BDNF mRNA expression in the hippocampus of AD patients.	The expression of NGF, BDNF and neurotrophins was assessed in the hippocampus regions of patients. The BDNF mRNA expression was decreased in the patients. No difference was in the levels of other neurotrophins [21].
To investigate the BDNF expression in AD patients at different stages of the disease.	BDNF expression was found to be increased in the early stages of the disease but as the stage of the disease progressed BDNF serum level started decreasing in correlation with dementia at the stage of the disease [22].
To quantitate the BDNF mRNA in human parietal cortex.	BDNF mRNA was found to be decreased by three folds in the parietal cortex of AD patients as compared to controls [23].
To investigate the BDNF mRNA expression and BDNF protein level in the human post-mortem hippocampi of AD patient.	A reduction in the BDNF expression was found in both the hippocampi and temporal cortices of patients as compared to controls [24].
To investigate the serum and CSF concentration of BDNF in AD patients.	Serum BDNF level was found to be decreased in AD patients as well as normal pressure hydrocephalus than controls. However, CSF was not found to be a good source for this analysis because of very low BDNF concentration [25]
To determine the pro-BDNF and mature BDNF protein levels in the parietal cortex of subjects with non-cognitive impairment, mild cognitive impairment and mild or moderate AD.	Both pro-BDNF and mature BDNF was found to be decreased in patients with MCI and AD as compared to subjects with non-cognitive impairment. The decrease in BDNF was also found to be correlated with the cognitive impairments [26].
To determine the BDNF mRNA expression in the Parkinson's disease Substantia Nigra.	BDNF level of substantia nigra pars compacta reduced by 70% in PD patients [27].
To investigate the concentration of BDNF and NGF in Parkinsonian patients.	The concentration of both BDNF and NGF was found to be decreased in patients than controls [28]
To determine the BDNF protein expression in the post-mortem mesencephalon of controls and Parkinson's disease patients.	Reduced expression of BDNF was there in this region [29].
To establish an association between Estrogen and BDNF in relation to neurodegenerative diseases.	Estrogen and BDNF both were found to be decreased in AD and PD patients [30].
To investigate the role of BDNF serum concentration as a marker related to the Huntington Disease patients' phenotype.	Serum BDNF concentration was decreased and was in correlation with the cognitive scores [31].

have also found that *wnt* and *p53* are important signaling molecules to be involved in the function of these molecules. Similarly, BDNF can also be found as a common link between both diseases. However, further investigations are required to examine BDNF as a common link [19].

3. BDNF AND NEURODEGENERATIVE DISEASES

The level of BDNF in several neurodegenerative diseases is found to be decreased. There are several studies which show that BDNFexpression decreases in AD patients and PD (Parkinson's disease) patients (Table **1**). The decreased BDNF level is responsible for cognitive impairment in these patients.

4. ANIMAL STUDIES SUPPORTING THE ROLE OF BDNF IN COGNITIVE IMPROVEMENT

Various studies conducted on animal models have already shown that if plasma BDNF levels increase, there can be a possible improvement in cognition. Jones *et al.* [32] transplanted neural stem cells in 3xTg-AD mice and assessed their cognitive improvement, memory latency, platform crosses and context-dependent recognition. All the symptoms were found to be improved but not due to altered amyloid-β or tau pathology. The cognitive impairment was accompanied by enhanced hippocampal synaptic density and elevated BDNF. One of the reasons for cognitive impairment in AD was reported due to abnormalities of immediate early genes such as cAMP response element binding protein (CREB*). Caccamo et al.* [33] also studied the effect of the transfer of CREB Binding Protein (CBP) gene to AD mice showing improved cognitive impairment in mice due to elevated levels of BDNF. A study from our lab also proposed that elevated levels of BDNF and CREB might improve cognitive impairment in amyloid-β injured mice [34]. The human umbilical cord blood-derived lineage negative stem cells transplanted in cognitively impaired mice enhanced the levels of BDNF mRNA expression and CREB that improved

their cognitive impairment. A recent study done by *Choi et al* showed the importance of BDNF as well as the exercise in improving cognition in Alzheimer's disease model. The enhancing of Adult Hippocampal Neurogenesis (AHN) was not found to be much helpful in improving cognition and memory but enhancing AHN along with exercise increased BDNF level as well as improved memory. Also, they have elevated the BDNF level to confirm its role. An elevation in BDNF has also improved memory [35]. *Tomi et al* highlight the importance of BDNF in cognitive pathways. They have used APdE9 mice model of Alzheimer's disease. The reduced BDNF in these transgenic mice has been shown to cause memory impairment. They have also analysed the level of BDNF along with age. It has been observed that the BDNF level was found increased in these mice with age but IHC studies show that the increased BDNF was found deposited around the proximity of the Ab-plaques. BDNF gene deficiency influenced spatial learning. BDNF plays an important role in cognitive improvement [36]. Jiao *et al* discovered that the transfer of BDNF gene *via* AAV-BDNF transfer system provided neuroprotection and improved the neuronal symptoms. BDNF gene improved tau proteinopathy mediated damage to neuronal cells, though it did not affect tau hyperphosphorylation [37].

5. ROLE OF BDNF IN CANCER

Colorectal cancer is one of the common causes of deaths all over the world [38]. Colon cancer pathology is still unclear. It has been shown that BDNF signalling protects cancer cells from EGFR inhibition and it is reported that the expression of BDNF is correlated with different types of carcinomas thus accelerating cell survival and proliferation [39]. Yang *et al* have demonstrated that ribozyme-based gene knockout of BDNF from colon cancer cell lines resulted in increased apoptosis and decreased rate of cell proliferation. They also concluded that the level of BDNF is increased at the time of diagnosis and modulates the cancer cells to become non-sensitive to chemotherapy [39]. In another cell line based study, it was shown that the presence of human BDNF significantly increases the migratory nature of colon cancer cells. The downstream pathway analysis also revealed that this migratory behaviour is induced by BDNF mediated upregulation of heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF) in these cells (Table **2**). The ERK, p38, and Akt signalling pathways were found to be involved in the faster migration of these cancerous cells and pathway inhibitors used in the study showed controlled regulation of BDNF induced VEGF/HO-1 activation [40].

5.1. Breast Cancer and Role of BDNF

Breast cancer is the second most common cause of death in women. There has been significant progress in screening and treatment strategies leading to improvement of the survival rates in the last couple of decades [41]. The role of neurotrophin family growth factors in the metastasis and progression of Breast cancer has been extensively investigated. A large number of neurotrophins such as NGF, BDNF and neurotrophin 4/5 are found to be expressed in breast tumors and linked to tumor growth and proliferation through various autocrine signalling loops such as tyrosine kinase pathway [42] (Table **2**). A high level of BDNF in tumors has been reported to worsen the clinical outcome and survival rates in breast cancer patients [43]. Anti-BDNF transgene strategy by systemic knockdown of BDNF in several breast cancer cell lines and their wild type counterparts expectedly showed a dampening effect in the proliferation and growth of tumor cells. Similarly, BDNF knockdown increased cell apoptosis in these cells. Some investigators examined the role of the receptor of BDNF and reported Tropomyosin-related brain through paracrine effects of BDNF-TrkB signalling. Interestingly, Kang *et al.* carried out a one year follow up study showing a significantly higher rate of depression in 309 breast cancer patients directly associated with higher level of methylation in the BDNF gene. This study further suggests that the cancer patients are more prone to depression through the methylation of one of the target genes associated with disease pathophysiology. Whether a holistic way of management by Mind Body techniques, Yoga or exercise can ameliorate the disease associated with stress and depression, mediated by methylation of BDNF, has not been investigated.

6. BDNF AS A MARKER FOR CANCER?

Recent developments have brought BDNF into the centre stage as a probable diagnostic marker for multiple cancer. Bronzetti *et al.* found that in prostate cancer, patients with raised BDNF level can be a target for the detection of cancer [44]. They recruited 16 patients with cancer, 20 with benign prostate hyperplasia and 4 whole prostates from four fresh male cadavers who had not died from the tumoral prostatic disease. Markers were measured immunohistochemically and the BDNF level was found to be significantly raised in patients with prostate cancer. The underlying mechanism was identified as the receptor for BDNF, p75NTR that mediated programmed cell death.

Similarly, Lai *et al.* [45] showed significantly overexpressed BDNF and TrkB in TCC (transitional cell carcinoma) samples compared to normal Urothelium. 12 normal urothelial tissues, 35 paired non-malignancy-involved bladder tissues from TCC patients and 65 TCC tissues were examined. Immunohistochemistry was carried out to analyse the expression of BDNF and TrkB expression which were found significantly overexpressed in TCC cells. BDNF is associated with a reduction in the apoptosis in Breast cancer cells. Higher levels of BDNF were associated with poor clinical outcome and survival [46]. These reports implicate that BDNF level could be an effective biomarker to analyse the stages and progression rates of various types of cancer.

7. BDNF AS A THERAPEUTIC TARGET IN CANCER

BDNF signalling acts as an anti-cancer target mediated by its receptor, TrkB. BDNF has been widely studied in the development and differentiation of fetal neurons and acts to produce anti-tumour immune response [1]. The role of BDNF/TrkB cascade in the pathogenesis of cancer has also been currently investigated. BDNF/TrkB cascade can even modulate a series of cell signalling pathways such as VEGF, Akt/PI3K, Wnt/β-catenin, Jak/STAT, NF-kB and UPAR/UPA pathways providing plausible links to predictive

Table 2. Various studies which suggest that BDNF and TrkB expression get upregulated in various types of cancers.

Aims	Outcome
To determine the expression of BDNF and TrkB in human bladder cancer cells.	BDNF and TrkB were found to be overexpressed in grade III and Grade I and III cancer, respectively [54].
To identify the relationship between BDNF and TrkB and prognosis in non-small cell lung cancer.	Overexpression of these molecules is related to a poorer prognosis. It was also found that coexpression of both molecules is responsible for poorer prognosis as compared to over-expression of one of these proteins [55].
To study the distribution of neurotrophins in normal, hyperplastic and prostate cancer cells.	BDNF and TrkB were found to be overexpressed whereas other NTs were not overexpressed. It was suggested that BDNF and TrkB have a possible predictive role in the diagnosis of prostate cancer [56].
To determine whether BDNF and TrkB can be the potential therapeutic target for peritoneal carcinomatosis.	Poor prognosis was there in the patients that either had a higher level of BDNF and TrkB or coexpression of both these markers [57].
To examine the BDNF and TrkB expression and function of their signalling in the small cell lung cancer	Co-expression was related to poor prognosis. TrkB can be the potential therapeutic target in small cell lung cancer [58].
To determine the expression of BDNF and TrkB in ovarian cancer patients.	Expression of both these molecules is related to the poor survival of ovarian cancer patients. BDNF /TrkB pathway is responsible for the cell migration [59].

biomarkers and therapeutic targets for several kinds of cancer [47]. Previously, it was reported that BDNF synthesis accelerates the growth and progression of cancerous tumors [43]. It was also implicated that TrkB and BDNF are upregulated in many types of cancers [48]. PI3K/AKT signalling pathway leads to the production of anti-apoptotic proteins through the binding of BDNF with its conjugate receptor, TrkB which initiates the signalling cascade for uncontrolled cell proliferation [49]. Studies implicate that TrkB receptor pathway evolves the phosphorylation of Tyrosine 705 stat 3 which transduces hypoxia-inducible factor1-alpha(HIF1α) mRNA levels [50]. EGFR and TrKB pathways are reported to be associated in many kinds of cancers and provide indirect inhibition [49]. In the case of lung cancer, TrkB response inhibits the effect of EGF administration and similarly, inhibition of EGF leads to the alteration of the effect of BDNF administration [50]. In case of ovarian cancer, excessive release of BDNF activates the TrkB pathway which results in the formation of zygotes into pre-implantation embryos and it also provides signals to granulosa cells for the immature upfolding of follicles [51, 52]. It has also been observed that in cases of bladder cancer, there was overexpression of BDNF and TrkB in cancerous tissue compared to normal samples at different stages and grades of metastasis. It is unambiguously indicated in these studies that BDNF and its signalling cascade play a dominant role in the proliferation of cancer making it amenable for either drug development or an important outcome measure from alternative interventions [45]. Many patients with cancer have also been reported to suffer from a series of health impairments when subjected to chemotherapy. These include cognitive impairment, memory loss, fatigue, restlessness and Depression [53]. Terence *et al.* has reported in Asian patients to develop cognitive impairment after radiotherapy.

8. ATTAINING BDNF HOMEOSTASIS THROUGH ALTERNATIVE THERAPIES: ROLE IN DISEASE MANAGEMENT

Cancer and its treatment cause depression, anxiety, fatigue, sleeplessness and pain to the patients. Alternative therapies which aim to improve the quality of life of the cancer patients have not examined the molecular outcome resulting from the interventions, often ignoring the reductionist approach. A study by Cohen *et al.* demonstrated that Tibetan Yoga (TY) helps in improving sleep-related outcomes in lymphoma patients. The authors studied the impact of Tibetan yoga (TY) practices of Tsa lung and Trulkhor, which integrate controlled breathing and visualization, mindfulness techniques, and low-impact postures. They recruited 39 lymphoma patients undergoing treatment or those who have completed treatment in the previous 12 months since recruitment [60]. Patients in the TY group reported significantly lower sleep disturbance scores as compared to patients in the non-TYgroup but did not analyse the spectrum of molecular markers including BDNF, leaving a void in the literature.

In another study reported by Danhauer *et al*., 51, ovarian cancer (n=37) and Breast cancer (n=14) patients participated in a weekly session of 75-minutes' Restorative yoga classes spanning 10 weeks that included physical postures, breathing, and deep relaxation. The authors reported significant improvement in depression, negative effect, anxiety, mental health, and overall quality of life. Fatigue levels were also found to be decreased in post-intervention follow-ups. This suggests that Yoga helps in improving the quality of life of cancer patients analysed by administering Questionnaires or documenting Neurophysiology correlates, completing ignoring the role of various cytokines and nerve growth factors in biofluids. However, Saligan *et al*. studied the effect of radio-

therapy on prostate cancer patients [61]. The association between plasma concentrations of three neurotrophic factors (BDNF, brain-derived neurotrophic factor; GDNF, glial-derived neurotrophic factor and SNAPIN, soluble N-ethylmaleimide sensitive fusion attachment receptor-associated protein) and initial fatigue intensification during external beam radiation therapy (EBRT) in euthymic non-metastatic prostate cancer men were investigated. Fatigue was measured by the 13-item Functional Assessment of Cancer Therapy-Fatigue (FACT-F), and plasma neurotrophic factors were collected at baseline (prior to EBRT) and mid-EBRT. Subjects who felt fatigued had a significantly reduced concentration of BDNF in plasma. BDNF reduced after treatment, causing stress, depression, fatigue and cognitive impairment. Similarly, Ng *et al.* suggested that Chemotherapy-associated with cognitive impairment (CACI) may be due to changes in plasma BDNF levels [62]. The Functional Assessment of Cancer Therapy-Cognitive Function (FACT-Cog) was done in chemotherapy receiving early stage breast cancer patients. Depression was also measured in patients. Plasma BDNF was found to be decreased in these patients with self-perceived cognitive decline. Despite these limited investigations, the role of mind-body techniques or Yoga on these changes was not analysed after its intervention. Studies have shown that practising Yoga improves brain plasticity, resulting in an increase in cognitive performance and mitigation of symptoms such as Depression and Post-Traumatic Stress Disorder (PTSD). In a study by Naveen *et al.,* consecutive out-patients of depression without suicidality were subjected to Yoga alone or with antidepressants [63]. The depression severity was rated on the Hamilton Depression Rating Scale (HDRS) before and at 3 months. BDNF levels were also estimated in the serum of patients. There was a positive association between fall in HDRS and rise in serum BDNF levels in Yoga-only group but not in those receiving Yoga and antidepressants or antidepressants-alone and found to be statistically significant. They reported that neuroplasticity may be related to the beneficial mechanisms of Yoga in Depression. In a study by Cahn *et al.* 2017, it was reported that there was an increase in BDNF levels along with the decrease in cortisol levels in 38 individuals who performed Yoga and meditation for 3 months [12]. The increased BDNF levels may be a prospective arbitrator between meditative practices and brain health.

Further, Tolahunase *et al.* 2017 studied the effect of Yoga and meditation on cellular ageing in apparently healthy individuals estimating levels of various biomarkers of ageing in blood which included DNA damage marker 8-hydroxy-2-deoxyguanosine (8-OH2dG), oxidative stress markers reactive oxygen species (ROS), and total antioxidant capacity (TAC), and telomere attrition markers telomere length and telomerase activity, metabotropic blood biomarkers associated with cellular aging were also assessed, which included cortisol, β-endorphin, IL-6, BDNF, and sirtuin-1. 96 recruits were subjected to Yoga and meditation Based Lifestyle Intervention (YMLI) programme [64, 65]. The mean levels of 8-OH2dG, ROS, cortisol, and IL-6 were significantly lower and mean levels of TAC, telomerase activity, β-endorphin, BDNF, and sirtuin-1 were significantly increased (all values $p< 0.05$) post-YMLI of 12 weeks. Authors proposed that YMLI significantly decreased the rate of cellular aging in an apparently healthy population [64, 65]. Above studies strongly suggest that alternative therapies such as Yoga and exercise exert beneficial effects in the cancer patients through the modulation of psycho-neuro-immunological pathways. BDNF/TrkB cascade might be an essential modulator in maintaining such homeostasis in the cancer patients. This can only be confirmed if large scale studies with Yoga intervention incorporating molecular analysis of the role of BDNF and its associated molecules in the pathway are conducted.

8.1. Herbs and BDNF

Literature is replete with the mentioning of herbs which have neuroprotective properties. *Ashwagandha* is used to treat forgetfulness since time immemorial. It is commonly known as *W. somnifera*. A study by Konar *et al* 2011 has shown that an alcoholic extract of *Ashwagandha* leaves reverses amnesia caused by scopolamine in mice. Scopolamine decreased BDNF which was reversed by the administration of *Ashwagadha* leaf extract [66]. An *in vitro* study by Shah *et al* on glioblastoma and neuroblastoma cells has shown that Ashwangha extracts, particularly active components with a one, at a low dose, is effective in protecting these cells from oxidative stress and further induces their differentiation [67]. Many studies have confirmed its anti amnesic properties. Alcoholic extract possesses cholinergic properties and prevents the amnesic effect of scopolamine in mice.

Bacopa monniera is another plant which is used to treat PC12, a cell line mimicking neuronal cells. Pre-treatment with *B. Moneira* extract was protective against the toxicity induced by Scopalamine by upregulating BDNF expression [68]. Lower BDNF levels are associated with depression-like behaviour in rats. However, if BME is administered daily, it restores BDNF levels [69]. *Curcuma longa* is frequently mentioned in the traditional system of Chinese medicine for anti-depressent properties. They subjected animals to stress for 20days and found lower levels of BDNF in hippocampus and prefrontal cortex whereas the administration of curcumin blocked such alteration in BDNF levels [70]. In another study by Dexiang *et al,* similar results were found where lower BDNF levels accompanied cognitive deficit induced by chronic unpredictable stress and curcumin reversed the alteration in BDNF level [71].

9. INFLUENCE OF PERSONALIZED EXERCISE PROGRAMS

Mind and body practices through mental and physical training can elicit improved brain networking in several Neurocognitive disorders such as AD and Schizophrenia. Whether exercise can increase the BDNF level in plasma and brain by strengthening the recovery of weakened neural connections in these disorders, needs further investigation. [72] Body mind practices and Yoga can improve self-perceived cognitive impairment as well as decreased stress and depression experienced by cancer patients. Zimmer *et al.* [73] measured the total metabolic rate, physical activity level, mean MET and steps, fatigue, self-perceived cognitive functioning, and biomarkers [C-reactive protein (CRP), interleukin 6, macrophage migration inhibiting factor (MIF), tumor necrosis factor (TNF)-α, BDNF, insulin-like growth factor 1

(IGF1)] in 60 patients with breast cancer. The stable rehabilitation program was administered for a long time. It was observed that there was a significant increase in BDNF and IGF1 levels, while CRP level was decreased. Fatigue and self-perceived cognitive functioning were found to be improved. Yoga increases the BDNF levels and decreases the inflammation markers. However, certain investigators refute these findings as the change was found to be significant. Others argue that in order to have desirable effects, Yoga and body-mind techniques can be personalised according to the patient's health conditions or *Triguna or Tridosha*. Desired results may be produced on the basis of the correct choice of alternative therapy given depending upon symptoms and pathology. It has been proposed that the well-managed rehabilitation programs and the personalised physical activities that also assesses BDNF levels can be more beneficial than the random exercise programs.

CONCLUSION

The recent findings have strongly suggested an inverse association between cancer and neurodegenerative disorders, especially AD. On one hand, the downregulation of BDNF is widely reported in many neurodegenerative disorders resulting in cognitive and other neuropsychological impairments [4, 74].On the other hand, the plasma BDNF level was found to be significantly upregulated in the patients causing faster tumour metastasis, accelerated tumor growth with poor survival rates [7, 42]. Therefore, it is hypothesized that BDNF may have a key role in the pathophysiology of cancers and neurodegenerative disorders. A recent article reported that BDNF/TrkB pathway facilitates brain metastasis in the breast cancer patients, suggesting that cancerous condition can trigger the brain infiltration through paracrine effects of BDNF/TrkB signalling [75]. Several reports suggest that in cancer, the BDNF homeostasis is disrupted through multiple channels such as BDNF/TrkB cascade, EGFR signalling and genetic polymorphisms [47]. Although, a recent large scale GWAS study revealed that there is both, a positive and negative correlation between AD and cancers. Several SNPs identified in these patients showed both positive and negative links to AD. It is believed that a similar set of genetic polymorphisms might alter the disease pathology either towards AD or cancer [10]. We further postulate that an alternative therapeutic strategy through the practice of Yoga, Mind-Body coordination and exercise can maintain the BDNF homeostasis and potentially ameliorate the disease and associated symptoms. Larger human studies along with supporting animal studies must be conducted to test this hypothesis and elucidate the underlying biochemical and molecular pathways. To understand whether or not BDNF is a diagnostic or prognostic or/and therapeutic marker for cancer and neurodegenerative diseases, extensive studies are required

AUTHOR'S CONTRIBUTION

All authors have contributed to the writing of this manuscript. All authors read and approved the final manuscript.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REVIEW
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Role of Ionizing Radiation in Neurodegenerative Diseases

Neel K. Sharma[1], Rupali Sharma[2], Deepali Mathur[3], Shashwat Sharad[4], Gillipsie Minhas[5], Kulsajan Bhatia[6], Akshay Anand[5] and Sanchita P. Ghosh[1]**

[1] Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [2] Center for Neuroscience and Regenerative Medicine, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [3] Neurobiology Laboratory, School of Biotechnology, KIIT University, Bhubaneswar, India, [4] Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, MD, United States, [5] Neuroscience Research Lab, Department of Neurology, Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh, India, [6] Government Medical College and Hospital, Chandigarh, India

Ionizing radiation (IR) from terrestrial sources is continually an unprotected peril to human beings. However, the medical radiation and global radiation background are main contributors to human exposure and causes of radiation sickness. At high-dose exposures acute radiation sickness occurs, whereas chronic effects may persist for a number of years. Radiation can increase many circulatory, age related and neurodegenerative diseases. Neurodegenerative diseases occur a long time after exposure to radiation, as demonstrated in atomic bomb survivors, and are still controversial. This review discuss the role of IR in neurodegenerative diseases and proposes an association between neurodegenerative diseases and exposure to IR.

Keywords: ionizing radiation, hypoxia, aging, neurodegenerative diseases, CNS

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Ramesh Kandimalla, Texas Tech University, United States

****Correspondence:***
Neel K. Sharma
neel.sharma.ctr@usuhs.edu;
kmlbio@yahoo.co.in
Sanchita P. Ghosh
sanchita.ghosh@usuhs.edu

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INTRODUCTION

The ionizing radiation (IR) is a group of subatomic particles and electromagnetic waves, or photons, which bear the capability to create electrically charged particles such as alpha, beta, gamma rays, and X-rays. The biological impact of IR on humans have already been reported a decade ago but recently there has been growing interest to understand the effect of radiation exposure in the central nervous system (CNS) in the clinical setting (Valentin, 2005). In today's world with global threat of radiation exposure from warfare, the neurobiological impact of high and low doses of IR and responses to biological systems needs to be revaluated. In the brain, the effect of IR is widely seen in the Hippocampus, a radio- sensitive region of the brain which hosts proliferating progenitor cells (Harada et al., 2014; Pospisil et al., 2015). It has been shown that differentiating cells amalgamated into the hippocampal network leads to apoptosis or dysfunction due to exposure to high doses of irradiation and lead to changes in synaptic protein levels, dendritic complexity, morphology and spine density alterations. (Parihar and Limoli, 2013). According to the data generated by radiation research, as well as the guidelines of regulatory bodies, the safe dose is considered to be acute exposure to less than 100 mSv, or 0.1 gray (Gy) (Morgan and Bair, 2013).

It has been established that radiation inhibits neurogenesis in a dose dependent manner (Low to High; >2 Gy to 45 Gy) through radiosensitive populations of neural stem and progenitor cells housing in the sub-granular zone of the dentate gyrus of the brain (Acharya et al., 2015). This can block the generation of new cells in the brain and cause neuroinflammation (Belarbi et al., 2013; Greene-Schloesser et al., 2013). Further, low dose exposures of IR have been shown to elevate the reactive oxygen and nitrogen species, which has the potential to initiate the changes

in the redox balance of the CNS microenvironment (Limoli et al., 2004). Acute exposure of IR has manifold effects on brain and cognitive functions (Loganovsky, 2009), which can directly act and manipulate the nervous system and indirectly damage the other systems through CNS reactivity (Kimeldorf and Hunt, 1965; Mickley, 1987). Both low and high doses of radiation can alter the function of CNS by oxidative stress, mitochondrial dysfunctions and protein degradation, leading to senescence or apoptotic cell death which can cause defects leading to neurodegenerative diseases (**Figure 1**). In brain, inflammatory reactions are induced by IR via microglia and endothelial cell activation. Microglial cells can be activated (MHC, CD68 upregulation) due to IR-induced double-strand breaks, which activates the NFκB pathway-mediated creation of proteins related to inflammation (**Figure 2**) (Lumniczky et al., 2017). High-mobility group protein 1 (HMGB1) in the extracellular environment are secreted by damaged neurons, which is a ligand for TLR4 on the activated microglia. Calreticulin is expressed by damaged neurons on the surface which is detected by activated microglia and produce phagocytosis of both healthy and damaged neurons. Activated microglia also increases the secretion of chemokine (C-C motif) ligand 2 (CCL2) and its receptor. The peripheral macrophages expressing C-C chemokine receptor type 2 (CCR2) penetrate the blood–brain barrier and the adhesion markers like intercellular adhesion molecule 1 (ICAM-1), P-selectin starts upregulating on endothelial cells of brain after radiation. HMGB1 and pro-inflammatory signals are emitted by impaired neurons and activated microglial cells activate brain-residing dendritic cells, which migrate to regional lymph nodes and induce immune activation in the brain (**Figure 2**) (Lumniczky et al., 2017). Neurogenesis in the hippocampus is inhibited by pro-inflammatory cytokines which are secreted by activated Microglial cells and disturb neurogenic signaling pathways. Kim et al. (2008) reported that acute radiation sickness in adult ICR mice disrupts the hippocampus functioning, which includes learning and memory, through neurogenesis inhibition. Raber et al. (2004), suggested that a decrease in neurogenesis in the pathogenesis of IR -induced cognitive impairments is associated with a decrease in proliferating Ki-67-positive cells and double cortin-positive immature neurons in the subgranular zone (SGZ) of the dentate gyrus.

There are different stress response pathways by which cells respond to different stressful environments, such as unfolded protein response, heat shock response, DNA damage response, oxidative stress response, which help to maintain the cellular homeostasis, either by pro-survival pathways or by killing the damaged cells through apoptosis or autophagy (Fulda et al., 2010). Accumulation of misfolded proteins, such as those observed in neurodegenerative diseases like Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis, is known to initiate stress response pathways to eliminate the aggregated proteins (Rao and Bredesen, 2004). It is also well established that the exposure to radiations causes misfolding and aggregation of proteins, which could further lead to activation of the unfolded protein response signaling pathway. There is evidence that radiation may speed up rates of folding and unfolding for globular proteins (de Pomerai et al., 2003). This can increase the chances of collision between partially unfolded molecules, leading to irreversible aggregation. The misfolding of protein leads to the protein aggregation which further develop the neurodegenerative disease. In our daily life, we are constantly exposed to the IR either from natural and/or man-made sources and it is known that IR exposure contributes to the etiology of neurodegenerative diseases (**Table 1**). In this review, we discussed the role of IR exposure with several of these neurodegenerative diseases including Multiple sclerosis, Age related macular degeneration, Amyotrophic lateral sclerosis, Ischemia related degeneration and Parkinson disease.

ROLE OF IONIZING RADIATION IN DNA DAMAGE AND EFFECT ON NEURODEGENERATION

Effect of IR on the human DNA have been implicated due to varying degree of penetrance. Exposure to IR directly damages DNA by inducing DNA breaks particularly double stranded breaks (DSBs), which prevent the DNA replication in growing cells and cause arrest in S-phase of the development cycle. DSBs are very much detrimental, which, if left unrepaired, may have harsh aftereffects and the cell cannot survive. It also leads to the formation of reactive oxygen species (ROS) which causes oxidative stress in cells and it is indirectly linked with DNA damage. However, there is a repair system in cell which is triggered when DNA is damaged, and it stops the cell cycle at specific checkpoints to repair DNA damage and prevent progression of the cell cycle. After DNA damage, signaling molecules like ataxia telangiectasia mutated (ATM) and RAD3-related (ATR) kinases are activated which are the central controller of DNA damage response signaling pathway. These kinases work collectively and regulate downstream processes. Furthermore, phosphorylation of the histone variant histone-2AX (H2AX), is activated. Phosphorylation of H2AX plays an essential role in DNA damage response and is needed for the aggregating DNA repair proteins at the sites containing damaged DNA as well as for activation of checkpoints proteins which arrest the cell cycle progression. Generation of ROS causes cell cycle dysregulation, decrease in cell viability and damages proteins and lipids by oxidizing them and eventually leading to cell death. Koturbash et al. (2016) reported that low dose exposure to IR results in DNA damage as indicated by increased occurrence of DSBs and also behavioral changes.

It has also been reported that low dose radiation changes the expression of genes implicated in cell cycle control and DNA synthesis/repair (Yin et al., 2003). Brain cells are non-proliferative in nature and may be susceptible to the progressive accumulation of unrepaired DNA lesions exposure to radiation at low doses (<50 cGy) significantly induces neurocognitive deficits, such as learning and behavioral changes. CNS behavioral changes such as chronic fatigue and depression occur in patients who undergoes irradiation for cancer therapy. At lower radiation doses, neurocognitive effects were especially observed in children. Radiation exposure is related to a decline in

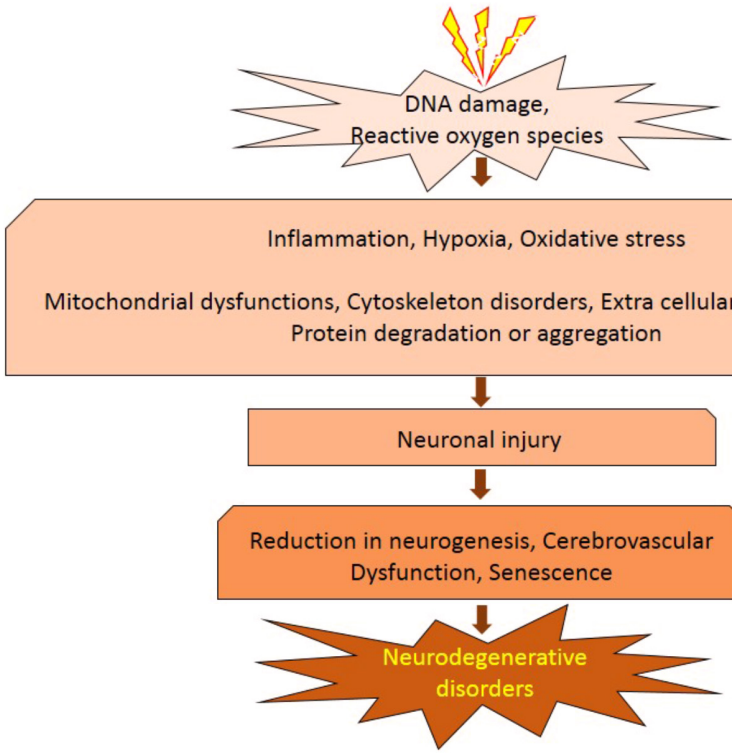

FIGURE 1 | Ionizing radiation cause neural injury leads to neurodegenerative diseases.

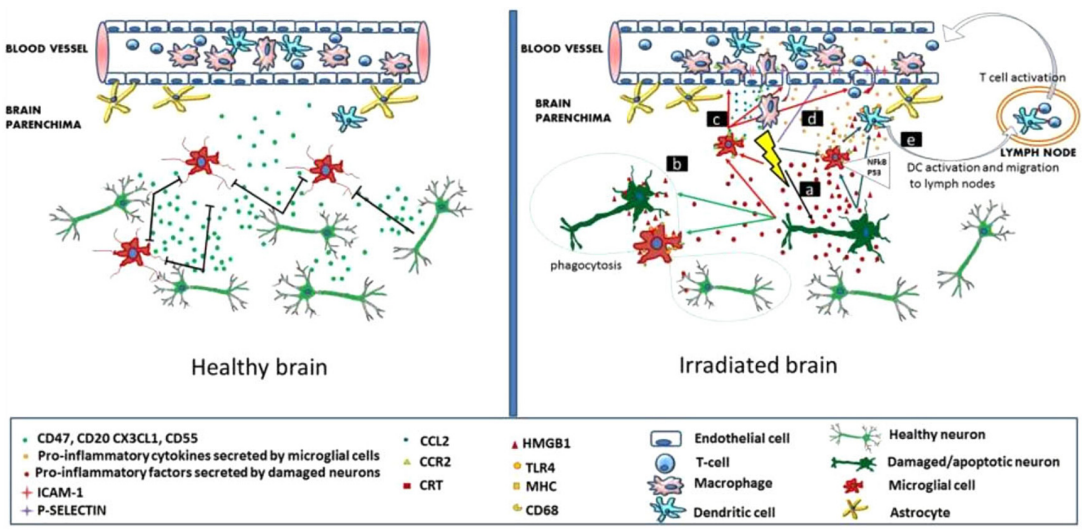

FIGURE 2 | Inflammation-mediated mechanisms in radiation induced brain injury. **(Left)** Healthy brain secretes CD55, CD47, CX3CL1, CD20 and have intact neurons. **(Right)** Radiations affect the neurons and microglia in brain. Reproduced with permission (Lumniczky et al., 2017).

academic achievement, intelligence and performance intelligence quotient (IQ). Increasing attention in recent years has been paid to the role of DNA damage and repair in neurological diseases. Many neurodegenerative diseases are related to defects in DNA single-strand break repair or double-strand break repair. In Parkinson's disease (PD) and Alzheimer's disease (AD) DNA repair defect could cause an abnormal accumulation of spontaneously occurring DNA damage in neurons *in vivo*, resulting in their premature death. There is reduction of DSB repair proteins like: DNA-PKcs and Mre11-Rad50-Nbs1 (MRN) as a result of high levels of DNA strand breaks and decreased base excision repair (BER) activity in AD patients (Jacobsen et al.,

2004; Shackelford, 2006). In Amyotrophic lateral sclerosis (ALS) patients, elevated levels of oxidative lesions and single-strand breaks (SSBs) have been reported in the neurons (Bender et al., 2006; Kraytsberg et al., 2006). To say that DNA damage has an underlying effect in the pathology of neurodegenerative diseases, requires stipulating what lesions and if any have tendency to accumulate in the sick neurons, they need to be classify with the molecular mechanisms that prevent the repair of these lesions.

ROLE OF IONIZING RADIATION IN MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a non-traumatic neurological disability commonly found among young people, with more than two million people affected worldwide (Sadovnick and Ebers, 1993). It's a complex and an unpredictable disease of the CNS with an unknown etiology. Immune mediated demyelination, gliosis and axonal degeneration constitute major pathological features of MS (Mayr et al., 2003; Ascherio and Munger, 2008). MS is generally regarded as an autoimmune disease, since the body's natural immunological defense destroys the body's native cells and damages the myelin sheath (Fernandes de Abreu et al., 2009) instead of destroying foreign cells. In the United States alone, it is estimated that 85 per every 100,000 people suffer from MS (Noonan et al., 2002) and the disease occurs twice as frequently in women compared to men (Ascherio and Munger, 2008). Studies have shown that myelin damage occurs in both white matter and the cortical gray matter of MS patients (Geurts and Barkhof, 2008; Calabrese et al., 2010). Since the prevalence of MS appears to be increasing worldwide, it is imperative to elucidate the various contributing factors and molecular mechanisms to

TABLE 1 | Late effects of radiation.

Source/Species	Late effects	Reference
Radiotherapy/human	Multiple lesions in periventricular area, centrum semiovale and corpus callosum were detected after magnetic resonance imaging. Developed Multiple sclerosis.	Shaygannejad et al., 2013
Radiotherapy/human	Magnetic resonance imaging showed new hyperintense lesions. Radiation treatment triggered an exacerbation of multiple sclerosis	Murphy et al., 2003
X-radiation /human	Activation of quiescent MS with plaques confined to the radiation fields. Multiple sclerosis activated by x-radiation	McMeekin et al., 1969
X-radiation /Between 4000 and 6000 rad (40–60 Gy)/human	Four patients who received radiation in full tumoricidal doses had unexpectedly poor clinical outcome, suggesting that radiation is especially injurious to patients with demyelinating disease.	Peterson et al., 1993
Gamma-irradiation (0.5-Gy) once a week for 4 weeks/mice	Findings demonstrated suppression of pro-inflammatory cytokines, reduction of cytotoxic T cells and induction of regulatory T cells in mice.	Tsukimoto et al., 2008
50 Hz magnetic fields at two intensities [100 and 1000 microT (rms)] for 7 weeks/mice	No link between exposure and ALS development	Poulletier de Gannes et al., 2009
X-ray irradiation at a dose of 0.8–1.5 Gy/min (Total dose of 4–16 Gy)/mice	No association between SOD1 mutation and radio-sensitivity	Wate et al., 2005
Dose-rate 1–2 Gy/min/Cells from ALS patients (Total 0 to 8 Gy)	No significant differences in production of DNA double-strand breaks	Mithal et al., 1999
Continuous radiation/mice (1.4 mGy/h) for 45 days	Chronic low-dose radiation exposure is genotoxic in mice	Graupner et al., 2016
Conventional radiotherapy in treatment/human	Direct relationship between radiation exposure and cerebrovascular events	Little, 2016
Gamma and x rays at dose greater than 0.1 Gy/human	Increased risk of stroke in those exposed to radiation more than 0.1 Gy	Azizova et al., 2014
X rays-0 to 30 Gy/human	Increased adhesiveness of human aortic endothelial cells which is chemokine mediated	Khaled et al., 2012
X rays and gamma rays in interventional procedures/human	Increased incidence of stroke noticed among workers	Rajaraman et al., 2016
Head and Neck Radiotherapy/human	Increased incidence of cerebrovascular events	Plummer et al., 2011
Longitudinal cohort studies of Japanese atomic bomb survivors, ionizing radiation	Increased incidence noted in cardiovascular diseases including stroke, Rheumatic heart disease (RHD), ischaemic heart disease (IHD), cardiomyopathy Heart failure and cerebral hemorrhage	Takahashi et al., 2013
Single radiation dose of 14 Gy/ApoE$^{-/-}$ mouse	Irradiation accelerates the development of macrophage-rich, inflammatory atherosclerotic lesions prone to intraplaque hemorrhage	Stewart et al., 2006
Mean dose 97 mv followed by max of 909 mv gamma radiations	Increased risk for death. cerebrovascular incidents was recorded as compared to other cardiovascular events	Kreuzer et al., 2013
Irradiation of CNS with doses 0, 5, 15, 25, and 35 Gy	Increased ICAM-1 expression. Suggests that increased leukocyte trafficking into the CNS may exacerbate the inflammation induced by radiation injury.	Olschowka et al., 1997
Cath lab radiation exposure	Decreased telomerase length and increased intima thickness of carotids	Andreassi et al., 2015

prevent the occurrence of MS. Environmental factors and genetic susceptibility are implicated in the pathogenesis of MS. However, IR is also contemplated as another potential factor of MS development (Flodin et al., 1988; Axelson et al., 2001; Bölviken, 2002). Although very few studies have been performed in the past to investigate the influence of IRs on the risk of MS development, existing studies suggests that IR may serve as a risk factor for MS.

Case control studies in Swedish population have demonstrated that individuals who are linked with radiological work and exhibit medical history of X-ray examinations have an increased risk for MS development (Bölviken, 2002). Tsukimoto et al. (2008) investigated the effect of low dose gamma irradiation (once a week for 4 weeks) on experimental allergic encephalomyelitis (EAE) animal model and observed a significant upregulation of regulatory T cells and suppression of proinflammatory cytokines such as inflammatory markers like interferon-γ (IFN-γ), interleukin 6 (IL-6), and interleukin 17 (IL-17). These findings imply that low dose gamma irradiation mitigates EAE through suppression of pro-inflammatory cytokines, reduction of cytotoxic T cells and induction of regulatory T cells.

A few reports have suggested that exposure to radiotherapy increases vascular permeability and may lead to the formation of demyelination lesions resulting in neuronal dysfunction (Lampert and Davis, 1964). In addition, chances of MS development may be elevated through exposure to cosmic rays, which are produced in northern and southern magnetic geographical areas (Barlow, 1960). Oldendorf and Cornford (1977) showed that spinal cord of rats exposed to X-radiation may aggravate symptoms of EAE (Oldendorf and Cornford, 1977).

Large numbers of individuals are exposed to natural sources of IR (e.g., cosmic radiation) as well as human-made radiation (e.g., X-rays and nuclear medicine) in day to day life. Accumulating data suggests that exposure to X-radiation may result in serious health concerns and adverse consequences if used intermittently and when doses of radiation exceed certain thresholds (Hammer et al., 2009; Bailey et al., 2010). Previous studies have investigated the potential hazards of IR and their effect on developing MS. In a case-control study, Motamed et al. (2014) determined the relationship between history of X-radiation and risk of MS. The investigators also determined whether the site and dosage of X-radiation would make any significant risk difference for MS (Motamed et al., 2014). Their findings revealed that patients who developed MS were previously exposed to X-ray radiation compared to controls, and that the difference was statistically significant. In addition, both cumulative number and dosage of X-radiation was significantly higher in MS patients compared to the controls. Their findings also demonstrated a link between X-ray radiation and risk of MS development in female patients, corroborating the notion that MS more frequently occurs in females. **Table 1** depicts source, dosage and effects of IR. A similar study from Sweden reported that MS patients prior to diagnosis had undergone X-ray examination several times during a 5-year period compared to controls (Flodin et al., 1988). In this study, only five MS patients were exposed to therapeutic X–ray as compared to controls. X-radiation was performed to diagnose individuals who were in the initial phases of the disease and also to identify the causes of the initial manifestations. This was considered as initial interpretation of these findings. Findings of Motamed et al. (2014) revealed that cumulative number of X-ray episodes was also significantly higher among the MS patients as compared to controls. Several lines of evidence have suggested interplay of immune system and oxidative stress underpinning the mechanism of MS pathogenesis (Lassmann et al., 2007). The cumulative dose and quality of radiation are crucial parameters to invoke immune and inflammatory reactions in brain (Amundson, 2008). Tsukimoto et al. (2008) investigated the effect of low dose gamma irradiation (once a week for 4 weeks) on an EAE animal model and observed a significant upregulation of regulatory T cells and suppression of proinflammatory cytokines such as IFN-gamma, IL6, and IL17 (Tsukimoto et al., 2008). These findings imply that low dose gamma irradiation mitigates EAE through suppression of pro-inflammatory cytokines, reduction of cytotoxic T cells and induction of regulatory T cells. Few other investigations have shown that TH1 cell activity is significantly enhanced and the levels of cytokines like IFN-γ, TNF-α, IL-2, are upregulated even with low dose radiation exposure. The level of other cytokines such as IL-10 is reduced which eventually results in free radical formation and oxidative damage of tissues (Liu, 2003). All of these perturbations in immune cell activity and cytokine levels disrupt blood brain barrier integrity, ultimately resulting in myelin and axonal damage which is observed in demyelinating MS disease pathology (Gilgun-Sherki et al., 2004). Furthermore, experimental and clinical data have reported that low dose radiation impedes tumor growth, diminishes metastasis, as well as alleviates the suppression of immunity due to tumor burden. Alike other immune related and neurodegenerative disorders (Taylor et al., 1975), it has been suggested that people suffering with MS have an increased sensitivity to IR (X-rays and gamma rays), which might elicit demyelination process in patients vulnerable to MS (Gipps and Kidson, 1981). Motamed et al. (2014) showed that patients diagnosed with MS were exposed to a considerable number of X-radiation and brain CT scanning in the past and that the cumulative dosage of IR is statistically related to the risk of MS. However, there are technical problems, which may be faced by the investigators in interpreting the results. For instance, there is a risk of misclassification bias. It is uncertain when exactly the pathological and molecular alterations appear in MS patients. Similarly, it is ambiguous that controls are devoid of any pathological alterations. These findings could result in reverse causation.

It has been observed that the chances of development of MS are more common in females upon exposure to any kind of X-ray radiation and imaging. It might be due to the smaller sample size of the male subgroup, which leads to a lower statistical significance. This difference in sex distribution is normal where MS is twice more common in females than males. However, MS-related genes present on the X chromosome have not been found by genome-wide association studies (GWAS). This suggests that preponderance of MS development in females is due to their female-specific physiology and hormones secreted in females (Orton et al., 2006). Higher prevalence of MS in females suggests that sex is a factor which makes females more susceptible to IRs. A recent study by Shaygannejad et al. (2013) has

reported a case of MS development after a patient was exposed to radiotherapy for the diagnosed meningioma. These results might be due to the influence of radiation on the blood brain barrier and the interaction between immune system antigens and white matter, and the formation of demyelination lesions. Their findings suggest that prevailing doses of radiation might trigger autoimmunity. In a similar case report, MS symptoms were elevated in a patient after radiotherapy for parotid carcinoma (Murphy et al., 2003). Moreover, a patient initially diagnosed for a glomus jugulare tumor showed an exacerbation of quiescent MS following radiotherapy (McMeekin et al., 1969). These results can be due to the reported development of disseminated plaques of demyelination, which is seen after radiotherapy (McMeekin et al., 1969). However, similar lesions can be seen in MS patients because of underlying a pre-disposition to demyelination.

The available data describing the effects of IR on the development of MS is meagre. Furthermore, the molecular mechanisms involved in radiation-induced MS are poorly understood. Some of the experiments conducted in animals and human case reports have suggested individuals who are considerably exposed to radiation are at a higher risk to develop MS symptoms. Even the impact of low dose of IR can be fatal and can cause serious health related problems if used intermittently. These findings indicate that IR may serve as confounding factor in MS disease. Therefore, it is of the utmost importance to better comprehend the possible relationship between exposure to IR and development of MS pathogenesis.

ROLE OF IONIZING RADIATION IN ALZHEIMER'S DISEASE

Five million people are estimated to live with Alzheimer's disease (AD) in the United States, and it is estimated that by 2025 there will be 50% increase in AD patients (Hebert et al., 2004). In aging population AD is a primary cause of dementia (Ashford, 2004). Patients with AD experience symptoms including memory loss, cognitive alterations and behavioral changes (Budson and Price, 2005).

As AD global prevalence is predicted to be double after 20 years, so it is crucial to recognize the molecular pathogenesis and different contributing factors for AD prevention strategy. There are numerous evidence describing the effects of IR on the brain, suggesting that IR exposure may eventually favor the progress of AD.

After cleavage, amyloid precursor protein (APP) produces 4.5-kDa peptides known as amyloid-β (Aβ) protein which is related to the pathogenesis of AD (Sisodia and Price, 1995). Due to imbalance between the levels of Aβ production and clearance, abnormal accumulation of Aβ occurs and is associated with oxidative stress, neurofibrillary tangle (NFT) formation, neuronal loss (Calissano et al., 2009), inflammation (Wyss-Coray and Rogers, 2012), and ultimately results in AD-related cognitive impairment. Since neurons are resistant to radiation-induced cell killing, brain is considered to be comparatively resistant to IR. Though, there are several studies describing the role of various cognitive and physiological effects of IR at various doses. Lower doses can lead to cognitive dysfunction without making significant morphological modification, however, at higher doses microscopic changes are visible (Abayomi, 1996).

Cherry et al. (2012) examined the effects of Fe particle irradiation in mouse model of AD (APP/PS1). Author had shown that after 6 months exposure with Fe radiation at 1 GeV/μ (10 and 100 cGy) APP/PS1 mice showed reduced cognitive abilities measured by novel object recognition tests and contextual fear conditioning. Increase of Aβ plaque pathology in male mice was observed and ICAM-1 immunohistology showed endothelial activation after 100 cGy in male mice which was suggesting potential modifications in Aβ trafficking through the blood brain barrier as a possible cause of plaque increase. The outcomes from these experiments showed that the high charged particle radiation may rise Aβ plaque pathology in APP/PS1 mouse model of Alzheimer's disease (Cherry et al., 2012).

Belka et al. (2001) suggested that IR leads to increased expression of IFN-γ and tumor necrosis factor-α (TNF-α), or adhesion molecules like ICAM-1 and E-selectin (Quarmby et al., 1999). Lowe et al. (2009) demonstrated that low dose of IR trigger gene modulation which is different than high dose and are associated with brain specific functions such as memory, learning and cognition. In agreement with the idea that IR low dose is a potential risk factor for AD, Lowe et al. (2009) have shown that the global gene variations in the irradiated mice brain were comparable to those detected in AD patients. Overall, changes by early IR involved ion regulation, signal transduction mechanisms and synaptic signaling; late changes involved metabolic functions, including myelin and protein synthesis.

Brain atrophy with neurologic and mental weakening has been recognized a few months later in radiation therapy patients without recurrent or residual brain tumors. Few reports suggest that dementia can be detected in 0% of long-term brain tumor survivors treated with radiotherapy (Imperato et al., 1990). Global damage of the cerebral white matter and progressive brain atrophy has been seen in magnetic resonance imaging (MRI) and computed tomography (CT) data (Asai et al., 1989). The neurological deficits of high-dose radiation are thought to be due to neural loss and demyelination with associated cognitive and neural deficiencies. Some of these cognitive defects after exposure to IR have been observed as a consequence of impaired neurogenesis.

Radiation effects in the CNS are more noticeable in kids than in adults; IR- induced cognitive effects comprise learning incapacities and are more pronounced in younger children. Studies from prenatally exposed atomic bombing of Hiroshima and Nagasaki particularly if that exposure occurs at critical stages in the growth of the neocortex stated that IR during gestation has harmful effects on the development of human brain.

Data on a variety of measures of cognitive function, including the occurrence of severe mental retardation as well as variation in the IQ and school performance, show significant effects on those survivors exposed 8–15 weeks and 16–25 weeks after ovulation. MRI from these mentally retarded survivors

has revealed a large abnormally situated gray matter which suggest an abnormal migration of neurons lead to cognitive function of the brain (Schull and Otake, 1999). Rola et al. (2004) have irradiated (2–10 Gy) 21-day-old C57BL/J6 male mice brain to determine the acute radiosensitivity of the dentate subgranular zone and performed immunohistochemistry of the tissues harvested 48 h after radiation. Histopathological analysis of the tissues showed dose dependent decrease in immature neurons. For analyzing long term effects of radiation in the brain, mice were given a single dose of 5 Gy whole brain irradiation. Radiations significantly decreased the production of new neurons after one and 3 months, however, glial cells showed no change. Three months later irradiation, changes were observed in spatial memory retention deficits which provide evidence that young animal irradiation induces a long-term impairment of subgranular zone neurogenesis that is associated with hippocampal-dependent memory deficits (Rola et al., 2004). Effects on adult neurogenesis within the hippocampus may be related to such deficits. To investigate this, Ben et al have irradiated adult mice brain with 4 Gy single dose and observed 80% decrease after 48 h in the cells immunoreactive for the proliferation marker 16, Ki67. The number of pyknotic cells increased approximately 2.5 fold after 16 h. However, all these levels came to normal after few days. The radiation effect was reversible on proliferation and neurogenesis in the dentate gyrus (Ben Abdallah et al., 2007). It was shown that irradiation of 10-day-old mice at 8 Gy resulted in decreased hippocampal neurogenesis and afterward increased the susceptibility of the adult brain to hypoxia-ischemia (Zhu et al., 2009). IR to the immature brain produced long-lasting changes, including resulting in larger infarcts, decreased hippocampal neurogenesis, increased hemispheric tissue loss and more inflammation than in non-irradiated brains. Other IR effects on the brain include severe interruption of the blood–brain barrier (BBB) which is resulting from apoptosis induced by radiation of microvascular endothelial cells, as detected in rats and mice exposed to 50 Gy dose (Li et al., 2003). The molecular and cellular events that subtend these defects are still unknown although some development toward understanding has been occurred. Currently, there is no strong human patient data linking low IR exposure to increased AD. Little is known concerning the molecular mechanisms involved in radiation-induced dementia. Therefore, understanding the biological effects of IR at high and low doses is developing as major concern for neurological health. Further, research is now needed to understand more about the association between IR and the risk of developing Alzheimer's.

ROLE OF IONIZING RADIATION IN AGE RELATED MACULAR DEGENERATION

Age related macular degeneration (AMD) is common cause of blindness in all over the world (Sharma et al., 2013a). It is a multifactorial disease with major risk factors like hypertension, environmental, smoking and aging (Sharma et al., 2012, 2013b,c). Problems in choroidal circulation and endothelial cells play important role in the pathogenesis of AMD. IR play an important role in the damage of endothelial cells. Radiation causes oxidative stress which leads to ROS, vascular abnormalities and cause choroid circulation reduction (Peiretti et al., 2006). ROS induce serious damage to biomolecules. ROS attack structural and enzymatic proteins by the oxidation of prosthetic groups, residual amino acids, protein aggregates and formation of cross links as well as proteolysis. In the vital metabolic pathways the inactivation of key proteins can have serious consequences and can evoke single and double stranded DNA breaks which can lead to cell death and can expedite the process of age related macular degeneration. In the radiation model for cataract, post-translational modifications resulted in altered protein–protein interactions, and the formation of high-molecular-weight aggregates that were enriched for αB-crystallin.

There are significantly less studies on the role of IRs in causing AMD and effects in later life. Potential increase in morbidity and stroke after IRs also raises worry about risk of AMD. There is one report on Hiroshima and Nagasaki atomic bomb survivors in later life in which Itakura et al. (2015) investigated the relationship between atomic bomb exposure and the frequency of AMD. In 2006 to 2008, Itakura et al. (2015) selected 1824 participants to assess the prevalence of AMD in atomic bomb survivors. The eye dose for individuals was analyzed with DS02 (a revised dosimetry system) which took account for shielding conditions and physical locations at the time of explosion. For eyes, the absorbed dose was used in gray (Gy), for an individual the dose corresponds to the total exposure in gamma-rays +10 X the smaller neutron dose (Cullings et al., 2006). The estimated exposure dose for 43.6% individuals was $\leq$0.005 Gy and 4.8% were exposed to more than 2 Gy. Though they did not get any significant association with radiation dose and AMD prevalence, which suggested that long term oxidative stress after radiation may lead to suppression of neovascularization in the retina. The prevalence of neovascular AMD in their study was lower as compared to general population in Japan (Itakura et al., 2015). However, high doses of IR has been known to be susceptible for retinal vasculature for some time. The role of IR in protection and cause of diseases can be understand from **Figure 3** (Betlazar et al., 2016). Radiation retinopathy is slowly progressive microangiopathy and was first described by Stallard (1933). The lethal effects of IRs on retina was recognized in Moore (1935). Moore (1935) described the ischemic retinal vasculopathy in retinoblastoma patients treated by radium seeds.

Neuronal cells, particularly photoreceptors, are resistant to irradiation as compared to vascular endothelial cells. A dose dependent loss of pericytes in capillary regions and endothelial cells has been shown by histology. In later stages of life, vision loss is caused by retinal ischemia and retinal edema by secondary conditions, like retinal detachment, neurovascular glaucoma and vitreous hemorrhage. Additional obliterations can be seen in choriocapillaries in addition to inner retina. Archer et al demonstrated that radiation causes an exaggerated vasculopathy in diabetes mellitus patients, and diabetic rats (streptozotocin induced) develop ischaemic retinopathy after exposing to 1500 centigray (cGy) of radiation (Archer et al.,

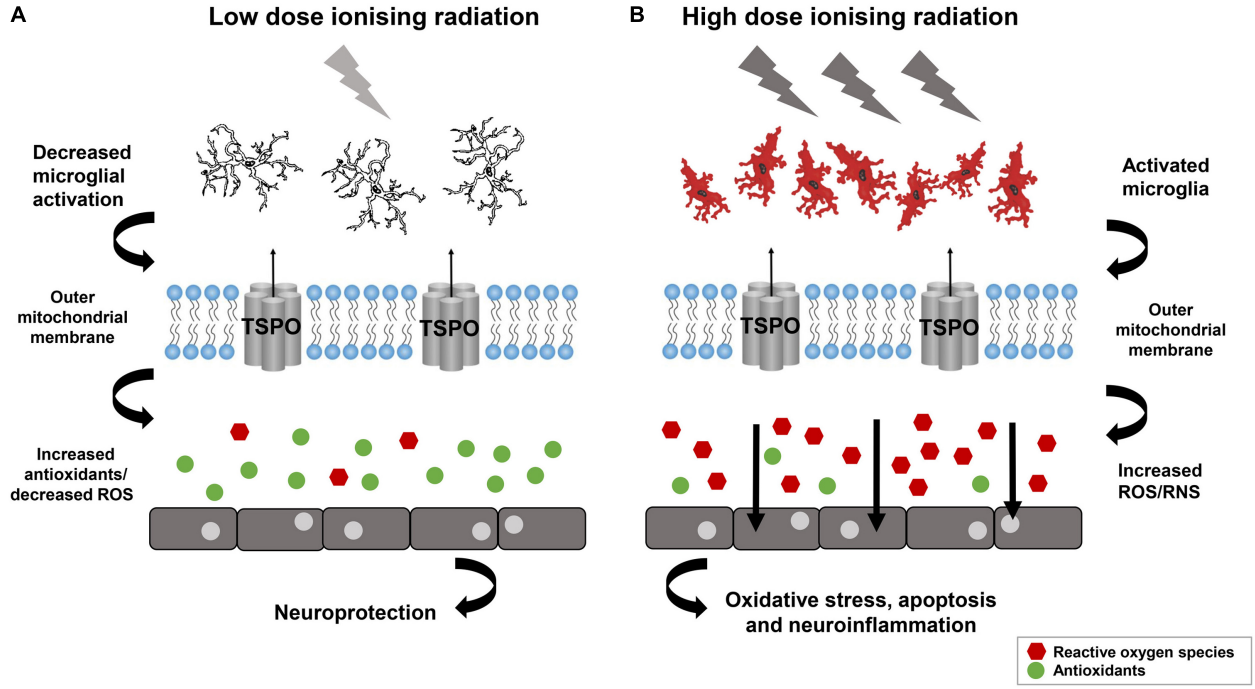

FIGURE 3 | Low dose Ionizing radiation confer not only the neuroprotection but also activate reparative mechanisms. **(A)** Neuroinflammation may decrease by low dose ionizing radiation and can lead in increased antioxidants and reduced oxidative stress. **(B)** Neuroinflammation can be provoked by high dose ionizing radiation lead to microglia activation and ROS which can affect the cell functioning. Reproduced with permission (Betlazar et al., 2016).

1991). Patients who receive a radiation dose of fewer than 2500 cGy in different fractions of 200 cGy are dubious to progress substantial retinopathy (Archer et al., 1991). Mild retinopathy patients progress gradually and visual functions remain almost normal, except if there is any substantial problem in macula. After irradiation at 10 or 15 Gy, wild type mice do not show any pathological change in the retina at any point in time, demonstrating that the wild type eye in mouse is not sensitive to single low dose of IR. However, after exposure to 18.5 Gy photoreceptors become obvious and with increase of dose at 22 and 25 Gy from an X-ray source, photoreceptors start terminating with damage to the retinal pigment epithelium containing large vacuoles (Gorgels et al., 2007). On the other hand, gamma rays at 10 Gy caused extensive retinal cell death in a Cockayne syndrome mouse model, a DNA repair disorder which suggests that oxidative DNA damage is responsible for the loss of photoreceptors (Gorgels et al., 2007). Amoaku et al. (1989) have demonstrated that the rat photoreceptor cells nuclei which are highly heterochromatic are intricately sensitive to IR as compared to those of the primate photoreceptors which are more euchromatic. Likewise, the inner retinal neurons – mainly ganglion-cells – are more euchromatic and revealed more resistant to radiation. It demonstrates that heterochromatin which is strongly packed is significantly more susceptible to damage after irradiation as compared to finely discrete euchromatin. This finding may clarify the effects of radiation on vascular endothelial-cells, whose nuclei are comparatively heterochromatic.

ROLE OF IONIZING RADIATION IN AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that has also been linked to radiation exposure. ALS is a motor-neuron disorder, where the neurons controlling the movement die. Various investigations have associated the risk of ALS with the exposure to radiation, primarily initiated with the high number of veterans reported with incidence of ALS related to exposure during service (Haley, 2003; Horner et al., 2003).

Majority of ALS cases reported are sporadic in nature, with just 10–20% cases connected with family history. In recent times, the studies of ALS and associated genetic mutations have gained pace, which has resulted in many discoveries. Mutations in different genes have been associated in past with the ALS, mainly in the gene encoding the antioxidant enzyme, superoxide dismutase 1 (SOD1), indicating important role of oxidative stress in ALS pathogenesis (Turner et al., 2013). Another gene that has been investigated with respect to ALS is the apurinic endonuclease or apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1), which has shown to be a part of DNA repair and plays a neuroprotective role against oxidative stress and exposure to IR (Vasko et al., 2011). The APEX1 mutants did not show any redox activity as evident from a Scottish population study, where sporadic ALS patients reported an amino acid change mutation in the APEX1 gene (Hayward et al., 1999). Another gene discovery that has associated IR

exposure to ALS is the FUS gene. The knock-out of the FUS gene in different studies has revealed aberrant DNA repair machinery and hence, sensitivity toward radiation exposure (Hicks et al., 2000; Kuroda et al., 2000). It has been postulated that the FUS gene interacts with IR responsive gene, such as cyclin D1 (*CCND1*) and blocks protein translation. Therefore, any mutation in the FUS gene will prevent it from binding with the genes such as *CCND1* and cause protein accumulation as well as further toxicity in motor neurons and cell death (Wang et al., 2008). Consequently, the major abnormality being reported in the ALS becomes the abnormal DNA repair machinery and accumulation of the damaged DNA (Bradley and Krasin, 1982).

Many epidemiological studies also report the disease hazard to the employees in the radiation related industries. The correlation between electrical exposure and occurrence of ALS was first reported in Haynal and Regli (1964). In a study pertaining to IR, it has been shown that the gamma-irradiation exposure perceived during occupation, diagnosis or accident has genotoxic effects in irradiated mice (Graupner et al., 2016). On the contrary, there are a few studies which nullify any involvement of radiation exposure with the disease emergence (Parlett et al., 2011).

Some familial cases (fALS) are associated with mutations in SOD1 which is an antioxidant enzyme whose action is conserved in many mutant forms, it was reported that in motoneurons wtSOD1 was present in cytoplasm and nuclei, while mutant SOD1 was mainly cytoplasmic (Sau et al., 2007). Any disruption in the SOD activity responsible for misfolded protein clearance in the two subcellular compartments. Cells with G93A-SOD1 mutation exhibited more DNA damage compared with those expressing wtSOD1 (Sau et al., 2007). Mutant SOD1 toxicity might therefore arise from misfolding or destabilization of the protein. However, there are no point mutations in SOD1 linked to fALS, suggesting that the whole SOD1 protein is involved (Khare et al., 2005). Protein misfolding may then activate a cascade of actions which include protein buildup, possibly followed mitochondrial or proteasome dysfunctions and axonal transport alterations. Additionally, these events may indirectly lead to activation of caspase and ROSs (Boillee et al., 2006). All these events might be interconnected and thus worsening the severity of the preliminary trigger, the protein misfolding. Poulletier de Gannes et al. (2009) investigated the role of chronic exposure to electromagnetic radiation on the SOD1 mutant mouse model, the model used to mimic ALS. The study did not reveal any difference between the extremely-low frequency (ELF) magnetic fields exposure and development of ALS. Similarly, cells derived from the SOD1 mutant ALS mouse model were subjected to X-ray irradiation and the radio-sensitivity was assessed, which indicated no association between the SOD1 mutation and radio-sensitivity (Wate et al., 2005). In an identical study, cells isolated from patients with SOD1 mutations, when compared to sporadic ALS patients, revealed no difference in the production of DNA double-strand breaks after irradiation (Mithal et al., 1999). Further investigations are required to reveal a concrete correlation between the radiation exposure and ALS.

ROLE OF IONIZING RADIATION IN ISCHEMIA RELATED DEGENERATION

Stroke is cerebrovascular disorder characterized by diminished blood supply to the brain. As a result, ischemic damage occurs, which is characterized by death of the brain cells resulting in plethora of signs and symptoms in concurrence with area of brain involved.

About 95 percent of stroke cases are present above the age of 65 years. Death rate and morbidity associated with stroke has increased with age and chances of recovery are minimal following a paralytic event. Since the incidence and prevalence of stroke has been increasing worldwide, it is important to highlight the emergent risk factors; especially targeting the role of IR. A case control showed direct causal relationship between the amount of dose and the cerebrovascular events in groups, which were treated by radiotherapy for non-malignant and malignant diseases and also in those exposed to environmental factors of radiation (Little, 2016). In another cohort study by Azizova et al. (2014) the incidence and mortality due to cerebrovascular diseases was assessed in 22,377 workers working in Mayak Production Association in 1948–1982 and were monitored up to 2008. These workers were subjected to occupational exposure to gamma and alpha rays. The mean plutonium body levels were calculated for both the types of radiation in males and females. Categorical analyses showed increased cerebrovascular disease incidence between employees with total absorbed external gamma-ray doses more than 0.1 Gy compared to lower doses exposed. The study shows direct linear relationship between risk of stroke and concentration of radiation exposure (Azizova et al., 2014). The immunological mechanism behind the pathogenesis of stroke is splendidly explained by Khaled et al. (2012). They exposed human aortic endothelial cells to 0–30 Gy x-rays and measured adhesiveness of endothelial cells using flow chamber assay. After 24 h of radiation, adhesiveness was increased with peak effect at 15 Gy. The study established a direct link between adhesiveness of chemokine associated endothelial cells which lead to subsequent increased risk of stroke (Khaled et al., 2012). In another study, Rajaraman et al. (2016) assessed the incidence and mortality among US medical radiation workers using fluorescent-guided interventional radiation procedures. A prospective cohort study was done with 90,957 technologists who were made to complete a survey regarding incidence of stroke among them spanning from 1994 to 2005. Thirty four percent increase in stroke was observed in technologists who were involved in fluoroscopically guided interventional procedures (Rajaraman et al., 2016). Apart from studies showing the environmental and occupational exposure of radiation various other studies indicated that routine treatment protocols have an equivocal say in the risk of stroke. As evident in study conducted by Plummer et al. (2011) demonstrated the effect of large dose radiotherapy to head and neck following head injury and trauma. Prospective and retrospective trials over past 30 years were conducted involving pathogenesis, imaging, epidemiology, and management of medium- and large-artery extra and intra cranial disease after neck and head radiotherapy.

They concluded that neck and head radiotherapy rises the risks of stroke and transient ischemic attacks in the survivors (Plummer et al., 2011). MicroRNA's (miRNA) are the non-specific RNAs, which cause post translational gene modification and increase the risk of certain malignancies. In a study conducted by Borghini et al. (2013), effect of low dose IRs in interventional cardiology were studied. These reports have demonstrated that using miRNA array, radiations cause dysregulation of brain specific miRNA and might prove to be having a role in pathogenesis of ischemic neurodegenration of brain tissue (Borghini et al., 2013). A study on German WISMUT uranium miners demonstrated the relationship among external gamma radiation and cerebrovascular diseases. The cohort study includes 58,982 former workers of the Wismut Company. During the follow up from 1946 to 2008 there were 9,039 deaths from cardiovascular diseases. Exposure to external gamma radiation was studied using job exposure matrix. The exposure was calculated using expert details till 1954 followed by measurements thereafter. Mean dose was 97 mv followed by max of 909 mv. Increased risk for death due to cerebrovascular incidents was recorded as compared to other cardiovascular events (Kreuzer et al., 2013).

Stewart et al. (2006) explained the effect of IR in developing atherosclerotic lesions in APOE mice and its risk of developing hemorrhage. They used a mouse model to study radiation induced atherosclerosis and compared it to age related plaque. Atherosclerosis prone APOE mice were subjected to radiation at the central vessels. At 22, 24, and 28 weeks, blood samples were taken and studied for changes in different markers. Cholesterol levels and inflammatory markers were not that much different from age-matched controls, however, there was increase in macrophages and marked influx of granulocytes predicting the role of inflammation. Intra-plaque hemorrhages and macrophage rich RBCs presence is prone to stroke like modality (Stewart et al., 2006). Injury to CNS results in inflammatory response, which is characterized by increased leukocyte activation and induction of cytokines. Injury model like stroke showed a marked influx of inflammatory mediators to CNS. This has been ascribed to increased levels of ICAM-1. In a mouse model, Olschowka et al. (1997) studied the relative induction of ICAM-1 using quantitative RT-PCR after 6 h following irradiation with either 0, 5, 15, 25, or 35 Gy and immunohistochemistry was done at 4, 24, 48 h and 7 days following 25 Gy irradiation. Results showed that the levels of ICAM1 and protein concentration increased (Olschowka et al., 1997) and suggested that activated ICAM-1 increases gene expression for proteins and resulted in increased risk for ICAM associated clotting which may increase risk of ischemic and hemorrhagic stroke.

After taking into consideration the human and natural sources of radiation, it is imminent to lay focus on the radiation exposure due to nuclear weapons detonation. In Japan, Takahashi et al. (2013) studied the impact of radiation on long-term survivors of atomic bomb at Hiroshima and the incidence of various cerebrovascular diseases in them. They documented that depending upon the age of exposure, density of exposure and the time of exposure to radiation from the atomic bombing, there was several fold increase in risk of certain cerebrovascular events like stroke, hemorrhages and myocardial infarction (MI) (Takahashi et al., 2013). A mathematical model constructed by researchers in the imperial college London predicts the role of low dose IR in cardiovascular events. They suggested that IR results in the killing of monocytes of the endothelial wall which would otherwise bind to MCP-1 whose levels increase in their absence. High levels of MCP-1 cause inflammation in the vessel wall resulting in damage to the cardiac tissue and may increase the risk of stroke via thrombus generation (Little et al., 2009). Stroke is a multifactorial disease, targeted by thrombotic, embolic and ischemic events. The risk of stroke and cerebrovascular insult increase with exposure to IR and the duration of exposure. Statistical studies discussed above have focused another aspect of stroke pathology related to radiation exposure. However, due to high background level of cerebrovascular accidents in civilian population, more emphasis should be laid on observing the overall impact of radiation by studying the animal models and co-relate them with human studies.

ROLE OF IONIZING RADIATION IN PARKINSON'S DISEASE

Parkinson's disease (PD) is the most common neurodegenerative disorder and its underlying molecular mechanisms are not fully understood. Aging in combination with the environmental and genetic factors plays a key role in the etiology of PD (Jenner et al., 2013; Xu and Chan, 2015). Environmental factors, such as pesticides, herbicide, metal irons and IR are the significant risk factors for PD (Xu and Chan, 2015). Inflammation and oxidative stress have also been associated with PD (Wang et al., 2013). The IR induces the inflammation and stress toward the neurobiological responses (Betlazar et al., 2016) and can cause PD. The most common defect of CNS is the neurogenic process in the brain and CNS is very sensitive to chemotherapy and IR (Acharya et al., 2015). IR exposure can lead to radiolytic lesions through various cellular process increasing the global stress response and impacting the DNA repair, cell cycle progression, and survival (Fike et al., 2007, 2009; Tseng et al., 2014). The IR response to neural precursor cells in the hippocampal dentate gyrus cell, altered neurogenesis suggesting to play a critical role in cognitive impairment (Mizumatsu et al., 2003). Further, the learning and memory deficiencies caused by the hippocampal dysfunction resulting into a long-term absence of normal stem/progenitor activity by IR (Monje and Palmer, 2003).

In our routine life, we are constantly exposed to the IR either from natural and/or man-made sources and it is known that IR exposure contributes to the etiology of neurodegenerative diseases (Kempf et al., 2013). PD is a progressive neurodegenerative disease and oxidative stress is involved in the progression of PD. The increase in ROSs damages the target neuronal cells (Hirsch, 1993) and dopaminergic neurons are more susceptible to oxidative stress. Further, the mitochondria plays an important role to maintain the cellular energy and calcium homeostasis. Any mitochondrial dysfunction results to energy and calcium imbalances, leads to cell death (Wang and Michaelis, 2010). It has been established that

dopamine metabolism and mitochondrial dysfunctions are the leading cause elevated ROS in PD progression (Greenamyre and Hastings, 2004; Licker et al., 2009).

The known etiology of PD is to bear the alterations at molecular level when and compared to effect of low dose IR such as less than 5 Gy (Kempf et al., 2013). The children and young adults are more prone to IR and the role of X-rays and CT scans cannot be ignored as one of contributor of IR (Kempf et al., 2013). Thus, it is very important to analyze the *in vitro* and/or *in vivo* radiation data regardless of radiation source and age of animal models or human subjects.

It has been known that low doses of IR leads to mitochondrial dysfunction either by interacting with mitochondrial DNA (mtDNA) or through the formation of reactive hydroxyl radicals (Prithivirajsingh et al., 2004). Both the IR-induced damage and mtDNA alterations are important and critical to induce mitochondrial impairment to cause neurodegeneration. Therefore, mitochondria is the marked target for the late onset damage of low dose radiation. It has also been shown that after low-dose gamma-ray irradiation (0.5 Gy) in C57BL/6 mice brain, the antioxidant molecules, glutathione (GSH) and thioredoxin remained, elevated upto 12 h (Prithivirajsingh et al., 2004). This also increases the oxidative stress in PD due to depletion of total GSH followed by mitochondrial dearth (Jenner, 1993). The radiation-induced damage to mtDNA affects the mitochondrial synthesis (Malakhova et al., 2005). Further, the protein thiol moieties are considered as a key target of radiation-induced oxidation via ROS pathway (Winterbourn and Hampton, 2008). The imbalance in mitochondrial activity leads to neurodegeneration in PD (Arduino et al., 2011) and this destruction develops at early stage of PD (Bueler, 2009). Recently, Kobashigawa et al. (2011) showed that dynamin-related protein-1 (Drp1), a major regulatory component to manage the mitochondrial fission rate accumulated in mitochondria of normal human fibroblast cell after exposure to 6 Gy gamma radiations (Kobashigawa et al., 2011). Furthermore, after IR exposure, the loss of mitochondrial membrane leads to neuronal cell death in PD due to impaired oxidative phosphorylation and increase in apoptosis (Kobashigawa et al., 2011).

In the neuropathology animal model system, low dose IR confer not only the neuroprotection but also activate reparative mechanisms (**Figure 3**) (Betlazar et al., 2016). The PD model, "1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)" showed increase in the levels of glutathione and catalase after 3 h exposure to 0.5 Gy gamma radiation (Yamaoka et al., 2002), which is further confirmed using mouse model of PD after whole body gamma radiation at 1.5 Gy (El-Ghazaly et al., 2015). The human epidemiological and animal model data suggest that the neurotoxin, MPTP and the pesticide, rotenone can trigger Parkinson like symptoms by inhibiting the mitochondrial Complex I and activating the ROS production demonstrating that the dysfunction of the mitochondria is a characteristic of PD (Coskun et al., 2012). The role of DNA damage in PD has also been shown in a cell culture based model system developed by Robbins et al. (1985). The lymphoblastoid cell line developed from the patient with sporadic PD were irradiated and they showed the genetic defect of DNA damage caused by somatic mutation during embryogenesis. This DNA repair defect leads to the abnormal accumulation of DNA damage in PD (Robbins et al., 1985). The radiosensitivity of sporadic PD patients' cell lines which does not involve the patients' germ cells showed premature death of their neurons *in vivo* due to genetic defect arising from dominant somatic mutation cropping up during embryogenesis (Robbins et al., 1985). The DNA damage caused by normally occurring DNA-damaging cellular metabolites, ROS, and spontaneous hydrolytic reactions provide the platform for the *in vitro* radiosensitivity and *in vivo* premature death of neurons in PD (Bankers Life and Casualty Company, National Cancer Institute (US), and International Symposium on Aging and Cancer, 1982). *In vitro* X-rays exposure of cells results in several unrepaired lethal lesions in DNA leads to PD. The *in vitro* radiosensitivity study of cultured non-neural cells from PD disease patients showed lethal abnormality elucidating the underlying mechanisms responsible for PD. The different clinical and neuropathological patterns of PD resulted from different defective repair processes originated from different mutations or DNA damage (Robbins et al., 1985). These findings indicate that mitochondria is a direct target of IR and mitochondrial defects leads to the development of PD.

Radiations may speed up the folding and unfolding of the proteins. It has long been known that the stability of proteins with respect to denaturation (as defined by aggregation) is lowered upon treatment with IR. α-synuclein, the main element of Lewy bodies, is highly conserved presynaptic protein linked to both familial and sporadic PD. The molecular mechanism for PD is strongly associated with α-synuclein aggregation (Breydo et al., 2012). The abnormal aggregation of α-synuclein in neurons leads to development of PD (Gundersen, 2010). Previously it was shown that α-synuclein protein is highly susceptible to dityrosine (DiY) crosslinking in protein by UV irradiation, resulting in DiY-modified α- synuclein monomers and dimers (Wordehoff et al., 2017). Radiation-induced oxidative stress can cause compromised mitochondrial functioning, protein misfolding and endoplasmic reticulum (ER) stress, besides DNA damage. Due to stress, parkin gene can also misfold the protein similar to α-synuclein. Martin et al. (1993) observed an increase of striatal D1 and D2 dopamin receptor density in discrete cerebral areas of rats, 2 h after exposure to (neutron-gamma) radiation at the dose of 5.5 or 7.5 Gy (Martin et al., 1993). The radiation may cause the α-synuclein aggregation, parkin gene misfolding and dopamine metabolism which may lead to inappropriate mitochondrial activity, Lewy bodies and cellular stress, the key component for the PD (Robinson, 2008).

CONCLUSION

More detailed epidemiological studies as well as a better understanding of biological mechanisms are needed according to the evidences presented here in this review, which may address the misclassifying factors. The risks of circulatory, age related and neurodegenerative diseases are similar to those of

radiation-induced cancers as reported for non-cancer diseases. Health effects after deep space radiation continue for long periods after exposure. Many circulatory diseases were observed in atomic bomb survivors in Japan at low doses as 0.5–2.0 Gy. Developing age related diseases after exposure to therapeutic and diagnostic purposes is always a health concern. However, slow growing tissues like brain require long exposure and high doses for developing degenerative symptoms. Different kinds of radiant energy cause diverse health effects ranging from birth defects to age related diseases decades after exposure, which depend up on the kind and conditions of radiation exposure. To understand the mechanism of radiation exposure in age-related and neurodegenerative diseases, further long term studies are needed.

AUTHOR CONTRIBUTIONS

NS and SG conceptualized, designed, edited the manuscript, and wrote the manuscript. RS edited the manuscript and contributed content for introduction and conclusion. DM contributed content for the Multiple sclerosis. GM contributed content for the ALS. SS contributed content for the PD. KB contributed content for the Stroke. AA edited and conceptualized the manuscript.

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Milk metabolites and neurodegeneration: Is there crosstalk?

Keshav Thakur, Akshay Anand

Neuroscience Research Lab, Department of Neurology, PGIMER, Chandigarh, INDIA

ABSTRACT

Milk has been considered as a natural source of nutrition for decades. Milk is known to be nutrient-rich which aids the growth and development of the human body. Milk contains both macro- and micronutrients. Breast milk is widely regarded as the optimal source of neonatal nutrition due to its composition of carbohydrates, proteins, minerals and antibodies. However, despite the wide use of milk products, investigations into the role of milk in degenerative diseases have been limited. This review will examine the relationship between the β-casein gene found in bovine milk and disease states by using age-related macular degeneration as an example.

KEYWORDS: Milk metabolism, A1/A2 alleles, β-casein, Opioid receptors, BCM-7

*Corresponding Author: Akshay Anand, PhD, Additional Professor, Neuroscience Research Lab, Department of Neurology, PGIMER, Chandigarh, 160012; Tel: +91-9914209090; E-mail: akshay1anand@rediffmail.com

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Introduction

Types of milk

Milk has been an important source of nutrition for several decades. Humans have been rearing animals for milk since the beginning of time/ beginning of history. Recently, interesting results from milk protein efficacy studies has reignited the need to examine the biological effects of proteins obtained from milk. RB Elliot and his group described that bovine milk may contribute to the risk of type 2 diabetes mellitus as well as heart disease. They conducted a study in Polynesian children with diabetes and found increased antibody levels against cow milk.[1] The suspected antigen was β-casein, a protein which is common constituent of milk from all species. A genetic study by same group showed the impact of different variants of β-casein gene.[2]

Immunoreactivity of milk metabolites

A review of the relevant literature indicates that the β-casein gene has 12 genetic variants, out of which the A1 and A2 genetic variants are most studied with respect to different diseases in human population. Although A1 and A2 differ from each other only by difference of one amino acid, their health outcomes are different.[3] In 2003, it became known to the public for first time that there are three types of cow'smilk, namely type A1, A2 and A1/A2 mixed. In 1995 theNew Zealand investigators of A2 Corporation Limited filed a patent (ID No: EP19950937232). This patentpostulated a mechanism to distinguish between the A1 and A2 milk types, which became known later as the diabetogenic and non-diabetogenic milk types. The patent applicationstarted a series of studies in milk research through the following claims.. The patent claimed that the A2, A3, D and E β-caseinvariants are non-diabetogenic variant of milk, with A2 being the preferred variant. The A1, B, C and F variants are diabetic, with the A1 variant being the most diabetogenic. A1 milk could be identified with chromatography screening for the presence of the hexapeptide Pro-Gly-Pro-Ile-His-Asn.. The nature of milk in a specific breed of cattle may be heritable. This patent lead to the practice of selling selected milk.[4] Later in 1998, another patent filed by New Zealand dairy board and New Zealand child health research foundation stated A1 milk triggers a beneficial immunogenic response.[5] These patents were the basis of separate marketing and selling ofA1 and A2 milk variants. Digestion of our food starts from our mouth. However, casein is not digested in mouth, but it immediately dissolves in gut enzymes. The peptides forming up after the breakdown of casein are shown to have positive immunoreactivity towards immunoglobulin (Ig) E,G,G4 or A. The levels of immunoreactivity after casein digestion may vary from variant to variant. As in case of Beta casein the reactivity levels are minimised towards the end of digestion, whereas it may be different story in case of alpha casein. As the immune reactivity depends on the epitopes, the changes in genetic variants of peptides arising out of casein digestions may modify the whole immunoreactions in body.[6,7] Recently as tudy in 2014 conducted a test to identify the role of IgA and TGF beta specific to casein in Food protein-induced enterocolitis syndrome (FPIES) to milk a gastro intestinal hypersentivity disorder in children. The study showed the minimal titer levels for IgG and IgA and absence of TGF beta levels stating tGF beta as a possible biomarker whose lowering levels may indicate the person being affect by (FPIES).[8]

Factors responsible for the A1 or A2 variant of milk

Genetic Variations

Over the last decade, various studies have documented the two types of milk. In 2003, Tailfor*det al.* reported that the presence of A1 and A2 variants is largely affected by thecattlebreed.[9] However, the role of epidemiological and etiological factors in milk type requires further investigation. In 2009, a group of researchers led by Olenski stated breeding as a reason for variation in β-casein milk protein. A regression model was used to study the association between the breeding values and milk variant. Genotyping of breeding bulls for A1 and A2 allele was suggested as an important precaution for lowering the risk of A1 allele in human health.[11] Below is the table consisting of studies carried out in different breeds animals.

Table 1. Table of studies involving different genes.

Is β-casein conserved in mammals?

Does β-casein gene impact all animalspecies ? In 1990 a Danish group published a report about the genetic polymorphisms for different proteins found in milk in 4 different breeds namely Danish Jersey,

Table 1: Studies depicting the analysis of polymorphisms in different genes for their polymorphisms

S. No.	Study	Animal Breed	Factor	Genes Scanned	Reference
1.	Polymorphism of the beta-casein gene and its association with breeding value for production traits of Holstein–Friesian bulls	Holstein–Friesian bulls	Breeding	None	[11]
2.	Milk protein polymorphism in Danish dairy cattle and the influence of genetic variants on milk yield	Danish Jersey, Red Danish Dairy Cattle (RDM), and Black and White Danish Dairy Cattle (SDM)	Genetic Polymorphism	αs1, β and K-cascin and β-lactoglobulin (β-Lg)	[12]
3.	Effects of genetic variants in milk protein on yield and composition of milk from Holstein-Friesian and Simmentaler cows	Holstein-Friesian (HF) and Simmentaler cows	Genetic polymorphism	αS1-, β- and κ-caseins (Cn) and β-lactoglobulin (-Lg)	[13]
4.	Milk protein genes CSN1S1, CSN2, CSN3, LGB and their relation to genetic values of milk production parameters in Czech Fleckvieh	Czech Fleckvieh	Genetic polymorphisms	alphaS1-casein (CSN1S1), beta-casein (CSN2), kappa-casein (CSN3) and beta-lactoglobulin (LGB)	[14]
5.	Detection of milk protein genetic polymorphisms in order to improve dairy traits in sheep and goats: a review	Sheep, Goat	Genetic polymorphisms	α-s1 and α-s2 casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin	[17]
6.	Milk protein polymorphisms in Holstein cattle	Holstein Cattle	Genetic Polymorphisms	α_{s1}-casein, β-casein, κ-casein and β-lactoglobulin	[18]

Red Danish Dairy Cattle (RDM), and Black and White Danish Dairy Cattle (SDM). The authors argued in the paper that the levels of 4 proteins i.e αs1, β and K-caesin and β-lactoglobulin (β-Lg) were variable in all the four breeds they studied.[11] However, from earlier studies it is also evident that the effects of β-casein also varies from animal to animal. In 1999, a study by the Elliot group showed the relationship between A1 β-casein form and incidence of diabetes. The group compared the data for diabetic patients, ranging from 0–14 years of age, from 10 different countries with a high cattle milk consumption. The group reported that the A1 variant formed Beta Casomorphin-7, which largely affected the opioid activity of different endogenous bio-chemicals. The A1 variant is absent in human and goat's milk – which are both rich in the A2 casein variant.[2] Some researchers have compared the effect of ' A1 and A2 milk metabolites on atherosclerosis in a rabbit model.[9] In 1986, a report by Obaid Ullah Beg confirmed the presence of β-casein protein in camel milk. β-casein Later,[10] Salami *et al.* reported camel milk β-casein as a good source of safe antioxidants which is easy to digestThe authors also postulated that camel milkinhibits the Angiotensin Converting Enzyme (ACE).[19] which regulates blood pressure.[20] Similarly in 2014 another group reported the increase ACE inhibitory activity of betacasein in bovine milk. The digestion of casein from bovine milk lead to increased levels of antioxidants as well as significant decrease in ACE expression thus pointing to conservatory phenomenon with respect to casein in mammals through evolution.[15] A comparison of the the α-s1, α-s2, β, and κ-casein, β-lactoglobulin and α-lactalbumin polymorphisms in sheep and goats show that. how the change in gene structure may impair the quality of cheese and milk product in both the animals. Polymorphisms are the quality determining factor of milk produced and they can impact the choice of breeding in mammals at invitro levels thus signifying the importance of common genetic pattern of casein gene in both mammals.[11] The phenomenon of casein common activity can be owed to the conservation of gene sequence in mammals. As reported by Kaimala in 2015 the author has identified the gene present in locus of casein. The authors reported the presence of Evolutionary conserved sequence in vicinity of ODAM gene in almost all the mammals.[16]

Milk metabolism and Opioid receptors

Human milk consists of approximately 20–40% casein protein.[3] Human milk is known to contain β-casein. β-casein has 13 allelic forms, out of which A1/A2 is most studied.[3] Polymorphisms in A1 β-casein are associated with risk factors of several syndromes and neurological disorders. Metabolism of A1 form of β-casein leads to formation of β-Casomorphin, also popularly called as BCM7.[21] BCM7 and lactoferrins are known to have opioid activity.[22,23] Lactoferrins are derived from whey proteins, another constituent of milk. The opioids are a class of psychoactive drugs which include morphine. Opioids are derived exogenously through food intake. Stimulation of the opioid receptors is responsible for its activity. These receptors are mostly found in both in central nervous system (CNS) and Peripheral Nervous

Systems (PNS) as well as the duct area regulated by mu opioid receptor.[23,24] Disruption of opioid activity may alter the the oxidation state of a cell. In 2006, Li *et al.* showed that endogenous opioids play a protective role in reducing the Low Density Lipoprotein (LDL)- associated oxidative stress levels in the human brain.[25–27] However, contrary to this, certain other studies show that opioid involvement hampers the oxidation balance and may lead to generation of oxidative stress.[28,29]

Cholesterol is one of the major risk factors of heart disease. Cholesterol is one of the main metabolites of milk protein. It takes on the form of either beneficial High Density Lipoprotein or damaging LDL. In 2003, Laugeseninvestigated the correlation between the milk metabolite A1, Ischemic heart disease and Type 1 diabetes. The study showed a strong correlation between A1 bovine milk and ischemic heart disease (IHD). The data from 20 different countrieswas comparable. However, a serum cholesterol analysis did not findasignificant relationshipbetween cholesterol and A1-rich bovine milk. The group showed that A1 bovinemilk had significant a correlation with IHD-related mortality, which was ascribed to genetic variability in the cow population.[26] Previously, in 1998, Estévez-Gonzálezanalyzed the LDL and serum cholesterol levels in population of with an age group of 3-9 years. The investigators showed that serum cholesterol levels and LDL levels, arehigher in the group which consumed whole milk. When the whole milk was replaced with fat free milk with oleic acid supplementation, serum cholesterol and LDL levels decreased. The caloric content of fat free milk and whole milk was comparable.

A1/A2 role in Age Related Macular Degeneration (AMD) pathogenesis

The A1 and A2 milk metabolites are associated with diabetes and age related disorders, such as.....reference. One such a disorder is age-related macular degeneration (AMD). AMD is the leading cause of blindness in the elderly. This disease is characterised by loss of vision in central field i.e. macula. This loss occure due to damage of retina. Retina is a structure in an eye which is composed of nerves and is responsible for sending light signals to brain. It is also called as part of CNS, thus bringing the AMD under the catogery of neurodegenerative disease.[30] Numerous studies have shown that inflammation plays a large role in the pathogenesis of AMD (Telander*et al.*, 2011). Although the relationship between the A1 andA2 alleles and AMD has not been established, studies have shown that A1/A2 to regulates the expression of AMD inflammatory markers. AMD is worsened by vascularization and this causes irreversible vision loss. Certain metabolites, such as (insert metabolite here) affects AMD progressionthrough altering the expression of Vascular Endothelial Growth Factor VEGF. Milk metabolites may have therapeutic benefits for retinal degeneration. Studying these effects with models of retinal degeneration will open a therapeutic window for the use of nutritional supplements in degenerative diseases.

AMD is a complex aging-related disorder, with a multifactorial progression profile. Investigating the role of environmental factors could shed light on this subjectA possible environmental cause are dietary lipids. Lipids are an important constituent of milk. Studies suggest that high lipid intake may increase the risk of developing AMD.[31–33] Mutations in the Lipase, Hepatic (*LIPC)* gene, which encodes triglyceride lipase, is a known risk factor for AMD.[34] Similarly, other genes like Apolipoprotien E *APOE*,[35] Cholesteryl ester transfer protein *CETP*[36] are also known to be involved in lipid metabolismand the pathogenesis of other age-related disorders such as Alzheimer's disease. APOE protien is derived in liver and is responsible for transport of cholesterol to neurons to provide the myelin covering sheath and protect them from degeneration. APOE has 4 main variants out of which variant 4 is found to be highly associated with patients of Alzheimer's disease. This may be due to APOE high binding affinity to Beta Amyloid plaques thus leading to memory loss. However in relation to AD amyloid beta plaques find a common role in both AD and AMD due to its presence in both brain as well as drusen an extracellular deposit between retinal pigment epithelium and Bruch's membrane.[37–39] For the last two decades the link between AMD and APOE has been a subject ofcontroversy. l. In 1998, a study linked theAPOE epsilon 2 variant with AMD at genetic level.[40] In 1998, Zarbin stated that APOE was involved in pathogenesis of AM D.[41] In 2011, Gareth *et al.* performed a pooled analysis to estimatethe risk conferredby APOE variants in pathogenesis of AMD. The study reported that the APOE 4 variant protects against AMD and APOE2 is a risk factor for late-onset AMD.[42] Other studies found similar results.[40,42–44] Mutations in these genes increases the levels of lipoproteins metabolized from lipids which increases cellular oxidative stress. These lipoproteins are further oxidisedby BCM7, thus increasing oxidative stress.[45] Reactive Oxygen Species (ROS) activate Hypoxia inducible factor 1α (HIF-1 alpha). HIF-1 alpha activates VEGF to initiate vascularisation which contributes to the pathology of AMD. Lactoferrin, another compound, from whey protein gives rise to Lactoferricin B LfcinB. LfcinBbinds to the heparin binding site of endothelial cells, through competitive inhibition of VEGF. This prevents angiogenesis,[46] which is beneficial for AMD. The matrix metalloproteinases (MMPs) are a group of compounds involved in the degeneration of the extra cellular matrix. These compounds are regulated by TIMP metallopeptidase inhibitor 3 TIMP3. TIMP3 is localized in humanretinal pigment epithelium (hRPE) AMD.[47] Dipeptidyl peptidase-4 (DPP4), is an enzyme involved in metabolism of β-casein. Inhibition of DPP4 has been shown to reduce the expression of MMP2 and thus DPP4 inhibition may have a protective role in AMD.[48] A reduction of DPP4 is also associated with improved cardiovascular health. Recently on 2014 an animal study in wistar rats reported the association of beta casein variant A1 with DPP4 activation and high inflammation, thus indicating that higher levels of DPP4 in association with Beta Casein A1 variant may lead to increased inflamtory response in body.[49] Several *invitro* studies have shown that DPP4 increases vascularisation through Neuropeptide Y (NPY) signalling. DPP4 converts NPY to a shorter form, NPY3-36, which is responsible for angiogenesis. This review of the literature indicates that the correlation between milk metabolism and age related disorders needs to be more thoroughly investigated. This review has focussed on vascularisation events which are important role in the pathogenesis of AMD. AMD is hypothesised to be regulated by metabolites of milk proteins. The casein and whey protein milk metabolites share acommon pathway in the molecular pathogenesis of AMD (Figure 1)

Conclusion

Vascularisation is an important event in AMD.[50–52] The review explores how the milk metabolites could influence vascularisation and cause AMD to improve the understanding of. role of nutrition in AMD. Vascularisation is involved in the pathogenesis of numerous degenerative

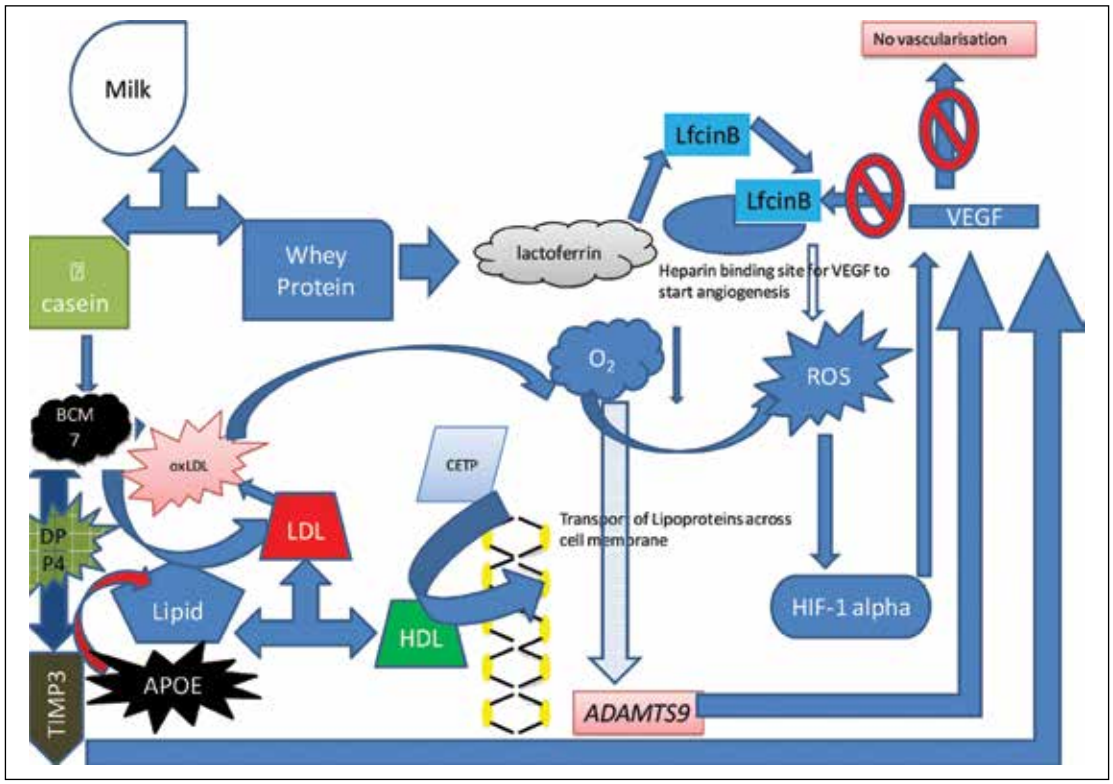

Fig. 1: Central role of vascularisation in crosstalk between different disease markers of AMD with milk protein.

disorders. One of these disorders, is amytrophic lateral sclerosis (ALS), a rapidly fatal neurodegenerative disease.[53–55] Vascularization may improve the survival of of dying neurons. The HIF-1 alpha pathway increases vascularization during times of oxidative stress y.[56] This review hypothesises the role of milk metabolites in hindering the expression of vascularisation. The milk metabolites may have different roles in the pathogenesis of different diseases and thus be beneficial in certain diseases and detrimental in others. There is an imperative need to study role of nutrition in different diseasesto understand the mechanisms by which nutrition alters the outcomes of aging.

Nutrition is a key constituent of our environment. Understanding of the role of nutrition is critical to improve understanding of degenerative diseases like AMD. Milk, which is widely regarded as an important constituent in early life, has been shown to deliberately affect our health. In order to ascertain the role of milk consumption in progression of this degenerative disease, it is imperative that the two alleles (A1 and A2) and their metabolic products be examined in relation with pathophysiology of the AMD.

Authors Contribution

Akshay Anand: Made substantial contributions to conception and design of manuscript, and gave final approval for publication of manuscript, **Keshav Thakur:** Has been involved in drafting the manuscript

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Review

Translation of Pre-Clinical Studies into Successful Clinical Trials for Alzheimer's Disease: What are the Roadblocks and How Can They Be Overcome?[1]

Avijit Banik[a,2], Richard E. Brown[b,2], James Bamburg[c], Debomoy K. Lahiri[d], Dheeraj Khurana[a], Robert P. Friedland[e], Wei Chen[f], Ying Ding[g], Amritpal Mudher[h], Ante L. Padjen[i], Elizabeta Mukaetova-Ladinska[j], Masafumi Ihara[k], Sudhir Srivastava[l], M.V. Padma Srivastava[m], Colin L. Masters[n], Raj N. Kalaria[j,*] and Akshay Anand[a,*]

[a]*Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India*
[b]*Department of Psychology and Neuroscience, Dalhousie University, Halifax, Nova Scotia, Canada*
[c]*Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA*
[d]*Departments of Psychiatry and of Medical & Molecular Genetics, Indiana University School of Medicine, Neuroscience Research Center, Indianapolis, IN, USA*
[e]*Department of Neurology, University of Louisville, School of Medicine, Louisville, KY, USA*
[f]*Division of Pulmonary Medicine, Allergy and Immunology, Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center, University of Pittsburgh, Pittsburgh, PA, USA*
[g]*Department of Biostatistics, University of Pittsburgh, 318C Parran Hall, Pittsburgh, PA, USA*
[h]*Southampton Neurosciences Group, University of Southampton, Southampton, UK*
[i]*Department of Pharmacology & Therapeutics, McGill University, Montreal, QC, Canada*
[j]*Institute of Neuroscience, Newcastle University, NIHR Biomedical Research Building, Campus for Ageing and Vitality, Newcastle upon Tyne, UK*
[k]*Department of Stroke and Cerebrovascular Diseases, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan*
[l]*Division of Toxicology, Central Drug Research Institute, Lucknow, India*
[m]*Department of Neurology, Neurosciences Centre, All India Institute of Medical Sciences, New Delhi, India*
[n]*Mental Health Research Institute, University of Melbourne, Royal Parade, The VIC, Australia*

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[2]These authors contributed equally to this work.

*Correspondence to: Dr. Akshay Anand, Neuroscience Research Laboratory, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India. Tel.: +91 172 2756090; Fax: +91 172 2744401; E-mail: akshay1anand@rediffmail.com and Prof. Raj N. Kalaria, Institute of Neuroscience, Newcastle University, NIHR Biomedical Research Building, Campus for Ageing and Vitality, Newcastle upon Tyne NE4 5PL, UK. E-mail: r.n.kalaria@ncl.ac.uk.

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Abstract. Preclinical studies are essential for translation to disease treatments and effective use in clinical practice. An undue emphasis on single approaches to Alzheimer's disease (AD) appears to have retarded the pace of translation in the field, and there is much frustration in the public about the lack of an effective treatment. We critically reviewed past literature (1990–2014), analyzed numerous data, and discussed key issues at a consensus conference on Brain Ageing and Dementia to identify and overcome roadblocks in studies intended for translation. We highlight various factors that influence the translation of preclinical research and highlight specific preclinical strategies that have failed to demonstrate efficacy in clinical trials. The field has been hindered by the domination of the amyloid hypothesis in AD pathogenesis while the causative pathways in disease pathology are widely considered to be multifactorial. Understanding the causative events and mechanisms in the pathogenesis are equally important for translation. Greater efforts are necessary to fill in the gaps and overcome a variety of confounds in the generation, study design, testing, and evaluation of animal models and the application to future novel anti-dementia drug trials. A greater variety of potential disease mechanisms must be entertained to enhance progress.

Keywords: Alzheimer's disease, animal model, dementia, memory disorder, pre-clinical, treatment

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. Over 7% of the world's population above 65 years of age (over 24 million people) suffer from dementia, with the number of cases estimated to double every twenty years [1–3]. In the most populous countries of the world, India and China, these numbers are estimated to be 3–8 million and are expected to double by the year 2030 [4, 5].

Translational medicine has emerged as a reaction to the slow speed by which new medical research findings are transformed to improved therapies of human disorders. The intent of this movement is to help bench and clinical researchers learn from each other and thus benefit patients [6] forming a "translational cycle" [7]. The most common use of the term translational research describes a "bench-to-bedside" flow, occasionally distinguishing two translational phases. T1 referring to "transfer of new understanding of disease mechanisms gained in the laboratory into development of new methods for diagnosis, therapy, and prevention and their first testing in humans"; T2, involves two phases, T2a, which describes the translation to patients or clinical care and T2b as translation to practice and heath decision making [8, 9] (Fig. 1). The focus of this review is on this arm of the translational research cycle; the contribution of recent studies on animals and more direct systems in the development of therapeutic targets and drugs for treatment of AD.

AD is a complex disease and more of a syndrome. The global threat of this syndrome and its associated social and economic burden has tremendously increased over last two decades [4, 5]. A variety of etiological factors has been proposed to contribute the pathogenesis of the disease. However, there is almost universal acceptance that proliferation of amyloid-β (Aβ) deposition is the root of AD. The central problem confronting AD research today is the failure to recognize other important causative pathological factors in parallel with Aβ pathology. Perhaps this alone has contributed to the failure of multiple drug trials. A new consensus hypothesis to replace one based on Aβ may not necessarily solve the problem, rather replicate the problem all over again. It is certain that Aβ plays a role in AD pathology but other factors ought to be equally investigated simultaneously for better preclinical outcomes. Such an approach may also provide better biomarkers for trials and result in patient cohorts that may be less variable in their response to treatments.

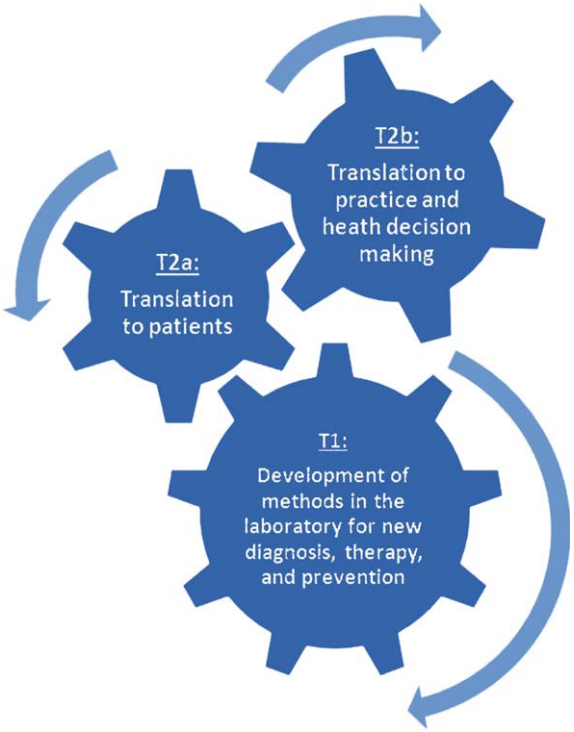

Fig. 1. Pathways from basic discoveries at the bench to clinical practice via essentially three stages (T1 to T2b).

New treatment strategies such as cell transplantation and immune-modulation have shown promising outcomes in various disease models. Their translation, however, into effective clinical treatments has generally not met expectations [10]. For example, the application of traditional herbal extracts such as *Bacopa monniera* [11–13] and traditional Chinese medicines [14] in animal models appear promising but these findings require further refining for translation to successful human trials [15–17]. Similarly, other studies based on phytochemicals, such as Nanocurcumin and S-Allyl-Cysteine (SAC), have also shown promising results in animal models [18, 19]. However, there is a general reluctance to launch new clinical trials based on herbal extracts as there are concerns about their safety, efficacy, and mode of action [20, 21].

The absolute reliance on animal models has been cited as a major factor in the impedance of drug discovery [22]. There are no animal models of any human disease, in particular of those that affect specifically human behavior, cognition, changes in mood and similar, and chronic in nature [23, 24]. Animal models at best mimic certain elements of human pathology. While it is imperative that animal models have added valuable information to our current understanding, the limitations in humanized disease models argue that the critical step in translation is the understanding of human disease pathology as a prerequisite for designing informative animal experiments.

Discovery of successful new drugs for clinical benefit of AD requires stringent analysis for safety and efficacy in suitable animal and cell-culture models. Over the last three decades, several animal models of AD have been developed to understand disease mechanisms and identify therapeutic targets, as well as screen novel drugs derived from synthetic chemistry and/or traditional herbal formulations [25]. Based upon different theories, multiple approaches have been used

Fig. 2. Road blocks in the translation of preclinical outcomes in Alzheimer's disease (AD) from animal to human.

for developing animal models ranging from traumatic brain injury [26] and neuronal cell death induced by intracranial delivery of neurotoxins to specific brain areas [27] and the generation of transgenic mouse models by genetic manipulation which impact upon molecular pathways involved in the pathogenesis of the disease [28, 29]. Naturally, these models need to be widely reproducible and validated, and have the characteristic neuropathological and behavioral/cognitive features of AD. However, it seems a daunting task to decipher which model(s) among the numerous produced may best simulate preclinical disease.

Given these impediments in translating basic research from animal models to treatments, a critical re-evaluation of current animal models as well as clinical trials methodology is imperative to understand the road blocks in the translation of preclinical knowledge for human benefit [30–32]. This review identifies key obstacles (Fig. 2) by use of examples where either central dogmas have been challenged or previous inexact research has established flaws in current understanding. We briefly review the impending limitations in diagnostic as well as treatment strategies, the weaknesses of the amyloid hypothesis of AD, and discuss why some other AD-related pathology should be considered. We then discuss the animal models and their inherent problems in recapitulating human disease, and the need for better integration of basic and clinical studies and for controlled and validated methods for design and analysis of clinical trials.

SLOW PROGRESS IN DIAGNOSIS OF AD

There have been increased efforts to discover unique biomarkers for early diagnosis and identify populations at high risk [33]. An optimal biomarker not only enhances the chance of early detection of the disease but also strengthens attempts to determine the prognosis, enabling disease monitoring. The current strategies do not focus on different biomarkers for different purposes but lump them together. Instead, it should depend upon the use at different stages of the disease management such as, detection of pathology, prediction of progression, surrogate marker of efficacy, or disease modification. Furthermore, if multiple biomarkers are found to reflect different cohorts of AD patients, they can be used to establish the optimal population for use in clinical trials of targeted therapeutics. There are several studies directed at identifying and validating biomarkers of AD in the blood or cerebrospinal fluid (CSF) [34–36] and at seeking neuroimaging, genomic, and epigenetic markers [37, 38], as would be expected of a multifactorial disease, but no single biomarker is reliable and valid [39]. The existing clinical accuracy, including sensitivity and specificity of the markers, remains relatively low [40].

CSF levels of the Aβ, total Tau, and phosphorylated Tau (pTau) in AD subjects are measured as potential diagnostic biomarkers [40–43]. Although these CSF markers enable categorization of the patients as mild cognitive impairment (MCI) or AD [43], they could only be established naturally by utilizing neuropsychological testing followed by biomarker evaluation and not *vice versa*. Aβ profiles may overlap considerably between non-cognitively impaired and AD subjects, even in subjects with the A673T mutation in AβPP gene. This suggests that it is not the amount of Aβ generated but post-cleavage processing that might be contributing to the differences between plaque formation (perhaps a beneficial factor to rid the brain of the soluble forms of Aβ) and processed soluble species (perhaps oxidized forms) in causing dementia [44, 45]. Hence, there is confusion whether the blood and CSF based biomarkers should be used for diagnosis or prognosis of the disease. Nevertheless, reliable biomarkers, which can differentiate AD from MCI, are warranted for appropriate treatment administered early in disease progression [46–48]. Standardization and validation of biomarkers thus play a critical role in the drug discovery process.

It is acknowledged that there is a high variability in the accuracy of CSF biomarkers which have been tested in various centers worldwide [39, 49]. While an association between disease progression and increased level of total Tau and pTau with concomitant decrease in $A\beta_{1\text{-}42}$ concentrations in CSF has been documented, the optimal reference range has not been defined due to variability in their levels. This variation could be due to the multifactorial nature of the disease in different populations or simply the use of various antibodies and ELISA sources in different laboratories. However, concerted efforts are needed to standardize procedures for biomarker assays and improve reproducibility between laboratories [39, 50].

There is also need for reliable clinically acceptable neuroimaging markers. Position emission tomography (PET) imaging biomarkers such as ^{11}C-labelled PiB (Pittsburgh Compound B) have been useful for *in vivo* imaging of Aβ distribution as a research tool [51]. The wide use and applicability of this compound in PET imaging is, however, limited due to its cost and short half-life (20 minutes), which mandates the availability of a cyclotron on-site for production of the isotope.

On the other hand, ^{18}F ligands (florbetapir, florbetaben, and flutemetamol) with a half-life of 110 minutes make Aβ PET imaging more attractive. A multicenter study has shown that florbetapir PET can identify individuals at increased risk of progressive cognitive decline [52]. All the three ligands are now approved by the US Food and Drug Administration (FDA) [53]. Subsequently, two more ^{18}F ligands, florbetaben and flutemetamol, were approved. Although these agents do not establish a positive diagnosis of AD due to their ability to identify individuals at high risk, they have potential for use in new drug development (Chase A, 2014). The use of these ligands along with other biomarkers including as magnetic resonance imaging (MRI), fluorodeoxyglucose (FDG) PET, CSF protein and clinical score of Alzheimer's Disease Assessment Scale (ADAS-Cog) will improve accuracy of diagnosis and predicting conversion from MCI to AD [54]. Combining the neuroimaging traits with epigenetic biomarkers is also under serious consideration to diagnose the disease even at the early stage, but these efforts are still in their infancy [55, 56].

Amyloid imaging and CSF screens for $A\beta_{1-42}$ and Tau protein levels could be of value in defining cognitively normal subjects who do not have preclinical AD pathology. For the past 50 years, studies have used controls that include subjects who had these early stages of the disease [57]. The use of "super-controls" could be of value in assessing premorbid anatomical and functional changes.

One problem in diagnosing AD is that the pathology changes over time, resulting in different stages of AD pathology [58], and the biomarkers used to detect AD may need to be matched to the pathological stages of AD [59, 60]. Measuring longitudinal patterns of changes in a set of different biomarkers may be the most reliable way to diagnose AD and measure its progression [60]. Relevant mouse models could provide vital information for translation to humans [61]. The use of both fluid (CSF and blood) biomarkers in combination with brain imaging and correlating changes with cognitive deficits would provide a means for the early detection of AD and for predicting which patients with MCI develop AD.

TREATMENT FOR AD: FOCUS BEYOND NEUROTRANSMITTERS

After several decades of research in the field of AD, there are, only two classes of FDA approved drugs for AD, namely acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine, and galantamine) and N-methyl-D-aspartate (NMDA) receptor antagonists (memantine). These are safe and efficacious but only offer short symptomatic relief without altering underlying disease pathology [62, 63].

Recent developments in treatment strategies include immune therapy consist of three approaches: active immunization, passive immunization, and immunomodulation. These approaches are mostly targeted against the insoluble oligomeric or fibrillar forms of the Aβ peptide. In addition, there are developments to counteract Tau pathology [64–66]. The humanized antibodies raised against these aggregated proteins or peptides are administered in passive immunization, whereas in active immunization the vaccine contains antigens, which generate antibodies in the recipient. The immunomodulation therapy consists of cytokine administration, which is able to alter the immune response in host against Aβ processing. Several animal studies have shown promising results after introduction of the immune therapies [66–68]. The positive outcome from preclinical immunomodulation studies has led to investigation of Aβ targeting molecules such as tarenflurbil (Myriad Genetics, USA), semagacestat (Eli Lilly and Company, USA), tramiprosate (Neurochem Inc., Canada), ELND006 and AN1792 (Elan Corporation, Ireland), and ponezumab (Pfizer, USA) in randomized, controlled trials but most of them could not successfully satisfy the safety and efficacy issues in human. None of these trials has proceeded beyond phase III due to negative primary outcomes [69]. Immune therapies showed worsening of cognitive performance and poor amyloid clearance [66]. These were accompanied by adverse events of microhemorrhages and increased deposition of Aβ in the vasculature causing harmful effects within the parenchyma [70]. The challenge of immunotherapy therefore, lies in the identification of the relevant antibody variants that can successfully clear the forms of Aβ responsible for the synaptic dysfunction with minimal adversity [70]. This approach requires the identification of the Aβ species, for which there is no current consensus.

There are also other strategies such as kinase inhibition [71], microtubule stabilization [72], vitamin supplementation [73], aggregate disintegration [74], among others, which have been tested in preclinical settings. Application of metal chelators such as clioquinol and PBT2 in arresting Aβ pathology also showed promising results in animal models [75]. Phase II clinical trials with PBT2 also improved cognitive functions in human subjects [76, 77]. It was further demonstrated that these metal ionophores have a strong

Table 1
Current list of clinical trials in Alzheimer's disease on compounds or strategies other than amyloid immunotherapy, acetylcholinesterase inhibition or glutamate antagonism (memantine). Data obtained from the site maintained by National Institute of Health; Web: www.clinicaltrials.gov

Molecule	Purpose	Sponsor/ Collaborator Countries	Study Design	Subject	Phase	Duration	Current Status	Outcome
TRx0237 [Trial ID: NCT01689233]	To assess the safety and efficacy of this Tau based new molecule	TauRx Therapeutics Ltd., USA	Double-blind, placebo-controlled, randomized	Mild to Moderate AD	Phase III	2012-	Ongoing but not recruiting participants	Study result not available
CERE-110 [Trial ID: NCT00876863]	An Adeno-associated virus to transfer the gene to make NGF in brain to protect nerve cells	Ceregene, USA	Double-blind, placebo-controlled (sham surgery)	Mild to Moderate AD	Phase II	2009-	Unknown	Study result not available
Semagacestat (LY450139) [Trial ID: NCT00762411]	A gamma-secretase inhibitor to lower beta amyloid in blood, spinal fluid and brain	Eli Lilly and Company, Asia, Europe	Double-blind, placebo-controlled, randomized	AD with MMSE score of 16	Phase III	2008-2011	Completed	Could not slow down the disease progression and worsened symptomatic outcomes compared to placebo
Exendin-4 [Trial ID: NCT01255163]	To assess safety and efficacy of this syntheticglucagon-like peptide-1 agonist to protect nerve cells	National Institute on Aging (NIA), USA	Double blind, randomized, placebo-controlled	Early-stage AD	Phase II	2010-	Recruiting participants	Study result not available
Bryostatin 1 [Trial No: NCT00606164]	To evaluate the safety, efficacy and pharmacokinetics of this PKC modulator	Blanchette Rockefeller Neurosciences Institute, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2008-	Unknown	Study result not available
EVP-6124 [Trial ID: NCT01073228]	To evaluate the safety and efficacy of this selective alpha7 nicotinic receptor agonist through cholinergic neurotransmission	EnVivo Pharmaceuticals, USA, Russia	Randomized, double-blind, placebo-controlled	Mild to Moderate probable AD	Phase II	2010-2012	Completed	The dose was safe and well tolerated and did not show any gender, age or food effects
ST101 (ZSET1446) [Trial ID: NCT00842673]	To evaluate the safety and efficacy of this azaindolizinone derivative compound	Sonexa Therapeutics, Inc. USA, Canada	Randomized, double-blind, placebo-controlled	AD	Phase II	2009-2011	Completed	Study result not available
Resveratrol (trans-3,4',5-trihydroxystilbene) [Trial ID: NCT01504854]	To evaluate the impact of this natural phenol extracted from plants in delaying or altering the mechanism of aging and dementia	Alzheimer's Disease Cooperative Study, National Institute on Aging, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2012-2014	Completed	Study result not available
Octagam [Trial ID: NCT00812565]	To evaluate the effect of intravenous immunoglobulin infusion on Aβ peptide levels in CSF and blood plasma	Octapharma, USA, Germany	Double-blind, randomized, placebo-controlled	Mild to Moderate AD	Phase II	2008-2011	Completed	Plasma Aβ level was found to be insignificant between placebo and treated among 5 out of 6 intervention groups

LY450139 Dihydrate (gamma-secretase inhibitor) [Trial ID: NCT00244322]	To evaluate the safety, tolerability and level of Aβ in plasma and CSF	Eli Lilly and Company USA	Randomized, double-blind, dose-escalation, placebo-controlled	Mild to moderate AD	Phase II	2005-2007	Completed	Doses well tolerated with reported adverse events. Decreased plasma Aβ concentration but no cognitive difference
Semagacestat (LY450139) (gamma-secretase inhibitor) [Trial ID: NCT00594568]	To evaluate the effect of γ-Secretase Inhibition on the progression of AD	Eli Lilly and Company USA	Randomized, double-blind, placebo-controlled safety/efficacy study	AD	Phase III	2008-2011	Completed	Did not slow disease progression and cognitive worsening with severe adverse events reported
NIC5-15 (Pinitol) [Trial ID: NCT01928420)	To evaluate the safety and efficacy of this plant produc with mild insulin sensitizing effects	James J. Peters Veterans Affairs Medical Center, USA; National Center for Complementary and Integrative Health (NCCIH), USA; Humanetics Corporation, USA	Randomized, double-blind, placebo controlled	AD	Phase II	2010-	Recruiting	Study result not available
ELND005 (scyllo-inositol) [Trial ID: NCT00568776]	To evaluate the dose-related safety and efficacy of this naturally occurring plant sugar alcohol	Transition Therapeutics Ireland Limited; Multi-center	Randomized, double-blind, placebo-controlled, dose-ranging	Mild to Moderate AD	Phase II	2007-2010	Completed	There was no significant improvement in cognitive measures compared to placebo
AADvac1 [Trial ID: NCT01850238]	To assess the safety and tolerability of this Tau peptide-KLH-conjugate active vaccine	Axon Neuroscience SE, Multi-center	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase I	2013-	Ongoing but not recruiting participants	Study result not available
Leukine/ Sagramostim [Trial ID: NCT01409915]	To assess the safety, tolerability and efficacy on cognitive function of this granulocyte-macrophage colony-stimulating factor	University of Colorado, Denver; The Dana Foundation, USA	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase II	2011-	Recruiting	Study result not available
Benfotiamine [Trial ID: NCT02292238]	To assess the efficacy of this synthetic S-acyl derivative of thiamine on cognitive function by minimizing the decrease in glucose utilization in brain	Burke Medical Research Institute, Columbia University, National Institute on Aging (NIA), Alzheimer's Drug Discovery Foundation, USA	Randomized, double-blind, placebo-controlled	Amnestic mild cognitive impairment and mild AD	Phase II	2014-	Recruiting	Study result not available
Azeliragon (TTP488) [Trial ID: NCT02080364]	To evaluate the safety and efficacy of this RAGE (receptor for advanced glycation endproducts) inhibitor	TransTech Pharma, LLC. USA, Canada	Randomized, double-blind, placebo-controlled	Mild AD	Phase III	2015-	Recruiting	Study result not available
Rebif (Interferon Beta-1a) [Trial ID: NCT01075763]	To evaluate safety, tolerability and clinical efficacy of this cytokine	Merck KGaA, Merck Serono S.P.A., Italy	Randomized, double-blind, placebo-controlled	Early-onset AD	Phase II	2004-2008	Completed	Dose well tolerated, No major side effects. No significant improvement reported in neuropsychological conditions

Table 1
(*Continued*)

Molecule	Purpose	Sponsor/ Collaborator Countries	Study Design	Subject	Phase	Duration	Current Status	Outcome
Dimebon [Trial ID: NCT00829374]	To evaluate safety, tolerability and clinical efficacy of this antihistamine drug	Medivation, Inc. Pfizer, Multi-center	Randomized, double-blind, placebo-controlled	Mild to Moderate AD	Phase III	2009-2011	Completed	Study result not available
Vitamin E and Selenium [Trial ID: NCT00040378]	To evaluate clinical efficacy of these anti-oxidants	University of Kentucky, National Institute on Aging (NIA), National Cancer Institute (NCI), USA, Canada, Puerto Rico	Randomized, double-blind, placebo-controlled	AD	Phase III	2002-	Current status unknown (Active but not recruiting in 2013)	Study result not available
E2609 [Trial ID: NCT02322021]	To evaluate safety, tolerability, and efficacy of this BACE1 inhibitor	Eisai Inc. Biogen Idec, USA	Randomized, double-blind, placebo-controlled	Prodromal AD and Mild AD	Phase II	2014-	Recruiting	Study result not available
AZD3293 [Trial ID: NCT02245737]	To evaluate safety and efficacy of this BACE1 inhibitor	AstraZeneca, Eli Lilly and Company, Multi-center	Randomized, double-blind, placebo-controlled	Early AD	Phase II Phase III	2014-	Recruiting	Study result not available
Resveratrol With Glucose and Malate (RGM) [Trial ID: NCT00678431]	To evaluate the efficacy of dietary supplement in slowing progression of AD	Department of Veterans Affairs, Alzheimer's Association, Mount Sinai School of Medicine, USA	Randomized, double-blind, placebo-controlled	AD	Phase III	2008-2011	Completed	Study result not available

affinity for synaptic metal ions and may restore the ion imbalance in the extracellular spaces of Aβ deposits and in turn restore the cognitive impairment [78]. However, the outcomes from animal studies remain mostly unreplicated in human trials [79].

Given the multifactorial nature of AD, it is likely that multiple approaches or a polypill is warranted for patient treatment until such a time as biomarkers allow for the selection of the appropriate cohort for new therapies. Additionally, in the absence of a reference anti-AD drug, even one that works on a limited cohort of patients, the comparative analysis of the efficacy of new therapeutic strategies remains difficult [80, 81]. There are several new molecules and compounds being tested on animal AD models and subsequently tested in human for their safety and efficacy (Table 1). It is pertinent that most of the clinical trials for AD are registered in the official site of the U.S. National Institute of Health, which has a tab for "results posted" but they are seldom reported (http://www.clinicaltrials.gov.). Ironically, only a few of these new agents have reached the stage of successful clinical translation. With the existing translational gap, a key question which remains unanswered is why many drugs which work in animals do not work in humans [32].

THE BLOOD-BRAIN BARRIER: OBSTACLE IN DRUG DISCOVERY

Delivery of therapeutic agents for most brain disorders remains a major cause of concern due to central nervous system (CNS) penetration across the blood-brain barrier (BBB) [82, 83]. The individual variability in absorption and dysfunction of the BBB [84, 85] could prove to be another significant barrier in the efficacy and brain bioavailability of novel CNS drugs. The CNS penetrability of drugs, their routes, and doses must be considered when formulating new treatment strategies. The same molecule may also not be equally effective in different pharmaceutical preparations. The current treatment strategy for AD overstates the systemic approach to the CNS rather than other approaches such as transdermal patches [86]. Other approaches including intranasal and intracerebroventricular delivery of therapeutic molecules, which can bypass the BBB, have been tested utilizing experimental animals to evaluate their safety and efficacy when compared to conventional approaches [87, 88]. The intranasal route was recently tested for its safety and efficacy in AD subjects [89]. It was also shown that intranasal delivery of insulin in AD and MCI patients improved memory performance and levels of CSF biomarkers [90]. Adeno-associated virus- and nanoparticle-based drug delivery systems also have been tested as alternative approaches for drug delivery to the brain with limited adverse effects [91]. Other animal studies have shown that drugs can be delivered orally in a hydration gel diet [92, 93]. These reports highlight the beneficial effects of novel drug delivery systems, albeit in animal models, but they need rigorous evaluation in humans. The outcome also looks promising but there are only a few studies in man. Further efforts are needed to validate other approaches in drug delivery.

TIME FOR REASSESSMENT OF THE ROLE OF Aβ

Aβ peptide has been implicated as a central feature in AD for more than two decades. The prevailing view is that Aβ peptide accumulation as etiological and implies that abnormal accumulation of $A\beta_{42}$ is an early event in the pathophysiologic cascade leading to the disease. Detergent extractable Aβ peptide and its fragments may also accumulate in different age-related dementias, particularly the oldest old, irrespective of the primary diagnosis [93]. There is also an overlap in the profiles of Aβ peptides pertaining to peptide solubility and oligomeric assemblies in brains from AD and normal aging subjects without any history of dementia [45]. Aβ subunits were shown to play a role in nucleation of Aβ aggregates, which has led to the proposal of AD being a prion disorder [94]. However, this is still controversial [95]. Recent studies on at least two mutations in human amyloid-β protein precursor (AβPP) have modified our understanding of the role of Aβ in brain aging and cognitive decline. The AβPP A673T mutation (an A2T change in Aβ) decreases Aβ production and confers resistance to AD and possibly to age-related cognitive decline, suggesting that such decline may also be Aβ-related [44]. The AβPP E693Δ mutation (E21Δ in Aβ) was discovered in subjects exhibiting AD-like dementia, but who were not diagnosed as AD because they lacked amyloid plaques [96]. Although total Aβ in E693Δ brain extracts was lower than from cognitively normal subjects, the majority of Aβ that was present was in the form of an SDS-stable dimer [96]. SDS-stable dimers are a major synaptotoxic form found in AD brain extracts [97] and, along with increased soluble Aβ, these correlate with the severity of dementia [98].

Rat and mouse Aβ differs from human Aβ by three residues, gly for arg at residue 5, phe for tyr at residue 10, and arg for his at residue 13. The three histidine residues at positions 6, 13, and 14 of Aβ provide the primary metal binding site of Aβ [99]. Binding of Cu^{1+} to this site is of very high affinity and its redox cycling between Cu^{1+} and Cu^{2+} can provide a source of reactive oxygen species (ROS) [100]. Furthermore, Cu depletion has been shown to down-regulate expression of the AβPP gene, which has an obvious consequence on amyloid deposition [101]. Aging related early changes in human brain associated with MCI include the accumulation of markers for oxidative stress [102]. Thus, it is plausible that increased ROS production contribute to synapse loss that accompanies early changes in cognitive ability. These early biochemical changes are predominant in pathological aging and validated by reduced expression of synaptic proteins such as synaptobrevin and synaptotagmin leading to cognitive decline [103].

Exposure of human but not rodent Aβ to low levels of Cu^{2+} (levels similar to those found associated with human Aβ plaques) in the presence of ROS, results in the formation of dityrosine linked Aβ covalent dimers. The dityrosine cross-linking may play significant role in Cu-mediated toxicity of Aβ [104]. The lack of tyrosine in rat and mouse Aβ explains its inability to form this product and its lack of neurotoxicity. The Aβ sequence from the naked mole rat differs from human Aβ in only a single residue, arg for his at position 13 [105]. This rodent species is very long lived (>30 years), has levels of Aβ in its brain that are equal or greater to those in the transgenic mouse AD models expressing mutant human AβPP, but the naked mole rats develop no cognitive behavioral deficits in assays for which the humanized transgenic mouse models show defects. Even though the naked mole rat Aβ has tyr10, an arg in place of the Cu^{2+}-chelating his13 would likely reduce its ability to form the Aβ dityrosine dimer. Thus, it is likely that specific modified forms of Aβ, and not simple Aβ oligomers or fibrils, are responsible for the early synaptic deficits that can be tested electrophysiologically in hippocampal slices for a loss of long-term potentiation [106]. Moreover, the Cu binding ability to Aβ differs from rat to human due to their differences in peptide sequences leading to a varied range of Cu^{2+}-induced Aβ aggregation and neurotoxicity in these species. The three amino acid residual changes at 5, 10, and 13 positions from human to rat Aβ peptides are also the Cu^{2+} binding domains influencing the aggregation process [107]. These examples show that we may need to redress the forms of Aβ that may be mediators of variable synaptotoxicity in AD in human and rodents.

In addition to better understanding of the forms of Aβ that mediate synapse loss, the targets of Aβ interaction leading to synapse dysfunction need to be fully evaluated. Aβ is a promiscuous protein with many different membrane proteins identified as binding partners [108]. Surprisingly, transgenic mice overexpressing human Aβ without one of the highly divergent Aβ receptors (e.g., cellular prion protein (PrP^C) [109]; PirB [110]; metabotropic glutamate receptor mGluR5 [111]) become resistant to memory and learning deficits. It is likely that the different receptors for Aβ are working through a common signaling pathway or a neuronal population involved in different aspects of memory and learning. The involvement of PrP^C, which is linked to the outer membrane leaflet via glycosylphosphatidylinositol, suggests that the different Aβ-binding transmembrane receptors participate in a membrane complex to generate a final common response. Indeed, a complex containing PrP^C and mGluR5 has been identified, and activation of the fyn non-receptor tyrosine kinase is one downstream target of the signaling pathway [111]. Although our current understanding on Aβ toxicity has contributed significantly toward corroborating the disease pathology, there is still much to investigate about the functional and structural differences in the peptide and their level of neurotoxicity in different species.

BEYOND Aβ HYPOTHESIS: OTHER CAUSATIVE AD PATHOLOGIES

Recent studies have found that amyloid deposits fail to induce AD-like symptoms in some mouse models [112, 113], leading to the hypothesis that non-amyloid targets may also underlie AD pathogenesis [114]. Over the past three decades, disease mechanisms other than those involving the amyloid hypothesis have been implicated in the pathogenesis of AD. The putative role of inflammatory-immune mechanisms in AD brain pathology has long been debated [115]. An increase in neuroinflammatory cytokines has been observed in AD subjects and considered to be another hallmark of AD pathogenesis, based on a careful analysis of the neuroinflammatory protein markers' phenotypes in sera of AD patients [116]. In keeping with the nomenclature used in phenotyping macrophages in which the M1 phenotype produces proinflammatory cytokines (e.g., IL-1β, IL-6, TNFα) and the M2 phenotype produces wound repair mediators (e.g., arginase-1) and

anti-inflammatory cytokines (e.g., IL-4 and IL-10), early AD subjects could be classified quite distinctly into each of these cohorts with 11 of 23 subjects assigned to the M1 cohort and remaining 12 to the M2 group, based upon 5 distinct markers for each group including interleukins-1β, −6, and −12, interferon-γ, and tumor necrosis factor-α [117]. The frontal cortex was used for these analyses, and the cerebellum, which showed no significant changes in more than a single marker, was used as control tissue. For late-stage AD subjects, both sets of markers were greatly increased and no distinction could be made. These results again point to the multifactorial nature of AD and the need to be able to categorize patients using biomarkers to test different therapeutic approaches.

Another example of change found in AD brain and explored in rodent neurons and organotypic slice cultures is that of cofilin-actin rods. These structures, detected by immunostaining for cofilin [118], contain linear arrays of cofilin-saturated actin filaments [119]. They form in both dendrites and axons and require oxidative stress to accompany the hyperactivation (dephosphorylation) of cofilin to form cofilin disulfide linked dimers [120]. Many changes that occur in AD brain, such as the increased production of Aβ [121], the decline in the p21 activated protein kinase (Pak1), which works upstream of cofilin and helps regulate its activity [122], the loss of two microRNAs (miR103 and 107) that control the expression of cofilin [123], the decrease in glutamate transporters leading to increased extracellular glutamate [124], and increased oxidative stress [102], all lead to cofilin-actin rod formation when studied in neuronal culture. Because rods sequester virtually all of the cofilin within the local region of the neurite [118], they inhibit synaptic function [125] because cofilin plays an important role in both AMPA channel insertion and dendritic spine enlargement associated with long-term potentiation [126]. Although rods are induced by oligomers of synthetic human $A\beta_{1\text{-}42}$ [121], Cu^{2+}-peroxide oxidation of synthetic human Aβ increases its rod inducing efficacy by about 600 fold [127]. The cofilin-actin rods contribute to synaptic loss as evident from cultured neurons subjected to excitotoxic stress and Aβ exposure. Exposure to proinflammatory cytokines also induced rod formation in these neurons. They have experimentally shown that both Aβ and proinflammatory cytokines induced rod formation in neurons are mediated through activation of prion protein-dependent NADPH oxidase pathway. This study links the Aβ and cytokine hypothesis in AD pathology and explains how complex and diverse mechanisms mediate synapse loss in AD [128]. Since rod pathology may be a common downstream effector of synapse loss for both familial and sporadic forms of AD, it is surprising that more studies have not examined the therapeutic potential for the elimination of cofilin-actin rods [129].

ANIMAL MODELS FOR AD: IN NEED OF BETTER CHARACTERIZATION

A range of animal species are used as experimental models for AD pathologies [129]. These models vary from invertebrate animals such as *Drosophila* [130], *C elegans* [131], and zebrafish [132] to rodents [133, 134] and non-human primates [135, 136], although mice are most often used to investigate AD pathogenesis. Even the most resilient transgenic mouse models for AD exhibit the rarer familial form of the disease, whereas sporadic AD is the most common [137]. Overall, no animal model is able to reproduce disease onset, progression, and relapse, reminiscent of AD patients [138]. The use of transgenic tau mice, either on their own or in association with AβPP mutations has not wholly enabled their purpose; characteristics of the phenotype depend on the transgenome and fall short of fully reflecting features of AD pathology. Thus, some of the available transgenic tau mice models, e.g., Tau4R-P301L and P301S, have shorter lifespan of 9 months, with most not surviving beyond 12 months. Others have age-associated impairment in retention of spatial memory, associative memory and cognition, or no abnormal behavior. Furthermore, they may also differ in respect to their motor phenotypes, which include severe and early onset of motor, gait, balance and behavioral disturbances, and the neuropathological features [139]. The highlighted cognitive behavioral, motor, and neuropathological heterogeneity of the transgenic tau animal models make them difficult to use in AD translational research, and much attention also needs to be paid in selecting an all-encompassing tau model to investigate the properties of novel drugs for treatment or neuroradiological tracers to diagnose AD [2, 140, 141].

Variables such as the strain, age, and expression level of the transgene and gender of animal models influence the disease progression and its pathophysiology, thereby confounding the results of studies seeking to discover novel biomarkers and therapies for AD. There are neural and behavioral differences between background strains used to produce transgenic mice [142, 143]. Strain differences in anxiety-like behavior

have been documented in mice tested in the open field and elevated plus maze [144]. For example, female C57BL/6J mice exhibit less anxiety-like behavior and more exploratory activity, compared to BALB/cJ mice [145]. Therefore, background strain effects should be considered when using transgenic mice to model neurodegenerative diseases (Table 2).

Since neurodegenerative disorders are age-dependent and physiological deficits may be age-specific [146], the age of the mice is another variable that must be considered. When age differences are taken into considerations in a transgenic (Tg2576) mice, FDG-PET reveals greater glucose metabolism in 7-month-old than in 19-month-old mice. However these differences were not supported by hemodynamic parameters when cerebral blood volume was measured by functional MRI [147]. One solution to this problem is to use senescence accelerated mice [148].

Some may argue that gender of animals does not pose differences in their brain architecture and related behavioral performances, but the current literature is contrary to this belief. There are gender differences in the brain and behavior of rodents [149] including transgenic mouse models of AD [150]. For example, mood disorders are reported to be more striking in female rodents [146, 151] and therefore the majority of behavioral experiments are performed using males. Some studies have reported differences in the biochemical parameters in the blood of Wistar rats as a function of gender and age [152]. The resulting gender bias in animal research is a serious concern. Researchers are encouraged to study both males and females of each animal model [153]. In many studies significant gender differences are reported [154], but these are not taken into consideration in the design of subsequent clinical trials [155]. Therefore, when developing an animal model of AD, either by inducing a specific genetic mutation or by electrical, mechanical, or pharmacological interventions, the validation of the model must consider the genetics of the strain used, expression level of the transgene, age, and gender differences. Such models should also address pathologies that are independent of or supplemental to the centrality of Aβ hypothesis. Given numerous existing models with none of them representing the true molecular, pathological, and behavioral traits of the human disease, it is unclear which one would best fit for evaluation. Considering the multifactorial nature of the disease, it is challenging to develop an AD model without any limitations. Therefore, it is imperative to test the safety and efficacy of any candidate molecule in multiple AD models before undertaking human studies.

PHYLOGENETIC DIVERSITY BETWEEN RODENTS AND HUMANS

As rodent models are extensively used in understanding the pathophysiology of AD, it is important to corroborate the basic differences between mouse and human nervous systems. The inter-species phylogenetic comparisons may reveal deeper insights into the cellular and genetic differences specific to neurodegenerative disorders [156]. For example, drug doses tested and validated in experimental animal models are not easy to extrapolate to humans [157]. In addition, the network of connections in the brain transcriptome differs among species and this is little considered. Although targeted gene mutations in animal models may mimic certain comparable phenotypes in humans, a single gene mutation in mouse models is often considered incapable of translating the same behavioral and pathological parameters observed in the human phenotype. While there is genetic similarity between mouse and human species, only a 10% homology for co-expressed genes has been reported [158]. A transcriptome analysis across human and mouse brains was developed by analyzing more than 1000 gene microarrays in both species. Among them, the species-specific gene modules described some highly conserved transcriptomes with an overlap of genetic networks between the two species [159]. When AD and other neurodegenerative disorders were specifically examined, the transcriptome revealed that the recruitment of microglial cells appeared to play a significant role in the development of AD. For instance, the human presenilin 1 mutation (PS1), which increases Aβ production, can increase the severity of the disease in man but was found to have a limited effect in mouse mutants. There is also a correlation between presenilin 1 and oligodendrocytes, which is selective in humans. It is evident that the number of oligodendrocyte progenitors increase significantly in brains of AβPP/PS1 transgenic mice while these cells are found to be limited in postmortem AD brains. Thus in similar pathological conditions, these cells have different repair mechanisms in humans and mice [160]

As microglia are an important sources of proinflammatory cytokines and major players in AD pathogenesis [116] that can trigger stress enhancing oxidation of the amyloid peptide, the differences between mice and humans could be dramatic for disease progression. Regardless of the dissimilarities, it is still possible to improve such animal models to incorporate the elements of AD pathogenesis. For example, oxidative stress can be co-induced in an AD transgenic model. Therefore, changes in expression patterns

Table 2
Impending limitations and plausible solutions in translation of pre-clinical studies in AD

Several animal models for AD but unable to completely reproduce disease onset, progression, relapse and reminiscent of human AD patients	Variables such as the strain, age, gender, genetics and expression level of the transgene should be taken into consideration
AD Models such as lesion induced by targeted brain injury or Aβ injection may only mimic the cognitive impairment and may not resemble the molecular mechanism behind disease pathogenesis	Such models must be critically examined for their pathological and molecular roles behind associated cognitive impairment
Transgenic mouse models mimic the familiar form of the disease, even though AD is mostly of sporadic origin	Expression patterns of such transgenes and their interrelated activities at cellular levels in comparison to man must be considered in animal testing
Most transgenic models focus on either the amyloid pathology or the neurofibrillary tangle (NFT) pathology alone and seldom both together	It must consider both Aβ and NFT at transgenic level and their synergistic effects in the progression of neural and behavioral pathologies
Rodent Aβ differs from human Aβ in structure as well as toxicity	Need to readdress the precise form and role of Aβ in AD pathology
Ignorance of associated risk factors on the occurrence of AD and its progression	Factors including increasing age, diet, cardiovascular disorders, diabetes, obesity, hypertension and other environmental factors must be evaluated in parallel to AD pathology
Ignorance of confounding effects in neurobehavioral studies	Variables such as genetic manipulation, stress, predatory effect, housing and test room environment, experimental design, test apparatus and experimenter effects should be taken into account
Compromised quality and reliability of the data obtained from animal studies	OECD Principles of Good Laboratory Practices must be applied to authenticate the data generated by establishment of Quality Assurance procedures in the test facilities
Due to lack of randomization and blinding in animal study design negative results are rarely published in journals, resulting in a bias in pre-clinical data	Standardized reporting through validated guidelines for animal experiments can improve the quality of the research and also contribute effectively to future research
Lack of consultation with statisticians with regards to the study design, conduct and analysis of animal trials	The statistical analysis plan (SAP) should be carefully developed before the initiation of the study. The SAP should determine various study protocol such as power analysis, sample size, randomization method, duration of trial and dropout animals information, etc.
Majority of investigations in animal models are cross-sectional in on the nature leading to lack of evidence for epigenetic influence pathogenesis of AD	Longitudinal animal studies, characterized by long-term follow up of exposure to drugs, nutrition, biotherapeutics or other non-pharmacological interventions can provide crucial evidence to delineate the causal factors in disease pathogenesis

of several genes related to AD and their interrelated activities at cellular levels play a unique and significant role in pathogenesis of disease in mouse models and patients, and these pose a serious challenge for experimental neurologists [159, 161–163].

PATHOPHYSIOLOGICAL DISSIMILARITIES

The pathological signature for AD is widely acknowledged to be the intracellular accumulation of hyperphosphorylated tau proteins into neurofibrillary tangles and neuropil threads and the extracellular insoluble deposits of Aβ peptide into neuritic plaques [164]. However, there is lack of a robust relationship between burden of senile plaques and cognitive impairment, but McDonald et al. [98, 165] have shown that the soluble forms of Aβ, particularly the SDS-stable covalent Aβ dimers, have a strong correlation with dementia progression. The Osaka mutation in AβPP (E693Δ) that gives rise to dementia in the absence of plaques [95], suggests that plaque formation, which is used to define AD, should not be highlighted as a critical pathological parameter. Mouse models expressing E693Δ mutant AβPP develop impaired hippocampal synaptic plasticity and memory impairment with no extracellular plaque formation [166]. Reduced total amount of Aβ but greater amounts of Aβ dimers are found in subjects with the Osaka mutation, again suggesting that it is the form of the Aβ present and not the total amount, which drive the synaptic dysfunction. Some animals develop plaques and tangles spontaneously, but for the majority of animal models, AD-like symptoms are induced by either pharmacological, neurochemical, electrolytic lesions, Aβ infusion, or genetic manipulations [127]. These manipulations are an attempt to induce the behavioral and pathological symptoms of AD in animal models without validating the critical pathophysiological details in the disease mechanism. For example, the lesion induced by a targeted brain injury or by Aβ injection may only mimic the behavior or cognitive impairment and may not resemble the molec-

ular mechanism responsible for disease pathogenesis. Similarly, injection of neurotoxins like amyloid peptide or ibotenic acid, which induce neuronal loss in defined regions of the brain, might mimic neuronal loss without providing reliable information about the pathophysiological mechanisms such as apoptosis and deposition of amyloid peptides [6]. Even the most promising transgenic models of AD have limitations [137, 167]. Although these models attempt to reproduce all the three aspects of the biological mechanisms for the pathogenesis of the disease viz. cause, symptoms, and pathology, the complexity of disease is difficult to capture through molecular pathways in a single model. For instance, the vast majority of the transgenic mouse models focus on either the amyloid pathology or the neurofibrillary tangle pathology alone and not both together which is more representative of the disease state [168]. Therefore, it is clear that such animal models cannot address all the aspects of the disease [169].

It is vital to understand the interaction between tau protein and amyloid peptide and their synergistic effect in the progression of neural and behavioral pathologies in transgenic animal models [170]. Nevertheless, newer transgenic rodent models that capture more of the pathology of human AD have been developed from which we can hope to learn more about the interactions between tau protein and amyloid peptide and their synergistic effects on the progression of neural and behavioral pathologies. However, even these models fail to include additional epigenetic influences that will help us better understand sporadic AD, the most common form. It is also pertinent to mention that these models have contributed enormously to the current progress of AD research and therefore the focus could be more on representing the human AD pathology and expressing newer human mutant proteins in mouse brain.

ANIMAL STUDIES AND ASSOCIATED RISK FACTORS OF AD

Proper assessment of risk factors has been instrumental in the discovery of disease mechanisms and drug discovery. By using case-control and disease cohort studies, multiple risk factors have been identified and assessed for their effects on the occurrence of the disease and its progression [171]. Increasing age, dietary factors, apolipoprotein E gene isoform 4 (*APOEε4*), cardiovascular disorders, diabetes, Down syndrome, MCI, traumatic brain injury, and multiple environmental factors are reported to be associated with pathogenesis of AD [172–176]. Unfortunately, the majority of these studies are retrospective in nature and none of these potential risk factors is routinely considered in experimental designs of animal studies as well as human trials.

Increasing age is considered the most important risk factor for sporadic AD [177]. However, old age is not the sole factor for development of disease. The vitality of all organs in the body during normal aging to ward off cardiovascular disease, hypertension, diabetes, and obesity plays an important role [178]. There appear to be seven key risk factors associated with AD. It is projected that an estimated 10–25% reduction in diabetes, obesity, hypertension, depression, smoking, and cognitive and physical inactivity could prevent around 3 million cases of AD in the global population [179].

Non-neuronal cells such as lymphocytes also show a high degree of susceptibility toward cellular apoptosis with increasing age [180]. Dietary deficiencies in folate, vitamin B6, and B12, which result in an increase in the level of circulating homocysteine, may also be a risk factor for developing AD. Transgenic AβPP mice fed on diets deficient in folate and vitamins B6 and B12 have an increase in the AD progression and pathological levels of amyloid in their brains [181]. Even wild-type mice develop atrophy and reduced metabolism in the hippocampus after long-term cerebral hypoperfusion [182].

The most prevalent genetic risk factor associated with sporadic AD is the inheritance of the ε4 allele of *APOE*. It is reported that the population carrying *APOEε4* alleles, increases the likelihood that they will eventually develop AD [183]. There are studies estimating *APOE* allele frequency to be about 12–14% for Caucasians and varies by ethnicity [184]. Type 2 diabetic patients are also at high risk of developing AD. Double transgenic mice for AD and diabetes ($A\beta PP^{+}$-ob/ob mice) exhibit significant cerebrovascular inflammation and extreme amyloid angiopathy compared to single $A\beta PP^{+}$ transgenic mice [185], and insulin deficiency may alter AβPP processing to result in an increase in Aβ plaques [186]. Among other factors, MCI is a significant risk factor for AD. In a multicenter study, Mattsson et al. [187] have shown that around 36% of the MCI population recruited in the study was eventually diagnosed with AD within 2 years follow up.

Individuals with risk factors identified for AD can modify their lifestyles to reduce the chances of developing AD. Some of the lifestyle factors include diet and burden of hyperglycemia as these may enhance the susceptibility for AD. The Mediterranean

diet has a significant role in lowering the risks of neurodegenerative disorders. This balanced diet, consisting of high amount of plant foods and olive oil and low consumption of dairy products and meat, when adhered to for a long time reduces the chance of developing AD [188]. Exercise and environmental enrichment may reduce the cognitive impairments caused by high fat diets in AβPP transgenic mice [189]. Future studies should, therefore, focus on rigorously identifying the lifestyle changes that reduce the risk factors for developing AD [178, 190–194]. In order to address such issues, many investigators have suggested the introduction of stress in transgenic animals across their lifespan.

LIMITED LONG TERM PROSPECTIVE STUDIES IN ANIMALS

The majority of investigations in both animal models and AD research are cross-sectional in nature. Longitudinal animal studies, characterized by long-term follow up of exposure to drugs, nutrition, biotherapeutics, or other non-pharmacological interventions, can provide crucial evidence to delineate the causal factors in disease pathogenesis. There is growing evidence that early exposure to environmental stimuli such as toxins, metals, and nutritional or educational exposure can exert epigenetic influence on the pathogenesis of AD [195–198]. Lahiri and colleagues [199, 200] have proposed the LEARn (Latent Early–life Associated Regulation) model highlighting the significance of long term follow up of animals exposed to subtoxic levels of lead in early life which resulted in the development of AD as they aged. A better understanding of epigenetic influences on neural disorders has come from studies in monkeys exposed to lead for 400 days in their infancy and evaluated 23 years later [201]. The expression of neuropathological genes related to AD, such as, AβPP, BACE1, and their transcriptional regulator (Sp1) were upregulated in the brains of the exposed monkeys compared to their control counterparts. Furthermore, the elevated levels of intracellular amyloid plaques and their increased distribution in cortical regions were observed in these monkeys. They concluded that alteration in DNA methylation levels through oxidative damage in early life resulted in the greater expression of AD related genes and disease pathogenesis at a later stage of life.

These findings signify the importance of early life regulation in AD. The FDA now prioritizes discovery of new drugs and institution of clinical trials in the early stages of AD. The Center for Drug Evaluation and Research, a US-FDA body, has recently released a draft guideline to assist the pharmaceutical industry in selecting early AD patients or patients with determined risk of developing AD for enrolment into the trials thus emphasizing the significance of early detection of the disease for effective intervention. A key reason for the failure in clinical trial of drugs effective in animal models is the recruitment of patients at an advanced disease state, further highlighting the need for early diagnosis and intervention. Therefore, the desirable characteristics of an animal model of late onset (sporadic) AD should incorporate not only the risk alleles but also the "age–related and environmentally induced epigenetic dysregulation" of AD [189] for effective translation of preclinical studies into tangible therapies.

CONFOUNDS IN THE NEUROBEHAVIORAL STUDY OF ANIMAL MODELS

AD is diagnosed on the basis of age-related cognitive and behavioral deficits of the patients as described in the DSM-IVR [202] and in more recent diagnostic manuals [203, 204], which can be correlated with biomarkers [60] as well as postmortem neuropathological abnormalities including Aβ plaques and neurofibrillary tangles [205]. Thus, mouse models of AD must demonstrate age-related cognitive and behavioral deficits of the type found in human AD, and these deficits must be shown to arise from neural degeneration of the AD type and no other confounding factors such as sensory-motor deficits or laboratory artifacts [206, 207]. However, many argue that animal models may not reproduce cognitive features of human disorders but only the pathological changes similar to human AD. The aim of using transgenic mouse models of AD is to demonstrate that AD transgenes and not some other confounding factors cause AD-like deterioration of behavior and brain. These confounding variables include the genetic manipulation and the housing environment of the mice, stress and the design of the experiments and tests conducted, the test room environment, test apparatus, and experimenter effects [206, 207].

When mice are genetically engineered as transgenic models of AD, genes are transfected into the mice and the behavioral changes of the transgenic mice are assumed to be due to these genes, but they can also be due to the background strain of mice used, to flanking genes or genes disrupted by transgene insertion

or to errors in genetic manipulation. Thus, one must know how the genome of the background strains of mice affects their behavior and be able to detect the effects of unwanted genes transfected along with the genes of interest. Since some strains of transgenic AD mice undergo retinal degeneration, using these as background strains means that the transgenic mice will be blind [208] or have age-related blindness which is not detected until mice are over 9 months of age [209]. Obviously, such age-related changes in vision will have profound effects on cognitive behavior measured using visual cues. Since water maze is often used to test memory in transgenic AD mice and to predict treatment response, it is important to analyze the effect of vision loss as a confounding effect on their cognitive performance. There can be profound effects of blindness on spatial memory deficits as demonstrated in a B6/SJL genetic background of widely used transgenic mice of AD [210]. The Tg2576 mice with visual impairment were unable to perform during acquisition trials in water maze when compared to transgenic alone. This confound was of no consequence when analyzed in memory retention trials [210]. One must also ensure that the mice being tested actually bear the genetic manipulation that they are supposed to have [211]. The source of the mice may also be a factor as mice from different breeders are not always the same [212]. Health status is also an important consideration as mice which have a peripheral infection of some sort may show abnormal behavior which is independent of the transgenes they express, or which exacerbate the effects of the transgene they express. The housing environment of mice is often ignored, but differences in the vivarium environment, home cage features, diet, social versus individual housing, and cage enrichment can significantly affect the behavior of mice. In addition, predatory stress of rat to mice (due to long-term housing of rats and mice in the same secondary enclosure) and some housing conditions may induce selective stress among the animals, altering their behavioral performance [212–214]. Even changing the mice from social housing to individual housing may be particularly stressful [215].

When testing mice behavior, the design of the experimental test is crucial. Which tests should be given, and in which order? Which strain of mice should be tested and what is the appropriate control strain? What sex and age of mice should be tested? Should tests at different ages be done longitudinally, with the same mice tested at each age, or in a cross-sectional study? How many subjects should be used in each group and what statistical analyses should be done? Behavioral testing is done on an apparatus in a test room. How should the test room be designed? How should animals be transported from their housing room to the test room, and when during the light-dark cycle, should mice be tested? Are all tests performed at the same time of the day? The type of apparatus used must be carefully considered as mice behave differently in different designs of the same apparatus. For example, different designs of the Barnes maze result in different types of learning and memory [216, 217]. Finally, one must ensure that the behavioral measures represent the psychological construct that they are meant to measure. For example, there are many tests of anxiety in mice but they may not be measuring the same psychological state or trait [145].

Many types of errors can occur. Thus test results must be examined for observer effects, observer error, and observer bias. Testing should be performed such that the observer is blind to the genotype of the mice being tested. Videotaping of each test is advised to check for procedural errors, equipment setup errors, and animal handling errors. Data recording errors must also be checked. To reduce experimenter error, test procedures may be automated, but automation may also introduce unexpected errors into the behavioral testing. Thus, uncontrolled and undetected confounds in the neurobehavioral study of mouse models may be a significant reason for the problems in the translation of pre-clinical studies into clinical trials [206].

NEED FOR RANDOMIZATION AND BLINDING IN STUDY DESIGN

Randomization of subjects to groups and blinding of experimenters as to which group each subject is allocated are essential elements of clinical trials, assuring quality trial performance and unbiased reporting of clinical outcomes. Even though the fundamental rationale for the animal experiments is similar to that of human randomized controlled trials, often the reporting of the methodology of animal studies is not sufficiently detailed to determine if randomization and blinding were appropriately done. Thus, the validity of the outcomes may be questionable, widening the chasm between preclinical and clinical investigations [218]. Rarely are negative results published in journals, resulting in a bias in reporting positive pre-clinical data [219, 220]. This is often argued as the primary reason why despite more than 1000 preclinical animal studies with positive neuroprotective interventions in stroke, none of the results could successfully achieve

clinical translation [221]. The situation is similar for preclinical studies in AD. A retrospective survey of 290 abstracts presented at a meeting reported that animal experiments without randomization and blinding are 5.3 times more likely to report positive outcomes [222]. Many surveys mention that most studies reveal inadequate reporting of the study design, sample size, randomization of the sample, blinding of the experimenter and statistical methods [218, 223, 224]. The sample size of experimental groups in a preclinical study rarely exceeds 10. It is difficult to imagine publishing a study on human AD with 6–10 patients.

Incomplete or inappropriate reporting may cause serious scientific, ethical, and economical implications. The situation is compounded by lack of guidelines for proper reporting of animal experiments unlike the CONSORT (Consolidated Standard of reporting trials) statement which is followed in most of the randomized controlled trials [225]. Standardized reporting through validated guidelines for animal experiments can improve the quality of the research and contribute effectively to future research. Kilkenny et al. [226] have taken the initiative to propose a draft guideline called ARRIVE (Animal Research: Reporting *In Vivo* Experiments) in order to strengthen preclinical reporting of data. Taking their cue from the CONSORT statement and after consultation with several eminent scientists, funding agencies and high impact factor journal editors have included essential requirements in study design and reporting such as randomization, blinding, sample size, inclusion and exclusion criteria, co-morbidities, and missing data. Compliance to new methods of reporting might increase the quality of investigations and hence the pace of clinical translation. Journals such as *Genes, Brain and Behavior* and *Nature Neuroscience* [227] have instituted new standards for reporting animal studies.

UNIVERSALIZATION OF GOOD LABORATORY AND CLINICAL PRACTICES

Although we have progressed reasonably in AD research, there is not enough information regarding key events in its causation so that they can be exploited for identifying suitable biomarkers for diagnosis or targets for its treatment. It is also obvious that animal studies have their own limitations in extrapolation of results to human. However, there is a third issue of quality and reliability of the animal and clinical studies which is essential for making these results meaningful for clinical translation. In a complex situation like the one being posed by AD, animal studies ought to be made more acceptable by applying the OECD Principles of Good Laboratory Practices (GLPs) [228, 229]. Briefly, these quality principles require use of "demonstrably" appropriate resources (manpower, infrastructure, equipment, laboratory facility, clinical facility, animal facility, and dose formulation facility) along with the use of "demonstrably" well characterized animals and dosing materials. Further, there should be well-written study plans, followed accurately with the help of documented standard operation procedures supported by detailed documentation of findings of the study [230] suitably archived in organized formats. GLPs also enjoin carefully secured and retrievable archiving of all study-related and facility-related data and materials so that any reasons for discrepancy in the findings of further studies can be traced back to their causation. Finally, the GLPs recommend establishment of Quality Assurance procedures in the test facilities with the help of staff not directly involved in the conduct of studies to ensure that all the work is done as per prior commitment, with true representation of all the study data in the final reports and acknowledgement of deviations from the planned activities and procedures (if any) [231]. It is hoped that if researchers and clinical scientists utilize GLP principles in planning, performing, recording, analyzing, and archiving of the animal and clinical studies with constant vigil of internal quality assurance, and make an averment to this effect while publishing their results, their data will be more reliable and acceptable for successful clinical translation.

CHALLENGES AND OPPORTUNITIES OF INTEGRATING GENETIC AND GENOMIC INFORMATION INTO CLINICAL PRACTICE

AD comprises familial and sporadic forms. The early-onset familial AD is an inherited disorder, caused by mutations in three major genes (*AβPP*, *PSEN1,* and *PSEN2*). The late-onset sporadic AD is a complex disease caused by multiple genetic and environmental factors. Great efforts have been made to understand the genetic causes of sporadic AD in the past decade [232, 233]. Recent candidate gene and genome-wide association studies (GWAS) have identified multiple genes associated with AD, such as *APOE*, bridging integrator 1 (*BIN1)*, clusterin (*CLU*,) complement receptor (*CR1)*, and phosphatidylinositol clathrin assembly

lymphoid-Myeloid Leukemia (*PICALM*). To increase study sample size, consortium meta-analysis combined multiple studies to further expand the gene list [234].

Despite the great progress on identifying disease-associated variants, it is still not clear how these identified genes affect the initiation and progression of AD pathology in the brain. The identified single nucleotide polymorphisms (SNPs) often fall into non-coding regions and have no obvious functional implication. With improving genotyping tools and technologies, there have been a series of SNP studies in AD conducted but the risk effects of these SNPs are small and have little contribution to disease prediction or diagnosis. Instead, these findings have led to a pre-debated possibility of highlighting false positive signals among the true disease polymorphisms [235].

At this stage, genetic information has not been widely integrated in clinical practice. Next-generation sequencing will be the next step to identify additional variants with less frequency (rare variants) but large effects [236]. Besides DNA variation, there is accumulating evidence of epigenetic effect contributing to AD, which implies the complex interplay between genetic and environmental factors [237–239]. However, most epigenetic studies so far in AD are limited by the sample size and the genome coverage. New technologies (e.g., whole-genome methylation chip) will help researchers assess the epigenetic mechanism systematically and bring new important insights to enhance our understanding of the pathogenesis of AD.

A potential benefit of emerging findings from genetic studies is to directly facilitate the design of clinical trials. Hu et al. [239] proposed a framework to integrate genetic risk scores that are based on the findings from GWAS, in clinical trials to reduce trial cost. The rationale is that using genetic information to enroll a subgroup of individuals can increase the disease rate, and thus reduce study duration and trial cost [240]. A few common complex diseases, such as type 1 and 2 diabetes, myocardial infarction, and macular degeneration have been examined. This approach can be applied analogously to AD. Furthermore, individual genetic profiles provide potentials for researchers to identify a subgroup of patients that have better drug response than general populations. Given the lack of new drug development, many researchers are examining how effective new pharmacological treatments are discovered [241] and returning to phenotypic screening in addition to target based drug discovery [242–244]. Studies of pharmacogenetics and pharmacogenomics on AD patients will help optimize drug use and improve drug efficacy [245]. We anticipate that the combined information of genetics, genomics, environmental factors, and drug response will yield a major change in clinical practice and facilitate the success of personalized medicine in near future.

AD DRUG TRIALS: GAPS IN DESIGN AND METHODS

Preclinical studies are generally not as highly regulated as clinical studies. For many preclinical studies, there is limited consultation with statisticians with regard to study design (such as sample size determination, randomization method, and duration of trial) and statistical analysis and interpretation of the data. Statistical support is believed to be less important and therefore less appreciated in the preclinical research phase. In the recommendations on best practice for animal studies of AD, jointly published by Alzheimer's Drug Discovery Foundation (ADDF) and Charles River Discovery Research Services [31], lack of standards in design, conduct, and analysis of animal trials is considered as one of the key challenges in translating preclinical studies to clinical trials for AD. Establishing rigorous preclinical standards cannot be accomplished without significant statistical contributions, especially for research on such a complex disease. Similar to the clinical studies, the hypotheses and objectives of the preclinical study must be prespecified using preferable statistical language so that they can be formally tested and evaluated. The statistical analysis plan (SAP) should be carefully developed before the initiation of the study. Power analysis and sample size estimation are recommended prior to the study even for the exploratory experiment. Randomization methods and blinding procedures should be carefully considered and stated in SAP. Appropriate procedures of handling the dropout animals (e.g., due to death or other adverse events) need to be specified in the SAP. By implementing these enhancements in the preclinical studies, the quality of the findings and the predictive value of the preclinical research can be improved and the translation to the clinical trials can be more reliable and efficient.

The design and implementation of clinical trials have also generated numerous obstacles (Table 3). According to ClinicalTrials.gov, there have been over 330 trials to understand and treat AD and about 30 among these are in Phase 3. To improve the probability of success in large late phase trials, AD clinical think-tank leaders have faced challenges on how best to design and evaluate early phase trials [246]. Although

some efforts have been already made to improve the quality of early clinical studies on AD, there is still considerable scope to introduce emerging statistical methods and advanced trial designs. Most of the AD clinical trials have used cognition as the primary outcome measure. However, it has been is suggested that ADAS-Cog (Alzheimer's Disease Assessment Scale – Cognitive) performs less than adequately in detecting patients at the mild stage of AD [247, 248]. The FDA Guidance Document on Alzheimer's Disease reiterates that most clinical evaluation criteria can only detect the disease in the presence of cognitive impairment, when it is generally late to prevent disease onset [249]. In addition, the primary outcomes in some trials are analyzed by univariate statistical methods to compare differences between treated and control groups that greatly limits the scope to interpret the complex multivariate data [247]. The outcomes of phase III bapineuzumab and solanezumab trials were critically reviewed by the EU/US/CTAD Task Force Members to evaluate the design methods and outcomes particularly for insights on future clinical trials. Remarkably, other factors contributing to the lack of efficacy include significant differences in actual binding and the cross-reactivity of anti-Aβ antibodies to amyloid and other proteins in humans. The lack of target engagement raises questions as to whether some of the monoclonal antibodies are suitable drug candidates for the preventative clinical trials for AD [250].

The task force came to a broad consensus that AD should be treated at early stages and a line of secondary prevention should be incorporated in the disease management. Based on the trial result, it is also realized that the trial outcomes should be measured primarily based on the cognitive outcomes irrespective of the changes in disease pathology. Other interesting recommendations made in this meeting were consideration of combining phase II and phase III trials, targeted and adaptive mode of trials, and measures of biomarkers at downstream levels for enhanced chance of success in trials [251].

AD is a complicated disease and there is considerable variability in disease symptoms, progression profiles, and responses to interventions among different populations. It is unlikely that a treatment can be effective in all populations. Dubois and colleagues [251] have suggested a revised definition of AD. One major impact from this new definition applies to the clinical trial design, indicating more targeted subpopulations of AD should be considered. This also implies that, as a result of targeted clinical trials, more advanced statistical methods for subgroup identification and evaluations have to be implemented in the analysis of such targeted trials. All of these could yield substantial improvements for assessing the efficacy of AD interventions.

COLLABORATIVE EFFORTS BETWEEN RESEARCH SCIENTISTS AND CLINICIANS

The crosstalk between basic scientists and clinicians is prerequisite for successful translation of preclinical findings into clinical prospects [252]. Unfortunately, this has not been the practice in most of the clinical or preclinical settings, creating a knowledge gap among the scientists and clinicians, and dampening the hope of promising clinical translation. Involvement of both can increase the transparency of the study design in animal models as well as clinical trials. Clinicians can have the opportunity to inform the animal modelers what kind and in which form they need the information from animal studies to benefit human trials. As an exemplary case, a group of basic scientists and clinical oncologists recently met at the Wistar Institute, Philadelphia, PA to discuss the outcome of preclinical mouse models of human melanoma for facilitating improved clinical trials [253]. The outcome of this exchange indicated that no human trial in melanoma should be planned without strong evidence of beneficial effects from progressively designed animal studies. Such meetings are important for ensuring optimal patient selection—many drugs have failed in clinical trials because the patients selected for trial were far too advanced for any disease-modifying therapies to be effective. This has clearly been demonstrated in numerous animal models but is not taken on board by investigators of clinical trials [254].

To tackle the problems of translation from animal studies of neuropsychiatric and neurodegenerative diseases, a number of new programs have been initiated. These include programs such as MITACS [255], CNTRICS [256], and PIvital [257] Others [258] have been developed to facilitate the development of new treatments for AD. The National Alzheimer's Coordinating Center (NACC) [259] has developed a "uniform data set" to submit the information related to neuropathological and epidemiological details of AD and propagate them among the basic researchers for a better development of preclinical studies. The Mary S. Easton Center for Alzheimer's Disease Research at UCLA has also been developed to co-ordinate research among the AD researchers and clinicians [260]. New programs such as these will train researchers who can integrate

Table 3
Limitations and plausible solutions in designing clinical studies

AD research has predominantly focused on Aβ pathology neglecting several other mechanisms	Consideration of other AD pathologies such as neuroinflammatory cytokines, cofilin-actin rods pathology
Pathological variations among different cohorts of AD patients are not considered in human trials	Variations such as AβPP mutation and plaque formation, which alter from cohort to cohort, should not be highlighted alone as a major pathological parameter
There are no standard biomarkers identified for early diagnosis of the disease and for the identification of populations with high risk or differentiate MCI from AD	Because of the multifactorial nature of AD, efforts should be made to establish a standardized diagnostic protocol by combining genetic analysis with neuroimaging traits and epigenetic biomarkers
AD pathology changes over time. The different stages of the disease are not considered in diagnostic criteria	Measuring longitudinal patterns of changes in a set of different biomarkers may be the most reliable way to diagnose AD and measure its progression
Majority of investigations in human trials are not longitudinal and patients recruited at an advanced disease state leading to lack of evidence for epigenetic influence on the pathogenesis of AD	Now FDA prioritizes the institution of clinical trials in the early stages of AD emphasizing the significance of early diagnosis for effective intervention
Conventional drug delivery system in AD remains a major cause of concern due to poor CNS penetration across the blood-brain barrier	Efficacious brain bioavailability by other approaches should be tested such as transdermal patches, intranasal, intracerebroventricular, adeno-associated virus- and nanoparticle-based drug delivery
Lack of crosstalk between basic researchers and clinicians creating a knowledge gap among them, dampening the hope of clinical translation	Involvement of both can increase the transparency and rationality of the study design in animal models as well as clinical trials
Recent genome-wide association studies (GWAS) have identified multiple genes associated with AD but these are not widely integrated in clinical practice	Emerging findings from GWAS studies should be integrated into the design of clinical trials, which may substantially reduce study duration and trial cost

basic research and mechanistic studies with clinical problems posed by patients as well as public-private partnerships [261].

CONCLUSIONS

Several factors have impeded the translation of basic bench research to effective treatment for AD (Tables 2, 3). It is indisputable that the development of animal models has paved the way to understanding the neurobehavioral outcomes, pathophysiology, and molecular events involved in the disease. Still it is apparent that human disease pathology cannot be replicated in animal models. The pathophysiological and phylogenetic differences between rodents and humans have made translation difficult. Preclinical studies involving animals seldom consider confounds, randomization, and blinding in their study designs. A variety of confounds in the generation, study design, and testing and evaluation of the models have also contributed to the limited success in the clinical translation of these findings (Fig. 2). In addition, the overemphasis on centrality of amyloid hypothesis to the exclusion of the non-amyloid mechanisms including early transgene changes, synapse loss, neuroinflammation, microvascular abnormalities that may trigger the cascade of cognitive decline, has hampered progress. The outcomes of the anti-Aβ drug trials in AD resonate with the outcome of Parkinson's disease trials, five decades ago, when the central hypothesis underlying Parkinson's disease research was tested: drugs that reversed the characteristic dopamine depletion in nigrostriatal neurons effectively ameliorated Parkinsonian signs and symptoms in most patients, even though the drugs had no discernible effect on the underlying disease process. Some hypotheses turn out to be correct; others do not. Hence, a broad range of putative underlying pathological mechanisms could be targeted for AD. A more balanced approach to disease treatment and prevention that includes the impact of nutritional and lifestyle changes should be considered in future direction for AD research.

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Translational Neurodegeneration

REVIEW Open Access

Role of early life exposure and environment on neurodegeneration: implications on brain disorders

Shweta Modgil[1,2], Debomoy K Lahiri[3], Vijay L Sharma[2] and Akshay Anand[1*]

Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) and retinal degeneration have been studied extensively and varying molecular mechanisms have been proposed for onset of such diseases. Although genetic analysis of these diseases has also been described, yet the mechanisms governing the extent of vulnerability to such diseases remains unresolved. Recent studies have, therefore, focused on the role of environmental exposure in progression of such diseases especially in the context of prenatal and postnatal life, explaining how molecular mechanisms mediate epigenetic changes leading to degenerative diseases. This review summarizes both the animal and human studies describing various environmental stimuli to which an individual or an animal is exposed during *in-utero* and postnatal period and mechanisms that promote neurodegeneration. The SNPs mediating gene environment interaction are also described. Further, preventive and therapeutic strategies are suggested for effective intervention.

Keywords: Aging, Metals, Epigenetics, LEARn, Methylation, Pesticides

Background

Early life plays an important role in health and development of an individual. Interactions between genes and environmental factors during early life are suggested to play role not only in human behavior but also in susceptibility to diseases. Surprisingly, in some individuals, onset of neurodegenerative disorders cannot be explained by family history. What triggers the sudden onset and rapid progression of these diseases still remains unexplained. Such sporadic diseases need to be studied in the context of early life environmental exposure. It is believed that environmental factors in childhood interact with the specific loci thereby modifying their expression and resulting in disease onset [1]. Epidemiological and animal based studies have also suggested a strong relationship between environmental factors and neurodegenerative disorders [2-8]. The effect of exposure to different environmental conditions during *in-utero* and developmental stages of life have been studied extensively and based on these studies various models have come into existence. A variety of agents including heavy metal exposure such as lead (Pb), manganese, mercury [9-11]; dietary habits [12,13]; pesticides [14-16]; stress [17] and other intrinsic factors such as inflammatory cytokines [18] affect early life and alter the regulation of gene expression. In this context, this review has been conceptualized to discuss the role of environmental cues that govern the onset of neurodegeneration. In addition, various single nucleotide polymorphisms (SNPs) associated with xenobiotic metabolizing enzymes (XMEs) have also been explained which may be useful for instituting preventive measures for adverse environmental stimuli.

Environmental factors in neurodegeneration

It is widely believed that environmental constituents such as food, metals, pollutants, microorganisms and lifestyle play a direct or indirect role in brain health. For example, environment to which a fetus is exposed during the gestational period plays a significant role in future health of an individual. Postnatal period is also crucial for rendering an individual susceptible to environmental

* Correspondence: akshay1anand@rediffmail.com
[1]Department of Neurology, Neuroscience Research Lab, Post Graduate Institute of Medical Education and Research, #3036, Research Block-B, 160012 Chandigarh, India
Full list of author information is available at the end of the article

influences. Adverse prenatal and postnatal environmental conditions disrupt the homeostasis and increase the risk of neurodegenerative disorders. Various animal and human studies have been discussed in this context.

In-utero conditions

Maternal environment affects the growing fetus as during *in-utero* stages, mother's body is the only environment to which fetus is exposed. Growth of fetus is generally proportionate to the mother's size and maternal constraint refers to the restriction provided to the growing fetus due to mother's body size [19,20]. The maternal restriction affects growth by limiting the size of placental connection between mother and fetus thereby affecting the supply of nutrients for growth. The restraint is increased with age of mother, short stature and multiple pregnancies [21].

Human studies

Human fetuses are generally exposed to chronic placental insufficiency (CPI), hypoxia, heavy metals or hormonal disturbances in the mother's womb. Studies have revealed that the chronic placental insufficiency (CPI) or umbilical cord occlusion to which fetus may be exposed to result in fatal hypoxenima [22] leading to synaptic dysfunction that triggers damage in neonates resulting in neurodegeneration [23]. Maternal hormonal disturbances also have adverse effect on fetus. Hormonal levels in fetus may be elevated if placental barrier between mother and fetus is compromised. For example, stress in mothers elevates glucocorticoid levels which travel through placenta adversely affecting fetus by programming the hypothalamus-pituitary-adrenal (HPA) axis due to change in number and affinity of glucocorticoid receptors in fetus [24]. Human studies showing the effect of gestational or *in-utero* exposure on neurodegeneration are limited. Most studies are either retrospective in nature, which imposes a recall bias in the study design, or if longitudinal studies are planned they are not of long duration.

Animal studies

As compared to human studies, animals provide an excellent model for longitudinal analysis of early life exposures due to comparatively small life cycle, easy maintenance and trackable follow up. Rat model of perinatal asphyxia has shown to affect retinal development by reduction in number of ganglion cells due to degenerative changes which lead to long term effects [25]. Similarly placental insufficiency was found to be associated to brain damage by impacting metabolic processes in rabbits [26]. Various mechanisms have been extensively reviewed by Johnston and coworkers [27] emphasizing that the developing brain is more vulnerable than the adult brain to the same insult. In an interesting study, pups of female exposed to lipopolysaccharide (LPS), a bacterial endotoxin, during pregnancy showed loss of dopaminergic neurons. This suggests that high LPS levels in mothers might interfere with the dopaminergic neurons in the fetus enhancing the susceptibility to PD [28]. Similarly, gestational exposure to metal toxins resulted in altered levels of various antioxidant enzymes in rats leading to oxidative stress [29]. Maternal hormones effect on newborns was reproduced in *in-vitro* studies on cerebral granular cells extracted from one week old pups of pregnant rats treated with dexamethasone and it was shown that oxidative stress due to glucocorticoids in cerebral regions is associated with neuronal apoptosis [30].

Together these studies not only highlight the importance of *in-utero* conditions in determining the health of fetus but also present an opportunity to increase the research investigations in this field of research.

Dietary exposure

Dietary habits have significant effect on the physiology and metabolism of an organism. Growth and development of fetus is dependent on nourishment which is provided by the maternal system, thus, any food restriction during pregnancy has a direct or indirect role on fetus development. Deficiency or excess of any nutritive supplement to the mother results in long term consequences to the offspring.

Human studies

The possible effect of fetal nutrition on the risk of degenerative disease in later life has generated interest in 1990s resulting in extensive studies which elucidated the positive relation between diet and disease onset [31]. Positive relation between maternal diet and neurodegeneration has been supported in some human studies. Vitamin B-12 for example, is important for maintaining homeostasis in body and studies have shown that Vitamin B-12 deficient diet to mother during pregnant adversely affects the myelination in nervous system of offspring [32]. Postulating the role of maternal micronutrients, Roy and coworkers have demonstrated that imbalanced micronutrient supplementation in mother affects the level of antioxidant enzymes in the offspring increasing the risk of neurodegenerative diseases [33].

Animal studies

Similar to human studies, correlation between maternal diet and fetal neurodegeneration was reported in animal studies as well. Performance in Morris maze experiments is affected in pups born to mice fed on high fat diet during gestational and lactation period and the results were attributed to decreased cell proliferation [34]. Similarly, studies have shown that maternal folate depletion results in oxidative stress and epigenetic changes in the offspring

[35] which ultimately lead to neurodegeneration. Further elevated levels of homocysteine in mother were shown to increase oxidative stress in pups brain leading to apoptosis, as marked by DNA fragmentation [36]. High dose of iron at neonatal stage has similarly been shown to result in neurodegeneration of midbrain at a later age. Pups with higher iron dose reduce dopaminergic neurons at age of 24 months as compared to that of 2 months old pups. This indicates that there are long term effects of neonatal iron exposure which are associated with degenerative changes [37]. Conversely, omega-3 fatty acid rich maternal diet is neuroprotective. This was shown by a study where omega 3 fatty acid supplementation to mother resulted in neonate protection from LPS induced brain injury [38]. Therefore, balanced diet during pregnancy has been suggested to protect offspring from neurodegenerative diseases.

Metal exposure

Heavy metals consist of toxic pollutants pervading the environment. They are widely distributed in the environment and poison the living systems, as they accumulate. Mature tissue is protected from metal toxicity by the blood–brain barrier which prevents the movement of heavy metals from the systemic circulation to brain and by the formation of metal-protein complexes rendering metals unavailable to exert its toxic effects. In fetal brain this sequestering mechanism is impaired [39].

Human studies

Various metals such as aluminium, zinc, iron, copper and mercury have been linked with the neurodegenerative diseases. However, in some cases results are controversial and no direct association between these metals and neurological diseases have been demonstrated. For example, high level of aluminium in drinking water has been shown as a risk factor of Alzheimer's disease in some studies while other studies fail to establish any such relation [40,41]. The reason for such contrary results includes inadequate aluminium analysis methods, improper selection of subjects and matching controls [42]. Transition metals like zinc and copper are other sources of brain toxicity and are believed to results in Aβ aggregation [43]. Like brain, retina is considered to be an immune privileged site due to presence of the blood-retinal barrier and has been found to be sensitive to metal toxicity. Metal exposure and its association with retinal degeneration has been examined in various studies [44-46]. Low and moderate level of gestational lead exposure (GLE) i.e. first trimester results in increased amplitude of a and b waves in 7–10 year old children [47]. Similarly high level of mercury and Pb in umbilical cord blood due to prenatal exposure impaired the visual processing as shown by visual evoked potential measurement in exposed children after 11 years [48].

Animal studies

Toxic effects of heavy metal exposure are also evidenced from animal studies. Long-term potentiation (LTP) which is responsible for enhancing the signal transmission between the neurons is considered as the major mechanism underlying information storage and memory formation, resulting in increased synaptic strength [49]. Enhancement in signal strength is dependent on two factors, one is the presynaptic increase in neurotransmitter release and other is enhanced function of glutamate receptor at the postsynaptic end. NMDA receptor function has been found crucial for the LTP induction in hippocampus [50,51]. Neonatal exposure to aluminium chloride has been shown to reduce the LTP amplitude in rats by affecting both presynaptic and postsynaptic signal transmission [52]. Heavy metal exposure such as zinc, copper and Pb have a negative effect on LTP during developmental stage as it reduces the potentiation magnitude and increases its decay time as well as the threshold level for induction in hippocampus [53,54].

Combined prenatal effects of arsenic, cadmium and Pb in rats exposed to metal mixture have been shown to disrupt blood–brain barrier and cause memory deficit [55]. Although various studies have focused on the role of different metals in pathogenesis of neurological disease, the role of Pb is most widely investigated. The early life exposure of Pb and its effect on adults has thus been a major area of investigation for past few years. Rats exposed to low Pb level during *in-utero* and lactation period have shown impaired learning and memory, hyperactivity and anxiety in adults [56]. *In vivo* studies of Pb exposure on various animal models, such as rats and monkeys, have revealed the role of developmental exposure of sub-toxic doses of Pb on neurodegeneration. It is evident from studies that the Pb exposure in developmental stages results in the increased level of beta amyloid in brain causing Alzheimer in later age [57,58].

Pesticides

Pesticides are other major pollutants or toxins to which living organisms are exposed. Health issues related to pesticides prevalence in environment are of major concern. These pesticides include insecticides, herbicides and fungicides. Insecticides such as organophosphates, organochlorines and carbamates are used more frequently and enter the living system through respiratory tract, gastrointestinal tract or through dermal contact [59,60]. Ocular exposure, although not a common route of exposure, may occur through accidental splashing of pesticides into eyes or through contact of hands with eye and further from ocular tissue to blood circulation [61]. β radiation based

radioactive studies have revealed movement of carbamate from the cornea to the retina via aqueous humor supporting the exposure of pesticide through ocular route [62].

Human studies

Exposure to pesticides is more prevalent in individuals working in agricultural sectors such as farmers, peasants, farm workers. They are at increased risk of direct exposure while others may be exposed due to food contamination [63]. Contaminants get accumulated in the body and change the gene expression profile in exposed tissues. Pesticides are thus believed to be one such contaminant that can alter the regulatory framework and lead to disease onset and progression through epigenetic changes [64]. Pesticide exposure has been shown to result in neuronal loss, cognitive impairment and motor dysfunction. These alterations in neurological behavior may be associated with neurodegenerative diseases.

Animal studies

Pesticides exposure studies in animals supported the adverse effect of early life exposure on later life. It was evidenced from study in which exposure to dieldrin during gestation and lactation has been reported to affect the dopaminergic responses in offsprings. Exposed mice showed elevated level of dopamine transporter and vesicular monoamine transporter 2 (VMAT) proteins. These alterations were persistent through later stages in life leading to dysfunction of dopamine making dopamine neurons more susceptible to damage in adulthood [65]. Another pesticide, paraquat in combination with maneb, has also been shown to be more destructive in animal studies and leads to PD by dysfunction of nigrostriatal dopaminergic system as well as motor response abnormalities [66]. Likewise, permethrin, when administered to rats at age of 6–21 day results in glutamate, NO and calcium imbalance in brain hippocampus [67]. Despite accumulating evidence of the effect of pesticides in pathogenesis of neurodegeneration, very only fewer studies have integrated this aspect of investigation in understanding of brain disorders.

Lifestyle, smoking and drug abuse

Lifestyle plays a central role in health and well being of organisms. With increased sedentary lifestyle and lack of physical activity the incidence of diseases is also increasing. Healthy lifestyle prevents disease occurrence whereas bad habits increase the susceptibility to disease. Exercise, in particular aerobic exercise, has a positive impact on brain functioning.

Human studies

Importance of healthy lifestyle in human life has been demonstrated. Childhood aerobics increases the resilience of the brain in later life [68]. Similarly, the association of caffeine, smoking and alcohol consumption has been well reported in neurodegenerative diseases [69-71]. Our SNP studies with patients of age related macular degeneration (AMD) showed higher frequency of TT genotype of CCL2 gene. Interestingly, the frequency of TT genotype was found to be higher in smoker AMD patients when compared to nonsmoker AMD patients [72] highlighting the role of smoking in exacerbating the pathogenesis of disease. Early life exposure to smoking with degenerative disease has not been investigated adequately and could be the subject of future research projects.

Animal studies

Studies carried out on animals further strengthen the correlation between lifestyle and neurodegeneration. In a study, the pups born to mothers underwent low intensity treadmill exercise during pregnancy were shown to have more hippocampal cell survival [73]. Similarly, pups performing treadmill exercise at postnatal day 21–60 showed enhanced spatial memory as compared to controls [74]. Drugs such as methamphetamine (MA), which is widely abused due to comparatively low prices in comparison to cocaine or heroin [75] have been studied for its role on retinal damage in rats born with prenatal and postnatal methamphetamine exposure. Female rats exposed to MA at gestational stage have shown altered optic nerve patterns in newborns with optic nerve diameter smaller than the controls. Furthermore, it has also been reported that optic nerves of MA exposed rats have reduced production of myelin basic protein and increased number of deformed axons, mean optic fiber area, less lamellar separation [76-78] (Figure 1; Table 1).

Mechanism, hypothesis and models

Epigenetics

Recent studies have focused on epigenetic mechanisms that modify the onset, latency period and progression of neurodegenerative diseases [91]. Epigenetics is an emerging field that focuses on the mechanisms that alter the function of genes. It generally takes into account the gene and environment interaction such that these changes are inherited. The epigenetic changes do not involve alteration in nucleotide sequences in the DNA but influence its functioning by controlling its expression by gene reprogramming [92]. The epigenome is therefore considered different from genome in being dynamic. It is altered by environmental signals, not only during the period of exposure but even later in life. It has been shown that fetal epigenetic patterns can be altered at later stages by environment exposures [93,94]. A traditional insight into the field is exemplified by the example of identical twins having same genotype but possessing different

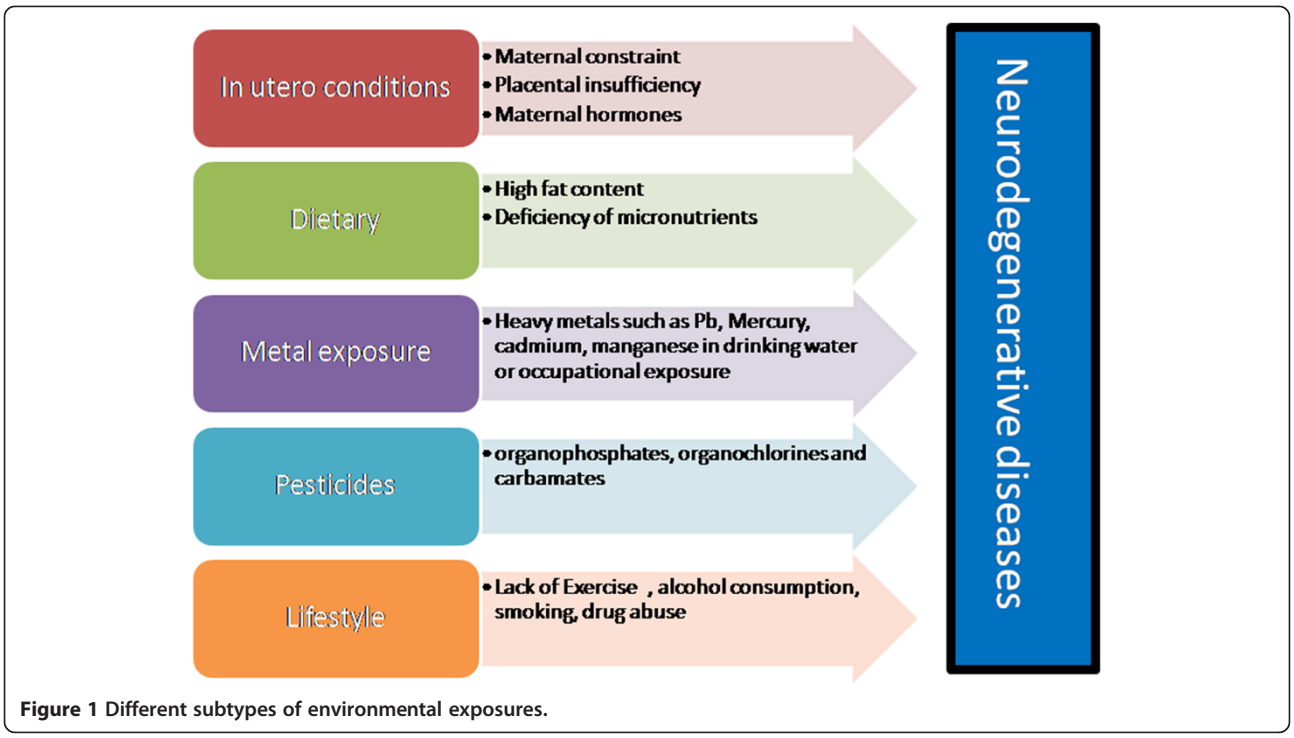

Figure 1 Different subtypes of environmental exposures.

Table 1 Spectrum of environmental stimuli and their effects on neurodegeneration

S. no.	Exposure	Subject/animals	Period of exposure	Effect	Reference
1.	Ethanol	Mice	Postnatal day 3-20	Decreased number of neurons in Retinal ganglion cell layer and dorsalateral geniculate	[79]
2.	Microwave irradiation	Mice	Prenatal + 4 months postnatal	Complete degeneration of RPE, nuclear pyknosis in photoreceptors, thinness of all layers	[80]
3.	Fried potato chips	Rats	Gestational day 6-postpartum day14	Vacuolization and apoptosis in GCL, swollen choriocapillaries, alteration in cellular organelles	[81]
4.	Lead (Pb)	Mice	Lactation period	Altered mitochondrial morphology, mitochondrial phosphorylation dysfunction	[82]
5.	Rotenone	Rats	Postnatal	Thinness of GCL, disruption of mitochondrial complex I, photoreceptor loss	[83]
6.	Cycus plant		Postnatal	ALS and PD	[84]
7.	Pesticide contaminated drinking water	Human	Postnatal	Inhibitory effect on antioxidant enzyme systems, mitochondrial and proteosome function (PD)	[85]
8.	1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine	Mice	Gestational day 8-12 and postnatal	Apoptosis of nigrostriatal dopamine neurons enhancing toPD risk	[86]
9.	Methamphetamine	Mice	Postnatal day 11-21	Altered level of muscarinic acetylcholine receptors in the hippocampus	[87]
10.	Cypermethrin	Rats	Postnatal day 5-19	Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) level in brain altered	[88]
11.	Aluminium	Mice	Pregnancy day 1-15	Neurotoxicity by affecting dopaminergic system	[89]
12.	Tobacco inhalation	Mice	Gestational day 6-17	Altered gene expression profile affecting morphology and function of hippocampus	[90]

epigenetic patterns in adulthood due to different environmental exposures leading to different epigenome and disease susceptibility [95-97]. Epigenomic variation leads to phenotypic diversity as well as susceptibility of individuals to disease. These changes are generally brought about by environmental influences. DNA methylation and histone acetylation have been recognized as epigenetic processes which regulate the functioning of gene. Histone acetylation controls the heterochromatic and euchromatic state of DNA wrapped around histones, and remaining in dormant state. Histone acetylation unwinds the DNA from histone and renders it available for transcription. Along with histone acetylation, DNA methylation plays an important role in regulating accessibility of DNA for transcription. Histone acetylase transferases (HAT) and Histone deacetylase (HDAC) controls histone modification in cell [98]. Animal studies have been used to describe the epigenetic pathways of disease etiology. It has been demonstrated that the early life exposure to various environment stimuli leads to methylation pattern changes in promoter region, resulting in altered gene expression in later stages. Methylation patterns have been found to be altered in mice offspring by methyl donors or low proteins in mother's diet [99]. Some sites in the genome are more susceptible to the epigenetic changes. It is, therefore, pertinent to note that C_pG islands are targeted more often for methylation [100]. Thus, switching on and off of expression is under the control of epigenetic patterns of histone acetylation and DNA methylation changes [98] which are influenced by early life exposure.

The non-coding RNA referred to as microRNA is believed to act at post transcriptional stage thereby exerting epigenetic regulation of such changes. MicroRNAs control the gene expression by interfering with the mRNA thereby destabilizing it and rendering it unavailable for translation. This unique property enables it to regulate many different mRNAs [101] (Figure 2).

Barker hypothesis or fetal basis of adult diseases (FeBAD)

Barker and coworkers have proposed the FeBAD model after their studies on adult cardiovascular diseases and

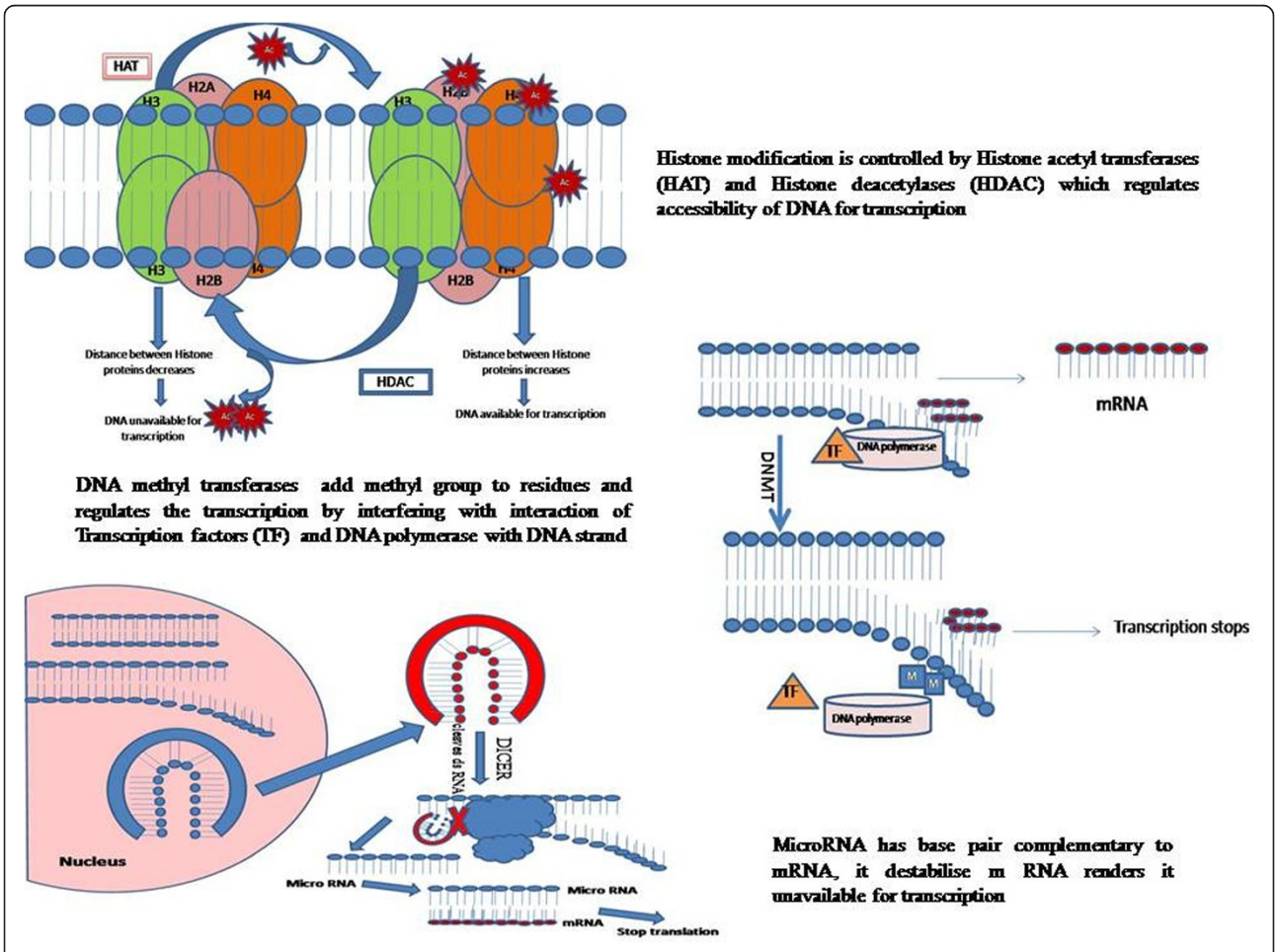

Figure 2 Epigenetic processes that regulate the gene expression at transcriptional and post-transcriptional level.

their fetal origin [31]. According to Barker's hypothesis adult diseases are more or less consequences of fetal adverse conditions. Although Barker's work was mainly confined to cardiovascular diseases, the hypothesis fits well to other diseases too. The fetus gets adapted to new environment depending on environmental stimuli in uterus by means of physiological and hormonal alterations and prepares itself to the upcoming conditions in postnatal life, a phenomenon called fetal programming. It takes cues from the maternal health status and show adaptive responses to survive in the maternal environment. Adaptive responses may be either in the form of metabolic changes, hormonal release or sensitivity of the target organs to hormones, which in turn affects the development of target organs, leading to physiologic and metabolic disturbances. Thus, the reduced growth or body size can be considered as a fetal adaptive response towards small uterus size of mother with no immediate consequences in the newborn but which may lead to physiologic changes that can cause diseases in later life [19].

Developmental origin of health and disease (DOHaD)

The DOHaD model was a modified version of FeBAD which postulated that postnatal period of development also plays an equal role as fetal life in health. According to DOHaD, the adaptive responses during developmental stages, which include not only embryonic development but also the period of development during infancy, are responsible for late life risk of diseases [20]. Environmental conditions prevailing during the infancy phase exert their influence on the genotype and alter the organism's ability to cope with its environment in later life. As compared to intra-uterine environment, which remains relatively constant throughout gestation, postnatal environment changes drastically. The DOHaD phenomenon explains how changing environmental factors affects the patterns of diseases.

Predictive adaptive response (PAR)

Gluckman and Hanson have suggested that when fetus is exposed to adverse conditions or stress it makes immediate changes which are often reversible, but if the stress conditions are prolonged, fetus undergoes irreversible changes which then persist throughout life and influence the adulthood. They coined term PAR for the phenomenon. The fetus predicts the extra-uterine environment from intrauterine conditions and makes changes for its better survival. These irreversible changes may or may not be useful to the fetus in the long run. If extra-uterine environment will be different from intrauterine, it will suffer from the physiological manifestations as changes in response to predictive environment will not match the actual environment [102]. If adaptations match the environment, then it leads to the better survival. For example, meadow vole pup born in autumn has thicker coat due to adaptive response to the signal emanating from maternal melatonin levels *in-utero* and thus has better survival [103].

LEARn model

LEARn (Latent early life associated regulation) model suggests the role of environmental factors in disease etiology. Lahiri *et al.* [94] have described the association of early environment with disease onset especially with respect to Alzheimer's disease. Due to lack of knowledge pertaining to disease cause and progression, the sporadic onset of several diseases have been believed to be associated with many environmental agents such as nutrition [104], head trauma [105], metal exposure [106] and lifestyle [107]. LEARn model describes these environmental exposures as 'hits'. The authors contrasted LEARn against different acute and chronic models of disease progression [94]. LEARn is distinct from these models in that it is neither acute nor chronic but acts through induced latent epigenetic changes. They further suggested that all neurodegenerative disorders come under the category of a 'n' hit latent model, according to which early life exposure leads to epigenetic perturbations in the genes but do not result in any disease symptom. A second trigger is required for the disease to develop and this time between first hit and disease onset is termed as latency period. Genes are divided into two categories the one which respond late in relation to early life responses (LEARned) and others which don't (unLEARNed). The process of responding to the early life environmental triggers after the long latency period is termed as LEARning [94] (Figure 3).

Prevention and reversibility

Reversal of induced changes may be possible if associated epigenetic (methylation, acetylation) and physiologic (gene expression) changes can be switched back to normal. Cognitive impairment because of imbalanced maternal diet has been tested by leptin treatment as leptin receptors are present in brain regions and known to regulate neuronal excitability and long term potentiation [108]. Peroxisome proliferator activator receptor α (PGC1α) regulates the expression of genes involved in bioenergetics. (PGC1α) expression in offspring of under-fed female rats returned to normal by exogenous supply of leptin [109]. Similarly folate deficiency related neurodegeneration is ameliorated by dietary S-adenosylmethionine (SAM) supplementation. Folate deficiency has been shown to result in neurodegeneration in mice due to reduced level of SAM which is attenuated by apple juice concentrate supplementation, containing high levels of SAM [110-112]. Likewise, polysaturated fatty acids exerts neuroprotective effect against neurodegeneration in PD and AD models by ameliorating the adverse effects of neuronal toxicity

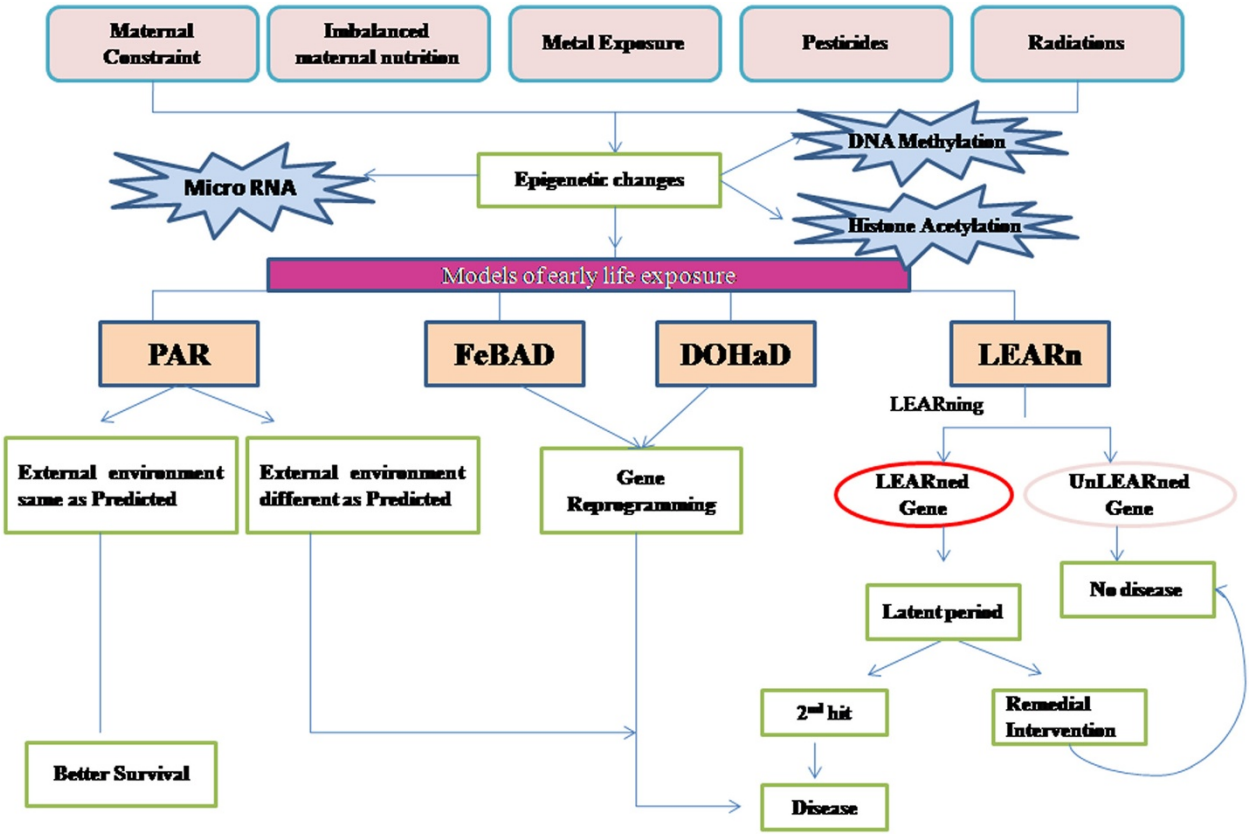

Figure 3 Effect of environmental factors on late-life disorders. The schematic summarizes the proposed mechanism and different models of early life exposures and how they operate.

[113,114] and creatine rich diet has also been shown to sustain the harmful effects of birth hypoxia [115]. These studies highlight the possibility of restoring altered epigenetic changes and provide scope for instituting therapeutic approaches for ameliorating degenerative diseases. Remedial intervention during latency period can prevent the disease onset by reversing the abnormal conditions back to normal for e.g. complete degeneration of inner retina by early life exposure to monosodium glutamate (MSG) [116] has been found to be reversed by enrichment of postnatal living conditions in rats. Provision of appropriate housing conditions such as larger cage size readily reversed the effect of MSG on retinal thickness [117]. Exercise is another preventive measure that has been shown to modulate the expression of genes regulating the methylation and acetylation of DNA and protein. Studies have shown decreased expression of DNA methyltransferases [118] and increased expression of HAT [119] in the hippocampus of rats which exert their epigenetic influence by increasing the expression of neurotrophic factors in brain. Further evidence was provided by Scopel *et al.* [120] by showing that exercise regime of 20 minutes for 2 weeks for wistar rat attenuates the damage in hippocampal slices submitted to ischemia *in-vitro* opening the field for further investigation.

Therapeutic interventions

While prevention is always better than cure, sometimes it is not feasible to prevent an environmental exposure due to occupational demand, as in pesticide exposure to farmers and metal exposure to workers in metallurgy industry is imminent. Similarly, if the sole source of water supply is contaminated, exposure to pollutants cannot be avoided. In such cases identification of targets for disease reversal are useful tools for pioneering therapies. The environmental agents modulate the normal functioning and physiology of central nervous system (CNS) by mechanisms that involve altered gene expression through modulation of signal pathways. These mechanisms, if explored, can provide a window of opportunity for therapeutic intervention during latency stage, thereby delaying or preventing the onset of disease. Recent studies have tried to elucidate the underlying mechanisms by which the environmental agents exert their toxic effects on CNS. Pb is reported to accumulate amyloid-β in brain tissue by decreasing the activity of insulin degrading enzyme (IDE)

and neprilysin (NEP), both known for amyloid beta degradation [121,122]. Exogenous administration of IDE and NEP may thus provide a good approach to prevent the lead induced toxicity. Another key factor involved in neurodegeneration is oxidative stress as is evident from studies related to AD and PD [123,124]. Environmental toxins such as heavy metals act as an electron acceptor or donor and result in formation of reactive oxygen species, leading to oxidative stress [125]. Therefore, antioxidants can be used for metal intoxification due to their property of ameliorating the oxidative stress. Certain antioxidants such as α-lipoic acid and vitamin E have already been reported to prevent neurotoxicity induced by copper [126]. Herbal extracts of Lutein, *Allium cepa,* and other natural antioxidants can similarly diminish the adverse effects of oxidative stress and prevent rapid disease progression [127,128]. By reducing the cause that results in neurodegeneration, remedial steps to reverse the effect can be evaluated. Metal exposure and drug abuse, for example, disrupts the signaling pathways, as manganese toxicity in striatum has been found to alter the AKT1/2 and ERK signal pathway [129] resulting in impaired VMAT and dopamine active transporter (DAT) regulation [130]. In such case the neuroprotective substance should be able to maintain the normal signaling pathway so that the expression of VMAT and DAT protein is not compromised. Trolox, has been found to reverse the adverse effect of manganese on ERK 1/2 pathway [130] while a Chinese prescription, Zhen Wu Tang (ZWT) ameliorates the neurodegenerative process by maintaining levels of VMAT-DAT mRNA [131]. Thus, both of them provide a therapeutic approach against metal toxicity. Similarly rotenone induced neurotoxicity was ameliorated by oxytocin by reducing the expression of various caspases which were responsible for apoptosis [132]. Likewise, targeting PGC 1α can be useful in PD patients as elevating PGC1α levels in *in-vitro* studies prevented dopaminergic neuron loss [133].

Metal induced neurotransmitters-receptor sensitivity and cause neurodegeneration. LTP has also been suggested to result from the malfunctioning of NMDA receptor [134,135]. NMDA receptor is a hetero-dimeric structure and the functionality of receptor depends on the proper assembly of subunits. Expression of NR2A subunit of receptor has been reported to be reduced due to Pb exposure resulting in altered LTP suggesting that NR1/NR2A receptor complex is required for the calcium mediated signaling to maintain the cognitive ability [136]. Taurine supplementation on the other hand was found to be protective against NMDA receptor malfunctioning by reducing calcium overload [137]. Therefore, for diseases related to NMDA receptor malfunctioning and calcium influx, taurine can be considered as neuroprotective.

Ubiquitin Proteosome Complex (UPC) maintains protein homeostasis in the body by degrading the misfolded, malfunctioned and accumulated proteins and inhibition of UPC results in aggregation and deposition of these malformed proteins in CNS leading to neurotoxicity [138]. As also described in epigenetics section that histone modification plays a major role in regulation of gene expression, HDAC inhibitors such as valproic acid, trichostatin and phenylbutyrate have been found to be neuroprotective. They exert neuroprotection by regulating the expression of neurotrophic factors such as glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and reducing inflammation and neuronal death [139,140]. Thus, therapeutic intervention by targeting these known processes can also prevent the progression of disease from environmental hazards. These neuroprotective agents thus help in disrupting the cascade of reactions that ultimately lead to cell loss by apoptosis (Figure 4).

Genetic susceptibility to environmental stimuli

Individuals exposed to same environment respond differently and this difference is attributed to differences in genetic make-up. SNP studies focus on the polymorphisms in genes which influence the susceptibility of individual to the environmental stimuli. Studies have been carried out to show that risk to the environmental toxins such as heavy metals and pesticides have positive correlation with gene polymorphisms. Polymorphism in XME genotype influences the metabolizing efficacy of enzyme. SNP variation effect the normal functioning of enzyme by altering the enzyme kinetics. One allele of glutathione synthetase *(GSS)* was found to be more interactive with metals over the other enhancing the risk of toxification [141]. Similarly, glutathione transferases *(GSTs)* are another group of enzymes involved in detoxification processes by ubiquitinization of pesticides and other toxicants. *GST* genotype and heavy metal metabolism have been studied and it was found that one form of gene readily metabolizes metals into non-toxic form and thus reduces the risk of toxicity [142,143]. Children of mothers with *GSTM1* and *GSTT1* allele, prenatally exposed to pesticides are at greater risk of fetal growth restriction [144]. Further studies on this gene revealed the positive associated of gene polymorphism with AD, PD and AMD [145]. Similar genotype study on human paraoxonase 1 *(PON1)* enzyme revealed that one form of gene is associated with increased susceptibility to pesticide related damage. Children of mothers with susceptible genotype have been found to be more prone to toxicity due to prenatal exposure of organophosphates [146]. N-acetyltransferase-2 *(NAT-2)* and Cytochrome P-450 *(CYP2C9)* are other XMEs that are studied for genetic susceptibility for DNA damage due to pesticide exposure. Singh and coworkers studied

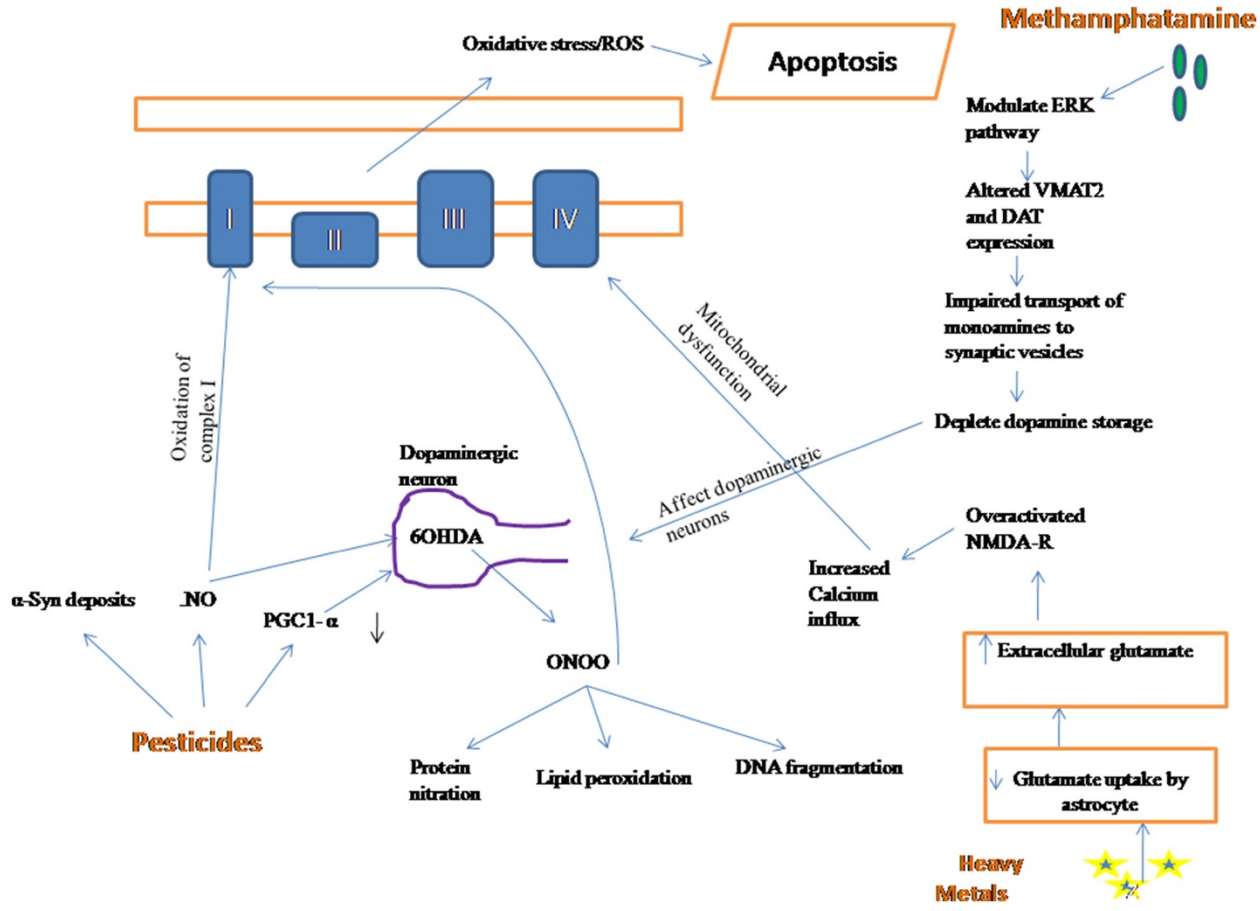

Figure 4 Schematic diagram representing the action of different environmental stimuli (metals, drug and pesticides) on dopaminergic neurons, glutaminergic neurons and mitochondria leading to neuronal apoptosis.

polymorphism of these enzymes in workers exposed to organophosphate pesticides and revealed that DNA damage was higher in persons with one particular allele as compared to the persons with another allele [147]. Pregnant women exposed to heavy metals have been reported to have placental accumulation of these metals which affects the transport of nutrients from mother to fetus. Metallothionein is involved in micronutrient transport and detoxification of placental toxins. Polymorphism in this gene results in differential accumulation of cadmium in placenta [148]. Similarly, SNPs in metallothionein *(MT)* gene have also been shown to be responsible for varying susceptility to ALS. Antioxidant enzymes help in preventing the oxidative stress and SNPs related to these enzymes also showed varied response to environment. Superoxide dismutase *(SOD)* genotype reconstruction showed that *SOD1* (GG) and *SOD2* (GT) alleles decrease the risk of retinopathy of prematurity in preterm babies [149]. The above studies have elucidated that certain alleles involved in xenobiotics metabolism make individual more susceptible to diseases, who can be counseled to adopt preventive measures to protect themselves from adverse environmental influences.

Conclusion

The present review emphasizes the importance of environmental cues and epigenetics on pathogenesis of neurodegenerative diseases. The role of early life exposure to environmental stimuli while ageing has largely remained underinvestigated which has been highlighted in this review. Present work postulates that the sporadic diseases can be considered as after effects of exposure in early life, in addition to prevalent theories of pathogenesis being investigated worldwide. Early life practices and environment determines physical and mental wellness in later stages due to genetic imprinting explained by epigenetics. Even though the bulk of research investigations have focused on molecular targets, the therapeutic outcome has not been very encouraging. A new focus on targeting the early life epigenetic mechanisms is imperative through larger studies. Whether developmental disorders and degenerative diseases have any epigenetic association could

be revisited though launch of longitudinal animal studies. Therefore, prevention of disease by preempting early life exposure should be tested by launching worldwide public health initiatives. The mechanistic understanding of neurodegeneration provided in the review will likely provide new insights important for healthy lifestyle in the individuals at risk for such diseases.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contribution

SM compiled the review of literature and wrote the manuscript, DKL provided the importance of pursuing early life exposure studies in degenerative diseases and called for review writing and edited the manuscript, VLS guided the first author in writing and compiling the manuscript; AA conceptualized the review writing, edited the manuscript and coordinated with various authors. All authors read and approved the final manuscript.

Authors' information

Shweta Modgil and Akshay Anand Equal first author.

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Author details

[1]Department of Neurology, Neuroscience Research Lab, Post Graduate Institute of Medical Education and Research, #3036, Research Block-B, 160012 Chandigarh, India. [2]Department of Zoology, Panjab University, Chandigarh, India. [3]Department of Psychiatry, Institute of Psychiatric Research, Indiana University Medical Center Indianapolis, Indianapolis, USA.

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Why AMD is a disease of ageing and not of development: mechanisms and insights

Kaushal Sharma[1], Neel Kamal Sharma[2] and Akshay Anand[1]*

[1] Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
[2] Neurobiology-Neurodegeneration and Repair Laboratory, National Eye Institute, Bethesda, MD, USA

Edited by:
Rodrigo Orlando Kuljiš, Zdrav Mozak Limitada, Chile

Reviewed by:
Francisco G. Wandosell, Centro de Biologia Molecular Seveo Ochoa "CSIC-UAM and CIBERNED", Spain
Robert Petersen, Case Western Reserve University, USA

***Correspondence:**
Akshay Anand, Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
e-mail: akshay1anand@rediffmail.com;
url: www.neurologypgi.org

Ageing disorders can be defined as the progressive and cumulative outcome of several defective cellular mechanisms as well as metabolic pathways, consequently resulting in degeneration. Environment plays an important role in its pathogenesis. In contrast, developmental disorders arise from inherited mutations and usually the role of environmental factors in development of disease is minimal. Age related macular degeneration (AMD) is one such retinal degenerative disorder which starts with the progression of age. Metabolism plays an important role in initiation of such diseases of ageing. Cholesterol metabolism and their oxidized products like 7-ketocholesterol have been shown to adversely impact retinal pigment epithelium (RPE) cells. These molecules can initiate mitochondrial apoptotic processes and also influence the complements factors and expression of angiogenic proteins like VEGF etc. In this review we highlight why and how AMD is an ageing disorder and not a developmental disease substantiated by disrupted cholesterol metabolism common to several age related diseases.

Keywords: age related macular degeneration, metabolism, 7-ketocholesterol, angiogenic proteins, VEGF, degenerative diseases, complement factors, developmental disorders

INTRODUCTION

Age related macular degeneration (AMD) is described by irreversible vision loss in older age. The disease pathology emerges with the degeneration of macula which forms the central part of retina. The macula consists of photoreceptor (rods and cones) important for central vision. As AMD symptoms appear, characteristic features such as formation of drusen, consisting of active and inactive complement associated inflammatory products, aggregate of lipoprotein, cell debris, oxysterols, oxidized phospholipids and *Alu* RNA deposits begin to emerge later in life and not during development. These aggregates deposit in the extra-cellular space between Bruch's membrane and retinal pigment epithelium cells (RPE). Gradual and consistent effects of these aggregates gradually cause degeneration of these cells followed by global atrophy of RPE cells, commonly known as geographic atrophy (GA). Besides, active inflammatory components of these deposits between Bruch's membrane and RPE, stimulate angiogenic factors (e.g., VEGF, TGFB etc.) which act on choriocapillary network beneath the Bruch's membrane and stimulate proliferation to new blood vessels (a process called neovascularization). These newly formed blood vessels can outgrow into the RPE cells and result in disruption of RPE cell integrity and function which is well preserved in early life. Understanding the complexity of mechanisms through genetics, epigenetics, metabolic pathways and risk factors have provided insight about the participation of cellular pathways that resemble aging, but not early or late development. Cells that lose their capacity to divide by a phenomenon called cell senescence undergo ageing. Several impaired cellular processes could lead to cellular and morphological changes in the cell over time in association with environmental factors in complex manner ultimately resulting in ageing. These cellular processes include: metabolic pathways (Uchiki et al., 2012), telomere shortening, impaired mechanism of autophagy, disrupted proteolytic and lysosomal function (Viiri et al., 2013); decline in ability to combat oxidative stress (Cutler et al., 2004) and enhanced mitochondrial dysfunction etc. all of which can disrupt homeostasis of the cell. Therefore, age related changes in the cell are the basis of several diseases which are termed as age related diseases. Hence, the age related diseases depend on the degree of ageing in cells. Several genetic loci have been postulated to drive age related changes in the organism even in pre-mature age (Friedman and Johnson, 1988; Kennedy et al., 1995; Hernandez et al., 2010). Therefore, such impaired cellular, genetic or molecular processes coupled with environmental changes result in age related disorders. Instead of age related disorders, the developmental disorders are mostly inherited when other cellular processes are intact. Infact the environmental factors also play an important role in developmental disorders but not in a manner representative of diseases of ageing. Therefore, future effective interventions to combat diseases of aging shall require comprehensive understanding of gene regulatory networks than single gene replacement strategies.

INTERACTION BETWEEN ENVIRONMENT FACTORS AND GENETIC LOCI

It is evident that the disease pathology of AMD is equally influenced by both environmental as well as genetic factors in a

complex manner such that the effects are not manifest early in life. Several environmental factors such as age, sex, diet, smoking and demographic distribution have been reported to be strongly associated with AMD pathology (**Figure 1**), unlike a developmental disorder.

EPIGENETIC CHANGES INTRODUCED BY ENVIRONMENTAL FACTORS IN AMD

The epigenetic changes in the genome have been well defined in several genetic diseases. These epigenetic alterations affect the 5 expression level of several important genes apart from exerting protective role on host genome by either preventing activation of restriction enzymes or other mechanisms. Several cancers are believed to progress through similar chemical modifications (epigenetic changes) of the genome which impact the expression pattern of regulatory genes. The fundamental chemical changes include methylation, phosphorylation, acetylation, *etc*. which are reportedly involved in eukaryotes. Several environmental factors like smoking, stress, dietary habits etc. have been well described in introducing chemical changes on the genome (Lim and Song, 2012). Ageing can also influence the methylation pattern of the genome (Bollati et al., 2009). These studies suggest that apart from the inherited epigenetic changes, these changes can also be introduced by several modulatory environmental factors especially smoking and deficiency of anti-oxidants in diets in a later stage of life than at the developmental stage.

Similarly, there are several co-morbid conditions which have been found to be associated with manifestation of AMD. Persistent hypertension, heart disease and diabetes, accelerate AMD pathogenesis. Lately, it has also been revealed that Alzheimer's disease appears to have concurrence with AMD pathology suggesting the role of several common gene loci indicating that their associated pathological mechanisms, underlying cascade of downstream events, converge to cause the two diseases of ageing. This review highlights the role of various candidate genes that play an independent role in progression of AMD and digresses on spectrum of gene association studies which modify AMD pathology in a manner not characteristic of a development disorder.

The genes associated with AMD can be conveniently categorized into following groups based on their direct or indirect association reported in various studies:

1. Complement system components: e.g., complement factor H (CFH), component C2, component C3 and complement factor I (CFI). Moreover, TLRs receptor components of innate immunity have also been found to be strongly associated with AMD pathogenesis. Therefore, in general the components of innate immunity have predominant role in progression of AMD.
2. Enzymes involved in metabolic processes: Hepatic Lipase (LIPC), cholesteryl ester transfer protein (CEPT) and Apolipoprotein E (APOE) are involved in different stages of lipid metabolism.
3. Angiogenic factors: VEGF has been reported as the strongest angiogenic factor. Several angiogenic factors which have been

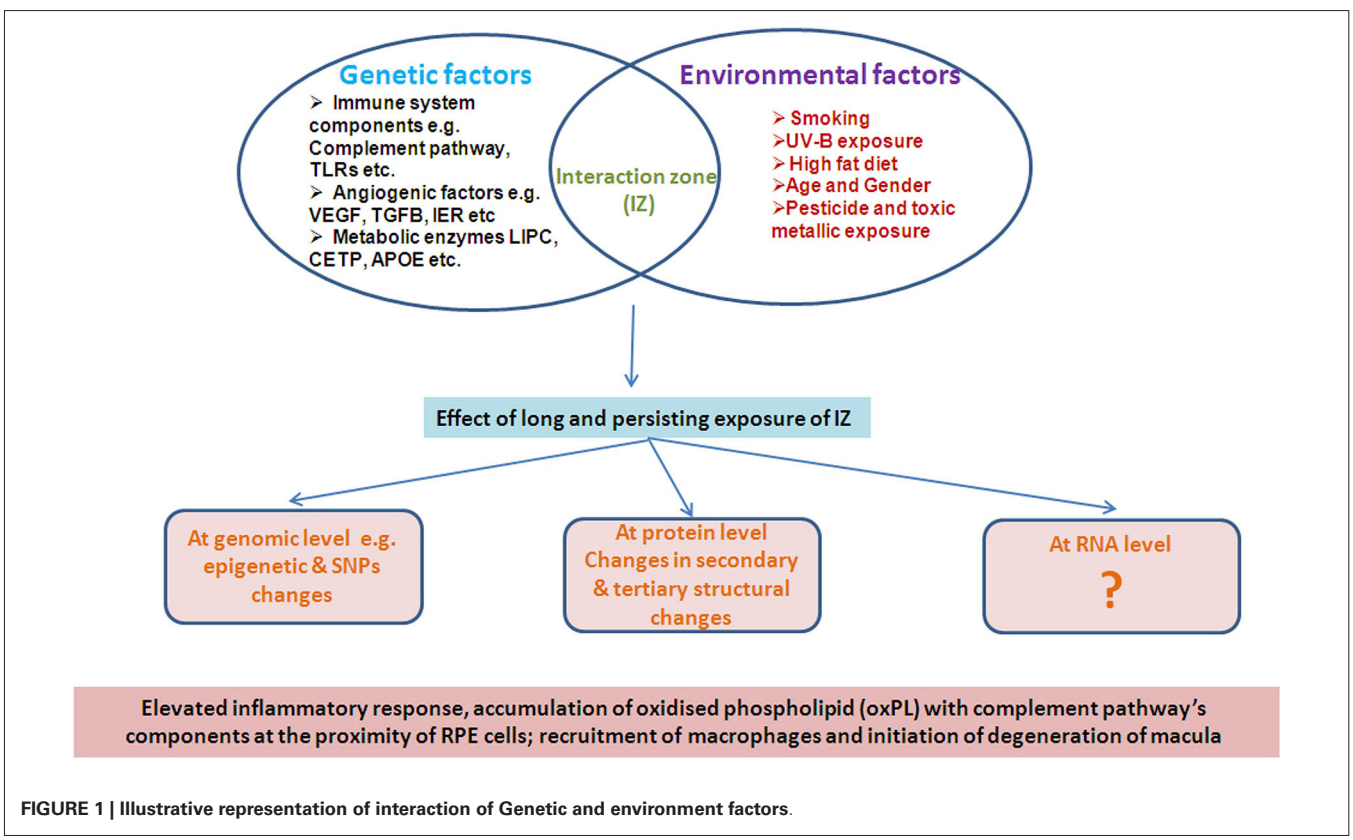

FIGURE 1 | Illustrative representation of interaction of Genetic and environment factors.

reported till now are either associated with AMD or are reported to be associated with degenerative and inflammatory diseases. e.g., VEGF, COL genes, TGFBR1, immediate early response (IER3) etc.

4. Proteases: Several proteases have been implicated in AMD pathophysiology including degradation of extracellular matrix. For example, Tissue inhibitor metalloprotenases 3 (TIPM3), IER3 and numerous multifunctional proteins which have several cellular functions like ADMS2, HTRA1 *etc*. play a non redundant role in pathologies of AMD.
5. Proteins in apoptosis and cellular regulation: e.g., *TNFRSF10A* and regulatory cellular enzymes like*DICER*.

AMD literature is replete with both confirmatory and conflicting reports of several genes and their polymorphisms which have been found to be associated with AMD.

PROTEINS INVOLVES IN METABOLIC PROCESSES

Metabolic processes constitute the normal biochemical pathways in the cell which are involved in transportation and biosynthesis of biomolecules including several regulatory processes of the cells. AMD can be regarded as a disease caused by disruption or abnormal metabolic processes especially lipid metabolism. The metabolic processes which are associated with lipid metabolism are one of the major factors in AMD pathogenesis. Several enzymes which participate in lipid and lipid associated metabolism have been found to be associated with initiation and progression of AMD pathology. Several population based genetic and animal based studies have revealed the role of proteins involved in lipid metabolism and their associated receptors in AMD. Important enzymes which participate in lipid metabolism, including human hepatic lipase gene (LIPC), lipoprotein lipase gene (LPL), cholesterol ester transferase gene (*CEPT*) and ABC binding cassettes A1 (*ABCA1*) gene have been found to be associated with AMD pathology. Lipoprotein lipase acts when systemic cholesterol levels fall along with the function of *ABCA1* leading to formation of *APOE*, *APOA1* and HDL. When cholesterol levels rise, the action of CEPT comes into play which removes the cholesterol ester (CE) from HDL and convert into LDL by enzymatic process. LIPC and LPL both have dual functions i.e., as triglyceride hydrolase as well as in receptor mediated endocytosis of cholesterol at different stages of metabolism.

Recent studies have demonstrated composition and substructures of drusen comprising of 3.2% dry weight of long fatty acid esterified and non-esterified cholesterol and about 30 different types of proteins, including apolipoproteins (apo E and apo B) (Burns and Feeney-Burns, 1980; Pauleikhoff et al., 1992; Crabb et al., 2002; Li et al., 2005). Lipid metabolizing enzyme human Lipase C (*LIPC*) is expressed in hepatic cells and adrenal gland whose principal function is to convert intermediate-density lipoprotein (IDL) to low-density lipoprotein (LDL) which have been widely investigated in AMD. The lipids and lipoprotein contents of drusen between Bruch's membrane and RPE are basically distinct from the plasma contents and are comprised of free and esterified cholesterol, phosphotidylcholine (PC), apolipoproteins B-100, A-I and ApoE (Curcio et al., 2001; Wang et al., 2010). Several genetic studies have investigated the association of LIPC with AMD pathogenesis. Recently, Neale et al. (2010) implicated the association between AMD and a variant in the hepatic lipase gene (*LIPC*) (rs493258). They found *LIPC* association was strongest for a functional promoter variant, rs10468017, that influences *LIPC* expression and serum HDL levels with a protective effect of the minor T allele (HDL increasing) for advanced wet and dry AMD.

ApoE, another protein, also involved in lipid transportation and metabolism has also been extensively investigated in AMD pathology. ApoE is a polymorphic protein which plays multiple roles in lipid homeostasis in central nervous system and metabolism of plasma lipid (Mahley, 1988). APOE gene has three different variants called APOE2, APOE3 and APOE4 which are due to the variation in two SNPs in the APOE gene, rs429358 and rs7412.Immunoreactivity of APOE in the eye has been restricted to Bruch's Membrane, Müller cells, RPE, drusen and basal deposits (Klaver et al., 1998; Anderson et al., 2001; Malek et al., 2003). mRNAs for APOE are synthesized by the RPE and by Muller cells in the neural retina (Anderson et al., 2001). Polymorphism in APOE gene is a risk factor for various neurodegenerative diseases, and the protein has been shown in the lesions of these disorders. APOE gene is also known to play a major role in AMD pathology. Some association studies have reported that APOE4 variant provides some protection from developing AMD. APOE2 variant is more common in individuals with AMD and may play a role in its progression (Klaver et al., 1998; Souied et al., 1998; Schmidt et al., 2002; Baird et al., 2004). These results are in variance to the animal studies in whichAMD severity has been found to be associated with ApoE4 expressing aged mice which were subjected to high fat cholesterol. Even the major complex diseases such as atherosclerosis, Alzheimer's disease, and stroke, are characterized by strong association betweenAPOE4allele and disease (Kalaria, 1997; Weller and Nicoll, 2003; Enzinger et al., 2004).

Several ApoE knockout mice studies have indicated the role of ApoE affecting systemic cholesterol levels (Plump et al., 1992; Zhang et al., 1992). Dithmar et al. have shown ultrastructural changes of the Bruch's membrane in ApoE deficient mice. They have found elevated fraction of membrane bound electron-lucent particle accumulation between two membranes in ApoE −/− mice as compared to control mice (Dithmar et al., 2000). Lately, it has been found that AMD pathology, like sub-retinal drusen deposition, drusenoid, thickening of BM, atrophy, hypo and hyper-pigmentation of RPE are dependent on ApoE isoforms. AMD severity has been found to be associated with ApoE4 over expressing senescent mice which were subjected to high fat cholesterol (Nguyen-Legros and Hicks, 2000). Moreover, cell-membrane remodeling is an essential mechanism for maintenance of retina and its normal functioning (Klaver et al., 1998), which may be regulated by APOE. Protective function of PAOE4 are believed to arise from its inability to form dimers as compared to APOE2 and APOE3 variants which may permit easier transportation of lipids through Bruch's membrane (Souied et al., 1998). Lipids accumulate with the advancement of age, which may lead to the creation of hydrophobic barrier in the Bruch's membrane causing AMD.

On the other hand, the involvement of APOE has also been widely explored in AD. It has been reported that severe oxidative

changes in protein and lipid have been shown in synaptosome of APOE knockout mice after Aβ induced insult (Lauderback et al., 2001). However, it has also been hypothesized that APOE may function as "pathological chaperon" in stabilizing the Aβ sheet structure and can also alter the normal Aβ in plaque (Ji et al., 2001). Moreover, it has also been investigated that the burden of amyloid-β-peptide was diminished in $APP^{V717F+/-}$ Apoe−/− as compared to $APP^{V717F+/-}$ $Apoe^{+/-}$ and Apoe−/− triglycerides (TG) mice in 20 month aged mice. The burden of Aβ in the hippocampal region was higher in Apoe+/+ transgenic mice as compared to Apoe+/− and Apoe−/− mice. This suggests that even though both pathologies implicated similar elements but the final effect are quite opposite (Bales et al., 1999). Therefore, these studies have exemplified the role of APOE in two major age related degenerative disease, suggesting that APOE may have the dual role in both AMD and AD.

It was also discovered that age and high fat cholesterol diet alone were not sufficient factors to stimulate AMD pathology apart from isoform dependent pathology. In experimental mice, advanced features of AMD i.e., neovascularization, with the presence of VEGF in drusen, has been found to be deposited in BM region, which was also reported earlier, implying the role of ApoE function in both types of AMD. The components responsible for dry AMD further stimulate genetic cascade which leads to activation of angiogenic molecules or metalloproteases (Malek et al., 2005). It is argued that these changes are not critical to developmental and the disorders of developmental. Rudolf et al. (2005) have further distinguished the role of lipid metabolism in stimulation of angiogenic factor (VEGF) in LDL-receptor knockout mice. Interestingly, it has been shown that in LDL- knockout mice, the expression of VEGF was high as compared to controls.

Another lipid metabolizing enzymes cholestryl ester transfer protein (CEPT) is a plasma protein that plays an important role in cholesterol metabolism by facilitating the transportation of triglyceride and cholesteryl ester from VLDL to HDL or vice versa. Several genetic studies have demonstrated conflicting results of CEPT association with AMD progression. Recently, Yu et al. (2011) reported no association between CEPT polymorphism and AMD pathology but demonstrated that other cholesterol metabolizing enzymes like LIPC and a cholesterol transporter protein *ABCA1* may have role in AMD pathophysiology such that the cumulative effect gets manifest in ageing and not earlier in life.

Several cholesterol metabolic intermediates have been found to stimulate pathological mechanisms involved in AMD like angiogenesis, inflammation etc. 7-ketocholesterol (7-Kch), one of the oxidized component of cholesterol metabolism, has been found to stimulate angiogenic factors and inflammatory processes (Ignacio and Larrayoz, 2010). Oxidation of cholesterol into 7-Kch is carried out by both enzymatic and non-enzymatic processes. Non-enzymatic processes are carried out by the involvement of singlet oxygen species and free radical mechanism. The mechanism mediated by singlet oxygen species requires photosensitizer (Thomas et al., 1987) and free radical based mechanism carried out by the involvement of metal catalysts like Cu^{+2}/Fe^{+2} (Dzeletovic et al., 1995; Brown et al., 1996). These studies have also suggested the probable role of Fe^{+2} and oxidation mediated processes in AMD pathophysiology. Currently, only free radicals based mechanisms have been found to be occur in retina, however, no evidence for singlet mediated cholesterol oxidation into 7-Kch has been reported.

Most important enzymatic oxidation of cholesterol takes place with the involvement of CYT46A1 and CYP27A1 loci of cytochrome P450 family which forms 24-hydroxycholesteorl (24-Hch) and 27-hydroxycolesterol (27-Hch), respectively (Björkhem et al., 2009). The high levels of 27-Kch have been reported in macrophages foam cells and in atherosclerotic plaque, but has not been documented in retina (Björkhem et al., 1994; Luoma, 2008). These population based genetic studies coupled with animal experiments indicate a prominent role of cholesterol metabolism in AMD pathology indicating the longitudinal and cumulative effects that do not alter phenotype earlier in life. Recently, it has been demonstrated that the metabolic components of lipid metabolism influence and activate the angiogenic factors, complement factors and other regulatory components by disrupting their regulatory pathways not critical in early life.

EFFECTS OF CHOLESTEROL METABOLISM INTERMEDIATES ON COMPLEMENT FACTORS

Data from the previous research strongly indicates that the dysregulation of innate innunity can induce and result in progression of AMD. Several GWAS and rare variants analysis studies have strongly specified the role of alternative complement pathway in AMD (Richards et al., 2007; Anderson et al., 2010; Raychaudhuri et al., 2011; Khandhadia et al., 2012; Tuo et al., 2012) and it has been well established that the dysregulation of complement pathways, especially alternative complement pathway is responsible for AMD pathophysiology and aberrant regulation of these factors may lead to progression of AMD.

Recently, several studies have demonstrated the effects of cholesterol metabolic components which influence these intermediate components like in case of atherosclerosis. It has been already established by Hasselbacher et al. in 1980 that cholesterol has high potential to activate the alternative complement pathways componetnts which have been demontrated by immunoelectrophoretic reactivity of C3 and properdin factor B (Hasselbacher and Hahn, 1980), however none of the the complement factors was activated when liposomes, containing sphingomyelin as a phospholipid (Alving et al., 1977), was used. Therefore, these studies have suggested the potetial role of cholesterol intermediates and alternative complement pathway in influencing disease outcome. The mechanism behind cholesterol dependent complement activation has been illustrated and found to be associated with C5/C3 conversion into cholesterol crystals which consequently induce alternative complement cascades which may be stabilized by factor B and D. It has also been examined that factor I (inactivator of factor C) shows affinity with cholesterol crystals thus indicating cholesterol mediated complement pathway activation proceeds by transformation activity not by the involvement of factor C (Vogt et al., 1985). Consistent with these studies the generation of anaphylatoxins and C5b-9 complex has also been observed and suggested to play a role in the inflammatory processes mediated by cholesterol and its intermediators, as seen in artherosclorosis, AMD and Alzheimers disease (Seifert and Kazatchkine, 1987). The accumulation of lipofuscin,

with several oxysterol, have been shown to have amplified CFH activity along with increased C3b suggesting lipid intermediate based complement dysregulation. This may lead to features reminscent of AMD pathology (Sparrow et al., 2012). Recently, it has been further reported that the CFH interaction with oxidized products of cholesterol can modulate the protective effect of CFH. The protective allele of CFH in AMD rs1061170 has been shown to exert strong affinity and inhibitory effect of CFH upon binding with oxidized phospholipid mediated by changes in the expression of genes responsible for neovascularization, inflammation and macrophages infiltration. This study has suggested the interaction of oxidized phospholipid could bring changes in the protective role of CFH by modulating other genes involving various processes associated with AMD pathology (Shawa et al., 2012). Additionally, Sharma et al. have also shown the reduced levels of CFH in AMD patients (Sharma et al., 2013b). Similarly the same group, also reported altered CCL-2 levels in AMD patients (Anand et al., 2012). Both results suggest that impaired macrophages recruitment in AMD pathology.

Apart from interaction and effects on complement factors, lipid metabolic intermediates have also accounted for their direct consequences on inflammatory responses by affecting gene regulation involved in such processes. Through *in vitro* studies by Shawa et al. (2012) it has been demonstrated that the interaction of oxidized phospholipids could upregulate the expression of CCR-2, IL-6, IL-8, CD-36 and VEGF. The increased expression of CCR-2 suggests augmented infiltration of macrophages at the site of accumulated oxidized phopholipids which further influence the intergrity of RPE cells by modulating the expression levels of proinflammatory, inflammatory and angiogenic factors through NFkB pathway. Recently, Amaral et al. have shown in a rat model that the administration of 7-Kch which induces the macrophage infiltration at site of accumulation, have displayed increased immunolocalization of CD68 (Amaral et al., 2013). This could result in enhanced expression of proinflammatory genes along with angiogenic factors in moncytic THP-1 cells (Erridge et al., 2007). Additionally, several *in vitro* studies have also shown that accumulated intermediates of lipid and cholesterol metabolism can induce the endoplasmic reticulum (ER) stress (Lee et al., 2009; Huang et al., 2012) by involvement of various NFkB pathways (Dugas et al., 2010; Larrayoz et al., 2010; Huang et al., 2012).

The role of macrophages in atherosclerosis against oxysterols have also been extensively investigated. These studies suggest that the regulation of synthesis of inflammatory factors like TNF-α, IL-8, IL-6 etc. is mediated by MEK/ERK and AP-1 processes (Lemaire-Ewing et al., 2005, 2009; Erridge et al., 2007). The inhibition of these proinflammatory factors induced by some oxysterols could be regulated via liver X receptor (LXR) possessing the capacity to suppress their synthesis at the time of atherosclerosis (Calkin and Tontonoz, 2010; Im and Osborne, 2011). Collectively, these studies indicate that macrophages mediated proinflammatory processes induced by accumulated lipid/cholesterols intermediates play a major role in diseases like Alzheimer's disease, Atherosclerosis and AMD all of which occur with ageing. Lately, an elaborate role of cholesterol in macrophages have been illustrated by participation of lipid metablic processes in AMD. Sene et al. have demonstrated that impaired cholesterol transport in macrophages could influence the macrophages polarization into particular cell type consequently impacting the pathological hallmarks in AMD (Sene et al., 2013).

Conclusively, these studies have suggetsed the importance of lipid/cholesterol metabolites in inducing the AMD pathological hallmarks by several regulatory components of innate immunity.

These studies demontrate that lipids/cholesterol metabolites have their impact on regulation of complement factors, interaction with protective variant of CFH and its modulation, macrophage infiltration, and regulation of proinflammatory/inflammatory genes suggesting the role of cholesterol metabolism and its metabolites on the complement pathway. These studies also suggest that cholesterol transport influences the role of macrophages in progression of AMD such that these effects do no operate early in life.

CHOLESTEROL METABOLITES AND ANGIOGENIC FACTORS

Lipids and choleserols are essential for biological functions ranging from membrane trafficking to signal transduction and can activate various important regulatory molecules which could be associated with several pathological hallmarks of different types of diseases. Several studies have increased our understanding of direct relation of lipids and cholesterol intermediates in pathogenesis of inflammatory diseases and diseases affected by lipids deposition, at the molecular and cellular level. Neovascularization is one of the common features which affects about 10% of total AMD and can be influenced by several intermediates of cholesterol metabolism. Both angiogenic factors as well as matrix metalloproteases (MMP) can contribute in the progression of pathophysiology of choroidal neovascularization (CNV) in AMD. VEGF expression, expression of proangiogenic factors, and activation of metalloproteases are believed to play an important role in the formation of CNV in AMD. It is well understood that the biologic activities of VEGF-A, proangiogenic molecules like (TGFB, TIMP, IER etc.) and proinflammatory cytokines can be influenced by the interaction of cells with the extracellular matrix (ECM). Cell-ECM interaction, useful for integrity and functionality of cell, are influenced by the expression of these factors (VEGF, angiogenic and proangiogenic factors). ECM protein channel-ECM communications influence tissue remodeling events, including angiogenesis. Several angiogenic factors have been widely studied as one of the cardial feature of AMD pathophysiology: "neovascularization". The neovascularization being the consequence of dysregulated action of genes involved in proangiogenesis, angiogenesis, extracellular proteins, and metalloproteases is the central thesis in wet AMD. These effects are additive, rarely affecting development milestones. *VEGF*, one of the potent angiogenic factor known, plays an important role in new vessels formation because it is involved in vascular development and have been strongly implicated and reported in the pathogenesis of AMD (Carneiro et al., 2012) as well as corneal neovascularization (Philipp et al., 2000). The *VEGF* family mainly binds with three types of *VEGF*Rs which include: *VEGF*R1, *VEGF*R2, and *VEGF*R3, as well as to

co-receptors [such as heparan sulphate proteoglycans and neurophilin] (Hiratsuka et al., 1998). It has been investigated that the expression of VEGF can be influenced by components of complement pathway. When RPE cell culture were exposed with C3a/C5a, also present in drusen, it can significantly increase the expression of VEGF (Nozaki et al., 2006). Recently, we also reported VEGFR2 in AMD patients which are found to be significantly associated with disease pathology (Sharma et al., 2012a).

Similarly, *TGFBR1* also possesses the property to exert proangiogenic effects with its encoded protein forming a heteromeric complex with type II *TGF*-beta receptors and transducing the TGF-beta signal from the cell surface to the cytoplasm when bound to TGF-beta. This protein is a serine/threonine protein kinase. This gene is expressed in all tissues but more abundantly expressed in brain and heart. This protein is involved in several biological processes like induction of apoptosis, response to hypoxia, epithelial to mesenchymal transition, artery morphogenesis, signal transduction, regulation of transcription, negative regulation of endothelial cell differentiation, positive regulation of cell proliferation, collagen fibril organization *etc*.

Apart from above mentioned angiogenic genes, *IER3* has been postulated to be responsible for regulating death receptor mediated apoptosis, interaction with NF-κB pathways, and upregulated at irradiation, ionizing radiation, viral infection and at the time of other cellular responses. Numerous functional data relying on cell culture based studies and knock-out mouse models has revealed the involvement of *IER3* expression in immune functions and in the physiology of the cardiovascular system. Arlt et al. showed that deficiency of *IER3* expression in mice results in an aberrant immune regulation and enhanced inflammation, hypertension or impaired genomic stability (Arlt and Schäfer, 2011). Another report has documented the genetic network in injured retina reporting increased expression of *Ier3* gene with other transcription factors such as *Crem*, *Egr1, Fos, Fosl1, Junb, Btg2, Atf3*, and *Nr4a1* etc. Together, these genes implicate an angiogenic pathway attributed to *CFH* independent mechanism, popularly accepted to be responsible for almost half of AMD cases. None of these gene loci or their networks have been reported to be involved in a development disorder. Sasada et al. has also reported the effect of *IER3* which interferes with certain signaling pathways specifically NF-kB, MAPK/ERK and PI3K/Akt and have found aberrant immune function and increased inflammation, with an alteration of blood pressure and impaired in genomic stability (Sasada et al., 2008). Numerous patients based studies have also indicated a role of *IER3* in tumorigenesis. In pancreatic cancer patients, significant negative correlations have been observed between the *IER3* expression and serosal or arterial invasion of tumors. Thus, a positive *IER3* expression in tumor tissues may be associated with a better prognosis in pancreatic cancer (Sasada et al., 2008).

Several studies have been carried out to examine the effect of deposited oxidized sterols and 7-Kch on the expression pattern of these angiogenic proteins like VEGF. The effect of oxidized cholesterol and 7-Kch has been widely investigated in both animal as well as *in vitro* studies being responsible for inflammtory processes at accumulation site. Moreover, recent studies have validated the reports that phospholipidosis could increase several times in retina when exposed with these cholesterol intermediates (Miguet-Alfonsi et al., 2002; Vejux et al., 2008). Even though the activation of complement factor can also alter the expression of metalloproteases 2/9 as well as their activity along with imbalance in VEGF to PEDF ratio, these results suggest the effect of activated complement which could further induce the proangiogenic environment for neovascularization in AMD (Bandyopadhyay and Rohrer, 2012). Therefore, the activated complement components with oxidized sterols and 7-Kch can directly influence the expression of angiogenic and proangiogenic molecules.

Knockout mice for ApoE and the mice feeding high fat diet have shown several AMD like characteristics features. Increased implicit time and reduced oscillation potential amplitude were observed in these mice. Moreover, the scanning electron microscopic examination has also revealed that thickening of Bruch's membrane and disorganized elastic lamina which results in stimulation of choroidal angiogenesis beneath the Bruch's membrane. Therefore, the such studies suggest the role of *ApoE* and cholesterol which may provoke the nearby environment of RPE, Bruch's membrane and molecules involved in adhesion of RPE and lamina by stimulating the angiogenic factors and matrix proteases (Ong et al., 2001).

Recently, the mechanistic view of activation of proangiogenic/angiogenic factors by oxidized sterols, 7-Kch or by impaired cholesterol transfer has been demonstrated by several investigators (**Figure 2**). Sene et al. (2013) had discovered the existence of impaired cholesterol transport in macrophages affecting ATP binding cassette A1 (ABCA1) transporter converting the naive macrophages into senescent macrophages. The effect of cholesterol accumulation inside macrophages has been found to be abnormal polarization of macrophages into two populations. The macrophage M1 only secretes proinflammatory and inflammatory components but polarization of M1 to M2 could pilot the secretion of angiogenic factor like VEGF, IL-8 *etc*. Therefore, these studies together signify the importance of cholesterol metabolism in AMD which is altered in neovascularization of choroid blood vessels.

The immediate effects of these deposited oxidized cholesterol components have also been examined in higher vertebrate i.e., monkey *etc*. The cholesterol intermediates 7-Kch have also shown the capacity to induce VEGF in monkeys. Moreira et al. have attempted to figure out the localization of 7-Kch in retina and examine the impending consequences by deposition of these oxidized cholesterol products in higher primates. The immunolocalization studies define the location of 7-Kch which was found to be deposited in between of choriocapillaries and Bruch's membrane besides the surface of vascular endothelial cells. This was found to be 4–5 times higher when compared to control monkey retina. Further, *in vitro* studies have demonstrated the induction of VEGF in ARPE-19 cells is 8–10 folds higher in 7-Kch induced retina as compared to those without induced cells. Moreover, they also found that the expression of VEGF was decreased when inhibitor

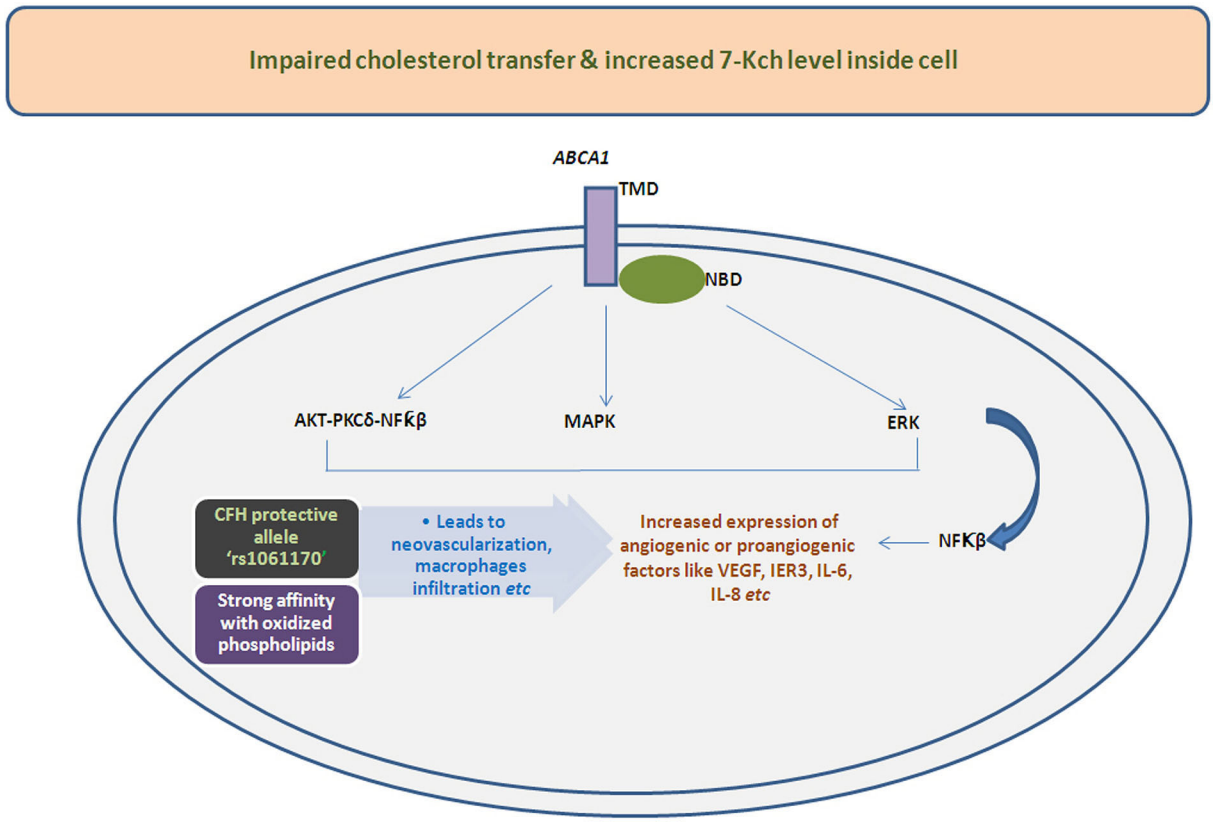

FIGURE 2 | Illustrative mechanistic flow chart functions of various signaling pathways leads to altered expression of angiogenic/proangiogenic factors influence by impaired transport and deposition of cholesterol or oxidized cholesterols due to hampered action of *ABCA1* transporter. The deposited oxidized cholesterols components also have shown higher affinity with protective allele of CFH i.e., rs1061170 which can also changes the expression of angiogenic molecules.

for LXR receptor was exposed with anatagonist, however, no change in expression was noticed when activity of NFkB was inhibited, except IL-8. The study implies the role of 7-Kch in CNV induced by VEGF may be partially regulated by LXR receptor but independent on HIF-1 α and NFkB (Moreira et al., 2009).

APOPTOSIS MEDIATED BY OXIDIZED CHOLESTEROL AND 7-Kch

The 7-Kch exerts toxic effects by inflammation (Vejux et al., 2008; Larrayoz et al., 2010) and subsequently induce apoptosis of RPE cells mediated by mitochondrial apoptotic pathway (Miguet-Alfonsi et al., 2002; Ignacio and Larrayoz, 2010). Accumulated oxidized sterols, 7-Kch and impaired cholesterol transport leads to increase the level of Ca^{+2} inside cell mediated by *Trp* channel. This increased Ca^{+2} levels can activate the two types of apoptotic mechanisms: (i) dephosphorylation of "BH3 only protein Bad" which leads to mitochndrial apoptotic pathway; and (ii) activation of "pro-apoptotic BH3 only protein Bim" ultimately inducing apoptosis by inhibiting the activity of Bcl-2 family members proteins along with microtubultes destabilization (**Figure 3**). Recently, we have reported the reduced DcR1 (TNF-related apoptosis-inducing-ligand receptor-3) levels in AMD patients (Anand et al., 2014) where the angiogenic factors like CCR-3, VEGFR2, and eotaxin-2 (Sharma et al., 2012a,b, 2013a) were found to be increased in serum of these patients. These reults suggested the role of angiogenic factors with the involvement of apoptotic molecules may contribute to the disease pathology in these patients.

CONCLUSION

Several age related disorders like AD, Parkinson's disease (PD), atherosclerosis and AMD can be distinguished by several pathological hallmarks that appear at the time of ageing. Collectively, these studies suggest the imperative role of metabolic processes and various oxidized products which exert their additive effect on both complement and proangiogenic/angiogenic molecules with time. Several such reports indicate that altered cholesterol metabolism leads to pathological changes which results in accumulation of sterols, APOE, cholesterol oxidase and oxidized cholesterols in these diseases of ageing (Kannel et al., 1971; Martins et al., 2006; Moreira et al., 2009). The altered levels of LDL, HDL,

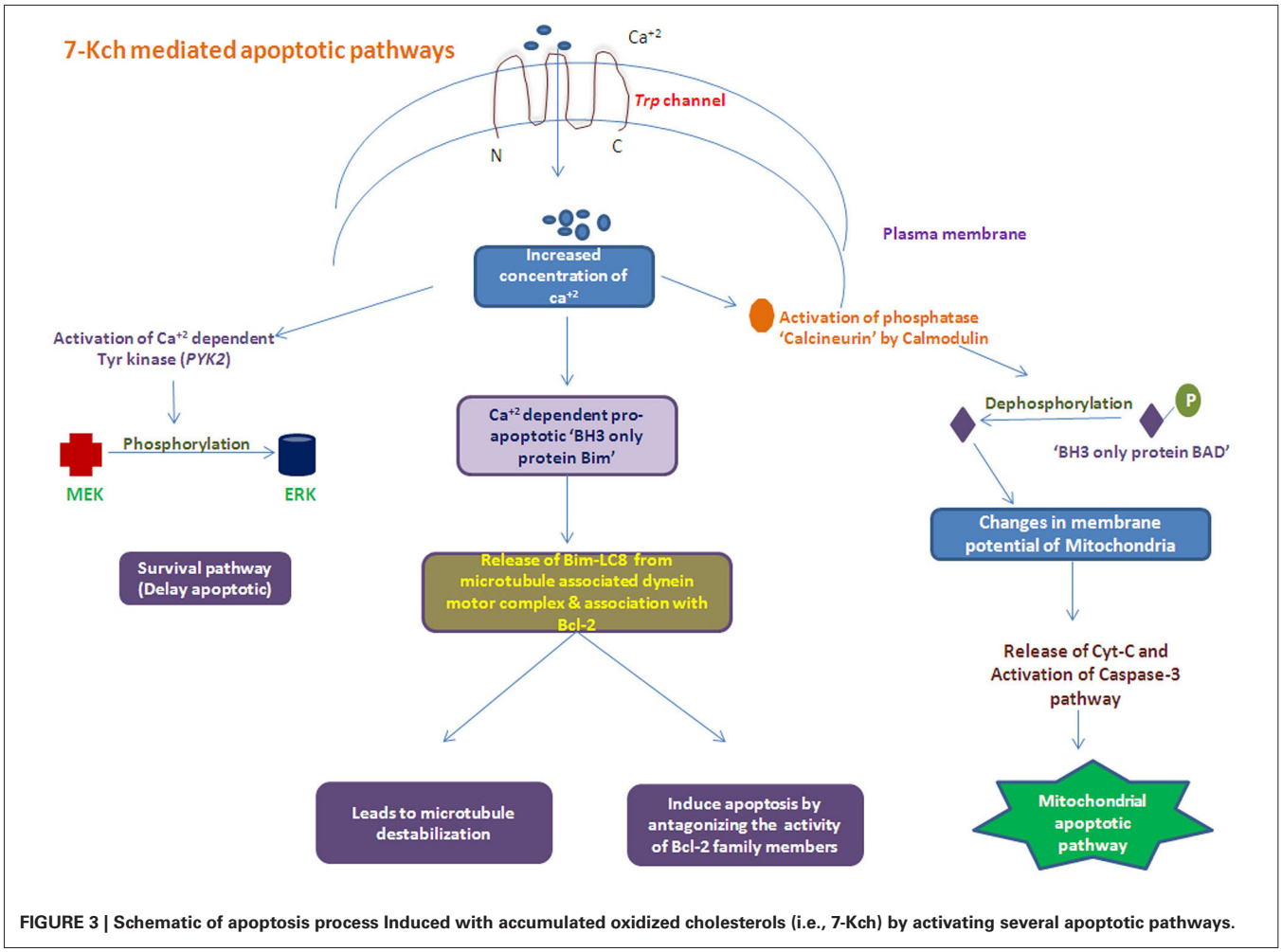

FIGURE 3 | Schematic of apoptosis process Induced with accumulated oxidized cholesterols (i.e., 7-Kch) by activating several apoptotic pathways.

ApoAI, ApoB and TG in AMD pathology is thus not a coincidence as these are rarely reported in developmental disorders (Nowak et al., 2005; Reynolds et al., 2010; Davari et al., 2013).

In this context, the generality of need to compare the critical mechanisms distinguishing an ageing disorder such as AMD from mechanisms akin to developmental disorders can provide insight for future interventions. Understanding the molecular mechanisms that prevent AMD from being a developmental disorder may therefore lead to effective therapies in future. For this, longitudinal comparative studies between developmental and degenerative disorders are warranted.

AUTHOR'S CONTRIBUTIONS

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Targeted Drug Delivery to Central Nervous System (CNS) for the Treatment of Neurodegenerative Disorders: Trends and Advances

Kritika Goyal[1,2], Veena Koul[2], Yashveer Singh[3] and Akshay Anand[1,*]

[1]*Neuroscience Research Lab., Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India;* [2]*Centre for Biomedical Engineering, Indian Institute of Technology Delhi, New Delhi 110016, India, All India Institute of Medical Sciences, Ansari Nagar-110029;* [3]*Department of Chemistry, Indian Institute of Technology Ropar, Nangal Road, Rupnagar, Punjab 140001, India*

Abstract: The treatment of brain diseases has been a major challenge since a long time. Although there are several potent drugs, which are highly therapeutic yet their efficiency is marred due to the presence of the Blood Brain Barrier (BBB). The BBB, which is present at the capillary level regulates and monitors the entry of all small and large molecules entering into the brain. Although this barrier is of immense importance to the brain in terms of safety, it becomes a hindrance when it comes to therapy because the drug molecules are unable to reach the brain. Various biomaterial-based strategies are being developed to overcome the BBB and deliver the drug into the brain. These include polymeric nanoparticles, liposomes, solid-lipid nanoparticles (SLNPs), nanogels, implants, etc. This review provides an overview on CNS disorders, BBB, and various delivery strategies available for biologists engaged in translational neuroscience, to target CNS.

Keywords: Blood-brain barrier (BBB), CNS drug delivery, drug transport, targeting.

1. INTRODUCTION

Brain is duly protected by the solid and fluidic processes [1]. Pia mater contains a large number of important blood vessels [2]. Cerebrospinal fluid (CSF) plays a major role by acting as a cushion for the brain and fills the space known as the sub arachnoid space (between the arachnoid and the pia mater). It is a watery fluid, which is synthesized from the blood by choroid plexus [3]. The purpose and function of all these protective layers and processes is to protect the brain from the harsh external environment. They provide lubrication and cushioning effects to the brain and also protect the brain from various injuries, which can cause damage to the brain [4]. Injury or trauma to the brain can originate in the brain itself in the form of 'neurodegeneration' [5, 6]. Neurodegeneration refers to the degeneration of neurons, which causes progressive damage in the neurons. This leads to structural and functional loss in the neurons and finally neuronal death [7]. The neurodegenerative diseases comprise a class of brain diseases, which include diseases like Alzheimer's Disease, Huntington's Disease, Amyotrophic Lateral Sclerosis (ALS), Parkinson's Disease, Pick's Disease, etc [8]. There are more than 1.5 billion people suffering from CNS disorders worldwide and CNS disorders constitute about 35% of the total burden of all diseases in Europe [9]. The problem becomes more acute for elderly population. At an advanced age of 70 years or more, about half of the population may exhibit symptoms of CNS disorders [10]. It has been estimated that the current burden of CNS disorder worldwide, which is believed to be 11%, will increase to 14% by 2020 [11]. The treatment of these neurodegenerative brain disorders is a challenging task because the initiation, cause and pathology of the neurodegeneration inside the brain remain undeciphered. The major cause for the failure in the treatment of the diseases is ineffective drug delivery to the central nervous system (CNS).

The problem of poor drug delivery arises because the brain is under the stringent surveillance of the blood brain barrier (BBB). BBB is a physical barrier, which is formed with the help of tight junctions between the endothelial cells of the capillaries (discussed in detail later). The tight junctions impart the BBB its impermeable nature. It protects the brain against the trespassing organisms and undesirable molecules and maintains the internal environment of the brain [12]. Unfortunately, this barrier also denies entry to drugs for CNS disorders. About 98% of the small molecular weight drugs and 100% of macromolecule drugs are unable to cross the BBB. Generally, the drugs with molecular weight <500 Da and high lipophilic character are able to overcome the BBB [13]. However, this general description of drug overcoming BBB does not hold true in the presence of membrane transporters.

This review provides an overview on the epidemiology and causes of the neurological disorders, stringency of the BBB, approaches to surmount the BBB, and the current advances made in the domain of targeted drug delivery to CNS.

2. NEUROLOGICAL DISORDERS

It has been estimated that approximately 35.6 million individuals are suffering with dementia worldwide [14]. The researchers speculate that these numbers will double almost every 20 years and may reach up to 65.7 million in 2030 and 115.4 million in 2050 [14].

*Address correspondence to this author at the Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India; Tel: +172-2748399;
E-mails: akshay1anand@rediffmail.com; akshay2anand@gmail.com

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The neurological diseases, which affect the brain and CNS, are generally caused due to two major reasons: neurodegeneration and neuroinflammation [15]. The cause and exact mechanism of neurodegeneration is still unknown but it can be well associated with the age. Individuals who have a family history of any neurodegenerative disease are more prone to such diseases [16]. Excitotoxicity and apoptosis are other reasons for neurodegeneration. Hyperactivity of glutamate receptors leads to deleterious effects on neurons, which can be attributed to the over production of free radicals. It is believed that p53 apoptotic pathway is activated at the time of stress, which further leads to programmed cell death [17, 18] The mutant Cu, Zn-superoxide dismutase 1 (SOD1) has been shown to exert toxic effect on motor neurons in *C. elegans*'s amyotrophic lateral sclerosis (ALS) model. When human wild type or G93A SOD1 was specifically expressed in motor neurons of *C. elegans*, it caused defects related to locomotion, which recapitulates some of the characteristic features of ALS, which include age-associated motor neuron dysfunction and motor neurons degeneration with respect to SOD1 aggregation [19]. The Bristrol strain of N2 worms was used in this study and standard *C elegans* techniques were employed [20]. Five different mutant strains were used in the study and the gene expression analysis was done by real time polymerase chain reaction (PCR) using respective forward and reverse primers. The locomotory analysis was done in the growth media. It was calculated as the distance travelled in 30 seconds, which was divided by the length of the body.

Recently, a group elucidated the role of Beclin 1 in neurodegeneration. Beclin 1 has a critical role to play in various autophagy pathways. Researchers showed that microglia, which lack the Beclin 1 expression, were defective in the phagocytic pathway and as a consequence the clearance of Amyloid Beta in mouse model of the Alzheimer's disease was impaired [21]. Neuroinflammation is characterized by the riposte of the glial cell, which are inflammatory in nature. This process is specific to the CNS and is portrayed by development of lesions, disorders related to neurite growth, and hyper-phosphorylation of tau proteins. These events are absolutely different to the inflammatory processes going on in the periphery. These characteristic features prompt the researchers to hypothesize that the glia, particularly the microglia primarily act as the immune cells of the brain. They react in an exclusive way to an insult induced by any inflammatory substance by activating cytokines and phagocytosis. Therefore, it can be concluded that the microglial cells are important brain immunity cells, which are involved in different types of neurological disorders like Parkinson's disease, Alzheimer's disease, Multiple Sclerosis, etc. [22].

3. BLOOD BRAIN BARRIER (BBB)

The brain is a well-protected organ. There are numerous gateways to enter brain parenchyma and the two most important gateways are blood circulation, that is the systemic route and CSF [23]. The BBB is present in most organisms in which the CNS is well-developed [24] and it protects the brain from the foreign matter and maintains the cerebral homeostasis [15]. The BBB is formed by the endothelial cells, which are the basic structural unit of the capillary walls. In human brain, there exist about 100 billion capillaries corresponding to total length of approximately 650 km and total surface area of approximately 20 m^2 [25]. Thus, the brain endothelium forms the anatomical and functional site of BBB (Fig. **1**). The characteristics features of the BBB are as follows [23]: (i) the lack of fenestrations with very few pinocytic vesicles [15, 26] (ii) existence of tight junctions

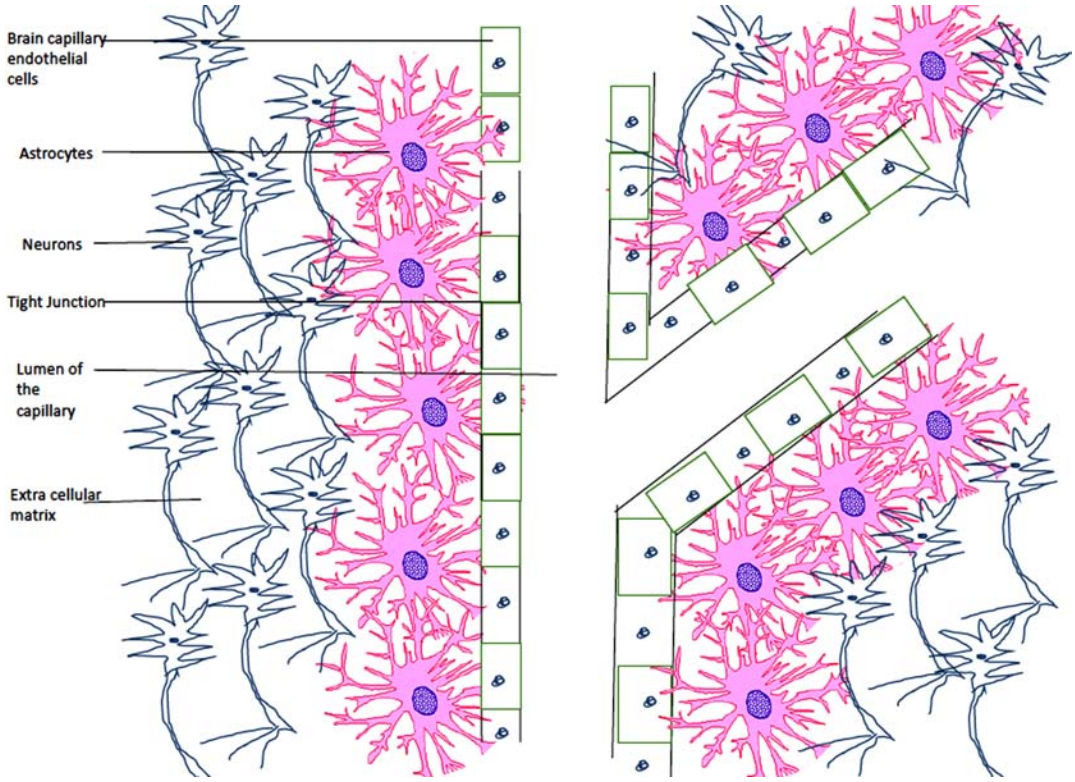

Fig. (1). The Blood Brain Barrier.

(TJ) between adjoining endothelial cells and associated complex of transmembrane proteins like junctional adhesion molecule 1 (JAM 1) [27], claudin [28], occludin [29], zona occludens etc. [30] (Fig. **2**); and (iii) limited passage of the immune cells due to paucity of lymphatic drainage and major histocompatibility complex (MHC) antigens. Apart from the brain capillary endothelial cells, the extracellular base membrane, astrocytes, adjoining pericytes, microglia, and the cerebral microvasculature endothelium are integral part of BBB support system [26].

The BBB thus acts as a barrier and prevents the entry of molecules in the brain parenchyma from the blood capillary network [31]. The BBB restricts the entry of almost all of the large molecules and 98% of the small sized molecules [32]. The maximum limit of transport at the BBB is 4 μmol $min^{-1}g^{-1}$ in rat, whereas in man it is 1 μmol $min^{-1}g^{-1}$ [33]. The BBB also contains efflux transporters like multi drug resistance proteins (MRPs) and p-glycoprotein (P-gp), which also remove the molecules entering the brain [23].

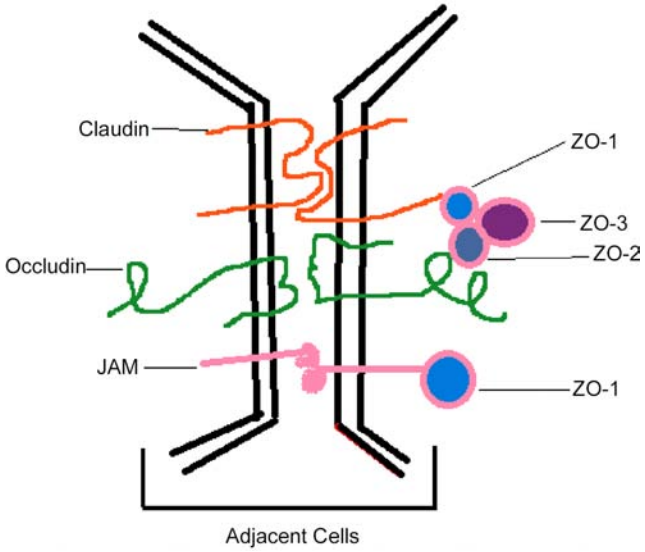

Fig. (2). The tight junctions (TJ) between the adjacent endothelial cells formed by the assembly of transmembrane proteins. Redrawn from Britta Engelhardt, Development of Blood Brain Interface [29].

A second barrier to the brain is also present, which is established by the epithelial cells of the choroid plexus that are in direct contact with the cerebrospinal fluid and it is known as blood cerebrospinal fluid barrier (BCSFB) [34]. It is secreted across the choroid plexus epithelial cells into the brain ventricular system. The third barrier to the brain is constituted by the avascular arachnoid epithelium, underlying the dura and it completely encloses the CNS, which in turn accomplishes the segregation between the extracellular fluids of CNS and the rest of the body [35]. The contribution of this barrier is less significant in comparison to the BBB and BCSFB.

Due to its stringency, these barriers do not allow even the therapeutic drugs to enter the brain, which renders various therapeutic strategies ineffective. Therefore, there is a significant research interest in developing strategies, in particular nanotechnology-based strategies to overcome the hurdle of BBB in CNS drug delivery.

4. ROUTES OF DRUG ADMINISTRATION TO CNS

The routes of drug administration to CNS can be broadly grouped into two categories: systemic administration and direct CNS administration (Table **1**) [36].

Table 1. Different routes of drug administration to CNS.

Systemic	Direct to CNS
Intravenous	Intra-cerebral
Intra-arterial	Intra-ventricular
Intranasal	Intra-thecal

4.1. Systemic Administration

For CNS drug delivery, systemic administration is achieved by following three methods: intravenous, intra-arterial, and intra-nasal administration.

4.1.1. Intravenous

The drugs, which are administered intravenously, are generally in the form of injectables. For CNS and brain drug delivery, intravenous route is the most widely used route of drug administration. Most of the polymeric and liposomal based drug formulations are delivered intravenously. For example, PLGA nanoparticles conjugated with cationized albumin [37] and PEG-PLGA nanoparticles decorated with a peptide, comprising of 12 amino acids [37], have been administered by the intravenous route. Other examples of intravenously administered polymeric formulations include polymeric nanoparticles conjugated with transferrin [38]; PLGA nanoparticles modified with trimethylated chitosan [39]; and tempol-loaded nanoparticles made using PLGA. These nanoparticles are aimed at treating the Alzheimer's and Parkinson's diseases [40].

4.1.2. Intra-arterial

It is similar to the intravenous route but not as widely used because it is easy to locate the veins in comparison to arteries. Intra-arterial administration is mainly used for the disruption of the BBB [41]. Doolittle *et al.* used the intra-arterial route to deliver methotrexate to CNS in conjunction with BBB disruption [42]. The intra-arterial route has been used to deliver stem cells to treat cerebral ischemia [43]. To study the effect on uptake of the hemispheres, a radiotracer was delivered intra-arterially [44].

4.1.3. Intranasal

Intranasal route of drug administration is used for drug delivery to brain because the intranasal route can sidestep the BBB and the drug can be delivered to brain with the help of cellular processes of the olfactory pathway. Administration through intranasal route requires expertise and specialized equipment to control the dosage. The advantage of this system is that it is a painless method and hence patient friendly. Microemulsions and nanogels are administered preferably through the intranasal route. Patel and coworkers administered paliperidone-loaded microemulsions *via* intranasal

route to deliver drug to the brain [45]. The intranasal drug delivery is primarily dependent on the interaction of the drug molecule with the olfactory epithelial cells. Chitosan, PLA and PLGA nanoparticles were investigated for the direct olfactory uptake in the olfactory ensheathing cell line [46]. Human acidic fibroblast growth factor fused with TAT peptide was also administered intranasally. This study also aimed at evaluation of safety issues of the intranasal delivery. The results confirmed the safety and efficacy of the system delivered *via* the intranasal route [47].

4.2. Direct Administration to CNS

The advantage of directly administering the drugs to CNS is that the availability of drug to the affected area it highly enhanced. It is a painful route as the patient has to go through a surgical intervention but the advantage is that the drug is not exposed to any of the cellular and physiological barriers. The bioavailability of drug is at maximum. This route is so robust that it can not only be used to administer drugs and therapeutics but also the diagnostics and imaging moieties. For instance, this route can be used to incorporate array of microelectrodes and optoelectronics. Optoelectronics, in particular, are combination of optical fibers, photodetectors, and light emitting diodes, and used to study, monitor and control brain activities [48].

4.2.1. Intracerebral

The intracerebral delivery of the drug into the brain involves the administration of drugs directly into the parenchymal space of the brain [49] and it involves the use of various catheters and matrices. The problem with the intracerebral administration is that the brain microenvironment is tightly packed. Because of this, the diffusion coefficient is quite limited, which leads to slow movement of the drug. Therefore, to maintain constant drug concentration, a large amount of drug is required. In one study, drug delivery catheters were used to infuse carboplatin to the pons of cynomoglus monkeys. The pumps, which infused the drug or saline, were placed in high thoracic/low cervical region. The cynomoglus monkeys (n = 5) were subjected to midline incision for placing the catheters. A 2.5 cm hole was made, through which a catheter was inserted into cerebellum, which further led the catheter to the pons. The saline infusing and carboplatin infusing pumps were placed in separate groups of animals. The study was used to carry out radiographic imaging and assess the neurotoxicity. Animals were assessed using the computed tomography scanning, histopathology, magnetic resonance imaging, and various neurological examinations [50]. Similar studies were done by Bernal *et al*., who used convection enhanced delivery to increase the bioavailability. This type of delivery makes use of hydrostatic pressure gradient to distribute drug in the brain tissue, through the implanted catheters [51].

4.2.2. Intraventricular

Similar to the intracerebral route of administration, this route of administration is used to deliver drug directly into the brain, especially in the ventricles of the brain as well as the sub arachnoid space. Since there is no tight packing as in the intracerebral route of administration, the drug moves at a higher pace and smaller amount of drug suffices the need. Saluja *et al.* used intracerebroventricular route to deliver the drug to brain and investigated the anti-inflammatory capacity of Dapsone loaded-chitosan nanoparticle in mouse model of dementia induced by streptozotocin. The chitosan nanoparticles were prepared by using the nano precipitation method with some modifications. Briefly, chitosan was used in an aqueous solution of acetic acid and polysorbate 80 was used as the surfactant. The nanoparticles were obtained by centrifugation. The morphology was studied by transmission electron microscopy (TEM) analysis, and size, polydispersity index, and surface charge were analyzed on a *Zetasizer*. The *in vitro* release studies were done in phosphate buffered saline (PBS) and the *in vivo* studies were carried out in Swiss Albino mice. The dapsone-loaded chitosan nanoparticles were successful in reversing the dementia [52]. In a case of subarachnoid hemorrhage, nicardipine releasing implants were placed intraventricularly in human subjects. The intraventricular delivery of the drug proved to be effective and well tolerated [53]. Recently, in a study of multiple sclerosis in an animal model of autoimmune encephalomyelitis, neural stem cells (NSC) were administered intraventricularly. The data proved the efficacy of the intraventricularly administered NSC in preventing remitting relapses [54].

4.2.3. Intrathecal

The intrathecal administration delivers the drug into the CSF through the intrathecal route. This method is relatively less invasive as compared to the other two methods of direct CNS administration. The disadvantage of this method is that the drug is unable to penetrate the deeper tissues of brain and therefore, it is mainly used in diseases related to spine [36]. The intrathecal route has been used to deliver arylsulfatase, a therapeutic enzyme, for the treatment of metachromatic leukodystrophy. The enzyme even though effective is unable to reach the brain due to the BBB and, therefore, was administered directly into the CSF *via* the intrathecal route [55].

5. DRUG TRANSPORT ACROSS THE BBB

As discussed above, BBB regulates the entry and exit of molecules across the BBB [56]. Transport of molecules across the BBB is possible by following three mechanisms: (i) passive diffusion; (ii) carrier-mediated transport; and (iii) receptor-mediated endocytosis/transcytosis (Fig. **3**). These transport mechanisms can be exploited to deliver drug across the BBB.

5.1. Passive Diffusion

Due to the existence of tight junction between the endothelial cells, the passive diffusion takes place by transcellular route and not paracellular route [24, 56]. In passive diffusion, molecules move across the membrane from a region of high concentration to that of low concentration without the input of energy, following Fick's law. However, due to the presence of tight junctions, only lipid soluble molecules with molecular weight of <500 Da are able to diffuse across the BBB passively. A high polar surface area, tendency to form hydrogen bonds (more than six), presence of rotatable bonds, and high affinity for plasma proteins will greatly reduce the ability to diffuse passively across the BBB [56]. Usually, molecules with

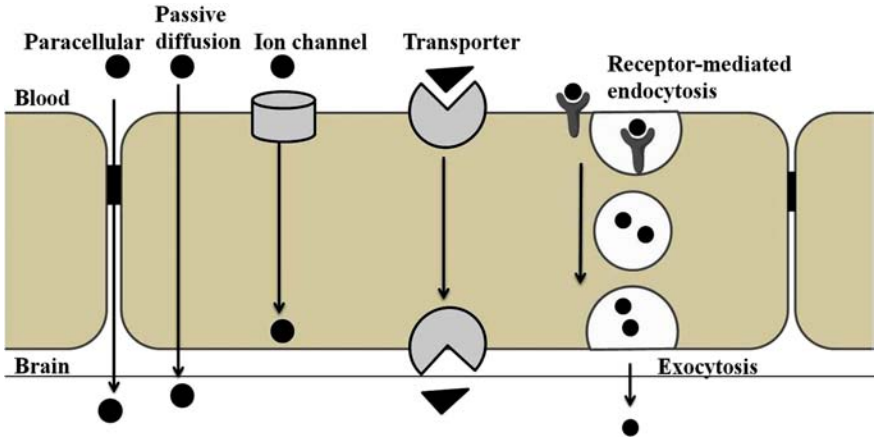

Fig. (3). Major routes of drug transport across the blood brain barrier. Redrawn from: Y. Chen, L. Liu, Modern methods for delivery of drugs across the blood brain barrier, *Adv. Drug Del. Rev.* 64 (2012) 640-665.

positive charge will have advantage over neutral or anionic molecules [56]. Example of molecules that cross the BBB by passive diffusion includes alcohol, steroidal hormones, blood oxygen etc.

5.2. Carrier-mediated Transport

Carrier-mediated transport is used for the polar molecules, which cannot passively diffuse across the BBB [24, 56]. It is achieved by either facilitated diffusion or active transport. In facilitated diffusion, transport proteins forming the membrane channel undergo conformational change, which allows specific molecules to pass through the membrane down the electrochemical gradient, without the input of energy [56]. Examples include glucose and equilibrative nucleoside transporters.

The brain endothelial cells which are the major constituents of the BBB express several carrier proteins. These proteins recognize, interact with, and internalize specific molecules in the cell by transporting it across the membrane against the eletrochemical gradient, using energy from ATP hydrolysis. The process is called active transport and two major classes of such carriers are solute carriers (SLCs) and ATP-binding cassette (ABC super family) transporter [24]. The SLCs are used to transport molecules like glucose (GLUT1); amino acids (LAT2, SNAT2-5, ASCT1-2, EAAT1-3, etc); nucleobases, nucleosides, and nucleotides (ENT1-2, CNT1-2); carboxylic acids (MCT1); organic anions and cations (OAT2-3, OCT2-3); etc. across the BBB [24]. The ABC transporters are further grouped into seven sub families. The major ABC class of transporters expressed in BBB are efflux transporters, which include p-glycoprotein (P-gp), multidrug resistance (MDR) protein (ABCB1), breast cancer resistance proteins (BRCP, ABCG2), etc. [24]. Unlike SLCs, the efflux transporters transport molecules out of the CNS and the brain capillary endothelium. This action protects the brain from toxic molecules but often responsible for removing the drug targeted to the CNS.

5.3. Receptor-mediated Endocytosis/Transcytosis

Transcytosis *via* endocytic route is used for the transport of macromolecules across the BBB [23, 24, 56]. In receptor-mediated transcytosis (RMT), the macromolecule ligand binds to the specific receptor on the cell surface (luminal membrane) and internalized into endocytic vesicles. Transcytosis is achieved, if the endocytic vesicle containing the macromolecule reaches the other end of the cell (basal membrane) without fusing with the lysosome, which may degrade the contents of endocytic vesicle. The macromolecules are finally exocytosed and released into the brain. In adsorptive-mediated transcytosis (AMT), the cationic macromolecule ligand interacts (electrostatic interactions) with anionic cell membrane and induces endocytosis and subsequent transcytosis [24]. Peptides like albumin, insulin, insulin growth factor, low density lipoprotein, ceruloplasmin, transferrin, etc. are transported across the BBB by receptor mediated endocytosis/transcytosis.

6. APPROACHES FOR DRUG DELIVERY TO CNS

Peptide-based delivery systems were most commonly employed for CNS drug delivery up till 1980s. Apart from peptide-based delivery, disruption and modification of the BBB was also used to deliver drugs across the BBB. The later method was discarded because disrupting the BBB or making it leaky in any way could further damage the brain, as other unwanted particle along with the drug molecules may enter the brain. For example, usage of bradykinin and its analogs increases the osmotic pressure of the cells, which in turn leads to opening of the tight junctions of the BBB [57]. In 1986, Neuwelt *et al.* used mannitol to modify the BBB in human subjects. Mannitol was administered intra-carotidally or intra-vertebrally along with the therapeutic agents [58]. Pardrigde used chimeric peptides to enhance the transport of the neuro-pharmaceuticals to the brain. The peptides, which had the capability to cross the BBB through transcytosis, were attached to the hydrophilic neuro-pharmaceuticals, to be transported into the brain. Insulin and insulin like growth factors were also used for drug transport [59-61]. Cationized albumin is another protein that was used to transport drugs across the BBB [62].

During the 1990s, antibodies were employed to deliver the drug across the BBB. OX-26, which binds to the transferrin receptor, was used for CNS drug delivery [63].

This approach was used to deliver nerve growth factor (NGF) across the BBB to treat dementia in AD patients. Delivery of NGF with the help of antibody resulted in the survival of both the cholinergic and the non-cholinergic neurons [64]. Higher costs and unstable nature of the antibodies led the scientists to look for robust alternatives. In the mid 1990s, came the remarkable era of CNS drug delivery with the help of nanoparticles. Nanoparticles of polybutylcyanoacrylate were made and coated with polysorbate 80, which were successful in transporting loperamide across the BBB [65]. The same formulation was also used to administer dalargin intravenously into the mice [66, 67]. Along with the prevalence of nanoparticles, the role of P-glycoprotein (P-gp) was also elucidated. P-gp is a transporter protein, which is present in the basal luminal membrane of the capillary endothelial cells, forming the BBB. P-gp is encoded by mdr1a gene, and when this gene was knocked out using the antisense technology, the drugs like digoxin, cyclosporine etc. exhibited higher brain accumulation rate in wild type mice [68].

Later period witnessed tremendous advancement in the usage of nanoparticles for drug delivery to brain (Fig. **4**). As the new millennium started, new approaches were developed for the drug delivery across the BBB. For instance, pluronic, which is a poloxamer, was shown to inhibit the P-gp in wild type animals. This opened up a new era of CNS drug delivery by inhibiting the P-gp, responsible for drug efflux [69]. In 2002, nanogels were used for the purpose of CNS drug delivery [70]. The current decade has seen significant advancement in the use of nanoparticles, nanogels, and lipid-based systems. An overview of various approaches presently under development for CNS drug delivery is provided below.

6.1. Biomaterial Based Strategies for Drug Delivery

Apart from performing as good carrier system for the delivery of drugs, peptides, oligonucleotides, growth factors etc. [71], biomaterials are also being used for repair and regeneration of nerve tissue [72]. For instance nerve conduits were developed to repair the nerve injury. In this study, poly [(lactic acid)-co-{(glycolic acid)-alt-(L-lysine)}] nerve conduits were developed by grafting the polymer with RGD peptide and NGF [73]. The biomaterials are being used in the treatment of various brain disorders. There are various strategies, which have been proven to be successful in delivering drugs across the BBB. The drug delivery systems based on polymers and lipid has gained much attention over a period of time and they still hold the limelight. Table **2** summarizes the application of various drug delivery strategies being used.

6.2. Polymeric Systems

The FDA approved polymers, like polylactide, polycaprolactum, polyglycolide, polydioxanone, etc., are gaining much importance in the field of CNS drug delivery. The polymer-based systems are generally used as hydrogels, implants, nanoparticle formulations, and so on. This particular section specifically deals with the use of polymers as nanoparticles for CNS drug delivery. Nanoparticles are the self-assembling structures whose size range is in nanometers. The size of the nanoparticles is of great importance as the particle size is one of the important parameters, which govern the cellular uptake and bio-distribution of the nanoparticles [95]. Calvo *et al.* developed polycyanoacrylate nanoparticles; the size range of these nanoparticles was in 130-150 nm range. The PEGylated nanoparticles with the size of 137 nm were the most efficient ones to reach the brain [96]. Later on it was established that the particle size should be below 100 nm for efficient drug delivery across the BBB. Koziara *et al.* developed polysorbate nanoparticles for brain delivery with size less than 100 nm [97]. The similar results were reproduced by Gao *et al.*, which firmly established that the particle size for brain delivery should be less than 100 nm. They developed polysorbate-80 coated polybutylcyanoacrylate nanoparticles to deliver methotrexate across the BBB. They had particles of various sizes viz., 70 nm, 170 nm, 220 nm and 354 nm. The results, which were obtained with the particles with size below 100 nm, were remarkable [98]. Since then the particle size for efficient

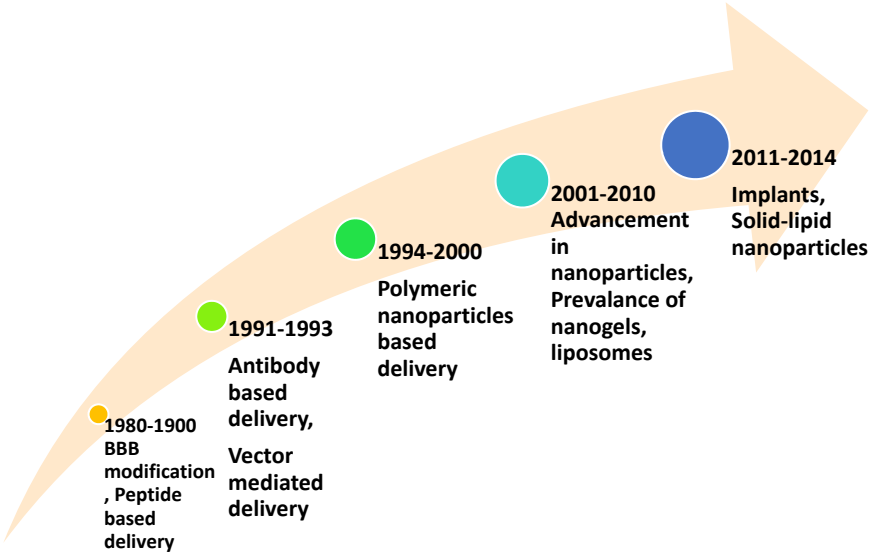

Fig. (4). Highlights of the major developments in the field of CNS drug delivery since 1980.

Table 2. A brief summary of various approaches for drug delivery to CNS.

Systems	Drug Loading	Neurological Applications
Polymeric Nanoparticles	Drug can be loaded into the core or matrix in solid state or solution; can also be attached to the surface of the nanoparticles	Various polymeric nanoparticles containing therapeutic drugs have been used for the treatment of Alzheimer's disease and Parkinson's disease [74-76] Antisense oligonucleotides have been delivered for muscular dystrophy [77, 78]
Liposomes	Water soluble drugs are entrapped in the core and the lipophilic drugs are embedded in the lipid bilayer	Liposomes have been used for Alzheimer's and Parkinson's disease in the form of Liposome-DNA complex; Active therapeutic agents have also been delivered in case of ALS [79-81]
Microemulsions	Drugs with poor solubility and bioavailability are delivered	Transdermal microemulsions have been used successfully for Parkinson's disease [82]. They have also found application in Muscular Dystrophy, Huntington's disease, and Alzheimer's disease [83-85]
Solid lipid nanoparticles	Drugs are adsorbed or dispersed in the hydrophobic core	SLNPs have been used in ALS and Parkinson's disease [86, 87]
Nanogels	Drugs are bound through ionic interactions	Naogels complexed with beta-amyloid have been proved to be successful in Alzheimer's disease [88, 89]
Implants	Implants act as drug reservoir, from which drug is eluted out at a controlled rate	Alzheimer's disease [90-92] and tumors [93, 94]

brain delivery is always taken below 100 nm. In a similar research, which elucidated the effect of size on effective brain delivery, it was confirmed that the polysorabte 80 nanoparticles coated with PEG-COOH in between the size range of 40-100 nm diffused to the brain tissue rapidly [99]. Similar results were confirmed by various other research groups [51, 100-103].

6.2.1. Methods of Preparation of Nanoparticles

Some of the most commonly used methods used to prepare nanoparticles are discussed below:

6.2.1.1. Nano-precipitation Method

In this method, the polymer is first dissolved in a water miscible solvent like acetone, tetrahydrofuran, methanol, ethanol, acetonitrile, DMF etc. The polymer dissolved in the organic phase is dispersed in the aqueous phase under constant stirring. The aqueous phase may or may not contain any surfactant. The organic phase is removed to obtain the nanoparticles in the aqueous phase [103]. The nanoparticles are quickly precipitated because the organic solvent is diffused in the aqueous phase. As the solvent diffuses from organic phase to the aqueous phase, it carries with it some polymeric chains, which aggregate to form the nanoparticles [104]. The concentration of polymer and the solvent system used effects the size of the nanoparticles [105, 106]. The higher the concentration of the polymer, the higher the size of the nanoparticles [103]. This method is being used in the latest researches to prepare nanoparticles [107-111].

6.2.1.2. Solvent Evaporation

The solvent evaporation method is similar to the nano-precipitation method. The difference lies in the organic solvent. In nano-precipitation the organic solvent, which is used, is miscible with water but the organic solvent used in the solvent evaporation method is immiscible with water. The organic phase is dispersed in the aqueous phase and it forms an oil-in-water emulsion. The emulsion is stabilized by using surfactants like polysorbate-80, poly vinyl alcohol (PVA), poloxamer-188, etc. Once the emulsion is stabilized, the organic phase is evaporated under constant stirring. The method is being used by various groups [95, 112-117].

6.2.1.3. Salting Out

This process involves the dissolution of a polymer in a water miscible organic solvent. The polymer dissolved in the organic solvent is slowly added to the aqueous phase. The aqueous phase contains high concentration of salt and emulsifiers due to which the mixing of organic phase and the aqueous phase is slowed down. These separate phases are emulsified by stirring. For the formation of nanoparticles, water is added to the system rapidly to reduce the ionic strength. The excess salt must be removed before applying it for therapeutic use [103, 118-121]. The main disadvantage of this process lies in its extensive washing steps to remove excess salts and stabilizing agents [122].

6.2.1.4. Emulsion Diffusion

In this process, the polymer is dissolved in an organic solvent, which is partially miscible with water (e.g. ethyl acetate, polyprolylene carbonate, etc.). Aqueous phase containing stabilizer is added to this system. The mixture is constantly stirred with simultaneous addition of water. Ample quantity of water is added, which leads to the formation of oil-in-water emulsion. The organic phase is later on removed by constant stirring, which results in the

formation of nanoparticles. This process is known for its high reproducibility. The stabilizers used in this system reduce the surface tension and makes the particle size smaller [103, 119, 123-127].

6.2.1.5. Emulsion Evaporation

This process is similar to emulsion diffusion method. An oil-in-water emulsion is made by dispersing the organic solvent containing polymer in the aqueous phase containing the stabilizers. A strong shear stress is applied to the emulsion droplets, which further breaks down the droplets into smaller size. Later on the organic phase is evaporated and polymer is precipitated to form the nanoparticles. The application of high shear stress is important to stop the migration of emulsion droplets towards each other and prevent their aggregation [103, 128, 129].

6.2.2. Nanoparticulate Formulations

In recent decade, the polymeric nanoparticulate systems have been used by various scientists to deliver drugs of various types to cure several diseases. The various nanoparticulate formulations pertaining to polymeric systems are generally of four types: micelles, nanospheres, nanocapsules and polymersomes.

6.2.2.1. Micelles

The development of the micelles dates back to late 1990s. At that time, the polymeric micelles were seen as an advancement of surfactant micelles owing to their stability [130]. The micelles are basically made of block copolymers, which self-assemble to from a dense hydrophobic core, which is surrounded by a hydrophilic brush like layer (Fig. **5**) [131].

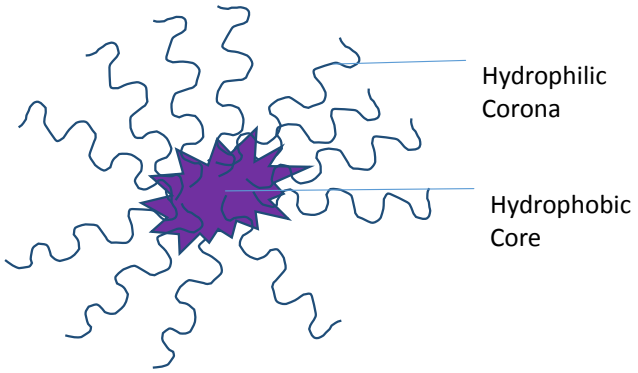

Fig. (5). Morphology of a polymeric micelle.

The brush like outer structure has a significant role to play. It prevents the micelles from adhering to proteins and cells. This conformation also prevents opsonization and hence gives the micelles the advantage of having long circulation time in the body. Other advantages of micelles are controlled drug release and good tissue penetrating ability [132]. The various hydrophobic core forming molecules, which are used frequently in micelle formation are poly(propylene oxide) (PPO), poly(D, L-lactic acid) (PLA), poly(ε-caprolactone) (PCL), poly(l-aspartate) and poloxamers. The hydrophobic core of the micelles provides an excellent environment for the encapsulation of lipophilic drugs. The disadvantages associated with most micelle carriers are the limited stability, difficult polymer synthesis, immature drug encapsulation technology, limitation to hydrophobic drugs, slow extravasation, and possible chronic liver toxicity due to slow metabolic processing [133-135].

6.2.2.2. Nanospheres

A polymeric nanosphere can be defined as a matrix which is enclosed by a polymeric membrane. The nanosphere forms the solid particle in which drugs can be dissolved, encapsulated, and entrapped. The drug molecules can also be attached to the polymeric matrix by adsorption or chemical bonding. These nanospheres are generally larger than micelles and have diameters between 100-200 nm. These particles also exhibit markedly higher polydispersity (Fig. **6**).

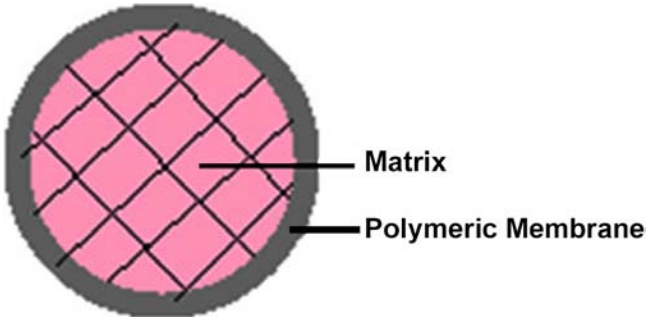

Fig. (6). Nanosphere.

The major concern with the use of nanospheres for CNS drug delivery is that it is not cleared from the body because it is captured by the reticuloendothelial system (RES), the sequestering particles within the organs like liver and spleen. The hydrophobic surface of the nanospheres is susceptible to opsonization and hence it does not have long circulation time [136].

6.2.2.3. Nanocapsules

Polymeric nanocapsules are nanoscale vesicular structures. They are made up of polymeric membrane, which encapsulates the inner core (Fig. **7**). The size of the nanocapsules generally ranges from 10-1000 nm. The drug is loaded into the inner core [137, 138]. Both lipophilic and hydrophilic drugs can be encapsulated in the inner core depending on the method of preparation of the nanocapsules [138].

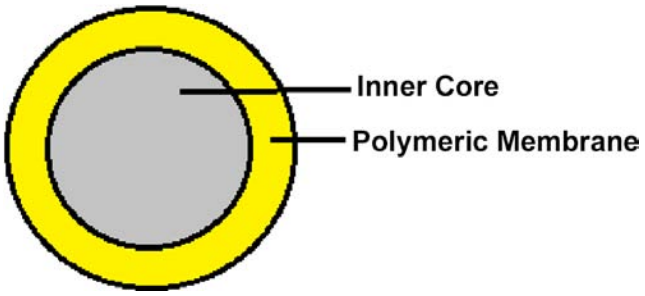

Fig. (7). Nanocapsule.

6.2.2.4. Polymersomes

Polymersomes are the vesicular structures, which are composed of an aqueous internal core surrounded by a polymeric membrane.

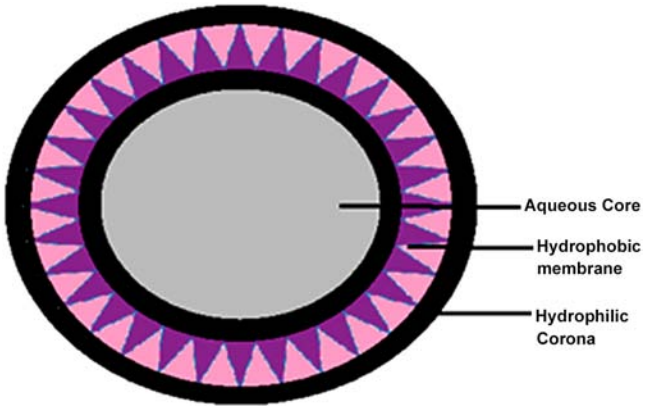

Fig. (8). Cross-sectional view of a polymersome.

The polymeric membrane can be viewed as a hydrophobic layer sandwiched between hydrophilic polymeric brushes (Fig. **8**). They are considered as more stable vesicles when compared to the lipid based vesicles due to their thicker outer membranes and, therefore, they are gaining more attention amongst the drug delivery community [139]. The size ranges from 5 nm-5 μm [136].

Biocompatibilty concerns limit the wide range use of polymeric systems as vehicles of transport. There are numerous biocompatible, FDA-approved polymers, which can be used as carriers for CNS drug delivery. Polyethylene glycol (PEG)-poly(D, L-lactide-co-glycolide) (PLGA) nanoparticles have been shown to successfully cross the BBB. The surfaces of these nanoparticles were modified with cationic bovine serum albumin (CBSA) [140] and it exhibited a good accumulation rate in the brain. It was tested in isolated brain capillaries and evaluated using internal carotid perfusion/ capillary depletion technique *in vivo*. It was observed that CBSA modified nanoparticles were transported to brain *via* the absorptive mediated transcytosis process through the BBB. The 6-coumarin was loaded into the nanoparticles and used as the fluorescent probe. The polymeric nanoparticles were administered through the caudal vein. The surface modification with CBSA remarkably increased the brain uptake of 6-Coumarin [140].

To enhance the drug delivery across the BBB, the nanoparticles were targeted against the active agents of the endothelial cells of the BBB. Li *et al.* in 2011 developed PEG-PLGA nanoparticles decorated with a peptide, comprising of 12 amino acids. The nanoparticles were synthesized by the solvent-emulsion evaporation method and loaded with coumarin-6. To study the *in vitro* cytotoxicity and the uptake by the brain cells, bEnd.3 cells were used and it was found that nanoparticles with the peptide showed better uptake in the bEnd.3 cells. Furthermore, these particles did not exhibit cytotoxic effects on the cells. For *in vivo* studies, nude mice were used and the amount of drug reaching the brain was analyzed using coumarin-6. It was observed that the peptide conjugated PEG-PLGA nanoparticles have a higher uptake in the cells *in vitro* and higher brain accumulation *in vivo*. Overall, it could be a potent drug delivery strategy for delivering the drug across the BBB [37].

To cross the BBB, Gan *et al.* developed polymeric nanoparticles conjugated with transferrin. They synthesized a diblock copolymer of poly (lactide)-D-α-tocopheryl and polyethylene glycol succinate. The nanoparticles were synthesized by nanoprecipitation method and were characterized for their size, surface charge, size distribution, surface morphology and encapsulation efficiency. The nanoparticles conjugated to transferrin demonstrated better uptake *in vitro* and higher accumulation in brain of the Sprague-Dawley rats. The IC_{50} data proved it to be an efficient formulation, which can take the drug across the BBB to cure neurodegenerative diseases [38]. Similarly, PLGA nanoparticles were modified with trimethylated chitosan to cross the BBB. The results proved that this formulation to be promising one, with low cytotoxicity [39]. In another study, tempol-loaded nanoparticles were made using PLGA for treating the Alzheimer's and Parkinson's diseases [40]. Thus, the polymeric nanoparticles have great potential for delivering drugs across the BBB. These nanoparticles can be modified with peptides, antibodies, ligands, etc. These nanoparticles are transported to the brain through either absorptive-mediated transcytosis or receptor-mediated transcytosis. The advantage of using the polymeric drug delivery system lies in its robustness and stability. It provides for high drug loading capacity and an opportunity to control the drug release kinetics of the system. Apart from being safe for human use, the polymeric systems are flexible systems, which can be modified to display various ligands and surface moieties for targeted and site-specific drug delivery.

6.3. Liposomes

The advantage of liposomes is that the liposome structure is similar to cells with regard to the lipid bilayer present in cell membranes. The lipid bilayer aligns itself in such a fashion that the hydrophobic end of the bilayer sticks to each other and hydrophilic ends come at the outer surface (Fig. **9a**). In this way a vesicle is formed, which has a hydrophilic core surrounded by a hydrophobic layer, decorated with hydrophilic groups on the outer surface. The bilayer is generally composed of the phospholipids (Fig. **9b**). Liposomes can successfully act as nanocarriers to deliver drugs across the BBB.

Liposomes were developed and conjugated with antibodies to work as immunoliposomes in the treatment of Alzheimer's disease [141]. In this study, the liposomes consisting of DSPC (l,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, DSPE-PEG2000 (l,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[amino(polyethylene glycol)-2000], and DSPE-PEG2000-mal (1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[maleimide(polyethylene glycol)-2000], were synthesized by thin film lipid hydration method. The antibodies against the transferrin receptor were conjugated onto the surface of the liposomes. Thus, a promising lipid based system was produced to cross the BBB [141]. Similarly, PEGylated liposomes were prepared with glutathione-modified surfaces [142]. Liposomes, comprising of sphingomyelin/cholesterol and phosphatidic acid, were

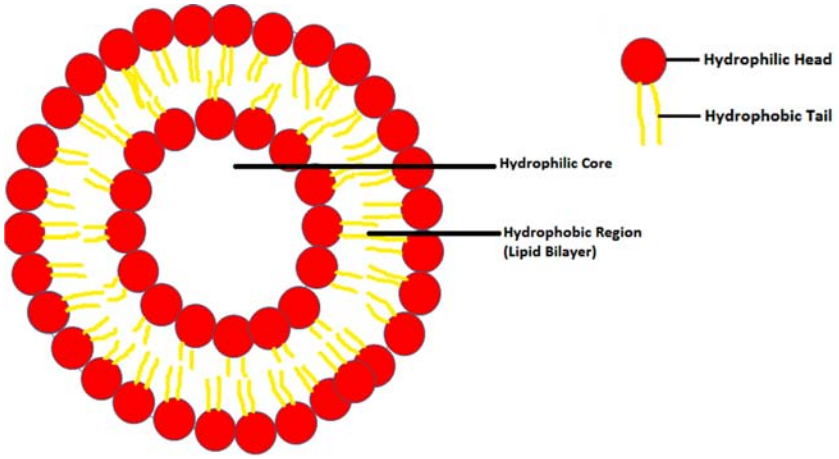

Fig. (9). a): Cross-sectional view of the liposome; and **b**) Single unit of a phospholipids.

developed to treat the Alzheimer's disease. The phosphatidic acid is known to have the property to bind to beta amyloid. The surface of the liposomes was modified using RI7217 antibody, which binds to the transferrin receptor on the BBB cells. The antibody was bound to the surface by biotin/ streptavidin linkage and/or thiol-maleimide coupling. Studies not only proved the efficiency of liposomes in transporting drugs across the BBB but also demonstrated the fact that the thiol-maleimide coupling is more efficient that the biotin/ streptavidin coupling [143]. Higher bioavailability, low cost, and simplicity of preparation, makes liposomes an attractive nanocarrier for CNS drug delivery. Another popular reason for liposomes to become so prevalent is that they are capable of transporting both hydrophobic and hydrophilic drugs. The disadvantages associated with liposomes lies in its size- the liposomes tend to have relatively higher size [144]. The liposomes can be used with or without surface modifications. For instance, the NGF is essential for the nerve repair in case of neurodegenerative diseases but the clinical use of NGF remains limited because it is unable to cross the BBB and reach the affected area. So the NGF was packed into the liposomes, which were modified on their surfaces, to transport NGF to the affected area more efficiently [145].

6.4. Microemulsions

Microemulsions are emulsions with droplet size at least 100 times less than the conventional emulsion system. These are thermodynamically stable blends of oil, water, and surfactant, and widely used for enhancing the bioavailability of the poorly soluble drugs. The small droplet size and low surface tension of microemulsions leads to higher absorption and permeation across the BBB [146, 147].

Cetyl trimethyl ammonium bromide (CTAB)-based microemulsions were developed for drug delivery across the BBB. These microemulsions were intended for rivastigmine delivery to treat AD. Rivastigmine is a reversible inhibitor of cholinesterase, and used for the treatment of AD. The disadvantage of using the conventional drug lies in its first pass metabolism in liver, which reduces the drug bioavailability from 100 to 36%. Rivastigmine is highly hydrophilic drug and its hydrophilicity hinders it penetration across the BBB. To overcome this problem, microemulsions and mucoadhesive microemulsions were developed to deliver the drug through the intranasal route. These microemulsions were synthesized by a titration method and characterized for various parameters. The microemulsions, which were not mucoadhesive were synthesized using capmul, labrosol, and distilled water, whereas the microemulsions with mucoadhesive properties consisted of chitosan and CTAB. Nonetheless, the promising properties of chitosan-based mucoadhesive microemulsions could be guaranteed only after the *in-vivo* studies [148]. Another study aimed at treating schizophrenia with the help of microemulsions, used microemulsions loaded with paliperidone. These studies proved the potential of microemulsions in transporting the drug into brain *via* the intranasal route. The microemulsions loaded drug had a greater extent of brain transport than the conventional drug [45]. Similarly, carbamazepine was also delivered using the microemulsions and extended drug transport was observed, when compared to the conventional drug [149]. Microemulsions are thermodynamically more stable and, therefore easy to store. They have higher skin penetration and diffusion rate [150]. Although microemulsions had made a mark in the field of CNS drug delivery, use of large amounts of surfactants to stabilize microemulsions is still a major concern.

6.5. Solid Lipid Nanoparticles (SLNPs)

Solid lipid nanoparticulate system is an emerging field in which the nanoparticles are formed with a solid core. The SLNPs are synthesized from the solid lipids i.e. the lipids, which remain solid at room temperature. The advantage of SLNPs lies in their very high drug loading capacities and various groups have used SLNPs to enhance the CNS drug delivery efficiencies.

The SLNPs were used to deliver the drug streptomycin sulfate, which in its conventional form is administered orally. Due to the poor absorption, the drug bioavailability is greatly reduced. Also, its chronic use leads to serious side effects, like ototoxicity and nephrotoxicity. Loading of the

SLNPs were synthesized in cationic microemulsion and later on converted into SLNPs by rapid cooling. Human brain-microvascular endothelial cells (HBMECs) were used to study the uptake to the drug loaded SLNPs. To synthesize the SLNPs, decyltrimethylammonium bromide (DTMAB) was mixed with sodium dodecylsulfate (SDS) in water, and to synthesize the lipid phase, dynasan 114, cacao butter, stearic acid, octadecanoic acid, hexyldecyl stearate, and ETP were dissolved in methanol [152]. The surface of SLNPs loaded with carmustine was modified with monoclonal antibody to enhance the drug delivery across the BBB. This research provided promising breakthrough to the use of SLNPs for CNS drug delivery [153]. The solid lipid nanoparticles are advantageous as they allow control over the drug release, both hydrophilic and lipophilic drugs can be incorporated. With the use of physiological lipids in the synthesis process, the risk of toxicity can also be reduced. But low concentration of the dispersed lipids is an issue of concern [154].

6.6. Nanogels

Nanogels are hydrogels with crosslinks of nanoscale size. The crosslinking is between hydrophilic or amphiphilic polymeric chains. The nanogel made from polyethyleneimine (PEI) and polyethylene glycol (PEG) swells by making crosslinks when dispersed in a solution. As the drug binds to it through electrostatic interaction, the nanogel collapses. PEG plays a major role in stabilizing the structure and nanogels form a stabilized dispersion [155]. Nucleoside reverse transcriptase inhibitors (NRTIs), which are used in antiviral therapy, are neurotoxic and less efficacious in eradicating HIV-1 that infects the CNS. In a recent study, it was shown that 5'-triphosphates of NRTIs encapsulated in cationized nanogels (nano-NRTIs) were more successful in suppressing the HIV-1 activity in CNS than NRTIs. The cationic drug-loaded nanogels exhibited less toxicity to the mitochondria [70]. Nanogels were prepared by the ionic gelation method using sodium tripolyphosphate and chitosan. The nanogels were modified with polysorbate 80. The drug transported through the nanogel was remarkably higher in the brain than the free drug [156].

6.7. Implants

Implants are the device used to replace a structure, support a damaged structure, or augment an existing structure in human body. The implants are also used for diagnostic and drug delivery applications.

The implants were traditionally made up of silicon- the microelectrode arrays, which were used for investigating the neuronal firing [157]. The concerning issue in this case was severe neuroinflammation caused by the implant [158]. Consequently, it did not succeed as efficacious implantable system. It was hypothesized that the mechanical incompatibility between the rigid implant and soft brain tissue was the significant reason for neuroinflammation. A nanocomposite based intracortical implant was developed by casting polyvinyl alcohol (PVA) in a solution of DMF based on the same hypothesis. The results proved the hypothesis, as the animals implanted with the mechanically adaptive implant exhibited improved neuronal proximity in terms of improved glial scar [159]. In case of intracortical implants, it was seen that the implant caused tissue reactivity, which was probably because of the oxidative stress created by the implant as well as its inflexibility. Its rigidity with respect to the softness brain tissue leads to the failure of the implant.

Recently, a group developed PVA based polymeric implants. These implants were mechanically adaptive and flexible, and designed to release curcumin. The two important parameters, which acted synergistically *in vivo* were: softening and flexibility of the implant and presence of an antioxidant. The PVA and curcumin stock solution were separately made in DMSO with constant stirring and 1% and 3% curcumin-loaded PVA hydrogels were made by adding the two stock solutions accordingly. The mixture was stirred continuously at room temperature and sonicated later. It was then cast into a petri dish. It was dried for 5 days and later on subjected to high vacuum. These films were pressed with the spacers between them. The films were tested for mechanical strength in terms of tensile strength. The antioxidant activity of the curcumin-loaded PVA films was assessed using 2,2-diphenyl-1-picrylhydrazil (DPPH). The *in vitro* studies were done in artificial cerebrospinal fluid and for *in vivo* assessment Sprague-Dawley rats were used [158]. The results of this investigation have opened up a new channel of flexible intracortical implants, where the neuroinflammation due to the rigidity of the implants has been minimized remarkably. The implants are advantageous as they provide with maximum bioavailability but their implantation and removal requires surgical intervention, which is troublesome for the patient.

7. DRUG DELIVERY APPROACHES FOR HERBAL MEDICINE

Synthetic drugs with immense potential for treating various CNS diseases also confer the patients with various adverse effects like bradycardia, nausea, diarrhea, anorexia, abdominal pain, etc. An overdose of such drugs may lead to collapse and convulsions. Herbal medicines which belong to the category of flavonoids, terpenes, triterpenoids, saponins, tannins and jojubogenins [160], are gaining importance due to their natural origin, low side effects, and more specifically due to their anti-oxidant activities in comparison to modern allopathic drugs. Additionally, these medicines are more easily metabolized in the body and are less toxic. However, these molecules have high molecular weight, complexity of their chemical composition, and water solubility, which makes their bioavailability a big challenge. Various approaches have been developed to overcome the hurdles in the effective use of herbal medicines for CNS disorders. An overview of various carrier systems employed to deliver herbal medicines for CNS disorders is given below [161, 162].

7.1. Phytosomes

Phytosomes are the recent addition to the field of CNS drug delivery. As the name suggests, these are vesicles, which contain plant extract bound by a lipid. Phytosomes contain advanced form of herbal extracts and advanced pharmacokinetics and pharmacodynamics properties, which increase the drug bioavailability by bypassing the lipid membranes. Phytosomes are prepared using a patented methodology [163]. The holistic plant extract or the active

constituents are water soluble, which are reacted with lipid, preferably the phosphatidyl choline, to produce a lipid and drug complex. The ratio of the plant extract to the lipid is generally 1:1. Various plant extract have been converted to their respective phytosome using this method. Examples include green tea, gingko, grape seed, milk thistle, etc. A commercial phytosme, which protects the brain and its vascular linings is available commercially with the trade name of Gingko Phytosome™. It contains 24% flavnoids *Gingko biloba* [164].

7.2. Pharmacosomes

Pharmacosomes are micelles of nanoscale range, which forms a colloidal suspension. In this case, herbal extract is attached to lipids through covalent linkages, unlike the phytosomes. Their characteristic features are their small size, high drug loading capacity, amphiphilicity, and stability [165]. The 3', 5'-dioctanoyl-5-fluoro-2'-deoxyuridine pharmacosomes have been prepared using a central composite design. The pharmacosome exhibited a great capacity to cross the BBB. The *in vivo* studies indicated its enhanced bioavailability and efficacy [165, 166].

7.3. Polymeric Systems

In 2012, Mathew *et al*., developed PLGA nanoparticles conjugated with Tet 1 peptide. These nanoparticles were loaded with curcumin for use in the treatment of Alzheimer's disease. Curcumin being hydrophobic in nature was encapsulated using the single solvent emulsion method. They studied the encapsulation efficiency of the nanoparticles, and the physicochemical properties of the nanoparticles were assessed by TEM, scanning electron microscopy (SEM) and atomic force microscopy (AFM). The Tet peptide was conjugated to the curcumin-loaded PLGA nanoparticles through EDC-NHS coupling. The PLGA-curcumin nanoparticles showed good results in the disaggregation profile of the beta-amyloid. Also, the conjugation of nanoparticles to the Tet peptide resulted into higher affinity of nanoparticles for GI 1 cells. This formulation successfully crossed the BBB [167]. To reduce the toxicity caused by levodopa, which is administered to the patients of Parkinson's disease, Lees *et al*., used *Mucuna pruriens* in polymer matrices. *Mucuna pruriens* is a legume, which is also known as velvet bean. Its seeds contain levodopa and, therefore, it has been used in the treatment and management of Parkinson's disease [168].

7.4. Liposomal Formulations

In a study, liposomes were used to encapsulate, breviscapine, a traditional drug of Chinese origin. *Scutellaria barbata* is an angiosperm from which the Breviscapine has been derived. This has been used clinically to cure ischemia and cerebrovascular disorders. The conventional drug had to be administered repeatedly. In order to make the drug stay in circulation for prolonged period, a multivesicular liposome based system was designed. This system is called DepoFoam. When compared with traditional drug, the drug loaded in multivesicular liposome exhibited longer stay both *in vitro* and *in vivo*. The pharmacokinetic results revealed the mean residence time, which was 16.6 folds higher than that of the conventional solution [169]. Another research group focused on *Bacopa monniera* and its anti-amnesic effects [170, 171]. Due to the inability of Bacopa to cross the BBB, it was complexed with phospholipids to create a novel phytoformulation by Habbu *et al*. The L-α-phosphatidyl choline was used to prepare the Bacopa-phospholipid complex. The Bacopa-phospholipid complex reversed the age induced amnesia in mice more efficiently than the conventional extract [172]. Bacoside is an active component of *Bacopa monniera*. The SLNPs were chosen to carry the bacosides for efficient delivery. The SLNPs were based on stearic acid and made using the emulsion method [173].

8. COMBINATORIAL APPROACH OF NANOSYSTEMS

The combinatorial approach of the nanosystems implies that the nanosystems are designed to act in a multifunctional manner. At times they can act as reservoir for two drugs simultaneously or they can help both in therapy as well as diagnostics, at the same time. Based on this very multifunctional strategy, Han *et al.* designed and constructed a novel gene delivery complex for brain tumor based on polyamidoamine (PAMAM). The polymer was modified with magnetosome and TAT peptide. This complex formed a novel non-viral transfecting system for gene therapy to brain, which could efficiently target, transport and transfect [174]. Angiopep-2 was used to develop dual targeting nanoparticles for brain as Angiopep-2 can concurrently activate the low density lipoprotein receptor related protein 1 on both the endothelial cells and the glial cells. So these nanoparticles can do both the tasks of crossing the BBB and targeting the glial cells [175].

9. CONCLUSIONS AND PERSPECTIVES

Brain is a complex organ and more complex are its diseases. The conventional drugs are either inefficacious or efficacious but hindered by various physiological and cellular barriers like the BBB and the BCSFB. These barriers greatly reduce the bioavailability of the drug. To increase the therapeutic dose, the patient is compelled to take the drug time and again, which adds to his discomfort. In addition, the repeated administration of the drug leads to toxicity due to the drug overdose and enhanced side effects associated with it. To overcome the hurdles associated with poor CNS drug delivery, various strategies have been devised through which the drug can be administered to CNS in safe and efficacious manner, with high patient compliance. Some of these approaches include the usage of polymer based drug carriers in the form of nanoparticles, polymersomes, hydrogels, etc. Others include liposomes, microemulsions, nanogels, and solid lipid nanoparticles (SLNPs). In parallel, rapid advancement has been made in the field of diagnostics through implantation of, for instance, optoelectronics. MRI contrast agents have been developed, which target CNS with high efficiency. All these drug delivery systems have the advantages of reduced drug intake, reduced toxicity, reduced side effects, and controlled and sustained drug delivery. Rate-controlled and feedback-regulated drug delivery systems constitute an emerging area of research. Even though the benefits derived by various drug delivery strategies have been well established and thoroughly explored, the safety and the toxicity concerns have been not fully addressed. The

conventional safety assessment cannot be efficiently applied to the nanoscale systems because of the peculiar physical-chemical properties they possess. Safety assessment protocols specific to the nanoscale systems need to be designed and validated. Another problem usually encountered is contamination with LPS (bacterial endotoxins). To avoid the LPS contamination, the synthesis of nanoparticles should take place in an endotoxin free environment [176].

There is no method to quantify nanoparticles entering the brain. From the *in vivo* biodistribution data, it has been observed that less than 1% of the brain targeted nanoparticles are able to cross the BBB. Majority of these nanoparticles are entrapped by the liver and RES [100, 177].

When the nanoparticles enter the neurons or the glial cells, they are directed to the lysosomal vesicles. There is a possibility that the nanoparticles remain in the cytoplasm and interact with other organelles of the cell. They may also interact with other cellular entities like the cytoplasmic proteins. Such interactions may lead to alterations in the structure of proteins and may also lead to aggregation, which in turn will alter the cellular functions that are dependent on those particular proteins [178]. Apart from organelles and proteins, the nanoparticles may also interact with cell membranes, which may lead to the change its permeability. This makes it extremely important for the researchers to investigate the potential toxicities caused by the nanoparticles. Therefore, more work is needed to fully address the safety and toxicity concerns associated with CNS delivery of nanoparticles.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Does toll-like receptor-3 (TLR-3) have any role in Indian AMD phenotype?

Neel Kamal Sharma · Kaushal Sharma · Amod Gupta · Sudesh Prabhakar · Ramandeep Singh · Pawan Kumar Gupta · Akshay Anand

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Abstract Age-related macular degeneration (AMD) is a devastating disease that results in irreversible central vision loss. TLRs signaling pathway has been found to play an important role in AMD pathogenesis as evidenced by several studies. The objective of the study was to determine the single nucleotide polymorphism (SNP) changes in TLR3 in North Indian AMD patients. We recruited 176 patients comprising 115 AMD patients and 61 controls. Real time PCR was used to evaluate the SNP changes at rs3775291 locus. Pearson's χ^2 test was used evaluate association between various groups. No significant association in genotype and allele frequency was found in AMD patients as compared to control. The results suggest that AMD pathology in North Indian AMD patients is not affected by TLR3 signaling but it could be influenced by other genetic or environmental factors unique to North India.

Keywords Age-related macular degeneration · TLR3 · Single nucleotide polymorphism · Signaling · Genotype · Allele frequency

Neel Kamal Sharma and Kaushal Sharma have contributed equally to the manuscript.

N. K. Sharma · K. Sharma · S. Prabhakar · P. K. Gupta · A. Anand (✉)
Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India
e-mail: akshay1anand@rediffmail.com
URL: http://www.neurologypgi.org

A. Gupta · R. Singh
Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

Introduction

Age is a risk factor for degenerative diseases like Age-related macular degeneration (AMD). AMD is an ocular disease that causes central vision loss in individuals with aging. It is considered as the main cause of irreversible blindness in the aged population, with a prevalence of 12 % after 80 years of age [1]. AMD is characterized by degenerative and neovascular changes occurring between the neural retina and the underlying choroid, which causes progressive loss of central vision. With increase in elderly population, it is estimated that by 2020 the number of AMD patients in the US and India will rise further [2]. The etiology of AMD is associated with both genetic as well as environmental risk factors which may vary between populations. The immune components, complement system, and Toll-like receptors (*TLRs*), which act as pattern recognition (PRR) molecules for innate immune system, may participate in progression of AMD. TLRs consist of 10–12 families of type-I integral membrane receptors which are known to be expressed on different cell types including eye tissues, and recognize pathogen-associated microbial pattern (PAMP) and consequently initiate inflammatory responses [3, 4]. These TLR receptors function in response to their respective ligands in two ways: by stirring up the phagocytosis of the target molecule and by prompting the signal pathways that can provoke the expression of cytokines and other inflammatory mediators [5, 6]. These augmented and persisting inflammatory responses in retinal pigment epithelium (RPE) cells, by the means of complement system, TLR signaling, or through the co-activation of both, can stimulate the drusen formation along with aggregation of other lipoproteins and activated immune system components in macula. Therefore, it remains a critical molecule, which facilitates the disruption of the inflammatory cascade which is responsible for AMD [7, 8]. Additionally, viral and bacterial entities

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may aggravate the inflammation that may initiate the progression of AMD pathogenic features. The TRIF-dependent process utilizes *TLR3* and TLR4 receptors activating the IRF3 which ultimately induces the *NFκB* in a similar manner [9]. Apart from the current AMD treatment, the preclinical study on mice, by knocking down the function of TLR3 with siRNA, efficiently suppressed the angiogenesis in these mice [10] in target and sequence independent manner. Similarly, when targeted siRNA was not internalized inside the cells, it stimulated the degeneration of retinal cells by TLR3-induced caspase-3 pathway [11] which highlights the importance of TLR3 in AMD. Additionally, expression pattern of TLR-3 was found to be high in wet AMD at both mRNA level as well as at protein level [12] and also on RPE cells obtained from donor AMD patients [13], which signifies the abnormal expression pattern of TLR 3 in AMD pathogenesis. The genetic changes at particular loci in these TLRs family receptor's genes are known to be associated with abnormal innate immunity regulation and progression of several diseases. Zareparsi et al. [14] have described strong association between *TLR4* (TLR4, a bacterial endotoxin receptor) variants and increased risk of AMD susceptibility [15]. The association of *TLR3* polymorphism and their role in AMD pathogenesis is not well defined because of the conflicting results in different populations. Yang et al. [16] showed that rs3775291 polymorphism in *TLR3* confers resistance against geographic atrophy (GA). However, Cho et al. [17] did not report any association between *TLR3* polymorphism and AMD pathogenesis. Edwards et al. reported that in rs3775291 the minor allele frequencies differed from those reported by Yang et al. [18]. We examined the *TLR3* polymorphism and their association with AMD progression or occurrence of AMD in North Indian AMD patients who have unique dietary life style; we performed the single nucleotide polymorphism (SNP) analysis of *TLR3* gene (rs3775291) by using real time PCR.

Materials and methods

Subjects

The Ethical Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India has approved the study vide letter No Micro/10/1411. A signed informed consent of participation in research was obtained from all subjects. Study included 176 case–control samples consisted of 115 AMD patients and 61 healthy controls.

AMD diagnosis

The diagnosis of AMD pathogenesis was substantiated by fluorescein fundus angiography (FFA) and optical coherence tomography (OCT). A retina specialist also performed visual assessment by ophthalmologic examination like dilated fundus examination, visual acuity measurement, and slit lamp biomicroscopy of anterior segment.

Table 1 Demographic characteristics of controls and AMD patients

Variables	AMD	Controls
Total	115	61
Wet AMD	84 (47.7 %)	–
Dry AMD	31 (17.6 %)	–
Minimal classic	7 (11.9 %)	–
Predominant Classic	16 (27.1 %)	–
Occult	36 (61.0 %)	–
Sporadic cases	105 (91.3 %)	–
Familial cases	10 (8.7 %)	–
Duration of disease[a]	23 ± 2.6 (M)	–
Smokers	50 (43.5 %)	11 (20 %)
Non-Smokers	65 (56.5 %)	44 (80 %)
Alcoholic	37 (32.2 %)	17 (30.9 %)
Non-alcoholic	78 (67.8%)	38 (69.1 %)
Vegetarian	61 (53%)	31 (56.4 %)
Non-vegetarian	54 (47%)	24 (43.6 %)
Age	64.97 ± 7.1	60.38 ± 13.2
Male	75 (65.2 %)	40 (65.6 %)
Female	40 (34.8 %)	21 (34.4 %)

Clinical and demographic details of subjects. *AMD* age-related macular degeneration, *M* Months, *Age* Age of onset, Values are mean ± SD or (percentage)

[a] Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset

Demographic characterization

The demographic information of participants was obtained by administering the standard AMD questionnaire which included all aspects of life style and other co-morbidities. We have categorized the AMD patients into smoker and non-smokers in order to assess the effect of smoking in TLR3 polymorphism. The patients who consumed at least 3 cigarettes in a day were defined as smokers. The comorbidity was defined by existence of cardiovascular, metabolic, or hypertension disorders communicated by the physician. The demographic characteristics of all participants have been summarized in Table 1.

Inclusion and exclusion criteria

The exclusion and inclusion criteria of participant were based on age of patient, size, and number of drusen. FFA examination was conducted to characterize the advanced

form of AMD i.e., choroidal neovascularization and geographic atrophy. The inclusion criteria for AMD group included those with an age of 50 years or more with choroidal neovascularization and/or dry AMD with >5 drusen in at least one eye. The participants included in control group included those with age of 50 years or older with no drusen or less than 5 drusen without fulfillment of diagnostic criteria for AMD.

The exclusion criteria included the occurrence of degenerative changes in photoreceptors due to other ocular diseases like myopia, retinal dystrophies, vein occlusion, diabetic retinopathy, uveitis, or other diseases. The participants were not included below the age of 50 years. Moreover, participants were excluded who had limitation of papillary dilation or other problems which prevent the adequate fundus photography [19].

DNA extraction

5 ml blood sample was withdrawn from all the subjects who participated in the study. The blood sample was further kept at room temperature for 3–4 h. The supernatant was collected and pipetted on Histopaque-1077 in equal volumes (Sigma, USA) to purify PBMCs from whole blood. PBMCs were processed to extract genomic DNA by extraction kit (INVITROGEN and QIAGEN) as per the instructions. The extracted DNA was stored in −20 °C until SNP analysis.

Real time PCR

Real time PCR was performed using 48 wells Step OneTM real time PCR (Applied Biosystems Inc., Foster city, CA). The genotyping was done using TaqMan® SNP Genotyping Assays (Applied Biosystems) as per the instructions of manufacturer. Real time PCR was performed for 20.0 µl of volume containing 10 µl of master mix; 5 µl Assay (Applied Biosystems); and genomic DNA added at the concentration of 20 ng/µl. The final volume of 20 µl was made up with molecular biology grade water. The reaction was carried out by using two reporter dyes VIC and FAM. PCR mix without DNA was defined as negative control. The amplification product-based SNP analysis was carried out by using the Software StepOneTM v 2.0 (Applied Biosystems Inc., Foster city, CA). The fluorescence measurement was calculated by Rn value generated from PCR amplification.

Statistical analysis

The association between various groups was studied by Pearson's χ^2 test. Odds ratios (ORs) with 95 % CI and genotypic associations were estimated by binary logistic regression. The level of significance at $p \leq 0.05$ was considered to be significant. Statistical analysis was performed with statistical package and service solutions (SPSS; IBM SPSS Statistics 20.0, Chicago, Illinois, USA) 20.0 software.

Results

The description of genotype and alleles frequency has been summarized in Tables 2 and 3. The genotype and allele frequency of *TLR3* was not found to be significant in AMD. GG or AG was not frequent in AMD (OR value = 0.093, $p = 0.112$, CI = 0.005–0.1.73, OR = 0.163 and $p = 0.222$, CI = 0.009–2.99, respectively, Table 2).

The other demographic variants like food and smoking habits and co-morbidity were not found to be associated with AMD phenotype (Table 4). There was no significant frequency of allele G in *TLR3* which was found to be

Table 2 Effect of TLR3 rs3775291 variant on disease phenotype

Genotype	Number (frequency)		Unadjusted p value			Multivariate analysis, adjusted for age and gender		
			OR	95 % CI	*p* value	OR	95 % CI	*p* value
TLR3 rs3775291								
	AMD	Controls						
AA	6 (0.05)	0	Reference			Reference		
AG	33 (0.30)	27 (.44)	0.093	0.005–1.73	0.112	*	*	*
GG	73 (0.65)	34 (.56)	0.163	0.009–2.99	0.222	*	*	*
	Wet AMD	Dry AMD						
AA	5 (0.06)	1 (.03)	Reference			Reference		
AG	22 (0.27)	11 (.37)	0.400	0.041–3.855	0.428	5.343	0.242–11.79	0.289
GG	55 (0.67)	18 (.60)	0.611	0.067–5.58	0.662	0.311	0.022–4.465	0.390

* The Mantel-Haenszel common odds ratio estimate is asymptotically normally distributed under the common odds ratio of 1.000 assumption. So is the natural log of the estimate

Table 3 Allele frequency of TLR-3 in AMD and normal controls

Allele	Number (frequency)		OR	95 % CI	*p* value
TLR3 rs3775291					
	AMD	Controls			
A	45 (0.20)	27 (0.22)	Reference		
G	179 (0.80)	95 (0.78)	1.130	0.660–1.936	0.655
	Wet AMD	Dry AMD			
A	32 (0.20)	13 (0.22)	Reference		
G	132 (0.80)	47 (0.78)	1.141	0.552–2.357	0.721

Table 4 Logistic regression of the association of TLR3 and AMD stratified by smoking and comorbidity

Genotype	Number (frequency)		Unadjusted *p* value			Multivariate analysis, adjusted for age and gender		
			OR	95 % CI	*p* value	OR	95 % CI	*p* value
TLR3 rs3775291								
	Non-vegetarian AMD	Vegetarian AMD						
AA	1 (0.02)	5 (0.09)	Reference					
AG	20 (0.38)	13 (0.22)	7.692	0.804–73.55	0.077	6.924	0.800–59.90	0.08
GG	32 (0.60)	41 (0.69)	3.902	0.434–35.08	0.224	0.196	0.019–2.035	0.172
	Smokers AMD	Non-Smokers AMD						
AA	4 (0.82)	2 (0.03)	Reference					
AG	16 (0.33)	17 (0.27)	0.479	0.075–2.932	0.419	2.112	0.368–12.13	0.402
GG	29 (0.59)	44 (0.70)	0.3295	0.056–1.917	0.216	0.333	0.046–2.431	0.279
	AMD with comorbidity	AMD without comorbidity						
AA	5 (0.06)	1 (0.03)	Reference					
AG	22 (0.28)	11 (0.37)	0.400	0.041–3.855	0.428	2.800	0.219–35.81	0.429
GG	53 (0.66)	18 (0.60)	0.588	0.064–5.382	0.639	0.551	0.048–5.071	0.551

The value could not be complied because of the equal frequencies

significant in AMD patients (OR = 1.130, $p = 0.655$, CI = 0.660–1.936, Table 3). This difference was not significant in wet and dry AMD when genotypes and allele frequencies were analyzed (Tables 2 and 3).

We analyzed both age and gender as a risk factor variants in AMD progression. The multiple regression analysis was carried out after adjusting the age, gender, and the difference was not significant between AMD and control participants for both AG and GG alleles (Table 2).

Discussion

TLR3 transmembrane protein transduces the signal generated by double stranded RNA and has been found to be responsible for alteration of the risk of AMD pathogenesis [16, 20]. TLRs mainly participate in recognition of infectious agents and clearance of highly potential infectious self immunogenic molecules [21, 22]. Both endogenous (e.g., phagocytose nucleosome, sn ribonucleoproteins, and necrotic cells) and exogenous (ssRNA & dsRNA) RNA can stimulate TLR signaling.

Effect of rs3775291 polymorphism

The Leu412Phe (rs3775291) polymorphism is localized in the coding region which forms the *TLR3* receptor ectodomain necessary for ligand binding and dimerization of domain after activation of *TLR3* receptor [23]. The structure of *TLR3* has revealed that Leu412Phe is near the site of glycosylation (Asn413) and is necessary for receptor activation and ligand-binding surface for dsRNA [24, 25]. Binding of ligand with TLR3 may lead to changes in conformation and promote dimerization of *TLR3* receptor. *TLR3* receptor is required for signal transduction [26, 27]. Therefore, the activation of *TLR3* and *TLR7* is changed by polymorphism in these amino acids through altered ligand binding or dimerization which has shown in the Fig. 1 representing the AMD pathogenesis mediated by *TLR3* signaling. The *TLR3*-mediated signaling may be activated

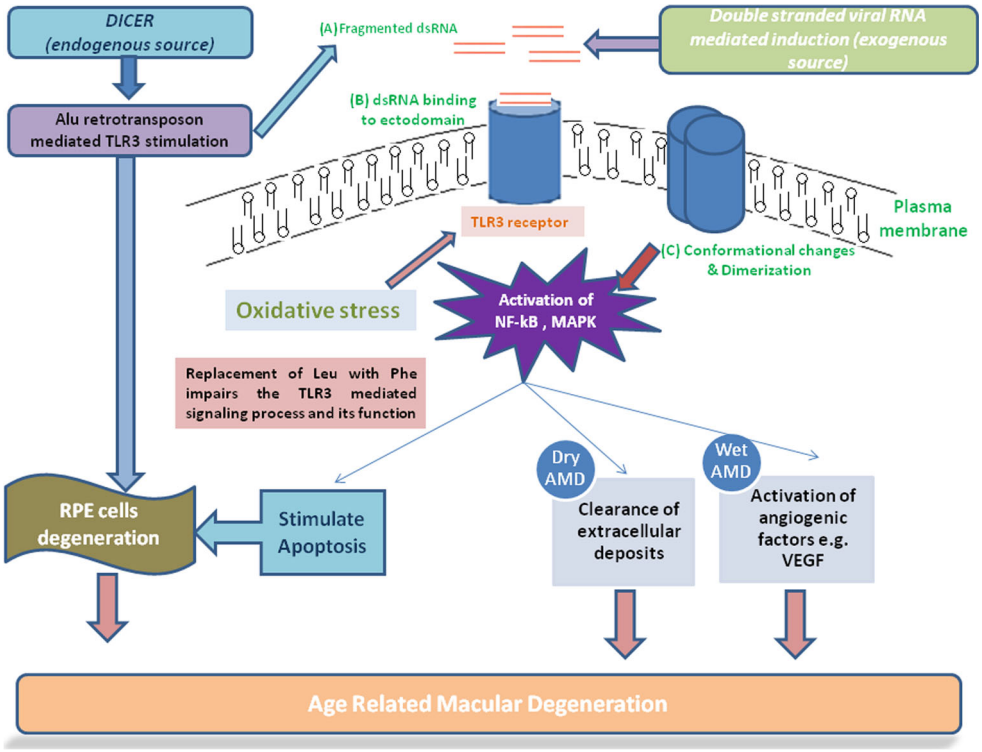

Fig. 1 Schematic representation of pathogenesis of AMD mediated by *TLR3* signaling process. **a** Fragmented dsRNA obtained from digestions of Alu retrotransposon and endogenous viral dsRNA genome. **b** Binding of fragmented dsRNA on ectodomain of *TLR3* rerceptor. **c** Conformational changes and dimerization of *TLR3* receptor induced by binding of dsRNA fragments

by pro-inflammatory molecules released by adjacent cells, by pathogen-associated molecules or by exogenous and endogenous sources of dsRNA. Many studies have shown the role of diet and smoking in AMD progression and found greater level of extracellular deposits (e.g., lipid peroxidation derivatives, autofluorescent byproducts of phototransduction, extracellular matrix, inflammatory proteins, and cellular debris) between Bruch's membrane (BM) and RPE cells [28]. These deposits accumulate and *TLR3*-mediated signaling enables clearance of these deposits by activation of macrophages.

Since the North Indian population catered to by this institute consists of non-smokers, we analyzed the *TLR3* polymorphism in AMD patients. Our findings did not show any significant *TLR3* polymorphism which suggests that the pathology of AMD is not influenced by *TLR3* signaling in North Indian AMD patients. We did not study the entire TLR3 signaling or the role of environmental stimuli but this could be the subject of future analysis. Therefore, at this time, this statement remains speculative in the absence of substantive data in its support. However, Yang et al. [16] have examined the effect of this polymorphism in American population of European descent. They genotyped the rs3775291 polymorphism for 441 patients with CNV, 232 patients for GA, 152 patients with soft confluent drusen, and 359 controls and found the significant association of "T" allele providing protection against GA with $p = 0.005$; OR for geographic atrophy in heterozygotes, 0.712; 95 % CI, 0.503–1.00; and OR for homozygotes, 0.437; 95 % CI 0.227– 0.839. The SNP was not found to be significantly associated with CNV and soft confluent drusen. These results revealed that this SNP is directly related to GA in AMD pathogenesis and may have protective effect by reducing the dsRNA-mediated RPE cells apoptosis. Meanwhile, Edwards et al. [18] have also reported the association of non-synonymous polymorphism rs3775291 for *TLR3* and rs179008 *TLR73* polymorphism with pathogenesis of AMD. They have found marginally associated alleles and genotype frequency for *TLR3*, *TLR4*, and *TLR7* and did not exclude the role of TLRs signaling in AMD pathogenesis. Apart from these studies, Cho et al. [17] have carried out *TLR3* polymorphism in two SNPs rs3775291 and rs4986790 by examining the case–control samples. They did not observe any statistically significant correlation in polymorphism and AMD pathology. There are other studies demonstrating the genotypic variation of complement components (*C2, C3, CFH*) and AMD susceptible genes (*LOC387715/ARMS2/HTRA1*) which have been found to be concurrent with the presence to GA but not with the progression of GA, on the contrary, genetic variation of *APOE* and *TLR3* did not show such relation with the presence of GA [29, 30].

Our finding is consistent with reports that do not suggest polymorphism of *TLR3* receptor to mediate signaling process in clearance of extracellular debris or apoptosis of RPE cells by *NF-kB*-mediated signaling [17, 20]. The

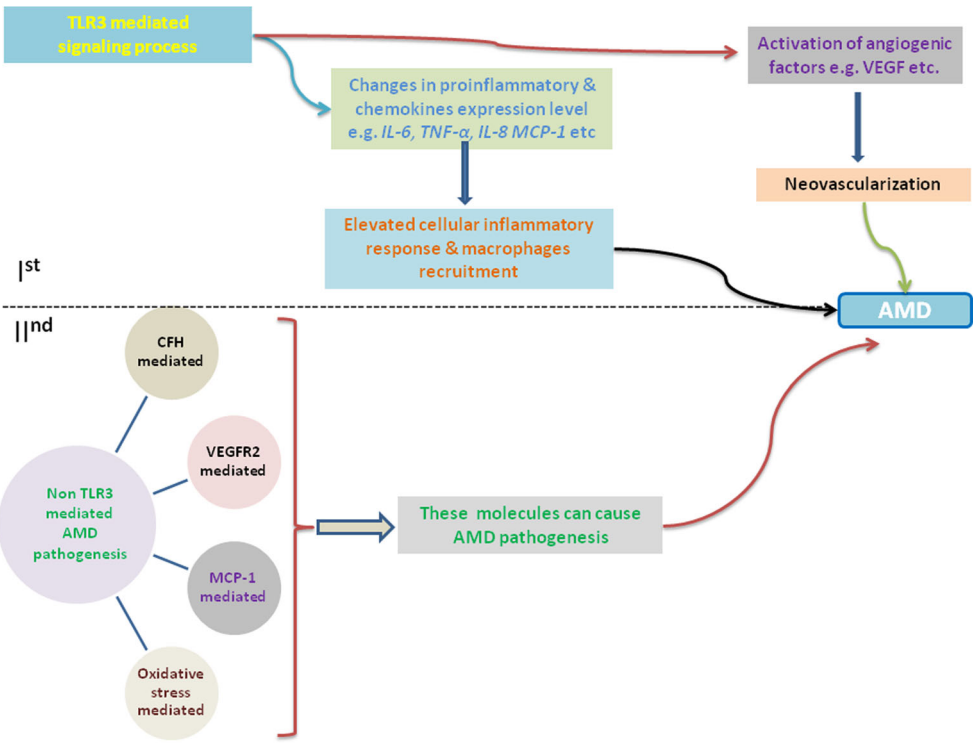

Fig. 2 AMD pathogenesis. Ist part showing the AMD pathogenesis mediated by *TLR3* by stimulating proinflammatory cytokines and angiogenic factors. IInd part conferring the role of other genetic factors can cause AMD pathology independent of *TLR3* mediated process

clearance of extracellular debris has been found to be mediated by recruiting macrophages at the site of deposition. Recently, Wornle et al. have reported the proinflammatory and chemokines response of *TLR3* signaling after stimulation with poly (I:C) RNA on human retinal pigment epithelial cells (ARPE-19) and found dose dependent increased expression of *TLR3* and RIG-I (retinoic acid inducing gene-I, cell receptor recognize viral ds RNA) concomitantly with increased expression of proinflammatory cytokines like *IL-6, TNF-α, IL-8, ICAM-1*, and *b-FGF*, but the expression of *VEGF* and *PEDG* was not found to be influenced [31]. As we correlate these findings with our previous work which revealed the elevated level of serum *MCP-1* in both wet and dry AMD patients [32], the absence of association with *TLR3* polymorphism suggests a dominant role of cellular inflammatory response and recruitment of macrophages independent of *TLR3* pathway. Klettner et al. [33] have also reported the increased expression of VEGF levels after being exposed to Poly I:C in a dose-dependent manner without involvement of *TLR3* signaling mechanism. Further studies should, therefore, examine the other gene loci in North Indian AMD phenotypes. Our previous studies strengthen the redundancy of *TLR3* [19, 32, 34, 35] (Fig. 2). The role of environmental factors has been implicated in AMD. Apart from age, several environmental factors such as body mass index (BMI), smoking, hypertension, alcohol consumption, sun light exposure, and diet habits have been found to be associated with AMD [36–38]. Environmental factors are known to introduce the epigenetic changes by regulating the protein or by altering the nucleotide sequence in the genome. It has also been documented that as the age progresses, the methylation is increased several folds in the genome [39]. The methylated promoter of receptor for advanced glycated end products (RAGE) may stimulate the formation of aggregates along with advanced glycated products which is the hallmark of age related diseases [40]. However, the precise mechanism of how environmental factors introduce changes in the gene remain unclear. Our analysis of environmental factors did not show any association with TLR3 polymorphism. Therefore, we propose that AMD pathology is predominantly influenced by genetic factors like *MCP*-1, *VEGFR2*, *CFH*, and oxidative stress and not by TLR3 as shown in our previous studies. However, additional studies with larger sample size can validate such studies.

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Conflict of interest The authors declare no conflict of interest.

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Review Article

ALS and Oxidative Stress: The Neurovascular Scenario

Akshay Anand, Keshav Thakur, and Pawan Kumar Gupta

Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012, India

Correspondence should be addressed to Akshay Anand; akshay1anand@rediffmail.com

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Oxidative stress and angiogenic factors have been placed as the prime focus of scientific investigations after an establishment of link between vascular endothelial growth factor promoter (*VEGF*), hypoxia, and amyotrophic lateral sclerosis (ALS) pathogenesis. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter and mutant superoxide dismutase 1 (*SOD*1) which are characterised by atrophy and muscle weakness resulted in phenotype resembling human ALS in mice. This results in lower motor neurodegeneration thus establishing an important link between motor neuron degeneration, vasculature, and angiogenic molecules. In this review, we have presented human, animal, and *in vitro* studies which suggest that molecules like *VEGF* have a therapeutic, diagnostic, and prognostic potential in ALS. Involvement of vascular growth factors and hypoxia response elements also highlights the converging role of oxidative stress and neurovascular network for understanding and treatment of various neurodegenerative disorders like ALS.

1. Introduction

At the developmental stages, the establishment of a neurovascular network, outside CNS, is crucial to the subsequent brain and spinal cord development. Molecules deserving special attention in the course of development and maintenance of neurovasculature include *VEGF* (especially *VEGF*-A)/*VEGF* receptors, Notch, ephrin, semaphorins/plexin receptors, latent transforming growth factor β's [*TGF*β's], and TGFβ receptors, $\alpha v\beta 8$ integrin, *neuropilins*, and FGF1 [1–3]. Any dysregulation in the pathways having the above mentioned factors (responsible for angiogenesis) which contributes to the development of this communication network has serious consequences manifesting in the form of CNS disorders. Hence angiogenesis is required for vasculature development and is governed by the gene expression of vascular molecules [4]. Abnormal expression and reduced levels of *VEGF* have been explored to account for devastating disorders of the CNS, especially in studies focused on ALS, which is designated by motor neuron degeneration and is fatal in nature [5]. Genetic studies in a transgenic mouse and rat model of ALS with mutated superoxide dismutase 1 $SOD1^{G93A}$ have indicated that inhibition of hypoxia response element (HRE) in the *VEGF* gene promoter may promote motor neuron degeneration (since HRE is responsible for inducing angiogenesis through *VEGF* as shown in Figure 1) whereas administration of *VEGF* prolongs survival [6]. Hence, here we review the role of neurotrophic and angiogenic factors like *VEGF* in the pathogenesis of ALS.

2. ALS: A Fatal Disease of the Motor Neurons

Motor neuron disease (MND) defines conglomerate of related and progressive degenerative disorders characterized by selective degeneration of upper motor and lower motor neuron located in the motor cortex and brain stem and spinal cord, respectively [4]. The disease may either affect lower motor neuron (progressive muscular atrophy) or upper motor neurons (primary lateral sclerosis) or both upper/lower motor neurons (amyotrophic lateral sclerosis); however, careful pathological and clinical studies in MND have shown that extra-motor parts of the central nervous system are also affected. ALS is the most severe MND where selective degeneration of motor neurons leads to atrophy of voluntary muscles followed by paralysis and may prove fatal [5]. Mechanisms of selective degeneration of motor neurons in ALS are obscure. Largely, ALS symptoms include weakness of muscles, especially those in the hands, arms, and legs with

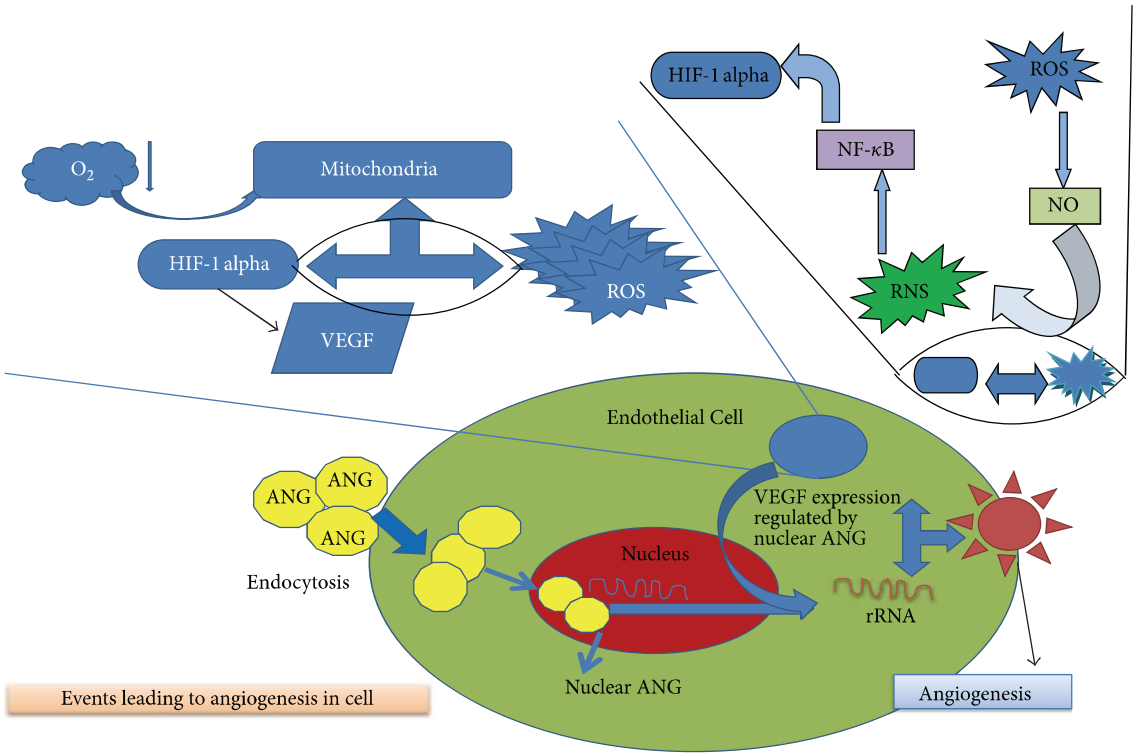

FIGURE 1: The role of hypoxia in stimulating the *VEGF* through an activation of HIF-1 alpha element. HIF-1 alpha gets activated in deficiency of oxygen in mitochondria leading to creation of oxidative stress. This involves the formation of reactive oxygen species which on reaction with free nitrogen forms NO ultimately leading to reactive nitrogen species (RNS). This RNS further activates NF-κB pathway which ultimately leads to activation of HIF-1 alpha factor. The activated form of HIF-1 alpha further leads to *VEGF* activation thus leading to angiogenesis.

or without dysarthria and dysphagia. Fasciculation or muscle twitching is also an important clinical finding [7].

3. ALS: Contributing Factors

ALS occurs in both sporadic and familial form at an incidence varying between 0.4 and 2.6 for every 100,000 individuals and a prevalence rate of 4–6 per 100,000 population per year [8]. The etiology of ALS has been elusive and believed to be multifactorial. Though causes of most cases of ALS are unknown, major factors include genetic factors like point mutations in superoxide dismutase 1 (SOD1) gene accounting for around 20% of familial ALS (fALS) cases [9]. The purely lower motor neuron (LMN) degeneration variant of ALS shows missense mutations in *CHMP2B* (charged multivesicular protein 2B; involved in cellular transport). In 10% cases of ALS, patients with *CHMP2B* mutations are shown to have lower motor neuron degeneration. Apart from this, other genes like vesicle-associated membrane protein B (*VAPB*) (which is involved in providing unfolded protein response to endoplasmic reticulum), senataxin (*SETX*) (gene present in central nervous system involving brain and spinal cord as well as muscle and play major role in DNA repair to maintain integrity of cell), and *dynactin 1* (involved in cellular transport during cell division and specially in axonal transport of nerve cells) with mutations have been shown to play role in aggregate formation and hampering the normal activity of the motor neurons thus contributing to the pathogenesis of ALS overall in subject's body [10–14]. Genes encoding angiogenin (*ANG*) having missense mutations have also been involved in the pathogenesis of ALS. Angiogenin, like *VEGF*, is produced in response to hypoxia and plays a role in neovascularisation as shown in Figure 1. Its importance further stems from the fact that it can regulate the expression of *VEGF* [15, 16]. Hypoxia takes place when oxygen availability is low in cell due to which the mitochondria produces ROS species which in turn reacts with nitric oxide (NO) to produce reactive nitrogen species RNS and activates HIF-α pathway through NF-κB pathway resulting in stimulation of *VEGF*. The expression of this *VEGF* is dependent on the nucleolar *ANG* which directly helps in stimulating the proliferation of epithelial cells and helps in angiogenesis [17]. However, this hypothesis raises a question whether angiogenin crosses the blood brain barrier or is retained in cerebrospinal fluid [18].

Apart from genetic factors, the presence of insoluble intracellular protein aggregates in motor neurons and reactive astrocytes are considered as the hallmarks for the disease (Figure 3). [19]. The other factors include glutamate toxicity [20], lack of trophic growth factors [6, 21], autoimmunity [22], toxin [23], and susceptibility of motor neurons to neurodegeneration because of their large size and high energy demands [24].

Currently, there is no treatment that could substantially alleviate the disease burden because of incomplete understanding of ALS etiology. Food and Drug Administration (FDA) has approved only single drug for the treatment of

ALS, a glutamate antagonist that is Riluzole [25, 26]. Riluzole has also been studied as a potential inhibitor of *VEGF* induced endothelial cell proliferation under both *in vitro* and *in vivo* conditions [27]. Its neuroprotective effect via sodium channel blockage is brought about by the fact that this mechanism increases resistance to hypoxia through a reduction in energy demands (a decreased cerebral glucose consumption) [28].

4. *VEGF*: The Neurotrophic and Angiogenic Family

*VEGF*A gene in humans is positioned at chromosome 6p21.3 with eight exons and is expressed as several isoforms of different amino acid chain lengths because of alternative splicing ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$) [29] that differ in their ability to bind heparin, *neuropilin*-1 (NP-1), and *neuropilin*-2 (NP-2). Two classes of receptors for *VEGF* are the tyrosine kinase and the nontyrosine kinase receptors. *VEGF*R1 (Flt-1 (fms-related tyrosine kinase 1)), *VEGF*R2 (KDR/Flk-1 (kinase insert domain receptor/fetal liver kinase-1), and *VEGF*R-3 (Flt-4) are three structurally related receptors present in tyrosine kinase class V, whereas *neuropilin*-1 (NP-1) and *neuropilin*-2 (NP-2) are part of nontyrosine kinase receptors. *VEGF* binds to NP 1 and 2 and *VEGF*R1 and 2 but not to *VEGF*R-3 as the latter one is not a receptor for *VEGF*. Studies indicate that for transmission of critical angiogenic signals in response to *VEGF VEGF*R2 plays the role of key mediator [30]. However in case of *VEGF*R1 the major function is prevention of *VEGF* binding to *VEGF*R1 thought to be done by a virtue of "decoy receptor" to negatively regulate angiogenesis [31]. *Neuropilins* (NP1 and 2) whose primary location is in central nervous system are described as receptor for collapsin/semaphorin family, which are responsible for controlling neuronal cell guidance [32, 33]. For *VEGF*165 and a coreceptor of *VEGF*R2 *Neuropilin-1* (NP-1), it is a specific receptor whereas *Neuropilin*-2 (NP-2) binds *VEGF*165 and *VEGF*145 in isoform specific manner. *VEGF* is the part of genes which accommodate placental growth factor (PLGF), *VEGF*B, *VEGF*C, *VEGF*D, and *VEGF*E including *VEGF*-A, out of which lymphatic vessels development is affected by *VEGF*-C [34]. Recent evidence from studies also indicates that neural cells are directly affected by *VEGF*-A, *VEGF*-B and *VEGF*-C [35]. In ALS, *VEGF* has been studied as an important member of gene families impacting the pathology of disease.

5. *VEGF*: Molecular Risk Factor in ALS

The lack of trophic (growth) factors has been hypothesized as probable cause of ALS. Since growth factors are neurotrophic and help in growth, survival, and maintenance of neuronal cells. The hypoxia response brings together a cascade of events involving angiogenic and inflammatory factors (Figures 1 and 3). Studies have focussed on predicting/correlating disease state with changing levels of such factors in body fluids even though these have been conducted utilising heterogeneous controls.

VEGF and its receptors are reported to be localised in neurons and astrocytes [36, 37] which, in case of ischemia or spinal cord injuries, provides neuroprotection and stimulates neuronal growth. Decreased *VEGF* levels may impair perfusion and induce ischemia of motor neurons, other than depriving cells of important survival and neuroprotective signals which are *VEGF* dependent [6].

Cronin et al. reported elevated levels of serum angiogenin, but no change in serum *VEGF* levels was observed. The authors also failed to observe any correlation between serum angiogenin and *VEGF* levels [16]. In another study, the patients with limb onset and long duration of ALS showed higher concentration of CSF *VEGF* as compared to those with bulbar onset of ALS and patients with short duration illness, respectively [38]. It may be possible that significant increase in cerebrospinal fluid (CSF) *VEGF* levels may have protective role against over-excitation of motor neurons (excitotoxicity). This overexitation may be mediated by excessive accumulation of glutamate at synaptic cleft in patients with limb onset of ALS and those with long duration of the disease, since it was suggested that the increased levels of *VEGF* account for a compensatory mechanism and may be required to stabilize neuronal excitation [39]. The rationale was further supported by Bogaert et al. who reported that *VEGF* protects motor neuron against excitotoxicity by upregulating Glutamate receptor 2 [40]. Significantly, lower baseline CSF *VEGF* levels in case of patients with ALS in comparison to normal controls and neurologic controls during early phase of disease have been observed, suggesting the possible link of ALS pathogenesis with *VEGF* gene regulation [41].

Moreau et al. demonstrated that hypoxaemic ALS patients had lower *VEGF* levels in CSF from normoxaemic ALS patients. This happened due to an early defect in hypoxia induced factor-1 (HIF-1) mediated regulation of *VEGF*. In contrast, higher levels of *VEGF* in CSF were demonstrated in hypoxaemic neurological controls than normoxaemic neurological controls. Hypoxaemia severity in ALS is explained by dysregulation of *VEGF* in ALS. This association of *VEGF* expression and hypoxia (Figure 1) in ALS introduced a concept of incongruous response [42]. Nagata et al. failed to reproduce the above results as no significant difference was observed in CSF *VEGF* levels between ALS patients, normal controls, and controls with other neurological disorders [43]. It was argued by Cronin and coworkers that the conflicting reports of elevated, normal, and decreased *VEGF* might have resulted from different study designs and ELISA kit employed with varying diagnostic criteria of ALS patients, diverse clinical details of ALS patients including definite and probable forms of disease [16]. In a unique histochemical study, a markedly elevated level of *VEGF* was detected in the skin of ALS patients when compared with normal subjects suggesting a positive correlation of *VEGF* levels in skin and severity of ALS patients [44]. The finding suggests systemic dysregulation of *VEGF* expression in ALS. Recently, it has been observed that elevated levels of *VEGF*A in CSF, serum, and peripheral blood mononuclear cells may account for substantially prolonged life span of Indian ALS patients as compared to their Western counterparts [45–47]. Surprisingly, longer survival is shown in Indian ALS patients after

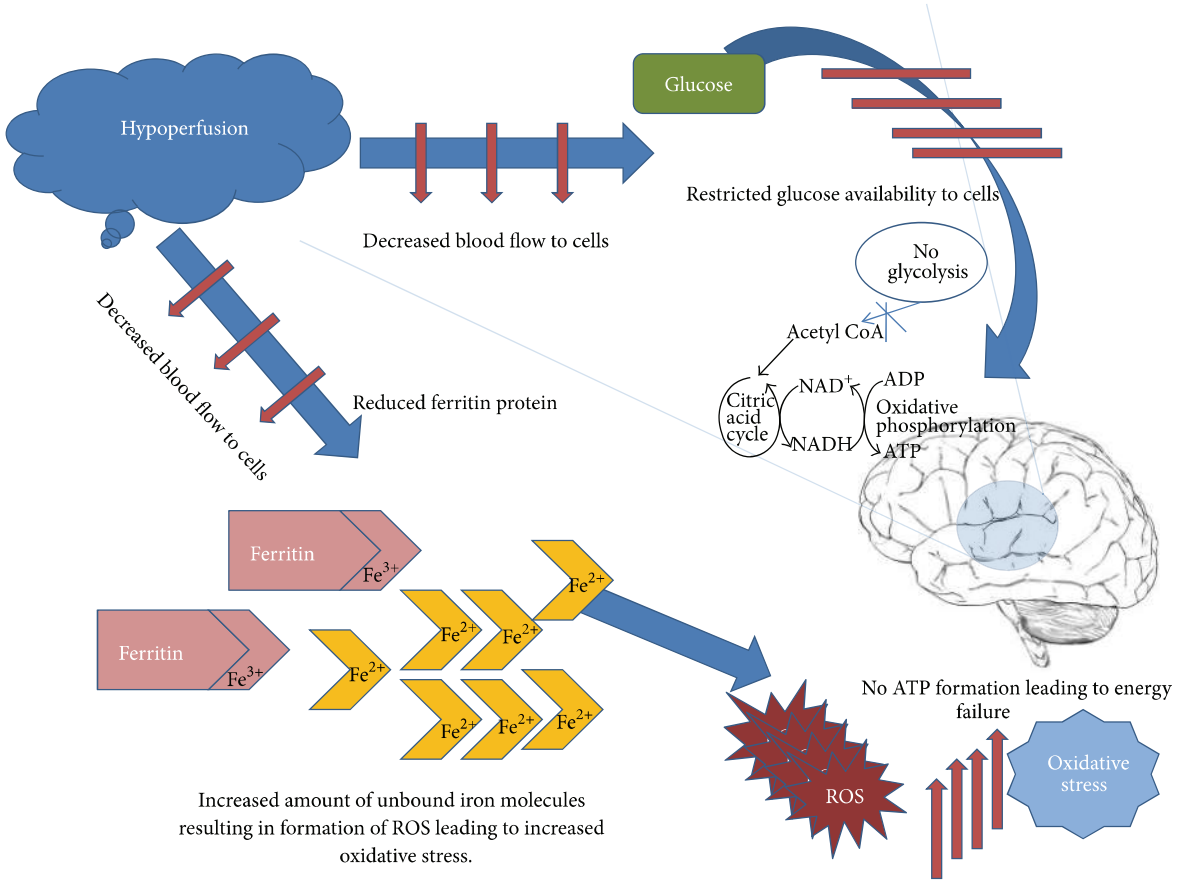

FIGURE 2: Role of hypoperfusion in elevation of oxidative stress and energy failure. As hypoperfusion reduces blood flow towards cells resulting in reduced ferritin Fe^{3+} protein, it releases unbound iron Fe^{2+} molecules resulting in formation of ROS thus increasing the oxidative stress. Hypoperfusion also leads to unavailability of glucose to brain cells thus leading to energy failure.

onset (~9 year) of ALS [45, 46, 48, 49]. Further, reduced levels of soluble *VEGF*R1 (s*VEGF*R1), an inhibitory receptor of *VEGF*, have been observed in these patients, supporting the neurotropic nature of *VEGF* [50]. However, these results need confirmation in comparable Caucasian ALS population.

6. ALS: *VEGF* and Oxidative Stress

Lowering of *VEGF* levels places neural tissue at the risk of limited perfusion thus making way for motor neuron degeneration [51]. This degeneration is a direct consequence of the fact that the deficient oxygen and glucose levels created as a result of decreased vascular perfusion can hardly meet the energy demands of motor neurons [52]. Oxidative stress due to hypoperfusion has been reported in cases of other neurodegenerative disorders such as Alzheimer's disease [53]. Oxidative stress is one of the outcomes of hypoperfusion apart from energy failure as blood is known to carry several vital components essential for cell survival including glucose and ferritin. As glucose is able to readily cross blood brain barrier (BBB), the deficiency of blood flow leads to reduced supply of glucose to brain resulting in limited energy production for cells. Similarly, the deficiency of ferritin, which is responsible for binding of free iron, results in formation of reactive oxygen species as shown in Figure 2 [54]. At least one study has reported that the variable levels of *VEGF* lead to altered ferritin levels [55]. Therefore, it is safe to say that oxidative stress deserves special significance in the pathogenesis of neurodegenerative diseases like ALS since motor neurons are particularly susceptible to oxidative damage.

This significance is born out of the fact that the first evidence of association between ALS pathology and *VEGF* came when Oosthuyse et al. created homozygous *VEGF* ($VEGF^{\delta/\delta}$) knock-in mice by introducing homozygous mutation of hypoxia response element (HRE) in the *VEGF* gene promoter to study angiogenic property of *VEGF*. They observed that almost 60% of mice did not survive before or around birth due to vasculature aberrations in lungs. The 40% who survived began to develop symptoms like classical ALS around five months of age [6]. This unusual finding compelled researchers to explore significance of growth factors in pathology of ALS utilising a variety of tools such as those discussed below.

6.1. Autopsy Based Studies. Spinal cord tissue analysis of ALS patients has revealed elevated dendritic cell marker transcripts (like CD83) and monocytic/macrophage/microglial transcripts [56], expression of cyclooxygenase-2 (*COX-2*)

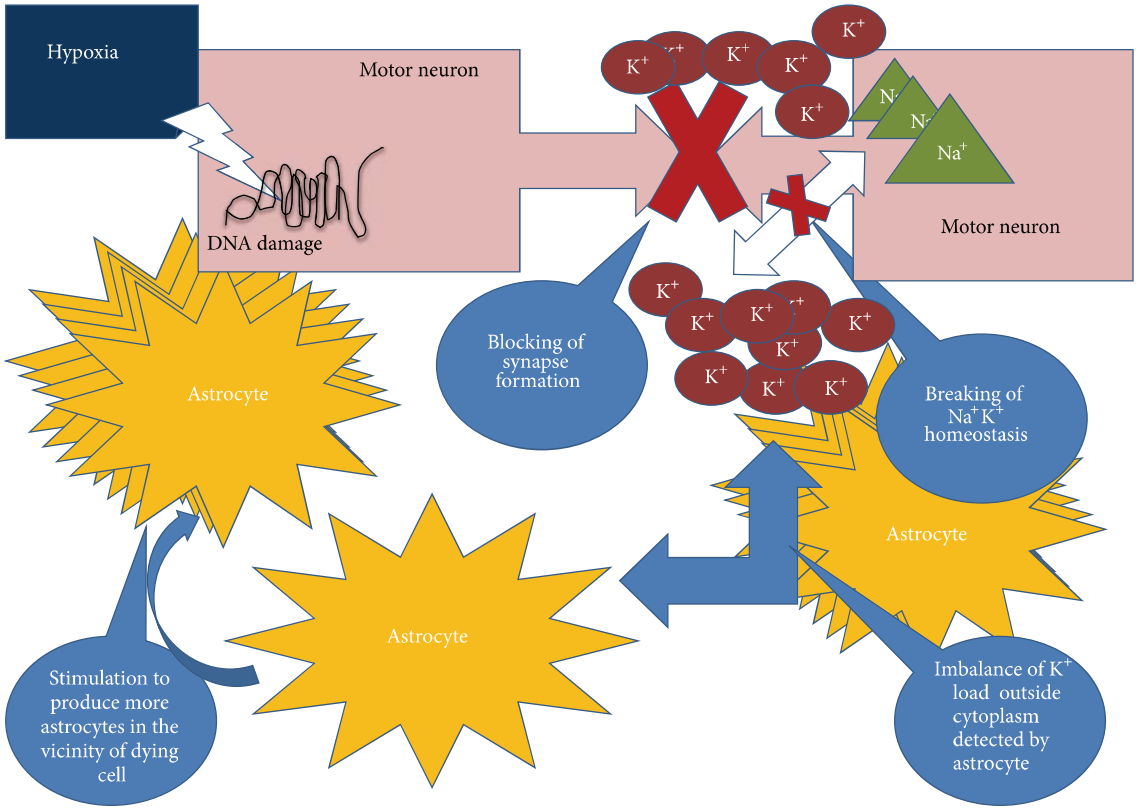

FIGURE 3: Hypoxia induced mobilisation of astrocytes. Astrogliosis is the result of aggressive increase of astrocytes number in the vicinity of damaged neuron cell. Synapse formation is hampered when there is neuronal damage thus leading to breakdown of Na^+K^+ homeostasis. This K^+ concentration is detected by the astrocytes.

[57], connective tissue growth factor (CTGF) [58], monocyte chemoattractant protein-1 (*MCP1*) [56] and *VEGF* receptor (*VEGF*R)-1 [59], and activity of glutamate dehydrogenase (*GDH*) accompanied by reduced levels of glutamate and aspartate [60].

The increase in CTGF expression is explained by the fact that CTGF plays an important role in astrogliosis which is often seen as a consequence of hypoxic conditions and is therefore a pathological hallmark of ALS [58]. As depicted in Figure 3 astrogliosis is the result of aggressive increase of astrocytes number in the vicinity of damaged neuron cell. Hypoxia generally induces damage in the DNA of the neuronal cells. Since the neuronal damage has taken place its normal activity of synapse formation is hampered affecting the Na^+K^+ activity in those cells leading to breakdown of Na^+K^+ homeostasis. This change in balance of K^+ concentration is detected by the astrocytes. This alteration results in the activation of astrocytes by initiation of clustering around the damaged cells in order to restore the functioning of those damaged cells [61–63].

Gliosis is also related to the enhanced GDH activity as reported by Malessa et al. [60]. The function of the GDH is to enhance the availability of the glutamate. This glutamate further acts as neurotransmitter or gliotransmitters since it increases the availability of Ca^+ required by glial cells to perform their normal function of providing protection, nutrition, and avoiding accumulation of any chemicals involved in synapse formation which may later lead to toxication of neuron cell. Recruitment of glial cells to the site of damage may be considered as the body's primary response to save the dying neurons [64, 65], and thus the fact may be related to the point of association of enhanced GDH activity to gliosis. The authors also suggested a disturbance in cholinergic transmission in ALS spinal cord thus contributing to the reduced amino acid levels [60]. Glutamate and aspartate amino acids are linked with the neurotransmitters in the body. They are mainly the excitatory neurotransmitters, which utilise the Na^+K^+ pump to maintain their flow to the postsynaptic cleft during the nerve transmission. Li and Zhuo demonstrated that cholinergic transmitters play a role in inhibiting the glutamate based transmission. Release of acetylcholine leads to the activation of the muscarinic receptors, resulting in an inhibition of AMPA receptors (also called as glutamate receptors), and it increases the nonavailability of glutamate. This evidence also supports the fact mentioned in the above study that disturbance in cholinergic transmission may lead to reduced amino acid levels [66].

VEGF was first measured in spinal cord and serum of ALS patients by Nygren and colleagues. Authors did not observe any significant alteration in spinal cord *VEGF* levels, but they were able to observe higher serum *VEGF* levels in ALS patients in comparison to controls similar to those later reported by Gupta et al. in case of Indian ALS patients [46]. Considering the higher levels of *VEGF* in serum suggests that

the cells other than central nervous system or which are not part of CNS are involved. In case of ALS skeletal muscles are the most affected region of body. Regional ischemia, a condition in which the blood supply is halted in specific region of brain, has been reported in case of ALS [67]. Rissanen et al. observed higher levels of *VEGF* in skeletal muscles with acute phase of ischemia [68]. Thus, it was hypothesized that *VEGF* is expressed in skeletal muscles in response to hypoxia and the increase was also reflected in serum [69].

The autopsy samples depict the terminal stage of the disease and provide a reliable proof of the disease and its signatures [70].

6.2. Muscle Biopsy Based Studies. In contrast to the increased cyclooxygenase (*COX*) activity in spinal cord of ALS patients, as discussed above, Crugnola et al. reported *COX* deficiencies in 46% patients, based on their histochemical analysis of muscle specimens. Moreover, molecular studies and biochemical analysis on the selected specimens displaying severe *COX* deficiencies even correlated with mutations in *SOD1* and *TARDBP* genes and mitochondrial DNA defects thus pointing towards the secondary nature of *COX* deficiencies in the pathogenesis of ALS in light of the genetic nature of defects [71]. This is also confirmed by the findings of Vielhaber et al. who observed mitochondrial DNA damage in skeletal muscle, along with lowered levels of mitochondrial Mn-SOD [72]. The specific nature of mitochondrial dysfunction is further revealed by studying mitochondrial markers like citrate synthase and succinate dehydrogenase in muscle, histochemically. However, such a study by Krasnianski et al. revealed that one cannot narrow down the observed mitochondrial changes to only depict ALS but in fact view them as an indication of other neurogenic atrophies too [73]. In view of neurotrophic support provided by muscle tissue, the findings by Küst et al. depicted enhanced expression of nerve growth factor (NGF) and neurotrophins such as brain-derived neurotrophic factor (BDNF), in postmortem bicep tissue of ALS patients. Even so, externally administered neurotrophins have not shown promising results in human trials or animal models of ALS [74].

6.3. Polymorphism Based Studies. Increased oxidative stress implies consequent increased oxidative damage for motor neuronal DNA. Such oxidative damage of DNA is driven by the base excision repair (BER) system. One such product of oxidative damage of DNA is 8-hydroxy-2′-deoxyguanosine (8-OHdG) which is regulated by two enzymes, namely, human 8-oxoguanine DNA glycosylase 1 (*hOGG1*) and apurinic/apyrimidinic endonuclease APE1. Consequently, mutations and polymorphisms in coding area of genes coding for both of these enzymes are of interest to researchers. Concurrent oxidative stress conditions and a faulty DNA repair system are a risk factor for motor neurons.

In most studies concerning *hOGG1* Ser326Cys polymorphism levels of 8-OHdG are taken into account as 8-OHdG is the product of DNA oxidation [75]. A study conducted by Chen et al. showed the reduced activity of *hOGG1* in patients with 326 CC polymorphisms ($P = 0.02$) as compared to those with 326 SC polymorphisms ($P = 0.05$) [76]. Similar observations were made by authors in current study. In a Caucasian study, Coppedè et al. studied the distribution of allele frequencies and genotypes in sALS patients and controls for the *hOGG1* Ser326Cys polymorphism in sALS patients and controls. The authors reported a significantly increased sALS risk associated with a combined Ser326Cys + Cys326Cys genotype. However, the Ser326Cys genotype showed nonsignificant results predicting that the *hOGG1* Ser326Cys polymorphism in patient also pose a risk factor for ALS. Ser326Cys polymorphism takes place when at exon 7, position 1245 C to G substitution occurs and as a result S is substituted to C in codon 326.

Another interesting observation (though not significant as the test group of subjects used for the study was small, more significant results can be obtained if the study with large number of patients is conducted) in the above study was the fact that sALS patients as opposed to those bearing one or two copies of the 326Cys mutant allele bearing the Ser326Ser genotype displayed lower levels of AOPP (advanced oxidation protein products; believed to be stable markers of oxidative damage to proteins) [77]. Since abnormal levels of *VEGF* are implicated as risk factor in ALS, it is evident that mice with hypoxia response element deletion in vascular endothelial growth factor gene develop features reminiscent of ALS [5] although no spontaneous mutations have been observed in HRE in ALS patients [78, 79]. Large family-based and case-control cohort of North American white subjects ($n = 1{,}603$) were studied for the association of sALS with promoter polymorphisms of three *VEGF* genes. *VEGF* promoter polymorphisms do not find their casual role in ALS in light of absence of their association with sALS [80]. Risk of developing ALS has been associated to *VEGF* due to alterations in sequence in the promoter region of gene. In The Netherlands, 373 patients with sporadic ALS along with 615 matched healthy controls were found to have *VEGF* promoter haplotypes. No significant association between the previously reported at-risk haplotypes and ALS was found [81]. However, in some studies ALS has been found to be associated with *VEGF* C2578A polymorphism. In a study of Chinese population by Zhang et al. 115 sALS patients with 200 healthy individuals were analyzed for C2578A polymorphism (by amplifying 2705 to 2494 bps of *VEGF* gene promoter). Reports were in disagreement to previous studies from Caucasian populations as Chinese population did not fall susceptible for ALS due to C2578A polymorphism (attributing the effect to different genetic background in Chinese population) [82]. No significant association of ALS with three common *VEGF* variations [-2578C/A, -1154G/A, and -634G/C] in original form or in haplotype combination in a recent meta-analysis study comprising of over 7000 individuals involving three North American population and eight European populations was reported. However, in males -2578AA genotype increased the risk of ALS in subgroup analyses by gender [83] in contrast to a German study which suggested that risk of ALS in case of female patients might be higher as the *VEGF* role might be gender dependent [84]. Oates and Pamphlett did not observe any alteration of functioning of motor neurons by epigenetic transcriptional silencing of *VEGF* gene by methylation [85]. Additionally,

screening of regulatory sequences of *VEGF*R2 found no association of polymorphism of *VEGF*R2 gene with risk of ALS [86]. Although association of *VEGF* with ALS has been well established by culture and animal studies, evidence from genetic studies in human cohorts suggests only a minor association between *VEGF* and the risk of developing ALS.

The role of *VEGF* involvement in ALS is questioned due to lack of association of *VEGF* genotypes and haplotypes in large meta-analysis study. Possibilities for *VEGF* role in predisposed patients to ALS cannot be ruled out. More studies are needed to discern the actual role of *VEGF* in pathogenesis of ALS.

6.4. Animal Model Based Studies

6.4.1. Primates. The concept of utilizing the cytotoxic properties of the extract obtained from the spinal cord of ALS patients was applied by Zil'ber et al. as early as in 1963 so as to reproduce the disease in rhesus monkeys. The authors could only conclude to a viral nature of this disease, but at the same time they recognised that the high incidence previously reported in the Chamorro tribe of Guam suggested a unique basis [87]. Another study conducted on rhesus monkeys attempted to validate the efficacy of bovine *SOD* as a therapeutic agent to compensate for the functions of the mutated form of the enzyme. SOD being a locally acting enzyme was administered intrathecally and intraventricularly so as to bypass the blood brain barrier. The injected bSOD showed commendable tolerance though its clearance was slower when compared with results obtained from rats. But the therapy when administered into a late stage FALS patient did not show promising results [88].

6.4.2. Rodents. The neuroprotective effect of *VEGF* suggests that exogenous *VEGF* administration may prevent degeneration of motor neuron. In a $SOD1^{Gly93Ala}$ rat model of ALS, it was shown that onset of paralysis was delayed by 17 days, improved motor performance, and extended lifespan by 22 days due to intracerebroventricular (i.c.v.) delivery of recombinant (*VEGF*). The study demonstrated the high scale effect in animal models of ALS achieved by protein delivery [89]. Intrathecal transplantation of human neural stem cells overexpressing VEGF increased the duration of survival of a transgenic ALS mouse model [90]. Similarly, mice after spinal cord ischemia showed susceptibility to paralysis in nervous tissue with reduced *VEGF*-A expression levels whereas after treatment with *VEGF*-A showed protective effect against ischemic motor neuron death [91]. These results unveil a therapeutic potential of *VEGF* for degenerating motor neurons in case of human ALS. In similar study authors investigating the protective role of *VEGF* during ischemia has shown to reduce infarct size, improve neurological performance, and enhance the survival of newborn neurons in the dentate gyrus and subventricular zone in adult rat brain with focal cerebral ischemia. Thus, *VEGF* shows acute neuroprotective effect, and prolongs survival of new neurons in the ischemic brain [92].

Zheng et al. demonstrated for the first time in Cu/Zn *SOD1* transgenic mouse model of ALS that *VEGF* delayed diseased symptoms progression and prolonged survival, suggesting the importance of *VEGF* or related compounds in the treatment of ALS patients [93]. Rats having *VEGF* treatment showed significantly improved performance up to 6 weeks after spinal cord contusion injury compared with control animals. Furthermore, the group showed that *VEGF* treated animals had increased amount of spared tissue in the lesion centre with higher blood vessel density in parts of the wound area compared to controls, proving neurogenic and angiogenic capacity of *VEGF* [94]. Enhanced expression of *VEGF* by intramuscular administration of zinc finger transcription factor in *SOD1* rats has been shown to improve functional disability [95]. Nitric oxide is known to decrease pressure in blood vessels [96], and it is possible that low *VEGF* adversely affects vasculature via changing the amount of nitric oxide released from endothelial cells, which further impairs perfusion and causes ischemic damage of motor neurons [91]. Moreover, decreased flow of blood has been observed in patients with ALS [97]. Both mechanisms may contribute to adult-onset progressive degeneration of motor neurons, muscle weakness, paralysis, and death, a typical feature of amyotrophic lateral sclerosis. It was earlier demonstrated that exposure to low levels of lead prolongs survival of ALS transgenic mouse, possibly mediated by upregulation of *VEGF*, which in turn reduces astrocytosis [98]. In another case retrograde delivery of lentivirus into mouse model of ALS prolonged survival in animals. Authors reported that lentivirus helped in stimulation of *VEGF* levels during diseased condition in animals [99]. Although in ALS animal models *VEGF* delivery has been successful, dose of delivery of *VEGF* should be adequately optimized to prevent adverse effects on the vascular system. It is possible that levels of *VEGF* higher than a certain threshold value may increase leakiness of blood vessels and modulate permeability of blood brain barrier [100] and therefore result in intrathecal accumulation of fluid. The presence of the blood breakdown product hemosiderin in and around spinal cord motor neurons supports increased leakiness and malformed blood vessels in ALS mouse models [101].

It must be noted that a drawback with using *SOD1* based transgenic models is that *SOD1* gene mutations represent only 20% of cases of familial ALS, which themselves represent just 10% of the total ALS cases. Therefore, remaining 90% of ALS cases, sporadic in nature, are difficult to mimic using such animal models [70].

6.5. Cell Culture Based Studies. Owing to a translational gap from animal models of ALS to humans, *in vitro* investigations utilising human motor neurons and astrocytes purified from the human embryonic spinal cord anterior horns allow for greater manipulations and are therefore a critical tool in discerning mechanisms pertaining to motor neuron degeneration in ALS [102].

The mRNA level of *VEGF* has been an important marker to analyse the role of *VEGF* in ALS. Destabilization and downregulation of *VEGF* mRNA with concomitant loss of protein expression in glial cells expressing mutant *SOD1* *in vitro* are in consensus with many reports on the role of reduced *VEGF* expression in ALS pathogenesis [103]. In

contrast, it was reported that hypoxia induced proteins bind and stabilize VEGF mRNA transcript resulting in increased expression of VEGF as a compensatory protective mechanism in later stages of disease [104].

The potential role of *VEGF* in preventing cell death by SOD-1 mutation has been studied in NSC-34 motor neuron cell line from mouse. Infection by adenovirus containing mutant Gly93Ala-*SOD1* was shown to increase cell death and cellular oxidative stress. However, *VEGF* showed a dose dependent resistance to oxidative damage from hydrogen peroxide, TNF-alpha, and mutant Gly93Ala-*SOD1* in NSC-34 cells treated with *VEGF*. Both phosphoinositide-3-kinase (PI3-K) and mitogen activated protein kinase (MAPK) activities in mouse NSC-34 motor neuron-like cells were activated by *VEGF* [105]. Recently, a culture study using primary culture of *SOD1* mutated rat motor neurons has shown that decrease in *VEGF* before or during motor neuron degeneration amplifies the risk of mutated *SOD1* induced toxicity in motor neurons [106]. Thus, the *in vitro* study shows *VEGF* as an antiapoptotic molecule. Overexpression of *VEGF* in the hippocampus using recombinant adeno associated virus vector in adult rats has been reported to result in improved cognition in association with approximately 2-fold increase in neurogenesis. Moreover, environmental induction of neurogenesis is completely blocked RNA interference based inhibition of *VEGF* expression. This data supports a model whereby *VEGF* acting via kinase insert domain receptor (KDR) is a mediator of the effect of the environment on neurogenesis and cognition [107]. Meng et al. investigated *in vitro* the proliferation and differentiation of subventricular zone neural progenitors of adult mouse by virtue of direct effect of *VEGF*. Downregulation of endogenous *VEGF* receptors 1 and 2, in association with reduced neural progenitor cell proliferation and enhanced neuronal differentiation, was reported as a result of high dose (500 ng/mL) of *VEGF*, whereas endogenous *VEGF* receptors 1 and 2 were significantly upregulated without increased proliferation and differentiation at low dose (50 ng/mL) of *VEGF*. Above given experiments suggest that *VEGF* regulates neurogenesis and its high dose enhances adult neural progenitor cell differentiation into neurons showing exogenous *VEGF* to exert a biphasic effect on the expression of endogenous *VEGF* receptors [108]. It has been shown that *VEGF* induces differentiation of stem cells in endothelial cells which in turn secrete various neurotrophic factors and infers a novel mechanism of neuroprotection by *VEGF* [109]. Apart from *VEGF*, recently, *VEGF*B was shown to protect cultured primary motor neurons. Further, it was observed that mutated SOD1 ALS mouse without *VEGF*B gene developed more severe form of ALS than ALS mouse with *VEGF*B [110].

7. *VEGF* in Blood Brain Barrier (BBB) and Blood Spinal Cord Barrier (BSCB)

Blood brain barrier (BBB) is the only checkpoint that stops inflammatory agents to reach central nervous system (CNS), as it contains a balanced interaction of microvascular endothelial cells and other components such as astrocytes, pericytes, neurons, and basement membrane. These components are collectively called as neovascular unit NVU. Tight junctions among NVU make the entry of undesirable components restricted to CNS [111]. BBB breakdown may lead to disruption of various biochemical reactions or may lead to accumulation of various inflammatory proteins that may aggravate the disease conditions of CNS [112–114]. Similarly, blood brain and spinal cord barrier which can be a morphological isotype for BBB performs same function in separating the spinal cord from all harmful components that may lead to diseased conditions of nervous system [115]. It has been observed that in case of human and animal model studies both the infiltration of brain and spinal cord with T cell, dendritic cells, or IgG have resulted in degeneration of motor neurons [116]. Claudins play a major role in forming tight junctions in the body among the cells to function as a barrier or act as a filter for these inflammatory factors to enter CNS [117]. Earlier studies have shown that astrocytes produce certain chemokines which play a role in attracting the dendritic cells to the CNS [118]. Recently, a link between the reactive astrocytes and disruption of these barriers has been reported. Argaw et al. tried to examine a link between astrocyte derived *VEGF*A and BBB permeability. Astrocytic expression of HIF-alpha and *VEGF*A leads to downregulation of claudins CLN-5 and their regulatory protein OCLN [119]. *VEGF*A, by the virtue of tyrosine phosphorylation, downregulates the expression of CLN ultimately resulting in disruption of permeability barrier. *VEGF* induces the migration among the endothelial cells and increasing the permeability to CNS [120] (Figure 4). However, theis link of *VEGF* is conflicting with the earlier reports in this paper regarding the protective role of *VEGF* in ALS pathogenesis.

8. Natural Products and Regulation of *VEGF* Expression

Naturally occurring compounds are also in current focus to examine their role in *VEGF* expression. The mechanism has been postulated to be common in all cases for those which are known to be responsible for increase in the expression of *VEGF*. All of them have been shown to affect the HIF pathway inducing the expression of *VEGF*. It is not clear how these natural compounds can be successfully translated for clinical use in near future which will need more studies. Certain extracts like *turmeric*, *gigko biloba*, and *ginseng* have been shown in mice studies to delay the disease onset or prolong survival in mice studies. However, recently a group from China reported a component *Baicalin* in the roots of plant *Scutellaria baicalensis* which enhances the expression of *VEGF* [121]. Although the HIF expression was less as compared to *VEGF*, authors reported that other transcription factors such as oestrogen-related receptors (EERs) exert their effects via *VEGF* promoters. Peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*), an important molecule independent of activator, is shown to interact with *ERRα* [122, 123]. These *PGC-1α* are shown to enhance expression of *VEGF* in cultured muscle cells *in vivo* in HIF independent pathway [121, 124]. In contrast, grape seed extract (GSE)

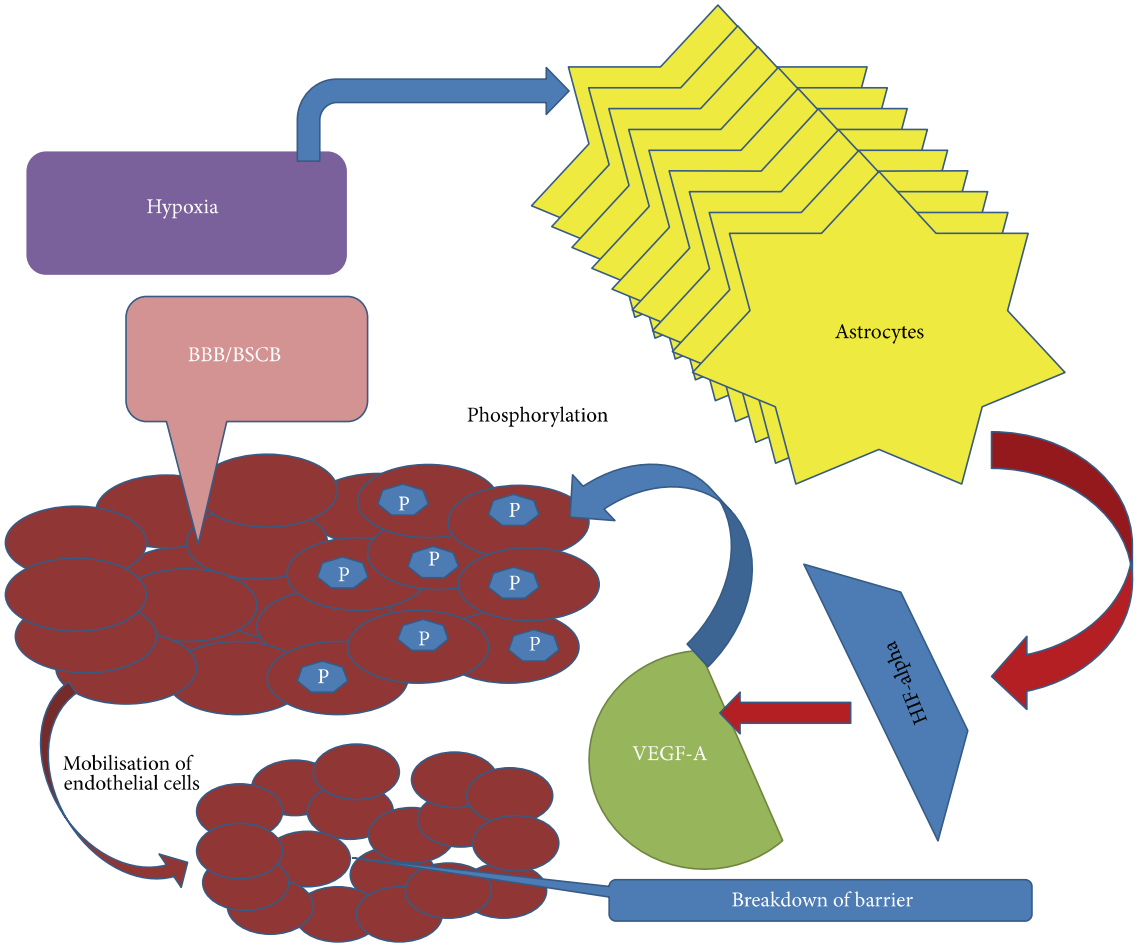

Figure 4: *VEGF* and permeability of blood brain barrier. Astrocytic expression of HIF-alpha and *VEGFA* leads to downregulation of claudins CLN-5 and their regulatory protein OCLN. *VEGFA*, by the virtue of tyrosine phosphorylation, downregulates the expression of CLN ultimately resulting in disruption of permeability barrier. *VEGF* induces the migration among the endothelial cells and increases the permeability to CNS.

is known for its antitumor properties and is shown useful in case of breast, lung, skin, or gastrointestinal cancer [125–127]. Lu and group recently showed that GSE reduced the *VEGF* expression by inhibiting the HIF expression in human breast tissue cancer cells. Authors argued that it involved the blockade of HIF expression by inhibiting AKT-3 pathway normally known for supporting the cell survival [128]. Apart from these other natural components have been shown to provide nonsatisfactory results in certain trials conducted in different human population. Vitamin E the most commonly studied antioxidant has been implicated with role of slowing down the disease progression in its severe form. Desnuelle C and colleagues conducted a study in French population of ALS patients. 289 patients were recruited for the study. All of them were randomly assigned the dose of Vitamin E and were assessed after every 3 months. The results did not show the effect in survival of muscle cells, except for the fact that patients who were administered the Vitamin E stayed in the milder form of disease for longer time [129]. Another study done in German population with high dose of Vitamin-E (5000 mg/day) showed ineffective results in comparison to the placebo effect [130]. However, in one of the meta-analyses of 23 studies published in year 2008, it was stated that antioxidants whether in combination or during individual administration do not show effective results [131]. Creatine one of the sports supplement has been known to increase the muscle strength. One study that came up in 1999 was conducted in animal model of ALS. Transgenic mice of ALS was administered with creatine dose. Authors reported that creatine was helpful in saving the mice neurons from dying in the age of 120 days. The group reported that creatine was also helpful in saving the mice from oxidative stress as well [132]. Later in 2004 a translational study performed with the same idea in human subjects demonstrated totally opposite results. 175 probable laboratory supported ALS patients were administered the 10 gm dose of creatine daily. The study showed no effect on survival rate neither it helped in reviving the rate of functional activities in patients [133]. Cannabinoid another naturally produced chemical present in humans as well as animals was studied by a group in 2004 in ALS mice

model. They reported that Cannabinoid helped in prolonging survival of animals. Authors also reported reduced oxidative damage in spinal cord cell cultures of ALS mice and showed that it acts as an antiexcitotoxic agent *in vitro* [134]. Similar results have been shown in case of synthetically produced chemical called as cannabinol with dosage of 5 mg/kg/day for over a period of 12 weeks although no effect was there on survival [135]. Details for the functioning of these agents have not been mentioned but all of them report to choose or affect the oxidative stress pathway, although the oxidative stress based stimulation pathway by these compounds for *VEGF* cannot be ignored. Several studies report a common path for *VEGF* enhanced expression, but validity of usefulness of these natural components in case of ALS still needed to be studied.

9. Concluding Remarks

The human, animal, and culture studies have shown that *VEGF* could be a promising therapeutic target in ALS. Upregulation of *VEGF* by different means such as genetic engineering, transplantation of stem cells overexpressing *VEGF*, and/or direct infusion of *VEGF* may rescue the damage of motor neurons and enhance the survival of patients with ALS either by increasing blood perfusion or direct neuroprotective effect on motor neurons. However, additional blinded preclinical studies of *VEGF*, particularly among primates, are still needed in ALS and other neurodegenerative disorders including Alzheimer's and Parkinson's disease before starting clinical trials. Regardless of the conflicting reports describing the role of oxidative stress and role of *VEGF* in various ALS investigations, both human and *in vivo* studies suffer from longitudinal analysis including the prospective nutritional interventional studies. Besides, the patient oriented genetic profiling studies have failed to include large cohort of homogeneous populations thus impacting the understanding of the demographic-SNP link in motor neuron degeneration. Nonpharmacological therapeutic approaches in ALS have not been adequately addressed and need new research focus for development of therapeutics.

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Association between CFH Y402H Polymorphism and Age Related Macular Degeneration in North Indian Cohort

Neel Kamal Sharma[1☯], Amod Gupta[2], Sudesh Prabhakar[1], Ramandeep Singh[2], Suresh Kumar Sharma[3], Wei Chen[4], Akshay Anand[1*☯]

1 Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, **2** Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, **3** Department of Statistics, Panjab University, Chandigarh, India, **4** Division of Pulmonary Medicine, Allergy and Immunology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania, United States of America

Abstract

The purpose of the study was to determine serum complement factor H (CFH) levels in patients of age related macular degeneration (AMD) and examine its association with CFH Y402H polymorphism. 115 AMD patients and 61 normal controls were recruited in this study. The single nucleotide polymorphism was assayed by real time PCR and serum CFH levels were measured by ELISA and standardized to total serum protein. Chi-square test was applied to polymorphism analysis while Mann Whitney U-statistic for CFH-levels. Mendelian randomization approach was used for determining causal relationship. The genotype frequency differed between the AMD patients (TT- 18.3%, TC-41.3% and CC-40.4%) and controls (TT-76.3%, TC-13.6%, and CC-10.1%) ($p = 0001$). The frequency of alleles was also significantly different when AMD (T-39% and C-61%) was compared to controls (T-83% and C-17%) ($p = 0.0001$). Level of serum CFH was significantly lower in AMD patients as compared to normal controls ($p = 0.001$). Our data showed that the CFH Y402H polymorphism is a risk factor for AMD in the North Indian population. Mendelian randomization approach revealed that CFH Y402H polymorphism affects AMD risk through the modification of CFH serum levels.

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* E-mail: akshay1anand@rediffmail.com

☯ These authors contributed equally to this work.

Introduction

AMD is a progressive disease of the retina and a leading cause of irreversible visual impairment [1,2]. AMD has two stages: early stage and advanced stage. In the early phase of disease there is presence of soft drusen with hyperpigmented and pigmented area. With time a few of early AMD may progress to advanced stage [1]. First is the dry AMD, which is marked by drusen or depigmentation caused by products of the photoreceptors and retinal pigment epithelium (RPE). The next phase of disease is called wet AMD because it is due to the growth of new abnormal blood vessels under the neurosensory retina and RPE, which results in subretinal bleeding and consequent scar formation. Both types of AMD may lead to central vision loss but 90% vision loss is known to be due to wet AMD. Fewer than 1% of the affected patients are under the age of 65 years, which increases with age, to 9% over 65 years and up to 30% over 70 years [3]. Therefore, the increasing population of elderly individuals impact health economics of every nation. The prevalence of AMD in India ranges from 1.84–2.7% [4].

AMD results from both environmental and genetic factors, even though its actual etiology remains unclear. CFH single nucleotide polymorphisms [SNPs] have been reported as the most important genetic risk factors for AMD pathogenesis.

Some independent studies have suggested that Y402H polymorphism in CFH gene plays an important role in determining AMD susceptibility (Y402H has a TrC substitution in exon 9 at 1277 nucleotide, which results in a tyrosine to histidine change) [5–7].

Another study from India has also reported significant association of Y402H among AMD patients ($p = 1.19 \times 10^{-7}$). They showed that persons homozygous for CC had a significantly higher risk ($p = 0.0001$) of AMD than heterozygous genotype [8].

CFH has been reported to be present in human and mouse ocular tissues such as RPE and choroid and is associated with drusen in AMD patients [9,10]. AMD is associated with complement dysregulation or activation of the spontaneously initiated alternative complement pathway leading to local inflammation, which is involved in pathogenesis of disease. CFH is known to be involved in maintaining homeostasis of complement system and any alteration in this system either in the form of altered functions of CFH variants or CFH expression could lead to activation of complement systems which triggers further events leading to cell damage of the RPE cells, formation of drusen and visual loss [11]. Complement components C3a and C5a are prominently involved in the AMD [12]. C3a deposition and C5a release after complement activation are inhibited by Complement factor H, any defect in CFH induces increased production of C3a and C5a frequently seen in AMD autopsies [13] thus confirming a

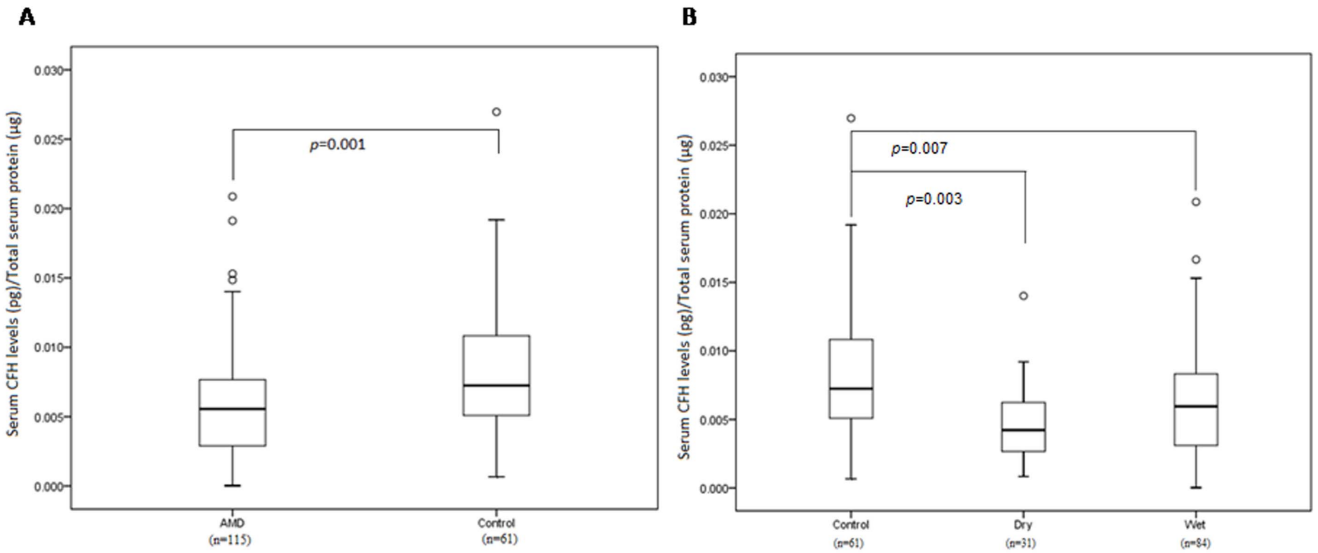

Figure 1. A) Serum levels of CFH in AMD and normal controls. B) Serum levels of CFH in Controls, Dry and Wet AMD. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Outliers and extreme values are shown in circles and asterisk respectively. Levels of CFH were standardized to total protein. AMD, Age Related Macular Degeneration; CFH, Complement Factor H; pg, picogram; µg, microgram.
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local role of inflammation and complement in the pathogenesis of AMD. We hypothesized that a mutation in CFH could affect the CFH protein levels.

The purpose of this study was to determine the frequencies of the CFH Y402H variants and the levels of serum CFH in AMD patients and normal controls in the north Indian population, a study which has not been undertaken earlier. In this study, we applied Mendelian randomization approach to test whether CFH polymorphism, CFH levels and other confounders have any role in the etiology of AMD.

Table 1. Demographic characteristics of Controls and AMD patients.

Variables	AMD	Controls
Number	115	61
Age	64.97±7.1	60.38±13.2
Duration of disease¥	23±2.6 (M)	–
Wet AMD	84 (73.04%)	–
Minimal Classic	7 (11.9%)	–
Predominant Classic	16 (27.1%)	–
Occult	36 (61.0%)	–
Familial Cases	10 (8.7%)	–
Bevacizumab Treated	55 (65.5%)	–
Smokers	50 (43.5%)	11 (20%)
Alcohol User	37 (32.2%)	17 (30.9%)
Vegetarian	61 (53%)	31 (56.4%)
Male	75 (65.2%)	40 (65.6%)

Clinical and demographic details of subjects. AMD, age related macular degeneration; M, Months; Age, Age of onset; Values are mean ± SD or (percentage), ¥ Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset.
doi:10.1371/journal.pone.0070193.t001

Materials and Methods

Patients and Control Individuals

Two independent groups of North Indian population including patients of AMD and controls were recruited in the study through the retina clinic, Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. The study was approved by institutional ethics review committee of PGIMER, Chandigarh (No. Micro/10/1411). Patients were enrolled in the study based on approved inclusion and exclusion criteria after written informed consent was obtained. We included 176 case-control samples consisting of 115 AMD patients along with 61 genetically unrelated healthy controls. We have excluded those cases in which any demographic detail was lacking.

Only those AMD patients were recruited who fulfilled the inclusion criteria such as those with an age 50 years or more with a diagnosis of AMD defined by dry and/or choroidal neovascularization with five large drusen or more [14]. The controls were of age 50 years or older with no drusen and absence of other diagnostic criteria defined for AMD.

All patients and controls were examined by a retina surgeon for visual acuity measurement, and dilated fundus examination. All patients underwent fluorescein fundus angiography. AMD diag-

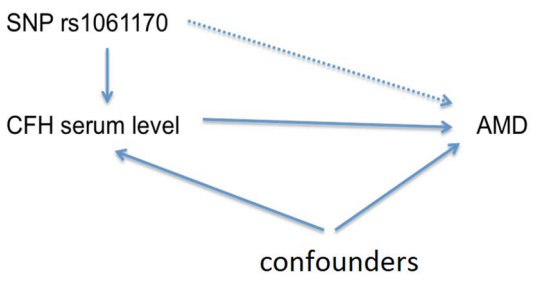

Figure 2. Mendelian randomization approach.
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Table 2. Genotype and allele frequency of CFH rs1061170 by Logistic Regression analysis.

			Unadjusted p value			Multivariate analysis, adjusted for age, gender, food habits, smoking and comorbidity		
Genotype	**Number (frequency)**		**OR**	**95% CI**	**p Value**	**OR**	**95% CI**	**P Value**
CFH rs1061170								
	AMD	**Controls**						
TT	20 (.183)	45 (.763)	Reference			Reference		
TC	45 (.413)	8 (.136)	12.65	5.05–31.69	0.0001	1.960	0.393–3.526	0.014
CC	44 (.404)	6 (.101)	16.5	6.05–44.96	0.0001	*	*	*
	Wet AMD	**Dry AMD**						
TT	16 (.20)	4 (.138)	Reference			Reference		
TC	33 (.412)	12 (.414)	0.69	0.191–2.471	0.566	1.447	0.897–3.791	0.226
CC	31 (.388)	13 (.448)	0.60	0.167–2.129	0.426	0.400	0.049–3.289	0.394
Allele	**Number (frequency)**		**OR**	**95% CI**	**p Value**			
	AMD	**Controls**						
T	85 (.39)	98 (.83)	Reference					
C	133 (.61)	20 (.17)	7.6	4.4–13.3	0.0001	-	-	-
	Wet AMD	Dry AMD						
T	65 (.41)	20 (.34)	Reference					
C	95 (.59)	38 (.66)	0.77	0.41–1.43	0.41	-	-	-

*The value could not be complied because of the equal frequencies.

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nosis was based on ophthalmoscopic and fluorescein angiographic findings.

A standardized risk factor questionnaire was used by a trained interviewer to interview all the subjects [15–17]. Demographic information such as alcohol intake, cigarette smoking, food habits and comorbidity were included in a questionnaire. Smokers were defined as those having smoked at least three cigarettes per day or 54 boxes for at least 6 months. Non vegetarian patients were defined as those having chicken, meat or fish for at least 6 months. Information about alcohol use for at least 6 months was also collected. Co-morbidities were determined based on the participant's answers to whether a physician had ever informed them for diagnosis of any main neurological, cardiovascular or metabolic illness.

Table 3. Logistic regression of CFH rs1061170 and AMD stratified by food habits, smoking and comorbidities.

			Unadjusted p value			Multivariate analysis, adjusted for age and sex		
Genotype	**Number (frequency)**		**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
CFH rs1061170								
	Vegetarian AMD	**Non Vegetarian AMD**						
TT	6 (0.10)	14 (0.28)	Reference			Reference		
TC	29 (0.50)	16 (0.31)	4.22	1.35–13.15	0.012	0.404	0.113–1.445	0.164
CC	23 (0.40)	21 (0.41)	2.55	0.83–7.86	0.102	2.579	0.589–11.29	0.209
	AMD Smokers	**AMD Non Smokers**						
TT	9 (0.19)	11 (0.18)	Reference					
TC	22 (0.47)	23 (0.37)	1.16	0.40–3.36	0.772	1.00	0.271–3.694	1.00
CC	16 (0.34)	28 (0.45)	0.69	0.23–2.04	0.512	0.412	0.70–2.42	0.327
	AMD with Comorbodities	**AMD without Comorbodities**						
TT	11 (0.14)	9 (0.32)	Reference			Reference		
TC	36 (0.46)	8 (0.29)	3.68	1.145–11.83	0.028	0.083	0.08–0.898	0.040
CC	32 (0.40)	11 (0.39)	2.38	0.779–7.265	0.127	1.836	0.462–7.29	0.388

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Table 4. Log CFH Serum levels according to AMD and control subtypes (Comparison using t-Statistic).

Subjects CFH	Mean	t-Value	p-value
AMD	−5.37		
Control	−4.94	−3.27	0.001
Dry	−5.49		
Wet	−5.32	−0.85	0.400
Bevacizumab treated	−5.34		
Not treated	−5.29	0.216	0.830
Minimal Classic	−5.50		
Predominant Classic	−5.59	0.124	0.871
Occult	−5.37	−0.327	0.806
Alcohol consumption	−5.46		
No Alcohol consumption	−5.32	0.670	0.50
Smokers	−5.39		
Non Smokers	−5.35	0.244	0.807
Vegetarian	−5.45		
Non Vegetarian	−5.28	0.98	0.331
Without comorbidities	−5.31		
With comorbidities	−5.39	0.43	0.670

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Sample Collection

Blood samples were collected from all subjects. Serum was separated from 4.0 ml of blood by using serum separator tubes (BD Biosciences, USA). Genomic DNA was extracted from the peripheral venous blood using a commercial kit (QIAGEN, Germany and INVITROGEN, USA) according to the manufacturer's protocol. The samples were coded, labeled and stored in −80°C freezer until assayed.

Protein Analyses

The quantification of serum total protein was done using Bradford assay in order to standardized CFH levels estimated from ELISA. The CFH protein levels were analysed using commercially available enzyme linked immunosorbant assay (ELISA; Cusabiotech; Catalog no. CSB-E08931h) according to manufacturer's procedure and the absorbance was taken at 450 nm by 680XR Microplate reader (Biorad, Hercules, USA). This assay recognizes recombinant and natural human CFH with detection range of 15.6 µg/ml-1000 µg/ml. All the samples were analysed in duplicates. The standard curve for CFH estimation was done by linear regression analysis. All the values were standardized with total serum protein.

Genotyping

The SNP (rs1061170) employed for analysis in our study was previously documented in other ethnic populations for involvement with AMD and was chosen based on its functional significance. It is defined as Y402H. The rs1061170 (T) allele encodes the more common Tyr (Y), while the generally rarer rs1061170(C) encodes the His (H). The SNP (rs1061170) assay was done by using Real time PCR (Applied Biosystems Inc., Foster city, CA) using published TaqMan® SNP Genotyping Assays (7). Real time PCR was carried out for 20.0 µl volume containing 10 ul master mix, 5 ul Assay (Applied Biosystems) and 20 ng DNA was added to make the volume 20.0 µl. TaqMan® SNP Genotyping Assays (Applied Biosystems) was used to carry out all reactions according to manufacturer's recommendations. Two reporter dyes FAM and VIC were used to label the Allele 1 and 2 probes and 5′ Nuclease Assay was carried out. PCR mix without DNA was used as negative control. StepOne^{TM} v 2.0 software (Applied Biosystems Inc., Foster city, CA) was used to perform the genotype calling and Sequence Detection System (SDS) Software was used to import the fluorescence measurements made during the plate read to plot fluorescence (Rn) values after PCR amplification (2).

Statistical Analysis

After taking the log of CFH ELISA values it was observed from Normal Quantile plot (Q-Q plot) that the data was approximately normally distributed. t-test was therefore, applied for comparing the two groups. For comparing more than two groups, One-way analysis of variance (ANOVA) followed by post-hoc was applied for multiple comparisons. The $p \leq 0.05$ was considered significant. The measure R^2 (Coefficient of determination) was used to determine the goodness of standard curve fit for ELISA and total protein. The linear and quadratic regressions with $R^2 > 0.80$ were considered to be a good fit. The genotypes were stratified for homozygosity and heterozygosity of the respective allelic variant. Association between various study groups was done by using Pearson's Chi-square test. Odds ratios (ORs) with 95% CI and genotypic associations were estimated by binary logistic regression. All statistical analysis such as linear regression, quadratic fit and test of significance were performed with statistical package and service solutions (SPSS; IBM SPSS Statistics 20.0, Chicago, Illinois, USA) 20.0 software. Mendelian randomization (MR) approach was used to investigate the CFH causal pathway in our study.

Results

Summary statistics of all important variables are reported in Table 1.

Single Nucleotide Polymorphism

We analyzed one polymorphism in the CFH gene by real time PCR. Genotype and allele frequencies of CFH have been listed in Tables 2. There was a significant difference between the homozygous genotype frequency for allele T, homozygous genotype frequency for allele C and heterozygous genotype frequency between AMD patients and normal controls. The CC and TC genotypes were more frequent in AMD patients than to controls (OR = 16.5, CI = 6.05–44.96, p = 0.0001, and OR = 12.65, CI = 5.05–31.69, p = 0.0001 respectively, Table 2). The C allele was more frequent in AMD cases than controls (OR = 7.6, CI = 4.4–13.3, p = 0.0001, Table 2). There was no significant difference in the genotype and allele frequencies between wet and dry AMD patients (Tables 2). Logistic regression analysis for eating habits, smoking and presence of comorbidity revealed that the TC genotype was more frequent in vegetarian AMD patients (OR = 4.22, CI = 1.35–13.15, p = 0.012, Table 3) and AMD patients with comorbodities (OR = 3.68, CI = 1.145–11.83, p = 0.028, Table 3). The difference was not significant when compared for bevacizumab treatment, the number of eyes affected and between wet AMD patients ie minimally classic, predominantly classic and occult (data not shown).

A logistic regression analysis was performed to analyze the association between the SNP and other risk factors with AMD simultaneously. We analyzed age, sex, food habits smoking and comorbidity as risk factors which have been shown to be

associated with AMD previously. When multiple logistic regression analysis was carried out with adjustment for age, sex, food habits, smoking and comorbidity, we found that TC genotype was at higher frequency in AMD patients than controls ($p = 0.014$, Table 2). Sex and age adjustment for AMD patients with comorbidity also showed higher frequency of the TC genotype than in AMD patients without comorbidity ($p = 0.040$, Table 3).

Serum Levels of CFH are Decreased in AMD Patients

We investigated the serum CFH levels in AMD and controls. We also examined the correlation between CFH genotype and protein expression in serum. The CFH levels in AMD were significantly lower than in controls (Figure 1A, Table 4, $p = 0.001$). However, we did not find significant difference in the CFH serum levels between wet and dry AMD patients, but both patients groups had significantly lower levels than controls (Figure 1B, $p = 0.007$, 0.003 respectively, Table 4). To estimate the predictive value of CFH, serum levels of CFH were again segregated into minimal classic, predominantly classic and occult AMD. The difference was not significant between the wet AMD subgroups (Table 4). We did not find any significant difference when the ELISA levels were compared to other parameters like smoking, alcohol, eating habit and bevacizumab treatment (Table 4). We did not find any significant correlation between CFH genotype and protein expression in serum.

Mendelian Randomization Approach

We used the Mendelian randomization (MR) approach [18,19] to investigate the potential causal pathway by including SNP, CFH serum levels, and AMD with other risk factors (food habit, comorbidity, and smoking). CFH rs1061170 was analyzed as an instrumental variable for CFH serum level. We found that allele C increased the risk of AMD and lower CFH serum level was observed in AMD patients. Allele C reduces the CFH serum level, but SNP was also associated with food habit and comorbidity. Therefore, the causal effect of CFH serum on the risk of AMD may require further studies. We illustrate this approach in Figure 2.

Discussion

The Y402H polymorphism in CFH is a major risk factor for AMD [6,7]. The non-synonymous variant (T-C) results in tyrosine to histidine transformation at codon 402 of this loci. Several studies have established an association of the CFH gene, which is an inhibitor of the alternative complement activation pathway to be responsible for AMD. Association of the Y402H (rs1061170) variant of CFH with AMD has been described in several populations worldwide [6,20], with TC and CC genotype being approximately 2.5 and 6 times extra likely to have AMD than patients having TT genotype [21], and this was later confirmed in Italian [22], French [7], British [6], Russian [14] and Icelandic [20] populations. However, it appears to be less common in Chinese [23], and is absent in Japanese [24,25] but no such study has been conducted in the homogeneous population from Northern India.

This study was therefore conducted to determine the prevalence of CFH polymorphism and to test whether differences in levels of serum CFH exist between Indian patients with AMD and healthy controls. We report significantly lower serum CFH levels in AMD patients as compared to controls and Y402H variant of CFH to be associated with AMD in this population. Homozygous CC and heterozygous TC genotypes were more frequent among AMD patients than controls. Moreover, the CC and TC genotypes conferred OR for AMD of 16.5 and 12.6, respectively. CFH is involved in the inflammatory response of the innate immune system. Low levels of CFH in North Indian population is consistent with other reports. Dhillon et al showed that the prevalence of factor H autoantibodies decreased in AMD patients as compared with normal controls [26]. Some investigators have shown that reduced serum CFH is associated with obesity, hypertension and smoking which are known risk factors for AMD [27,28]. In a recent study, Silva et al observed significant differences in the plasma levels of the alternative pathway proteins i.e. Factor D (FD) and Factor I (FI) between the AMD patients and control. They showed significantly lower FD plasma levels and higher FI levels in AMD patients and also identified a significant decrease in CFH plasma levels in AMD females patients in relation to normal females [29].

Several studies have previously examined the role of CFH Y402H polymorphism in the AMD subtypes such as geographic atrophy (GA) or choroidal neovascularization (CNV). The weakly regulated complement cascade, due to CFH polymorphism, might enhance cellular damage, ultimately leading to atrophy or neovascular response [30]. In the patients investigated the Y402H polymorphism was not predictive for either of these AMD phenotypes. This supports the concept that it could be involved in both dry and wet AMD variants [31]. It is pertinent to note that conflicting results exist where such associations have been investigated wherein some groups have suggested that neovascular AMD to be at a higher risk of this genotype variant [32,33] while others noting that atrophic AMD represents a higher risk of this polymorphism [34,35], however, there are many others who have reported it to bear no variation with AMD phenotype [17]. Our results are not consistent with those that suggest association with neovascular or dry AMD.

There are certain reports indicating increased risk for each successive stage of AMD associated with the CFH polymorphism [36]. Our findings do not show any difference between minimal classic, predominantly classic and occult AMD in the association with the CFH Y402H genotype. Interestingly, our findings also raise questions about the role of eating habits and other co-morbidities on individual genotype. We, however, note that AMD has previously been reported to be associated with other diseases such as stroke and depression [37,38]. Vegetarian diet and existence of co-morbidities in AMD patients seemed to suggest a non redundant association with the TC genotype and the risk of developing AMD with OR = 4.22 and 3.68, respectively. The importance of this association is unclear due to limited data. However, those on vegetarian diet including those not consuming fish, may be deficient in a essential nutrients – especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) the long-chain omega-3 fatty acids. Alphalinolenic acid (ALA) is an omega-3 fat and is the precursor of the longer chain omega 3 fats EPA and DHA, i.e. ALA in the body can form EPA and to a lesser extent DHA. Some fish and seafood are the major dietary sources of these fatty acids. As a result, vegetarian diets provides little DHA and EPA. Kornsteiner et al showed that vegetarians are left with less omega-3 levels [39]. In addition, ALA, DHA, and EPA are particularly important for the prevention of AMD [40]. Some studies have reported that fish consumption and omega-3 fatty acid intake reduces the risk of AMD [41,42]. However, some studies suggest an inverse relation between regular dietary intake of DHA, EPA, fish and risks of advanced AMD [43,44]. Recent unpublished reports from Punjab, India have also shown correlation between excess use of pesticides in agricultural crops and incidence of cancer and other degenerative disorders (http://health.india.com/diseases-conditions/are-the-farmers-in-punjab-paying-a-price-for-the-green-revolution/).

Using a Mendelian randomization approach, our results show strong evidence that CFH serum levels are causal to AMD, which strengthens the study. This implies: Allele C increases the risk of AMD; lower CFH serum level is observed in AMD patients; Allele C reduces the CFH serum level. This evidence strengthens the argument that increasing CFH serum level might lower the risk of AMD. SNP was also found to be associated with food habit and comorbidity. Therefore, the correlation of CFH serum on the risk of AMD may require further studies. The key limitation of the study was the lack of local tissue.

Conclusion

Our study demonstrated that the CFH Y402H polymorphism with a higher frequency of the allele may affect the CFH serum levels resulting in AMD.

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Author Contributions

Conceived and designed the experiments: AA. Performed the experiments: NKS. Analyzed the data: AA SS NKS WC. Contributed reagents/materials/analysis tools: SP AG RS. Wrote the paper: AA NKS.

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Clinical Study

Superoxide Dismutase1 Levels in North Indian Population with Age-Related Macular Degeneration

Akshay Anand,[1] Neel K. Sharma,[1] Amod Gupta,[2] Sudesh Prabhakar,[1] Suresh K. Sharma,[3] and Ramandeep Singh[2]

[1] *Department of Neurology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India*
[2] *Department of Ophthalmology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh 160012, India*
[3] *Department of Statistics, Panjab University, Chandigarh 160014, India*

Correspondence should be addressed to Akshay Anand; akshay1anand@rediffmail.com

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Aim. The aim of the study was to estimate the levels of superoxide dismutase1 (SOD1) in patients of age-related macular degeneration (AMD) and examine the role of oxidative stress, smoking, hypertension, and other factors involved in the pathogenesis of AMD. *Methods.* 115 AMD patients and 61 healthy controls were recruited for this study. Serum SOD1 levels were determined by ELISA and were correlated to various risk factors. Logistic regression model of authenticity, by considering SOD1 as independent variable, has been developed along with ROC curve. *Results.* The SOD1 levels were significantly higher in AMD patients as compared to those of the controls. The difference was not significant for wet and dry AMD. However, the difference was significant between wet AMD subtypes. Nonsignificance of the Hosmer-Lemeshow goodness of fit statistic ($\chi^2 = 10.516$, df $= 8$, $P = 0.231$) indicates the appropriateness of logistic regression model to predict AMD. *Conclusion.* Oxidative stress in AMD patients may mount compensatory response resulting in increased levels of SOD1 in AMD patients. To predict the risk of AMD on the basis of SOD1, a logistic regression model shows authenticity of 78%, and area under the ROC curve (0.827, $P = .0001$) with less standard error of 0.033 coupled with 95% confidence interval of 0.762–0.891 further validates the model.

1. Introduction

AMD is the most important cause of blindness which is characterized by progressive degeneration of macula leading to severe irreversible loss in vision [1]. The vision loss results either from retinal degeneration (dry AMD) or from the choroidal neovascularization (wet AMD). The clinical manifestation of AMD includes drusen, geographic atrophy, hyperplasia of the retinal pigment epithelium (RPE), and angiogenesis of choroidal vessels (CNV) [2].

Smoking, alcohol, oxidative stress, and genetic factors are implicated in the pathogenesis of AMD [3], but the exact cause of AMD remains complex. It has been reported that aging is associated with pathological and biochemical changes in the eye. In general, aging and AMD are believed to result from cumulative and increased oxidative damage [4]. Oxidative stress can exert molecular or cellular damage mediated by reactive oxygen species (ROS) which has been earlier shown to be implicated with diseases of ageing [5]. The elevated levels of endogenously synthesized ROS are known to be regulated by various antioxidant, enzymatic, and nonenzymatic protective biochemical mechanisms like Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) [6]. ROS which includes free radicals, nascent oxygen, hydrogen peroxide and the by-products of oxygen metabolism are deleterious for eye pathophysiology. Due to the high consumption of O_2, the high concentration of polyunsaturated fatty acid and direct exposure of light render retina susceptible to oxidative stress [7]. Various factors are responsible for oxidative stress generated from aging; these include decreased levels of vitamin C and vitamin E in plasma [8]. It has also been shown that oxidized glutathione levels

increase in plasma and that glutathione levels decrease with the age [9]. Increased lipid peroxidation is also reported in aging [10], and the consequences of these imbalanced biochemical changes lead to increased susceptibility of retinal pigment epithelium cells (RPE) to oxidative damage with aging. Even catalase activity and vitamin E levels have been reported to decrease with aging in RPE cells [11]. There are several pathological features which accompany aging. These include increased volume of lipofuscin contents (increased lipid and protein contents) which enhance the oxidative damage susceptibility and decreased optical density of macular pigment [12] which results in membrane blebbing of RPE cells, a phenomenon observed in AMD eyes and aging [13].

We hypothesized that oxidoreduction alteration in the eye might result from deranged SOD1 levels. We, therefore, analysed the expression of superoxide dismutase1 in patients of AMD as compared to controls. The major antioxidant system in the retina consists of three superoxide dismutase (SOD) isoenzymes that catalyse dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2) [14]. SOD is an antioxidant enzyme useful in the defense system against ROS. Superoxide dismutase catalyzes the dismutation reaction of O_2^- (superoxide radical anion) to H_2O_2, which is then catalyzed to H_2O and O_2 by catalase and glutathione peroxidase. There are three major families of superoxide dismutase, depending on metal cofactors: Cu-Zn SOD (SOD1), present in cytosol, Mn (Fe)-SOD (SOD2) present in mitochondrial matrix, and the extracellular SOD (SOD3) interstitium of the tissues as a secretary form [15].

The amount and activity of the Cu-Zn SOD (SOD1) are the highest among the three isoenzymes in human retina, so it seems reasonable to screen SOD1 for possible role in accelerating age-linked changes in the retina [15].

Currently, there is no study examining the role of SOD1 in Indian AMD patients, and this investigation will likely provide the substrate for future therapies in AMD.

2. Methodology

2.1. Study Participants. This study was approved by Institute Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India (letter no. Micro/10/1411). Patients and controls were first informed about the study and thereafter enrolled in patient/control group after obtaining written proforma from all participants. All enrolled participants were recruited from the Department of Ophthalmology, PGIMER, Chandigarh, India, in which phenotypic criteria were strictly followed. A retina specialist carried out ophthalmic examination of all AMD patients for best corrected visual acuity, dilated fundus examination, and slit lamp biomicroscopy of anterior segment. All patients underwent optical coherence tomography (OCT) and fluorescein fundus angiography (FFA). AMD diagnosis was based on FFA and ophthalmoscopic findings.

We included a total of 176 case-control samples consisting of 115 AMD patients from PGIMER, Chandigarh, India, with 61 genetically distinct healthy controls as per inclusion and exclusion criteria. However, some demographic details were not available for some subjects.

2.2. Inclusion and Exclusion Criteria. 50 years or older AMD patients with more than five drusen in case of dry AMD in at least one eye and/or choroidal neovascularization in case of wet AMD were incorporated in the study [16, 17]. The controls in the study included those with age 50 years or more with the absence of other diagnostic criteria for AMD.

The exclusion criteria excluded the retinal diseases involving the outer retinal layers and/or photoreceptors other than AMD loss, such as central serous retinopathy, high myopia, diabetic retinopathy, retinal dystrophies, uveitis, and vein occlusion, or similar outer retinal diseases that have been present earlier to the age of 50 and opacities of the ocular media, or other problems enough to preclude satisfactory stereo fundus photography. These situations contain occluded pupils due to cataracts and opacities and synechiae due to ocular diseases.

2.3. Baseline Examination. A trained interviewer collected the information about medical history, demographic characteristics, and lifestyle risk factors like smoking, alcohol, and so forth, using a standard risk factor questionnaire [18, 19]. Smokers were defined as those having smoked at least three cigarettes per day or 54 boxes for at least 6 months and were segregated further into smokers and nonsmokers. Nonvegetarian patients were defined as those having chicken, meat, or fish for at least 6 months, and alcohol consumers were defined as those having whiskey, rum, wine, or homemade alcohol for at least 6 months. Hypertension was defined as diastolic blood pressure ≥90 mm Hg and systolic blood pressure ≥140 mm Hg at the time of examination and for this condition whether they had ever taken medications. Similar practices have been used in previous studies [20]. Participants were also asked to report any previous diagnosis of migraine, use of antihypertensive medications, stroke, diabetes, or history of heart diseases.

2.4. Collection of Blood and Serum Separation. 4.0 mL of blood was collected in serum separator tube (BD Biosciences, USA) from both AMD and controls and left for 30 minutes at 37°C to allow it to clot according to the standard procedures. Serum was subsequently separated by centrifugation for 30 minutes at 3000 rpm. The separated serum was frozen at −80°C until analysis [21, 22].

2.5. Total Protein Estimation. The Bradford assay was used to estimate the total serum proteins for normalization of SOD1 levels analysed by ELISA as per the manufacturer's recommendations [23, 24].

2.6. SOD1 Expression. Serum from AMD patients and controls was used to carry out the quantitative detection of SOD1 using commercially available enzyme-linked immunosorbant assay (ELISA) (AB Frontier; Catalog no. LF-EK0101) as per the instructions from manufacturer, and absorbance was taken at 450 nm using 680XR Microplate reader (Biorad,

Hercules, USA). Sample assays were carried out in duplicate. The procedure to analyse the SOD1 levels was provided by manufacturer of the kit. This assay recognizes native and recombinant human SOD1 with the detection of more than 12.5 pg/mL. The standard curve was generated by linear regression analysis for SOD1 in both controls and patients. All the values were normalized with total serum protein.

2.7. Statistical Analysis. All statistical calculations were carried out by statistical product and service solutions SPSS (IBM SPSS Statistics 20.0, Chicago, IL, USA) software. The assumption of normality was tested with the help of Normal Quantile plot (Q-Q plot), and it was observed that data were not normally distributed. Therefore, the Mann-Whitney U-test was applied to compare the two groups. For comparing more than two groups, the Kruskal-Wallis oneway analysis of variance (ANOVA) followed by post hoc for multiple comparisons was applied. The $P \leq 0.05$ was considered significant. The measure R^2 (coefficient of determination) was used to determine the goodness of standard curve fit for ELISA and total protein. The linear and quadratic regressions with $R^2 > 0.80$ were considered to be of a good fit. In order to identify the risk factors associated with AMD, a logistic regression was carried out, and adjusted odds ratios were also obtained. ROC (receiver operating characteristics) curve defines the sensitivity/specificity of the experiment. The ROC curve is basically important for the evaluation of diagnostic tests. The true positive rate (sensitivity) is plotted as the function of the false positive rate (100-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal). ROC curve for predicted model was mapped [25, 26].

Table 1: Clinical and demographic details of subjects.

Variables	AMD	Controls
Total	115	61
Wet AMD	84 (73.04%)	—
Dry AMD	31 (26.96%)	—
Minimal classic	7 (11.9%)	—
Predominant classic	16 (27.1%)	—
Occult	36 (61.0%)	—
Avastin treated	55 (65.5%)	—
Not treated with Avastin	29 (34.5%)	—
Duration of disease[¥]	23 ± 2.6 (M)	—
Smokers	50 (43.5%)	11 (20%)
Nonsmokers	65 (56.5%)	44 (80%)
Alcoholic	37 (32.2%)	17 (30.9%)
Nonalcoholic	78 (67.8%)	38 (69.1%)
Vegetarian	61 (53%)	31 (56.4%)
Nonvegetarian	54 (47%)	24 (43.6%)
Hypertension	52 (45.2%)	10 (16.4%)
Nonhypertensive	61 (53%)	45 (73.8%)
Heart disease	16 (13.9%)	—
No heart disease	60 (52.2%)	55 (100%)
Age	64.97 ± 7.1	60.38 ± 13.2
Male	75 (65.2%)	40 (65.6%)
Female	40 (34.8%)	21 (34.4%)

AMD: age-related macular degeneration; M: months; Age: age of onset. Values are mean ± SD or percentage, [¥]Duration of disease is the interval between appearance of the first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease onset.

3. Results

Summary statistics of important variables have been shown in Table 1. 115 AMD patients were recruited for the study with average age of 64.97 ± 7.1, whereas 61 controls were recruited with an average age of 60.38 ± 13.2. The AMD population was divided according to presence of clinical features and Avastin treatment. The recruited patients and controls were further classified based on their food habits and smoking as well as alcohol consumption and the presence of other associated diseases like hypertension, heart disease, and so forth in order to estimate the levels of SOD1 among these groups. The serum SOD1 level was found to be significantly elevated in AMD subjects as compared to normal controls (Figure 1, Table 2, $P = 0.0001$). However, there was no significant difference between the levels of dry and wet AMD (Table 2, $P = 0.117$). Moreover, in the wet AMD subgroups, significant difference was found. The levels of SOD1 in predominantly classic ($P = 0.022$) and occult AMD patients ($P = 0.023$) were significantly higher as compared to those of minimal classic (Figure 2(a)). An independent analysis was carried out while adjusting the risk factors to AMD. Important risk factors like smoking, alcohol, food habits, gender, hypertension, and heart diseases were analyzed to examine their association with SOD1. The SOD1 levels were found to be higher among hypertensive patients (Figure 2(b), Table 2, $P = 0.015$), those with heart disease (Figure 2(c), Table 2, $P = 0.002$) and male AMD patients (Figure 2(d), Table 2, $P = 0.035$), as compared to nonhypertensive patients or those without heart disease and female AMD patients, respectively. However, the difference was not significant between AMD smokers and AMD nonsmokers, alcohol consumers and alcohol nonconsumers, and vegetarian and nonvegetarians (Table 2). The levels were not found to be significant when compared among Avastin treated AMD patients versus untreated AMD patients (Table 2). It has been observed that there was a significant association of levels of SOD1 with AMD subtypes ($\chi^2 = 6.326$, $P = .042$), gender ($\chi^2 = 6.860$, $P = .032$), and smoking ($\chi^2 = 6.291$, $P = .043$). The prediction equation for AMD, by considering SOD1 as independent variable, shows that 78% of the cases have been correctly classified (model authenticity 78%) with attending confidence intervals for ROC curve. The area under ROC was 0.827 ($P = .0001$) with standard error of 0.033 and confidence interval of 0.762–0.891 (Figure 3).

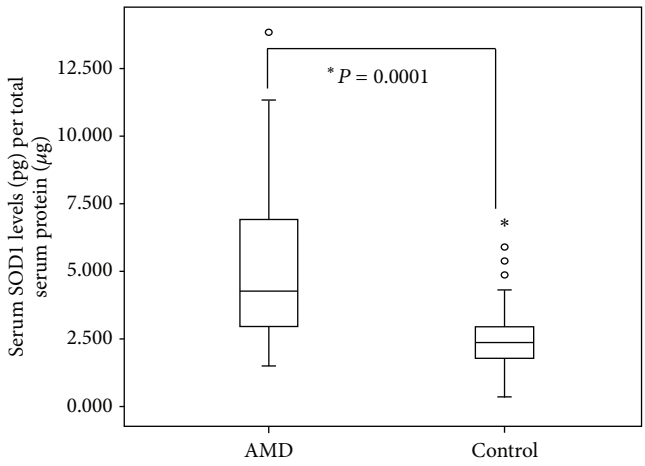

Figure 1: Serum levels of SOD1 in AMD and normal controls. Boxes include values from the first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk, respectively. Levels of SOD1 were normalized to total protein. Data was analyzed by using the Mann-Whitney *U* test.

Table 2: SOD1 levels according to different subtypes. ELISA levels were compared by applying the nonparametric Kruskal-Wallis *H* test followed by the Mann-Whitney *U* test.

Subjects	Mean rank	*Z* value	*P* value
AMD	107.61	7.08	0.0001*
Control	50.42		
Dry	49.97	1.56	0.117
Wet	60.96		
Minimal classic	15.29		
Predominant classic	35.75	2.272	0.022*
Occult	30.31	2.270	0.023*
Avastin treated	43.71	0.626	0.532
Not treated	40.21		
Alcoholic	60.57	0.569	0.570
Nonalcoholic	56.78		
Smokers	59.26	0.355	0.722
Nonsmokers	57.03		
Vegetarian	60.13	0.729	0.466
Nonvegetarian	55.59		
Hypertensive	65.13	2.437	0.015*
Nonhypertensive	50.07		
Heart disease	93.62	3.107	0.002*
No heart disease	62.16		
Male	62.80	2.114	0.035*
Female	49.00		

*Significant.

4. Discussion

The major reason for vision loss in elderly population is accounted for by AMD [27]. To understand the mechanism of AMD pathogenesis, several studies have attempted to correlate various targets and biomarkers with conflicting reports and unverified data from the Caucasian population. Facts suggest that oxidative stress plays a major role in the pathogenesis of AMD [28, 29].

This study was carried out to determine whether the serum SOD1 levels are altered in AMD patients as compared to normal controls as this region is characterized by unique dietary habits. Results from this study indicate that the SOD1 levels were elevated significantly in AMD as compared to normal controls. To our knowledge, this non-Caucasian study is first to demonstrate elevated SOD1 serum levels in Indian AMD patients. However, several other studies have been carried out to estimate the activity (not levels) of SOD along with other biomarkers associated with oxidative stress in various population [30–32].

Retina is very susceptible for lipid peroxidation [11, 12] which increases with age in macular region [11]. This is associated with cellular damage which involves decreased cellular antioxidants [13]. In our results, the high levels of SOD1 indicate that lipid peroxidation and oxidative stress are involved in tissue damage in AMD patients. Whether increased SOD1 levels in our study are indeed due to compensatory regulation or causative of AMD can be determined by conducting longitudinal study performed on intermediate or early AMD patients.

SOD1 levels of occult and predominantly classic AMD patients were found to be higher as compared to those of minimally classic AMD patients. This corresponds to disease severity whether induced by SOD1 or resulting from disease. Interestingly, it has been shown previously that the protein content of SOD1 and SOD2 in RPE homogenates increases in the later stages of AMD [33].

The fact that SOD1 levels were found to be higher among male, hypertensive, and heart disease patients could be ascribed to high oxidative stress in these patients. It was shown that the oxidative stress could be involved in the cardiovascular diseases and hypertensive patients [34, 35]. It is pertinent to point out that although SOD1 is an antioxidant, its over expression can lead to increased oxidative stress. Studies on transgenic animals have shown that increased levels of SOD1 lead to more hypersensitivity to oxidative stress [36, 37]. It is possible that the negative effects seen with high levels of SOD1 are caused by an increased level of the product of the dismutation reaction which yields hydrogen peroxide [38]. Kowald et al. have defined such situation by deriving mathematical equations and proposed the three alternative mechanisms driven by SOD1: (i) reaction of hydrogen peroxide with CuZnSOD that leads to formation of hydroxyl radicals, (ii) superoxide radicals acting as chain breaker, and (iii) interchange between oxidized and reduced form of SOD while detoxifying superoxide radicals [38]. These studies do not demonstrate the dual function of SOD, but instead they indicate an alternative pathway which is driven by the free radical burden inside the cell. Recently, it has been found that peroxidase activity of superoxide dismutase depends on CO_2. The generation of free radicals by the peroxidase activity of superoxide dismutase was found to be higher in the presence of bicarbonate-carbon dioxide.

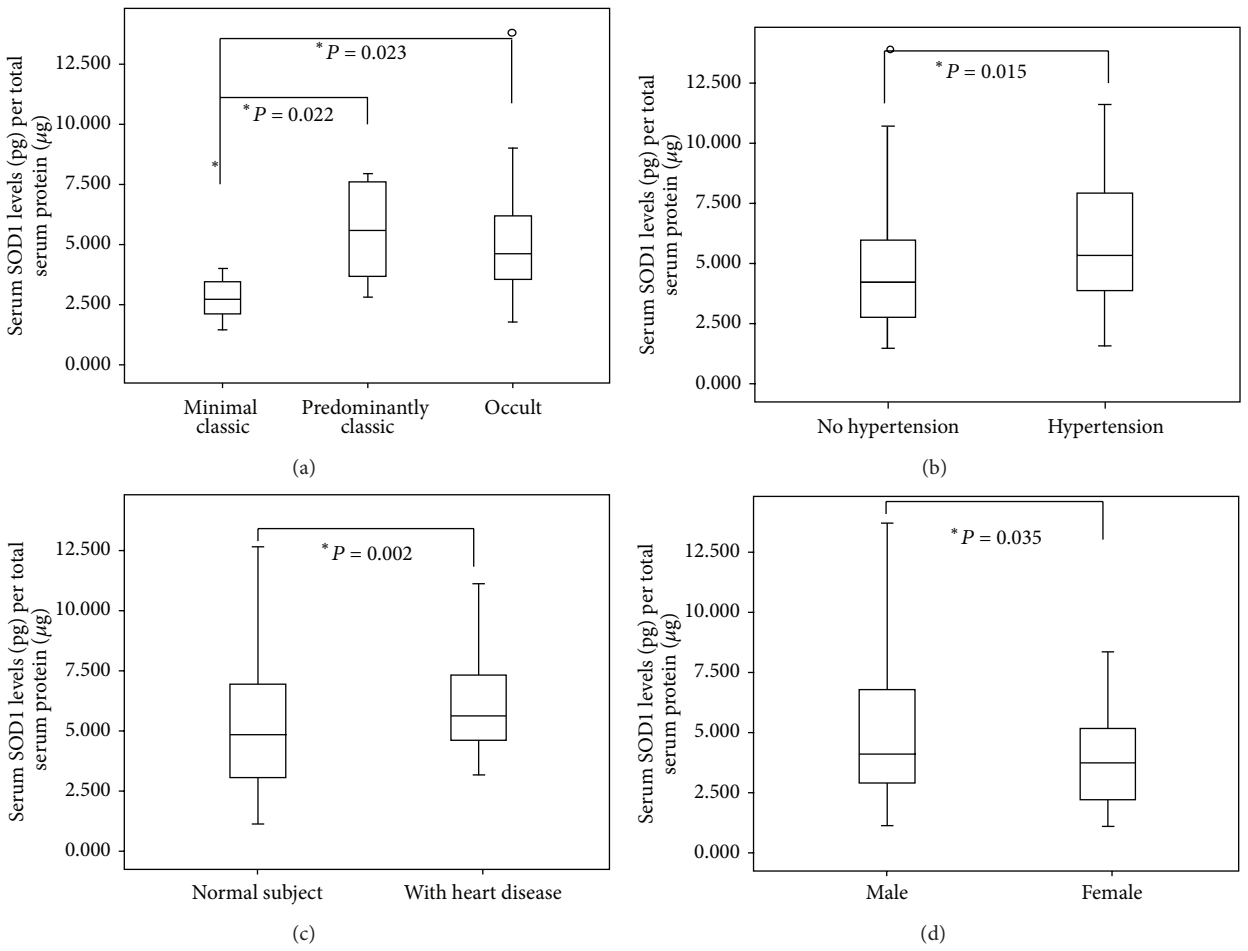

Figure 2: (a) Serum levels of SOD1 in minimal classic, predominant classic, and occult AMD. (b) Serum levels of SOD1 in hypertensive and nonhypertensive AMD patients. (c) Serum levels of SOD1 in heart disease and no heart disease patients. (d) Serum levels of SOD1 in male and female AMD patients. Boxes include values from the first quartile (25th percentile) to the third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisks, respectively. Levels of SOD1 were normalized to total protein. Data was analyzed by using the Mann-Whitney *U* test.

This mechanism explains why strong oxidant is generated during peroxidase activity of SOD which is followed by CO_2 oxidation to carbonate radicals. These free carbonate radicals have tendency to oxidize various biomolecules inside the cell [39].

Moreover, we have earlier reported that the VEGFR2 levels increased significantly in the AMD patients as compared to those in normal control [40]. We hypothesized that there is positive correlation between the increased SOD1 and VEGFR2 levels because under *in vitro* conditions oxidative stress has been correlated previously with upregulation of VEGF and is thought to be involved in the increased expression of VEGF [41, 42]. In addition, several studies involving tissue culture and animal models have pointed out that oxidative stress is a critical moderator in the transduction of the mitogenic effects of VEGF [43, 44].

We have attempted to predict AMD based on SOD1 using logistic regression, which showed 78% model predictivity, and area under curve is 0.827. The high value of AUC may be used to diagnose AMD patients with very less standard error. The association with gender, smoking, and AMD types means that the increased levels of SOD1 are associated with these factors.

Therefore, the oxidative stress is considered as an important causative factor for AMD, which can lead to induced apoptosis of RPE and result in impairment of RPE function [45–47], and, hence, its analysis in larger AMD cohort is imperative.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Akshay Anand and Neel K. Sharma contributed equally to the paper.

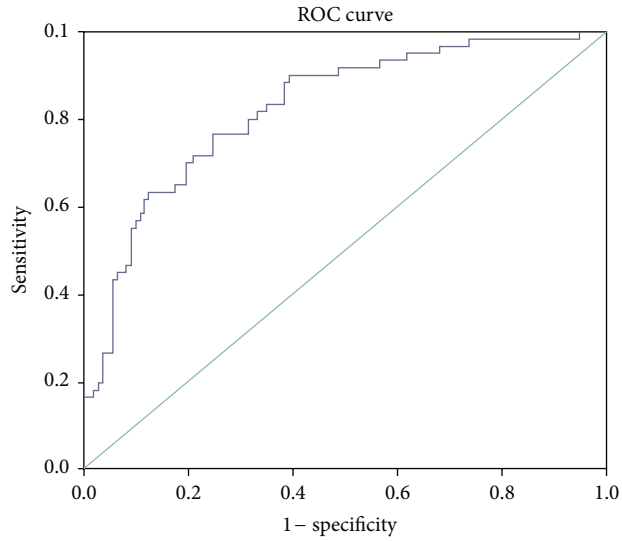

Figure 3: Receiver operating characteristic (ROC) obtained from binary logistic regression model which generates significant predictors of AMD. Area under the curve is reported to be 82.7%.

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Single Nucleotide Polymorphisms in MCP-1 and Its Receptor Are Associated with the Risk of Age Related Macular Degeneration

Akshay Anand[1*☯], Neel Kamal Sharma[1☯], Amod Gupta[2], Sudesh Prabhakar[1], Suresh Kumar Sharma[3], Ramandeep Singh[2], Pawan Kumar Gupta[1]

1 Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, **2** Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, **3** Department of Statistics, Panjab University, Chandigarh, India

Abstract

Background: Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population. We have shown previously that mice deficient in monocyte chemoattractant protein-1 (MCP1/CCL2) or its receptor (CCR2) develop the features of AMD in senescent mice, however, the human genetic evidence so far is contradictory. We hypothesized that any dysfunction in the CCL2 and its receptor result could be the contributing factor in pathogenesis of AMD.

Methods and Findings: 133 AMD patients and 80 healthy controls were enrolled for this study. Single neucleotid Polymorphism for CCL2 and CCR2 was analyzed by real time PCR. CCL2 levels were determined by enzyme-linked immunosorbent assay (ELISA) after normalization to total serum protein and percentage (%) of CCR2 expressing peripheral blood mononuclear cells (PBMCs) was evaluated using Flow Cytometry. The genotype and allele frequency for both CCL2 and CCR2 was found to be significantly different between AMD and normal controls. The CCL2 ELISA levels were significantly higher in AMD patients and flow Cytometry analysis revealed significantly reduced CCR2 expressing PBMCs in AMD patients as compared to normal controls.

Conclusions: We analyzed the association between single neucleotide polymorphisms (SNPs) of CCL2 (rs4586) and CCR2 (rs1799865) with their respective protein levels. Our results revealed that individuals possessing both SNPs are at a higher risk of development of AMD.

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* E-mail: akshay1anand@rediffmail.com

☯ These authors contributed equally to this work.

Introduction

Age related macular degeneration is the leading cause of irreversible blindness in the elderly population [1,2]. AMD is of two types: early and late. In the early stage of disease there is presence of drusen with pigmented and hyperpigmented area. After the disease progresses with time, it enters into the second stage i.e. the late stage. The early one is dry or atrophic AMD, which is marked by geographic atrophy or sharply demarcated area of depigmentation caused by waste by products of the retinal pigment epithelium (RPE) and photoreceptors. The late stage of disease is called wet AMD as it occurs because of the growth of new blood vessels under the RPE and neurosensory retina, which results in subretinal bleeding and subsequent scar formation [3]. The complete mechanism of age-related macular degeneration (AMD) is not well understood. In recent years, there has been increasing evidence of an inflammatory component in AMD. It has been found to be associated with polymorphism of complement factor H (CFH) [1,2], a polymorphism which leads to an overactivation of the complement system [3], emphasizing the importance of inflammatory mediators in AMD.

During past few years, certain studies have also focused on the role of chemokines in the progression of AMD. Although the mechanisms underlying the regulation of these cytokines in the eye of patients with AMD remain unclear, chemokines like MCP-1, while acting in concert with receptor CCR2, promote recruitment of macrophages [4]. We hypothesized that any dysfunction in the CCL2 and CCR2 results in impaired macrophage recruitment and debris formation under the retinal pigment epithelium (RPE) contributions to AMD. CCL2 gene is located on chromosome 17q11.2 while CCR2 is located on chromosome 3p21.31.We previously described the spontaneous development of CNV in senescent mice deficient in CCL2 or its CCR2 receptor [4]. Besides, many recent reports have suggested that inflammation is the major cellular process that plays main role in the pathogenesis of AMD [5] and its development to CNV [6]. Some RPE cells play essential role in the maintenance of outer retina by secreting cytokines including CCL2 [7], which have been suggested to be

Table 1. Description of SNPs genotyped.

Gene (RefSeq)	SNP	Chromosome position	Position in reference to 5′ UTR	Amino acid translation	Minor Allele
CCL2 (NM_0029823)	rs4586	17q11.2	+T974C	Cys35Cys	C
CCR2 (NM_0006482)	rs1799865	3p21.31	+T4439C	Asn260Asn	C

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implicated in the pathogenesis of AMD [8]. RPE cells can secrete CCL2 in the direction of choroidal blood vessels during inflammatory reaction suggesting that RPE cells might promote macrophage recruitment to the choroid from circulating monocytes.

There are a few studies which have examined SNPs of the chemokine system with AMD susceptibility but did not find any evidence of association between CCL2, CCR2 and AMD [9,10]. The absence of any such genetic association studies between CCL2 or CCR2 and AMD from Indian patients prompted us to explore the role of these chemokines in these patients. We analyzed whether single nucleotide polymorphism (SNP) variants in the CCL2 or CCR2 loci independently or in combination are associated with AMD as different ethnic groups may exhibit a varying spectrum of SNPs.

Methods

Study Population

The study was approved by the Ethics Committee of Post-Graduate Institute of Medical Education and Research, Chandigarh, India vide letter No Micro/10/1411. The written informed consent was obtained from participants for the study, as well as for the publication of the data obtained after retrieval of medical records, besides use of blood and DNA for AMD related research project. All the patients were scored at the base line. Individuals with AMD in at least one eye were recruited between 2008 to 2011 from Advanced Eye Centre, Post-Graduate Institute of Medical Education and Research, Chandigarh (PGIMER), India.

We included 213 case-control samples consisting of 133 AMD patients from Eye Centre, PGIMER, with 80 genetically unrelated healthy controls as per inclusion and exclusion criteria described below. Out of 133 AMD and 80 control samples, about nine samples were not included in the analysis due to delayed refrigeration. The limited sample size of this study needs to be addressed by larger studies even though many previous investigators have examined comparable sample size [11,12]. The strength of our study, however, lies in the ethnically homogeneous nature of population which was enrolled from a single largest tertiary care centre in the region catering to over 1,50,000 general patients annually.

Table 2. Demographic characteristics of Controls and AMD patients.

Variables	AMD	Controls
Total	133	80
Wet AMD	95 (71.4%)	–
Dry AMD	38 (28.6%)	–
Avastin treated	68	
Not treated with Avastin	27	
Duration of disease[¥]	23 ± 2.6 (M)	
Age†	66.56 ± 7.6	54.24±7.01
Male	88 (66.2%)	57 (71.2%)
Female	45 (33.8%)	23 (28.7%)

Clinical and demographic details of subjects. AMD, age related macular degeneration; M, Months; Age, Age of onset; Values are mean ± SD or (percentage),

†Unpaired, independent 2-tailed student t test analysis showed that mean age differ significantly among the groups ($p = 0.02$),

¥Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset.

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Inclusion and Exclusion Criteria

The inclusion criteria for patients in both groups included those with age 50 years or older with the diagnosis of AMD. AMD was defined by geographic atrophy and/or choroidal neovascularization with drusen more than five in at least one eye. The controls constituting the study included those that were of age 50 years or older and had no drusen or no more than 5 drusen with absence of other diagnostic criteria for AMD.

The exclusion criteria included the retinal diseases involving the photoreceptors and/or outer retinal layers other than AMD loss such as high myopia, retinal dystrophies, central serious retinopathy, vein occlusion, diabetic retinopathy, uveitis or similar outer retinal diseases that have been present prior to the age of 50 and opacities of the ocular media, limitations of papillary dilation or other problems sufficient to preclude adequate stereo fundus photography. These conditions include occluded pupils due to synechiae, cataracts and opacities due to ocular diseases.

Diagnosis of AMD

A retina specialist diagnosed all patients by ophthalmologic examination for best corrected visual acuity, slit lamp biomicroscopy of anterior segment and dilated fundus examination. All AMD patients were subjected to optical coherence tomography (OCT) and fluorescein fundus angiography (FFA). The diagnosis of AMD was based on FFA and ophthalmoscopic findings.

Demographic Information

The demographic details were obtained by a trained interviewer using a standardized risk factor questionnaire. A written informed consent form signed by each participant, which included the written risk factor questionnaire was taken from each participant. The details such as age, sex, race, smoking etc as self reported by participants were entered in the data base for analysis. Smokers were defined as having smoked at least 1 cigarette per day for at least 6 months and divided into smokers and never smokers. Comorbidity was determined based on the participant's responses

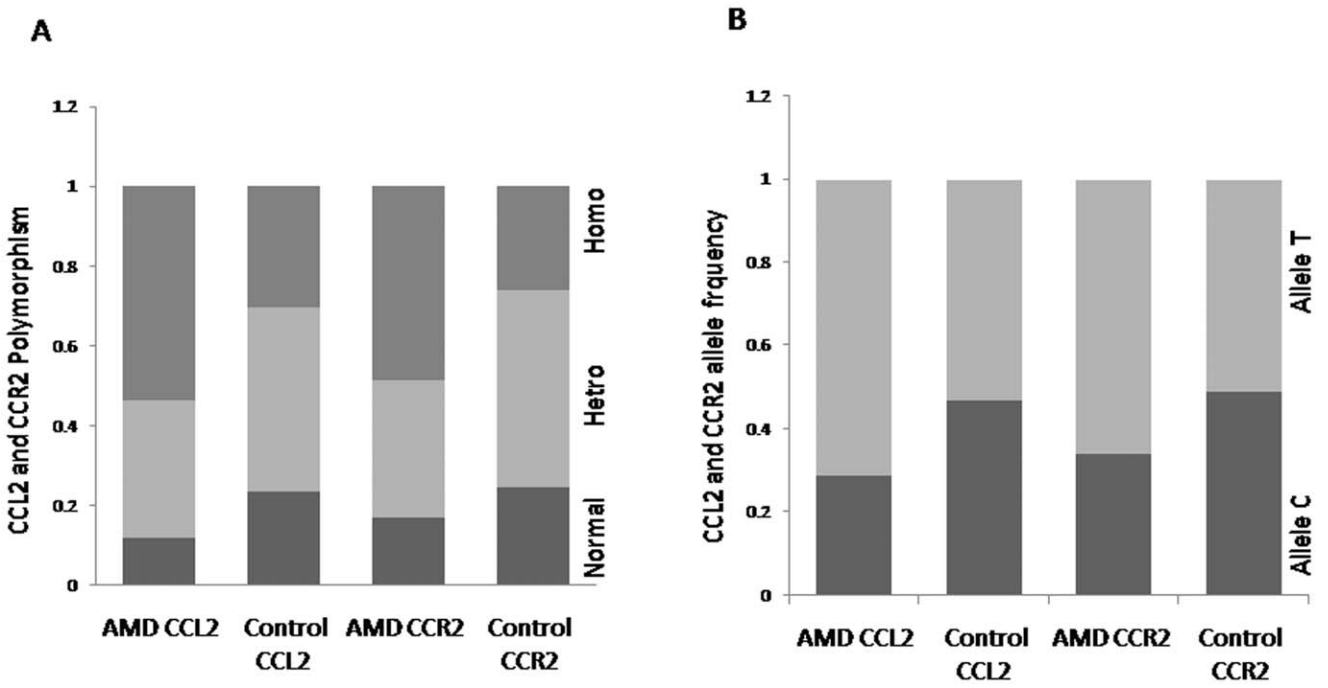

Figure 1. A) Genotype distribution (y-axis) of CCL2 and CCR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages. B) Allele frequency (y-axis) of CCL2 and CCR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages.
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to whether a physician had ever told them for diagnosis of any major neurological, metabolic or cardiovascular illness.

Selection of Single-nucleotide Polymorphisms

The selected single-nucleotide polymorphisms (SNPs) in our study were either previously studied in other ethnic populations for association with AMD or other inflammatory diseases and chosen due to their reputed functional significance. The details are enumerated in Table 1.

Serum, PBMCs and DNA Isolation

About 8.0 ml of blood sample was collected from all subjects. About 3.0 ml of blood sample was left for 1 hour at 37°C and allowed to clot. Serum was subsequently separated in serum separator tube (BD Biosciences, USA) after centrifugation at 3000 rpm for 30 minutes. From rest of the blood PBMCs were isolated as per Histopaque-1077 (Sigma, USA) instruction sheet provided by the vendor. Briefly, 5.0 ml blood was layered on equal volume of Histopaque-1077 followed by centrifugation at 1800 rpm for 30.0 mins at room temperature. PBMCs were collected from plasma/Histopaque-1077 interface. Aliquots of PBMCs were stored in 90% fetal bovine serum (FBS, HiMedia, India) + 10% dimethyl sulphoxide (DMSO, Sigma, USA) and kept at −80°C until flow cytometry was done. Genomic DNA was extracted from PBMCs using a commercially available genomic DNA extraction and purification kit (INVITROGEN and QIAGEN) according to the manufacturer's protocol. The samples were labeled, coded and stored.

Real Time PCR

SNP (Single neucleotide polymorphism) was analyzed by using real time PCR, and was performed in the 48 wells model Step One™ (Applied Biosystems Inc., Foster city, CA) using published TaqMan® SNP Genotyping Assays. Real time PCR was carried out for 20.0 µl containing 10 ul master mix, 5 ul Assay (Applied Biosystems), 20 ng DNA and molecular biology grade water was added to make the volume 20.0 µl. All reactions were carried out using TaqMan® SNP Genotyping Assays (Applied Biosystems) according to manufacturer's recommendations. Two reporter dyes VIC and FAM were used to label the Allele 1 and 2 probes and a 5′ Nuclease Assay was carried out. Negative controls included the PCR mix without DNA. Software StepOneTM v 2.0 (Applied Biosystems Inc., Foster city, CA) was used to perform amplification and to estimate SNP. After PCR amplification the Sequence Detection System (SDS) Software was used to import the fluorescence measurements made during the plate read to plot fluorescence (Rn) values.

Total Protein Estimation

Total protein was estimated using Bradford assay. The estimation of total protein was performed according to manufacturer's recommendations. Briefly, serum samples were diluted 1500 times in double distilled water. Bovine Serum Albumin (BSA) served as the standard. Diluted samples and BSA standard protein were mixed with coomassie brilliant blue G–250 dye (Bradford reagent) in 4:1 ratio followed by incubation at room temperature for 10–15 minutes. The absorbance was read at 595 nm in Microplate reader (680XR Biorad, Hercules, CA, USA). The standard curve of BSA was estimated with linear or quadratic fit models.

Enzyme Linked Immunosorbant Assay (ELISA)

The expression of CCL2 was analyzed using commercially available enzyme linked immunosorbant assay (RayBio, Norcross, Cat#: ELH-MCP1-001) as per manufacturer's protocol and absorbance was read at 450 nm in Microplate reader (Biorad

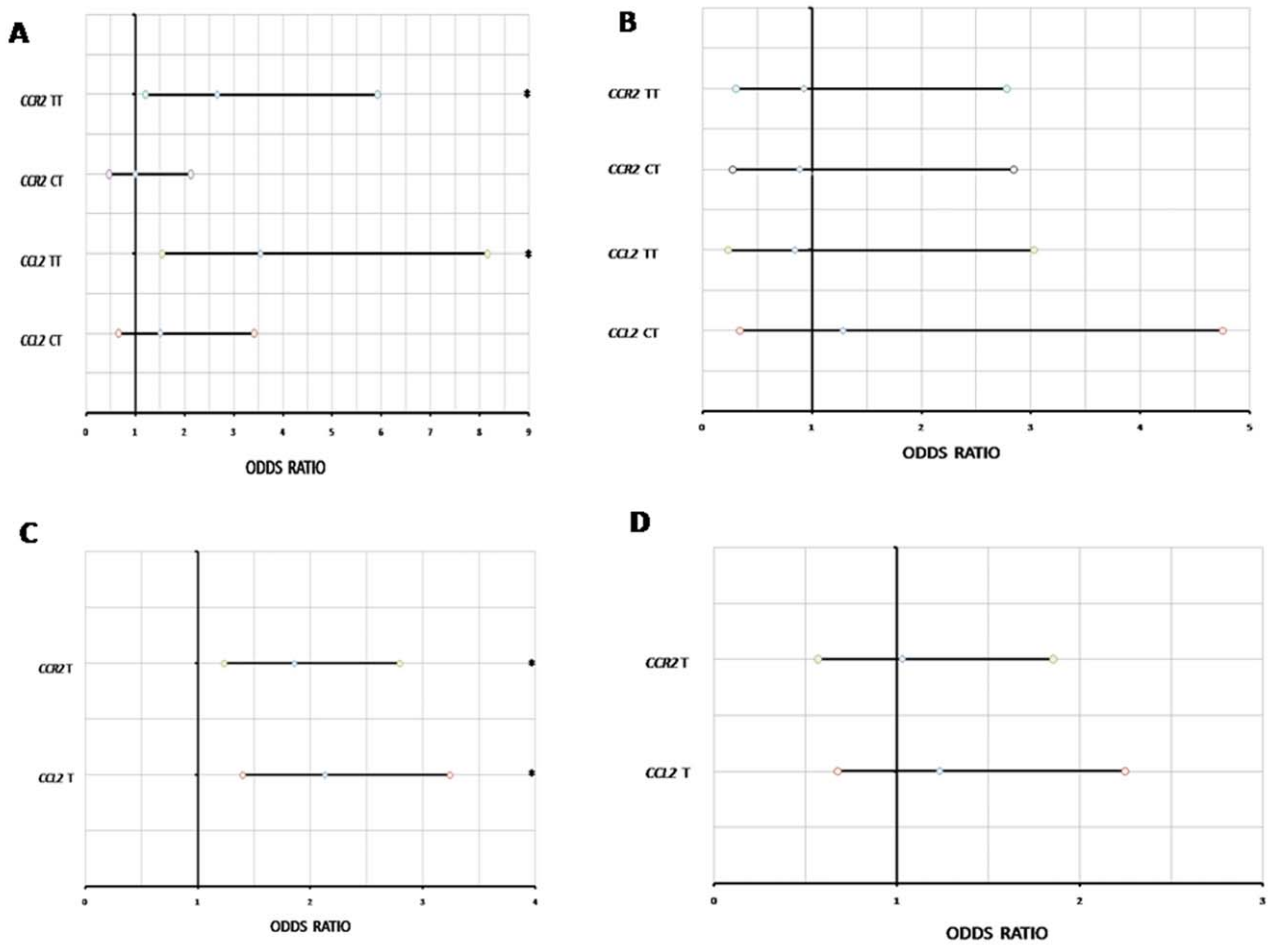

Figure 2. A) Univariate logistic regression analysis in AMD patients with CCL2 and CCR2 polymorphisms as independent and normal controls as a dependent variable. B) Univariate logistic regression analysis in Wet AMD patients with CCL2 and CCR2 polymorphisms as independent and Dry AMD as a dependent variable. C) Univariate logistic regression analysis in AMD patients with CCL2 and CCR2 alleles frequency as independent and normal controls as a dependent variable. D) Univariate logistic regression analysis in Wet AMD patients with CCL2 and CCR2 alleles frequency as independent and Dry AMD as a dependent variable. *p,0.05.
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680XR, Hercules, CA, USA). Sample assays were performed in duplicate. This assay recognizes recombinant human CCL2 with minimum detectable dose of CCL2 typically less than 2 pg/ml. The standard curve was plotted using linear model and results were obtained after normalization with total protein.

Flow Cytometry

Flow cytometry was used to study the expression levels of surface receptors namely hCCR2 in PBMCs of normal subjects and AMD patients. $\sim 3\times10^5$ PBMCs were initially processed for blocking with Fc blocker (1.0 µg, purified human IgG, R&D Systems Inc., Minneapolis, MN, USA) for 15 mins at room temperature with 0.2 ml of 0.1% sodium azide (Sigma, Germany) in 1× Ca^{2+} and Mg^{2+} free phosphate buffer saline (PBS) (HiMEDIA, India, pH = 7.2–7.4). Cell suspension was then incubated with primary labeled anti-hCCR2 - Allophycocyanin (0.1 µg, R&D Systems Inc., Minneapolis, MN, USA) antibody for 45 mins on ice in dark in 0.2 ml of fluorescence-activated cell sorter (FACS) buffer. Labeled antibody incubation was followed by two washings with 1× PBS at 5,000 rpm for 5 mins at 4°C. Finally, the cells were reconstituted in 250.0 µl of 1X PBS and analyzed in flow cytometer. Approximately 10,000 viable PBMCs were gated based on their forward and side scatter profile, and acquired in each run. PBMCs gate was set to include both lymphocytes and monocytes where maximum CCR2 fluorescence was observed. Same gating was used between the experiments. Background signal was measured for each sample by acquiring unlabeled PBMCs as negative controls and normalized to the signal obtained from anit-hCCR2 labeled PBMCs. Acquired cells were then verified for expression of CCR2. All the analysis was done by acquisition of data within one hour of incubation on FACS CANTO (BD Biosciences, San Jose, CA) flow cytometer using FACS DIVA software (Becton Dickinson).

Statistical Analysis

In order to see whether the data is normally distributed, Normal-quantile (Q-Q) plots were constructed. After establishing the normality for wet AMD cases, a parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post-hoc test was applied to compare multiple groups. For comparison of two groups unpaired, student-t test with equal or unequal variance (Welch's correction) was applied. For

Table 3. Effect of CCL2 rs4586 and CCR2 rs1799865 variants on disease phenotype.

			Unadjusted p value			Multivariate analysis, adjusted for age			Multivariate analysis, adjusted for gender		
Genotype	**Number (frequency)**		**OR**	**95%CI**	**p-Value**	**OR**	**95%CI**	**p- Value**	**OR**	**95%CI**	**p- Value**
CCL2 rs4586											
	AMD	**Controls**									
CC	15 (0.118)	18 (0.236)	Reference			Reference			Reference		
CT	44 (0.346)	35 (0.461)	1.509	0.667–3.413	0.324	0.950	0.227–3.980	0.944	1.523	0.665–3.486	0.320
TT	68 (0.536)	23 (0.303)	3.548	1.543–8.157	0.003	0.517	0.107–2.494	0.411	0.300	0.129–0.695	0.005
	Wet AMD	**Dry AMD**									
CC	11(0.118)	4 (0.118)	Reference			Reference			Reference		
CT	30 (0.323)	14 (0.412)	1.283	0.347–4.749	0.709	2.450	0.388–15.46	0.340	1.254	0.335–4.686	0.737
TT	52 (0.559)	16 (0.471)	0.846	0.237–3.026	0.797	0.334	0.051–2.191	0.253	1.338	0.364–4.915	0.661
CCR2 rs1799865											
	AMD	**Controls**									
CC	22 (0.172)	19 (0.246)	Reference								
CT	44 (0.344)	38 (0.494)	1.00	0.472–2.121	1.00	2.147	0.558–8.232	0.267	1.00	0.472–2.212	0.999
TT	62 (0.484)	20 (0.260)	2.677	1.210–5.924	0.015	0.126	0.023–0.679	0.016	0.379	0.171–0.840	0.017
	Wet AMD	**Dry AMD**									
CC	16 (0.168)	6 (0.182)	Reference								
CT	33 (0.347)	11 (0.333)	0.889	0.279–2.836	0.842	0.404	0.072–2.249	0.301	0.875	0.275–2.789	0.822
TT	46 (0.484)	16 (0.485)	0.928	0.310–2.779	0.893	1.058	0.210–5.330	0.945	1.108	0.376–3.261	0.853

This table summarizes the genotype frequencies for the single-nucleotide polymorphisms (SNPs) in CCL2 rs4586 and CCR2 rs1799865 among patients with age-related macular degeneration (AMD) and control subjects. Genotype distributions were in Hardy-Weinberg equilibrium. The p-value represents comparison of risk significance between AMD cases and controls. OR indicates odds ratio and CI refers to confidence interval.
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Table 4. Allele frequency of CCL2 and CCR2 in AMD and Normal controls.

Allele	**Number (frequency)**		**OR**	**95%CI**	**p- Value**
CCL2 rs4586					
	AMD	**Controls**			
C	74 (0.29)	71 (0.47)	**Reference**		
T	180 (0.71)	81 (0.53)	2.132	1.403–3.238	0.0003
	Wet AMD	Dry AMD			
C	52 (0.28)	22 (0.32)	**Reference**		
T	134 (0.72)	46 (0.68)	1.232	0.676–2.246	0.49
CCR2 rs1799865					
	AMD	**Controls**			
C	88 (0.34)	76 (0.49)	**Reference**		
T	168 (0.66)	78 (0.51)	1.86	1.237–2.796	0.002
	Wet AMD	Dry AMD			
C	65 (0.34)	23 (0.35)	**Reference**		
T	125 (0.66)	43 (0.65)	1.028	0.571–1.852	0.92

This table summarizes the allele frequencies for the single-nucleotide polymorphisms (SNPs) in CCL2 rs4586 and CCR2 rs1799865 among patients with age-related macular degeneration (AMD) and control subjects. The p-value represents comparison of risk significance between AMD cases and controls. OR indicates odds ratio and CI refers to confidence interval.
doi:10.1371/journal.pone.0049905.t004

non-normal data, a non-parametric Kruskal-Wallis H test followed by Mann-Whitney-U test was applied. The real time PCR estimated genotypes for each mutation were stratified for heterozygosity, and homozygosity for the respective allelic variant. Pearson's Chi-square test was applied to study the association between various groups. Genotype distributions were analyzed by logistic regression, integrating adjustments for age and gender. Genotypic associations and odds ratios (ORs) with 95% confidence intervals (CI) were estimated by binary logistic regression. The $p \leq 0.05$ was considered to be significant. Statistical analysis was performed with the help of SPSS 16.0 software.

Results

Summary statistics of all-important variables have been obtained and reported in Table 2.

rs4586 and rs1799865 Polymorphism in AMD Patients

To analyze the spectrum of polymorphism in CCL2 and CCR2 gene, real time PCR was used. The genotypes were in Hardy-Weinberg equilibrium. Genotype and allele frequencies of the polymorphisms of the genes CCL2 and CCR2 have been listed in the Table 3, 4 and Figure 1. The genotype and allele frequency for both CCL2 and CCR2 was found to be significantly different between AMD and normal controls. The TT genotype was more frequent in AMD patients than in controls for both CCL2 and CCR2 (OR = 3.548, p = 0.003, CI = 1.543–8.157 and OR = 2.677, p = 0.015, CI = 1.210–5.924, respectively, Table 3; Figure 2A). The study showed that the TT risk variant of CCL2 and CCR2 is associated with AMD (Figure 2A). The individuals having CT genotype in CCL2 and CCR2 revealed no risk of

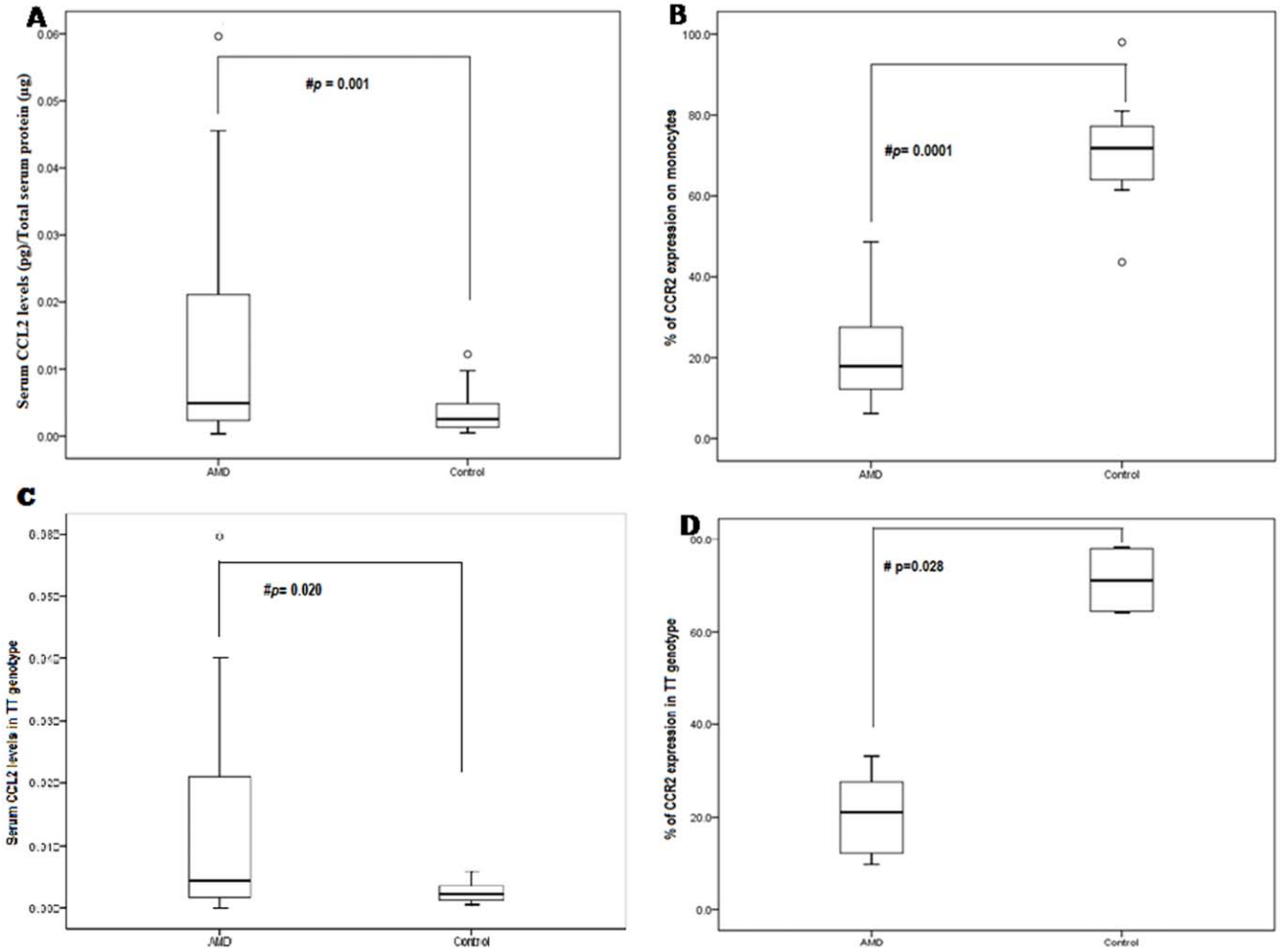

Figure 3. A) Serum levels of CCL2 in AMD and normal controls. B) Percentage (%) of PBMCs expressing CCR2 protein in AMD patients and Normal controls. C) Serum levels of CCL2 in TT genotype of AMD and normal controls. D) Percentage (%) of PBMCs expressing CCR2 protein in TT genotype of AMD patients and Normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Levels of CCL2 were normalized to total protein. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. AMD, Age Related Macular Degeneration; CCL2, Chemokine ligand 2; CCR2, Chemokine Receptor 2; pg, picogram; µg, microgram.
doi:10.1371/journal.pone.0049905.g003

developing AMD (Figure 2A). Logistic regression analysis for food habits, existence of comorbidity and smoking habit revealed no significant difference between vegetarian/non-vegetarian, existence of comorbidity/without comorbidity and smokers/non-smokers AMD patients. However, when the comparison was done between AMD and controls, we found that TT genotype was more frequent among vegetarian AMD individuals than in vegetarian controls for CCL2 (OR = 5.574, p = 0.010, CI = 1.510–20.572, Table S1), TT genotype was more frequent in Non-vegetarian AMD than in Non-vegetarian controls for CCR2 (OR = 6.629, p = 0.008, CI = 1.652–26.59 Table S1) emphasizing the association of TT genotype in AMD. The AMD smokers and AMD never smokers showed significant TT frequency as compared to control smokers and control never smokers for CCL2 (OR = 5.80, p = 0.040, CI = 1.081–31.112 and OR = 3.380, p = 0.019, CI = 1.223–9.347, Table S2) and TT frequency was significantly higher in AMD smokers as compared to control smokers for CCR2 (OR = 15.6, p = 0.016, CI = 1.662–146.4, Table S2). However, there was no significant difference on the basis of comorbidity for CCL2 and CCR2 genotypes (Table S3). The frequency of allele T in CCL2 (rs4586) was found to be significantly higher in AMD patients (0.71%) as compared to the controls (0.53%) (OR = 2.132, p = 0.0003, CI = 1.403–3.238, Table-4, Figure 2C). CCR2 (rs1799865) allele frequency of allele T was also significantly higher in AMD patients (0.66%) as compared to the controls (0.51%) (OR = 1.86, p = 0.002, CI = 1.237–2.792, Table 4, Figure 2C). We did not find any significant difference in genotype and allele frequency between wet and dry AMD patients (Table 3&4; Figure 2B&D). The difference was also not significant when compared between wet AMD patients ie minimally classic, predominantly classic and occult (data not shown). There was no significant difference when compared between those wet variant of AMD patients who received Avastin treatment (dose 1.25 mg in 0.05 ml) and those that did not (data not shown).

Multiple Logistic Regression Analysis

To analyze the association of genetic polymorphism and other risk factors with AMD simultaneously, we performed uncondi-

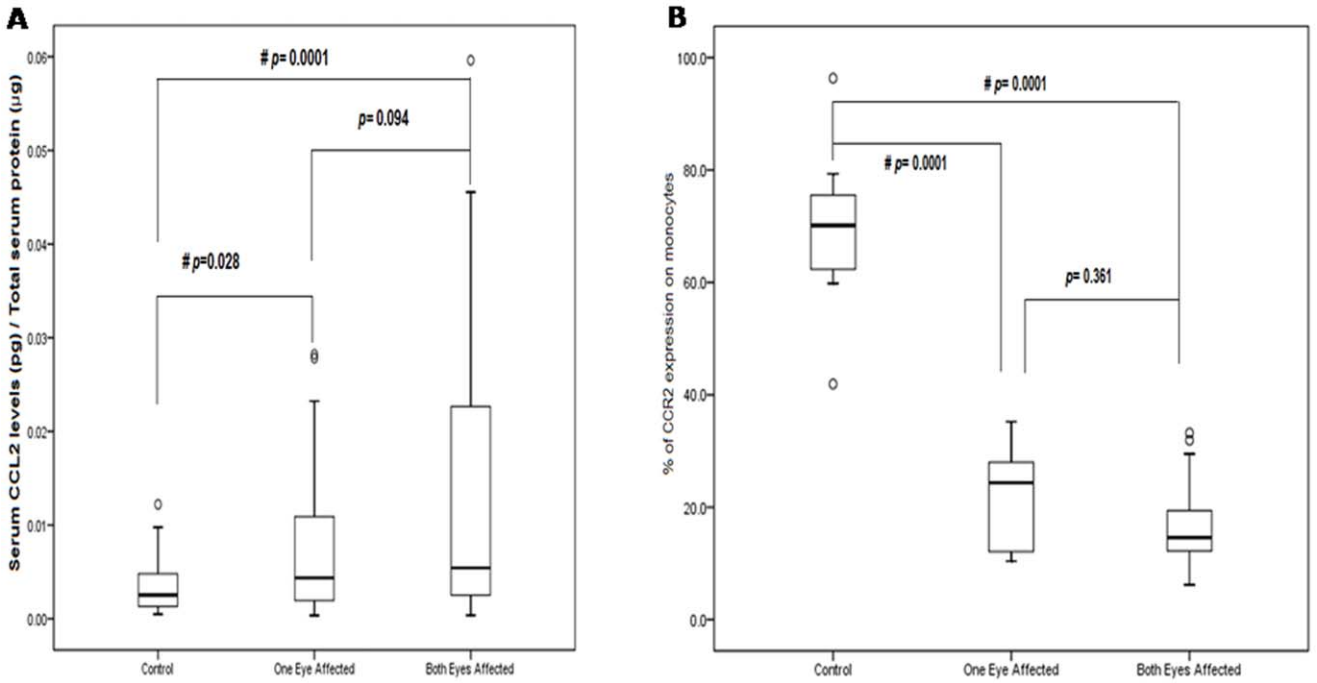

Figure 4. A) Serum levels of CCL2 in normal controls, AMD patients affected in one eye and AMD patients affected in both eyes. B) Percentage (%) of PBMCs expressing CCR2 protein in normal controls, AMD patients affected in one eye and AMD patients affected in both eyes. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Levels of CCR2 were normalized to total protein. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. CCL2, Chemokine ligand 2; CCR2, Chemokine Receptor 2; pg, picogram; µg, microgram.
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tional logistic regression analysis and obtained optimized model. We analyzed both age and gender as risk factors which have been shown to be associated with AMD previously. The Hosmer-Lemenshow test shows that the data fits well to the logistic regression ($p = 0.70$). When multiple logistic regression analysis was carried out for age adjustment, we found that TT genotype showed significantly higher frequency for CCR2 rs1799865 in AMD as compared to controls (OR = 0.126, $p = 0.016$, and CI = 0.023–0.679, Table-3) and multiple logistic regression adjustment analysis for gender showed that TT genotype was at significantly higher frequency for CCL2 rs4586 and CCR2 rs1799865 for AMD patients (Table-3). Gender adjustment also showed significant difference in genotype TT for Vegetarian AMD, never smokers AMD (CCL2 rs4586) and comorbidity and smoker AMD (CCR2 rs1799865 Table S1, S2, S3).

Decreased CCR2 and Increased CCL2 Levels

ELISA estimation revealed elevated levels of serum CCL2 in AMD patients as compared to normal controls (Figure 3 A; $p = 0.001$). No difference was observed in CCL2 levels for wet and dry AMD ($p = 0.327$). CCL2 concentration was significantly elevated in the patients affected in one or both eyes with AMD as compare to controls (Figure 4A). However, flow cytometry analysis of PBMCs of AMD patients and normal controls indicates a significant decrease in proportion of CCR2 expressing PBMCs from AMD patients than those from normal controls (Figure 3B & 5; $p = 0.0001$). We found no significant difference in their expression between Dry and Wet AMD samples ($p = 0.934$). CCR2 expression was significantly lower in the patients affected in one eye or both eyes with AMD as compared to controls but the difference was not significant between one eye affected and both eyes affected (Figure 4B). The CCL2 ELISA and CCR2 FACS levels were not significant when compared between avastin treated & untreated wet AMD patients and between different classes of wet AMD i.e. minimally classic, predominantly classic and occult (data not shown). No association of cigarette smoking, alcohol and meat consumption with CCR2 and CCL2 levels in serum was observed upon univariate and multivariate analysis. The levels of CCL2 determined by ELISA and CCR2 expression estimated by FACS were corresponded to the TT polymorphism in CCL2 and CCR2 in between AMD and controls (Figure 3C&D).

Discussion

The current study suggests that inflammation is essential part of the pathogenesis of AMD in the Indian AMD patients. After examining the involvement of gene polymorphism and levels of inflammatory genes with the risk of AMD, it is suggested that genetic variations in the genes encoding the inflammatory processes might confer susceptibility to AMD by altering the expression of these cytokines. The presence of risk genotype of these genes may increase the risk of AMD.

We examined the levels of CCL2, percentage of cells expressing CCR2 and two variants of these pro-inflammatory cytokine genes which have been studied for other ethnic populations for AMD [9] and shown to be linked with inflammatory diseases [13,14] and were functional variants affecting expression or function of these genes. It must be mentioned that SNPs from CCL2 are previously known to affect CCL2 protein levels [15]. In acute inflammation expression of CCL2 in the retina and RPE increases [16–18], with oxiative stress in the RPE [19]. A recent study had shown that subretinal microglial cells (MCs) induce CCL5 and CCL2 in the

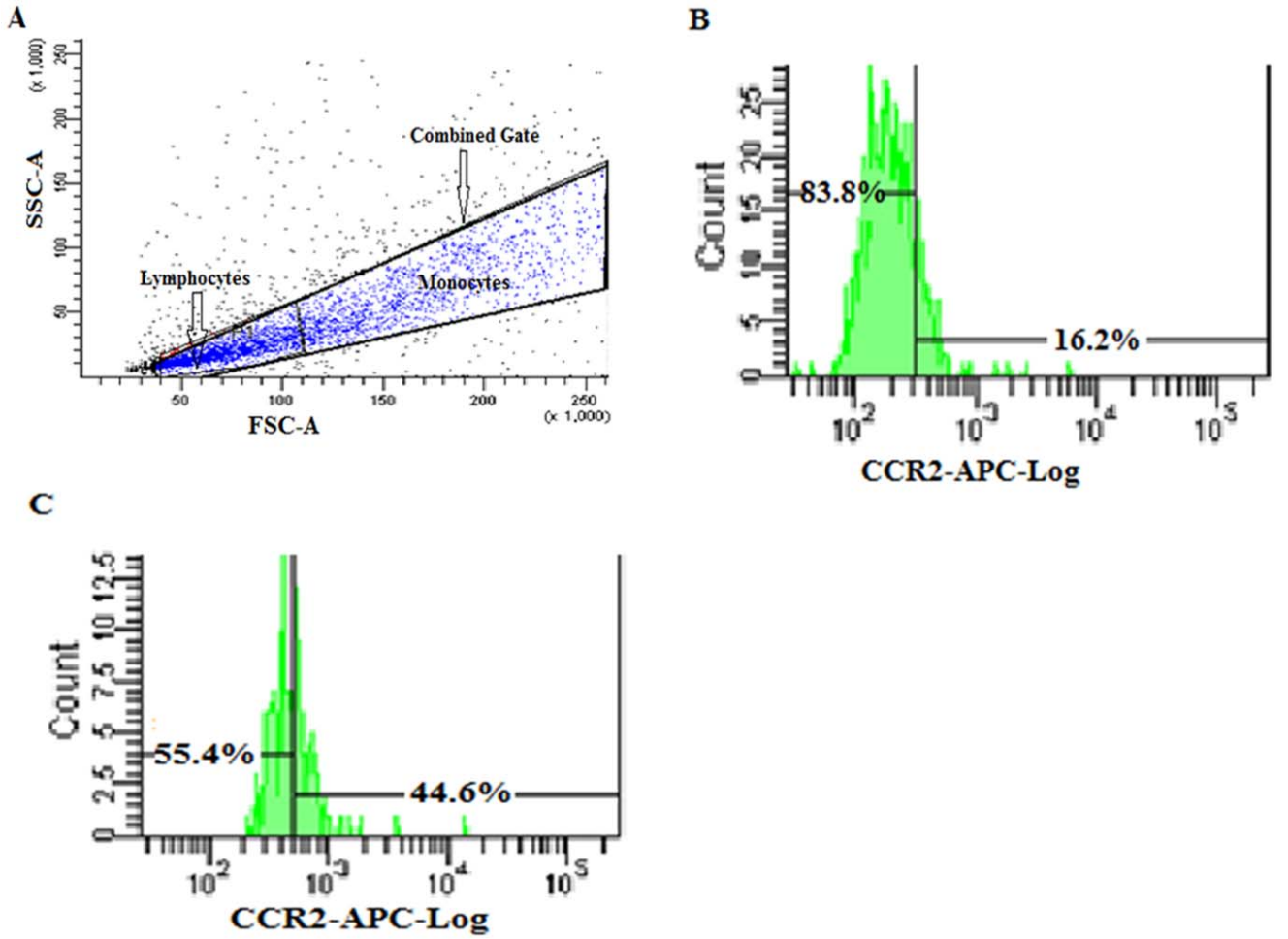

Figure 5. Percentage (%) of CCR2 + PBMCs in AMD patients and normal control subjects as measured by Flow Cytometry. (A) Dot plot showing side and forward scatter analysis of purified unlabeled PBMCs (large combined gate) from a AMD patient. PBMCs consists of two distinct populations namely lymphocytes and monocytes. Approximate lymphocytes and monocytes populations are indicated as smaller gates. Events outside the PBMCs gate represent cell debris and granulocytes. Same gating has been used for PBMCs from each AMD and normal control sample. ~10,000 events have been acquired in each experiment. X-axis represents population cell size in forward scatter (FSC) and y-axis represents population cell granularity in side scatter (SSC). (B,C) Single parameter representative histogram of flow cytometric expression pattern of CCR2 on gated PBMCs is showing decreased number of CCR2 expressing PBMCs in AMD (16.2%; B) as compared to normal control (44.6%; C). Number of cells is represented along y-axis and blue APC fluorescence along x-axis. Appropriate unlabeled PBMCs were used to set marker in histogram and measure background fluorescence. APC, allophycocyanin; CCR2, chemokine receptor 2; PBMCs, peripheral blood mononuclear cells; AMD, Age related macular degeneration.
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RPE [20]. CCL2 mainly signals through CCR2 [21]. It has been shown that CCL2/ CCR2 signaling is involved in monocyte or microglial cells enrollment after laser injury [22]. Microglial cells or CCR2-expressing monocytes are present at some point in these models. In a clinical study Jonas et al showed that elevated intraocular levels of CCL2 are associated with exudative AMD [23] and in a mouse model of CNV [16]. CCL2 might therefore play a role in monocyte and MC recruitment to the subretinal space in AMD.

Besides our own work there are numerous reports using CCL2−/− or CCR2−/− mice in an attempt to translate the inflammatory mechanisms of AMD. Recently Chen et al has also shown that aged CCL2 or CCR2 deficient mice develop certain features of atrophic, but not angiogenic AMD-like changes, and represent an animal model for early stage human geographic atrophy [24]. Several studies have examined AMD susceptibility and analyzed SNPs from chemokine family. However, no evidence has been found for an association between common genetic variations of CCR2 and CCL2 with the etiology of AMD [9,10] but this did not include North Indian patients. However, functional polymorphisms in these genes has been found to play a significant role in the development of other inflammatory diseases [13,25,26]. A family of structurally related chemotactic cytokines comprise chemokines that direct the migration of leukocytes throughout the body, both under pathological and physiological conditions [27]. CCR2 and CCL2 are key mediators in the infiltration of monocytes into foci of inflammation from blood. The CCL2 protein is expressed ubiquitously and exerts its effect after binding to its receptor CCR2 which leads to shape change, actin rearrangement and monocytes movement [28]. As CCL2 and CCR2 genes were considered as potential candidates genes in AMD animal model studies, we analyzed the evidence from genetic variation of CCL2 and CCR2 in human despite conflicting reports. The results of these finding support the

postulation that mice deficient in these genes develop hallmarks of AMD [4] (i.e. lipofuscin, accumulation of drusen, photoreceptor atrophy, and CNV). The presence of AMD-like disease in these knockout mice had raised questions of whether CCR2 and CCL2 play a role in human AMD. On examining the two variants of these inflammatory cytokines it was found that these alleles and genotypes are in Hardy-Weinberg Equilibrium in AMD and control subjects. Earlier studies in animal models have shown that CCL2 and CCR2 are involved in the pathogenesis of AMD [4,29,30]. We have examined single polymorphism for CCL2 (rs4586) and CCR2 (rs1799865) with their levels for susceptibility of AMD. The CCL2 transcription may be influenced by the CCL2 (rs4586) SNP, which may act in association with the CCR2 (rs1799865) SNP, impacting the biological activity of the CCR2 receptor, and the CCL2/CCR2 messenger system.

Our study has revealed that the levels of CCL2 were higher and number of cells expressing CCR2 were lower in AMD patients as compared to controls which could be ascribed to the varying physiology of primates and rodents. This might be explained by proposing the activation of a negative feedback seeking to limit the inflammation caused by extravasations of activated monocytes/lymphocytes at the site of macular degeneration. We also found that the levels of CCL2 or percentage of cells expressing CCR2 did not significantly increase or decrease in the patients affected in one eye or those affected in both eyes. We are unable to rule out the local difference in CCL2 and CCR2 because we did not analyze the respective autopsies. The levels of CCL2 in TT genotype of rs4586 was significantly higher in AMD patients as compared to normal controls and the percentage of cells expressing CCR2 were significantly lower in TT genotype of rs1799865 in AMD patients as compared to normal controls which we are unable to explain. The risk of disease increases in individuals 2.6–3.5 times in those who present with genotype TT as compared to CC within both CCR2 (rs1799865) and CCL2 (rs4586) respectively. Individuals with T allele have higher risk of 1.8–2.1 times for developing AMD as compared to C allele for both CCR2 (rs1799865) and CCL2 (rs4586) respectively. We did not find any significant difference between food habit, comorbidity and smoking for AMD patients which indicates no association with disease.

To the best of our knowledge this is the first study suggesting synergy between the SNPs of CCL2 (rs4586) and its receptor CCR2 (rs1799865) with their protein levels in the development of AMD. Additional studies in larger populations comparing Asian and African and North Americans are needed to validation with larger sample size to allow for the confirmation or negation of an independent role of each of these SNPs on the risk of AMD development or verifying their mutual properties.

Supporting Information

Table S1 Logistic regression of the association CCL2, CCR2 and progression of AMD stratified by food habits.
(DOC)

Table S2 Logistic regression of the association CCL2, CCR2 and progression of AMD stratified by smoking.
(DOC)

Table S3 Logistic regression of the association CCL2, CCR2 and progression of AMD stratified by comorbidity.
(DOC)

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Author Contributions

Conceived and designed the experiments: AA. Performed the experiments: NKS. Analyzed the data: AA NKS SKS PKG. Contributed reagents/materials/analysis tools: AA SP. Wrote the paper: AA NKS. Inclusion of patients and clinical scoring: AG RS.

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Single Nucleotide Polymorphism and Serum Levels of VEGFR2 are Associated With Age Related Macular Degeneration

Neel Kamal Sharma[1], Amod Gupta[2], Sudesh Prabhakar[1], Ramandeep Singh[2], Suresh Sharma[3] and Akshay Anand[1,*]

[1]*Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India*

[2]*Department of Ophthalmology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India*

[3]*Department of Biostatistics, Panjab University, Chandigarh, India*

Abstract: Age-related macular degeneration (AMD) is a leading cause of blindness and is the third leading cause of blindness. Genetic factors are known to influence an individual's risk for developing AMD. Linkage has earlier been shown to the vascular endothelial growth factor 2 (VEGF2) gene and AMD. To examine the role of VEGFR2 in north Indian population, we conducted a case control study. Total 176 subjects were enrolled in a case-control genetic study. Real-Time PCR was used to analyze the SNPs (rs1531289 and rs2305948) of VEGFR-2 gene. ELISA was conducted to determine the levels of VEGFR2. A non-parametric Mann-Whitney-U test was applied for comparison of the ELISA levels and pearson's Chi-square test was applied to study the association of polymorphism between various groups. The single SNP (rs1531289) AG genotype was significantly associated with AMD (OR= 2.13, 95%CI= 1.011-4.489, P=0.047). VEGFR2 levels were found to be increased significantly in AMD patients as compared to normal controls. We also found significant increase in the levels of wet AMD as compared to dry AMD. This study demonstrates higher levels of VEGFR2 and frequency of AG (rs1531289) genotype in AMD patient population, suggesting the role of VEGFR-2 in pathogenesis of AMD.

Keywords: Angiogenesis, genotype, macular degeneration, single-neucleotide polymorphism, VEGFR2.

INTRODUCTION

Age-related macular degeneration (AMD) is leading cause of visual impairment and blindness in older population [1]. AMD is of two types i.e. dry and wet AMD. A typical sign of dry AMD is the presence of drusen, and retinal pigment epithelium (RPE) abnormalities in the form of geographic atrophy and areas of hyperpigmentation. A severe visual loss occurs in wet AMD in which there is growth of abnormal blood vessels through Bruch's membrane and they penetrate the RPE and sub-retinal space [2]. This process can cause hemorrhagic retinal detachment, and may develop into scarring on retinal outer layer [3]. Imbalance between angiogenic and anti-angiogenic factors and due to defect in the retinal pigmented epithelium (RPE) results in choroidal neovascularization (CNV). Currently, there are some risk factors reported with AMD; like age, heredity, gender, smoking and high body mass index (BMI) [4, 5]. The results from multiple genetic screening indicates that AMD involves multiple genes, risk factors, and interactions [6].

VEGF plays an important role in vascular development and has been strongly implicated and reported in the pathogenesis of age-related macular degeneration [7], and corneal neovascularization [8]. There are several VEGFs isoforms that have been reported which are products of alternative exon splicing. The VEGF family mainly binds with three types of VEGFRs which are: VEGFR1, VEGFR2, and VEGFR3, as well as to co-receptors [such as heparan sulphate proteoglycans nad neurophilin] [9]. VEGF regulates angiogenesis in the vascular endothelium through the high-affinity receptor tyrosine kinases VEGFR-1 and VEGFR-2 [10].

VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF [11]. VEGFR2 is main receptor by which VEGF mediates its permeability and angiogenic activities [12]. We hypothesized that levels and individual functional single nucleotide polymorphisms (SNPs) in VEGFR2 might be associated with AMD for its role in CNV.

VEGF gene polymorphisms have been investigated in AMD yet the data seems to be controversial [13]. Recently, Boekhoorn and colleagues [14] did not find any association between AMD and polymorphisms of the VEGF gene. On the contrary, in a study of Taiwan Chinese and English population found to have an association between SNPs of VEGF-A gene and AMD [15,16]. Fang et al found no association for VEGFR-2 tSNPs by allele or genotype analysis. Haplotype analysis, however, did show a single rare haplotype to be mildly associated with AMD [17].

Little data is currently available about VEGFR-2 polymorphisms and AMD. To our knowledge, until now, there has been no study which reported the VEGFR2 gene polymorphisms and serum VEGFR2 levels in Indian AMD patients. Therefore, in order to test whether VEGFR2 is a

*Address correspondence to this author at the Associate Professor, Department of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh, India; Tel: +919914209090, +911722756094, E-Mail: akshay2anand@gmail.com

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Table1. Description of SNPs Genotype.

SNP	Chromosome	Location in Gene	Genomic Location	Variation	Minor Allele
rs1531289	4	Intron 25	55649989	A to G	A
rs2305948	4	Exon 7	55674315	C to T	C

major genetic determinant of AMD in Indian population, we compared the VEGF genotype and allele frequencies between a series of unrelated AMD patients and a control group of individuals without AMD. The SNP selected in our study were previsouly studied in other ethnic populations and were chosen due to their functional significance in the gene. Vascular endothelial growth factor receptor type 2 (VEGFR2), or kinase insert domain-containing receptor (KDR), consists of 1356 amino acids. VEGFR2 gene is located in 4q11–q12 and consisted of 26 exons.

MATERIALS AND METHODS

This study was approved by the Institute Ethics Committee, Post-Graduate Institute of Medical Education and Research, Chandigarh, India vide letter No Micro/10/1411. A signed informed consent was obtained from patients in the prescribed format endorsed by the Institute Ethical Committee.

The inclusion criteria for AMD patients was 50 years or older with the diagnosis of advanced AMD as defined by geographic atrophy and/or choroidal neovascularization with drusen more than five in at least one eye. The controls in the study included those above 50 years with no drusen and absence of other diagnostic criteria for AMD. The exclusion criteria included the retinal diseases involving the photoreceptors and/or outer retinal layers other than AMD loss such as high myopia, retinal dystrophies, central serous retinopathy, vein occlusion, diabetic retinopathy, uveitis or similar outer retinal diseases that have been present prior to the age of 50 and opacities of the ocular media, limitations of pupillary dilation or other problems sufficient to preclude adequate stereo fundus photography. These conditions include occluded pupils due to synechiae, cataracts and opacities due to ocular diseases.

We included 176 cases which contained 115 AMD samples and 61 normal healthy controls after getting a signed informed consent. All enrolled participants were referred from Eye Center, PGIMER, Chandigarh (India). All patients and controls received a standard examination protocol including comprehensive medical and ophthalmic history review. In briefly, all AMD patients underwent for ophthalmic examination by a retina specialist for best-corrected visual acuity, slit lamp biomicroscopy of anterior segment and dilated fundus examination. All AMD patients were subjected to fluorescein fundus angiography (FFA) and optical coherence tomography (OCT). The diagnosis of AMD was based on ophthalmoscopic and FFA findings.

DEMOGRAPHIC INFORMATION

Signed informed consent form with written risk factor questionnaire related to demographic and environmental risk factors was obtained by measurement and questionnaire in both patient and controls. The detail was (age, sex, race, smoking etc) self reported by participants. Smokers were defined as having smoked at least 1 cigarette per day for at least 6 months and divided in to smokers and never smokers. The patients with heart disease were segregated on the basis of their cardiac reports, whether they have any problems related to heart. Subjects were also asked to report any prior diagnosis of stroke, use of antihypertensive medications, diabetes, migraine and history of heart diseases.

DNA EXTRACTION

The genomic DNAs were extracted from the whole blood of AMD cases as well as controls. The genomic DNA extraction has been done by commercially available genomic DNA extraction and purification kit (INVITROGEN and QIAGEN) according to the manufacturer's protocol.

SERUM EXTRACTION

Collected 4.0 ml of blood sample in serum separator tube (BD Biosciences, USA), left for 1 hour at 37°C to allow it to clot and serum was subsequently separated after centrifugation at 3000 rpm for 30 minutes.

TOTAL PROTEIN

Total protein was estimated using Bradford assay according to manufacturer's recommendations. Briefly, serum samples were diluted 1500 times in double distilled water. Bovine Serum Albumin (BSA) served as the standard. Diluted samples and BSA standard protein were mixed with coomassie brilliant blue G – 250 dye (Bradford reagent) in 4:1 ratio followed by incubation at room temperature for 10 mins – 15 mins. The absorbance was read at 595nm in Microplate reader (680XR Biorad, Hercules, CA, USA). The standard curve of BSA was estimated with linear or quadratic fit models.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The human VEGFR2 ELISA kit [Raybio Cat No # ELH VEGFR2-001] was used to estimate the levels of VEGFR2 according to the manufacturer's instructions and absorbance was read at 450 nm using 680XR model of Microplate reader (Biorad, Hercules, USA). Sample assays were performed in duplicate. This assay recognizes recombinant human VEGFR2 with minimum detection range less then 70 pg/ml. The linear regression analysis was used to generate the standard curve for VEGFR2 estimation in both patients and controls. All the values were normalized to total serum protein. The final concentration was shown as VEGFR2 serum concentration (pg) normalized to total protein concentration of serum (μg).

SNP SELECTION

We have selected two SNPs of VEGFR2 gene which were related to AMD and cardiovascular diseases i.e. rs1531289 & rs2305948 in VEGFR2. The detail of each SNP are described in Table **1**.

Table 2. Demographic Characteristics of Controls and AMD Patients.

Variables	AMD	Controls
Total	115	61
Wet AMD	84 (47.7%)	----
Dry AMD	31 (17.6%)	----
Minimal Classic	7 (11.9%)	----
Predominant Classic	16 (27.1%)	----
Occult	36 (61.0%)	----
One eye Affected	31 (27%)	----
Both eyes Affected	84 (73%)	----
Sporadic Cases	105 (91.3%)	----
Familial Cases	10 (8.7%)	----
Duration of disease[¥]	23 ± 2.6 (M)	----
Smokers	50 (43.5%)	11 (20%)
Non Smokers	65 (56.5%)	44 (80%)
Alcohalic	37 (32.2%)	17 (30.9%)
Non-alcohalic	78 (67.8%)	38 (69.1%)
Vegetarian	61 (53%)	31 (56.4%)
Non-vegetarian	54 (47%)	24 (43.6%)
Age	64.97 ± 7.1	60.38±13.2
Male	75 (65.2%)	40 (65.6%)
Female	40 (34.8%)	21 (34.4%)

GENOTYPING

Allelic discrimination for SNPs rs1531289 and rs2305948 was performed by real-time PCR (RT-PCR) on a 48 wells model Step OneTM (Applied Biosystems Inc., Foster city, CA) using published TaqMan® SNP Genotyping Assays for each of the polymorphisms mentioned above. Real time PCR was carried out for 20.0μl containing 10ul master mix, 5ul Assay (Applied Biosystems), 20ng DNA and molecular biology grade water was added to make the volume 20.0μl. TaqMan® SNP Genotyping Assays (Applied Biosystems) was used for all reactions according to manufacturer's recommendations. Two fluorescence signal detectors dyes VIC and FAM were used to label the Allele 1 and 2 probes and a 5´ Nuclease Assay was carried out. PCR mix without DNA served as negative control. The cycling program for Real Time PCR was as: preread 50°C, 1 minute; 95°C, 10 minutes, 1 cycle; 92°C, 15 seconds, 60°C 1 min, 40 cycles; postread 50°C, 1 minute. Software StepOneTM v 2.0 (Applied Biosystems Inc., Foster city, CA) was used to perform amplification and to calculate SNP. After PCR amplification the Sequence Detection System (SDS) Software imports the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well.

STATISTICAL ANALYSIS

Statistical analysis was performed with the help of SPSS 20.0 software. A non-parametric Kruskal-Wallis test followed by Mann-Whitney-U test was applied for comparison of the ELISA levels. The real time PCR estimated genotypes for each mutation were stratified for heterozygosity, and homozygosity for the respective allelic variant. Pearson's Chi-square test was applied to study the association between various groups. Genotype distributions were analyzed by logistic regression, integrating adjustments for age and gender. Genotypic associations and odds ratios (ORs) with 95% confidence intervals (CI) were estimated by binary logistic regression. The $p < 0.05$ was considered to be significant.

RESULTS

Summary statistics of all-important variables have been obtained and reported in Table **2**.

LEVELS OF VEGFR2 IN AMD AND CONTROLS

ELISA indicated significantly elevated levels of VEGFR2 in AMD patients as compared to normal controls (Fig. (**1**), Table **3**, p=0.0001) and the difference in the serum levels was also significant when compared between wet and dry AMD patients (Fig. **2**, Table **3**, p=0.048). AMD patients affected with heart diseases also showed significantly higher levels of VEGFR2 as compared to AMD patients without heart diseases. (Fig. **3**, Table **3**, p=0.001). No association was found between one eye affected and both eyes affected, alcoholic and non-alcoholic, smokers and non-smokers, vegetarian and non vegetarian as well as subtypes of wet AMD i.e minimal classic, predominant classic and Occult AMD patients (Table **3**). The difference was also not significant between male/female and familial/sporadic cases (data not shown).

Clinical and demographic details of subjects. AMD, age related macular degeneration; M, Months; Age, Age of

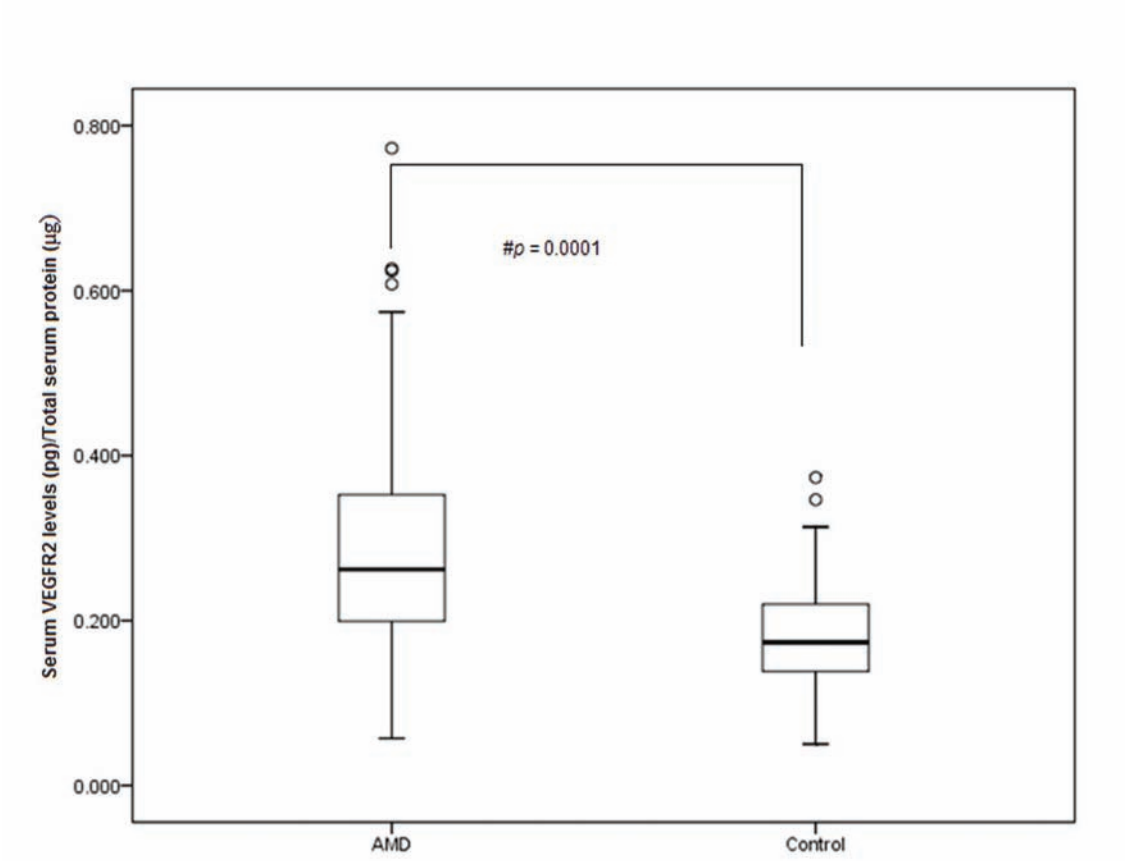

Fig. (1). Serum levels of VEGFR2 in AMD and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Levels of VEGFR2 were normalized to total protein. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by using Mann-Whitney-U test. AMD, Age Related Macular Degeneration; VEGFR2, Vascular endothelial growth factor 2; pg, picogram; µg, microgram.

Table 3. VEGFR2 Levels According to Different Subtype. Comparison of ELISA Levels Using Mann-Whitney-U Test.

Subjects	Mean Rank	Z- Value	p- Value
Control	50.33		
AMD	95.61	5.61	0.0001*
Dry	47.10		
Wet	60.74	1.976	0.048*
Minimal Classic	18.43		
Predominant Classic	31.75	1.737	0.082
Occult	31.47	1.809	0.070
One Eye Affected	47.77		
Both Eyes Affected	60.49	1.723	0.066
No heart Disease	55.12		
Heart Disease	87.44	3.514	0.001*
Non Alcoholic	60.21		
Alcoholic	51.86	1.262	0.207
Non Smokers	60.73		
Smokers	53.36	1.182	0.237
Vegetarian	61.97		
Non Vegetarian	51.38	1.86	0.086

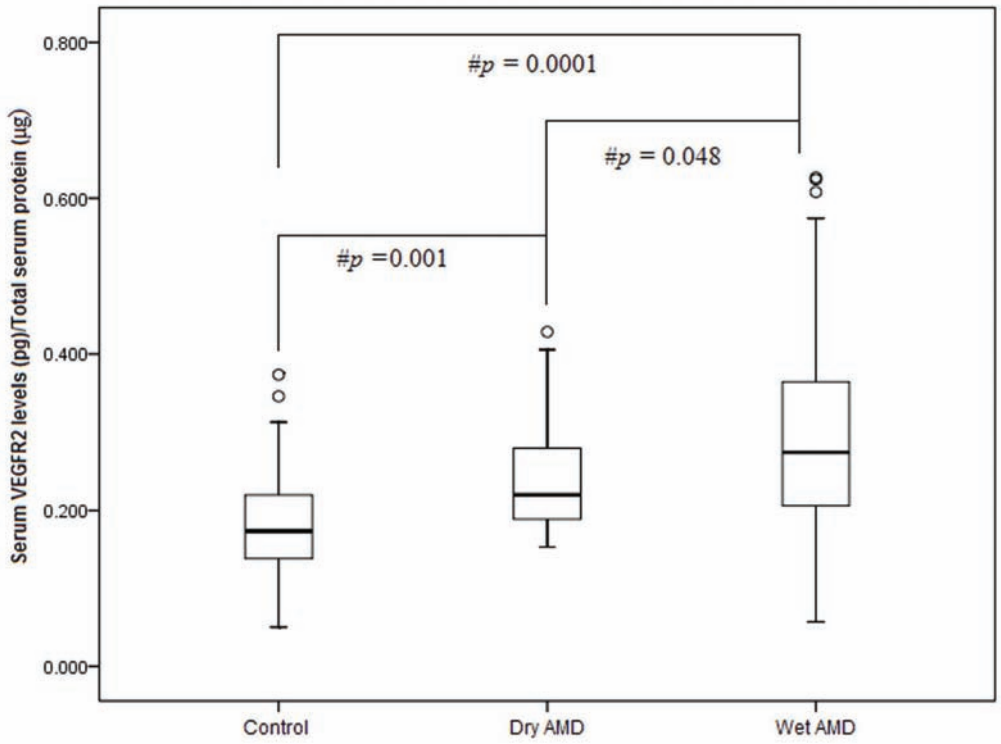

Fig. (2). Serum levels of VEGFR2 in normal controls, Dry AMD and Wet AMD. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Levels of VEGFR2 were normalized to total protein. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by using Mann-Whitney-U test. AMD, Age Related Macular Degeneration; VEGFR2, Vascular endothelial growth factor 2; pg, picogram; µg, microgram.

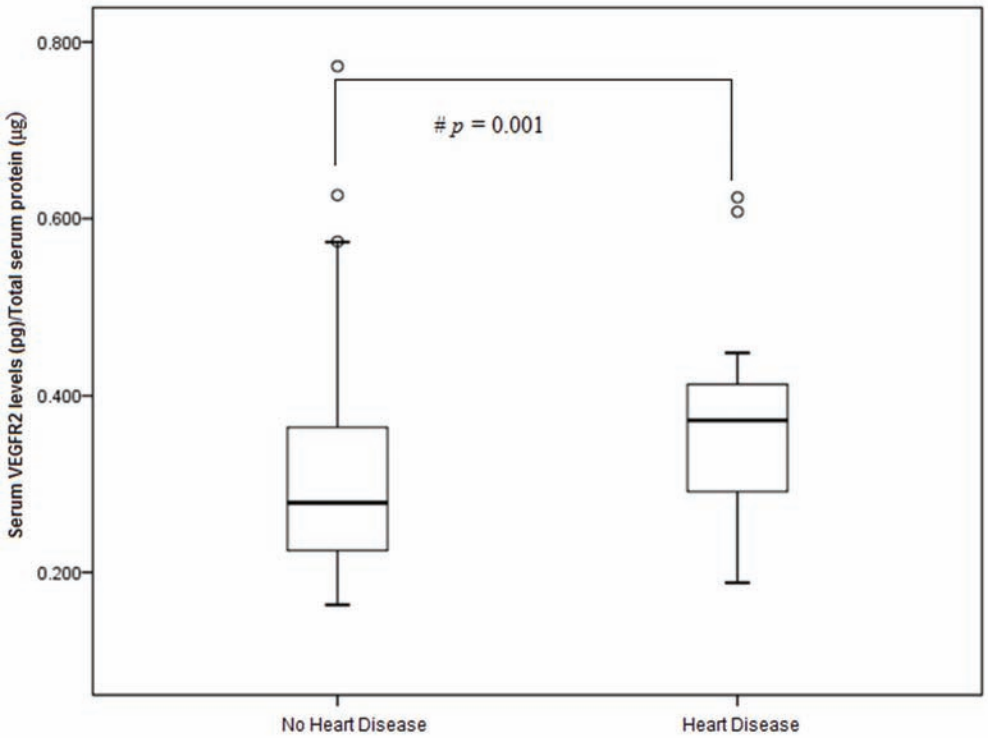

Fig. (3). Serum levels of VEGFR2 in AMD patients with heart disease and AMD patients without heart disease. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Levels of VEGFR2 were normalized to total protein. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by using Mann-Whitney-U test. AMD, Age Related Macular Degeneration; VEGFR2, Vascular endothelial growth factor 2; pg, picogram; µg, microgram.

Table 4. Effect of rs1531289 and rs2305948 Variants on Disease Phenotype.

			Unadjusted p Value			Multivariate Analysis, Adjusted for Age and Gender		
Genotype	**Number (Frequency)**		**OR**	**95%CI**	**P Value**	**OR**	**95%CI**	**P Value**
rs1531289								
	AMD	Controls						
AA	49 (.44)	34 (.65)	Reference			Reference		
AG	43 (.38)	14 (.26)	2.13	1.011-4.489	0.047	1.152	0.141-0.589	0.811
GG	20 (.17)	5 (.09)	2.77	0.949-8.117	0.062	0.975	0.211-4.501	0.974
	Wet AMD	Dry AMD						
AA	36 (.44)	13 (.44)	Reference			Reference		
AG	33 (.40)	10 (.33)	1.192	0.461-3.082	0.718	0.932	0.234-3.710	0.920
GG	13 (.16)	7 (.23)	0.678	0.220-2.048	0.483	1.015	0.166-6.217	0.987
rs2305948								
	AMD	Controls						
CC	98 (.86)	52 (.87)	Reference			Reference		
CT	16 (.14)	8 (.13)	1.061	0.425-2.644	0.898	0.556	0.090-3.437	0.527
TT	0	0	0	0	0	0	0	0
	Wet AMD	Dry AMD						
CC	73 (.88)	25 (.81)	Reference			Reference		
CT	10 (.12)	6 (.19)	0.571	0.188-1.731	0.322	1.205	0.198-7.321	0.839
TT	0	0	0	0	0	0	0	0

Table 5. Allele Frequency of rs1531289 and rs2305948 in AMD and Normal Controls.

Allele	**Number (Frequency)**		**OR**	**95%CI**	**p- Value**
rs1531289					
	AMD	Controls			
A	141 (0.63)	82 (0.77)	Reference		
G	83 (0.37)	24 (0.23)	2.011	1.184-3.415	0.009*
	Wet AMD	Dry AMD			
A	105 (0.64)	36 (0.60)	Reference		
G	59 (0.36)	24 (0.40)	0.842	0.459-1.54	0.580
rs2305948					
	AMD	Controls			
C	212 (0.93)	112 (0.93)	Reference		
T	16 (0.07)	8 (0.07)	1.056	0.438-2.544	0.902
	Wet AMD	Dry AMD			
C	156 (0.94)	56 (0.90)	Reference		
T	10 (0.06)	6 (0.10)	0.598	0.207-1.722	0.340

onset; Values are mean ± SD or (percentage), ¥ Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset.

GENETIC POLYMORPHISMS

After investigating the outcomes of VEGFR2 ELISA, we further analyzed the SNP by real time PCR. The genotype frequencies were in Hardy-Weinberg equilibrium. The genotype and allele frequencies in AMD patients and controls have been reproduced in Tables **4**, **5** and Fig. (**4**). The AG genotype of rs1531289 was more frequent in AMD patients as compared to normal controls (Table **4**, Fig. (**5A**), Univariate analysis OR=2.13, CI=1.011-4.489, p=0.047). The G allele of rs1531289 was also significantly different in AMD patients (Table **5**, Fig. (**5C**), OR=2.011, CI=1.184-3.415, #p=0.009). In rs2305948 we did not found GG genotype in AMD patients and normal controls (Table4). There was no significant difference in the genotype and allele frequency of rs2305948 (Table **4** & **5**, Fig. **5 A** & **C**, p=0.892 and 0.902). The study showed that the AG risk variant of rs1531289 is associated with the progression of AMD (Fig. **5A**, p=0.047). The individuals having GG genotype revealed no risk of developing AMD (Fig. **5A**, p=0.062). We did not find any significant difference in genotype and allele frequency for wet and dry AMD patients (Table **4** & **5**; Fig. **5B** & **D**). Logistic regression analysis in

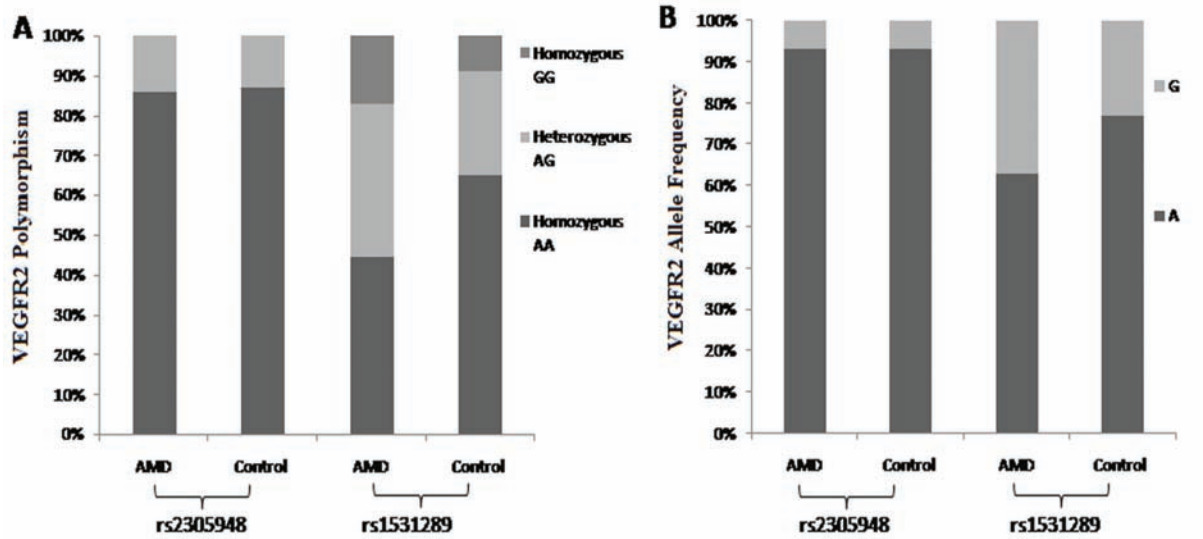

Fig. (4A). Genotype distribution (y-axis) of VEGFR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages (**B**) Allele frequency (y-axis) of VEGFR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages.

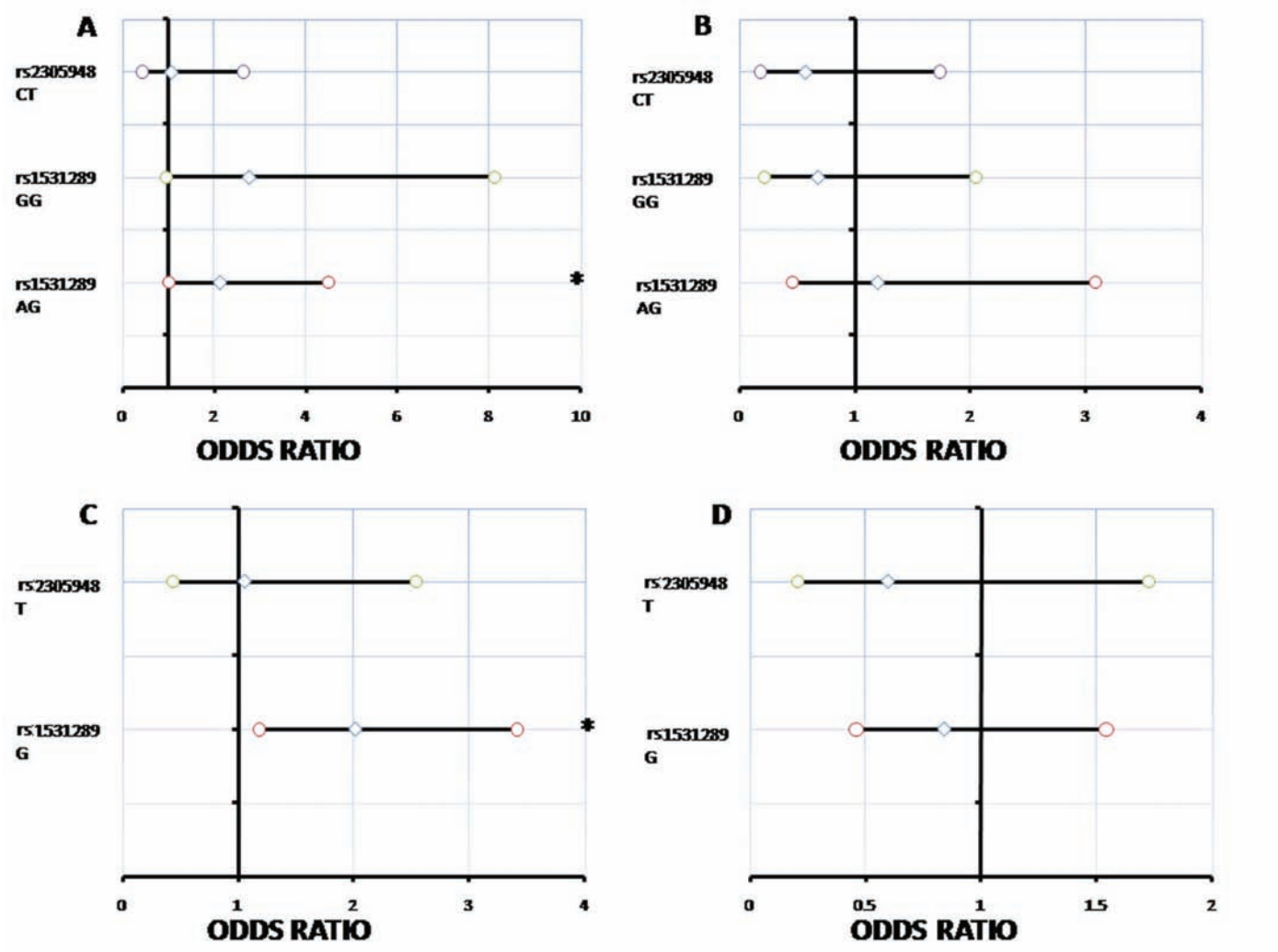

Fig. (5A). Univariate logistic regression analysis in AMD/Control as dependent variable and VEGFR2 polymorphism as independent variable. **B**) Univariate logistic regression analysis in Wet/Dry as dependent variable and VEGFR2 polymorphism as independent variable. **C**) Univariate logistic regression analysis in AMD/Control as dependent variable and VEGFR2 alleles frequency as independent variable. **D**) Univariate logistic regression analysis in Wet/Dry as dependent variable and VEGFR2 polymorphism as independent variable. *p≤0.05.

Table 6. Logistic Regression of the Association of rs1531289, rs2305948 and Progression of AMD Stratified by Food Habits, Smoking and Heart Disease.

			Unadjusted p Value			Multivariate Analysis, Adjusted for Age and Gender		
Genotype	**Number (Frequency)**		**OR**	**95%CI**	**p-Value**	**OR**	**95%CI**	**p-Value**
rs1531289								
	Non Vegetarian AMD	Vegetarian AMD						
AA	18 (0.35)	31 (0.51)	Reference					
AG	24 (0.46)	19 (0.32)	2.175	0.942-5.02	0.068	1.829	0.693-4.829	0.223
GG	10 (0.19)	10 (0.17)	1.722	0.601-4.92	0.310	0.762	0.180-3.229	0.713
	Smokers AMD	Non Smokers AMD						
AA	17 (0.35)	32 (0.50)	Reference					
AG	20 (0.40)	24 (0.37)	1.568	0.680-3.617	0.290	0.610	0.221-1.686	0.341
GG	12 (0.25)	8 (0.13)	2.823	0.967-8.237	0.057	4.894	0.921-26.01	0.062
	AMD with Heart disease	AMD without Heart disease						
AA	8 (0.53)	25 (0.40)	Reference					
AG	4 (0.27)	28 (0.44)	0.446	0.119-1.664	0.229	3.227	0.581-17.92	0.180
GG	3 (0.20)	10 (0.16)	0.9375	0.205-4.269	0.933	0.833	0.107-6.496	0.862
rs2305948								
	Non Vegetarian AMD	Vegetarian AMD						
CC	50 (0.93)	48 (0.80)	Reference					
CT	4 (0.7)	12 (0.20)	0.320	0.096-1.061	0.062	0.312	0.078-1.248	0.100
TT	0 ()	0	0	0	0	0	0	0
	Smokers AMD	Non Smokers AMD						
CC	41 (0.82)	57 (0.89)	Reference					
CT	9 (0.18)	7 (0.11)	1.78	0.615-5.191	0.285	0.768	0.250-2.360	0.644
TT	0	0	0	0	0	0	0	0
	AMD with Heart disease	AMD without Heart disease						
CC	12 (0.75)	54 (0.84)	Reference					
CT	4 (0.25)	10 (0.16)	1.800	0.482-6.721	0.381	0.200	0.017-2.313	0.198
TT	0	0	0	0	0	0	0	0

both SNPs for smokers, food habit and heart disease did not show any differences (Table **6**). The difference was also not significant when compared between alcoholic patients, number of eyes affected, familial patients, gender and wet AMD patients ie minimally classic, predominantly classic and occult (data not shown).

To analyze the association of genetic polymorphism and other risk factors with AMD we performed unconditional logistic regression analysis and obtained optimized model. As age and gender were reported as risk factors for AMD so we analyzed both as risk factors. When age and gender were adjusted by multiple logistic regression, we did not find significant difference between any group (Table **4** & **6**). The VEGFR2 ELISA levels were not correspondent to the polymorphism of SNPs (Table **7**).

DISCUSSION

Vascular endothelial growth factor is an important regulator of vasculogenesis and angiogenesis with a specific mitogenicity for endothelial cells. This study was conducted to determine whether there is any relation of VEGFR2 and AMD disease in North Indian population. After examining the involvement of levels and VEGFR2 gene polymorphism with the risk of AMD, it is suggested that genetic variations in the gene (rs1531289) encoding the angiogenic processes might confer susceptibility to AMD. The significant relationship between the rs1531289 AG VEGFR-2 genotype and AMD could open novel perspectives in the etiology and associated risk factors in AMD.

Our results indicate that the levels of VEGFR2 increased significantly in AMD patients as compared to normal controls and the difference was pronounced in wet AMD patients as compared to dry AMD. VEGFR-2 mediates the majority of the angiogenic and permeability-enhancing effects of VEGF [12]. The levels of VEGF increases under hypoxic circumstances [18]. Atrophy of the choriocapillaris and atherosclerosis resulting in relative ischaemia of the retina are assumed to be involved in the development of AMD [19]. The increased levels of VEGFR2 in AMD might be caused due to hypoxia in the retina. Recently, a

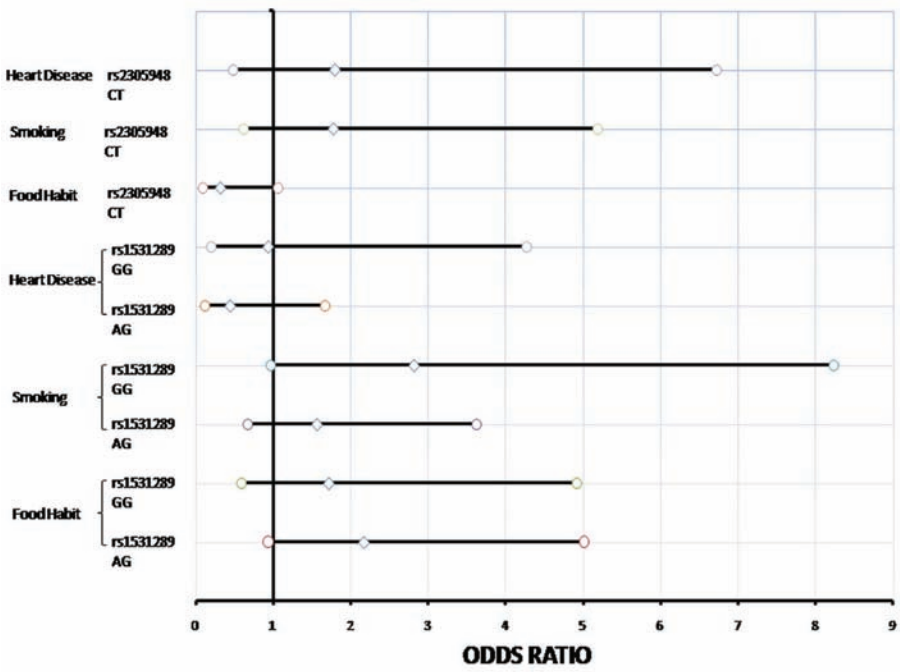

Fig. (6). Univariate logistic regression analysis in non-vegetarian, smokers and patients with comorbidity as dependent variable and VEGFR2 polymorphisms as independent variable.

pioneering retrospective study on neovascular AMD cohort and VEGFR-2 SNPs has been published [17]. Levels of VEGFR2 were higher in the AMD patients affected with heart diseases as compared to those without heart diseases. It is known that variants in VEGFR-2 are linked with heart disease [20]. Previously, it was shown that AMD and cardiovascular disease share common background [21]. Variants in VEGFR-2 may even influence the risk of developing breast cancer [20]. Recently, polymorphisms in VEGFR-2 and VEGFR-1 were reported to be associated with sarcoidosis, an inflammatory condition with a hypothesized antigenic stimulus and [22]. This study also found association of SNP (rs1531289) in AMD patients as compared to normal controls but did not report any association with rs2305948 SNP. All the alleles and genotypes were in Hardy-Weinberg Equilibrium in both AMD and control subjects. VEGFR2 signalling plays very important role during expansion of neovascularization in pathological or physiological conditions [12]. VEGFR2 have the autophosphorylation capability after the stimulus with VEGF ligand as compared to VEGFR1, and numerous phosphorylated tyrosine residues have been assigned in this receptor. For maximum kinase activity of VEGFR2, phosphorylation of two Tyr1054 and Tyr1059 is necessary [23] which provides the docking site for other proteins leading to activation of phospholipase C (PLC) and phosphatidylinositol 3'-kinase (PI3K) thus affecting gene expressions.

Recurrently, agents that block the effects of vascular endothelial growth factor [VEGF] are emerging as the most successful treatment for AMD, including anti-VEGF aptamer [3] and anti-VEGF monoclonal anti- body [24]. Results obtained in this study showed that VEGFR2 gene polymorphism and serum VEGFR2 levels have a relationship with AMD, VEGFR2 gene polymorphism has a relationship with increasing levels of serum VEGFR2, which in turn is co-related to the incidence of age-related macular degeneration. From the results obtained in this study, it can be concluded that the VEGFR2 gene polymorphism (rs1531289) had a significant relationship to the incidence of AMD. Levels of serum VEGFR2 were higher in wet as compared to dry AMD and both were higher as compared with the control group. There was a significant correlation between serum VEGFR2 levels of patients with AMD and controls. It would be interesting to examine if VEGFR2 levels vary among responders and non responders to Avsatin, the drug of choice in wet AMD.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

FINANCIAL DISCLOSURE

The funders (F.No. SR/SO/HS-109/205 dated 1-05-2007) had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR'S CONTRIBUTIONS

NKS Data acquisition and writing of manuscript; AA Conceptualisation, writing of grant application, editing and interpretation of results; AG editing and supply of patients: RD patient selection and clinical evaluation; SP editing of manuscript; SS Statistical analysis.

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Possible Association between Expression of Chemokine Receptor-2 (CCR2) and Amyotrophic Lateral Sclerosis (ALS) Patients of North India

Pawan K. Gupta, Sudesh Prabhakar, Neel K. Sharma, Akshay Anand*

Neuroscience Research Laboratory, Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

Abstract

Background and Objectives: We earlier reported elevated chemokine ligand-2 (CCL2) in Indian amyotrophic lateral sclerosis (ALS) patients. We now analysed chemokine receptor-2 (CCR2), the receptor of CCL2, in these ALS patients.

Methods: Indian sporadic ALS patients (n = 50) were included on the basis of El Escorial criteria. Percentage (%) of CCR2 expressing peripheral blood mononuclear cells (PBMCs) was evaluated using Flow Cytometry. Real Time Polymerase Chain Reaction (PCR) was used to quantitate CCR2 mRNA expression in PBMCs. Normal controls (n = 40) were also included for comparison.

Results: Flow Cytometry revealed significantly reduced CCR2 expressing PBMCs in the ALS patients. We also found a significant decline in number of CCR2 expressing PBMCs in limb onset ALS when compared to bulbar onset ALS. PBMCs from ALS patients showed substantial down-regulation of CCR2 mRNA. CCR2 mRNA expression was found to be decreased among limb ALS patients as compared to bulbar onset ALS. Further, the count of CCR2+ PBMCs and CCR2 mRNA transcript in PBMCs was significantly lower in severe and moderate ALS as compared to ALS patients with mild impairments.

Conclusions: Downregulation of PBMCs CCR2 may indicate its etio-pathological relevance in ALS pathogenesis. Reduced PBMCs CCR2 may result in decreased infiltration of leukocytes at the site of degeneration as a compensatory response to ALS. CCR2 levels measurements in hematopoietic stem cells and estimation of comparative PBMCs count among ALS, disease controls and normal controls can unveil its direct neuroprotective role. However, the conclusions are restricted by the absence of neurological/non-neurological disease controls in the study.

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* E-mail: akshay1anand@rediffmail.com

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by typical participation of inflammatory cascade. Interaction of chemokine ligand-2 (CCL2), a small chemokine belongs to C-C subfamily with its receptor chemokine receptor-2 (CCR2) strongly regulate these inflammatory changes. CCL2/CCR2 pathway is known to drive circulating leucocytes and resident immune cells of brain, including microglial cells, towards the site of neurodegeneration. Studies have shown that CCL2 and CCR2 knock out transgenic mouse exhibit reduced infiltration of blood mononuclear, natural killer cells and dendritic cells at the site of inflammation and these mice are resistant to experimental autoimmune encephalomyelitis (EAE) [1,2]. Furthermore, elevated CCL2 levels in biofluids from ALS patients have been reported earlier [3–13]. Contrary, reduced CCR2 expression in peripheral blood monocytes of ALS patients has also been observed [7,14] and could be argued as conflicting with postulated role of CCR2 in inflammation in ALS pathogenesis. Therefore more studies in other populations with varying clinical phenotype are imperative to uncover the role of interplay of these molecules in the ALS disease.

Whether CCL2/CCR2 alteration is neurotoxic or provides neuroprotection at a given stage of ALS disease remains unclear as CCL2/CCR2 pathway is also reported to impart neuroprotection besides mediating inflammation [15,16]. For instance, it has been demonstrated that CCL2 rescues fetal neurons and astrocytes in a mixed culture from N-methyl-D-aspartate (NMDA) induced apoptosis by reducing glutamate and NMDA receptor-1 (NMDAR1) [15]. Additionally, CCL2/CCR2 has also been reported to prevent HIV-tat induced damage of rat midbrain neurons [16].

We recently reported higher CCL2 in bio-fluids from Indian ALS patients and postulated that this may contribute towards extended survival reported in these patients [12,13]. A major study from India reported significantly longer survival duration among ALS patients when compared to Western ALS populations [17]. In this study, we present an indirect evidence of reduced mRNA and protein CCR2 levels in peripheral blood mononuclear cells

(PBMCs) of Indian ALS patients suggesting its etio-pathological association with ALS.

Materials and Methods

Ethics Statement

All subjects were included in the study after obtaining written informed consent as outlined in the research protocol. Ethical approval for the study was obtained by institute ethical committee, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India–160012 (No. 7055-PG-1Tg-05/4348-50).

Subjects

Fifty patients, born in North India and diagnosed with ALS by El Escorial criteria at Neurology outpatient, PGIMER, Chandigarh, India were recruited in the study. ALS patients with history of diabetic neuropathy, glaucoma, pre-eclampsia, stroke and those receiving Riluzole, anti inflammatory drugs, antioxidants or other treatment were excluded from the study. The ALS functional rating score-revised (ALSFRS-R) was measured to evaluate severity of disease and overall functional status of patients. This revealed 11 patients which presented with respiratory symptoms such as orthopnea, dyspnea and other respiratory insufficiencies even though none of the patients were on respiratory support [18]. At the time of blood collection, 15 ALS patients presented with mild neurological impairment [ALSFRS-R_{range} = 36–45; ALSFRS-R_{mean} = 40±0.5(SE)], 30 ALS patients with moderate impairment [ALSFRS-R_{range} = 24–36; ALSFRS-R_{mean} = 32.5±0.4(SE)] while 5 ALS patients with severe clinical phenotype [ALSFRS-R_{range} = 16–24; ALSFRS-R_{mean} = 18.5±1.5(SE)] as indicated by ALSFRS-R criteria. The ALS patients had an overall mean ALSFRS-R score of 34.4±0.8(SE) with a range of 16 to 45. Disease duration (interval between appearance of first ALS symptom and sample collection) for patients with mild, moderate and severe impairments is reported to be 16.6±11.6(SD), 18.4±11.9(SD) and 28.8±23.0(SD) months respectively. Of 8 bulbar onset ALS patients, 3 patients exhibited severe neurological impairments and remaining 5 were presented with moderate impairments. Of 42 limb onset ALS cases, 2 patients were severe, 15 cases were presented with mild deficit and 25 were presented with moderate neurological deficit. Based on disease duration, ALS patients were divided in two groups, disease duration≤19 months (n = 34) and disease duration>19 months (n = 16). The mean disease duration of ALS patients was 19.0±12.7(SD) months and therefore set as cutoff. The reference group for comparisons consisted of 40 genetically unrelated; sex and age matched normal controls without any apparent health problems such as hypertension, diabetes, heart disease etc. Since there were no neurological deficits upon examination, the ALSFRS-R score of each normal control individual was considered as 48. No neurological/nonneurological disease controls were included in the study. The clinical details of subjects which are published earlier [12], have also been reproduced here in Table 1.

PBMCs Isolation

PBMCs were isolated as per Histopaque-1077 (Sigma, USA) instruction sheet provided by the vendor. Briefly, 6.0 ml blood was collected from each subject and layered on equal volume of Histopaque-1077 followed by centrifugation at 1800 rpm for 30.0 mins at room temperature. PBMCs were collected from plasma/Histopaque-1077 interface. Aliquots of PBMCs were stored at −80°C in *RNA later* (Sigma, USA) until used for total RNA extraction. Some aliquots of PBMCs was stored in 90% fetal bovine serum (FBS, HiMedia, India) +10% dimethyl sulphoxide (DMSO, Sigma, USA) and kept at −80°C until flow cytometry was done.

RNA Extraction

Total RNA was extracted using RNAeasy columns (Qiagen, USA). RNA was quantitated by taking absorbance at 260.0 nm. About 5000.0 ng total RNA was used to synthesize cDNA according to RevertAid^{TM} first strand cDNA kit (Fermentas, USA).

Real Time Polymerase Chain Reaction (PCR)

Real Time PCR was used to quantitate expression of CCR2 mRNA in PBMCs and was performed in the 48 wells version of Step One^{TM} (Applied Biosystems Inc., USA) using CCR2 specific forward (5′-AGT TCA GAA GGT ATC TCT CGG TC-3′) and reverse primer (5′-GGC GTG TTT GTT GAA GTC ACT-3′) sequences available at primer bank (http://pga.mgh.harvard.edu/cgi-bin/primerbank). PCR reactions were carried out in duplicates using SYBR green real time PCR kit (Invitrogen, USA) according to manufacturer's recommendations. The cycling conditions consisted of initial denaturation step for 10.0 mins at 95°C followed by 40 cycles of denaturation at 95°C for 1.0 min, annealing at 52°C for 1.0 min and extension at 72°C for 1.0 min. Relative expression was analyzed using $2^{-\Delta\Delta Ct}$ or comparative Ct (threshold cycle) method after normalization with β-actin [4] and fluorescence data were obtained at annealing step.

Table 1. Characteristics of subjects.

Subjects	†Age (y)	M/F (n)	Age of onset (y)	‡,†Disease duration (mo)	El Escorial criteria at the time of sample collection	†Total serum protein (g/l)
Total ALS	47.4±12.4	38/12	46.2±12.8	19.0±12.7	25 Definite, 15 Probable, 10 Possible	48.2±26.7
Limb onset ALS	47.0±13.3	33/9	45.5±13.8	19.5±14.0	20 Definite, 15 Probable, 7 Possible	47.6±26.7
Bulbar onset ALS	49.7±7.5	5/3	48.3±7.3	15.7±6.3	5 Definite, 3 Possible	51.6±28.4
Normal controls	46.0±10.9	30/10				49.9±28.2

Clinical details of subjects. ALS, amyotrophic lateral sclerosis; F, female; g, grams; l, litre; n, Number; M, male; mo, months; y, years. Age, age of onset, duration of disease and total serum protein are indicated as mean ± standard deviation (SD).

‡Duration of disease is the interval between appearance of first symptom of ALS and collection of sample.

†*One-way analysis of variance* (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis showed that mean age, mean disease duration and mean total serum protein did not differ significantly among the given conditions ($p>0.05$). ALS subjects were asked to provide all clinical details at the age of onset of disease.

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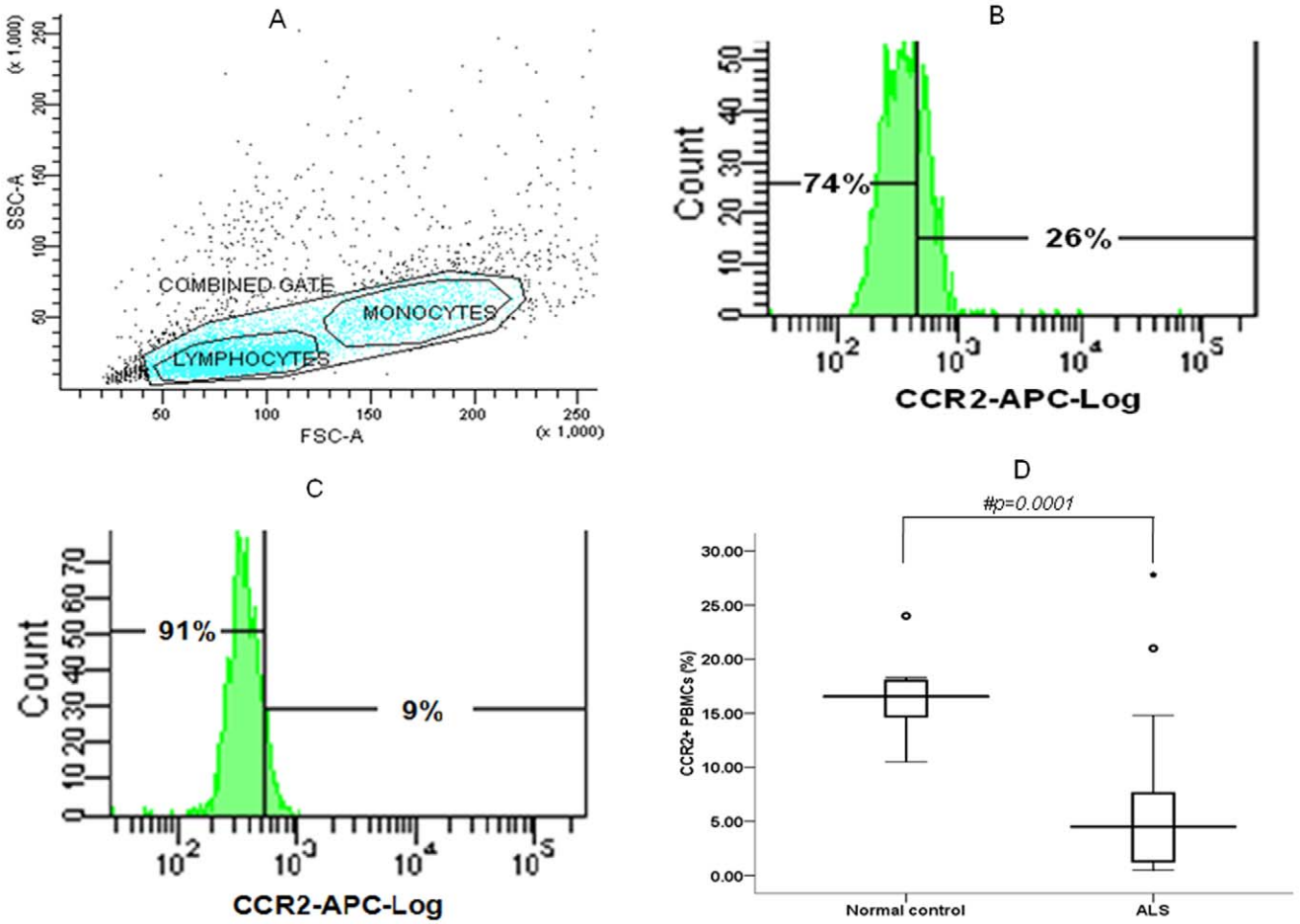

Figure 1. Percentage (%) of CCR2+PBMCs in ALS patients and normal control subjects as measured by Flow Cytometry. (A) Dot plot showing side and forward scatter analysis of purified unlabeled PBMCs (large combined gate) from a normal control. PBMCs consists of two distinct populations namely lymphocytes and monocytes. Approximate lymphocytes and monocytes populations are indicated as smaller gates. Events outside the PBMCs gate represent cell debris and granulocytes. Same gating has been used for PBMCs from each ALS and normal control sample. ~10,000 events have been acquired in each experiment. x-axis represents population cell size in forward scatter (FSC) and y-axis denotes population cell granularity in side scatter (SSC). (B,C) Single parameter representative histogram of flow cytometric expression pattern of CCR2 on gated PBMCs is showing decreased number of CCR2 expressing PBMCs in ALS (9%; C) as compared to normal control (26%; B). Number of cells is represented along y-axis and blue APC fluorescence along x-axis. Appropriate unlabeled PBMCs were used to set marker in histogram and measure background fluorescence. (D) Box plot compares CCR2 expressing PBMCs between ALS and normal subjects. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. Nonparametric *Mann Whitney U* test indicates significant difference betweesn the given conditions ($p<0.05$). ALS, amyotrophic lateral sclerosis; APC, allophycocyanin; CCR2, chemokine receptor 2; PBMCs, peripheral blood mononuclear cells.
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Flow Cytometry

Flow Cytometry was used to study CCR2 protein levels on PBMCs surface. ~ 3×10^5 PBMCs were blocked with Fc blocker (1.0 µg, purified human IgG, R&D Systems Inc., USA) +0.1% sodium azide (Sigma, Germany) +1X Ca^{2+} and Mg^{2+} free phosphate buffer saline (PBS, HiMEDIA, India), for 15.0 mins at room temperature. Cell suspension was then stained with anti-human CCR2 primary antibody labeled with allophycocyanin (0.1 µg, R&D Systems Inc., USA) +0.5% bovine serum albumin (BSA, Sigma, Germany) +0.1% sodium azide +1X PBS for 45.0 mins on ice in dark followed by two washings with 1X PBS at 5,000 rpm for 5.0 mins at 4°C. Finally, the cells were reconstituted in 250.0 µl of 1X PBS and analyzed in FACSCANTO (BD Biosciences, USA) flow cytometer using FACS DIVA software with in 1 hr. Approximately 10,000 viable PBMCs were gated based on their forward and side scatter profile, and acquired in each run. PBMCs gate was set to include both lymphocytes and monocytes where maximum CCR2 fluorescence was observed. Events outside the PBMCs gate represent cell debris and any contaminated granulocytes while separating PBMCs from whole blood. Same gating was used between the experiments. Since purified population of PBMCs was used during flow cytometry experiments, no additional surface marker labeling was done to identify such populations. Background signal was measured for each sample by acquiring unlabeled PBMCs as negative controls and normalized to the signal obtained from anit-hCCR2 labeled PBMCs.

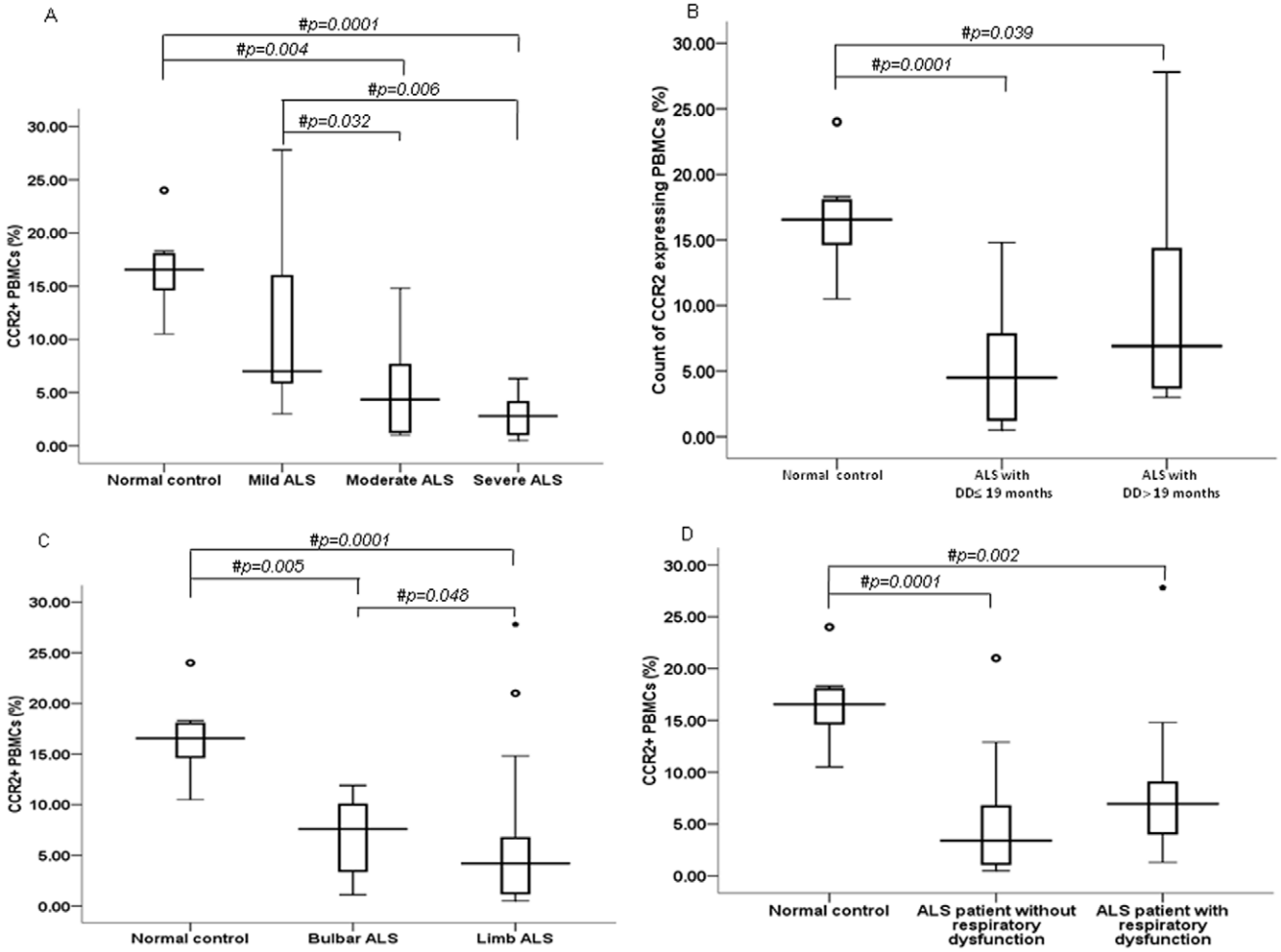

Figure 2. CCR2+ PBMCs in ALS patients with varying clinical characteristics. (A) Percentage (%) of PBMCs expressing CCR2 protein in ALS patients with mild, moderate and severe neurological impairments as indicated by ALSFRS-R. (B) Count (%) of CCR2+ PBMCs in ALS subjects with disease duration (DD) ≤19 months and DD>19 months. (C) Percentage (%) of CCR2 expressing PBMCs in bulbar and limb onset ALS patients. (D) CCR2+ PBMCs in ALS patients with respiratory dysfunction. In each box plot (A–D), boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Data was collected using Flow Cytometry. A non-parametric *Kruskal-Wallis H* test followed by *Mann Whitney U* test was used to analyze the data. # indicates significant difference among the groups ($p<0.05$). Outliers and extreme values are shown in circles and asterisk respectively. ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating score-revised; CCR2, chemokine receptor 2; DD, disease duration; PBMCs, peripheral blood mononuclear cells.
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Statistical Analysis

For skewed data and multiple comparisons, a non-parametric *Kruskal-Wallis H* test followed by *Mann-Whitney U* test was applied. For skewed data and comparison of two groups, a nonparametric *Mann-Whitney U* was used to score the level of significance.

A parametric *one-way analysis of variance* (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test was applied to compare multiple groups with normal distribution. However, if data was normally distributed with two groups to compare, unpaired, independent 2-tailed student *t* test with equal or unequal variance (Welch's correction) was applied. Quantile-quantile (Q-Q) plot was used to understand whether data is normally distributed or skewed. Skewed and normally distributed data is shown as median (10^{th} percentile -90^{th} percentile) and mean ± standard error (SE) respectively. The *p*-value was considered significant at ≤0.05. All statistical analysis was performed by statistical package and service solution (SPSS) 16 software.

Results

Flow Cytometry analysis of PBMCs of ALS and normal controls indicates a significant decrease in proportion of CCR2 expressing PBMCs in ALS patients than normal controls (Figure 1A–1D; $p = 0.0001$). CCR2 expressing PBMCs were found to be lower in severe ALS than ALS patients with mild and moderate neurological impairment (Figure 2A; $p = 0.006$ and $p = 0.032$ respectively). No such difference was observed in ALS patients with varying duration (Figure 2B; $p>0.05$). Reduced CCR2+ PBMCs was observed in bulbar and limb variants of ALS as compared to control group (Figure 2C; $p = 0.005$ and $p = 0.0001$ respectively). Moreover, CCR2+ PBMCs were found to be lower in limb onset ALS than bulbar onset ALS patients (Figure 2C; $p = 0.048$). In order to examine any possible association with hypoxia the CCR2 levels were also analysed in ALS patients with respiratory dysfunction and those without respiratory dysfunction,

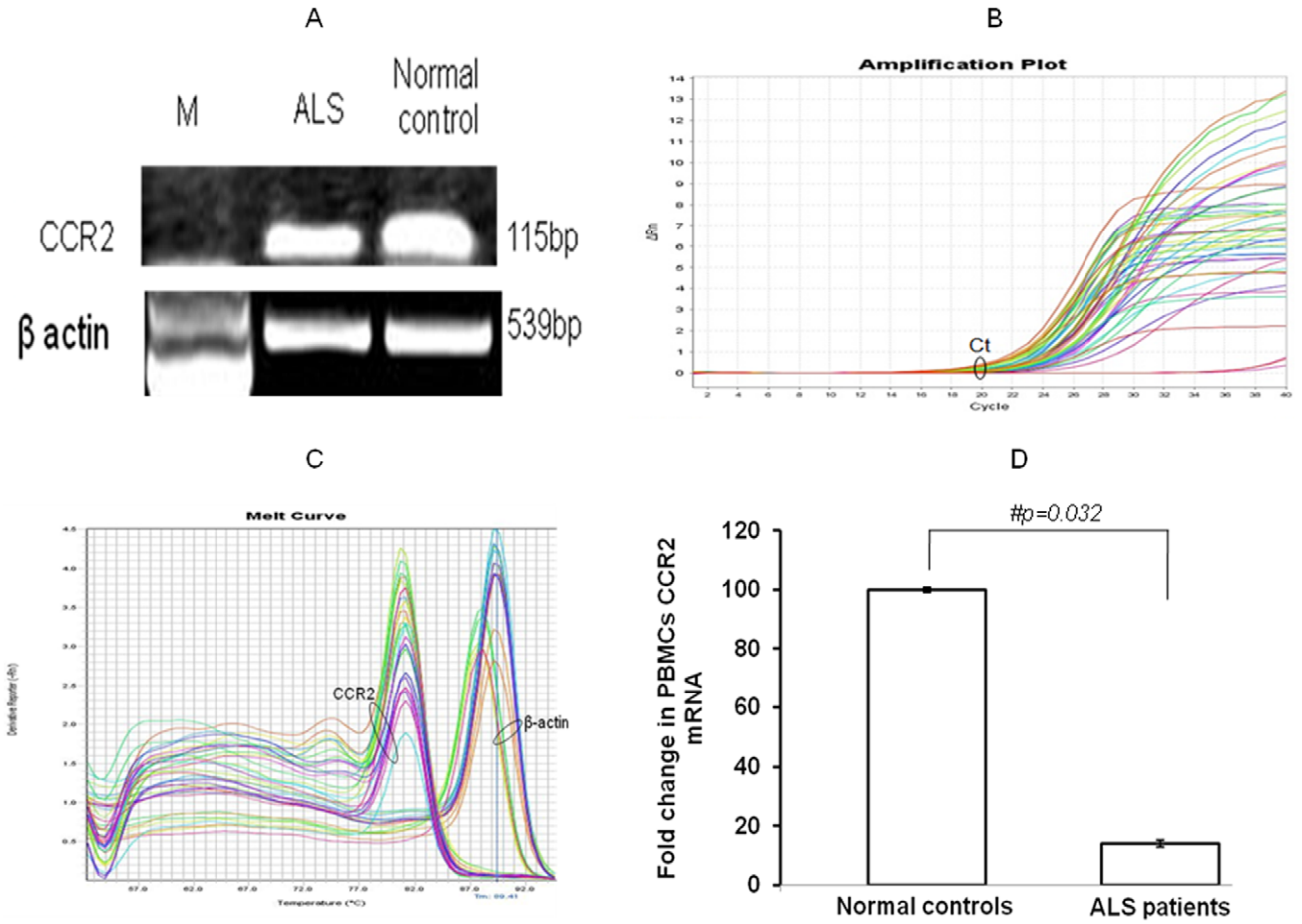

Figure 3. Real Time PCR analysis of relative mRNA expression of CCR2 in PBMCs of subjects. (A) Agarose gel electrophoresis of Real Time PCR products of target gene CCR2 and endogenous control β-actin. Reactions were performed with mRNA isolated form PBMCs of ALS patient and normal control. (B) Representative amplification curves depicting increase in fluorescence of CCR2 and β-actin from cDNA of same sample. No increment in fluorescence of negative controls was observed. The x-axis indicates cycle number and the y-axis shows intensity of relative fluorescence in linear scale. Ct is threshold cycle where normalized fluorescent signal of SYBR green intersect with threshold line. (C) Melt curve profile of Real Time PCR products of CCR2 and β actin clearly indicates the absence of any non specific amplification. The x-axis indicates negative derivative of change in amount of fluorescence per unit change in temperature (dF/dT) and the y-axis represents temperature in Celsius (°C). (D) Bar diagram showing fold change in CCR2 mRNA expression PBMCs from ALS and normal subjects. Values are plotted as mean ± SE (Standard error). Data was analyzed by unpaired, independent 2-tailed *student t* test with equal variance. # indicates significant difference among the groups ($p<0.05$). Expression of CCR2 were normalized to expression of endogenous control β-actin. ALS, amyotrophic lateral sclerosis; CCR2, chemokine receptor 2; PBMCs, peripheral blood mononuclear cells.
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however, no difference in CCR2 was been observed between these ALS groups (Figure 2D; $p>0.05$).

Real Time PCR analysis also indicated a 7.1-fold down-regulation of CCR2 mRNA expression in PBMCs of ALS patients as compared to normal subjects (Figure 3A–3D; $p = 0.032$). A 27-fold and 20-fold reduction was observed in severe ALS as compared to normal and mild ALS respectively (Figure 4A; $p = 0.009$ and $p = 0.023$ respectively), however, mRNA levels were comparable across moderate and severe ALS patients (Figure 4A; $p>0.05$). In addition, there was no significant reduction of CCR2 mRNA in PBMCs from ALS patients with disease duration >19 months in comparison to ALS patients with ≤19 months (Figure 4B; $p>0.05$). There was a significant decrease of 8.0-fold in PBMCs CCR2 mRNA in limb onset ALS when compared with bulbar onset ALS patients (Figure 4C; $p = 0.009$). PBMCs CCR2 transcript expression was comparable between ALS patients with respiratory dysfunction and without respiratory problems (Figure 4D; $p>0.05$).

Discussion

It has earlier been established that chronic activation of PBMCs via CCL2/CCR2 signaling pathway mediates inflammation in many neurological disorders. It has been observed that infiltration of PBMCs in the central nervous system (CNS) of mouse model of EAE is mediated by CCR2 [19,20]. A profound reduction in infiltration of leukocytes has been reported around denervated hippocampus following axonal injury in CCR2 deficient mice [21]. Mahad et al., showed that the *in vitro* model of blood-brain barrier (BBB) was selectively permeable for migrating CCR2+ lymphocytes and monocytes, and suggest the pathological importance of infiltrated CCR2 expressing PBMCs in multiple

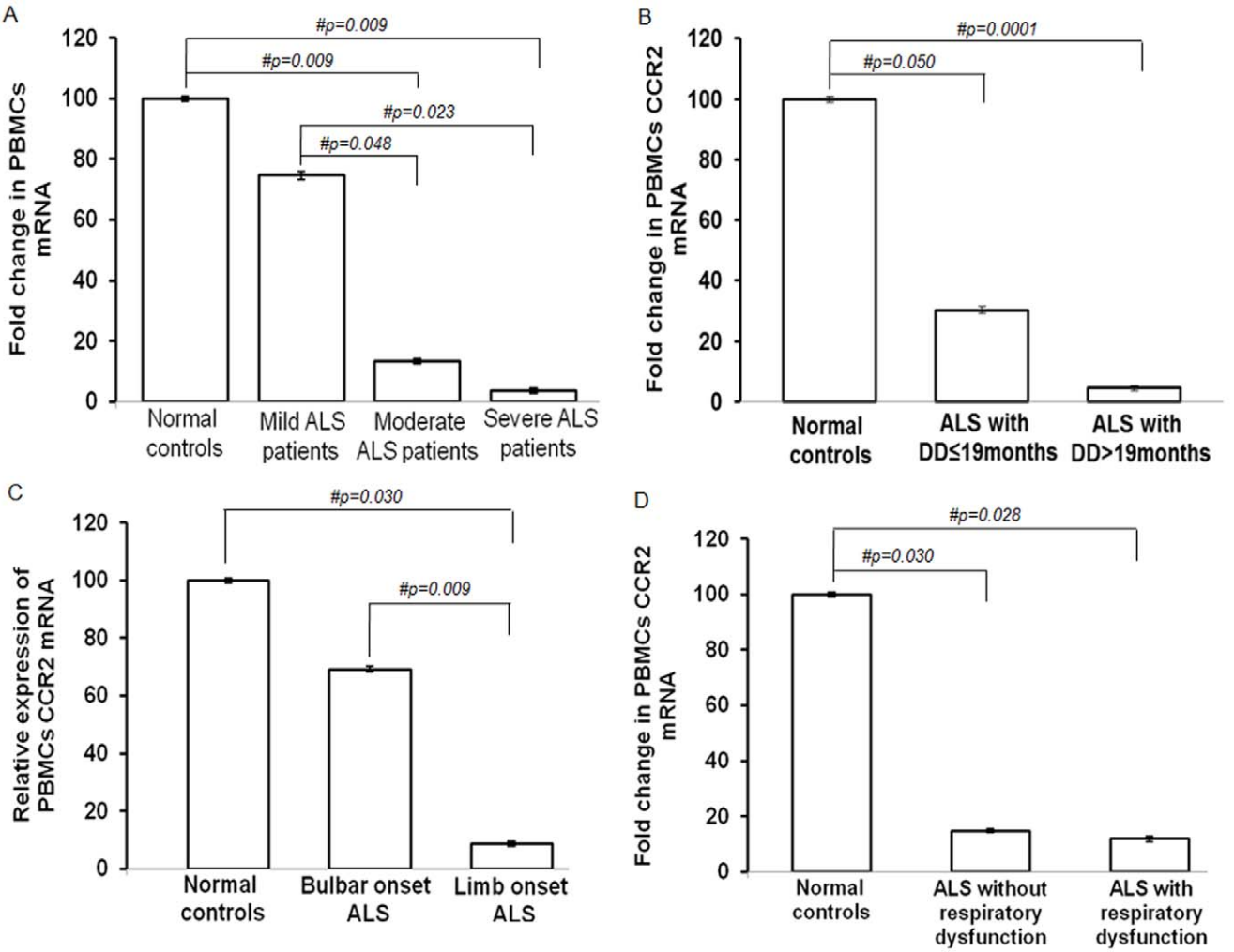

Figure 4. CCR2 mRNA in PBMCs of ALS patients with different clinical states. (A) Relative mRNA expression of CCR2 in PBMCs from ALS patients with mild, moderate and severe neurological impairments as indicated by ALSFRS-R. (B) Fold change in expression of PBMCs CCR2 mRNA in ALS subjects with disease duration (DD) ≤19 months and DD>19 months. (C) Relative CCR2 mRNA expression in PBMCs of bulbar and limb onset ALS patients. (D) Comparison of PBMCs CCR2 transcripts in ALS patients with respiratory dysfunction. In each bar diagram (A–D), values are plotted as mean ± SE (standard error). Data was collected using Real Time PCR and analyzed by *one-way analysis of variance* (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis. # indicates significant difference among the groups ($p<0.05$). Expression of CCR2 was normalized to expression of endogenous control β-actin. ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating score-revised; CCR2, chemokine receptor 2; DD, disease duration; PBMCs, peripheral blood mononuclear cells.
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sclerosis (MS) [22]. The concept is furthered by the observation that the ablation of CCR2 in HexB−/− mouse model of Sandhoff disease results in reduced PBMCs infiltration in the brain parenchyma and ameliorates the clinical progression of the disease by reducing neuroinflammation, and hence links reduction in CCR2+ PBMCs with neuroprotection [23]. Increased production of inflammatory chemokines has recently been reported in PBMCs of Alzheimer's disease (AD) patients [24]. With this background, we measured the levels of CCR2 in PBMCs of Indian ALS patients as these patients exhibit substantially extended survival duration of ~115 months after onset of disease [17].

The reduced systemic expression of CCR2 in PBMCs of these ALS patients reported here suggests its etio-pathological relevance to ALS pathogenesis. Earlier elevated levels of abnormally activated and differentiated monocytes/macrophages in sporadic ALS patients [25] were found to be associated with down regulation of CCR2 on circulating monocytes [7]. Furthermore, a significant reduction of CD14+ and CCR2 expressing monocytes in ALS patients, particularly with less severe form of disease, has been suggested to drive the recruitment of activated monocytes CNS in the early stages of the disorder [14].

We propose that the decrease in PBMCs CCR2 and previously reported elevated CCL2 in our ALS patients [12,13] may indicate an activation of a negative feedback regulation serving to alleviate the inflammation caused by extravasation of activated monocytes/lymphocytes at the site of CNS injury and denervated neuromuscular junction. Significantly reduced CCR2 levels in moderate and severe ALS patients and not in mild variants show that CCR2 may not be causally associated with primary motor neuron degeneration and its pathophysiological involvement could be secondary to neurodegeneration. However, our finding of unaltered CCR2 levels across ALS patients with varying disease duration is

explained by absence of information about disease progression rate and actual survival duration after onset of disease. Therefore, at this time, direct association of reduced CCR2 with extended survival duration of these ALS patients awaits further analysis through multi ethnic and multi cultural studies.

Reduced CCR2+ PBMCs in peripheral blood at the time of sample collection may raise the possibility of their migration and extravasation in CNS through damaged blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) in ALS pathogenesis [26,27,28,29], however, anatomical and histopathological analysis of BBB and BSCB was not conducted. Our report, therefore, suggests the need of future autopsy studies where brain and spinal cord tissue from Indian ALS patients can be analysed for presence of CCR2+ PBMCs. The massive infiltration of immature blood dendritic cells, CD4+ and CD8+ T-lymphocytes in spinal cord parenchyma has earlier been observed in Western ALS cases [4] and in superoxide dismutase 1 (SOD1) mutated transgenic ALS mouse model [30].

The unaltered levels of total serum protein in the ALS patients studied (Table 1) suggest that these findings are specific to CCR2+ PBMCs. However the expression of CCR2+ immune cells other than PBMCs including natural killer cells, dendritic cells and macrophages in these patients were not separately performed. Significantly elevated PBMCs CCR2 in bulbar ALS versus limb variant appears to be in contrast to the theory developed in the paper. For instance, the overall functional deterioration was found to be higher in bulbar [Mean ALSFRS-R = 28.7±3.2(SE)] when compared to the limb ALS [Mean ALSFRS-R = 35.4±0.8(SE)]. We speculate that higher levels of PBMCs CCR2 reported in bulbar variants may result from reduced disease duration and severity of these cases. Hence, future longitudinal studies for estimation of PBMCs CCR2 in higher number of bulbar Indian ALS patients and its possible association with clinical progression of the ALS disease may provide useful information about role of CCR2 in ALS. Since lower PBMCs CCR2 may facilitate neuroprotection, at the moment, lower CCR2 levels among limb ALS than bulbar ALS patients may account for relatively slower progression of disease and longer survival duration in limb ALS cases [17,31]. These findings are consistent with existing literature where limb Indian ALS patients were reported to exhibit significantly higher median survival duration [177.9±3.2(SE)] after disease onset as compared to bulbar group [55.9±2.9(SE)] [17].

We further propose that the downregulation of CCR2 mRNA in ALS subjects may indicate the underlying genetic/epigenetic abnormalities in regulatory elements of CCR2 gene including its post transcriptional deregulation necessitating detailed analysis of CCR2 domain in ALS population. CCR2 reduction in response to environmental cues can also not be ruled out in present ALS patients. Even though respiratory dysfunction did not impact CCR2 levels in the present study (Figure 2D & 4D), role of respiratory impairment should be addressed in larger ALS cohort with respiratory complications as downregulation of monocytic CCR2 under hypoxia has earlier been observed in *in vitro* conditions [32].

Because of lack of neurological disease controls having overlapping or distinct clinical symptoms with ALS, the differences observed in CCR2 levels in the study may reflect the molecular change relevant to diseases of CNS in general, as opposed to ALS disease in specific. Therefore, the absence of such control group is an important caveat of the study and further investigations should focus on the use of neurological controls in the analysis.

In conclusion, although causal association of reduced CCR2 with increased survival in Indian ALS patients remains speculative, the present findings may suggest an etio-pathological and possible immunomodulatory importance of PBMCs CCR2 in pathogenesis of ALS. Whether the blocking or reducing glial and leukocyte CCR2 (by intracerebral and/or systemic injection of its antagonists or synthetic siRNA against CCR2 mRNA) will lead to any therapeutic efficacy in ALS will be determined by further preclinical studies.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: PKG SP AA. Performed the experiments: PKG. Analyzed the data: PKG NKS AA. Contributed reagents/materials/analysis tools: SP AA. Wrote the paper: PKG AA.

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A predictive model for amyotrophic lateral sclerosis (ALS) diagnosis

P.K. Gupta [a,1,2], S. Prabhakar [a,3], S. Sharma [b,1,4], A. Anand [a,*]

[a] Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh-160012, India
[b] Department of Statistics, Panjab University, Chandigarh-160012, India

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ABSTRACT

Objective: The clinical diagnosis of amyotrophic lateral sclerosis (ALS) usually takes several months. The delay in diagnosis compromises the effective therapeutic interventions. Therefore, the present study was aimed to develop a statistical model for predicting the risk of ALS at earlier stages for better management of ALS patients.
Methods: The study recruited 44 sporadic ALS patients and 29 normal controls. Thirteen different independent variables (predictors) which were believed to be associated with ALS were included in the study. Forward stepwise (likelihood ratio) binary logistic regression was used to find significant variables and probability of disease prediction.
Results: The Hosmer–Lemeshow goodness of fit statistic ($\chi^2=4.379$, df=8, $p=0.821$) indicate the appropriateness of forward stepwise (likelihood ratio) binary logistic regression model. Serum chemokine ligand-2, chemokine ligand-2 mRNA, vascular endothelial growth factor-A mRNA, smoking and alcohol consumption are the independent variables found significant to predict risk of ALS ($p<0.05$). The current model yielded 93.2% sensitivity and 86.2% specificity with 90.4% overall validity of correct ALS prediction.
Conclusion: Forward stepwise (likelihood ratio) binary logistic regression model is an accurate method to predict ALS in the presence of serum CCL2, CCL2 mRNA, VEGFA mRNA, smoking and alcohol consumption with high sensitivity and specificity. However, bed side diagnostic utility of these variables needs to be validated further in larger ALS cohorts.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that affects both upper and lower motor neurons. Etiology of ALS is complex and believed to be multifactorial. ALS is known to have strong hereditary components. Sporadic ALS, without any known genetic link, accounts for 90% of cases while the remaining 10% of cases are of familial type. Nearly, 20% of familial ALS (fALS) cases and 4% of sporadic ALS (sALS) are known to be caused by toxic gain of function missense point mutations in superoxide dismutase-1 (SOD1) gene [1,2]. The other contributing factors include glutamate toxicity, reduced level of neurotrophic factors such as vascular endothelial growth factor-A (VEGFA), disruption of vital proteins and organelles, environmental factors (e.g. smoking, alcohol etc.) and relatively enhanced vulnerability of motor neurons to degeneration. The relative contribution of these factors, however, is likely to differ in patients. The diagnosis of ALS is difficult at the earlier stages because the symptoms can be similar to many other treatable neuromuscular disorders and is based on complete neurological examination, electrophysiological and radiological investigations. Usually these investigations to diagnose ALS take several months to complete and long delay between onset of symptoms and diagnosis affects the possibilities of effective therapeutic interventions [3]. Hence, earlier and effective diagnosis of ALS for better management of ALS patients needs the development of a statistical model in the absence of any suitable genetic and/or protein diagnostic biomarker.

2. Methods

2.1. Subjects

The study recruited 44 incidental sporadic ALS (sALS) cases diagnosed by 'El Escorial' criteria after obtaining informed consent from each patient as per guidelines prescribed by the institute ethical committee (No. 7055-PG-1Tg-05/4348-50). ALS patients with history of stroke, pre-eclampsia, diabetic neuropathy, glaucoma, pulmonary hypertension or those receiving anti inflammatory drugs (e.g. aspirin, Ibuprofen, naproxen etc.), antioxidants or riluzole, or those that have

* Corresponding author. Tel.: +91 1722748399.
E-mail address: akshay1anand@rediffmail.com (A. Anand).
URL: http://www.neurologypgi.org (A. Anand).
[1] These authors contributed equally to the manuscript.
[2] Tel.: +91 1722756090.
[3] Tel.: +91 1722756691.
[4] Tel.: +91 9815911381.

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Table 1
Clinical and biochemical details of subjects.

Subjects	Age (y)	M/F (n)	Age of onset (y)	Disease duration (mo)‡	El Escorial criteria	B/L(n)	Total protein (g/l)†	
							CSF	Serum
ALS	47.2 ± 12.4	34/10	45.6 ± 13.4	19.2 ± 13.5	20 definite 14 probable 10 possible	34/7	0.43 ± 0.2	48.2 ± 26.7
Control	38.1 ± 2.1	23/06					0.42 ± 0.1	48.7 ± 28.7

Clinical and biochemical summary of ALS cases and Controls. n, number; M, male; F, female; y, years; mo, months; B, bulbar; L, limb; g, gram; l, liter; CSF, cerebrospinal fluid; Age, age of onset, duration of disease, CSF and serum total protein are indicated as mean ± standard deviation (SD). ‡Duration of disease is the interval between appearance of first symptom of ALS and collection of sample. ALS subjects were asked to provide all clinical details at the age of disease onset. †Unpaired, independent 2-tailed Student's *t*-test showed that mean concentration of total protein in serum and CSF did not differ significantly among the groups ($p>0.05$).

documented history of hypersensitivity for riluzole were excluded from the study. We included 29 genetically unrelated controls in the study for comparisons. Control subjects were defined as healthy with no complaints of hypertension, diabetes or heart problem. The clinical details of subjects have been listed in Table 1.

2.2. Collection of demographic data

ALS patients were asked to provide demographic details (e.g. age, sex, cigarette smoking status, alcohol consumption, diet and body mass index) at the age of onset of disease using a questionnaire. The subjects were categorized as smoker and alcohol consumer, based on smoking and alcohol intake habits respectively. Individuals who either never smoked or never consumed alcohol in their life time or in the last 20 years were considered never smokers and nonalcoholic respectively [4]. Same criteria were applied to establish two categories of diet 1) vegetarian and 2) nonvegetarian (or meat consumers). Weight (in Kg) and height (in meters) was measured for each patient to calculate body mass index (BMI). Subjects were categorized into four standard weight classes based on value of BMI: 1) under weight – BMI below 18.5, 2) normal – BMI 18.6 to 24.9, 3) overweight – BMI 25 to 29.9 and 4) obese – BMI above 30.0. The demography of subjects has been reproduced in Table 2.

2.3. Peripheral blood mononuclear cells (PBMCs) and serum separation

6.0 ml of blood was used to isolate PBMCs as per Histopaque (Sigma, USA) datasheet. Serum was separated from 4.0 ml of blood collected in serum separator tube (BD Biosciences, USA).

2.4. Cerebrospinal fluid (CSF) preparation

1.0 ml–2.0 ml CSF was drawn from lumbar region of subjects in a sterilized container and stored at –80 °C in crude state.

2.5. Real time PCR

Real time PCR was used to quantitate expression of vascular endothelial growth factor (VEGFA) and chemokine ligand-2 (CCL2) mRNA in PBMCs and was performed in the 48 wells version of Step One™ (Applied Biosystems Inc., USA).

2.6. Enzyme linked immunosorbent assay (ELISA)

Levels of CCL2 in both serum and CSF, and serum VEGFA was measured using commercially available Quantikine sandwich ELISA kits (R & D systems, USA) as per manufacturer's instructions and read at 450 nm using 680XR Microplate reader (Biorad, Hercules, USA).

2.7. Lipid peroxidation

Lipid hydroperoxides (LPO) levels were measured in serum and CSF of ALS patients using a commercially available kit (Calbiochem San Diego, USA) as per manufacturer's instructions.

2.8. Statistical analysis

A forward stepwise (likelihood ratio) binary logistic regression was applied in order to compute the predicted risk of ALS. Thirteen independent demographic and biochemical variables (or risk factors), which were believed to be associated with ALS, were included in the study (Table 3). Each qualitative risk factor was given a numerical code (Table 3) in statistical product and service solutions (SPSS) 16.0 software to apply statistical tools. Quantitative variables were entered in original values obtained by instrument. It has been observed that the assumption of normality was lacking for most of the variables; we preferred to use logistic regression rather than probit or any other model. For calculating predicted risk of ALS in our case, logistic regression model is more appropriate than "logit model". Logistic regression connects dichotomous outcome variable (0 for control subjects and 1 for ALS patients) to a set of independent variables on which ALS depends. The unknown parameter β's in the regression model have been estimated using maximum likelihood method. Xi's represent 13 risk factors in the form of (X1, *X*2, ————————X13) as follow, X1: serum CCL2; *X*2: CCL2 mRNA; X3: CSF CCL2; X4: serum VEGFA; X5: VEGFA mRNA; X6: serum LPO; X7: CSF LPO; X8: age; X9: gender; X10: diet; X11: smoking status; X12: alcohol intake; X13: BMI. *p*-value was considered significant at <0.05.

Table 2
Demographic characteristic of subjects.

	Smoking		Alcohol consumption		Diet		BMI			
	Smoker	Neversmoker	Alcoholic	Nonalcoholic	Vegetarian	Meat consumer	Under	Normal	Over	Obese
ALS (n)	10	34	11	33	33	11	09	26	07	02
Control (n)	05	24	11	18	11	18	04	21	03	01

Demographic details of ALS patients and controls. ALS, amyotrophic lateral sclerosis; n, number of subjects in each category; BMI, body mass index.

Table 3
Statistics of risk factors.

	ALS	Control	*p*- value
Quantitative variables			
CCL2 mRNA (ΔCt)	6.1 ± 0.97	11.7 ± 0.56	0.0001
Serum CCL2 (pg/μg)	0.010 ± 0.0014	0.012 ± 0.002	0.569*
CSF CCL2 (pg/μg)	2.0 ± 0.50	0.89 ± 0.21	0.009
Serum LPO (μM/μg)	0.0013 ± 0.0002	0.001 ± 0.0001	0.112*
CSF LPO (μM/μg)	0.10 ± 0.01	0.031 ± 0.0057	0.208*
VEGFA mRNA (ΔCt)	3.0 ± 0.70	10.3 ± 0.54	0.0001
Serum VEGFA (pg/μg)	0.018 ± 0.0020	0.006 ± 0.0012	0.044
Age (y)	47.2 ± 2.0	38.1 ± 2.1	0.003
Numerical codes for qualitative variables			
Gender	Female - 0	Male −1	
Smoking status	Neversmoker - 0	Smoker −1	
Alcohol intake	Nonalcoholic - 0	Alcohol consumer - 1	
Diet	Meat consumer - 0	Vegetarian - 1	
BMI	Under weight - 0	Normal - 1 Overweight - 2	Obese - 3

Statistical details of risk factors. ALS, amyotrophic lateral sclerosis; BMI, body mass index; CCL2, Chemokine ligand 2; Ct, threshold cycle (the cycle where first fluorescence was detected by real time PCR machine); pg, pictogram; μg; microgram; μM, micromolar; LPO, lipid hydroperoxides; VEGFA, Vascular endothelial growth factor A; y, years. Quantitative data are indicated as Mean ± SE (standard error). Mann Whitney U- test was used to calculate p-values. p-values were considered significant at ≤0.05.
* Nonsignificant values.

3. Results

3.1. Logistic regression model development

Out of the 13 independent variables, only 5 variables viz serum CCL2, CCL2 mRNA, VEGFA mRNA, smoking and alcohol consumption were found to be significant ($p<0.05$) after applying forward stepwise (likelihood) binary logistic regression analysis. In logistic regression model, we computed the predicted risk (P) with the help of the following equation

$$P = \left(\frac{1}{1 + e^{-\left(\beta 0 + \sum_{i=1}^{K} \beta i Xi\right)}} \right)$$

However, it is possible to obtain a link function in which the model can act as generalized linear model (GLM) by taking log odds of the predicted risk i.e.

$$Y = Log\left(\frac{P}{1-P}\right) = \beta O + \sum_{i=1}^{K} \beta i Xi$$

From the equation, it is clear that log odds have linear relationship. The predicted risk of ALS can be computed, using

$$P = \left(\frac{1}{1 + e^{-Y}}\right)$$

In order to test whether the given data fits well to the logistic model, first of all, we have tested the following hypothesis:

Null hypothesis (H_0): The given data fits well to the logistic model;
Alternate hypothesis (H_1): The given data does not fit well to the model.

The Hosmer–Lemeshow goodness of fit statistic was applied and it indicates a good fit if the significance value is more than 0.05. In our case, chi square (χ^2) = 4.379, degree of freedom (df) = 8 and p = 0.821. Since p-value is greater than 0.05 therefore, the logistic model is adequately in agreement with the null hypothesis and fits the data. Additionally, according to Omnibus tests of model coefficients, chi square (χ^2) = 76.8, degree of freedom (df) = 5 and p = 0.0001, suggests that forward stepwise (likelihood) binary logistic regression is highly appropriate.

Table 4
Special features and level of significance of selected independent variables by maximum likelihood method for logistic regression equation.

Variable	β (Beta)	S.E.	Wald	df	*p*-value
Serum CCL2	−97.707	49.238	3.938	1	0.047
CCL2 mRNA	−0.591	0.240	6.080	1	0.014
VEGFA mRNA	−0.763	0.233	10.733	1	0.001
Smoking	−3.618	1.653	4.790	1	0.029
Alcohol consumption	3.985	1.563	6.504	1	0.011
Constant	13.001	4.201	9.577	1	0.002

CCL2, Chemokine ligand 2; VEGFA, Vascular endothelial growth factor A; mRNA, messenger ribonucleic acid; S.E., standard error; df, degree of freedom.

On the basis of special features of independent variable as presented in Table 4, the Wald test clearly shows that there are 5 significant variables (or predictors) which can predict the ALS risk and following logistic regression equation was obtained for the same.

$$\begin{aligned}\text{Model score (Y)} = {} & 13.001 - 97.7 \text{ serum CCL2} - 0.591 \text{ CCL2 mRNA} \\ & - 0.763 \text{ VEGFA mRNA} - 3.618 \text{ smoking} \\ & + 3.985 \text{ alcohol consumption}\end{aligned}$$

The 5 variables chosen by the forward stepwise (likelihood) method also show significant changes in −2 log-likelihood method as shown in Table 5 and further support the adequacy of model.

To check the association of variables in current logistic regression model, coefficient of determination (R^2) was computed using Cox and Snell's, and Nagelkerke's tests. It was observed that, in our case, Cox and Snell's $R^2 = 0.605$ and Nagelkerke's $R^2 = 0.818$ which is close to 1 and indicates a strong association of selected independent variables with dependent variables [5,6].

3.2. Logistic regression model validity

The validity of correct classification of ALS using forward stepwise (likelihood) method has been reported to be 90.4% as shown in classification table (Table 6). To see the proportion of actual positives which are correctly identified positive and actual negatives which are identified as such, sensitivity and specificity of the model have been tested. It was observed that the model yielded 93.2% sensitivity and 86.2% specificity. Based on five predictors, receiver operating characteristic (ROC) curve (Fig. 1) offers an excellent visual performance of the model, and the area under the curve (83.7%) suggests that the model works very well with minimum standard error of 0.048 and close to 95% confidence intervals for area.

Table 5
Change in −2 Log-likelihood.

Model if Term Removed				
Variable	Model Log Likelihood	Change in −2 Log Likelihood	df	*p*-value of change
Serum CCL2	−18.088	5.882	1	0.015
CCL2 mRNA	−22.873	15.453	1	0.0001
VEGFA mRNA	−33.788	37.283	1	0.0001
Smoking	−18.350	6.407	1	0.011
Alcohol consumption	−20.270	10.247	1	0.001

CCL2, Chemokine ligand 2; VEGFA, Vascular endothelial growth factor A; mRNA, messenger ribonucleic acid; df, degree of freedom.

Table 6
Classification table.

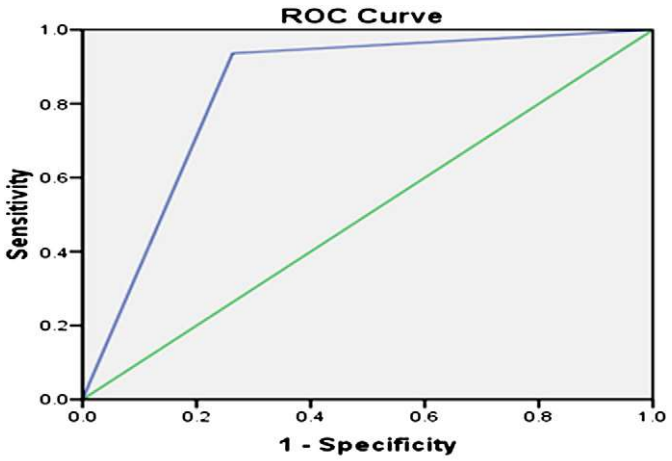
Observed		Predicted		
		ALS		% Corrected
		0	1	
ALS	0	25	04	
	1	03	41	
Overall %		90.4%		

Classification table showing overall % for correct prediction of ALS. Normal control individuals are given an arabic numeral code '0' and ALS patients were represented as '1'.

4. Discussion

ALS is a rare neurologic problem with an incidence that varies between 0.4 to 2.6 per 100,000 population and a prevalence of 4–6 per 100,000 population per year [7]. Since clinical manifestation of ALS is heterogeneous and often unpredictable, it is not possible to diagnose ALS accurately at early stages of disease based on neurological, radiological and electrophysiological findings. Simple risk factor identification through the proposed statistical equation provides a powerful and easy to understand solution to diagnose ALS at early or preclinical stage enabling patients to make informed recommendation regarding their future health.

We have attempted for the first time to provide a binary logistic regression equation based on five independent variables for those at risk of ALS development including either demographic (smoking and alcohol consumption) and/or molecular (Serum CCL2, PBMCs CCL2 mRNA and PBMCs VEGF-A mRNA) predictors which can be obtained easily by personal interviews or by way of non invasive estimation of CCL2 and VEGF-A using established methods. Such regression model had earlier been used in other neurological and non neurological disorders such as stroke, chronic obstructive pulmonary disease (COPD), coronary and liver diseases, etc. [8–11]. The North Indian ALS patient population was sampled in this study which offers a unique genetic perspective with significantly extended survival duration after the onset of disease and it will be interesting to test this model in other populations [12].



Area under the curve

Area	S.E.*	p-value	Asymptotic 95% Confidence Interval (CI)
0.837	0.048	0.0001	(0.742 – 0.931)

* S.E., Standard Error

Fig. 1. Receiver operating characteristic (ROC) for the forward stepwise (likelihood ratio) binary logistic regression model generated for significant predictors for ALS risk. Area under the curve is reported to be 83.7% and suggests that current model is good enough to differentiate ALS individuals from normal controls.

ALS is usually diagnosed in middle age and occurs more frequently in men as compared to women with an approximate ratio of 1.3 to 2.1 suggesting age and gender as a potential risk factors among others [13,14]. However, we did not find age and gender as significant predictors of ALS disease. The present study found serum CCL2, CCL2 mRNA, VEGFA mRNA, smoking and alcohol consumption as significant predictors for the risk of ALS ($p>0.05$). The earlier molecular studies of ALS are in consensus with our significant predictors. Elevated CCL2 protein and mRNA have already been observed in serum and spinal cord autopsy of ALS patients [15]. Likewise, increased serum VEGFA was observed previously [16,17]. Demographic studies have shown that current smokers are at 1.4 to 3-fold increased risk of ALS development as compared to never smokers [18,19], although association of alcohol with development of ALS is still under question.

Risk factors identification by logistic regression model is not only an effective method for predicting ALS diagnosis (90.4%) with excellent sensitivity and good specificity as shown by area under the ROC curve (Fig. 1), but also an important step in translating genetic investigations for strengthening diagnostics and public health initiatives. The clinical applicability and bed side utility of the model should, nevertheless, be checked with prospective inclusion of ALS patients and predictive accuracy of the current model must be cross validated in other larger populations where apart from confirmed ALS patients, subjects with other neurological disorders and ALS-like symptoms should also be measured.

Though the statistical model is quite versatile, inclusion of probable and possible cases of ALS along with definite ALS may represent a potential limitation. However, because of higher proportion of definite ALS, this limitation does not seem to affect validity of logistic regression model.

The logistic regression equation serves to influence policy and health economics given the weak network of specialists at primary care centers in India as no radiological or electrophysiological investigations are proposed in the equation. This will revitalize the telemedicine initiatives of third world countries and boost the South Asian Association for Regional Cooperation (SAARC) health initiative for South East Asia.

At the moment, it is not possible to state how the current logistic regression model changes the course of treatment or prescription of drugs, however, it may indeed facilitate diagnosis at preclinical stage when the primary motor neuron degeneration and severity of ALS would not be significant. The current statistical approach may lead to cost reduction and early therapeutic intervention with standard treatment strategies prevalent worldwide. This may also enable transparent, improved and effective management of ALS for both patient and disease managers.

In conclusion, our model may be applied for identification of individuals who are at high risk of ALS development with satisfactory sensitivity and specificity in the presence of serum CCL2, CCL2 mRNA, VEGFA mRNA, smoking and alcohol consumption. Future studies are, however, required to further validate its diagnostic utility.

Competing interest

None.

Author contribution

P.K.G and S.S. contributed equally to the manuscript. P.K.G. acquisition of data and writing of manuscript, S.P. inclusion of patients, grant PI and clinical scoring: S.S. statistical analysis: A.A. interpretation and analysis of data, grant co PI and editing of manuscript.

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SHORT REPORT Open Access

Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients

Pawan K Gupta†, Sudesh Prabhakar†, Chandrika Abburi, Neel K Sharma and Akshay Anand*

Abstract

Background: We have earlier shown that protein levels of vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) were elevated in Indian amyotrophic lateral sclerosis (ALS) patients. Here, we report the mRNA levels of VEGF-A and CCL2 in Indian ALS patients since they display extended survival after disease onset.

Methods: VEGF-A and CCL2 mRNA levels were measured in peripheral blood mononuclear cells (PBMCs) of 50 sporadic Indian ALS patients using Real Time Polymerase Chain Reaction (PCR) and compared with normal controls (n = 50). Their levels were adjusted for possible confounders like cigarette smoking, alcohol and meat consumption.

Results: VEGF-A and CCL2 mRNA levels were found to be significantly elevated in PBMCs in ALS patients as compared to controls. PBMCs from definite ALS revealed higher VEGF-A mRNA expression as compared to probable and possible ALS. CCL2 mRNA levels were found to be unaltered when definite, probable and possible ALS were compared. PBMCs from patients with respiratory dysfunction showed much higher VEGF-A and CCL2 elevation when compared to patients without respiratory dysfunction. No association of smoking, alcohol and meat consumption with VEGF-A and CCL2 was observed after analyzing the data with univariate and multivariate analysis.

Conclusion: VEGF-A and CCL2 mRNA upregulation in PBMCs may have a clinico-pathological/etiological/epidemiological association with ALS pathogenesis. The cross-cultural and cross-ethnic investigations of these molecules could determine if they have any role in enhancing the mean survival time unique to Indian ALS patients.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by selective loss of motor neuron. Vascular endothelial growth factor-A (VEGF-A) is a dimeric secreted polypeptide that was discovered first in the VEGF family which also includes placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF-A stimulates growth of blood vessels during embryonic development and helps in proliferation of blood collaterals in diseased conditions including ALS through a tyrosine kinase dependent VEGF receptor-2 (VEGFR2) [1]. Apart from angiogenesis, VEGF-A is suggested to exert direct neuroprotection via VEGFR2 and neuropilin-1 (NP-1) in animal models and patients of various neurodegenerative disorders [2]. Mice having homozygous deletion in hypoxia response element (HRE) of VEGF-A promoter ($VEGF^{\delta/\delta}$) were reported to develop symptoms like classical ALS [3] and conversely, intrathecal transplantation of stem cells overexpressing VEGF-A delays the onset and progression of ALS in superoxide dismutase-1 (SOD1) mutated transgenic mouse by downregulating proapoptotic proteins and activating

* Correspondence: akshay1anand@rediffmail.com
† Contributed equally
Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh-160012, India

phosphatidylinositol 3-kinase/protein kinase B (PI3-K/Akt) anti apoptotic pathway [4]. On the other hand, chemokine ligand-2 (CCL2), a proinflammatory molecule, may impart neuroprotection in ALS against glutamate induced excitotoxicity either by reducing release of glutamate and/or increasing efficiency of astrocytes to clear glutamate at synapses [5].

Indian ALS patients are known to exhibit significantly extended survival duration after disease onset as compared to Western ALS patients [6-8]. We recently reported that augmented biofluids VEGF-A and CCL2 protein may be associated with increased survival duration of Indian ALS patients [9]. We now measured the mRNA expression of VEGF-A and CCL2 in peripheral blood mononuclear cells (PBMCs) of these patients.

Subjects and methods

50 patients, born in North India and diagnosed with ALS were included from a convenience sample of Neurology outpatient, post graduate institute of medical education and research (PGIMER), Chandigarh after obtaining informed consent as a part of research protocol as per institute ethical committee guidelines (No. 7055-PG-1Tg-05/4348-50). Based on the "El Escorial criteria", there were 25 definite ALS patients, 15 individuals were probable ALS and remaining 10 were possible ALS at the time of sample collection. ALS-functional rating score-revised (ALSFRS-R) revealed that 11 patients had respiratory dysfunction such as orthopnea and dyspnea accompanied with other respiratory insufficiencies, although none of the patients needed respiratory support [10]. ALS patients with history of diabetic neuropathy, glaucoma, pre-eclampsia, stroke, those receiving riluzole, anti inflammatory drugs, antioxidants or other treatment were excluded. 50 genetically unrelated healthy normal controls without any apparent health problems such as hypertension, diabetes, heart disease etc were included for comparison. The subjects were categorized as cigarette smokers and never smokers, alcohol consumers and nonalcoholics, vegetarian and non-vegetarian (or meat consumers) using a standard questionnaire as per published criteria [11]. The clinical and demographic details of subjects published earlier [9] have also been reproduced here in Table 1.

PBMCs were isolated as per Histopaque-1077 (Sigma, USA) datasheet. Briefly, 6.0 ml blood was collected from each subject and layered on equal volume of Histopaque-1077. It was then centrifuged at 1800 rpm for 30.0 mins at room temperature and PBMCs were collected from plasma/Histopaque-1077 interface and preserved in RNA later (Sigma, USA) at -80°C until used.

Total RNA was extracted from PBMCs using RNAeasy columns (Qiagen, USA). RNA concentration was measured by taking absorbance at 260.0 nm. About 500.0 ng - 5000.0 ng total RNA was used to synthesize cDNA according to RevertAid™ first strand cDNA kit (Fermentas, USA).

Real Time Polymerase Chain Reaction (PCR) was used to quantitate expression of VEGF-A and CCL2 mRNA using published primers [12-14]. Methodology of Real Time PCR has been elaborated in "Additional File 1".

Because the data was normally distributed as indicated by quintile-quintile (Q-Q) plot, unpaired, independent, 2-tailed student *t* test and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis was applied for statistical comparisons. Crude and adjusted odds ratio (OR) was evaluated by univariate and multivariate logistic regression respectively to check any possible influence of smoking, alcohol and meat consumption on VEGF-A and CCL2 mRNA levels and χ^2 (chi square) test was performed to find significance level.

p-value was considered significant at ≤0.05. Statistical analysis was performed by statistical package and service solutions (SPSS) 16 software. Results were analyzed by two independent and masked researchers.

Results

Real Time PCR indicates that VEGF-A expression is 77-fold higher in ALS than controls (Figure 1A; p = 0.0001). CCL2 mRNA has shown an increment of 9.5-fold in ALS than controls (Figure 1B; p = 0.005). There was elevated VEGF-A mRNA expression in definite ALS patients in comparison to controls, probable and possible ALS (Figure 2A; p = 0.0001, p = 0.029 and p = 0.018 respectively). Further, both probable and possible ALS patients were shown to have higher VEGF-A than controls (Figure 2A; p = 0.0001 and p = 0.0001 respectively). However, CCL2

Table 1 Characteristics of the subjects

Subjects	Age (y)†	M/F (n)	Age of onset (y)	Disease duration‡ (mo)	B/L (n)	Smokers (n)	Alcohol consumers (n)	Non-vegetarian (n)
ALS	47.4 ± 12.4	38/12	46.2 ± 12.8	19.0 ± 12.7	8/42	12	12	20
Controls	40.0 ± 12.8	39/11				10	14	27

Clinical and demographic details of subjects. ALS, amyotrophic lateral sclerosis; n, Number; M, male; F, female; y, years; mo, months; B, bulbar; L, limb; Age, age of onset, duration of disease are indicated as mean ± standard deviation (SD). † Unpaired, independent 2-tailed student *t* test analysis showed that mean age differ significantly among the groups (p = 0.004). ‡ Duration of disease is the interval between appearance of first symptom of ALS and collection of sample. ALS subjects were asked to provide all clinical and demographic details at the age of disease-onset.

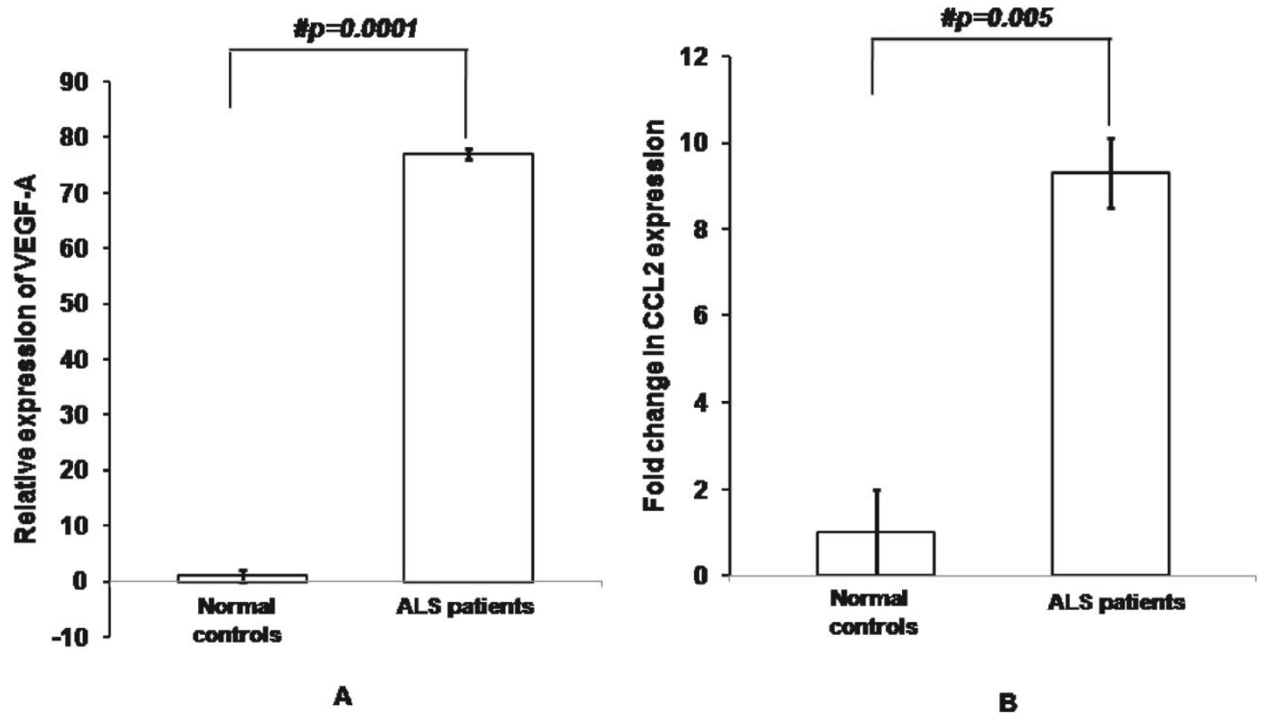

Figure 1 Relative mRNA expression of VEGF-A (A) and CCL2 (B) in PBMCs of ALS patients. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by unpaired, independent 2-tailed student *t* test. # indicates significant difference among the groups ($p < 0.05$). Expression of VEGF-A and CCL2 were normalized to expression of endogenous control b-actin. ALS, amyotrophic lateral sclerosis; VEGF-A, vascular endothelial growth factor-A; CCL2, chemokine ligand-2; PBMCs, peripheral blood mononuclear cells.

levels did not vary between definite, probable and possible ALS cases (Figure 2B; $p > 0.05$).

To find association of respiratory dysfunction, VEGF-A and CCL mRNA levels were reanalyzed among ALS patients with respiratory dysfunction and those without respiratory dysfunction. Significantly increased VEGF-A and CCL2 was observed in ALS patients with respiratory dysfunction as compared to patients without respiratory dysfunction (Figure 3A-B; $p = 0.045$ and $p = 0.021$ respectively)

No association of cigarette smoking, alcohol and meat consumption with VEGF-A (Table 2) and CCL2 (data not shown) mRNA was observed upon univariate and multivariate analysis.

Discussion

It has been reported that median survival duration of Indian ALS patients is ~9 years after disease onset which is significantly higher as compared to their Western counterparts who survive for 3-6 years after disease onset [6-8]. Because of this contradicting presentation, we investigated the levels of VEGF-A and CCL2 among the Indian ALS patients.

The increased PBMCs VEGF-A and CCL2 expression in our patients may suggest the pathophysiological involvement of circulating monocytes and lymphocytes in ALS. The elevated PBMCs VEGF-A is in contrast to previous reports where a profound downregulation of VEGF-A mRNA in SOD1G93A ALS mouse and significantly reduced serum and cerebrospinal fluid (CSF) VEGF-A in ALS patients was observed possibly because of genetic changes in promoter regions [15-17]. Increased serum and CSF VEGF-A reported earlier in ALS and in its different clinical subtype with limb onset and extended disease duration are in agreement with current results [18,19]. However, some studies have failed to detect significant change in serum, plasma and CSF VEGF-A in ALS patients [20,21]. It is believed that the variable study designs including different molecular tools, study power, diverse clinical and genetic spectrum of ALS patients may account for conflicting VEGF-A levels. The increased PBMCs CCL2 is consistent with reports where elevated CCL2 mRNA was observed in spinal cord and skeletal muscles of ALS patient's autopsies and SOD1 mutated ALS mice [14,22].

As VEGF-A and CCL2 are neurotrophic, Indian ALS patients may enhance VEGF-A and CCL2 expression in an attempt to ameliorate excitotoxicity through upregulation of glutamate receptor as reported earlier [5,23]. Increased VEGF-A and CCL2 may promote migration

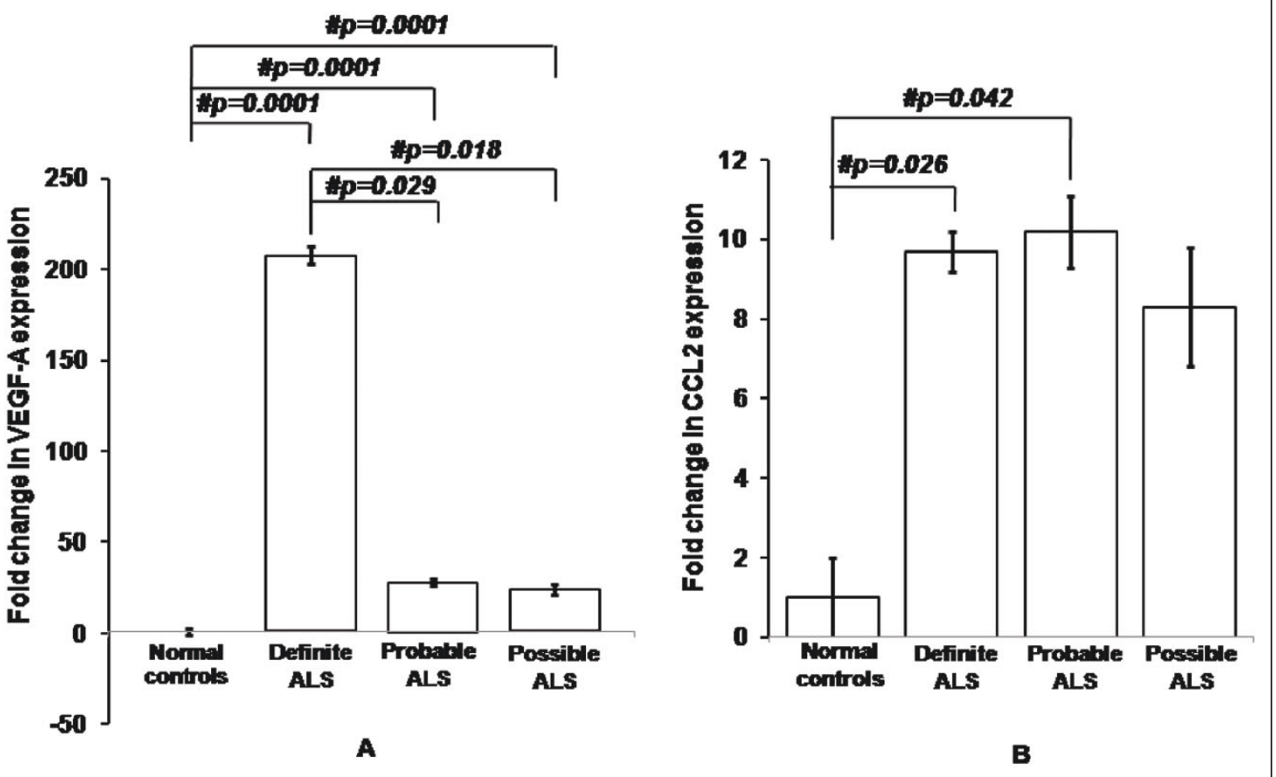

Figure 2 Relative mRNA expression of VEGF-A (A) and CCL2 (B) in PBMCs of definite, probable and possible ALS patients. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test. # indicates significant difference among the groups ($p < 0.05$). Expression of VEGF-A and CCL2 were normalized to expression of endogenous control b-actin. Basal VEGF-A mRNA expression in normal control group is represented by x-axis in part "A". ALS, amyotrophic lateral sclerosis; VEGF-A, vascular endothelial growth factor-A; CCL2, chemokine ligand-2; PBMCs, peripheral blood mononuclear cells.

and differentiation of VEGF receptor 1 (VEGFR1), VEGFR2 and chemokine receptor 2 (CCR2) expressing adult neural progenitor cell into neuronal and glial phenotypes at the site of injury [24,25]. Whether their upregulation represent any compensatory response towards extended survival of Indian ALS patients should be evaluated in future comparable cross-cultural and cross-ethnic ALS population where survival is longer. It must be emphasized that mean survival duration of reported ALS patients could not be ascertained.

Since elevated CCL2 initiates inflammatory reaction by increasing production of nitric oxide and other inflammatory chemokines from unregulated monocytes/macrophages [26] and VEGF-A is known to recruit leukocytes at the site of brain injury by increasing vascular permeability [27], it is possible that the high VEGF-A and CCL2 in our ALS patients may exert limited inflammatory responses associated with neuroprotection [28].

At this moment, we are not able to state whether the increased VEGF-A and CCL2 mRNA is a consequence of genetic and/or epigenetic changes of upstream regulatory sequences, altered transcriptional regulation or amyotrophy and thus the present report lays the foundation for future studies to screen promoter elements of VEGF-A and CCL2 in Indian ALS population for subtle genetic differences. The stress conditions, like respiratory problems, may also modify transcriptional gene regulation as indicated by increased VEGF-A and CCL2 mRNA expression in the 11 ALS patients with respiratory dysfunction and signifies a possible association with hypoxia (Figure 3).

Based on existing literature [29,30], elevated VEGF-A and CCL2 in definite ALS may represent the possibility of relatively extensive extra central nervous system (CNS) involvement and higher degree of nerve endings arborization at neuromuscular junction than probable and possible ALS, however, neuroanatomical architecture of neuromuscular junction has not been evaluated. The possibility of increased VEGF-A and CCL2, in definite ALS due to respiratory dysfunction, may not be ruled out even though only 28% of all definite ALS cases presented with respiratory symptoms.

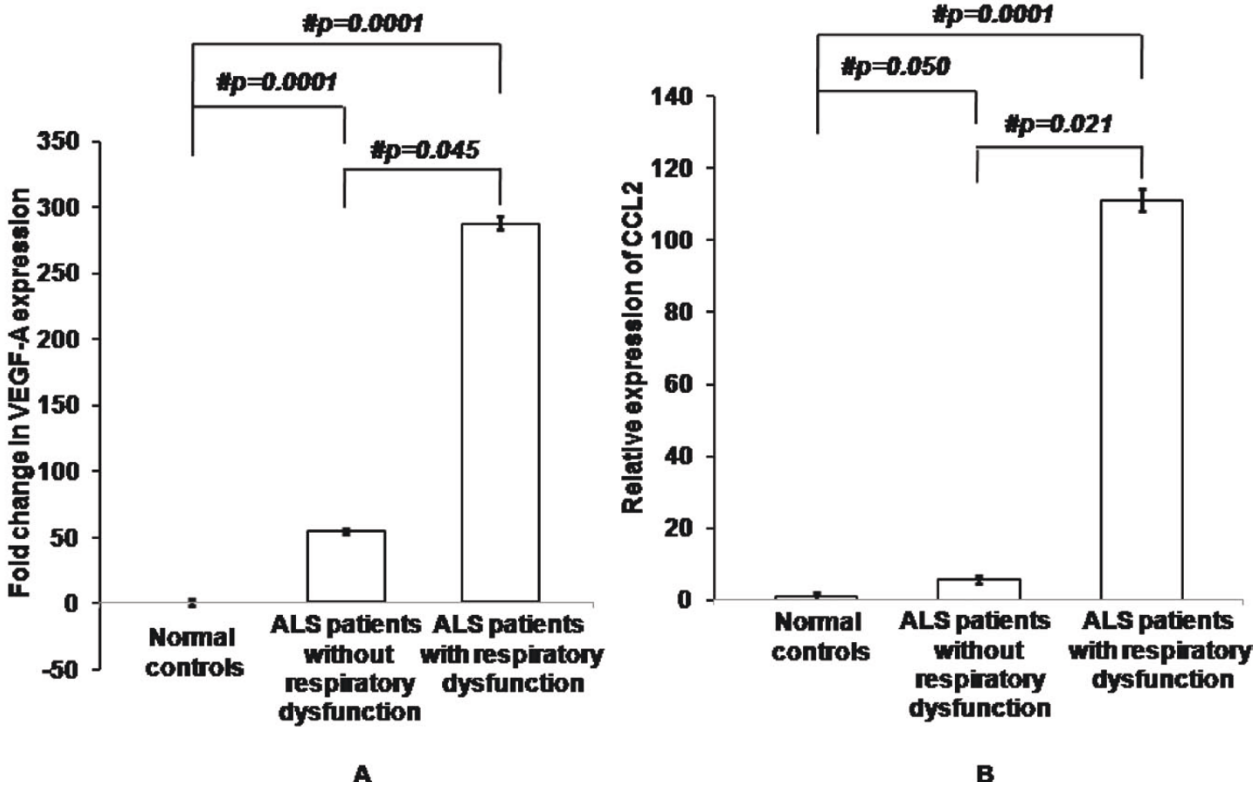

Figure 3 Fold change in expression of VEGF-A (A) and CCL2 (B) in PBMCs of ALS patients with respiratory dysfunction. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* test. # indicates significant difference among the groups ($p < 0.05$). Expression of VEGF-A and CCL2 were normalized to expression of endogenous control b-actin. Basal VEGF-A mRNA expression in normal control group is represented by x-axis in part "A". ALS, amyotrophic lateral sclerosis; VEGF-A, vascular endothelial growth factor-A; CCL2, chemokine ligand-2; PBMCs, peripheral blood mononuclear cells.

Conclusion

Although it can not be concluded that increased VEGF-A and CCL2 expression contributes towards enhanced survival yet the importance of clinico-pathological, etiological and epidemiological association of increased VEGF-A and CCL2 with survival of Indian ALS patients may not be underestimated and needs further investigations.

Table 2 Crude and adjusted OR for VEGF-A mRNA

	OR (95% CI)†	*p**	Adj. OR (95% CI)‡	*p**
VEGF-A mRNA				
Smoking	0.8 (0.2-4.3)	0.8	1.1 (0.2-6.3)	0.8
Alcohol consumption	1.0 (0.2-4.3)	0.9	0.9 (0.2-4.5)	0.9
Meat consumption	0.8 (0.2-3.0)	0.8	0.8 (0.2-3.3)	0.8
Never smoking/ Nonalcoholic/ Vegetarian**	1.0		1.0	

† Univariate logistic regression was used to calculate crude OR. ‡ Multivariate logistic regression was used to adjust the effect of smoking on VEGF-A mRNA levels with alcohol and meat consumption as covariates. Likewise, effect of alcohol and meat consumption on VEGF-A is also adjusted for covariates. *χ^2 (chi square test) was used to test the level of significance. ** Never smoking, nonalcoholic and vegetarian diet is considered as reference group. VEGF-A, vascular endothelial growth factor-A; OR, odds ratio; CI, confidence interval; Adj, adjusted.

Ethical approval

Ethical approval was obtained by institute ethical committee, PGIMER, Chandigarh, India - 160012 (No. 7055-PG-1Tg-05/4348-50).

Additional material

Additional file 1: Real Time Polymerase Chain reaction (PCR). Methodology of Real Time PCR; PCR cycling conditions and amplicon size of VEGF-A and CCL2; sequences and references of primers used.

Abbreviations

ALS: amyotrophic lateral sclerosis; ALSFRS-R: ALS functional rating score-revised; ANOVA: analysis of variance; CCL2: chemokine ligand-1; CCR2: chemokine receptor-2; CNS: central nervous system; CSF: cerebrospinal fluid; EDTA: ethylene diamine tetraacetate; HRE: hypoxia response element; LSD: least significant difference; mRNA: messenger ribonucleic acid; NMDA: N-Methyl-D-aspartate; NP-1: neuropilin-1; OR: odds ratio; PBMCs: peripheral

blood mononuclear cells; PCR: polymerase chain reaction; PI3-K: phosphatidylinositol 3-kinases; SOD1: superoxide dismutase 1; VEGF: vascular endothelial growth factor; VEGFR1: VEGF receptor-1; VEGFR2: VEGF receptor-2.

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Authors' contributions
PKG Acquisition of data and writing of manuscript. SP inclusion of patients, grant PI and clinical scoring. CA Acquisition of data. NKS Statistical analysis. AA Interpretation and analysis of data, grant co PI and writing and editing of manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Regular paper

Altered oxidative stress levels in Indian Parkinson's disease patients with *PARK2* mutations

Monika Vinish★, Akshay Anand★ and Sudesh Prabhakar✉

Department of Neurology, PostGraduate Institute of Medical Education and Research (PGIMER), Sector-12, Chandigarh, India

The aim of this pilot study was to determine the baseline state of oxidative stress indices in patients with Parkinson's disease (PD). Peripheral blood samples of 15 PD subjects were analyzed and compared with ten age matched healthy controls. Patients with *PARK2* mutations were also compared with PD patients without mutations. There was significant increase in malondialdehyde content and superoxide-dismutase (SOD) activity in peripheral blood parameters in PD patients ($P < 0.05$) in comparison to controls. These findings suggest an important role of oxidative stress in Parkinson's disease evolution and progress. No changes were observed in glutathione peroxidase and nitric oxide levels. We found significant correlation between SOD activity and lipid peroxidation when the biochemical data was further analyzed. In addition, significant increase in the levels of SOD among the PD patients with *PARK2* mutations was observed, which can be ascribed to chronic oxidative stress induced by *PARK2* mutations.

Key words: oxidative stress, mutations, Parkinson's disease, *PARK2*

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INTRODUCTION

Parkinson's disease (PD) is characterized by a loss of dopaminergic neurons in the substantia nigra, leading to the major clinical and pharmacological abnormalities that characterize the disease. Although the pathogenesis of PD remains ambiguous, oxidative stress (OS) to dopaminergic neurons in the substantia nigra pars compacta (SNpc) has been reported to be one of the leading causes of neurodegeneration in PD (Bahmann *et al.*, 2004). The human body has evolved several defense mechanisms to counteract OS such as vitamin E, vitamin C, vitamin A, glutathione and various antioxidant enzymes, but the brain appears to be more susceptible to these assaults than other organs because of its low antioxidant capacity. Being highly metabolic, brain tissue generates more oxyradicals. Alteration in the oxidative stress has been proposed to cause the loss of dopaminergic neurons in PD patients (Hung & Lee, 1998). Although the changes in lipid peroxidation and antioxidant defenses are documented in the SNpc of PD patients but there is difficulty in obtaining a brain biopsy, until after the death of the afflicted individual. It is therefore crucial to develop suitable peripheral markers, which can help in the diagnosis of PD during life.

The etiology of Parkinson's disease is unknown although both genetic susceptibility and environmental factors appear to play an important role in its development. Parkin is a Parkinson disease-related E3 ubiquitin ligase; parkin-deficient animals exhibit mitochondrial degeneration and increased oxidative stress vulnerability, and both mice and flies lacking DJ-1 are hypersensitive to environmental toxins associated with PD (Palacino *et al.*, 2004; Shen & Cookson, 2004). Currently, accumulating evidence indicates that parkin may play a role in maintaining mitochondrial function and preventing oxidative stress (Hyun *et al.*, 2005). We therefore examined if *PARK2* deletions alter the antioxidant profile of Indian PD patients.

MATERIAL AND METHODS

Patients. The study group included 15 sporadic or non-consanguineous PD patients visiting the Neurology Clinic at the PostGraduate Institute of Medical Education and Research (Chandigarh, India) and ten healthy controls. The diagnosis of Parkinson's disease was made on the basis of the UK Parkinson's Disease Society Brain Bank Research criteria, London (Hughes *et al.*, 1992). Clinical diagnosis was established with the presence of at least two of the cardinal symptoms, i.e., tremors, muscular rigidity, bradykinesia and postural instability while patients with vertical gauge impairment, marked autonomic disturbances, atypical Parkinsonism and those on antipsychotic drugs were excluded from the study (Lang & Lozano, 1998). Written informed consent was obtained from all patients and controls as per the Institute Ethics Committee guidelines. Genetically unrelated controls were also examined for the absence of extra-pyramidal signs, which included spouse of the patient and other age, sex and ethnicity matched healthy individuals. The mean age of onset for patients recruited for the study was 46.5 ± 2.1 years while that for healthy volunteers was 43.4 ± 2.2. About 10 mL of venous blood was drawn for genetic analysis from these patients and controls. All the biochemical assays were performed in duplicates.

Superoxide dismutase (SOD) assay. Determination of Cu,Zn-SOD activity was performed using a commercial kit (Ransod; Randox, CrumLin, UK) based on the method developed by McCord and Fridovich (1988). Coupling of $O_2^{-\bullet}$ generators (xanthine and xanthine oxi-

✉e-mail: pgineurology@gmail.com
★These authors contributed equally to this work

Abbreviations: Gp_x, glutathione peroxidase; INT, (2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride LRRK2, leucine rich repeat kinase; MDA, malondialdehyde; PD, Parkinson's disease; *PARK2,* Parkin gene; SNc, substantia nigra; SOD, superoxide dismutase; PINK-1, PTEN-induced putative kinase 1

dase) with an $O_2^{\bullet}$ detector INT (2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride) leads to formation of red formazan dye. One unit of SOD activity was defined as the amount of protein that inhibits the rate of INT reduction by 50%. Enzyme activity was measured in SOD units/mL of whole blood.

Glutathione peroxidase (Gp_x) assay. Measurement of glutathione peroxidase (Gp_x) activity was performed using Ransel reagents (Randox Laboratories, UK) and is based on the method of Paglia and Valentine (1967). Gp_x catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted into the reduced form with a simultaneous oxidation of NADPH to $NADP^+$. For the Gp_x assay, hemolysates (50 µL) were diluted with 1.0 mL of Ransel diluting agent and incubated for 5 minutes, followed by the addition of 1.0 mL of the Drabkin reagent. The decrease in absorbance at 340 nm was measured.

Nitric oxide estimation. Nitric oxide estimation was done by the method of Titheradge (1998). About 100 µL of the sample (plasma) was added to 400 µL of distilled water, 500 µL of freshly prepared solution C (Griess reagent) was then added to the vials containing sample. The reaction mixture was incubated at room temperature for 10 minutes and absorbance was read at 546 nm.

Lipid peroxidation. Brain tissues are rich in phospholipids and vulnerable to attack by oxygen-derived free radicals to initiate lipid peroxidation. Lipid peroxidation was evaluated as the concentration of malondialdehyde (MDA), a lipid peroxidation end product that reacts with TBA (thiobarbituric acid) to form a conjugate. MDA levels thus provide valuable information for evaluation of oxygen radical-induced oxidative stress. In our study, MDA was assayed by the method of Buege and Aust (1978). About 1.0 mL of plasma was mixed with 2 mL of mildly heated reagent (15% (w/v) trichloroacetic acid, 0.375% (w/v) TBA, 0.25 M HCl). The solution containing plasma and reagent was heated in boiling water bath for 15 min and then cooled. The flocculation precipitate was removed by centrifugation at 1 000 r.p.m. for 10 min. The supernatant was collected and the absorbance was read at 535 nm against appropriate blank. The amount of MDA was calculated using molar absorption coefficient of MDA ($1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$). The results were expressed as mmol of MDA/L.

***PARK2* analysis.** Genomic DNA was extracted from the sporadic PD patients and controls. All exons of *PARK2* were amplified by PCR using parkin specific primers (Kitada *et al*., 1998). PCR products were visualized on 2% agarose gel for absence or presence of amplified product. Gene dosage analysis was not carried out because lack of real-time PCR device in our institute. Therefore this study was restricted to qualitative analysis alone.

In order to determine the mutations in *PARK2*, PCR amplified exons were subjected to SSCP and band pattern was obtained by silver staining. Sequencing was performed for all those PCR products of patients and controls that showed mobility shift by SSCP analysis.

Statistical analysis. The mean values of various antioxidants were compared using Student's *t*-test. Correlation between different variables was tested using nonparametric Spearman's coefficient and statistical significance was considered at $P < 0.05$. (SPSS 17.0, Chicago, IL, USA).

RESULTS

Central Nervous System related studies have been confronted with the complexity of direct investigation because of difficulty in obtaining biopsies. It is therefore crucial to investigate peripheral markers, which can help in the early diagnosis of Parkinson's disease (PD). Our recent study has revealed upregulated levels of SOD and lipid peroxidation in PD patients (Sharma *et al*., 2008). Molecular studies in familial forms of the disease have identified genes: α-synuclein, parkin, *DJ-1* (*PARK7*), *PINK-1* (PTEN-Induced putative kinase 1) and *LRRK2* (leucine rich repeat kinase) encoding key proteins involved in PD pathogenesis, and support a major role for mitochondrial dysfunction and oxidative stress (Thomas & Beal, 2007). Here we studied the antioxidant profile (SOD, Gp_x, nitric oxide and lipid peroxidation (MDA) in blood of PD patients and controls using spectrophotometric analysis.

Superoxide dismutase (SOD)

SOD is a Cu,Zn containing enzyme responsible for catalytic dismutation of highly reactive and potentially toxic superoxide radicals ($O_2^{-\cdot}$) to H_2O_2 (McCord and Fridovich, 1988). $O_2^{-\cdot}$ is constantly generated in the body tissues and failure in its removal can initiate a damaging effect on polyunsaturated fatty acids and structural proteins of plasma membranes. We estimated SOD levels among PD patients and controls using spectrophotometric analysis. The results showed significantly increased SOD levels ($P = 0.026$) in PD patients as compared to controls ($n = 10$) (Fig. 1).

Glutathione peroxidase (Gp_x)

Gp_x has been previously reported as an important hydroperoxide-degrading enzyme and the importance of glutathione reductase (GR) lies in its ability to keep glutathione in its reduced (biologically active) form. Numerous chemical processes in aerobic cells lead to the production of peroxides by activated forms of oxygen. These peroxides by their decomposition to free radicals and other reactive chemical species cause oxidative damage in biological tissues. The simplest hydroperoxides such as H_2O_2 and lipid peroxides can be detoxified by the selenium-dependent Gp_x. The activity was estimated by following the oxidation of NADPH, required for the reduction of GSSG to GSH. We only found an insignificant decrease in the Gp_x levels (699 ± 59.4) in patients as compared to controls (730 ± 79.0) (Fig. 2).

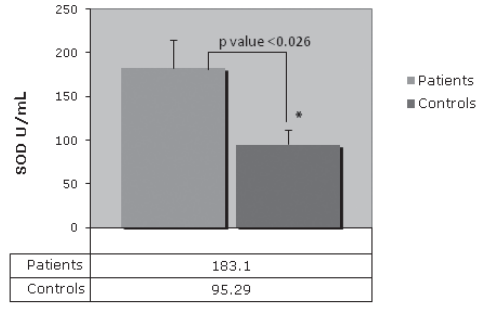

Figure 1. SOD levels in PD patients and controls.

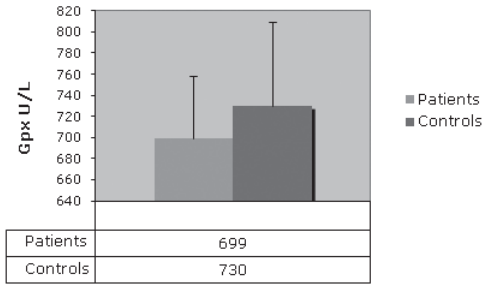

Figure 2. Glutathione peroxidase (Gp_x) levels in PD patients and controls

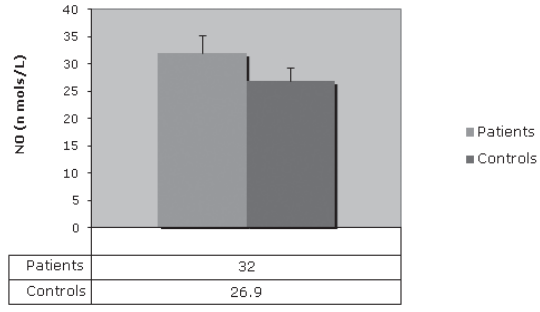

Figure 3. Nitric oxide levels in PD patients and controls

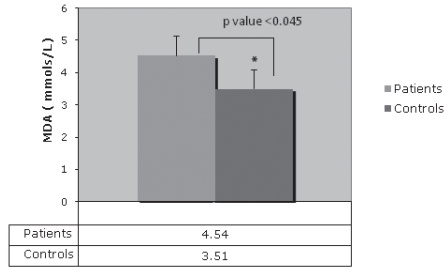

Figure 4. Lipid peroxidation (MDA) levels in PD patients and controls

Nitric oxide estimation

Nitric oxide is a unique biological messenger molecule which plays diverse physiologic roles. NO mediates blood vessel relaxation, immune activity of macrophages and neurotransmission of central and peripheral neurons. Our results revealed a slight increase in NO levels in PD patients (32.0 ± 3.3) *vs* controls (26.9 ± 2.4) but this was statistically insignificant (Fig. 3).

Lipid peroxidation

Lipid peroxidation reflects oxidative deterioration of polyunsaturated fatty acids, important constituents of biological membranes and is measured in terms of nmols of MDA formed/mg protein. Higher levels of MDA, a marker of oxidative stress, have been reported in the SNpc of PD patients (Dexter *et al.*, 1989). Increased lipid peroxidation is well reported in neurodegenerative diseases (Dei *et al.*, 2002). Similarly, we found a significant increase in the plasma MDA levels of PD patients as compared to controls (Fig. 4). Thus these increased lipid peroxidation products suggest that ROS have an important role to play in the pathogenesis of neurodegenerative diseases.

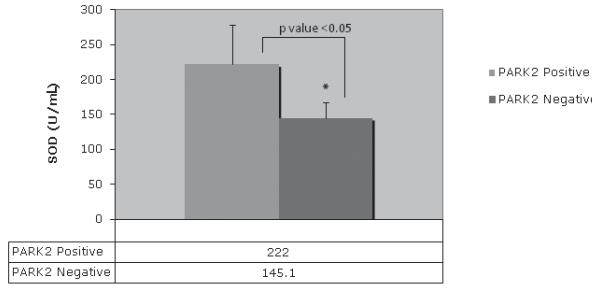

Figure 5. SOD levels in PD patients with and without *PARK2* mutations

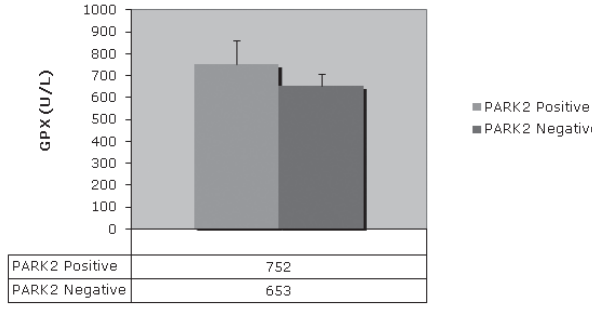

Figure 6. Glutathione peroxidase (Gp_x) levels in PD patients with and without *PARK2* mutations

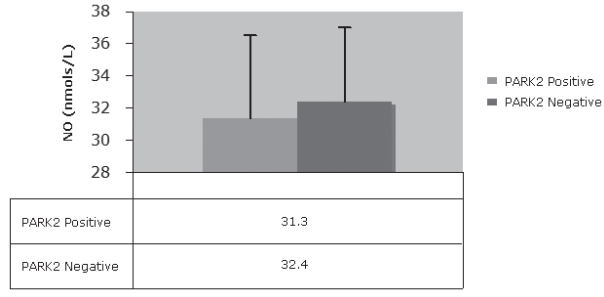

Figure 7. Nitric oxide levels in PD patients with and without *PARK2* mutations

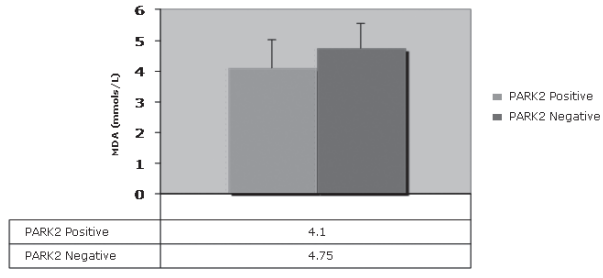

Figure 8. Lipid Peroxidation (MDA) levels in PD patients with and without *PARK2* mutations.

PARK2 analysis

All the 12 exons of *PARK2* were amplified in PD patients and healthy controls. Absence of a band was confirmed by repeating PCR and revalidated by *GAPDH*

Table 1. Correlation among the various biochemical variables

Variables	Sex	Age	SOD	Gp_x	Lpx	NO
Sex	1.00	0.175	0.456	0.171	−0.116	0.463
Age	0.175	1.0	−0.378	−0.113	−0.507	0.031
SOD	−0.456	−0.378	1.0	−0.093	0.797**	−0.187
Gp_x	0.171	−0.113	−0.093	1.0	−0.225	0.341
Lpx	−0.116	−0.507	0.797**	−0.225	1.0	−0.279
NO	0.463	0.031	−0.187	0.341	−0.279	1.0

**Correlation is significant at $P < 0.01$ level (2-tailed)

(positive control) amplification of the same template. *PARK2* analysis in these patients revealed exonic deletions in exons 1, 2, 3 and 12 by PCR and SSCP analysis. The exons were amplified thrice under same set of PCR conditions. In addition, when the biochemical values of these PD patients carrying *PARK2* mutations and those without mutations were compared (Figs. 5–8), a significant increase in the levels of SOD among the PD patients with *PARK2* mutations was observed, which can be attributed to chronic oxidative stress induced by *PARK2* mutations.

Correlation between biochemical parameters

Spearman correlation analysis was performed to study the association between the biochemical parameters. A significant positive correlation between superoxide dismutase and MDA levels was found ($P = 0.001$) (Table 1).

DISCUSSION

Oxidative stress represents one of the risk factors that can promote neurodegeneration in PD. Recent evidence suggests that several known mutations cause familial Alzheimer disease (AD) (amyloid β protein precursor, presenilin-1, or presenilin-2 gene) while familial PD genes such as *Parkin, PINK-1*, or *DJ-1* are associated with increased oxidative stress. Also, several known genetic (e.g., apolipoprotein Eε4 variant) and environmental (e.g., metals or pesticides exposure) risk factors of sporadic AD and/or PD are associated with increased oxidative stress (Dei *et al*., 2002).

Our recent study revealed high mutation frequency in North West Indian PD population (Vinish *et al*., 2010) and also recently reported alterations in the lipid peroxidation and SOD profile in a separate set of PD patients, suggesting that these mutations may be related to mitochondrial dysfunction and oxidative stress. We therefore extended our study to understand if *PARK2* mutations affect the antioxidant profile of such patients (Sharma *et al*., 2008) and thus evaluated the oxidative stress in these patients and tested whether *PARK2* mutations contribute to alterations in antioxidant profile. We found that SOD levels and lipid peroxidation were higher in PD patients as compared to controls. This increase may be a compensatory response of the body to counteract the increased superoxide radicals, which are generated in these patients. We also found slight decrease in Gp_x activity in patients as compared to controls, which could be a result of disturbed oxidative stress (OS). Brain contains large amounts of unsaturated fatty acids, which are target for lipid peroxidation. Enhanced lipid peroxidation levels have been well reported in the postmortem brain of PD patients. MDA has also been previously reported as a potential peripheral oxidative stress marker in plasma of PD patients (Llic *et al*., 1999; Serra *et al*., 2001; Younes-Mhenni *et al*., 2007). Our study also revealed increase in plasma MDA levels in PD patients as compared to healthy controls, which reflects a state of OS in these patients. Therefore, the increased SOD and lipid peroxidation levels in PD patients appear to play an important role in PD pathogenesis. The elevated SOD levels have been earlier reported in blood of PD patients (Llic *et al*., 1999; Serra *et al*., 2001; Younes-Mhenni *et al*., 2007; Sharma *et al*., 2008). Besides, we also found excellent correlation between SOD and lipid peroxidation levels in the blood of these patients which can serve as a biomarker in the blood (Table 1).

PARK2, DJ-1 and *PINK-1* gene mutations have been previously reported (Thomas & Beal, 2007) in the elevation of oxidative stress. Our results partly match such antioxidant profile. Although the antioxidant profile was altered in PD patients with mutations when compared to those without mutations a larger study will determine the importance of these results. Nevertheless, this pilot study provides preliminary information which forms the basis of the investigations to follow. Based on the analysis, we conclude that alterations in SOD and lipid peroxidation levels are possibly due to increased oxidative stress in these patients or as a general compensatory response. Mutations in *PARK2* might affect the oxidative machinery of PD patients but a larger study can establish this. Our study supports the involvement of oxidative stress that is implicated in the pathogenesis of PD. Perspectives for treatment of PD in the future should investigate the role of antioxidant therapy.

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Occurrence of *PARK2* Mutations in a Never-Smoker Population with Parkinson's Disease in North India

Sudesh Prabhakar Monika Vinish C.P. Das Akshay Anand

Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Key Words
Parkinson's disease · *PARK2* mutation · Sikhs · Never-smokers · Socio-economic status

Abstract
Background: Parkinson's disease (PD) is a complex neurological disorder without any well-documented genotype-demography associations among sporadic variants. We recently reported *PARK2* mutations to be constituting 40% of PD in this region and thus analysed how demographic variables associate with *PARK2* mutations in 70 of these patients. ***Methods:*** PD samples were screened by PCR single-strand conformation polymorphism (SSCP) and sequencing and their demographic data collected. Demographic and religion data was obtained from 1,010 randomly selected individuals of 120,000 patients visiting the Neurology Clinic and was compared with state database and PD patients. ***Results:*** Sikhs from a rural background exhibited the majority of *PARK2* mutations. The frequency of *PARK2* mutations among females was significantly higher as compared to males ($p < 0.015$). The age of onset of PD patients with a rural background was found to be significantly lower as compared to patients with an urban background ($p < 0.004$). The demographic spectrum of the 1,010 randomly selected patients and the background population was found to be comparable. ***Conclusions:*** As PD patients with *PARK2* mutations were found to be of sporadic origin and never-smokers, a non-redundant inverse relationship between founder *PARK2* mutations and smoking is implicated to account for its high frequency. The predisposition of Sikhs to *PARK2* mutations necessitates a larger study among its familial variants and a control smoker PD population. The spectrum of *PARK2* mutations among Sikh smokers is difficult to study because of the religion-based aversion to smoking.

Introduction

PARK2 mutations have been implicated in parkinsonism and reported in several studies [1, 2]; however, there is a lack of demography association studies among non-familial variants of Parkinson's disease (PD). The epidemiological investigations have earlier shown a high frequency and mortality of PD patients in Caucasians when compared to African-Americans [3]. It is also reported to be more common in men than women [4, 5] with a higher incidence in older people, but its spectrum on the Indian subcontinent has not been adequately addressed.

It is widely acknowledged that both genetic susceptibility and environmental factors play an important role in the development of PD. For instance, history of smok-

S.P. and M.V. contributed equally to this work.

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Assist. Prof. Akshay Anand, PhD
Department of Neurology
Postgraduate Institute of Medical Education and Research
Chandigarh (India)
Tel. +91 991 420 9090, E-Mail akshay1anand@rediffmail.com

ing, sleeping patterns and other gene loci are reported to play a cardinal role in the progression of PD [6–8]. Interestingly, nicotine lowers the incidence of PD in smokers [9], and brain expression of cytochromes P450, 2B6, 2D6 and 2E1 is also found to be higher in smokers, apparently induced by nicotine. This can lead to disruption of the neurotoxins associated with PD [10]. The association of *PARK2*-related PD and smoking has not been explored earlier even though these mutations have been extensively reported in different parts of the world. North-West India provides a culturally unique place with the highest percentage of religion-based never-smokers and an ideal sample to answer some of these outstanding questions. We examined the prevalence of such mutations across traditionally conservative communities inhabiting this place and studied their correlation between the nature and location of *PARK2* mutations with disease phenotype, smoking habits and demographic origin of patients. We recently reported that 40% of the PD patients possess *PARK2* mutations in this region [11]. We now report that the majority of these mutations are harboured by the never-smoker Sikh community fuelling speculations whether *PARK2* susceptibility is enhanced among communities that never smoke.

Materials and Methods

Participants

The diagnosis of 70 PD cases was made on the basis of established UK PD Society Brain Bank clinical diagnostic criteria [12]. The written informed consent was obtained from all patients and genetically unrelated age-, sex- and religion-matched controls following the Institute of Ethics committee guidelines. Out of 120,000 patients attending the Neurology Outpatient Clinic, 70 PD patients were screened on the basis of at least 2 of the following symptoms: tremors, muscular rigidity, bradykinesia and postural instability. Patients with vertical gaze impairment, marked autonomic disturbances, atypical parkinsonism and those on antipsychotic drugs and anti-oxidants were excluded from the study [13]. Although our inclusion criteria did not allow exclusion of smokers, all the patients recruited in the study turned out to be never-smokers based on the last 20-year smoking habits. This has limited the comparison between smokers and never-smokers. Normal healthy never-smoker controls were examined for the absence of extrapyramidal signs which included the spouse of the patient and other age-, sex- and ethnicity-matched healthy individuals.

Procedure

Genetic Analysis

The mutations in the *PARK2* gene were detected by using PCR single-strand conformation polymorphism (SSCP) and direct sequencing of the purified PCR products. PCR products were resolved on 8% non-denaturing polyacrylamide gel followed by silver staining. The SSCP variants were identified and sequenced. Gene dosage analysis was not carried out because of lack of facility for real-time PCR in our institute, and therefore this study was restricted to qualitative analysis alone.

Sociodemography Comparison of Patients and Background Population

In order to substantiate the postulated inverse role of never-smoker Sikhs with a high frequency of *PARK2* mutations and to validate the homogeneity in sampling, it was necessary to demonstrate the lack of bias in the sampling of 70 PD cases while screening the 120,000 patients visiting the Neurology Clinic. About 25,000–30,000 patients visit the clinic every year. Our data was, therefore, stratified to minimize confounding e.g. by religion, sex, age or socio-economic background. For this purpose, we analysed 1,010 randomly selected patients visiting our clinic for different variables and tested if these patients adequately represented the socio-economic and demographic spectrum of the general population from the adjoining three states of North-West India (Punjab, Haryana, Himachal Pradesh and Union Territory of Chandigarh) from where these patients were recruited. These 1,010 patients who also included non-PD patients and smokers were therefore analysed for smoking habits, sex ratio, rural-urban residence, demographic origin, socio-economic status and religion before we normalized the findings to the background population. There were certain variables such as average income, which could not be compared due to lack of information in the state databases or published reports.

Statistical Analysis

We performed statistical analysis with SPSS 16 (Chicago, Ill., USA). The χ^2 analysis and t test were used to address the hypothesis if there were significant differences in the variables between those with or without mutation and those with varying nature (heterozygous or homozygous) and number of mutations. We also compared the demographic and socio-economic spectrum of the representative sample (n = 1,010) with the background population by χ^2 analysis. A binary logistic regression model was applied to predict the probability of occurrence of an association between covariates.

Results

Genetic analysis of 70 sporadic PD patients revealed a higher number of exonic deletions in *PARK2* (28/70; 40%) including point mutations (6/70; 8.5%) as determined by PCR-SSCP and sequencing (table 1). This frequency of exonic deletions is perhaps the highest ever reported among sporadic PD patients. None of the controls exhibited any mutations in the *PARK2* gene.

Rural Sikhs Have Higher PARK2 *Deletions*

All the recruited patients were found to be never-smokers without any family history of the disease. Patients were categorized as either with or without *PARK2*

Table 1. Distribution of exonic deletions among PD patients by PCR analysis (number of samples analysed)

	Exon 1	Exon 2	Exon 3	Exon 6	Exon 7	Exon 8	Exon 9	Exon 10	Exon 12
Sporadic PD	12 (17.1%)	2 (2.8%)	8 (11.48%)	4 (5.7%)	1 (1.4%)	1 (1.4%)	5 (7.14%)	1 (1.4%)	8 (11.4%)

Total = 28/70 (40%); deletions may occur in several exons.

Table 2. Demographic variables as percentage of mutation-positive PD patients with Parkin mutations, number and nature

Variables	Parkin mutations			Number of Parkin mutations			Nature of mutation		
	positive	negative	p value	1	>1	p value	homozygous	heterozygous	p value
Socio-economic status									
Urban	11/39 (28.2)	28/39 (71.8)	<0.001[a]	7/28 (25)	4/28 (14.28)	0.095	10/28 (35.7)	1/28 (3.57)	0.214
Rural	17/31 (54.8)	14/31 (45.16)	OR = 0.32	11/28 (39.28)	6/28 (21.4)		12/28 (42.8)	5/28 (17.8)	
			CI = 0.51–0.6						
Communities									
Hindus	14/46 (30.4)	32/46 (69.6)	<0.001[a]	8/28 (28.5)	6/28 (21.4)	0.428	12/28 (42.8)	2/28 (7.14)	0.019[a]
Sikhs	12/20 (60)	8/20 (40)	OR = 0.29	7/28 (25)	5/28 (17.8)		10/28 (35.7)	2/28 (7.14)	
Muslims[b]	2/4 (50)	2/4 (50)	CI = 0.15–0.53	0	2/28 (7.14)		NA	2/28 (7.14)	
Clinical score									
HY score	1.8	1.4	0.45	1.61	1.76	0.798	1.75	1.68	0.76
Sex									
Males	17/49 (34.7)	32/49 (65.3)	0.015[a]	10/28 (35.7)	7/28 (25)	0.45	14/28 (50)	3/28 (10.7)	0.03[a]
Females	11/21 (52.3)	10/21 (47.6)		8/28 (28.5)	3/28 (10.7)		8/28 (28.5)	3/28 (10.7)	

Data was analysed by χ^2 test at the significance level of $p < 0.05$. NA = Not applicable; HY = Hoehn-Yahr. Values are shown as absolute figures along with their proportion in parentheses.

[a] Value was significant because of absence of Muslims in the homozygous group.

[b] Due to very few Muslim PD patients, these were not considered.

Table 3. Variables as percentage of PD patients across communities and demographic status

Variables	Residence		
	urban	rural	p value
Socio-economic status			
Urban	NA		
Rural			
Communities			
Hindu	3/70 (44.28)	15/70 (21)	0.48
Sikhs	7/70 (10)	13/70 (18)	0.004
Muslims[1]	1/70 (1.4)	3/70 (4.3)	0.5

NA = Not applicable. Values are shown as absolute figures along with their proportion in parentheses.

[1] Due to very few Muslim PD patients, these were not considered.

Table 4. Age of onset and duration comparisons among PD patients at demographic/residence level (means ± SE)

Variables	Age, years	p value	Duration, years	p value
Urban	50.4 ± 1.6	0.004	3.74 ± 0.53	0.89
Rural	42.7 ± 2.08		3.65 ± 0.42	
Males	47.6 ± 1.53	0.47	3.83 ± 0.41	0.58
Females	45.6 ± 2.78		3.4 ± 0.65	
Hindu	48.7 ± 1.6	0.12	3.4 ± 0.42	0.45
Sikh	44 ± 2.6		4 ± 0.58	

mutations and analysed for their age of onset, duration of disease, male-female preponderance, Hoen-Yahr score, rural-urban residency, religion and demography. The patients living in rural areas were found to be significantly susceptible to *PARK2* mutations as compared to those living in the urban areas irrespective of the number of mutations (table 2; $p < 0.001$; odds ratio, OR = 0.32; confidence interval, CI = 0.50–0.61). Interestingly, a community-based analysis showed a significant preponderance of *PARK2* mutations among Sikhs (table 2; $p < 0.001$; OR = 0.29, CI = 0.15–0.53). Conversely, there were significantly fewer Sikh PD patients (40%) who exhibited PD features without *PARK2* mutations (table 2). Besides, the majority of these Sikhs were found to belong to rural residence after normalizing the findings to the rural-urban ratio (table 3). Additionally, the prevalence of PD was found to be more in males (70%) than females (30%) after adjusting to the male-female ratio at the time of inclusion. However, the occurrence of *PARK2* mutations among females was 52.3% as compared to males (34.7%), which was significantly higher ($p = 0.015$; table 2). We found that the number and character of mutations did not bear any correlation with religion, Hoen-Yahr score or demographic origin of PD patients (table 2).

The mean age of onset of disease among sporadic PD patients was 47 ± 1.36 years. The mean age of the male patients was 47.6 ± 1.53 years while it was 45.57 ± 2.78 years for females, which is comparable. The age of onset of patients with rural background was 42.7 ± 2.08 years, which is significantly lower as compared to patients with an urban background (50.4 ± 1.6 years; $p = 0.004$; table 4). This is consistent with national statistics. A binary logistic regression model corroborated these results and predicted the association of age and rural residency with the onset of *PARK2* mutations.

PD Patients Typify the Randomly Sampled Patients and the Background Population in Their Demographic and Socio-Economic Characteristics

Demographic and socio-economic characteristics of the randomly selected 1,010 of 120,000 patients who visited the Neurology Clinic when compared with 59 PD patients and the background population of three states (including the Union Territory of Chandigarh based on the 2001 census) was found to be comparable, indirectly validating the homogeneity of samples and uniqueness of the Institute's catchment capacity; the proportion of patients from Punjab, Chandigarh (OR = 2.1, CI = 1.14–3.88), and Haryana (OR = 0.2, CI = 0.09–0.44) figured more than those from Himachal Pradesh (OR = 2.07, CI = 0.91–4.78), and the proportion of PD patients (59/70) visiting the Institute was similarly higher from Chandigarh, Punjab (OR = 1.79, CI = 0.97–3.30), and Haryana (OR = 0.37, CI = 0.16–0.82) when compared to the sample of 1,010 subjects (table 5). This could be due to the central location of the Postgraduate Institute in relation to Punjab and Haryana. The analysis was restricted to only 59 out of 70 patients in order to adjust for a relatively large background population and size of Uttar Pradesh and Uttranchal states from where the remaining 11 patients originated. Likewise (23/28), 5 patients belonging to Uttar Pradesh and Uttranchal in the *PARK2* mutation group were also excluded. Male-female patient distribution among the population of 1,010 individuals was comparable with the background population. The socio-economic status of the random sample was in favour of the urban population (OR = 3.97, CI = 2.21–7.50) despite the existing 69% rural residents (OR = 0.26, CI = 0.14–0.49). This further validates the preponderance of *PARK2* mutations among the rural population (OR = 0.26, CI = 0.14–0.49). Similar community-wise comparisons reveal homogeneity in sampling, highlighting the higher prevalence of *PARK2* mutations among Sikhs (OR = 0.34, CI = 0.18–0.64; table 5). It may be pertinent to point out that the proportion of smokers was higher in the random sample when compared to the background population (OR = 2.43, CI = 0.55–12.28). We further analysed PD *PARK2*-negative patients for any possible demographic associations. Forty-two PD *PARK2*-negative patients were characterized by an absence of correlation between any of the above variables. This partly compensates for the absence of a PD *PARK2* smoker group. [The significance values ($p < 0.05$) imply a lack of association (homogeneity) between the 1,010 subjects and the background or PD population, while higher p values ($p > 0.05$) indicate good homogeneity in sampling.]

Discussion

Despite a population of 1.1 billion and constituting one sixth of humanity, the lack of epidemiological data for PD in India is unfortunate. The Indian population is remarkably diverse with more than 2,000 ethnic groups and, representing every major community in the population, with an average life span of 63 years (census 2001). Our study provides a unique opportunity to study not only the newly generated Parkin mutations, but also investigates their religion-demographic association. The *PARK2* analysis has revealed a distinct genetic suscepti-

Table 5. Normalization of 59/70 PD patients with randomly sampled 1,010/120,000 visiting the Neurology Outpatient Clinic with the background population by demography, sex, socio-economic status, community and lifestyle

Variables	Proportion of 1,010 randomly sampled patients from Neurology Outpatient Clinic	Proportion of background population (52,350,447)	p value (1,010 and population)	OR	95% CI	Proportion of 59 PD patients	p value (comparison of 59 PD and 1,010 patients)	OR	95% CI
Demography									
Chandigarh/Punjab	670/1,010 (0.66)	25,190,210 (0.48)	0.01	2.1	1.14–3.88	31/59 (0.52)	0.044	1.79	0.97–3.30
Haryana	119/1,010 (0.12)	21,082,989 (0.40)	<0.001	0.2	0.09–0.44	16/59 (0.27)	0.007	0.37	0.16–0.82
Himachal Pradesh	221/1,010 (0.22)	6,077,248 (0.12)	0.0597	2.07	0.91–4.78	11/59 (0.18)	0.479	1.28	0.61–2.73
Sex									
Males	596/1,010 (0.59)	27,884,500 (0.53)	0.393	1.28	0.70–2.32	40/59 (0.67)	0.241	0.71	0.38–1.31
Females	414/1,010 (0.40)	24,465,947 (0.46)	0.391	0.78	0.43–1.43	19/40 (0.32)	0.238	1.42	0.76–2.64
Socio-economic status									
Rural	373/1,010 (0.37)	36,587,065 (0.69)	<0.001	0.26	0.14–0.49	29/59 (0.49)	0.086	0.61	0.33–1.12
Urban	637/1,010 (0.63)	15,763,382 (0.30)	<0.001	3.97	2.12–7.50	30/59 (0.51)	0.086	1.61	0.33–1.13
Literate	0.94	0.64	<0.001	0.09	0.03–0.27	NA			
Illiterate	0.052	0.36	<0.001		5.74–52.8				
Communities									
Hindus	723/1,010 (0.71)	34,162,067 (0.65)	0.66	0.73	0.37–1.45	37/59 (0.62)	0.177	1.5	0.80–2.83
Sikhs	275/1,010 (0.27)	15,980,579 (0.30)	0.638	0.86	0.45–1.67	20/59 (0.33)	0.354	0.75	0.39–1.44
Muslims	12/1,010 (0.011)	1,760,021 (0.033)	0.312	0.33	0.01–3.60	2/59 (0.034)	0.312	0.33	0.01–3.6
Lifestyle									
Smoker	97/1,010 (0.096)	1,679,498 (0.032)	0.0446	2.43	0.55–12.28	NA			
Never-smoker	913/1,010 (0.90)	50,670,949 (0.96)	0.096	0.38	0.10–1.36				

Analysis for literacy and annual income has not been separately done for PD patients, it was included in that of the 1,010 randomly selected patients.
The figures for the background population have been cited from state economic surveys of 2001. NA = Not applicable. Values are shown as absolute figures along with their proportion in parentheses.

bility among never-smoker Sikhs. This analysis resulted after multiple comparisons of socio-economic and demographic characteristics of the 1,010 randomly sampled patients with the background population, which was found to be comparable. It is speculated that the higher occurrence of *PARK2* mutations [14, 15] could be due to the higher proportion of never-smokers in this region. Almost 15% of the randomly sampled 1,010 patients were found to be smokers, which is comparable to previous reports [16]. Although there are several reports that support nicotine stimulation of dopamine release in the ventral and dorsal striatum, through the activation of somatodendritic nicotinic receptors on nigral and ventral tegmental area dopaminergic neurons [17], the association between mutations in PD-specific genes and never-smokers has not been studied earlier. There is growing evidence from various case-control and cohort studies that smoking is inversely related to the risk of developing PD [10, 18–20]. In addition, a recent study demonstrated that smoking and caffeine should be important covariates to consider in genetic studies of PD, which coincided with our findings [21]. Although there is epidemiological support for environmental factors responsible for neuroprotection, the biological mechanisms of protection have always eluded investigations. This report highlights the importance of gene environment interaction in determining PD susceptibility.

The higher occurrence of mutations reported among North Indians is a glaring indication of their increased susceptibility to *PARK2* mutations. The exonic deletions among PD patients are acceptable features for the disease development as reported earlier [2, 22]. The results of the present study show an increased occurrence of exonic deletions in exons 1, 3 and 12 among sporadic PD patients. In this study 66% of Sikhs were found to harbour *PARK2* deletions. However, deletions in exons 3–5 have been earlier reported to be more frequently involved in Japanese PD patients [2, 22]. The earlier mutation studies have, however, been restricted to ethnically mixed patient populations [23–25], and data on the occurrence of mutations in homogeneous patient groups is very limited. We believe that the ethnicity of the patients is an important confounding factor that results in variable mutation rates such as those ranging from 66% in patients from Japan

Proportion of *PARK2*-positive 23 PD patients	p value (comparison of 23 PD and 1,010 patients)	OR	95% CI	Proportion of *PARK2*-negative 36 PD patients	p value (comparison of 36 PD and 1,010 patients)	OR	95% CI
14/23 (0.6)	0.379	1.29	0.70–2.40	18/36 (0.5)	0.0218	1.94	1.06–3.58
4/23 (0.17)	0.315	0.67	0.28–1.58	13/36 (0.36)	<0.001	0.24	0.11–0.53
5/23 (0.21)	0.863	1.06	0.51–2.20	5/36 (0.13)	0.0939	1.89	0.84–4.28
14/23 (0.6)	0.885	0.96	0.52–1.76	26/36 (0.72)	0.053	0.56	0.30–1.05
9/23 (0.39)	0.885	1.04	0.57–1.91	10/36 (0.27)	0.051	1.8	0.95–3.42
16/23 (0.69)	<0.001	0.26	0.14–0.49	12/36 (0.33)	0.553	1.19	0.64–2.22
7/23 (0.3)	<0.001	3.97	2.12–7.50	24/36 (0.66)	0.657	0.88	0.47–1.63
9/23 (0.39)	<0.001	3.83	2.04–7.23	28/36 (0.77)	0.333	0.73	0.37–1.45
12/23 (0.52)	<0.001	0.34	0.18–0.64	8/36 (0.22)	0.411	1.31	0.65–2.63
2/23 (0.086)	0.0094	0.1	0–0.81	0	0.999		

[26] to <4% in a US early-onset PD population [27]. In addition, variations in the genetic mutations are also restricted among ethnically different populations, possibly contributing to different modes of pathogenesis. For example, *LARK2* mutations account for 40% of PD patients of Arab descent and 20% of Ashkenazi Jewish subjects [28–31], *PINK1* among Italian PD populations, *DJ1* in Dutch and Italian PD patients and *UCH-L1* in German populations [32].

The phenomenal genetic variability has been well documented among different populations from diverse ethnic origins [23–28], accompanied by different mechanisms of disease in these populations. Our discovery that the Sikh community, which is known for their aversion to smoking and which constitutes 60% of those PD patients that carry *PARK2* mutations, offers strong support to the above theory. Small populations such as those of Sikhs are known to be more susceptible to genetic drifts. The linkage disequilibrium in such populations may result in limited recombination or non-random distribution of alleles leading to a higher frequency of Parkin mutations than would be expected from a random formation of haplotypes from alleles. Such a high frequency of mutations could therefore be ascribed to founder effects manifest due to genetic drifts. It is well known that Sikhs, who inhabit this region, consider smoking as a social and religious taboo. It has also been reported earlier that the Sikh community is found to have the lowest tobacco consumption [33]. This is due to the strongly embedded sociocultural belief enshrined in Sikh religious texts which bans smoking. Combined with their strong adherence to intracommunity marriages, the existence of founder effects cannot be ruled out. In this context, it can further be speculated that *PARK2* loci may be especially vulnerable to mutations (knowing that smoking is inversely related to PD). It is possible that the perpetuation of *PARK2* modifications could have progressed over a long time accounting for linkage between the aberration of nicotine metabolism and mutations (which may have got incorporated into Sikh religious practice due to acquired 'repulsion' for nicotine tolerance). However, additional studies need to be carried out to verify this.

Another important finding of this study is the frequent occurrence of mutations observed in the rural pop-

ulation where most of the Sikh population is known to reside (table 2). Patients from rural areas, which is one of the important recognized risk factors for PD onset, had a mean age of 42.7 years in contrast to 50.4 years (table 4) of those from an urban background. This is predicted by a binary logistic regression model validating the association of age and rural residency with the onset of mutation. Since there is no obvious genotype-phenotype correlation in this study, the decision to perform Parkin analysis as a diagnostic tool in such populations can only be based on the frequency of the Parkin mutation as a function of age at onset that sharply decreases after 30 years of age.

Contrary to the widespread perception that free or subsidized treatment attracts rural populations, the majority of the patients representing the sample of 1,010 individuals in our study were urban (rural: OR = 0.26, CI = 0.14–0.49; urban: OR = 3.97, CI = 2.12–7.50; table 5). This may partly validate the significant preponderance of *PARK2* gene mutations in the rural population (OR = 0.26, CI = 0.14–0.49). We also found that the majority of the patients visiting the clinic were literate. This may be because they are better informed about neurological disorders. This trend can also be related to a positive sociocultural perception of PD among the literate population of this region. PD is known to occur more frequently among men than women [34]. Similarly, we also reported that 70% males (49/70) and 30% females (21/70) constituted our PD study population. These findings suggest that, although prevalence of PD was high in males, the occurrence of *PARK2* mutations was more frequent in females even though the female-to-male ratio in total PD patients and controls was 1:2.6 and 1:2.3, which is comparable. In this context, it is also worthwhile to note that females, like their male counterparts, generally smoke less.

As mentioned earlier, we could not analyse *PARK2*-positive smokers because we did not encounter them. The discovery of this unique association is serendipitous, and the results of this study should prompt larger *PARK2* studies among both sporadic and familial PD never-smokers, particularly Sikhs. It should also prompt further studies among hospitals located in areas where PD smokers can be analysed for *PARK2* mutations even though it is virtually impossible to find Sikh smokers. Identification and removal of subjects with *LARK2, PINK* and *DJ1* mutations from a subsequent genome-wide analysis will further reduce genetic heterogeneity in samples, thereby increasing the power to detect linkage to other genes *(PARK2)* and their susceptibility to never-smoker PD. We are also unable to rule out that compound heterozygous mutations are unrelated to disease or that PD smokers may have an equally high occurrence of mutations even though several reports have ruled out the role of heterozygous mutations in the pathogenesis of PD [35–37].

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the long and short of it

This editorial is being written at a time when Indian (neuro)science is witnessing a major policy shift in redefining the Indian research. Today we are confronted with a much awaited *avatar* of Bayh-Dole Act allowing for the transfer of exclusive control over many government funded inventions to universities, Institutions and businesses for the purpose of further development and commercialization. This bold initiative communicated by Ministry of Science and Technology seeks to transcend mere licensing of inventions to third parties to strengthening scientists' claim of holding equity in scientific enterprises and creating spin offs, exempting them from the draconian cluthches of CCS conduct rules. This recent Govt order is meant to ensure the continued involvement of researchers in translating the inventions or innovations, at any stage of development, through investment of their own money. This has been elaborated in the draft that sets the guidelines about creation of scientist based companies thereby permitting researchers to play a defining role in commercialization of knowledge products while being in professional employment. The entire draft has been published at www.dsir.gov.in for the information of its readers. The new law is applicable to all Institutes falling under Ministry of Health and Family welfare, besides other scientific establishments, providing a unique opportunity for medical institutes to mobilize its intellectual resources to generate wealth, an effort never envisioned earlier. Some feel that this is an opportunity to initiate incubation centres in their campuses and mobilise its human resources to industry and recall them back later to establish their own business development offices for generation of research funds. Both these measures have been sanctioned in the government order with the expressed purpose of supporting internal fund generation projects[1]. Such new wealth can be harnessed for rationalization of patient care costs as well as funding more R&D projects within the Institute. The potential of this approach in transforming regional economies (and that of the Institute) by creation of new companies is borne out of the examples set by MIT, IIT, IIM, Stanford and University of Cambridge, many of which are based on biomedical inventions. By promoting science and engineering based enterprises it has not only opened vistas for Institutes to impact society directly but also paved way for those who possess considerable intellectual portfolios and are ready to seek new frontiers in the field of technology and national wealth creation endeavours[2]. Many view this new regulation as serving to create a distinct reward system for over performers over average workers and may have potential to reverse brain drain. This could serve as a good tool for growing Institutions that are apparently not able to support their own thought leaders for want of stringent service conduct rules. The leadership displayed by IIT entrepreneurship schemes is laudable for others to emulate. Nusrat shafiq argues in her commentary in this issue why there is lackadaisical approach in asking good research questions, makes one think why the Indians who are settled abroad perform better than those who live in this country. Perhaps the right individuals with right questions can now expect to be rewarded from taking risks of asking the right questions and creating spin offs. Supten, on the other hand, argues in his commentary why number of years in service should be a necessary replacement for merit in science. Like developed nations, where excellence is the hallmark of career(read national) advancement, India too needs to experiment with its policies such that many Gokhales are given leadership roles of research organizations, journals and academies. It is therefore not difficult for India to regain its global leadership in the field of Science and Education. It would, therefore, be very interesting to see how the new regulation is shaped in the context of authority (read seniority) patterns identified in Supten's commentary.

Given the impact stem cell research is making in transforming regenerative medicine, this new law can enable researchers to create practical solutions by meeting social challenges and to directly participate in creating and sustaining competitive, self financing and low cost patient care industry for decades to come. This can be conceptualized by mobilising investment of several business houses who might want to set up their centres within the institutes, particularly medical organisations, and fund research in return for commercialisable products. At a time when organisations like CSIR, IIT and IISc have devised mechanisms to open incubation centres, medical institutes in the country have a unique opportunity to make impact in the field of biomedical innovation and not be left behind in the race.

The new law provides opportunities to aggressively establish for itself a corpus of individuals which can generate wealth for it. The new provision allowing mobility of faculty to and fro from industry, can accelerate seamless transfer of knowedge from one organization to other and industry. This regulation can now provide competitive edge to an Institute which is bestowed with faculty with special skills and entrepreneurial drive than those Institutions that don't. Therefore, an early composition of task forces to oversee the establishment of such offices will be the key to consolidation of an early lead in the field that will now become intensely competitive and attractive. Such a set up is bound to stimulate product (or patient) oriented research in anticipation of profits. Many see a dream opportunity for medical Institutes to translate this regulation into establishment of Business Development office comprising people from Knowledge management (such as IP management), informatics, quality control and as venture capitalists.

Young entrepreneurs in scientific journalism too have a new opportunity to combine their enthusiasm, ideas and productivity with advisory role of accomplished scientists so that a new order of thought leadership is created in running journals. Journalism is likely to gain because many may opt to start new journals as a spin off. New ideas of enhancing visibility to journals will include experiments with its design, content, enhanced investments, new breed of publishers, venues and leadership roles[3]. This will eventually lead to new dimension of neuroscience publications in the country. Some will question publication ethics, others the national funding mechanisms and policies, some the value of research audit, some the scientific dogmas and yet others the science leaders themselves.

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Akshay Anand

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