COFFEE BOOK

on

BIOMARKER DISCOVERY IN BRAIN DISORDERS

Neuroscience Research Lab

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Foreword

It gives me immense pleasure to write the foreword for this coffee book entitled "Biomarker discovery in Brain disorders". This coffee book is an initiative by the Neuroscience Research Lab (NRL) to compile the various studies and research whether at the literature review level or at the original bench work research level. This is an innovative idea of Dr. Akshay Anand to have a collection of all the researches done in the NRL.

The Brain disorders or also popularly known as Neurodegenerative disorders have great impact on overall health of the world. People with the disease suffers a lot. Also, the care givers around them have to do lot for maintaining their decline health. These disorders posit great threat to the human health and further his/her relationships.

Finding a treatment for these disorders is really warranted and need of the time. A lot of research is being done in area of therapy or treatment development for the diseases. NRL has also done elite research in the field. In case of brain disorders, along with finding suitable treatment, another big challenge is correct diagnosis of the disease or at least to know how the disease progresses. Some brain disorders can be identified correctly but most other brain disorders have same kind of pathophysiology, closely related symptoms and are really difficult to categories in one or the other category of disorder.

This similarity in disorder symptoms makes the early diagnosis of disease quite difficult. For better treatment and prognosis of the disease, early diagnosis of disease is really important. Biomarkers are the molecules found in the common bio fluids such as plasma and serum that reflects a lot about the body conditions. Biomarker discovery program of NRL has targeted for finding such effective biomarkers for the early diagnosis and correct prognosis of the brain disorders.

In this particular book, all the research articles of NRL regarding the biomarker discovery are compiled. The biomarker discovery is backed up by the Predictive regression models that can help to predict the risk of the disease in relation to various factors such as aging, smoking etc. Molecules related to neuroprotection and neurodegeneration have been explored for their potential of biomarkers. Along with protein levels, Single Nucleotide Polymorphisms (SNPs) have also been analyzed for their relation with brain disorders in North Indian population. These SNPs have been further explored for their associations with different molecules whose biomarker potential is being explored.

NRL really hopes to find diagnostic and prognostic biomarkers for the brain disorders in future and to contribute to the early diagnosis of diseases. So that diseases can be targeted better for therapeutics. We are hopeful that this book will give you insights about the biomarker discovery program and glimpses of various methods how to find suitable biomarker.

Happy Reading!!

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CONTENT

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

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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

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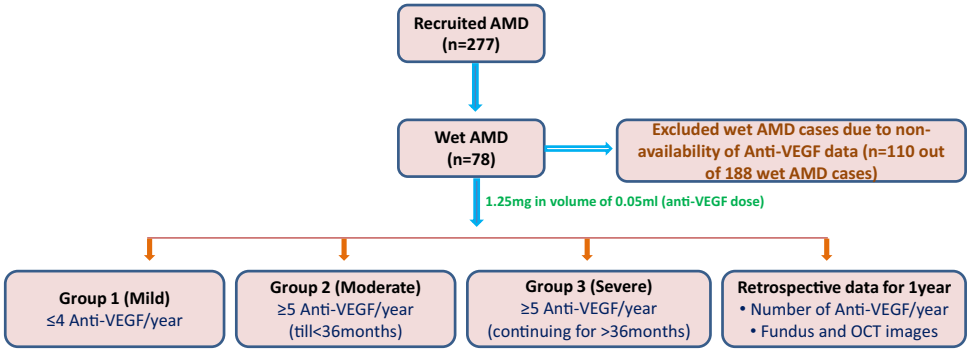


Figure 1. Schematic representation of groups categorised in study.

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-

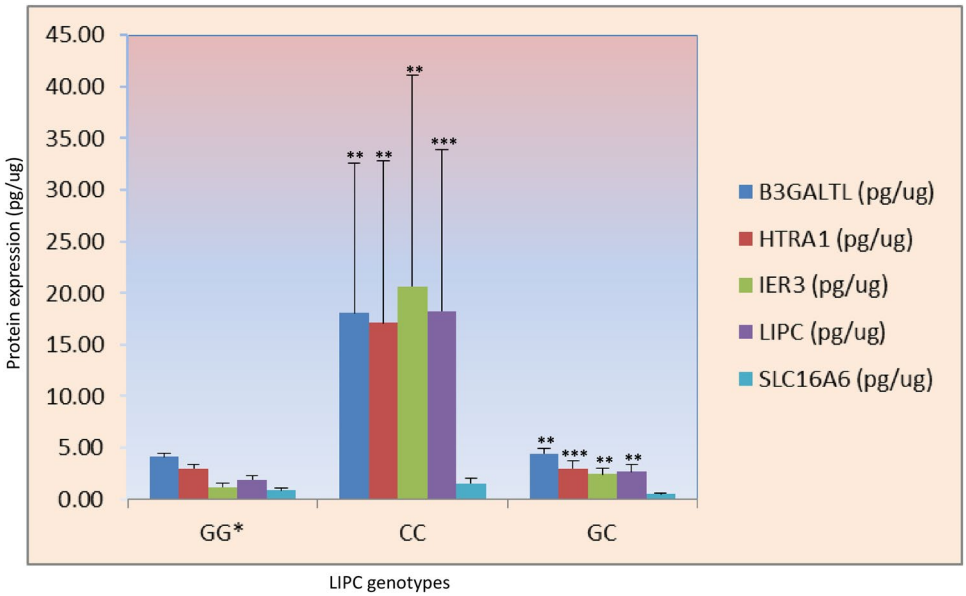


Figure 2. Impact of LIPC genotype on protein expression. Significant elevated expressions of B3GALTL, HTRA1, IER3 and LIPC were seen in 'CC' genotype of LIPC genetic variant (rs920915) as compared to both reference 'GG*' and heterozygous 'GC' alleles. *GG** Reference allele. Bar is representing SEM;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

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

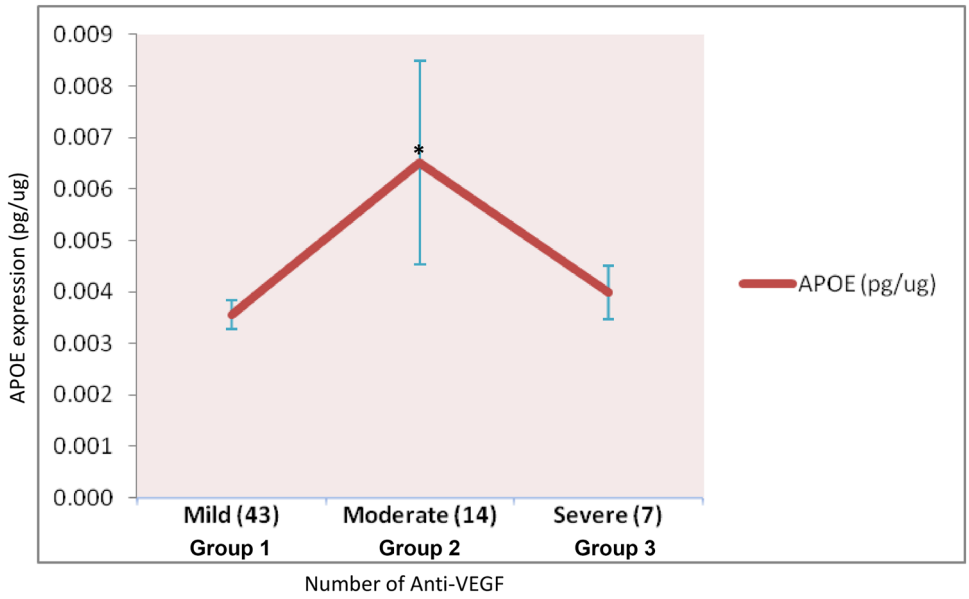


Figure 3. APOE expression in mild, moderate and severe groups of anti-VEGF response is based on the number of injections in wet AMD patients. Significantly higher levels of APOE were seen in moderate group as compared to mild group. Bar is representing SEM; $P < 0.05$.

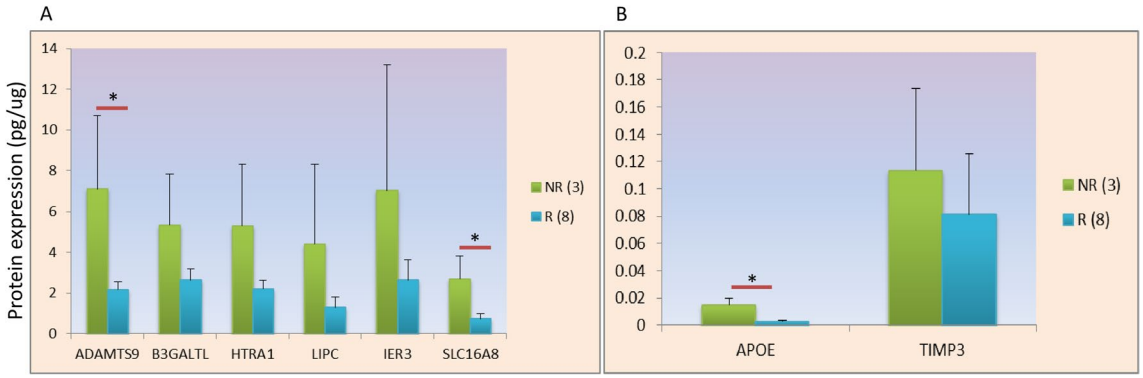


Figure 4. Differential expression of proteins in retrospectively group (Group 4). (**A**) Significant higher expression of ADAMTS9 and SLC16A8 in anti-VEGF non-responder (≥ 5 anti-VEGF injections/year), as compared to responders (≤ 4 anti-VEGF injections/year) in wet AMD patients. (**B**) APOE expression 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.

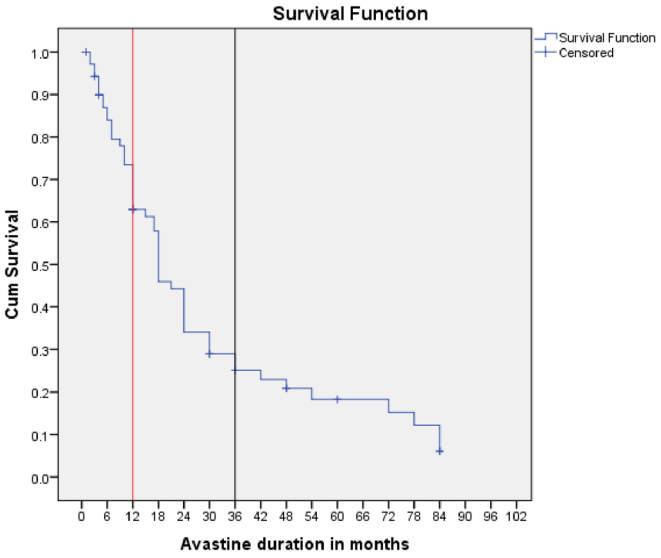
We wanted to examine the progress of disease in patients as with the duration of disease (in months), such as the effect of anti-VEGF treatment, until the occurrence of the AMD pathology. For this purpose, survival analysis was performed and Kaplan–Meier survival curve revealed that at 12 months anti-VEGF 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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References

1. Sharma, K., Sharma, N. K. & Anand, A. Why AMD is a disease of ageing and not of development: Mechanisms and insights. *Front. Aging Neurosci.* **6**, 151 (2014).

2. Fritsche, L. G. *et al.* A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat. Genet.* **48**(2), 134–143 (2016).
3. Sharma, K., Singh, R., Sharma, S. K. & Anand, A. Sleeping pattern and activities of daily living modulate protein expression in AMD. *PLoS ONE* **16**(6), e0248523 (2021).
4. Martin, D. F. *et al.* Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N. Engl. J. Med.* **364**(20), 1897–1908 (2011).
5. Rich, R. M. *et al.* Short-term safety and efficacy of intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. *Retina* **26**(5), 495–511 (2006).
6. Michels, S., Rosenfeld, P. J., Puliafito, C. A., Marcus, E. N. & Venkatraman, A. S. Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration twelve-week results of an uncontrolled open-label clinical study. *Ophthalmology* **112**(6), 1035–1047 (2005).
7. Hwang, R. Y., Santos, D. & Oliver, S. C. N. Rates of exudative recurrence for eyes with inactivated wet age-related macular degeneration on 12-week interval dosing with bevacizumab therapy. *Retina* **40**(4), 679–685 (2020).
8. Lorés-Motta, L. *et al.* Association of genetic variants with response to anti-vascular endothelial growth factor therapy in age-related macular degeneration. *JAMA Ophthalmol.* **136**(8), 875–884 (2018).
9. Anand, A. *et al.* Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. *PLoS ONE* **7**(11), e49905 (2012).
10. Sharma, N. K. *et al.* CC chemokine receptor-3 as new target for age-related macular degeneration. *Gene* **523**(1), 106–111 (2013).
11. Sharma, N. K., Sharma, K., Singh, R., Sharma, S. K. & Anand, A. CCL2 single nucleotide polymorphism of rs1024611 implicates prominence of inflammatory cascade by univariate modeling in Indian AMD. *PLoS ONE* **13**(4), e0193423 (2018).
12. Sharma, K., Sharma, N. K., Singh, R. & Anand, A. Exploring the role of VEGF in Indian Age related macular degeneration. *Ann. Neurosci.* **22**(4), 232–237 (2015).
13. Sharma, N. K. *et al.* Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. *Curr. Neurovasc. Res.* **9**(4), 256–265 (2012).
14. Battu, P., Sharma, K., Rain, M., Singh, R. & Anand, A. Serum levels of ARMS2, COL8A1, RAD51B, and VEGF and their correlations in age-related macular degeneration. *Curr. Neurovasc. Res.* **2**, 2 (2021).
15. Anand, A. *et al.* Superoxide dismutase1 levels in North Indian population with age-related macular degeneration. *Oxid. Med. Cell Longev.* **2013**, 365046 (2013).
16. Sharma, N. K. *et al.* Does toll-like receptor-3 (TLR-3) have any role in Indian AMD phenotype?. *Mol. Cell Biochem.* **393**(1–2), 1–8 (2014).
17. Sharma, K., Sharma, N. K., Singh, R., Sharma, S. K. & Anand, A. Gene networks determine predisposition to AMD. *Genomics* **113**(1 Pt 2), 514–522 (2021).
18. Anand, A., Sharma, K., Chen, W. & Sharma, N. K. Using current data to define new approach in age related macular degeneration: Need to accelerate translational research. *Curr. Genom.* **15**(4), 266–277 (2014).
19. Cheng, S. & Leng, T. Factors associated with poor response to aflibercept after switching from ranibizumab or bevacizumab in neovascular age-related macular degeneration. *Ophthalmic Surg. Lasers Imaging Retina.* **47**(5), 458–465 (2016).
20. Cabral, T. *et al.* Bevacizumab injection in patients with neovascular age-related macular degeneration increases angiogenic biomarkers. *Ophthalmol. Retina.* **2**(1), 31–37 (2018).
21. Habibi, I. *et al.* Effect of risk alleles in CFH, C3, and VEGFA on the response to intravitreal bevacizumab in tunisian patients with neovascular age-related macular degeneration. *Klin Monbl Augenheilkd.* **233**(4), 465–470 (2016).
22. Rodríguez, F. J. *et al.* Genetic association with intravitreal ranibizumab response for neovascular age-related macular degeneration in Hispanic population. *Taiwan J. Ophthalmol.* **9**(4), 243–248 (2019).
23. Sharma, K., Tyagi, R., Singh, R., Sharma, S. K. & Anand, A. Serum Levels of TIMP-3, LIPC, IER3, and SLC16A8 in CFH-Negative AMD cases. *J. Cell Biochem.* **118**(8), 2087–2095 (2017).
24. Anand, A. *et al.* AMD genetics in India: The missing links. *Front. Aging Neurosci.* **8**, 115 (2016).

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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

The authors declare no competing interests.

Additional information

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RESEARCH ARTICLE

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

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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

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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

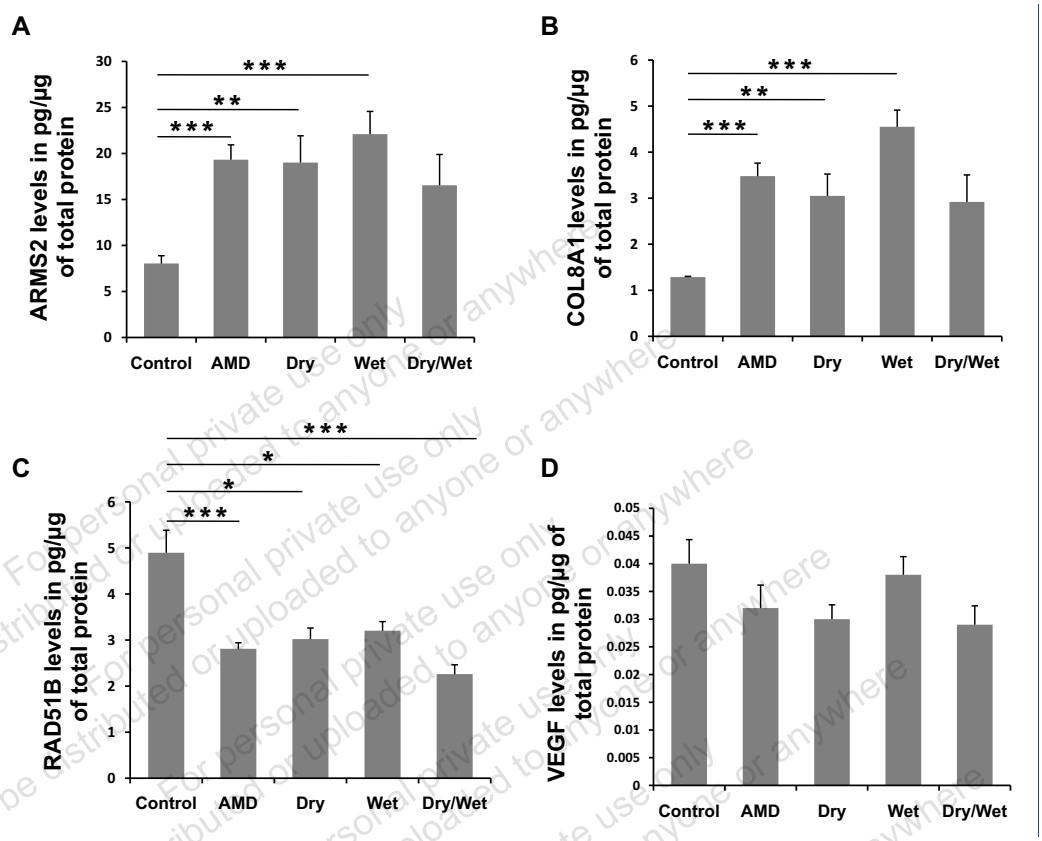

Fig. (1). Estimation of protein concentration by ELISA. (**A**) ARMS2 levels were significantly higher in the AMD group than in the control group. Also, its level was significantly higher in wet and dry subgroups than in the control group. (**B**) COL8A1 levels were also higher in the AMD group than 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.

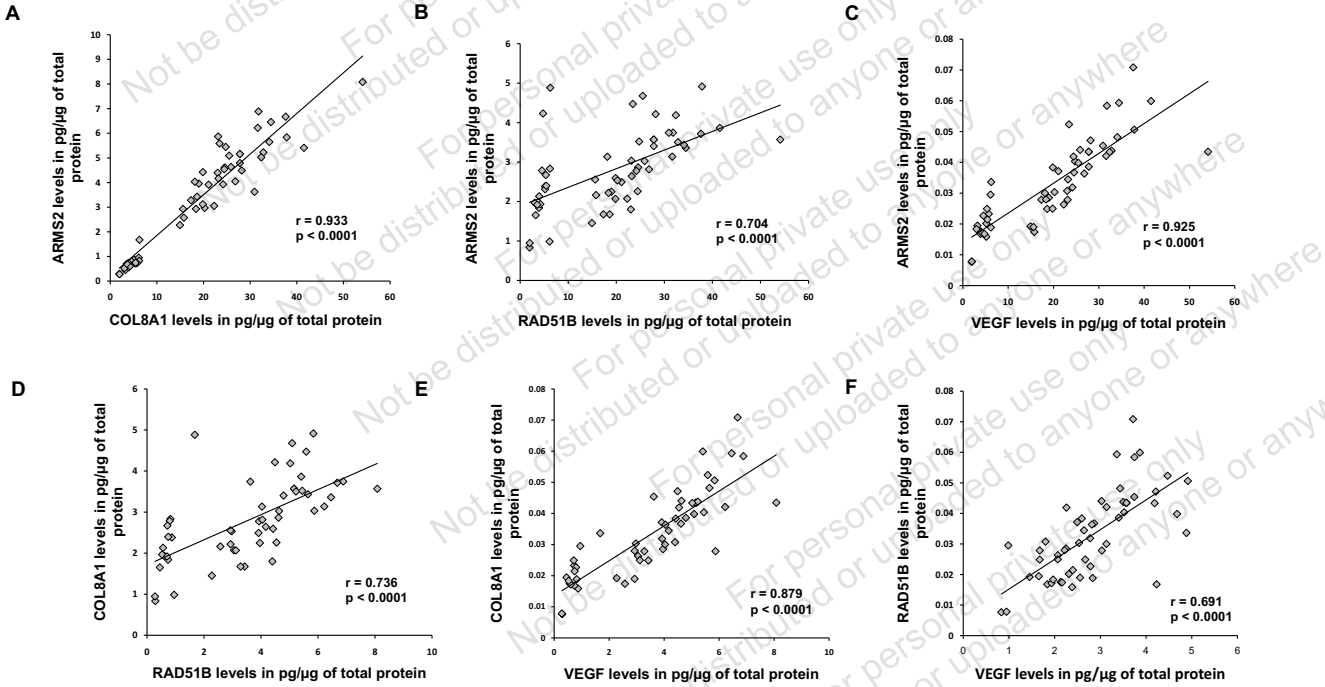

Fig. (2). Correlation of serum protein concentration of ARMS2, COL8A1, RAD51B, and VEGF in AMD group. (**A**) ARMS2 and COL8A1, (**B**) ARMS 2 and RAD51B, (**C**) ARMS2 and VEGF, (**D**) COL8A1 andRAD51B, (**E**) COL8A1 and VEGF, and (**F**) RAD51B and VEGF. Pearson's correlation was performed for RAD51 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.

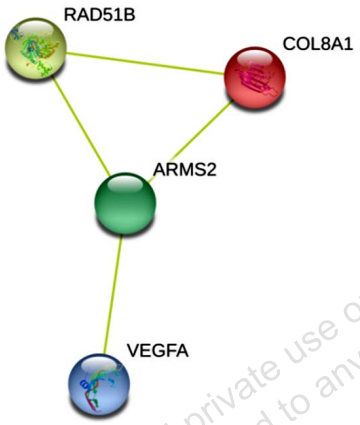

Fig. (3). Interaction among the four proteins shows 'text mining, represented by green interconnections. Abbreviations: ARMS2, Age-Related Maculopathy Susceptibility 2; COL8A1, Collagen type VIII Alpha 1 chain; RAD51B, Rad 51 paralog; VEGFA/VEGF, Vascular Endothelial Growth Factor. (*A higher resolution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

[1] de Jong EK, Geerlings MJ, den Hollander AI. Age-related macular degeneration. In: Genetics and Genomics of Eye Disease. Elsevier 2020; pp. 155-80. http://dx.doi.org/10.1016/B978-0-12-816222-4.00010-1

[2] García-Onrubia L, Valentín-Bravo FJ, Coco-Martin RM, *et al.* Matrix metalloproteinases in age-related macular degeneration (AMD). Int J Mol Sci 2020; 21(16): 5934. http://dx.doi.org/10.3390/ijms21165934 PMID: 32824762

[3] Fritsche LG, Igl W, Bailey JNC, *et al.* A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. Nat Genet 2016; 48(2): 134-43. http://dx.doi.org/10.1038/ng.3448 PMID: 26691988

[4] Shin H-T, Yoon BW, Seo JH. Comparison of risk allele frequencies of single nucleotide polymorphisms associated with age-related macular degeneration in different ethnic groups. BMC Ophthalmol 2021; 21(1): 97. http://dx.doi.org/10.1186/s12886-021-01830-9 PMID: 33618707

[5] Cascella R, Strafella C, Longo G, *et al.* Uncovering genetic and non-genetic biomarkers specific for exudative age-related macular degeneration: significant association of twelve variants. Oncotarget 2017; 9(8): 7812-21. http://dx.doi.org/10.18632/oncotarget.23241 PMID: 29487693

[6] Sharma K, Sharma NK, Singh R, Sharma SK, Anand A. Gene networks determine predisposition to AMD. Genomics 2021; 113(1 Pt 2): 514-22. http://dx.doi.org/10.1016/j.ygeno.2020.09.044 PMID: 32979492

[7] Sundaresan P, Vashist P, Ravindran RD, *et al.* Polymorphisms in ARMS2/HTRA1 and complement genes and age-related macular degeneration in India: findings from the INDEYE study. Invest Ophthalmol Vis Sci 2012; 53(12): 7492-7. http://dx.doi.org/10.1167/iovs.12-10073 PMID: 23060141

[8] van Asten F, Simmons M, Singhal A, *et al.* A deep phenotype association study reveals specific phenotype associations with genetic variants in age-related macular degeneration: Age-related eye disease study 2 (AREDS2) report no. 14. Ophthalmology 2018; 125(4): 559-68. http://dx.doi.org/10.1016/j.ophtha.2017.09.023 PMID: 29096998

[9] Micklisch S, Lin Y, Jacob S, *et al.* Age-related macular degeneration associated polymorphism rs10490924 in ARMS2 results in deficiency of a complement activator. J Neuroinflammation 2017; 14(1): 4. http://dx.doi.org/10.1186/s12974-016-0776-3 PMID: 28086806

[10] Hopfer U, Fukai N, Hopfer H, *et al.* Targeted disruption of Col8a1 and Col8a2 genes in mice leads to anterior segment abnormalities in the eye. FASEB J 2005; 19(10): 1232-44. http://dx.doi.org/10.1096/fj.04-3019com PMID: 16051690

[11] Takata M, Sasaki MS, Sonoda E, *et al.* The Rad51 paralog Rad51B promotes homologous recombinational repair. Mol Cell Biol 2000; 20(17): 6476-82. http://dx.doi.org/10.1128/MCB.20.17.6476-6482.2000 PMID: 10938124

[12] Albala JS, Thelen MP, Prange C, *et al.* Identification of a novel human RAD51 homolog, RAD51B. Genomics 1997; 46(3): 476-9. http://dx.doi.org/10.1006/geno.1997.5062 PMID: 9441753

[13] Seddon JM, Reynolds R, Yu Y, Rosner B. Three new genetic loci (R1210C in CFH, variants in COL8A1 and RAD51B) are independently related to progression to advanced macular degeneration. PLoS One 2014; 9(1): e87047. http://dx.doi.org/10.1371/journal.pone.0087047 PMID: 24498017

[14] Ferrara N. The role of the vegf signaling pathway in tumor angiogenesis. Tumor angiogenesis: A key target for cancer therapy. Expert Rev Anticancer Ther 2019; 18(3): 251-66.

[15] Jin Y. Effect of intravitreal injection of anti-VEGF on choroidal thickness and blood flow in posterior ciliary artery in patients with wet ARMD. International Eye Science 2018; 18(12): 2244-7.

[16] Pugazhendhi A, Hubbell M, Jairam P, Ambati B. Neovascular macular degeneration: A review of etiology, risk factors, and recent advances in research and therapy. Int J Mol Sci 2021; 22(3): 1170. http://dx.doi.org/10.3390/ijms22031170 PMID: 33504013

[17] Keir LS, Firth R, Aponik L, *et al.* VEGF regulates local inhibitory complement proteins in the eye and kidney. J Clin Invest 2017; 127(1): 199-214. http://dx.doi.org/10.1172/JCI86418 PMID: 27918307

[18] Foxton RH, Uhles S, Grüner S, Revelant F, Ullmer C. Efficacy of simultaneous VEGF-A/ANG-2 neutralization in suppressing spontaneous choroidal neovascularization. EMBO Mol Med 2019; 11(5): e10204. http://dx.doi.org/10.15252/emmm.201810204 PMID: 31040126

[19] McKibbin M, Ali M, Bansal S, *et al.* CFH, VEGF and HTRA1 promoter genotype may influence the response to intravitreal ranibizumab therapy for neovascular age-related macular degeneration. Br J Ophthalmol 2012; 96(2): 208-12. http://dx.doi.org/10.1136/bjo.2010.193680 PMID: 21558292

[20] Corominas J, Colijn JM, Geerlings MJ, *et al.* Whole-exome sequencing in age-related macular degeneration identifies rare variants in COL8A1, a component of Bruch's membrane. Ophthalmology 2018; 125(9): 1433-43. http://dx.doi.org/10.1016/j.ophtha.2018.03.040 PMID: 29706360

[21] Ma Z-H, Ma J-H, Jia L, Zhao Y-F. Effect of enhanced expression of COL8A1 on lymphatic metastasis of hepatocellular carcinoma in mice. Exp Ther Med 2012; 4(4): 621-6. http://dx.doi.org/10.3892/etm.2012.652 PMID: 23170115

[22] Imai H, Honda S, Kondo N, Ishibashi K, Tsukahara Y, Negi A. The upregulation of angiogenic gene expression in cultured retinal pigment epithelial cells grown on type I collagen. Curr Eye Res 2007; 32(10): 903-10. http://dx.doi.org/10.1080/02713680701604749 PMID: 17963110

[23] Blasiak J, Pawlowska E, Sobczuk A, Szczepanska J, Kaarniranta K. The aging stress response and its implication for AMD pathogenesis. Int J Mol Sci 2020; 21(22): 8840. http://dx.doi.org/10.3390/ijms21228840 PMID: 33266495

[24] Pires E, Sung P, Wiese C. Role of RAD51AP1 in homologous recombination DNA repair and carcinogenesis. DNA Repair (Amst) 2017; 59: 76-81. http://dx.doi.org/10.1016/j.dnarep.2017.09.008 PMID: 28963981

[25] Kaplan AR, Gueble SE, Liu Y, *et al.* Cediranib suppresses homology-directed DNA repair through down-regulation of BRCA1/2 and RAD51. Sci Transl Med 2019; 11(492): eaav4508. http://dx.doi.org/10.1126/scitranslmed.aav4508 PMID: 31092693

[26] Zhao W, Steinfeld JB, Liang F, *et al.* BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. Nature 2017; 550(7676): 360-5. http://dx.doi.org/10.1038/nature24060 PMID: 28976962

[27] Kang HJ, Kim HJ, Rih J-K, *et al.* BRCA1 plays a role in the hypoxic response by regulating HIF-1α stability and by modulating vascular endothelial growth factor expression. J Biol Chem 2006; 281(19): 13047-56. http://dx.doi.org/10.1074/jbc.M513033200 PMID: 16543242

[28] Kawai H, Li H, Chun P, Avraham S, Avraham HK. Direct interaction between BRCA1 and the estrogen receptor regulates vascular endothelial growth factor (VEGF) transcription and secretion in breast cancer cells. Oncogene 2002; 21(50): 7730-9. http://dx.doi.org/10.1038/sj.onc.1205971 PMID: 12400015

[29] Singh KK, Shukla PC, Quan A, *et al.* BRCA1 is a novel target to improve endothelial dysfunction and retard atherosclerosis. J Thorac Cardiovasc Surg 2013; 146(4): 949-960.e4. http://dx.doi.org/10.1016/j.jtcvs.2012.12.064 PMID: 23415688

[30] Guimaraes TAC, Georgiou M, Bainbridge JWB, Michaelides M. Gene therapy for neovascular age-related macular degeneration: Rationale, clinical trials and future directions. Br J Ophthalmol 2021; 105(2): 151-7. http://dx.doi.org/10.1136/bjophthalmol-2020-316195 PMID: 32269060

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

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Abstarct

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

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.

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 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 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 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.

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Footnotes

NoteAuthors' ContributionsConflict of Interest 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.

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.

None declared.

Supplementary Material

References

1. Boillée S, Vande Velde C, Cleveland D W. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron.* 2006;52(01):39–59. [PubMed: 17015226]

2. Renton A E, Chiò A, Traynor B J. State of play in amyotrophic lateral sclerosis genetics. *Nat Neurosci.* 2014;17(01):17–23. [PMCID: PMC4544832] [PubMed: 24369373]

3. Logroscino G, Traynor B J, Hardiman O et al. EURALS. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *J Neurol Neurosurg Psychiatry.* 2008;79(01):6–11. [PubMed: 18079297]

4. Talbott E O, Malek A M, Lacomis MD. Vol. 138. Amsterdam, The Netherlands: Elsevier; 2016. The epidemiology of amyotrophic lateral sclerosis; pp. 225–238. [PubMed: 27637961]

5. Raj P. Amyotrophic Lateral Sclerosis. Rare Diseases India. Available at: http://www.rarediseasesindia.org/. Accessed June 11, 2020

6. Manjaly Z R, Scott K M, Abhinav K et al. The sex ratio in amyotrophic lateral sclerosis: a population based study. *Amyotroph Lateral Scler.* 2010;11(05):439–442. [PMCID: PMC6485484] [PubMed: 20225930]

7. Zarei S, Carr K, Reiley L et al. A comprehensive review of amyotrophic lateral sclerosis. *Surg Neurol Int.* 2015;6:171. [PMCID: PMC4653353] [PubMed: 26629397]

8. Corcia P, Pradat P F, Salachas F et al. Causes of death in a post-mortem series of ALS patients. *Amyotroph Lateral Scler.* 2008;9(01):59–62. [PubMed: 17924236]

9. Borrás-Blasco J, Plaza-Macías I, Navarro-Ruiz A, Perís-Martí J, Antón-Cano A. [Riluzole as a treatment for amyotrophic lateral sclerosis] (in Spanish) *Rev Neurol.* 1998;27(160):1021–1027. [PubMed: 9951030]

10. Turner M R, Kiernan M C, Leigh P N, Talbot K. Biomarkers in amyotrophic lateral sclerosis. *Lancet Neurol.* 2009;8(01):94–109. [PubMed: 19081518]

11. Liscic R M, Breljak D. Molecular basis of amyotrophic lateral sclerosis. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011;35(02):370–372. [PubMed: 20655970]

12. Verstraete E, Kuiperij H B, van Blitterswijk M M et al. TDP-43 plasma levels are higher in amyotrophic lateral sclerosis. *Amyotroph Lateral Scler.* 2012;13(05):446–451. [PubMed: 22873561]

13. Iłzecka J. Cerebrospinal fluid vascular endothelial growth factor in patients with amyotrophic lateral sclerosis. *Clin Neurol Neurosurg.* 2004;106(04):289–293. [PubMed: 15297002]

14. Nygren I, Larsson A, Johansson A, Askmark H. VEGF is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis. *Neuroreport.* 2002;13(17):2199–2201. [PubMed: 12488796]

15. Gao L, Zhou S, Cai H, Gong Z, Zang D.VEGF levels in CSF and serum in mild ALS patients *J Neurol Sci* 2014346(1-2)216–220. [PubMed: 25204587]

16. Gupta P K, Prabhakar S, Sharma S, Anand A. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. *J Neuroinflammation.* 2011;8(01):47. [PMCID: PMC3117705] [PubMed: 21569455]

17. Cronin S, Greenway M J, Ennis S et al. Elevated serum angiogenin levels in ALS. *Neurology.* 2006;67(10):1833–1836. [PubMed: 17130418]

18. Chou C-C, Zhang Y, Umoh M E et al. TDP-43 pathology disrupts nuclear pore complexes and nucleocytoplasmic transport in ALS/FTD. *Nat Neurosci.* 2018;21(02):228–239. [PMCID: PMC5800968] [PubMed: 29311743]

19. Armon C. How are ALS Functional Rating Scale (ALSFRS) scores interpreted in the assessment of amyotrophic lateral sclerosis (ALS)? Available at: https://www.medscape.com/answers/1170097-81928/81928-print. Accessed June 11, 2020

20. Cedarbaum J M, Stambler N, Malta E.BDNF ALS Study Group (Phase III). The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function *J Neurol Sci* 1999169(1,2)13–21. [PubMed: 10540002]

21. WFN Research Group on ALS/MND Agosta F, Al-Chalabi A, Filippi M.The El Escorial criteria: strengths and weaknesses *Amyotroph Lateral Scler Frontotemporal Degener* 201516(1,2)1–7. [PubMed: 25482030]

22. Oosthuyse B, Moons L, Storkebaum E et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet.* 2001;28(02):131–138. [PubMed: 11381259]

23. Lambrechts D, Storkebaum E, Morimoto M et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat Genet.* 2003;34(04):383–394. [PubMed: 12847526]

24. Mackenzie I R, Neumann M, Bigio E H et al. Nomenclature and nosology for neuropathologic subtypes of frontotemporal lobar degeneration: an update. *Acta Neuropathol.* 2010;119(01):1–4. [PMCID: PMC2799633] [PubMed: 19924424]

25. Liscic R M, Grinberg L T, Zidar J, Gitcho M A, Cairns N J. ALS and FTLD: two faces of TDP-43 proteinopathy. *Eur J Neurol.* 2008;15(08):772–780. [PMCID: PMC2801606] [PubMed: 18684309]

26. Kwong L K, Neumann M, Sampathu D M, Lee VM-Y, Trojanowski J Q. TDP-43 proteinopathy: the neuropathology underlying major forms of sporadic and familial frontotemporal lobar degeneration and motor neuron disease. *Acta Neuropathol.* 2007;114(01):63–70. [PubMed: 17492294]

27. Feneberg E, Gray E, Ansorge O, Talbot K, Turner M R. Towards a TDP-43-based biomarker for ALS and FTLD. *Mol Neurobiol.* 2018;55(10):7789–7801. [PMCID: PMC6132775] [PubMed: 29460270]

28. Colombrita C, Onesto E, Megiorni F et al. TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. *J Biol Chem.* 2012;287(19):15635–15647. [PMCID: PMC3346140] [PubMed: 22427648]

29. Cicardi M E, Cristofani R, Rusmini P et al. Tdp-25 Routing to Autophagy and Proteasome Ameliorates its Aggregation in Amyotrophic Lateral Sclerosis Target Cells. *Sci Rep.* 2018;8(01):12390. [PMCID: PMC6098007] [PubMed: 30120266]

30. Brockington A, Wharton S B, Fernando M et al. Expression of vascular endothelial growth factor and its receptors in the central nervous system in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol.* 2006;65(01):26–36. [PubMed: 16410746]

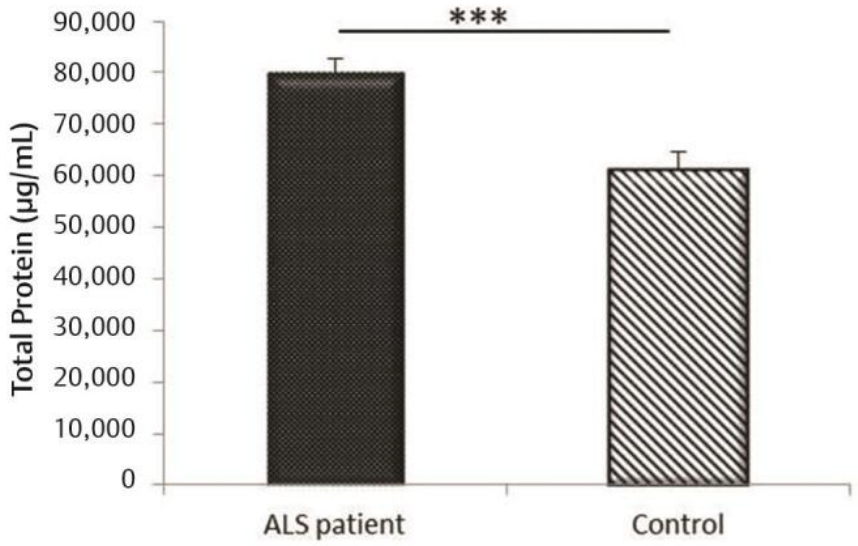
31. Anand A, Gupta P K, Sharma N K, Prabhakar S. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. *Eur J Neurol.* 2012;19(05):788–792. [PubMed: 21978169]

32. Nakazawa S, Oikawa D, Ishii R et al. Linear ubiquitination is involved in the pathogenesis of optineurin-associated amyotrophic lateral sclerosis. *Nat Commun.* 2016;7(01):12547. [PMCID: PMC4999505] [PubMed: 27552911]

33. Slowicka K, van Loo G. Optineurin functions for optimal immunity. *Front Immunol.* 2018;9:769. [PMCID: PMC5902560] [PubMed: 29692786]

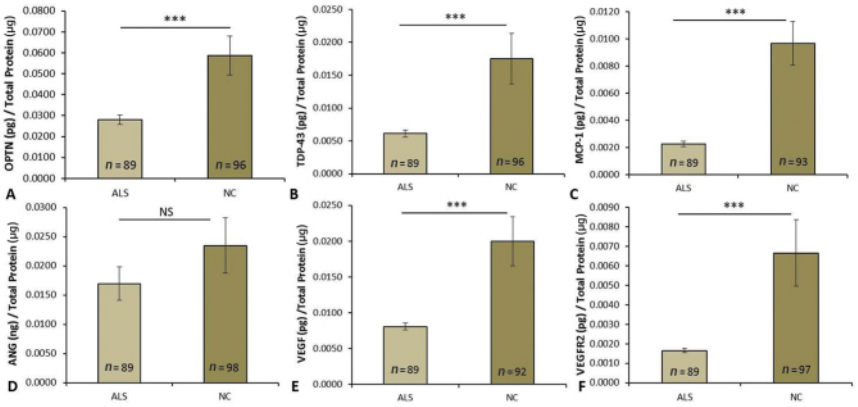
34. Arai T, Hasegawa M, Akiyama H et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun.* 2006;351(03):602–611. [PubMed: 17084815]

Fig. 1



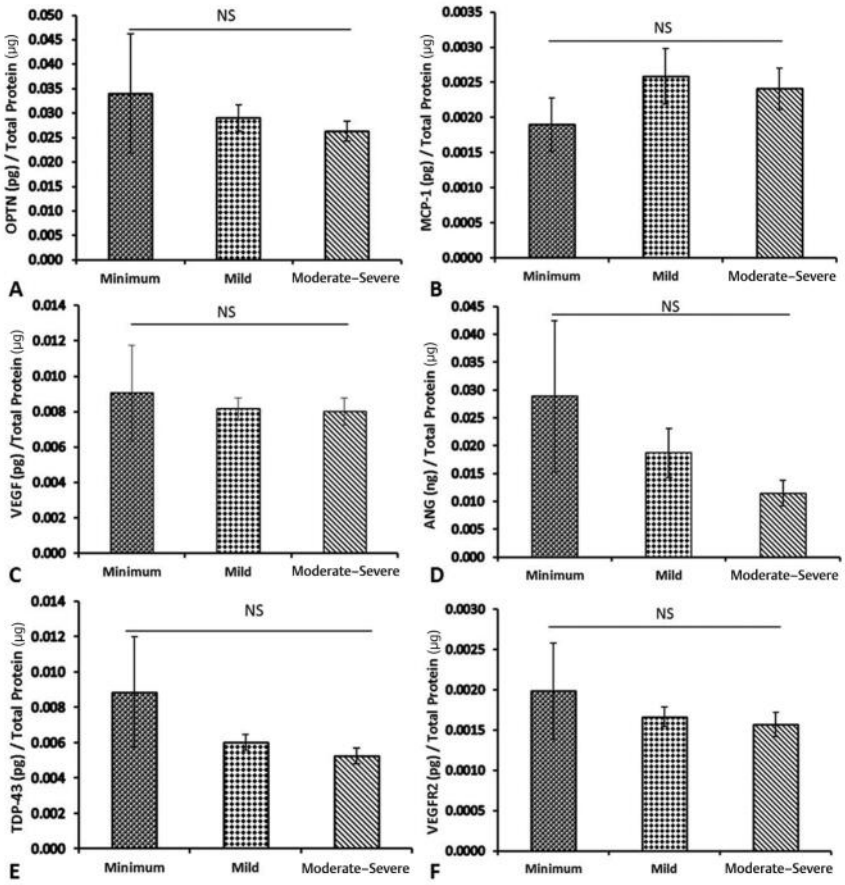
Total protein expression and comparison of ALS patients and normal controls. ALS, amyotrophic lateral sclerosis.

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.

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.

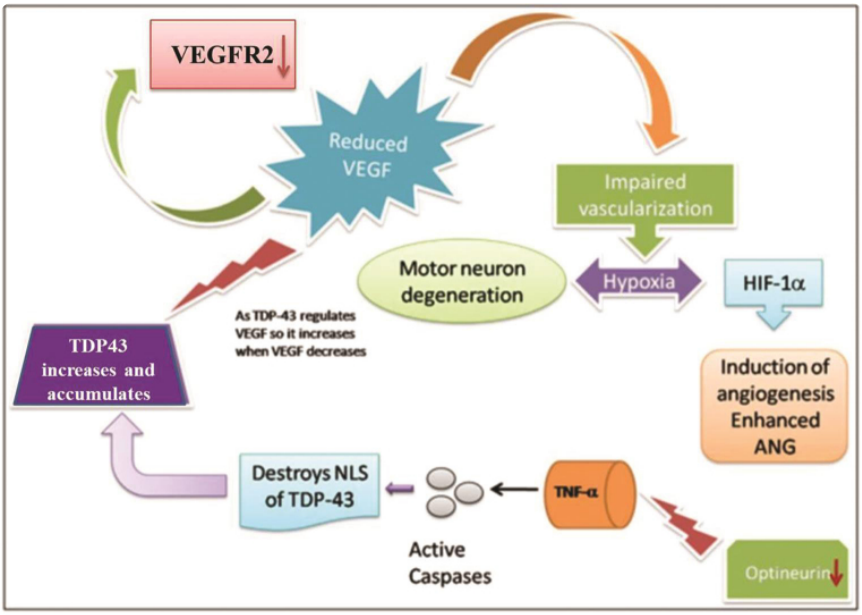
Table 1

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.

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.

Identifying Putative Cerebrospinal Fluid Biomarkers of ALS in a North Indian population

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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.

Disclosure of Conflicts of Interest

None of the authors has any conflict of interest to disclose.

Abstract

Title: Identifying Putative Cerebrospinal Fluid Biomarkers of ALS in a North Indian population

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 VEGFR2, optineurin (OPTN), monocyte chemoattractant protein 1 (MCP-1), angiogenin (ANG), TAR DNA-binding Protein (TDP-43) were quantified by ELISA in the CSF of 54 patients with sporadic ALS and 32 controls in a case-control study design.

Results: CSF levels of VEGF ($p=0.014$) and ANG ($p=0.009$) were decreased whereas VEGFR2 was higher ($p=0.002$) in patients with ALS than in controls. TDP-43 positively correlated with MCP-1 ($p=0.003$), VEGF ($p<0.001$) and VEGFR2 ($p<0.001$) in patients with ALS.

Discussion: Our findings suggest possible utility of VEGF, VEGFR-2 and ANG as biomarkers for ALS treatment trials.

Keywords: Amyotrophic lateral sclerosis, Neuromuscular disorder, Muscle wasting, Cerebrospinal fluid, Angiogenesis.

INTRODUCTION

A growing body of evidence points to involvement of growth factors such as vascular endothelial growth factor (VEGF)[1-3] and its receptor 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, 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 nucleus. Impaired functioning of TDP-43 leads to formation of TDP inclusions.[20]

Another important molecule 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 NF-κB-mediatedregulation 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 the present study, we have 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 have 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.

METHODS

Subjects

Our study received approval from the Institutional Ethics Committee of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. All the study participants gave written informed consent prior to enrollment. CSF samples were collected from ALS patients in the Neurology Inpatient Department of PGIMER Chandigarh and stored at -80ºC for >2 years. Inclusion criteria included a diagnosis of definite/probable/possible ALS by revised El-Escorial criteria (as

diagnosed by experienced neurologists based on clinical assessment),[22] aged 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 revised ALS Functional Rating Scale (ALSFRS-R) to evaluate the function of ALS patients. ALS patients were graded as minimum (ALSFRS-R>40), mild to moderate (ALSFRS-R 30-39), moderate to severe (ALSFRS-R20-29) and advanced disease (ALSFRS-R<20).[22]

Exclusion criteria included hepatic, renal, gastroentrological, respiratory, cardiovascular, endocrinological, neurological, immunological or hematological diseases, hypothyroidism or hyperthyroidism, cognitive impairment with significant decision making incapacity, major depression, schizophrenia or dementia including Alzheimer's disease and frontotemporal degeneration (as determined empirically by the study neurologists).

Controls samples were collected from Institute's trauma center. Inclusion criteria allowed for otherwise healthy subjects with limb injury, aged 20 to 65 years. 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.

ELISA

Human VEGF, VEGFR2, OPTN, TDP-43, ANG and MCP-1 levels were obtained using the sandwich ELISA kits. Kits for VEGF, VEGFR2, OPTN, TDP-43 and ANG was procured from Qayee

Biological Tehnology Co. Ltd. (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 HRP conjugate, the plate was incubated. Then the plate was 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, California, USA). A standard curve was plotted for each experiment and values of respective proteins for all the samples were calculated. R^2 value ≥ 0.97 was considered appropriate for the test.

Statistical Analysis

Statistical analyses were performed using SPSS v23 (IBM Inc., Armonk, NY, USA). Kolmogorov-Smirnov test was initially 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. ANCOVA was applied to control p-values for age by using General Linear model. Significance was considered at $p \leq 0.05$. Pearson's or Spearman's rho test statistics were used to correlate the datasets, based on the applicability. There were some patients whose socio-demographic 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. Bonferroni's correction test for multiple comparisons was applied to obtain significant correlations. The cut-off p-value after Bonferroni's correction for correlation between biomarkers and clinical severity or duration of illness was ≤ 0.004 and among the biomarkers was ≤ 0.003.

RESULTS

Clinical and demographic characteristics

No FALS patients were enrolled. 54 SALS patients and 32 control subjects were enrolled in the study. The socio-demographic features of participants are summarized in Table 1.

Quantitative protein expression

ELISA analysis 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 to the control group (Tables 2 and 3).

Correlation analyses

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

DISCUSSION

The current study found that levels of CSF VEGF in patients with ALS were decreased as compared to controls; VEGFR2 was significantly elevated and ANG was decreased in the CSF of ALS patients. Despite significant p-values, a substantial overlap between the values of control and ALS group was present. 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, though this has not been ascribed to the 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 has yielded benefit in these studies.[27] Our study is consistent with these findings, as we report lower levels of VEGF in the ALS group along with a concomitant rise in CSF levels of VEGFR2. Studies involving knockout mice have shown that low VEGF levels in mice with $Vegf^{\delta/\delta}$ could result in motor neuron degeneration owing to two possible pathways. Firstly, due to peripheral arteriolar dysregulation there is a decline in vascular supply and prolonged chronic ischemia. Endothelial cell dysfunction may also impair 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] Secondly, 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 SOD1G93A 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 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 explore 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 report 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.

Additionally, 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] Since ANG activates the NF-κB-mediated cell survival pathway and Bcl-2-mediated anti-apoptotic 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, 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 reason for non-participation were not recorded. Thus, the patient population included in the study may not represent the overall ALS population. The number 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 might exist between ALS patients and controls that could affect biomarker outcomes. Further, there were differences in age between ALS and controls that could not be controlled for; thus, adjusted and non-adjusted p-values are presented. Another limitation is that cognitive impairment and frontotemporal degeneration were diagnosed by neurologists using empirical means, rather than specific instruments.

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 neurologic disease.

ABBREVIATIONS

ANG, angiogenin

ALS, Amyotrophic lateral sclerosis

ALSFRS-r, Amyotrophic lateral sclerosisfunctional rating scale

BMI, body mass index

CCR2, chemokine receptor-2

CNS, central nervous system

CSF, cerebrospinal fluid

FALS, Familialamyotrophic lateral sclerosis

IEC, Institutional Ethics Committee

MCP-1, monocyte chemoattractant protein 1

M:F, Male:Female

OPTN, optineurin

PGIMER, Post Graduate Institute of Medical Education and Research

RNase, Ribonuclease

SALS, Sporadic amyotrophic lateral sclerosis

TDP-43, TAR DNA-binding Protein

VEGF, vascular endothelial growth factor

VEGFR2, vascular endothelial growth factor receptor 2

REFERENCES

1. Just N, Moreau C, Lassalle P, Gosset P, Perez T, Brunaud-Danel V, et al. High erythropoietin and low vascular endothelial growth factor levels in cerebrospinal fluid from hypoxemic ALS patients suggest an abnormal response to hypoxia. Neuromuscul Disord 2007;**17**:169-173.
2. Pronto-Laborinho AC, Pinto S, de Carvalho M. Roles of vascular endothelial growth factor in amyotrophic lateral sclerosis. Biomed Res Int 2014;**2014**:947513.
3. Gupta PK, Prabhakar S, Sharma S, Anand A. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. J Neuroinflammation 2011 May 13;**8**:47.
4. Wu D, Yu W, Kishikawa H, Folkerth RD, Iafrate AJ, Shen Y, et al. Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. Ann Neurol 2007;**62**:609-17.
5. Millecamps S, Salachas F, Cazeneuve C, Gordon P, Bricka B, Camuzat A, et al. SOD1, ANG, VAPB, TARDBP, and FUS mutations in familial amyotrophic lateral sclerosis: genotype–phenotype correlations. J Med Genet 2010;**47**:554-60.
6. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, et al. Mutations of optineurin in amyotrophic lateral sclerosis. Nature 2010;**465**:223-226.
7. Toth RP, Atkin JD. Dysfunction of optineurin in amyotrophic lateral sclerosis and glaucoma. Front Immunol 2018;**9**:1017.
8. Williams SM, Khan G, Harris BT, Ravits J, Sierks MR. TDP-43 protein variants as biomarkers in amyotrophic lateral sclerosis. BMC Neurosci 2017;**18**:20.

9. Steinacker P, Hendrich C, Sperfeld AD, Jesse S, von Arnim CA, Lehnert S, et al. TDP-43 in cerebrospinal fluid of patients with frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Arch Neurol 2008;**65**:1481-1487.
10. Noto Y, Shibuya K, Sato Y, Kanai K, Misawa S, Sawai S, et al. Elevated CSF TDP-43 levels in amyotrophic lateral sclerosis: specificity, sensitivity, and a possible prognostic value. Amyotroph Lateral Scler 2011;**12**:140-143.
11. Kasai T, Tokuda T, Ishigami N, Sasayama H, Foulds P, Mitchell DJ, et al. Increased TDP-43 protein in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. Acta Neuropathol 2009;**117**:55-62.
12. Nagata T, Nagano I, Shiote M, Narai H, Murakami T, Hayashi T, et al. Elevation of MCP-1 and MCP-1/VEGF ratio in cerebrospinal fluid of amyotrophic lateral sclerosis patients. Neurol Res 2007;**29**:772-776.
13. Baron P, Bussini S, Cardin V, Corbo M, Conti G, Galimberti D, et al. Production of monocyte chemoattractant protein-1 in amyotrophic lateral sclerosis. Muscle Nerve 2005;**32**:541-544.
14. Wang Y, Kilic E, Kilic Ü, Weber B, Bassetti CL, Marti HH, et al. VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena. Brain 2004;**128**:52-63.
15. Brockington A, Heath PR, Holden H, Kasher P, Bender FL, Claes F, et al. Downregulation of genes with a function in axon outgrowth and synapse formation in motor neurones of the VEGF delta/delta mouse model of amyotrophic lateral sclerosis. BMC Genomics 2010;**11**:203.

16. Vijayalakshmi K, Ostwal P, Sumitha R, Shruthi S, Varghese AM, Mishra P, et al. Role of VEGF and VEGFR2 receptor in reversal of ALS-CSF induced degeneration of NSC-34 motor neuron cell line. Mol Neurobiol 2015;**51**:995-1007.
17. Ogunshola OO, Antic A, Donoghue MJ, Fan SY, Kim H, Stewart WB, et al. Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. J Biol Chem 2002;**277**:11410-11415.
18. Lambrechts D, Lafuste P, Carmeliet P, Conway EM. Another angiogenic gene linked to amyotrophic lateral sclerosis. Trends Mol Med 2006;**12**:345-347.
19. Kishimoto K, Liu S, Tsuji T, Olson KA, Hu G-f. Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis. Oncogene 2005;**24**:445.
20. Winton MJ, Igaz LM, Wong MM, Kwong LK, Trojanowski JQ, Lee VM. Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation. J Biol Chem 2008;**283**:13302-9.
21. Akizuki M, Yamashita H, Uemura K, Maruyama H, Kawakami H, Ito H, et al. Optineurin suppression causes neuronal cell death via NF-κB pathway. J Neurochem 2013;**126**:699-704.
22. Brooks BR, Miller RG, Swash M, Munsat TL; World Federation of Neurology Research Group on Motor Neuron Diseases. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor Neuron Disord 2000;**1**:293-9.

23. Gupta PK, Prabhakar S, Sharma NK, Anand A. Possible association between expression of chemokine receptor-2 (CCR2) and amyotrophic lateral sclerosis (ALS) patients of North India. PloS one 2012;**7**:e38382.
24. Gao L, Zhou S, Cai H, Gong Z, Zang D. VEGF levels in CSF and serum in mild ALS patients. J Neurol Sci 2014;**346**:216-220.
25. Devos D, Moreau C, Lassalle P, Perez T, De Seze J, Brunaud-Danel V, et al. Low levels of the vascular endothelial growth factor in CSF from early ALS patients. Neurology 2004;**62**:2127-2129.
26. Guo J, Yang X, Gao L, Zang D. Evaluating the levels of CSF and serum factors in ALS. Brain Behav 2017;**7**:e00637.
27. Keifer OP Jr, O'Connor DM, Boulis NM. Gene and protein therapies utilizing VEGF for ALS. Pharmacol Ther 2014;**141**:261-271.
28. Shim JW, Madsen JR. VEGF signaling in neurological disorders. Int J Mol Sci 2018;**19**:E275.
29. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011;**473**:298-307.
30. Li B, Xu W, Luo C, Gozal D, Liu R. VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death. Brain Res Mol Brain Res 2003;**111**:155-164.
31. Tolosa L, Mir M, Olmos G, Llado J. Vascular endothelial growth factor protects motoneurons from serum deprivation–induced cell death through phosphatidylinositol 3-kinase-mediated p38 mitogen-activated protein kinase inhibition. Neuroscience 2009;**158**:1348-1355.

32. Bogaert E, Van Damme P, Poesen K, Dhondt J, Hersmus N, Kiraly D, et al. VEGF protects motor neurons against excitotoxicity by upregulation of GluR2. Neurobiol Aging 2010;**31**:2185-2191.

33. Storkebaum E, Lambrechts D, Dewerchin M, Moreno-Murciano MP, Appelmans S, Oh H, et al. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. Nat Neurosci 2005;**8**:85-92.

34. Lambrechts D, Poesen K, Fernandez-Santiago R, Al-Chalabi A, Del Bo R, Van Vught P, et al. Meta-analysis of vascular endothelial growth factor variations in amyotrophic lateral sclerosis: increased susceptibility in male carriers of the -2578AA genotype. J Med Genet 2009;**46**:840-846.

35. Vijayalakshmi K, Alladi PA, Sathyaprabha T, Subramaniam JR, Nalini A, Raju T. Cerebrospinal fluid from sporadic amyotrophic lateral sclerosis patients induces degeneration of a cultured motor neuron cell line. Brain Res 2009;**1263**:122-133.

36. Ivanov P, Emara MM, Villen J, Gygi SP, Anderson P. Angiogenin-induced tRNA fragments inhibit translation initiation. Mol Cell. 2011;**43**:613-23.

37. Sheng J, Xu Z. Three decades of research on angiogenin: a review and perspective. Acta Biochim Biophys Sin (Shanghai) 2015;**48**:399-410.

38. Xia W, Fu W, Cai X, Wang M, Chen H, Xing W, et al. Angiogenin promotes U87MG cell proliferation by activating NF-κB signaling pathway and downregulating its binding partner FHL3. PloS one 2015;**10**:e0116983.

39. Sadagopan S, Veettil MV, Chakraborty S, Sharma-Walia N, Paudel N, Bottero V, et al. Angiogenin functionally interacts with p53 and regulates p53-mediated apoptosis and cell survival. Oncogene 2012;**31**:4835-4847.
40. Subramanian V, Crabtree B, Acharya KR. Human angiogenin is a neuroprotective factor and amyotrophic lateral sclerosis associated angiogenin variants affect neurite extension/pathfinding and survival of motor neurons. Hum Mol Genet 2008;**17**:130-149.
41. Colombrita C, Onesto E, Megiorni F, Pizzuti A, Baralle FE, Buratti E, et al. TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. J Biol Chem 2012;**287**:15635-15647.
42. Mitra J, Guerrero EN, Hegde PM, Liachko NF, Wang H, Vasquez V, et al. Motor neuron disease-associated loss of nuclear TDP-43 is linked to DNA double-strand break repair defects. Proc Natl Acad Sci U S A 2019;**116**:4696-4705.

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)	0.003
BMI (kg/m^2)	22.09 ± 4.13 (n=49)	24.25±5.40 (n=28)	0.082
Gender (Male/Female)	43/10 (n=53)	28/4 (n=32)	0.554
Duration (months)	19.34 ± 15.13 (n=46)	-	-
ALSFRS-R score	34.37 ± 6.17 (n=48)	-	-

The data are presented as mean ± standard deviation except gender, which is represented as number of samples. Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-r, Amyotrophic Lateral Sclerosis Functional Rating Scale - Revised; BMI, body mass index; n, number of samples.

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	0.019 (1.36 to 14.47)	0.014 (1.83 to 15.80)
VEGFR2 (pg/ml)	37.35±2.19 (n=48)	35.46±2.29 (n=24)	3.390	0.001 (0.78 to 3.00)	0.002 (0.71 to 3.10)
TDP-43 (pg/ml)	105.70±24.22 (n=48)	112.07±13.88 (n=30)	-1.313	0.193 (-16.06 to 3.30)	0.230 (-16.63 to 4.06)
OPTN (pg/ml)	619.92±45.82 (n=46)	606.20±27.77 (n=31)	1.491	0.140 (-4.61 to 32.05)	0.083 (-2.28 to 36.73)

The data is presented as mean ± standard deviation.. Abbreviations: 95% CI, 95% confidence interval; ALS, amyotrophic lateral sclerosis; 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

Statistical Measures	ANG (ng/ml)		MCP-1 (pg/ml)		MCP-1/VEGF	
	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	0.009		0.074		0.304	

The data is presented 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 among the biomolecules in ALS patients.

MCP-1	r = 1					
VEGF	r = -0.006 p = 0.963	r = 1				
VEGFR2	r = 0.268 p = 0.065	r = 0.325 p* = 0.024	r = 1			
ANG	r = 0.062 p = 0.676	r = 0.230 p = 0.120	r = 0.077 p = 0.609	r = 1		
TDP-43	r = 0.425 p = 0.003	r = 0.719 p*<0.001	r = 0.545 p*<0.001	r = 0.332 p = 0.024	r = 1	
OPTN	r = 0.188 p = 0.211	r = 0.021 p* = 0.887	r = 0.060 p* = 0.693	r = 0.218 p = 0.151	r = 0.148 p* = 0.328	r = 1
Biomolecules	**MCP-1**	**VEGF**	**VEGFR2**	**ANG**	**TDP-43**	**OPTN**

P-values are significant at $p \leq 0.05/15 = 0.003$ after Bonferroni's correction. Abbreviations: ANG, angiogenin; MCP-1, monocyte chemoattractant protein 1; OPTN, optineurin; p, p-value for Spearman's rho correlation; p*, p-value for Pearson's correlation; r, correlation coefficient; TDP-43, TAR DNA-binding Protein; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2

Angiogenesis-Centered Molecular Cross-Talk in Amyotrophic Lateral Sclerosis Survival: Mechanistic Insights

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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

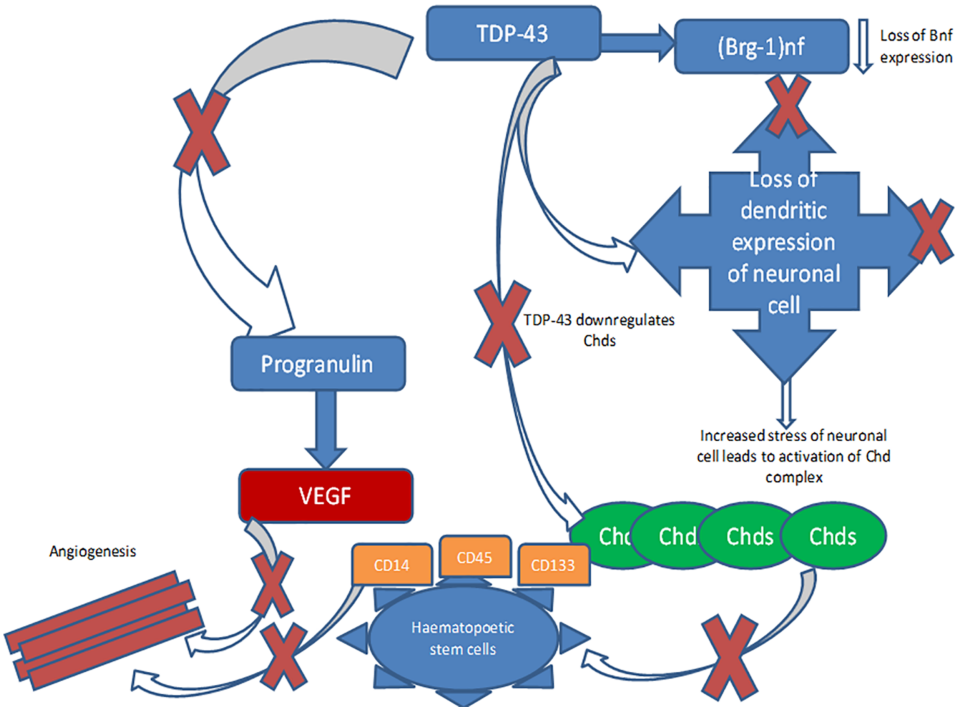

FIG. 1: Epigenetic dysregulation affecting angiogenesis in ALS

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

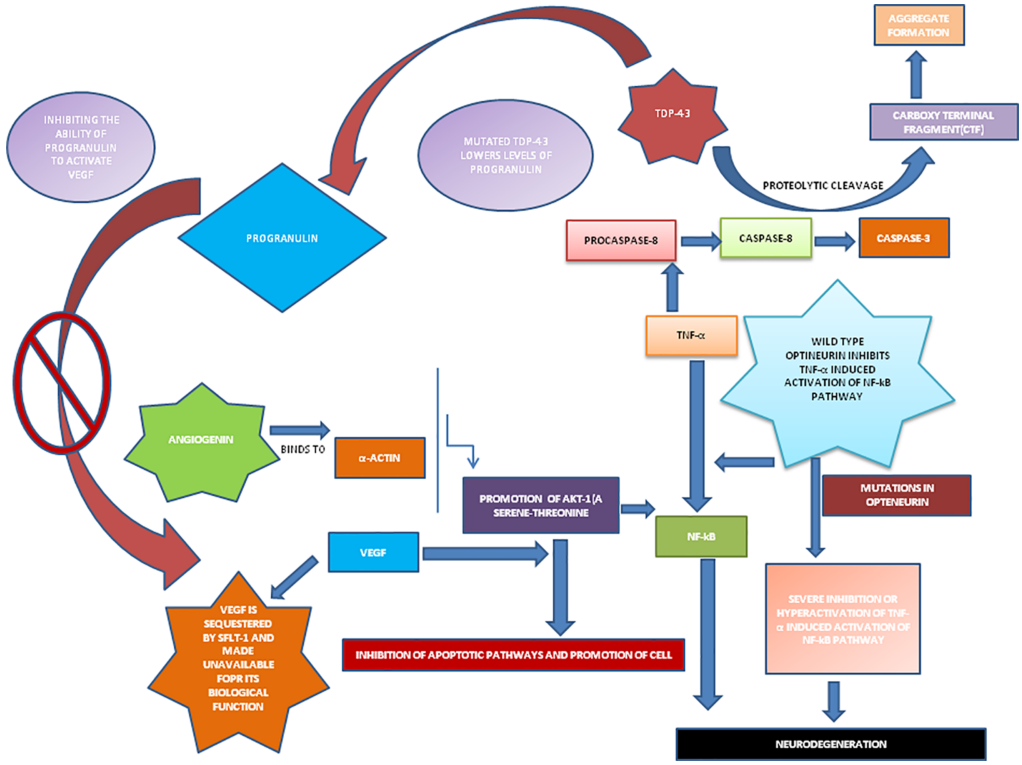

FIG. 2: Schematic representation of the cross-talk between candidate molecules and known pathways like Akt-1, caspases, and neurodegeneration

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.

REFERENCES

1. Aran F. Research on an as yet undescribed disease of the muscular system (progressive muscular atrophy). Arch Gén Méd. 1848;24:15–35.
2. Charcot JM. Lectures on the diseases of the nervous system. Delivered at La Salpêtrière: London: New Sydenham Society; 1877.
3. Chiò A, Benzi G, Dossena M, Mutani R, Mora G. Severely increased risk of amyotrophic lateral sclerosis among Italian professional football players. Brain. 2005;128(Pt 3):472–6.
4. Armon C. Sports and trauma in amyotrophic lateral sclerosis revisited. J Neurol Sci. 2007;262(1-2):45–53.
5. Andersen PM, Al-Chalabi A. Clinical genetics of amyotrophic lateral sclerosis: what do we really know? Nat Rev Neurol. 2011;7(11):603–15.
6. Jacobsson J, Jonsson PA, Andersen PM, Forsgren L, Marklund SL. Superoxide dismutase in CSF from amyotrophic lateral sclerosis patients with and without CuZn-superoxide dismutase mutations. Brain. 2001;1 424(7):1461–6.
7. Simpson E, Henry Y, Henkel J, Smith R, Appel SH. Increased lipid peroxidation in sera of ALS patients:

a potential biomarker of disease burden. Neurology. 2004;62(10):1758–65.
8. Storkebaum E, Quaegebeur A, Vikkula M, Carmeliet P. Cerebrovascular disorders: molecular insights and therapeutic opportunities. Nat Neurosci. 2011;14(11):1390–7.
9. Gupta PK, Prabhakar S, Sharma S, Anand A. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. J Neuroinflammation. 2011;8(1):47.
10. Gupta PK, Prabhakar S, Abburi C, Sharma NK, Anand A. Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients. J Neuroinflammation. 2011;8(1):114.
11. Gupta PK, Prabhakar S, Sharma NK, Anand A. Possible association between expression of chemokine receptor-2 (CCR2) and amyotrophic lateral sclerosis (ALS) patients of North India. PLoS One. 2012;7(6):e38382.
12. Raab S, Beck H, Gaumann A, Yüce A, Gerber H-P, Plate K, Hammes H-P, Ferrara N, Breier G. Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. Thromb Haemost. 2004;91(03):595–605.
13. Ogunshola OO, Stewart WB, Mihalcik V, Solli T, Madri JA, Ment LR. Neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain. Develop Brain Res. 2000;119(1):139–53.
14. Carmeliet P, Ruiz de Almodovar C. VEGF ligands and receptors: implications in neurodevelopment and neurodegeneration. Cell Mol Life Sci. 2013;70(10):1763–78.
15. Bautch VL, James JM. Neurovascular development: the beginning of a beautiful friendship. Cell Adh Migr. 2009;3(2):199–204.
16. Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, Thijs V, Andersson J, van Marion I, Al-Chalabi A, Borrnes S, Musson R, Hansen V, Beckman L, Adolfsson R, Pall HS, Prats H, Vermiere S, Rutgeerts P, Katayama S, Awata T, Leigh N, Lang-Lazdunski L, Dewerchin M, Shaw C, Moons L, Vlietinck R, Morrison KE, Robberecht W, Van Broeckhoven C, Collen D, Andersen PM, Carmeliet P. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet. 2003;34(4):383–94.
17. Lambrechts D, Carmeliet P. VEGF at the neurovascular interface: therapeutic implications for motor neuron disease. Biochim Biophys Acta. 2006;1762(11-12):1109–21.
18. Kishimoto K, Liu S, Tsuji T, Olson KA, Hu G-F. Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis. Oncogene. 2005;24(3):445–56.
19. Greenway MJ, Andersen PM, Russ C, Ennis S, Cashman S, Donaghy C, Patterson V, Swingler R, Kieran D, Prehn J, Morrison KE, Green A, Acharya KR, Brown Jr RH, Hardimon O. ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. Nat Genet. 2006;38(4):411–3.
20. Fernández-Santiago R, Hoenig S, Lichtner P, Sperfeld A-D, Sharma M, Berg D, Weichenrieder O, Illig T, Eger K, Meyer T, Anneser J, Münch C, Zierz S, Gasser T, Ludolph A. Identification of novel Angiogenin (ANG) gene missense variants in German patients with amyotrophic lateral sclerosis. J Neurol. 2009;256(8):1337–42.
21. Paubel A, Violette J, Amy M, Praline J, Meininger V, Camu W, Corcia P, Andres CR, Vourc'h P; French Amyotrophic Lateral (ALS) Study Group. Mutations of the ANG gene in French patients with sporadic amyotrophic lateral sclerosis. Arch Neurol. 2008;65(10):1333–6.
22. Gellera C, Tiloca C, Del Bo R, Corrado L, Pensato V, Agostini J, Cereda C, Ratti A, Castellotti B, Corti S, Bagarotti A, Cagnin A, Milani P, Gabelli C, Riboldi G, Mazzini L, Sorarù G, D'Alfonso S, Taroni F, Comi GP, Ticozzi N, Silani V; SLAGEN Consortium. Ubiquilin 2 mutations in Italian patients with amyotrophic lateral sclerosis and frontotemporal dementia. J Neurol Neurosurg Psych. 2013;84(2):183–7.
23. Gellera C, Colombrita C, Ticozzi N, Castellotti B, Bragato C, Ratti A, Taroni F, Silani V. Identification of new ANG gene mutations in a large cohort of Italian patients with amyotrophic lateral sclerosis. Neurogenetics. 2008;9(1):33–40.
24. Conforti FL, Sprovieri T, Mazzei R, Ungaro C, La Bella V, Tessitore A, Patitucci A, Magariello A, Gabriele AL, Tedeschi G, Simone IL, Majorana G, Valentino P, Condino F, Bono F, Monsurrò MR, Muglia M, Quattrone A. A novel Angiogenin gene mutation in a sporadic patient with amyotrophic lateral sclerosis from southern Italy. Neuromuscul Disord. 2008;18(1):68–70.
25. Bhutani H, Anand A. Biomarkers in amyotrophic lateral sclerosis: is there a neurovascular pathway? Curr Neurovasc Res. 2012;9(4):302–9.
26. Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bioessays. 2004;26(9):943–54.
27. Gao X, Xu Z. Mechanisms of action of angiogenin. Acta Biochim Biophys Sin (Shanghai). 2008;40(7):619–24.
28. Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet. 2001;28(2):131–8.
29. Subramanian V, Crabtree B, Acharya KR. Human angiogenin is a neuroprotective factor and amyotrophic lateral sclerosis associated angiogenin variants affect neurite extension/pathfinding and survival of motor neurons. Hum Mol Genet. 2008;17(1):130–49.
30. Krüssel JS, Casañ EM, Raga F, Hirchenhain J, Wen Y, Huang H-Y, Bielfeld P, Polan ML. Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sflt

in cycling human endometrium. Mol Hum Reproduct. 1999;5(5):452–8.
31. Birkenhäger R, Schneppe B, Röckl W, Wilting J, Weich HA, Mccarthy JE. Synthesis and physiological activity of heterodimers comprising different splice forms of vascular endothelial growth factor and placenta growth factor. Biochem J. 1996;316(Pt 3):703–7.
32. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem. 1994;269(41):25646–54.
33. Ahmad S, Hewett PW, Al-Ani B, Sissaoui S, Fujisawa T, Cudmore MJ, Ahmed A. Autocrine activity of soluble Flt-1 controls endothelial cell function and angiogenesis. Vasc Cell. 2011;3(1):15.
34. Anand A, Gupta P, Sharma N, Prabhakar S. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. Eur J Neurol. 2012;19(5):788–92.
35. Kishikawa H, Wu D, Hu G-F. Targeting angiogenin in therapy of amyotropic lateral sclerosis. Expert Opin Ther Targets. 2008;12(10):1229–42.
36. Li B, Xu W, Luo C, Gozal D, Liu R. VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death. Mol Brain Res. 2003;111(1-2):155–64.
37. Romashkova JA, Makarov SS. NF-κB is a target of AKT in anti-apoptotic PDGF signalling. Nature. 1999; 401(6748):86–90.
38. Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, Mann D, Tsuchiya K, Yoshida M, Hashizume Y, Oda T. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun. 2006;351(3):602–11.
39. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, MacKenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. 2006;314(5796):130–3.
40. Winton MJ, Igaz LM, Wong MM, Kwong LK, Trojanowski JQ, Lee VM-Y. Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation. J Biol Chem. 2008;283(19):13302–9.
41. Grossman M, Wood EM, Moore P, Neumann M, Kwong L, Forman MS, Clark CM, McCluskey LF, Miller BL, Lee VM-Y, Trojanowski JQ. TDP-43 pathologic lesions and clinical phenotype in frontotemporal lobar degeneration with ubiquitin-positive inclusions. Arch Neurol. 2007;64(10):1449–54.
42. Gitcho MA, Baloh RH, Chakraverty S, Mayo K, Norton JB, Levitch D, Hatanpaa KJ, White III CL, Bigio EH, Caselli R, Baker M, Al-Lozi MT, Morris JC, Pestronk A, Rademakers R, Goate AM, Cairns NJ. TDP-43 A315T mutation in familial motor neuron disease. Ann Neurol. 2008;63(4):535–8.
43. Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Velde CV, Bouchard J-P, Lacomblez L, Pochigaeva K, Salachas F, Pradat PF, Camu W, Meininger V, Dupre N, Rouleau GA. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet. 2008;40(5):572–4.
44. Van Deerlin VM, Leverenz JB, Bekris LM, Bird TD, Yuan W, Elman LB, Clay D, Wood EM, Chen-Plotkin AS, Martinez-Lage M, Steinbart E, McCluskey L, Grossman M, Neumann M, Wu IL, Yang WS, Kalb R, Galasko DR, Montine TJ, Trojanowski JQ, Lee VM, Schellenberg GD, Yu CE. TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. Lancet Neurol. 2008;7(5):409–16.
45. Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, Ackerley S, Durnall JC, Williams KL, Buratti E, Baralle F, de Belleroche J, Mitchell JD, Leigh PN, Al-Chalabi A, Miller CC, Nicholson G, Shaw CE. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science. 2008;319(5870):1668–72.
46. Yokoseki A, Shiga A, Tan CF, Tagawa A, Kaneko H, Koyama A, Eguchi H, Tsujino A, Ikeuchi T, Kakita A, Okamoto K, Nishizawa M, Takahashi H, Onadera O. TDP-43 mutation in familial amyotrophic lateral sclerosis. Ann Neurol. 2008;63(4):538–42.
47. Wegorzewska I, Bell S, Cairns NJ, Miller TM, Baloh RH. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. Proc Natl Acad Sci U S A. 2009;106(44):18809–14.
48. Wils H, Kleinberger G, Janssens J, Pereson S, Joris G, Cuijt I, Smits V, Ceuterick-de Groote C, Van Broeckhoven C, Kumar-Singh S. TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. Proc Natl Acad Sci U S A. 2010;107(8):3858–63.
49. Wang P, Wander CM, Yuan C-X, Bereman MS, Cohen TJ. Acetylation-induced TDP-43 pathology is suppressed by an HSF1-dependent chaperone program. Nat Commun. 2017;8(1):82.
50. Butovsky O, Siddiqui S, Gabriely G, Lanser AJ, Dake B, Murugaiyan G, Doykan CE, Wu PM, Gali RR, Iyer LK, Lawson R, Berry J, Krichevsky AM, Cudkowicz ME, Weiner HL. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. J Clin Invest. 2012;122(9):3063–87.
51. Sako W, Ito H, Yoshida M, Koizumi H, Kamada M, Fujita K, Hashizume Y, Izumi Y, Kaji R. Nuclear factor κB expression in patients with sporadic amyotrophic lateral sclerosis and hereditary amyotrophic lateral sclerosis

with optineurin mutations. Clin Neuropathol. 2012;31(6): 418–23.
52. Cicardi ME, Cristofani R, Rusmini P, Meroni M, Ferrari V, Vezzoli G, Tedesco B, Piccolella M, Messi E, Galbiati M, Boncoraglio A, Carra S, Crippa V, Poletti A. Tdp-25 routing to autophagy and proteasome ameliorates its aggregation in amyotrophic lateral sclerosis target cells. Sci Rep. 2018;8(1):12390.
53. Seilhean D, Cazeneuve C, Thuriès V, Russaouen O, Millecamps S, Salachas F, Meininger V, LeGuern E, Duyckaerts C. Accumulation of TDP-43 and α-actin in an amyotrophic lateral sclerosis patient with the K17I ANG mutation. Acta Neuropathol. 2009;118(4):561–73.
54. Yamasaki S, Ivanov P, Hu G-F, Anderson P. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J Cell Biol. 2009;185(1):35–42.
55. Colombrita C, Onesto E, Megiorni F, Pizzuti A, Baralle FE, Buratti E, Silani V, Ratti A. TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. J Biol Chem. 2012;287(19):15635–47.
56. Irwin D, Lippa C, Rosso A. Progranulin (PGRN) expression in ALS: an immunohistochemical study. J Neurol Sci. 2009;276(1-2):9–13.
57. Eguchi R, Nakano T, Wakabayashi I. Progranulin and granulin-like protein as novel VEGF-independent angiogenic factors derived from human mesothelioma cells. Oncogene. 2017;36(5):714–22.
58. Chang MC, Srinivasan K, Friedman BA, Suto E, Modrusan Z, Lee WP, Kaminker JS, Hansen DV, Sheng M. Progranulin deficiency causes impairment of autophagy and TDP-43 accumulation. J Exp Med. 2017;214(9):2611–28.
59. Feneberg E, Steinacker P, Volk AE, Weishaupt JH, Wollmer MA, Boxer A, Tumani H, Ludolph AC, Otto M. Progranulin as a candidate biomarker for therapeutic trial in patients with ALS and FTLD. J Neural Transm (Vienna). 2016;123(3):289–96.
60. Tangkeangsirisin W, Serrero G. PC cell-derived growth factor (PCDGF/GP88, progranulin) stimulates migration, invasiveness and VEGF expression in breast cancer cells. Carcinogenesis. 2004;25(9):1587–92.
61. Kumar-Singh S. Progranulin and TDP-43: mechanistic links and future directions. J Mol Neurosci. 2011;45(3): 561–73.
62. Ugarte F, Forsberg EC. Haematopoietic stem cell niches: new insights inspire new questions. EMBO J. 2013; 32(19):2535–47.
63. Berson A, Sartoris A, Nativio R, Van Deerlin V, Toledo JB, Porta S, Liu S, Chung C-Y, Garcia BA, Lee VM-Y, Trojanowski JQ, Johnson FB, Berger SL, Bonini NM. TDP-43 promotes neurodegeneration by impairing chromatin remodeling. Curr Biol. 2017;27(23):3579–90. e6.
64. Gómez-Del Arco P, Perdiguero E, Yunes-Leites PS, Acín-Pérez R, Zeini M, Garcia-Gomez A, Sreenivasan K, Jiménez-Alcázar M, Segalés J, López-Maderuelo D, Ornés B, Jiménez-Borreguero LJ, D'Amato G, Enshell-Seijffers D, Morgan B, Georgopoulos K, Islam AB, Braun T, de la Pompa JL, Kim J, Enriquez JA, Ballestar E, Muñoz-Cánoves P, Redondo JM. The chromatin remodeling complex Chd4/NuRD controls striated muscle identity and metabolic homeostasis. Cell Metab. 2016;23(5):881–92.
65. Arany Z, He H, Lin J, Hoyer K, Handschin C, Toka O, Ahmad F, Matsui T, Chin S, Wu P-H, Rybkin II, Shelton JM, Manieri M, Cinti S, Schoen FJ, Bassel-Duby R, Rosenzweig A, Ingwall JS, Spiegelman BM. Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle. Cell Metab. 2005;1(4):259–71.
66. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuura S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R, Kawakami H. Mutations of optineurin in amyotrophic lateral sclerosis. Nature. 2010;465(7295):223–6.
67. Deng H-X, Bigio EH, Zhai H, Fecto F, Ajroud K, Shi Y, Yan J, Mishra M, Ajroud-Driss S, Heller S, Sufit R, Siddique N, Mugnaini E, Siddique T. Differential involvement of optineurin in amyotrophic lateral sclerosis with or without SOD1 mutations. Arch Neurol. 2011;68(8):1057–61.
68. Nagabhushana A, Bansal M, Swarup G. Optineurin is required for CYLD-dependent inhibition of TNFα-induced NF-κB activation. PLoS One. 2011;6(3):e17477.
69. Swarup G, Nagabhushana A. Optineurin, a multifunctional protein involved in glaucoma, amyotrophic lateral sclerosis and antiviral signalling. J Biosci. 2010;35(4):501–5.
70. Wu D, Yu W, Kishikawa H, Folkerth RD, Iafrate AJ, Shen Y, Xin W, Sims K, Hu GF. Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. Ann Neurol. 2007;62(6):609–17.
71. Lee EB, Lee VM-Y, Trojanowski JQ. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. Nat Rev Neurosci. 2011;13(1):38–50.
72. Freischmidt A, Wieland T, Richter B, Ruf W, Schaeffer V, Müller K, Marroquin N, Nordin F, Hübers A, Weydt P, Pinto S, Press R, Millecamps S, Molko N, Bernard E, Desnuelle C, Soriani MH, Dorst J, Graf E, Nordström U, Feiler MS, Putz S, Boeckers TM, Meyer T, Winkler AS, Winkelman J, de Carvajho M, Thal DR, Otto M, Brännström T, Volk AE, Kursula P, Danzer KM, Lichtner P, Dikic I, Meitinger T, Ludolph AC, Strom TM, Andersen PM, Weishaupt JH. Haploinsufficiency of TBK1 causes familial ALS and fronto-temporal dementia. Nat Neurosci. 2015;18(5):631–6.
73. Wong YC, Holzbaur EL. Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. Proc Natl Acad Sci U S A. 2014;111(42):E4439–E48.
74. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P,

Zaffagnini G, Wild P, Martens S, Wagner SA, Youle RJ, Dikic I. Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. Proc Natl Acad Sci U S A. 2016;113(15):4039–44.
75. Kwok CT, Morris A, de Belleroche JS. Sequestosome-1 (SQSTM1) sequence variants in ALS cases in the UK: prevalence and coexistence of SQSTM1 mutations in ALS kindred with PDB. Eur J Hum Genet. 2014;22(4):492–6.
76. Goode A, Rea S, Sultana M, Shaw B, Searle MS, Layfield R. ALS-FTLD associated mutations of SQSTM1 impact on Keap1-Nrf2 signalling. Mol Cell Neurosci. 2016;76:52–8.
77. Goode A, Butler K, Long J, Cavey J, Scott D, Shaw B, Sollenberger J, Gell C, Johansen T, Oldham NJ, Searle MS, Layfield R. Defective recognition of LC3B by mutant SQSTM1/p62 implicates impairment of autophagy as a pathogenic mechanism in ALS-FTLD. Autophagy. 2016;12(7):1094–104.
78. Czabanka M, Korherr C, Brinkmann U, Vajkoczy P. Influence of TBK-1 on tumor angiogenesis and microvascular inflammation. Front Biosci. 2008;13:7243–9.
79. Korherr C, Gille H, Schäfer R, Koenig-Hoffmann K, Dixelius J, Egland KA, Pastan I, Brinkmann U. Identification of proangiogenic genes and pathways by high-throughput functional genomics: TBK1 and the IRF3 pathway. Proc Natl Acad Sci U S A. 2006;103(11):4240–5.
80. de Majo M, Topp SD, Smith BN, Nishimura AL, Chen H-J, Gkazi AS, Miller J, Wong CH, Vance C, Baas F, Ten Asbroek ALMA, Kenna KP, Ticozzi N, Redondo AG, Esteban-Pérez J, Tiloca C, Verde F, Duga S, Morrison KE, Shaw PJ, Kirby J, Turner MR, Talbot K, Hardiman O, Glass JD, de Belleroche J, Gellera C, Ratti A, Al-Chalabi A, Brown RH, Silani V, Landers JE, Shaw CE. ALS-associated missense and nonsense TBK1 mutations can both cause loss of kinase function. Neurobiol Aging. 2018;71:266.
81. van der Zee J, Gijselinck I, Van Mossevelde S, Perrone F, Dillen L, Heeman B, Bäumer V, Engelborghs S, De Bleecker J, Baets J, Gelpi E, Rojas-Garcia R, Clarimón J, Lleó A, Diehl-Schmid J, Alexopoulos P, Perneczky R, Synofzik M, Just J, Schöls L, Graff C, Thonberg H, Borroni B, Padovani A, Jordanova A, Sarafov S, Tournev I, de Medonça A, Miltenberger-Miltényi G, Simões do Couto F, Ramirez A, Jessen F, Heneka MT, Gómez-Tortosa E, Danek A, Cras P, Vandenberghe R, De Jonghe P, De Deyn PP, Sleegers K, Cruts M, Van Broeckhoven C, Goeman J, Nuytten D, Smets K, Robberecht W, Damme PV, Bleecker J, Santens P, Dermaut B, Versijpt J, Michotte A, Ivanoiu A, Deryck O, Bergmans B, Delbeck J, Bruyland M, Willems C, Salmon E, Pastor P, Ortega-Cubero S, Benussi L, Ghidoni R, Binetti G, Hernández I, Boada M, Ruiz A, Sorbi S, Nacmias B, Bagnoli S, Sorbi S, Sanchez-Valle R, Llado A, Santana I, Rosário Almeida M, Frisoni GB, Maetzler W, Matej R, Fraidakis MJ, Kovacs GG, Fabrizi GM, Testi S. TBK1 mutation spectrum in an extended European patient cohort with frontotemporal dementia and amyotrophic lateral sclerosis. Hum Mutat. 2017;38(3):297–309.
82. Oakes JA, Davies MC, Collins MO. TBK1: a new player in ALS linking autophagy and neuroinflammation. Mol Brain. 2017;10(1):5.
83. Mrak RE, Griffin WST. Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging. 2005;26(3):349–54.
84. Boillée S, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW. Onset and progression in inherited ALS determined by motor neurons and microglia. Science. 2006;312(5778):1389–92.
85. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A. 2007;104(5):1604–9.
86. Yin F, Banerjee R, Thomas B, Zhou P, Qian L, Jia T, Ma X, Ma Y, Iadecola C, Beal MF, Nathan C, Ding A. Exaggerated inflammation, impaired host defense, and neuropathology in progranulin-deficient mice. J Exp Med. 2010;207(1):117–28.
87. Liu-Yesucevitz L, Bilgutay A, Zhang Y-J, Vanderwyde T, Citro A, Mehta T, Zaarur N, McKee A, Bowser R, Sherman M, Petrucelli L, Wolozin B. Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue. PLoS One. 2010;5(10):e13250.
88. Mosley RL, Gendelman HE. Control of neuroinflammation as a therapeutic strategy for amyotrophic lateral sclerosis and other neurodegenerative disorders. Exp Neurol. 2010;222(1):1–5.
89. Muessel MJ, Klein RM, Wilson AM, Berman NE. Ablation of the chemokine monocyte chemoattractant protein-1 delays retrograde neuronal degeneration, attenuates microglial activation, and alters expression of cell death molecules. Brain Res Mol Brain Res. 2002;103(1-2):12–27.
90. Henkel JS, Engelhardt JI, Siklós L, Simpson EP, Kim SH, Pan T, Goodman JC, Siddique T, Beers DR, Appel SH. Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. Ann Neurol. 2004;55(2):221–35.
91. Maihöfner C, Probst-Cousin S, Bergmann M, Neuhuber W, Neundörfer B, Heuss D. Expression and localization of cyclooxygenase-1 and -2 in human sporadic amyotrophic lateral sclerosis. Eur J Neurosci. 2003;18(6):1527–34.
92. Spliet WG, Aronica E, Ramkema M, Aten J, Troost D. Increased expression of connective tissue growth factor in amyotrophic lateral sclerosis human spinal cord. Acta Neuropathol. 2003;106(5):449–57.
93. Malessa S, Leigh PN, Bertel O, Sluga E, Hornykiewicz O. Amyotrophic lateral sclerosis: glutamate dehydrogenase and transmitter amino acids in the spinal cord. J Neurol Neurosurg Psychiatry. 1991;54(11):984–8.

94. Lu L, Wang S, Zheng L, Li X, Suswam EA, Zhang X, Wheeler CG, Nabors L, Filippova N, King PH. Amyotrophic lateral sclerosis-linked mutant SOD1 sequesters Hu antigen R (HuR) and TIA-1-related protein (TIAR). Implications for impaired post-transcriptional regulation of vascular endothelial growth factor. J Biol Chem. 2009;284(49):33989–98.
95. Lunn JS, Sakowski SA, Kim B, Rosenberg AA, Feldman EL. Vascular endothelial growth factor prevents G93A-SOD1-induced motor neuron degeneration. Dev Neurobiol. 2009;69(13):871–84.
96. Bannwarth S, Ait-El-Mkadem S, Chaussenot A, Genin EC, Lacas-Gervais S, Fragaki K, Berg-Alonso L, Kageyama Y, Serre V, Moore DG, Verschueren A, Rouzier C, Le Bar I, Augé G, Cochaud C, Lespinasse F, N'Guyen K, de Septenville A, Brice A, Yu-Wai-Man P, Sesaki H, Pouget J, Paquis-Flucklinger V. A mitochondrial origin for frontotemporal dementia and amyotrophic lateral sclerosis through CHCHD10 involvement. Brain. 2014;137(Pt 8):2329–45.
97. Purandare N, Somayajulu M, Hüttemann M, Grossman LI, Aras S. The cellular stress proteins CHCHD10 and MNRR1 (CHCHD2): partners in mitochondrial and nuclear function and dysfunction. J Biol Chem. 2018;293(17):6517–29.
98. Brenner D, Müller K, Wieland T, Weydt P, Böhm S, Lulé D, Hübers A, Neuwirth C, Weber M, Borck G, Wahlqvist M, Danzer KM, Volk AE, Meitinger T, Strom TM, Otto M, Kassubek J, Ludolph AC, Andersen PM, WeisHaupt JH. NEK1 mutations in familial amyotrophic lateral sclerosis. Brain. 2016;139(Pt 5):e28.
99. Fang X, Lin H, Wang X, Zuo Q, Qin J, Zhang P. The NEK1 interactor, C21ORF2, is required for efficient DNA damage repair. Acta Biochim Biophys Sin. 2015;47(10):834–41.
100. Kenna KP, Van Doormaal PT, Dekker AM, Ticozzi N, Kenna BJ, Diekstra FP, Van Rheenen W, Van Eijk KR, Jones AR, Keagle P. NEK1 variants confer susceptibility to amyotrophic lateral sclerosis. Nat Genet. 2016;48(9):1037–42.
101. Patil M, Pabla N, Huang S, Dong Z. Nek1 phosphorylates Von Hippel-Lindau tumor suppressor to promote its proteasomal degradation and ciliary destabilization. Cell Cycle. 2013;12(1):166–71.
102. Ruf M, Mittmann C, Nowicka AM, Hartmann A, Hermanns T, Poyet C, van den Broek M, Sulser T, Moch H, Schraml P. pVHL/HIF-regulated CD70 expression is associated with infiltration of $CD27^+$ lymphocytes and increased serum levels of soluble CD27 in clear cell renal cell carcinoma. Clin Cancer Res. 2015;21(4):889–98.
103. Haase VH. The VHL tumor suppressor: master regulator of HIF. Curr Pharm Des. 2009;15(33):3895–903.
104. Van Rheenen W, Shatunov A, Dekker AM, McLaughlin RL, Diekstra FP, Pulit SL, Van Der Spek RA, Võsa U, De Jong S, Robinson MR, Yang J, Fogh I, van Doormaal PT, Tazelaar GH, Koppers M, Biokhuis AM, Sproviero W, Jones AR, Kenna KP, van Eijk KR, Harschnitz O, Schellevis RD, Brands WJ, Medic J, Menelaou A, Vajda A, Ticozzi N, Lin K, Rogelj B, Vrabec K, Ravnik-Glavač M, Koritnik B, Zidar J, Leonardis L, Grošelj LD, Millecamps S, Salachas F, Meininger V, de Carvalho M, Pinto S, Mora JS, Rojas-Garcia R, Polak M, Chandran S, Colville S, Swingler R, Morrison KE, Shaw PJ, Hardy J, Orrell RW, Pittman A, Sidle K, Fratta P, Malaspina A, Topp S, Petri S, Abdulla S, Drepper C, Sendtner M, Meyer T, Ophoff RA, Staats KA, Wiedau-Pazos M, Lomen-Hoerth C, Van Deerlin VM, Trojanowski JQ, Elman L, McCluskey L, Basak AN, Tunca C, Hamzeiy H, Parman Y, Meitinger T, Lichtner P, Radivojkov-Blagojevic M, Andres CR, Maurel C, Bensimon G, Landwehrmeyer B, Brice A, Payan CA, Saker-Delye S, Dürr A, Wood NW, Tittmann L, Lieb W, Franke A, Rietschel M, Cichon S, Nöthen MM, Amouyel P, Tzourio C, Dartigues JF, Uitterlinden AG, Rivadeneira F, Estrada K, Hofman A, Curtis C, Blauw HM, van der Kool AJ, de Visser M, Goris A, Weber M, Shaw CE, Smith BN, Pansarasa O, Cereda C, Del Bo R, Comi GP, D'Alfonso S, Bertolin C, Sorarù G, Mazzini L, Pensato V, Gellera C, Tiloca C, Ratti A, Calvo A, Moglia C, Brunetti M, Arcuti S, Capozzo R, Zecca C, Lunetta C, Penco S, Riva N, Padovani A, Filosto M, Muller B, Stuit RJ; PARALS Registry; SLALOM Group; SLAP Registry; FALS Sequencing Consortium; SLAGEN Consortium; NNIPPS Study Group, Blair I, Zhang K, McCann EP, Fifita JA, Nicholson GA, Rowe DB, Pamphlett R, Kiernan MC, Grosskreutz J, Witte OW, Ringer T, Prell T, Stubendorff B, Kurth I, Hübmer CA, Leigh PN, Casale F, Chio A, Beghi E, Pupillo E, Tortelli R, Logroscino G, Powell J, Ludolph AC, Weishaupt JH, Robberecht W, Van Damme P, Franke L, Pers TH, Brown RH, Glass JD, Landers JE, Hardiman O, Andersen PM, Corcia P, Vourc'h P, Silani V, Wray NR, Visscher PM, de Bakker PI, van Es MA, Pasterkamp RJ, Lewis CM, Breen G, Al-Chalabi A, van den Berg LH, Veldink JH. Genome-wide association analyses identify new risk variants and the genetic architecture of amyotrophic lateral sclerosis. Nat Genet. 2016;48(9):1043–8.
105. Le NT, Chang L, Kovlyagina I, Georgiou P, Safren N, Braunstein KE, Kvarta MD, Van Dyke AM, LeGates TA, Philips T, Morrison BM, Thompson SM, Puche AC, Gould TD, Rothstein JD, Wong PC, Monteiro MJ. Motor neuron disease, TDP-43 pathology, and memory deficits in mice expressing ALS–FTD-linked UBQLN2 mutations. Proc Natl Acad Sci U S A. 2016;113(47):E7580–E9.
106. Fahed AC, McDonough B, Gouvion CM, Newell KL, Dure LS, Bebin M, Bick AG, Seidman JG, Harter DH, Seidman CE. UBQLN2 mutation causing heterogeneous X-linked dominant neurodegeneration. Ann Neurol. 2014;75(5):793–8.
107. Williams KL, Warraich ST, Yang S, Solski JA, Fernando R, Rouleau GA, Nicholson GA, Blair IP. UBQLN2/ubiquilin 2 mutation and pathology in familial amyotrophic lateral sclerosis. Neurobiol Aging. 2012;33(10):2527.e3–10.

108. Edens BM, Yan J, Miller N, Deng H-X, Siddique T, Ma YC. A novel ALS-associated variant in UBQLN4 regulates motor axon morphogenesis. Elife. 2017;6:e25453.
109. Bakkar N, Kovalik T, Lorenzini I, Spangler S, Lacoste A, Sponaugle K, Ferrante P, Argentinis E, Sattler R, Bowser R. Artificial intelligence in neurodegenerative disease research: use of IBM Watson to identify additional RNA-binding proteins altered in amyotrophic lateral sclerosis. Acta Neuropathol. 2018;135(2):227–47.
110. Münch C, Sedlmeier R, Meyer T, Homberg V, Sperfeld A, Kurt A, Prudlo J, Peraus G, Hanemann C, Stumm G, Ludolph AC. Point mutations of the p150 subunit of dynactin (DCTN1) gene in ALS. Neurology. 2004;63(4): 724–6.

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REVIEW ARTICLE

Is Brain-Derived Neurotrophic Factor: A Common Link Between Neurodegenerative Disorders and Cancer?

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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

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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

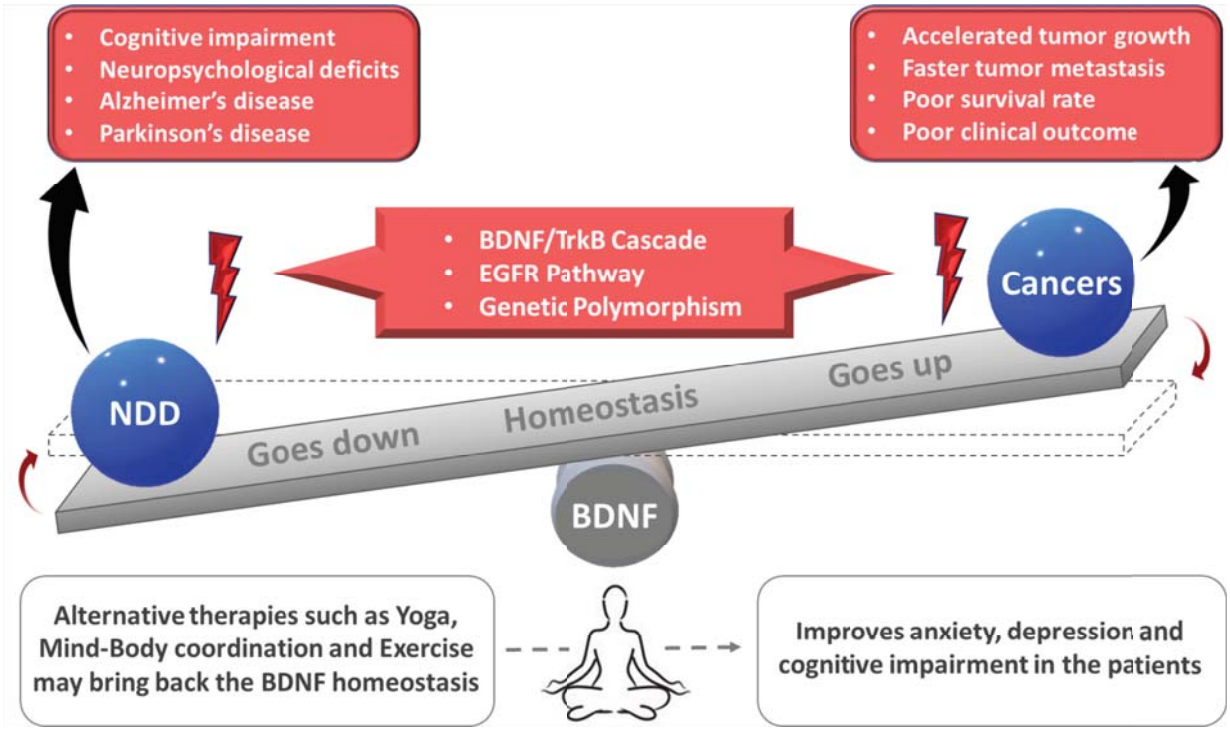

Fig. (1). Schematic representation shows the association between cancer, neurodegenerative disorders and BDNF induced pathology that how an alternative therapeutic approach may manage these diseases/disorders by maintaining the BDNF homeostasis. NDD: neurodegenerative disorders.

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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REFERENCES

[1] Mehler MF, Kessler JA. Growth factor regulation of neuronal development. Developmental Neurosci 16(3-4): 180-95 (1994).
[2] Trujillo CA, Schwindt TT, Martins AH, Alves JM, Mello LE, Ulrich H. Novel perspectives of neural stem cell differentiation: from neurotransmitters to therapeutics. Cytometry Part A: J Intl Soc Analy Cytol 75(1): 38-53 (2009).
[3] Oliveira SL, Pillat MM, Cheffer A, Lameu C, Schwindt TT, Ulrich H. Functions of neurotrophins and growth factors in neurogenesis and brain repair. Cytometry A 83(1): 76-89 (2013).
[4] Nagahara AH, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. NatRev Drug Discov 10(3): 209-19 (2011).
[5] Gupta VK, You Y, Gupta VB, Klistorner A, Graham SL. TrkB receptor signalling: implications in neurodegenerative, psychiatric and proliferative disorders. Intern J Mol Sci 14(5): 10122-42 (2013).
[6] Carlino D, De Vanna M, Tongiorgi E. Is altered BDNF biosynthesis a general feature in patients with cognitive dysfunctions? The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry. 19(4): 345-53 (2013).
[7] Radin DP, Patel P. BDNF: an oncogene or tumor suppressor? Anticancer Res 37(8): 3983-90 (2017).
[8] Glazner GW, Mattson MP. Differential effects of BDNF, ADNF9, and TNFalpha on levels of NMDA receptor subunits, calcium homeostasis, and neuronal vulnerability to excitotoxicity. Exp Neurol 161(2): 442-52 (2000).
[9] Driver JA. Inverse association between cancer and neurodegenerative disease: review of the epidemiologic and biological evidence. Biogerontology 15(6): 547-57 (2014).
[10] Feng YA, Cho K, Lindstrom S, Kraft P, Cormack J, Liang L, *et al*. Investigating the genetic relationship between Alzheimer's disease and cancer using GWAS summary statistics. Human Genet 136(10): 1341-51 (2017).
[11] Cao L, During MJ. What is the brain-cancer connection? Ann Rev Neurosci 35: 331-45 (2012).
[12] Cahn BR, Goodman MS, Peterson CT, Maturi R, Mills PJ. Yoga, meditation and mind-body health: increased bdnf, cortisol awakening response, and altered inflammatory marker expression after a 3-month yoga and meditation retreat. Front Human Neurosci 11: 315 (2017).
[13] Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, *et al*. Exercise induces hippocampal BDNF through a PGC-1alpha/FNDC5 pathway. Cell Metabol 18(5): 649-59 (2013).
[14] Driver JA, Beiser A, Au R, Kreger BE, Splansky GL, Kurth T, e*t al*. Inverse association between cancer and Alzheimer's disease: results from the Framingham Heart Study. BMJ 344: e1442 (2012).
[15] Roe CM, Fitzpatrick A, Xiong C, Sieh W, Kuller L, Miller J, *et al*. Cancer linked to Alzheimer disease but not vascular dementia. Neurology 74(2): 106-12 (2010).
[16] Li JM, Liu C, Hu X, Cai Y, Ma C, Luo XG, *et al*. Inverse correlation between Alzheimer's disease and cancer: implication for a strong impact of regenerative propensity on neurodegeneration? BMC Neurol 14: 211 (2014).
[17] Driver JA, Kurth T, Buring JE, Gaziano JM, Logroscino G. Prospective case-control study of nonfatal cancer preceding the diagnosis of Parkinson's disease. Can Causes Control 18(7): 705-11 (2007).
[18] Xie X, Luo X, Xie M. Association between Parkinson's disease and risk of colorectal cancer. Parkinsonism Related Disord 35: 42-7 (2017).
[19] W Nixon D. The inverse relationship between cancer and Alzheimer's Disease: a possible mechanism. Curr Alzheimer Res14(8): 883-93 (2017).

[20] Yu H, Zhang Z, Shi Y, Bai F, Xie C, Qian Y, *et al*. Association study of the decreased serum BDNF concentrations in amnestic mild cognitive impairment and the Val66Met polymorphism in Chinese Han. J Clin Psychiat 69(7): 1104-11 (2008).

[21] Phillips HS, Hains JM, Armanini M, Laramee GR, Johnson SA, Winslow JW. BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. Neuron 7(5): 695-702 (1991).

[22] Laske C, Stransky E, Leyhe T, Eschweiler GW, Wittorf A, Richartz E, *et al*. Stage-dependent BDNF serum concentrations in Alzheimer's disease. J Neural Transm 113(9): 1217-24 (2006).

[23] Holsinger RM, Schnarr J, Henry P, Castelo VT, Fahnestock M. Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease. Brain Res Mol Brain Res 76(2): 347-54 (2000).

[24] Connor B, Young D, Yan Q, Faull RL, Synek B, Dragunow M. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. Brain Res Mol Brain Res 49(1-2): 71-81 (1997).

[25] Laske C, Stransky E, Leyhe T, Eschweiler GW, Maetzler W, Wittorf A, *et al.* BDNF serum and CSF concentrations in Alzheimer's disease, normal pressure hydrocephalus and healthy controls. J Psychiatr Res 41(5): 387-94 (2007).

[26] Peng S, Wuu J, Mufson EJ, Fahnestock M. Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. J Neurochem 93(6): 1412-21 (2005).

[27] Howells DW, Porritt MJ, Wong JY, Batchelor PE, Kalnins R, Hughes AJ, *et al.* Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. Exp Neurol 166(1): 127-35 (2000).

[28] Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, *et al*. Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neuroscience Lett 270(1): 45-8 (1999).

[29] Parain K, Murer MG, Yan Q, Faucheux B, Agid Y, Hirsch E, *et al*. Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 10(3): 557-61 (1999).

[30] Sohrabji F, Lewis DK. Estrogen-BDNF interactions: implications for neurodegenerative diseases. Front Neuroendocrinol 27(4): 404-14 (2006).

[31] Ciammola A, Sassone J, Cannella M, Calza S, Poletti B, Frati L, *et al.* Low brain-derived neurotrophic factor (BDNF) levels in serum of Huntington's disease patients. Am J Med Genet 144b(4): 574-7 (2007).

[32] Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Müller F-J, Loring JF, *et al.* Neural stem cells improve cognition *via* BDNF in a transgenic model of Alzheimer disease. Proc Nat Acad Sci P106(32): 13594-9 (2009).

[33] Caccamo A, Maldonado MA, Bokov AF, Majumder S, Oddo S. CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. Proc Nat Acad Sci 107(52): 22687-92 (2010).

[34] Banik A, Prabhakar S, Kalra J, Anand A. Effect of human umbilical cord blood derived lineage negative stem cells transplanted in amyloid-β induced cognitive impaired mice. Behav Brain Res 291: 46-59 (2015).

[35] Choi SH, Bylykbashi E, Chatila ZK, Lee SW, Pulli B, Clemenson GD, *et al.* Combined adult neurogenesis and BDNF mimic exercise effects on cognition in an Alzheimer's mouse model. Science 361(6406) (2018).

[36] Rantamäki T, Kemppainen S, Autio H, Staven S, Koivisto H, Kojima M, *et al.* The impact of Bdnf gene deficiency to the memory impairment and brain pathology of APPswe/PS1dE9 mouse model of Alzheimer's disease. PLoS One 8(7): e68722 (2013).

[37] Jiao S, Shen L, Zhu C, Bu X, Liu Y, Liu C, *et al.* Brain-derived neurotrophic factor protects against tau-related neurodegeneration of Alzheimer's disease. Transl Psychiat 6(10): e907 (2016).

[38] Baghestani AR, Daneshvar T, Pourhoseingholi MA, Asadzade H. Survival of colorectal cancer patients in the presence of competing-risk. Asian Pac J Cancer Prev 15(15): 6253-5 (2014).

[39] Yang X, Martin TA, Jiang WG. Biological influence of brain-derived neurotrophic factor (BDNF) on colon cancer cells. Exp Therap Med 6(6): 1475-81 (2013).

[40] Huang SM, Lin C, Lin HY, Chiu CM, Fang CW, Liao KF, *et al.* Brain-derived neurotrophic factor regulates cell motility in human colon cancer. Endocrine-related Can 22(3): 455-64 (2015).

[41] Tichy JR, Lim E, Anders CK. Breast cancer in adolescents and young adults: a review with a focus on biology. J Nat Compren Can Network 11(9): 1060-9 (2013).

[42] Hondermarck H. Neurotrophins and their receptors in breast cancer. Cytokine Growth Factor Rev 23(6): 357-65 (2012).

[43] Patani N, Jiang WG, Mokbel K. Brain-derived neurotrophic factor expression predicts adverse pathological & clinical outcomes in human breast cancer. Cancer Cell Intern 11(1): 23 (2011).

[44] Bronzetti E, Artico M, Forte F, Pagliarella G, Felici L, D'Ambrosio A, *et al.* A possible role of BDNF in prostate cancer detection. Oncology Rep 19(4): 969-74 (2008).

[45] Lai PC, Chiu TH, Huang YT. Overexpression of BDNF and TrkB in human bladder cancer specimens. Oncology Rep 24(5): 1265-70 (2010).

[46] Yang X, Martin TA, Jiang WG. Biological influence of brain-derived neurotrophic factor on breast cancer cells. Intern J Oncol 41(4): 1541-6 (2012).

[47] Tajbakhsh A, Mokhtari-Zaer A, Rezaee M, Afzaljavan F, Rivandi M, Hassanian SM, *et al*. Therapeutic potentials of BDNF/TrkB in breast cancer; current status and perspectives. J Cell Biochem 118(9): 2502-15 (2017).

[48] Tsai YF, Tseng LM, Hsu CY, Yang MH, Chiu JH, Shyr YM. Brain-derived neurotrophic factor (BDNF) -TrKB signaling modulates cancer-endothelial cells interaction and affects the outcomes of triple negative breast cancer. PLoS One 12(6): e0178173 (2017).

[49] Puehringer D, Orel N, Lüningschrör P, Subramanian N, Herrmann T, Chao MV, *et al.* EGF transactivation of Trk receptors regulates the migration of newborn cortical neurons. Nat Neurosci 16(4): 407 (2013).

[50] Chen B, Liang Y, He Z, An Y, Zhao W, Wu J. Autocrine activity of BDNF induced by the STAT3 signaling pathway causes prolonged TrkB activation and promotes human non-small-cell lung cancer proliferation. SciRep 6: 30404 (2016).

[51] Au CW, Siu MK, Liao X, Wong ES, Ngan HY, Tam KF, *et al*. Tyrosine kinase B receptor and BDNF expression in ovarian cancers–Effect on cell migration, angiogenesis and clinical outcome. Cancer Lett 281(2): 151-61 (2009).

[52] Kawamura K, Kawamura N, Mulders SM, Gelpke MDS, Hsueh AJ. Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. Proc Nat Acad Sci USA 102(26): 9206-11 (2005).

[53] Kang HJ, Kim JM, Kim SY, Kim SW, Shin IS, Kim HR, *et al.* A longitudinal study of BDNF promoter methylation and depression in breast cancer. Psychiat Investig 12(4): 523-31 (2015).

[54] Lai PC, Chiu TH, Huang YT. Overexpression of BDNF and TrkB in human bladder cancer specimens. Oncol Rep 24(5): 1265-70 (2010).

[55] Okamura K, Harada T, Wang S, Ijichi K, Furuyama K, Koga T, *et al*. Expression of TrkB and BDNF is associated with poor prognosis in non-small cell lung cancer. Lung Cancer (Amsterdam, Netherlands). 78(1): 100-6 (2012).

[56] Bronzetti E, Artico M, Forte F, Pagliarella G, Felici LM, D'Ambrosio A, *et al*. A possible role of BDNF in prostate cancer detection. Oncology Rep 19(4): 969-74 (2008).

[57] Tanaka K, Okugawa Y, Toiyama Y, Inoue Y, Saigusa S, Kawamura M, *et al*. Brain-derived neurotrophic factor (BDNF)-induced tropomyosin-related kinase B (Trk B) signaling is a potential therapeutic target for peritoneal carcinomatosis arising from colorectal cancer. PLoS One 9(5): e96410 (2014).

[58] Kimura S, Harada T, Ijichi K, Tanaka K, Liu R, Shibahara D, *et al*. Expression of brain-derived neurotrophic factor and its receptor TrkB is associated with poor prognosis and a malignant phenotype in small cell lung cancer. Lung Cancer (Amsterdam, Netherlands) 120: 98-107 (2018).

[59] Au CW, Siu MK, Liao X, Wong ES, Ngan HY, Tam KF, *et al*. Tyrosine kinase B receptor and BDNF expression in ovarian cancers - Effect on cell migration, angiogenesis and clinical outcome. Cancer Lett 281(2): 151-61 (2009).

[60] Cohen L, Warneke C, Fouladi RT, Rodriguez MA, Chaoul-Reich A. Psychological adjustment and sleep quality in a randomized trial of the effects of a Tibetan yoga intervention in patients with lymphoma. Cancer100(10): 2253-60 (2004).

[61] Saligan L, Lukkahatai N, Holder G, Walitt B, Machado-Vieira R. Lower brain-derived neurotrophic factor levels associated with worsening fatigue in prostate cancer patients during repeated stress from radiation therapy. World J Biol Psychiat17(8): 608-14 (2016).

[62] Ng T, Lee YY, Chae J-w, Yeo AHL, Shwe M, Gan YX, *et al*. Evaluation of plasma brain-derived neurotrophic factor levels and self-perceived cognitive impairment post-chemotherapy: a longitudinal study. BMC Cancer 17(1): 867 (2017).

[63] Naveen G, Thirthalli J, Rao M, Varambally S, Christopher R, Gangadhar B. Positive therapeutic and neurotropic effects of yoga in depression: a comparative study. Ind J Psychiat 55(Suppl 3): S400 (2013).

[64] Tolahunase M, Sagar R, Dada R. Impact of yoga and meditation on cellular aging in apparently healthy individuals: a prospective, open-label single-arm exploratory study. OxiMed Cell Longev 2017: 7928981 (2017).

[65] Tolahunase M, Sagar R, Dada R. Erratum to "impact of yoga and meditation on cellular aging in apparently healthy individuals: a prospective, open-label single-arm exploratory study". Oxidative medicine and cellular longevity 2017 (2017).

[66] Konar A, Shah N, Singh R, Saxena N, Kaul SC, Wadhwa R, *et al.* Protective role of Ashwagandha leaf extract and its component withanone on scopolamine-induced changes in the brain and brain-derived cells. PloS One 6(11): e27265 (2011).

[67] Shah N, Kataria H, Kaul SC, Ishii T, Kaur G, Wadhwa R. Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: combinational approach for enhanced differentiation. Cancer Sci 100(9): 1740-7 (2009).

[68] Pandareesh M, Anand T. Neuromodulatory propensity of Bacopa monniera against scopolamine-induced cytotoxicity in PC12 cells *via* down-regulation of AChE and up-regulation of BDNF and muscarnic-1 receptor expression. CellMol Neurobiol33(7): 875-84 (2013).

[69] Hazra S, Kumar S, Saha GK, Mondal AC. Reversion of BDNF, Akt and CREB in hippocampus of chronic unpredictable stress induced rats: effects of phytochemical, Bacopa Monnieri. Psychiat Investig 14(1): 74-80 (2017).

[70] Xu Y, Ku B, Tie L, Yao H, Jiang W, Ma X, *et al*. Curcumin reverses the effects of chronic stress on behavior, the HPA axis, BDNF expression and phosphorylation of CREB. Brain Res 1122(1): 56-64 (2006).

[71] Liu D, Wang Z, Gao Z, Xie K, Zhang Q, Jiang H, *et al*. Effects of curcumin on learning and memory deficits, BDNF, and ERK protein expression in rats exposed to chronic unpredictable stress. BehavBrain Res 271: 116-21 (2014).

[72] Foster PP. Role of physical and mental training in brain network configuration. Frontaging Neurosci 7: 117 (2015).

[73] Zimmer P, Baumann FT, Oberste M, Schmitt J, Joisten N, Hartig P, *et al.* Influence of personalized exercise recommendations during rehabilitation on the sustainability of objectively measured physical activity levels, fatigue, and fatigue-related biomarkers in patients with breast cancer. IntegrCancer Ther 1534735417713301 (2017).

[74] Gupta PK, Prabhakar S, Abburi C, Sharma NK, Anand A. Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients. J Neuroinflammation 8: 114 (2011).

[75] Choy C, Ansari KI, Neman J, Hsu S, Duenas MJ, Li H, *et al*. Cooperation of neurotrophin receptor TrkB and Her2 in breast cancer cells facilitates brain metastases. Breast Cancer Res 19(1): 51 (2017).

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Altered Expression of Heat Shock Protein-27 and Monocyte Chemoattractant Protein-1 after Acute Spinal Cord Injury: A Pilot Study

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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

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] [13] [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).

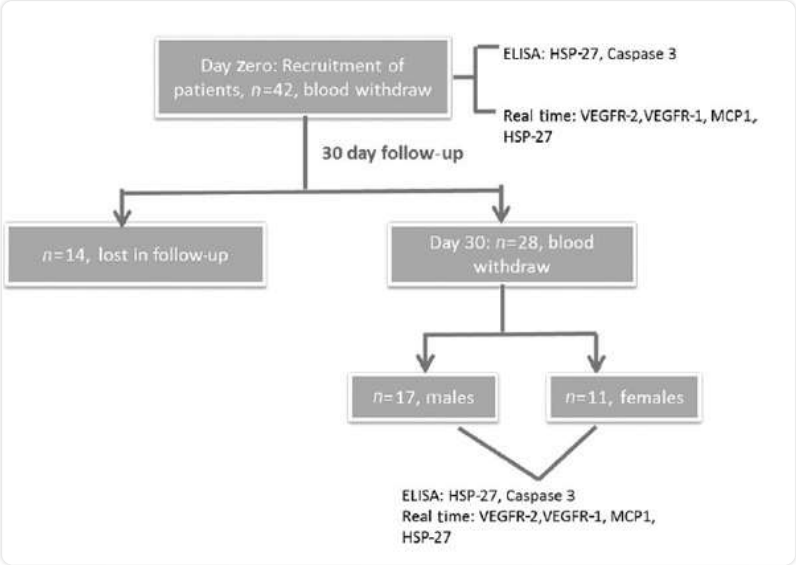

Fig. 1

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.

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.

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 Schmittgen19. 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 .

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.

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).

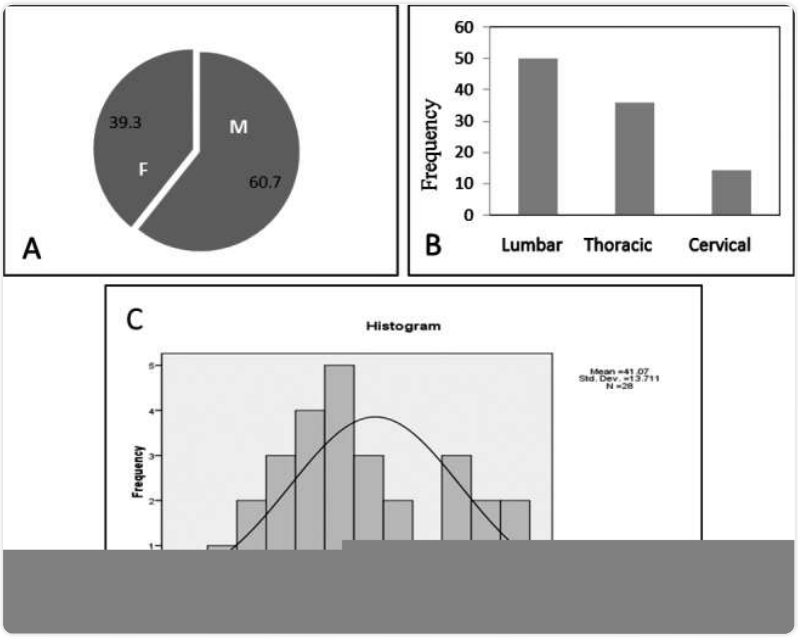

Fig. 2

(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.

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).

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.

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 ex-

pression 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).

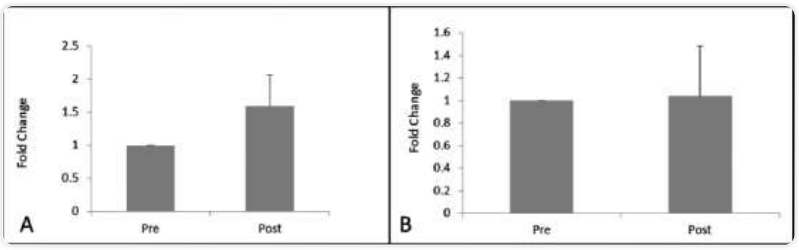

Fig. 4

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.

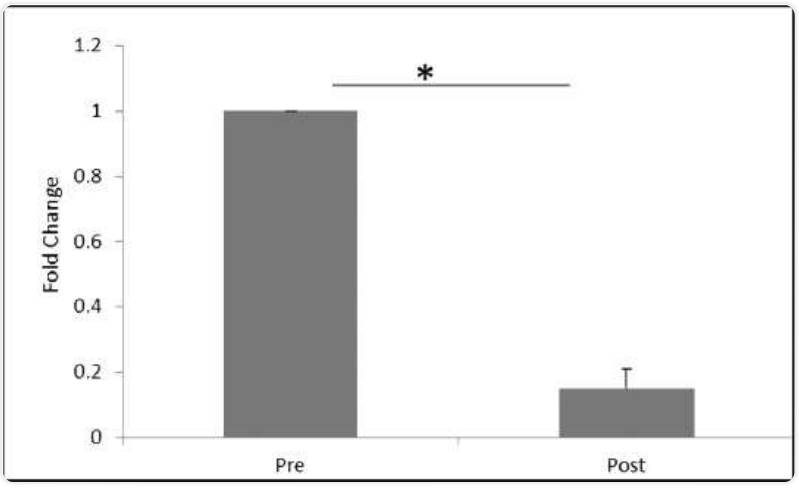

Fig. 5

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. 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

Discussion

SCI is a devastating condition with serious consequences. Proinflammatory and anti-inflammatory mechanisms participating in the secondary phase play a decisive role 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 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.

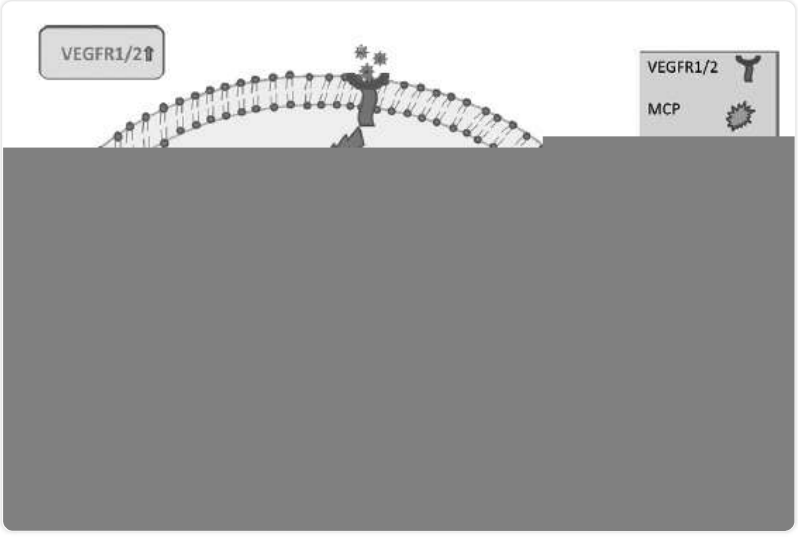

Fig. 7

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.

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 Statement

Funging None.

Footnotes

Conflict of Interest None declared.

References

1. Sekhon L H, Fehlings M G.Epidemiology, demographics, and pathophysiology of acute spinal cord injury *Spine* 20012624(suppl)S2–S12. [PubMed] [Google Scholar]

2. Fehlings M G, Tetreault L A, Wilson J R . Los Angeles, CA: SAGE Publications; 2017. A Clinical Practice Guideline for the Management of Acute Spinal Cord Injury: Introduction, Rationale, and Scope. [PMC free article] [PubMed] [Google Scholar]

3. Devivo M J. Epidemiology of traumatic spinal cord injury: trends and future implications. *Spinal cord.* 2012;50(05):365. [PubMed] [Google Scholar]

4. Anwar M A, Al Shehabi T S, Eid A H. Inflammogenesis of secondary spinal cord injury. *Front Cell Neurosci.* 2016;10:98. [PMC free article] [PubMed] [Google Scholar]

5. Siddiqui A M, Khazaei M, Fehlings M G. Translating mechanisms of neuroprotection, regeneration, and repair to treatment of spinal cord injury. *Prog Brain Res.* 2015;218:15–54. [PubMed] [Google Scholar]

6. Wilson J R, Forgione N, Fehlings M G. Emerging therapies for acute traumatic spinal cord injury. *CMAJ.* 2013;185(06):485–492. [PMC free article] [PubMed] [Google Scholar]

7. Profyris C, Cheema S S, Zang D, Azari M F, Boyle K, Petratos S. Degenerative and regenerative mechanisms governing spinal cord injury. *Neurobiol Dis.* 2004;15(03):415–436. [PubMed] [Google Scholar]

8. Gurtner G C, Werner S, Barrandon Y, Longaker M T.Wound repair and regeneration *Nature* 20084537193314–321. [PubMed] [Google Scholar]

9. Faden A I, Wu J, Stoica B A, Loane D J. Progressive inflammation-mediated neurodegeneration after traumatic brain or spinal cord injury. *Br J Pharmacol.* 2016;173(04):681–691. [PMC free article] [PubMed] [Google Scholar]

10. Kim H M, Hwang D H, Lee J E, Kim S U, Kim B G. Ex vivo VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. *PLoS One.* 2009;04(03):e4987. [PMC free article] [PubMed] [Google Scholar]

11. Welti J, Loges S, Dimmeler S, Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest.* 2013;123(08):3190–3200. [PMC free article] [PubMed] [Google Scholar]

12. Franklin T B, Krueger-Naug A M, Clarke D B, Arrigo A P, Currie R W. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Int J Hyperthermia.* 2005;21(05):379–392. [PubMed] [Google Scholar]

13. Chen Y, Voegeli T S, Liu P P, Noble E G, Currie R W. Heat shock paradox and a new role of heat shock proteins and their receptors as anti-inflammation targets. *Inflamm Allergy Drug Targets.* 2007;06(02):91–100. [PubMed] [Google Scholar]

14. Reddy S J, La Marca F, Park P. The role of heat shock proteins in spinal cord injury. *Neurosurg Focus.* 2008;25(05):E4. [PubMed] [Google Scholar]

15. Carr M W, Roth S J, Luther E, Rose S S, Springer T A. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A.* 1994;91(09):3652–3656. [PMC free article] [PubMed] [Google Scholar]

16. David S, Kroner A. Repertoire of microglial and macrophage responses after spinal cord injury. *Nat Rev Neurosci.* 2011;12(07):388–399. [PubMed] [Google Scholar]

17. Pineau I, Sun L, Bastien D, Lacroix S. Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion. *Brain Behav Immun.* 2010;24(04):540–553. [PubMed] [Google Scholar]

18. Springer J E, Azbill R D, Knapp P E. Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nat Med.* 1999;05(08):943–946. [PubMed] [Google Scholar]

19. Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. *Methods.* 2001;25(04):402–408. [PubMed] [Google Scholar]

20. Devivo M J. Epidemiology of traumatic spinal cord injury: trends and future implications. *Spinal Cord.* 2012;50(05):365–372. [PubMed] [Google Scholar]

21. Dumont R J, Okonkwo D O, Verma S et al. Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clin Neuropharmacol.* 2001;24(05):254–264. [PubMed] [Google Scholar]

22. Hagg T, Oudega M.Degenerative and spontaneous regenerative processes after spinal cord injury *J Neurotrauma* 200623(3/4)264–280. [PubMed] [Google Scholar]

23. Widenfalk J, Lipson A, Jubran M et al. Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience.* 2003;120(04):951–960. [PubMed] [Google Scholar]

24. Voss O H, Batra S, Kolattukudy S J, Gonzalez-Mejia M E, Smith J B, Doseff A I. Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. *J Biol Chem.* 2007;282(34):25088–25099. [PubMed] [Google Scholar]

25. Olsson A K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling—in control of vascular function. *Nat Rev Mol Cell Biol.* 2006;07(05):359–371. [PubMed] [Google Scholar]

26. Qaum T, Xu Q, Joussen A M et al. VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci.* 2001;42:2408–2413. [PubMed] [Google Scholar]

27. Herkowitz H N, Garfin S R, Eismont F J, Bell G R, Balderston R A. Philadelphia Elsevier Health Sciences; 2011. (Rothman-Simeone The Spine E-Book: Expert Consult, Vol. 01.). [Google Scholar]

28. Vaquero J J, Zurita M, de Oya S, Coca S. Vascular endothelial growth/permeability factor in spinal cord injury. *J Neurosurg.* 1999;90 02:220–223. [PubMed] [Google Scholar]

29. Herrera J J, Nesic O, Narayana P A. Reduced vascular endothelial growth factor expression in contusive spinal cord injury. *J Neurotrauma.* 2009;26(07):995–1003. [PMC free article] [PubMed] [Google Scholar]

30. Lee H, Kang J E, Lee J K, Bae J S, Jin H K. Bone-marrow-derived mesenchymal stem cells promote proliferation and neuronal differentiation of Niemann-Pick type C mouse neural stem cells by upregulation and secretion of CCL2. *Hum Gene Ther.* 2013;24(07):655–669. [PMC free article] [PubMed] [Google Scholar]

31. Park S W, Yi J H, Miranpuri G et al. Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther.* 2007;320(03):1002–1012. [PubMed] [Google Scholar]

32. Thuringer D, Jego G, Wettstein G et al. Extracellular HSP27 mediates angiogenesis through toll-like receptor 3. *FASEB J.* 2013;27(10):4169–4183. [PubMed] [Google Scholar]

33. Jin C, Cleveland J C, Ao L et al. Human myocardium releases heat shock protein 27 (HSP27) after global ischemia: the proinflammatory effect of extracellular HSP27 through toll-like receptor (TLR)-2 and TLR4. *Mol Med.* 2014;20:280–289. [PMC free article] [PubMed] [Google Scholar]

34. Dierick I, Irobi J, Janssens S et al. Genetic variant in the HSPB1 promoter region impairs the HSP27 stress response. *Hum Mutat.* 2007;28(08):830. [PubMed] [Google Scholar]

35. Zhang X, Chen C, Ma S, Wang Y, Zhang X, Su X. Inhibition of monocyte chemoattractant peptide-1 decreases secondary spinal cord injury. *Mol Med Rep.* 2015;11(06):4262–4266. [PubMed] [Google Scholar]

36. Hong K H, Ryu J, Han K H. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. *Blood.* 2005;105(04):1405–1407. [PubMed] [Google Scholar]

37. Sköld M, Cullheim S, Hammarberg H, Piehl F, Suneson A, Lake S, Sjögren A et al. Induction of VEGF and VEGF receptors in the spinal cord after mechanical spinal injury and prostaglandin administration European. *J Neurosci.* 2000;12(10):3675–3686. [PubMed] [Google Scholar]

38. Stammers A T, Liu J, Kwon B K. Expression of inflammatory cytokines following acute spinal cord injury in a rodent model. *Journal Neuroscience Res.* 2012;90(04):782–790. [PubMed] [Google Scholar]

Article

Serum levels of TIMP-3, LIPC, IER3 and SLC16A8 in CFH negative AMD cases†

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Key words: Age related macular degeneration, IER-3, SLC16A8, TIMP-3, LIPC, CFH negative, MLPA

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ABSTRACT

AMD is a complex eye disease predominantly occurring in aged population. Till now about 53 genetic loci have been found to be associated with the AMD pathology. AMD pathogenesis is being increasingly known to progress through mechanisms independent of the CFH dependent pathway. Therefore, our aim for current study was to examine the genes by analyzing their expression levels in AMD. We recruited about 50 AMD and same number of age matched controls. We analysed the CFH duplication and deletion by multiplex ligation probe amplification (MLPA) and found no duplication and deletion in CFH gene in AMD patients. We also estimated the IER-3, SLC16A8, LIPC and TIMP-3 expression levels in both CFH negative AMD cases (i.e. no duplication and deletion in CFH gene) besides examining these in AMD and controls. We found that the expression level of *LIPC*, *SLC16A8* and *TIMP*-3 was significantly associated with AMD pathology in both groups (*LIPC*: p=0.008, *SLC16A8*: p<0.001, TIMP-3: p<0.001, respectively). However, we did not find any significant difference in IER-3 levels in AMD and controls. Therefore, the evidence from current study, suggests that AMD pathology may be mediated through mechanistic pathways linked to other genetic loci.

INTRODUCTION

Age related macular degeneration (AMD) is a progressive eye disease of retina normally occurring in aged population. Macula is structural component of human retina which consists of rod and cone cells required for good vision. It has been widely believed that various cellular mechanisms that regulate the angiogenesis, apoptosis, inflammation and oxidative levels in AMD pathology are hampered resulting in degeneration of macula causing AMD. It has been described that both environmental and genetic factors contribute equally in the pathogenesis of AMD (Seddon et al., 2006). However, it has not been investigated how environmental factors impact the transcriptional or translation of above mentioned processes resulting in AMD.

AMD pathology could be categorized into three pathological conditions based on their clinical features of retina namely dry, wet and geographical atrophy (complete degeneration of photoreceptors) (Ferris et al, 2013). Drusen formation in the space between retinal pigment epithelial (RPE) and Bruch's membrane (BM) is common pathological hallmark in AMD. When the chemical composition of drusen is analysed it is found to consist of lipoproteins, oxidized lipids, activated complement factor's components, lipofuscin, A2E, and pigmented components *etc*. Deposition of drusen results in malfunctioning of cellular mechanisms due aging and disruption of molecular-genetic homeostasis mediated by various genetic factors. Wet form of AMD is characterized by new blood vessel formation in between the retinal space. These blood vessels sprout from choroids and percolate the BM. These new vessels can disrupt the RPE integrity and its functions. Moreover, these blood vessels could even release their fluid contents in between the space which can create haemorrhage like conditions. Neovascularization (new blood vessels formation) is well controlled process which is regulated though genes that regulate mechanisms involved in angiogenesis (MMPs and *TIMP*-3) and its associated receptors (Sharma et al., 2015; Sharma et al., 2012). Remodeling of extracellular matrix (ECM) is imperative process in neovascularization. Collagenase, angiogenic and matrix metalloproteases play an important role in this process. Expression of *TIMP*-3 has been found to be co localized with soft drusen and significantly raised in the BM of AMD patients as

compared to controls, however, it is lacking where RPE degenerated (Kamei and Hollyfield, 1999). Similarly, Leu *et al* have also demonstrated the MMP expression throughout the RPE choroids but *TIMP*-3 expression is only confined to accumulating drusen. Conclusively both studies signify the *TIMP*-3 role in AMD pathology by protecting MMPs mediated proteolytic cleavage of the deposited drusen (Leu et al., 2002). Consequently, neovascular formation and their disposes can stimulate the apoptosis of photoreceptors cells and lead to complete degeneration (GA condition). Immediate early response-3 (*IER-3* or *IEX-1*) have recently been found to be strongly associated with AMD pathology (Fritsche et al., 2013). IER-3 is involved in wide range of cellular functions including apoptosis, hypertension, regulation of immune system, tumorigenesis and provide the stability of genome. *IER-3* regulates these cellular and physiological processes through MAP kinase/ERK and PI3K/Akt pathways (Arlt and Schäfer, 2011). Injured retina has also been found to show express IER-3 in astrocytes, microglial, neuronal and lens epithelial cells (Vazquez-Chona et al., 2005). These studies suggest imperative role of *IER-3* in regulation of various cellular processes and may have their impact in progression of AMD pathology by inducing apoptosis and neovascularization. Additionally, our previous study on DcR1 (TNF-related apoptosis inducing ligand Receptor 3) which is an anti-apoptotic protein, has shown lower level of *DcR1* in AMD patients as compared to control population indicating an active role of apoptosis and associated proteins (Anand et al., 2014).

On the other hand, SLC16 gene family is proton coupled transporter protein which transports the monocarboxylic acids (lactate, pyruvic acid, and ketone bodies etc). *SLC16A8* is predominantly expressed in RPE cells which regulates the pH of the RPE by transportation of pyruvic acid in such metabolically active tissues (Halestrap et al., 2004). Expectedly, mouse deficit with *SLC16A8* has been shown two fold decrease of *a*-wave amplitude in ERG as compared to control mice. Moreover, decreased pH in outer retina has also observed along with 4-fold higher concentration of lactate in RPE cells (Daniele et al., 2008).

In the context of the above mentioned role of molecules, it must not be ignored that metabolism of cholesterol and lipoproteins are regulated by hepatic lipase (HL) which is prominently synthesized by hepatic cells and adrenal gland. The conversion of low density

lipoprotein (LDL) from intermediate density lipoprotein (IDL) is mediated by HL and regulates the cholesterol and lipoproteins levels in the plasma. Moreover, HL regulates the hydrolysis of triglyceride and phospholipids in high density lipoprotein (HDL) and it also facilitates the uptake of cholesterol ester from HDL (Thuren, 2000). HL genetic variant rs10468017 has found to be significantly associated with the progression of AMD pathology ($p < 0.001$) (Wang et al., 2015). Similarly, Lee *et al* performed cohort study on 1626 advanced AMD participants and demonstrated two genetic variants s493258 and rs10468017 significantly correlated with the advanced AMD pathology (Lee et al., 2013).

Amongst the various genetic factors, CFH is widely explored and universally accepted genetic component which is found to be associated with AMD pathology (Maller et al., 2006; Klein et al., 2005). Interesting findings by Toomey *et al* (2015) have fed high cholesterol diet to CFH-/+ and CFH-/- mice. Surprisingly, the mice with CFH -/+ were found to have more drusen deposits between BM and RPE. Findings indicate the role of CFH in progression of dry AMD phenotypes mediated though CFH. Similarly, when we explored the CFH Y402H polymorphism in North Indian AMD patients, we found the genetic association between CFH gene and AMD pathology. Moreover, the expression of CFH was also shown to be higher in controls as compare to both types of AMD pathology (Sharma et al., 2013). These studies indicate the complexity of AMD and the role of environment and genotype on AMD, with a dominant role of CFH in AMD.

Among techniques used to probe AMD genotype complexity, Multiplex Ligation-dependent Probe Amplification (MLPA) is a useful multiplex PCR based technique to amplify multiple allelic locations (up to 40 locations) in a single PCR reaction. MLPA is now widely used as a sensitive tool to identify copy number changes in the genes chiefly because of deletions and duplications (White et al., 2007; White et al., 2004). MLPA is also used to detect the methylation patterns, and real time MLPA (RT-MLPA) for mRNA profiling. MLPA utilizes property of probe-template hybridization to amplify the probes by universal primers. Hence, instead of template DNA, hybridized probe is amplified to detect the copy number variations. Probe is made up of two oligonucleotides, one of which is chemically synthesized and other one is derived from M13 vector. Oligonucelotides are ligated to make a range of 200-500 nucleotide fragments based on stuffer sequences attached with the oligonucleotides. We aimed to assess the CFH

duplication/deletion using MLPA in about 30 AMD patients, out of which 24 AMD patients did not possess CFH deletion/duplications. Further, we also estimated the expression of LIPC, IER-3, SLC16A8 and TIMP-3 in the CFH negative cases to examine if other genetic factors, as explained above, are involved in AMD pathology independent of CFH.

MATERIALS AND METHODS

Recruitment of participants

50 AMD and 50 controls participants were recruited from the Advanced Eye Centre, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India after obtaining their written approval on informed consent form. The ethical approval for the recruitment of the participants and to conduct the study was taken from the Institutional Ethical Committee (IEC), PGIMER, Chandigarh, India vides letter number NK/558/Res; dated: 04.02.2014.

Socio-demographic details

Socio-demographic and clinical details were obtained by administering the standard questionnaire amongst the participants at the time of recruitment. The details, include the co-morbidity (hypertension, heart problem, diabetes, migraine *etc*), smoking habits, food habit (vegetarian and non-vegetarian), and history of any prior surgery was taken.

Inclusion and Exclusion criteria

The inclusion and exclusion was based on the age, clinical features of drusen and neovascularization and/or photoreceptor degeneration. Minimum age of the participants was 50 years for both AMD and controls. AMD participants having at least <5 drusen with 60 micron in size were considered. Participants lacking any traces of drusen were included in control group. Patients who has very few drusen with <63 micron in size and drusen size in between to 64 to 124 micron were included in AREDS1 and AREDS2, respectively. The patients who have

intermediate drusen but at least one drusen with >125 micron were considered as AREDS 3. Patients who appeared with wet and GA phenotypes were included in ADRES 4 and AREDS 5 (Hoffman et al, 2016).

Those participants that were having AMD like pathological features due to some other associated disease pathologies like uveitis, diabetic retinopathy, vein occlusion and myopia were excluded from the study.

The clinical observation and measurements were done by ophthalmologist/retinal expert. AMD patients were evaluated on the basis of drusen shape and size, presence of neovascular area and apoptosis in macular region (by decrease in thickness of macula) evident in fundus imaging. The thickness of the macula in patients of wet and geographic atrophy (GA) was measured with Optical Coherence Tomography (OCT) imaging. Clinical scoring of the AMD pathology was based on AREDS criteria (Figure 7). Table 1 has shown the age and gender distribution of these participants.

PBMC isolation

4ml of blood sample from each of above mentioned subjects was taken in EDTA vial and left it for 1-2 hrs at room temperature to allow settle down of the RBCs. Upper separated layer were collected and placed gently on equal volume of histopaque (Sigma-Aldrich) and subjected to centrifugation at 1800rpm for 30 minutes. Middle buffy layer were collected in eppendorf tube and further washed with 1X PBS at 5000rpm for 5 minutes at 4^0C. Washing was repeated twice and dissolved pellet of PBMCs was stored at -80 for further use.

DNA isolation

The extraction of genomic DNA was done from the stored PBMCs by using the commercially available kits (Qiagen, Germany). The integrity and concentration of genomic DNA was determined by absorbance at 260nm though UV spectrophotometer. Genomic DNA was further labeled and stored at -20^0C for further research work.

Serum isolation

3ml of blood samples were taken in gel-clot activator vaccutainer (BD Biosciences, USA). The samples were centrifuged at 2500rpm for 30 minutes at room temperature. The serum were collected from vaccutainer and stored at -80^0 for further use after appropriate labeling.

Total protein estimation

Total protein estimation of serum samples was done by using Bradford's method. The dilution of serum samples ranged from 400 to 600 times. The Bovine serum albumin was used as standard in thsese experiments. Bradford's reagent (Sigma, USA) was used in 1:4 dilution and the absorbance was taken using 495nm filter ELISA reader (Biorad). The values obtained from total protein were used to normalise the ELISA values of LIPC, IER-3, SLC16A8 and TIMP-3.

ELISA

The expression levels of *LIPC (*YHB, China)*, IER-3 (*eBioscience, USA) *SLC16A8* (YHB, China) and *TIMP*-3 (YHB, China) in the serum of the participants were measured through the commercial ELISA kits. The ELISA protocol was followed as per the manufacturer's instructions. The dilution of samples was standardized before performing ELISA. The final absorbance was taken at 450nm on ELISA reader (Biorad, Hercules, USA). Standard curve was plotted based on values obtained from standard of the ELISA experiment. The ELISA values were further normalized to total protein counts.

Multiplex Ligation-dependent Probe Amplification (MLPA) for CFH gene

DNA was obtained from the peripheral blood leukocytes using the standard procedure (Qiagen mini kit). Quality and quantity of DNA was analysed through UV spectrophotometer and Gel electrophoresis. MLPA was performed to analyse the copy number status (duplication or deletion) of targeted exons of CFH gene from P236 probe set as per manufacturer's instructions (MRC Holland, Netherlands). 50 ng/µl of AMD DNA samples along with 3 reference samples were hybridized with probes by carrying out denaturation at 95^{0}C for 1 minute, followed by incubation at 60^{0}C for 16-20 hours. Ligation reaction of hybridized probes was performed at 54^{0}C and 98^{0}C for 5 minutes by incubating the mixture in ligase buffer and ligase enzyme. Amplification conditions were as follow: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s,

followed by 20 min at 72 °C and a pause at 15 °C for 35 cycles. Fragment analysis of PCR amplified products was conducted through capillary electrophoresis. The work flow of the experiment explained in Figure 1.

Assay condition for capillary electrophoresis

1 µl of amplified products were plated with mixture of 8.6 µl Hi-Di formamide and 0.40µl parts GeneScan LIZ 500 size standard in the well plate. After brief centrifugation, the well plate was kept at 95°C for 10mins followed by immediate ice cooling. Ice cooled amplified sample mixture was added in the ABI platform for capillary electrophoresis and raw data (.fsa) was obtained for further analysis.

Data Analysis

Peaks obtained through capillary electrophoresis were analysed by coffalyser. Net software (MRC, Holland). The peak height was normalized by internal control used in the experiment and reference probes present in the probe set. Ratios obtained from the software were used to determine the copy number changes in the CFH exons located in 1q32 region. Ratio between 0.70-1.30 was considered in the normal range while ratio of <0.50 and >1.50 was considered as deletion and duplication respectively (Figure 2).

Statistical Analysis

MLPA peaks were normalized through *coaffalyser. NET* software through intra-sample and inter-sample normalization. In Intra-sample normalisation each sample peak was compared with each reference probe peak to generate preliminary ratios. Final ratios were determined after inter-sample normalization with reference samples. Median of normalized values were used to obtain the Dosage Quotients. The statistical analysis of expression changes of *LIPC, IER-3, SLC16A8* and *TIMP*-3 comparison between two groups (AMD versus control) was carried out with independent t-test and Mann-Whitney test where the values were not normal. The normality of the data was analysed using Kolmogorov-Smirnov's test. p value <0.05 was considered as significant for experiments. The expression level of each protein were neutralized

with the total protein content of the respective sample. IBM SPSS 16 software was used to conduct the statistical analysis.

RESULTS

MLPA analysis

MLPA was performed in 30 AMD patients. MLPA analysis of CFH gene revealed copy number status of 13 target exonic and intronic regions as mentioned in Table 2. Normalized ratios between 0.70-1.30 were obtained from the fluorescent intensities of individual peaks through capillary electrophoresis, followed by analysis through *coffalyser.NET* software. No deletions/duplications were detected in the 13 target regions of the CFH gene and shown normal copy number status in 24 out of 33 AMD patients.

Expression level of *LIPC, IER-3, TIMP-3* and S*LC16A8*

ELISA revealed changes in the expression patterns of several proteins responsible for various mechanisms of AMD pathology. The expression levels of *LIPC, TIMP-3* and S*LC16A8* in serum of AMD patients (found to be negative for CFH) were further compared with the controls. *LIPC*, *SLC16AB* and *TIMP*-3 expression were significantly elevated in AMD (Figure 3, 4 and 5, respectively) as compared to the controls (*LIPC*: p=0.008, *SLC16A8*: p<0.001, TIMP-3: p<0.001) after normalization to total protein, while IER3 expression was found to be not significant in AMD patients in comparison to control population (P=0.437 (CFH negative) and p=0.193) as shown in Figure 6.

DISCUSSION

AMD is heterogenic disease and represents the genetic as well as environmental complexity (Anand et al., 2016). We have earlier reported the role of angiogenesis (Sharma et al., 2015; Sharma et al., 2012) as well as the role of chemokine and its receptor in AMD pathology in Indian population (Anand et al., 2012). TIMP-3, being involved in ECM remodeling and sprouting of new blood vessels in AMD progression, is also described in various genetic (Chen et al., 2010; Warwick et al., 2016) and histological studies (Leu et al., 2002; Kamei and Hollyfield, 1999) on

AMD patients revealing its role in pathology. In this study, we found elevated levels of systemic TIMP-3 expression in serum of North Indian AMD patients. Similarly, since SLC16A8 is known to play an important role in maintaining the pH of metabolically active RPE cells by transporting the monocarboxylic acids, the genetic variant rs8135665 of SLC16A8 was found to be strongly associated with Indian AMD pathophysiology (Fritsche et al., 2013; Fritsche et al., 2016). Knockout study of SLC16A8 in mice has earlier revealed the well defined role of this transporter protein in functional aspect of RPE (Daniele et al., 2008) but its human correlate has not been described earlier. Our report of elevated levels of SLC16A8 in serum of AMD patients signifies perturbation of the transportation of monocarboxylic acid and pH maintenance required for functional RPE activity in AMD pathology. We are unable to explain how this could be related to AMD pathology for which additional studies may be required.

As described earlier, oxidized lipid and cholesterol metabolites have been found to induce the age related changes in AMD resulting in apoptosis of RPE cells (Sharma et al., 2014). Based on the previous genetic studies (Thuren, 2000; Wang et al., 2015; Lee et al., 2013; Chen et al., 2010), we explored the levels of hepatic lipase (HL) in AMD and found high levels of HL in AMD patients in comparison to controls. This signifies that the lipid metabolism and its association with AMD pathological is quite significant.

IER3 is known to have an important role in the inflammation, immune regulation, cell cycle and survival pathways (Arlt et al., 2011). Recently, IER3 has been shown to be associated with tumorigenesis including cervical cancer (Jin et al., 2015). IER3 has also been linked as a susceptible genetic factor in the pathology of AMD (Fritsche et al., 2013). Our analysis of IER3 in these AMD patients did not reveal any significant changes in expression levels of IER-3 in AMD comparison to controls. IERs may, therefore, play a pivotal role in the manifestation of AMD pathology mainly by cell survival mechanisms that could be estimated in larger population (Figure 8).

We propose that AMD pathology is mediated through the *TIMP-3*, LIPC, and *SLC16A8* independent of *CFH* gene. A larger study is being proposed to Department of Biotechnology (DBT), New Delhi, India for funds to carry out the analysis in larger cohort.

CONFLICT OF INTEREST:

All authors of the manuscript have revealed no potential conflict of interest with this study.

ACKNOWLEDGEMENT:

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AUTHOR CONTRIBUTION:

AA: Principal Investigator of the study; **KS**: Sample collection, conducted the experiments of the study and writing of the manuscript; **RT**: conducted the experiments of the study and writing of the manuscript; **RS**: clinical evaluation and scoring of AMD patients; **SSK**: Statistical analysis and power calculation of the study.

REFERENCES

1. Anand A, Sharma K, Sharma SK, Singh R, Sharma NK, Prasad K. 2016. AMD Genetics in India: The Missing Links. Front Aging Neurosci 8:115.
2. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, Gupta PK. 2012. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PLoS One 7(11):e49905.
3. Anand A, Sharma NK, Singh R, Gupta A, Prabhakar S, Jindal N, et al. 2014. Does DcR1 (TNF-related apoptosis-inducing-ligand Receptor 3) have any role in human AMD pathogenesis? Sci Rep 4:4114.
4. Arlt A, Schäfer H. 2011. Role of the immediate early response 3 (IER3) gene in cellular stress response, inflammation and tumorigenesis. Eur J Cell Biol 90(6-7):545-52.
5. Chen W, Stambolian D, Edwards AO, Branham KE, Othman M et al. 2010. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. Proc Natl Acad Sci USA 107(16):7401-6.
6. Daniele LL, Sauer B, Gallagher SM, Pugh EN Jr, Philp NJ. 2008. Altered visual function in monocarboxylate transporter 3 (Slc16a8) knockout mice. Am J Physiol Cell Physiol 295(2):C451-7.
7. Ferris FL 3rd, Wilkinson CP, Bird A, Chakravarthy U, Chew E, Csaky K, Sadda SR; Beckman Initiative for Macular Research Classification Committee 2013. Clinical classification of age-related macular degeneration. Ophthalmology.120(4):844-51.
8. Fritsche LG, Chen W, Schu M, Yaspan BL, Yu Y, et al. 2013. Seven new loci associated with age-related macular degeneration. Nat Genet 45(4):433-9.
9. Fritsche LG, Igl W, Bailey JN, Grassmann F, Sengupta S, Bragg-Gresham JL et al. 2016. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. Nat Genet 48(2):134-43.

10. Halestrap AP, Meredith D. 2004. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 447(5):619-28.

11. Hoffman JD, van Grinsven MJ, Li C, Brantley M Jr, McGrath J, Agarwal A, Scott WK, Schwartz SG, Kovach J, Pericak-Vance M, Sanchez CI, Haines JL 2016. Genetic Association Analysis of Drusen Progression. Invest Ophthalmol Vis Sci. 57(4):2225-31.

12. Jin H, Suh DS, Kim TH, Yeom JH, Lee K, Bae J. 2015. IER3 is a crucial mediator of TAp73β-induced apoptosis in cervical cancer and confers etoposide sensitivity. Sci Rep 5:8367.

13. Kamei M, Hollyfield JG. 1999. TIMP-3 in Bruch's membrane: changes during aging and in age-related macular degeneration. Invest Ophthalmol Vis Sci 40(10):2367-75.

14. Klein RJ, Zeiss C, Chew EY, Tsai JY, et al. 2005. Complement factor H polymorphism in age-related macular degeneration. Science 308(5720):385-9.

15. Lee J, Zeng J, Hughes G, Chen Y, Grob S, Zhao L, et al. 2013. Association of LIPC and advanced age-related macular degeneration. Eye (Lond) 27(2):265-70.

16. Leu ST, Batni S, Radeke MJ, Johnson LV, Anderson DH, Clegg DO. 2002. Drusen are Cold Spots for Proteolysis: Expression of Matrix Metalloproteinases and Their Tissue Inhibitor Proteins in Age-related Macular Degeneration. Exp Eye Res 74(1):141-54.

17. Maller J, George S, Purcell S, Fagerness J, Altshuler D, Daly MJ, Seddon JM. 2006. Common variation in three genes, including a noncoding variant in CFH, strongly influen ces risk ofage-related macular degeneration. Nat Genet 38(9):1055-9.

18. Seddon JM, George S, Rosner B, Klein ML. 2006. CFH gene variant, Y402H, and smoking, body mass index, environmental associations with advanced age related macular degeneration. Hum Hered 61(3): 157-65.

19. Sharma K, Sharma NK, Anand A. 2014. Why AMD is a disease of ageing and not of development: mechanisms and insights. Front Aging Neurosci 6:151.

20. Sharma K, Sharma NK, Singh R, Anand A. 2015. Exploring the role of VEGF in Indian Age related macular degeneration. Ann Neurosci 22(4):232-7.

21. Sharma N. K., Gupta A., Prabhakar S., Singh R., Sharma S., Anand A. 2012. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Curr Neurovasc Res 9(4):256-65.

22. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma SK, Chen W, Anand A. 2013. Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. PLoS One 8(7):e70193.

23. Thuren T. 2000. Hepatic lipase and HDL metabolism. Curr Opin Lipidol 11(3):277-83.

24. Toomey CB, Kelly U, Saban DR, Bowes Rickman C. 2015. Regulation of age-related macular degeneration-like pathology by complement factor H. Proc Natl Acad Sci USA 112(23):E3040-9.

25. Vazquez-Chona FR, Khan AN, Chan CK, Moore AN, Dash PK, Hernandez MR, et al. 2005. Genetic networks controlling retinal injury. Mol Vis 11:958-70.

26. Wang YF, Han Y, Zhang R, Qin L, Wang MX, Ma L. 2015. CETP/LPL/LIPC gene polymorphisms and susceptibility to agerelated macular degenerati on. Sci Rep 5:15711.

27. Warwick A, Gibson J, Sood R, Lotery A. 2016. A rare penetrant TIMP3 mutation confers relatively late onset choroidal neovascularisation which can mimic age-related macular degeneration. Eye (Lond) 30(3):488-91.

28. White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, et al. 2004. Two-color multiplex ligation-dependent probe amplification: detecting genomic rearrangements in hereditary multiple exostoses. Hum Mutat 24:86–92.

29. White SJ, Vissers LE, Geurts van Kessel A, de Menezes RX, Kalay E, Lehesjoki AE, et al. 2007. Variation of CNV distribution in five different ethnic populations. Cytogenet Genome Res.; 118:19–30.

Figure legends:

Figure 1: The work flow of the study to conduct the expression levels of different genes in serum of the participants in CFH negative cases (done by MLPA) and, comparison between AMD and controls alone.

Figure 2: Electropherogram of MLPA fragments of a patient with AMD (A) and reference sample (B) were analyzed through coaffalyser.net software to generate ratios after normalizing the sample peak with the relative peak intensities of reference sample and reference probes. Range of 0.70-1.30 was chosen as normal copy number status. Y axis: Relative Fluorescent Units (RFU), X axis: Length of fragments (in nucleotide).

Figure 3: Human hepatic lipase (LIPC) expression in serum of AMD and controls. Figure 3A comparison between CFH negative AMD cases (p=0.008) and 3B comparative analysis of LIPC between AMD and controls alone (p=0.002). pg: pictogram; µg: microgram; AMD: age related macular degeneration. Bar is representing the standard error.

Figure 4: Comparative analysis of expression levels of SLC16A8 in AMD and controls. Figure 4A is showing comparison between CFH negative AMD cases (p<0.001) and 5B showing the comparative analysis in AMD and controls alone (p<0.001). SLC16A8: Solute Carrier Family 16 Member 8; ng: nanogram; µg: microgram; AMD: age related macular degeneration. Bar is representing the standard error.

Figure 5: Serum levels comparison of TIMP-3 between AMD and controls. Figure 5A representing the expression level changes in CFH negative cases (p<0.001) and figure 5B is normal comparison between AMD and controls (p<0.001). TIMP-3: tissue inhibitor of metalloprotease-3; pg: pictogram; µg: microgram; AMD: age related macular degeneration. Bar is representing the standard error.

Figure 6: IER-3 expression levels in serum of the AMD and control. (A) CFH negative cases (p=0.437) (B) comparison between AMD and controls alone (p=0.193). In both cases, the IER-3 levels were not significantly changed. IER-3: Immediate early response gene-3; pg: pictogram; µg: microgram; AMD: age related macular degeneration. Bar is representing the standard error.

Figure 7: Diagram showing the representative FFA and OCT image of different types of AMD phenotypes.

Figure 8: Plausible depiction of CFH independent AMD pathology due to genetic interaction of LIPC, SLC16A8 and TIMP-3 genes in Indian AMD patients.

Table 1: Age, gender and clinical scoring of the participants of the study

Table 2: Tabular representation of CFH target regions in MPLA analysis.

Table 1: Age, gender and clinical scoring of the participants of the study

Group	Average age	Gender		AREDS score
		Male	Female	
AMD	68.48 ± 8.09	29	21	AREDS 3: 06 AREDS 4: 25 AREDS 5: 19
Control	57.66 ± 9.73	32	18	

Table 2: Tabular representation of CFH target regions in MPLA analysis

No. of Participants	CFH Target regions	Targeted CFH Exons status
33	Exon 1-4, 6, 9, 13, 18, 23. Intron 10, 12, 15	Normal copy number status (0.70-1.30)

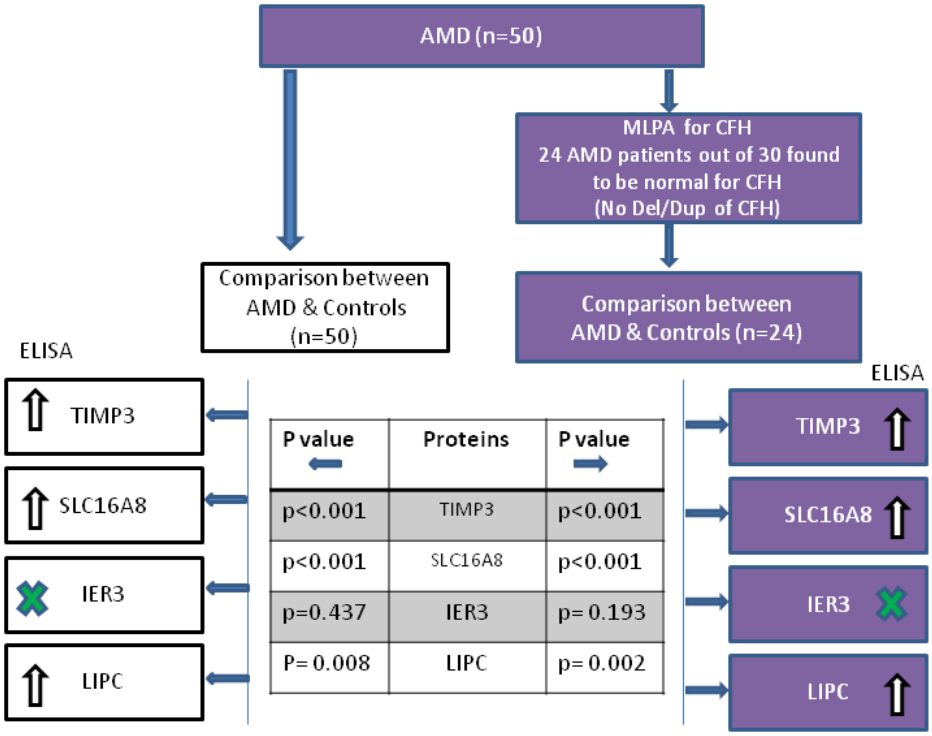

Figure 1

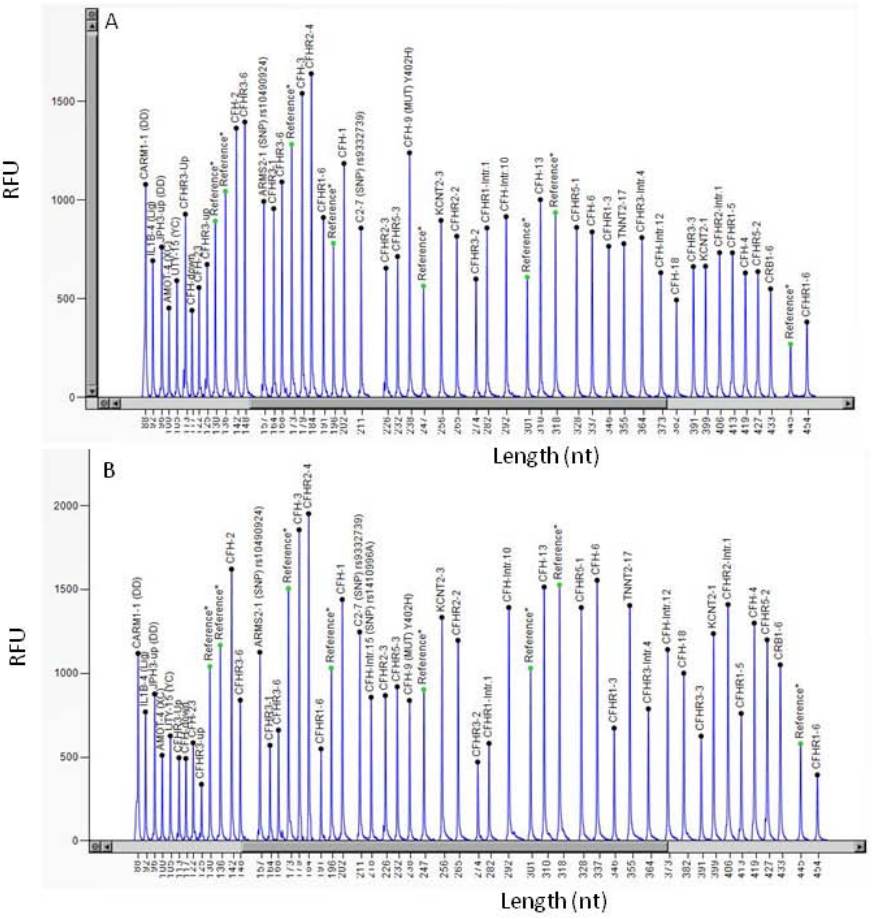

Figure 2

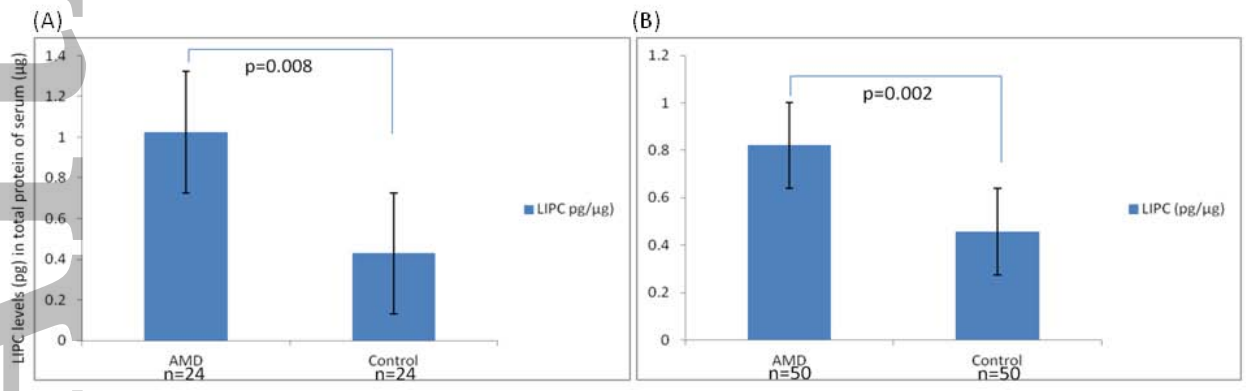

Figure 3

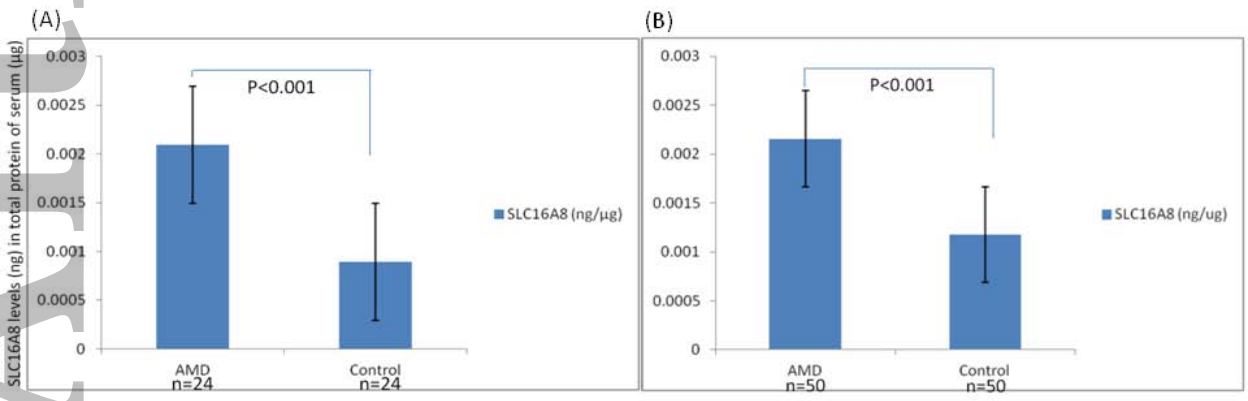

Figure 4

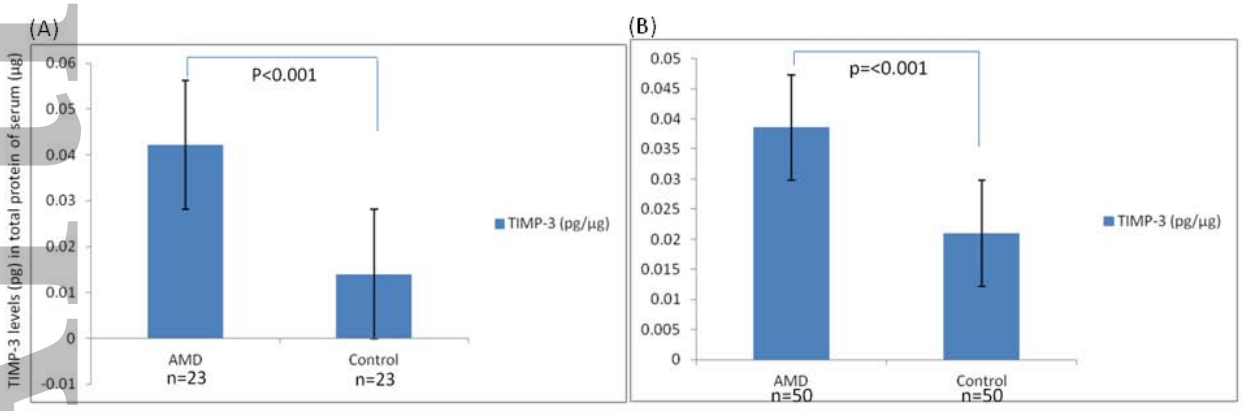

Figure 5

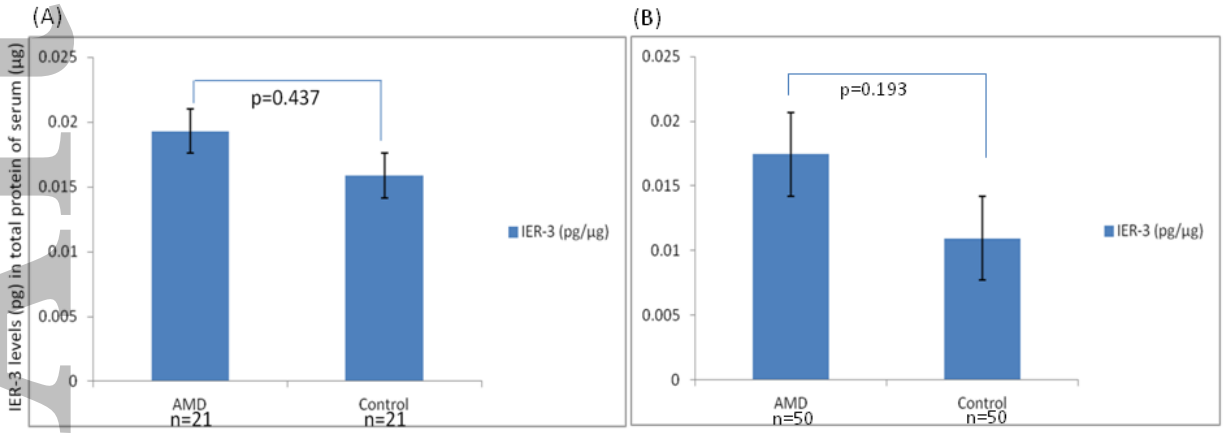

Figure 6

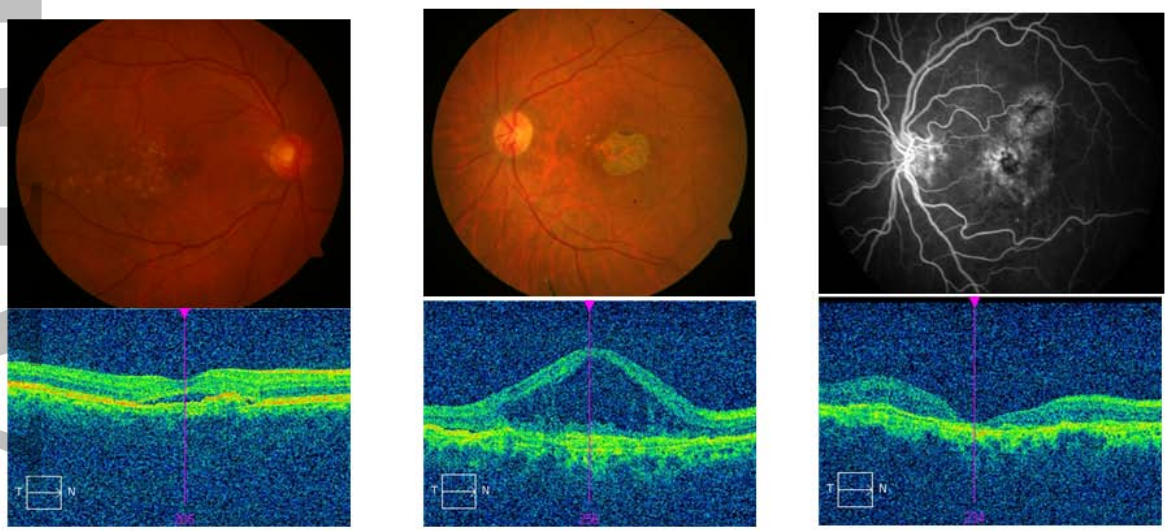

Figure 7

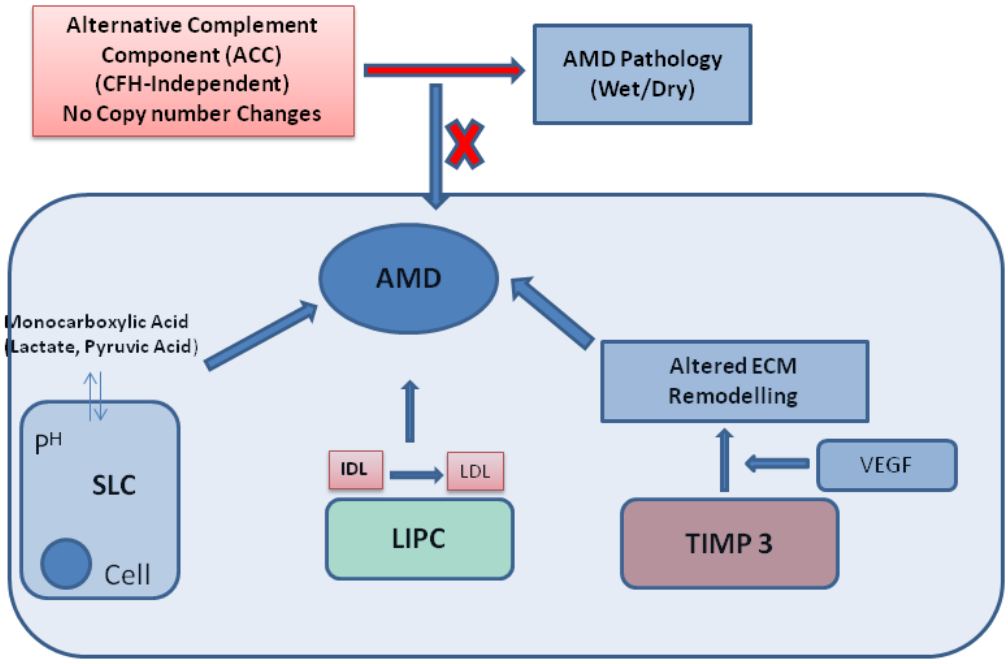

Figure 8

Exploring the role of VEGF in Indian Age related macular degeneration

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*Both contributed equally to the manuscript.

KEY WORDS

VEGF
Age related
macular degeneration
Anti-VEGF
Heart ailment

ABSTRACT

Background: Age related macular degeneration (AMD) is major devastating neurodegenerative disorder characterized by progressive irreversible vision loss in the elderly persons. In spite of several genetic and environmental factors, the role of VEGF and CFH predispose the pathological phenomenon in the AMD patients.
Purpose: The aim of the study was to estimate the VEGF levels in the serum of AMD patients and its correlation with co-morbidity of the participants.
Methods: The study recruited the 98 AMD patients and 59 controls with proper consent of the participants as per the exclusion-inclusion criteria. The co-morbidity and socio-economic details were obtained by introducing the standard questionnaire amongst the participants. Serum levels of vascular endothelial growth factor (VEGF) was estimated by ELISA and compared with the control population of the study. The levels of VEGF in the serum of AMD patients and controls were compared with Mann-Whitney *U*-test. Kruskal Wallis one-way analysis of variance (ANOVA) was employed to analyze more than two variables in the study.
Results: Elevated level of VEGF was found in AMD patients as compared to controls. Surprisingly, we did not find significant changes among wet AMD subtypes i.e. minimal, predominant and classic wet AMD. However, we have demonstrated the intravitreal anti-VEGF treatment (avastin) in AMD patients could reduce the systemic VEGF levels although it was not significant. Moreover, the heart ailment in the AMD patients could also influence the VEGF levels.
Conclusion: Our study is consistent with previous studies describing the imperative significance of VEGF in AMD pathology. However, our study did not reveal the role of VEGF in wet AMD progression but it is well established causative agent for the same. The increased levels of VEGF in heart ailment among AMD patients are significant.

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Introduction

Neovascularization (new blood vessel formation) is a characteristic feature of several disease pathologies including cancer and age related macular degeneration (AMD). These newly formed blood vessels have tendency to spread the disease condition in the tissue due to their leaky nature. Neovascularization underlying choroid can percolate the Bruch's membrane and disrupt the integrity of retinal pigment epithelium (RPE) layer. Neovascularization process is guided by several angiogenic factors including Platelet-derived growth factor (PDGF)[1,2], matrix metalloproteinases (MMPs),[3,4] Pigment epithelium-derived factor (PEDF)[5], TGF-β and its receptors.[6,7] Among all, vascular endothelial growth factor (VEGF) is more potent to lead angiogenic process. Although, it has been found that TGF-β could induce the VEGF expression in RPE cells, with the involvement of MAP kinases, signifies the role of TGF beta as the pro-angiogenic protein in enabling angiogenic process.[8] Apart from these angiogenic factors, several pro-angiogenic factors (TIMPs family and FGF)[3,4] are also involved in the process. Hence, neovascularization is a complex process having role of both angiogenic and pro-angiogenic factors. Uncontrolled phenomenon of angiogenesis leads to the pathological condition like in cancer and AMD.

AMD is the leading cause of irreversible blindness in the elderly people and is characterized by age related changes in the retina like formation of drusen and new vessels in between the retinal pigment epithelial (RPE) layer and Bruch's membrane.[9] The components of drusen can also promote the angiogenic factors to perform their action and consequently result in wet AMD.[10] It has already been investigated that AMD is complex disorder and, both environmental and genetic factors equally distribute in the disease pathology. The various studies on AMD suggested the role of VEGF in disease pathology.[5,11–12] VEGF ligands regulate its functions through binding with tyrosine kinase receptors VEGFR-1 and VEGFR-2.[13] Receptor VEGFR-2, plays important role in angiogenesis of endothelial cells amongst other receptors.[14,15]

The significant associations have been shown in several genetic studies by SNP analysis of VEGF promoter and 5' UTR sequences with the AMD pathology. It has been demonstrated that the 'CC' genotype could influence the VEGF expression in serum of diabetic retinopathy patients in Japanese population.[13] It has also been found that the AMD pathology was associated with the +405C allele in AMD cohort.[13] Moreover, there are several studies which have demonstrated the conflicting results with CG genotype of VEGF gene and its association with diabetic retinopathy but the levels of VEGF was found to be higher in individuals carrying +405GG genotype in various populations.[16,17] Haines et al have shown the correlation of VEGF, low density lipoprotein receptor- related protein 6 (LRP 6), and very low density lipoprotein receptor (VLDLR) with AMD pathology

in Caucasian population suggesting the role of metabolizing genes and angiogenic genes in AMD pathophysiology.[13] Meanwhile Churchill et al have also screened the 14 SNPs of VEGF promoter. The genotype +674 CC was found to be significantly associated with disease pathology. Moreover, the sequences 'CTCCT' and 'TCACC' of SNPs +674, +4618, +5092, +9162 and +9512 of VEGF gene by haplotype analysis have shown significant correlation with AMD in 45 patients as compared to age matched controls.[18]

Recently, increased mRNA expression of VEGF-A121 isoform was found in excised choroidal neovascular membrane tissue from AMD patients as compared to control. Moreover, the study has also suggested the other isoform of VEGF i.e. VEGF-A165 in AMD cases.[19] The expression of VEGF-A could progressively affect the function of RPE cells in the mice. Mice with increased VEGF-A were found with abnormal morphology of RPE cells, reduced levels of retinal rhodopsins and impaired transport of retinoic acid between RPE and photoreceptors. The alfa and beta waves analyzed were also found to be distorted as compared to control mice.[20] Similarly, Marneros et al have also demonstrated the altered morphology of RPE cell layer and distorted visual function in VEGF rpe-/- mice and demonstrated that VEGF function is independent on hypoxia inducing factor-1α (HIF-1 α).[21] Several genetic polymorphism studies have also shown the association with AMD pathology. Several anti-VEGF treatments are available currently which includes aflibercept, bevacizumab, pegaptanib, ranibizumab etc. targeting different sites of VEGF protein to inhibit its functions.

Previously, we had shown the increased expression of VEGF receptor2 (VEGFR2) alongwith single nucleotide polymorphism (SNP) which were also found to be associated with AMD pathology.[22] In this study we have estimated the levels of VEGF in AMD patients.

Methodology

Participants

We have recruited 98 AMD and 59 control participants for this study from outdoor patient care facility of Advanced Eye Centre, PGIMER, Chandigarh, INDIA. The participants were recruited after getting their approval and sign of consent form. The ethical approval of the study has been taken from Institute Ethical Committee (IEC) vide letter No PGI/IEC/2015/881; dated 29.01.2015.

AMD diagnosis

The AMD participants were included after their proper AMD diagnosis, which included fluorescein fundus angiography (FFA) and optical coherence tomography (OCT).[23–26] The retinal specialist examined all the ophthalmic parameters like visual acquity, dilated fundus examination and slit lamp biomicroscopy of anterior chamber of eye.

Demographic information

Demographic parameters of subjects were collected by administrating the standard questionnaire. The questionnaire includes demographic as well as general life style details of the participants.[27–28] The participants were characterized on the basis of their smoking habit and associated co morbidity like cardiovascular problems, hypertension *etc* and summarized in table 1.

Table 1: Demographic characteristics of Controls and AMD patients

Variables	AMD	Controls
Total	98	59
Male	61	38
Female	37	21
Duration of disease¥	25.6 M	
Dry	29	
Wet	69	
Avastin treated	40	
Not treated	29	
Minimal Classic	7	
Predom Classic	14	
Occult	29	
One Eye Affected	29	
Both Eyes Affected	69	
Alcoholic	31	15
Non Alcoholic	67	38
Smokers	41	10
Non Smokers	57	43
Vegetarian	51	29
Non Vegetarian	47	24
Age	65.31 ± 6	60 ± 13

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.

Inclusion and exclusion criteria

The criteria were based on the number and size of drusen, and age of the participants. The subjects with age of 50 years or more were included in the study. The AMD participants who were having choroidal vascularization and/or geographic atrophy were included in the study after FFA examination. In case of dry AMD, the participants who had >5 drusen in at least one eye were recruited in AMD group. The control subject included those with age of 50 or more and with <5 drusen and lacking other diagnostic parameters of AMD pathology.

The degenerative changes in their photoreceptors and retinal layers due to other ocular pathological condition like myopia, uveitis, retinal dystrophies, vein occlusion, diabetic retinopathy were excluded from the study. Moreover, those below 50 years of age were also excluded from study.

Serum separation

The serum samples were obtained from the 4 ml blood collected in serum separator tubes (BD bioscience, USA) and allowed the tube to coagulate for 30 minutes. Further, the tubes were centrifuged at 1800rpm for 30 minutes at room temperature. The separated serum samples were collected. The samples were labeled and stored at –80^{0}C to perform ELISA.

Total protein estimation

Total protein estimation was done with the standard procedure of Bradford's method as per the manufacturer's instructions. The serum samples were diluted with distilled water upto 1500 times. The standard curve was made using Bovine serum albumin (BSA). The coomassie brilliant blue G-250 dye (Bradford reagent) was used in 1:4 ratio. The absorbance was taken at 595 nm using the ELISA reader (680XR model of Microplate reader, Biorad, Hercules, CA, USA). Quadratic fit or linear method was used to obtain standard curve. Total protein was used to normalize the VEGF levels.

VEGF ELISA

VEGF level in serum was analyzed by ELISA as per the manufacturer's instructions (R&D, USA & Ray Bio, USA). The procedure was performed in duplicates. OD was taken at 450 nm in ELISA reader (Bio Rad, USA).

Statistics

Normality of the data was tested with the help of Q-Q plot and data was found not normally distributed. Two groups were compared by Mann-Whitney *U*-test and Kruskal Wallis one-way analysis of variance (ANOVA) followed by post-hoc was applied for more than two comparisons. Goodness of standard curve fit for ELISA and total protein were measure by R^2 (Coefficient of determination). All statistical analysis were performed with SPSS 20.0 software.

Results

All the demographic characteristics of studied population are in Table 1. AMD patients were both segregated as dry and wet AMD. The wet AMD patients were further subdivided into minimal classic, predominant classic and occult. Several other demographic factors were taken into consideration which included food habits (vegetarian vs non-vegetarian), smoking, co-morbidity (history of heart disease) *etc*. (Table 2). Serum VEGF expression ($p = 0.034$; z value = 2.11) was found to be higher in AMD cases as compared to controls (Figure 1). There was no significant difference in VEGF expression among dry and wet AMD patients ($p = 0.187$). Again the difference was not significant between minimal, predominant and classic wet AMD patients ($p = 0.079$). Moreover the history of heart disease was found to be associated with VEGF levels in AMD patients. VEGF level was found to be elevated in AMD patients as compared to controls ($p = 0.031$; Figure 2). The other factors like smoking history ($p = 0.974$), alcohol ($p = 0.912$), Food habits ($p = 0.076$) and use of anti-inflammatory drugs ($p = 0.912$) did not show any association with VEGF level in AMD patients. Not surprisingly, the anti-VEGF treatment (Avastine treatment) was found to have reduced VEGF levels at the systemic levels but it was not significant ($p = 0.058$).

Table 2: Serum VEGF levels comparison in AMD and controls

Subjects VEGF	No.	Mean Rank	Z Value	P value
AMD	98	84.96		
Control	59	69.10	2.11	0.034
Dry	29	43.66		
Wet	69	51.96	1.36	0.187
Male	61	49.95		
Female	37	48.76	0.202	0.840
Avastin treated	40	38.90		
Not treated	29	29.62	1.89	0.058
Minimal Classic	7	7.57		
Predom Classic	14	12.71	1.791	0.079
Occult	29	19.86	1.579	0.121
One Eye Affected	29	53.59		
Both Eyes Affected	69	47.78	0.922	0.356
Alcoholic	31	49.03		
Non Alcoholic	67	49.72	0.111	0.912
Smokers	41	49.42		
Non Smokers	57	49.61	0.032	0.974
Vegetarian	51	54.39		
Non Vegetarian	47	44.19	1.774	0.076
Anti inflammatory use	63	47.78		
No Anti inflammatory	32	48.44	0.110	0.912
Heart Disease	11	40.09		
No heart Disease	48	27.69	2.16	0.031

Discussion

The expression of VEGF in serum has been found to be elevated in AMD patients as compared to controls but no significant difference was observed between wet and dry AMD patients which suggests that VEGF is involved in both pathologies i.e. wet and dry form of AMD or possibly in progression of dry form of these patients into wet AMD. Functional studies of VEGF gene have revealed that the AMD progression could be successfully altered through targeting of this gene in mouse model of wet AMD.[28,29] These studies guide AMD research such that VEGF mediated pathology of wet AMD could be understood with additional evidence from various populations across the world. Consequently, most of AMD treatment strategies are only confined to targeting of VEGF molecules in order to suppress the disease phenotypes. The induction of human RPE and choroidal cells by chemokine cocktail (IFN-γ+ TNF-α+IL-1β) has been known to increase the production of VEGF-A and VEGF-C by over 10 times with the activation of proteins involves in JAK-STAT and NF-β pathways. Such studies suggest that the chemokines have important role in the regulation of angiogenic pathways and their molecules to influence pathological changes in the RPE cells and choroidal tissue.[9,30] We have

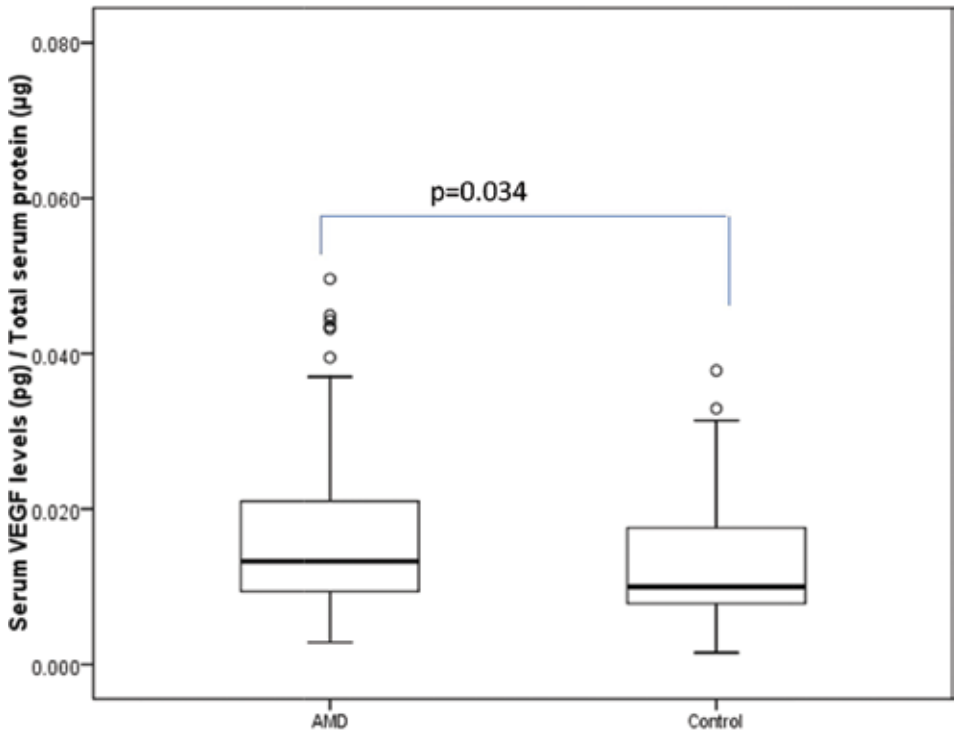

Fig. 1: Expression levels of VEGF in serum of AMD patients with comparison of controls. Representative boxes include the values from first quartile (25%) to third quartile (75%). The outliers in the experiments are shown with circles. The values of VEGF levels normalized with protein of the serum. AMD: age related macular degeneration; pg: picogram; µg: microgram; VEGF: vascular endothelial growth factor.

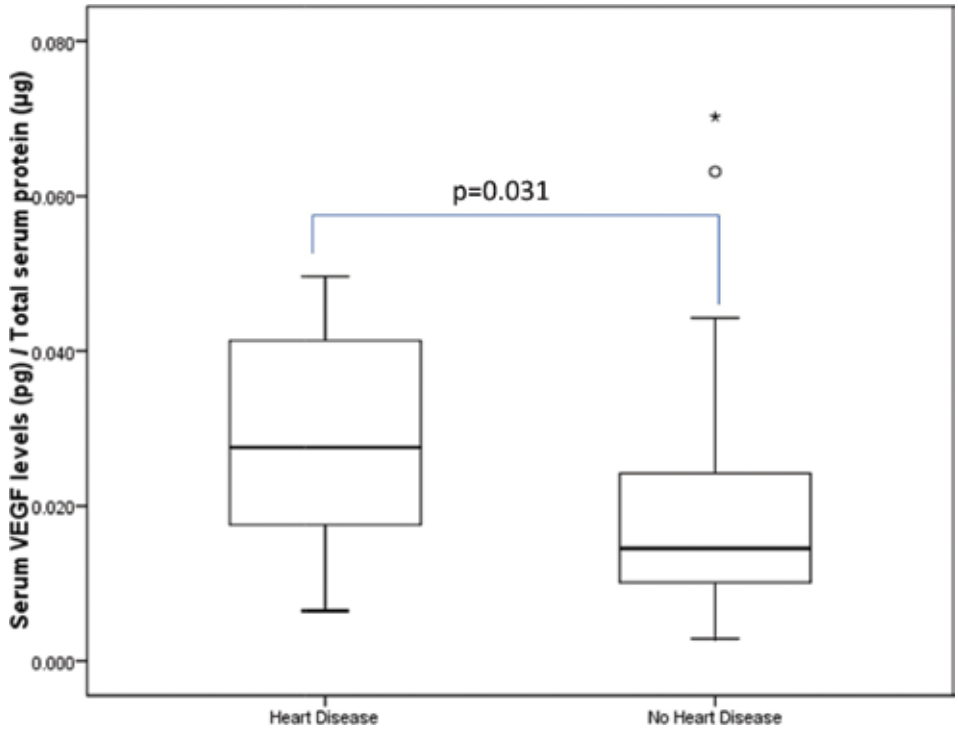

Fig. 2: VEGF Serum levels in heart ailment of AMD patients. The plotted values in the boxes includes from first quartile (25%) to third quartile (75%). The outliers and extreme values are designated with circles and asterisk respectively. The expression levels of VEGF (pg) standardized with total protein (µg). AMD: age related macular degeneration; pg: picogram; µg: microgram; VEGF: vascular endothelial growth factor.

investigated the monocyte chemoattractant protein-1 (MCP-1 or CCL2) and its receptor (CCR2) and reported its association with Indian AMD. We demonstrated the CCR2 (rs1799865) and CCL2 (rs4586) polymorphism was significantly associated with AMD pathogenesis. Moreover, the expression of both genes was also found to altered in AMD patients as compared to controls.[31] Additionally, we have also examined the other chemokines like CCR-3 (G-protein coupled receptor) and eotaxin-2 in Indian AMD patients and reported these to be associated with disease phenotype.[32,33] Both chemokines are involved in recruitment of eosinophils and other inflammatory cells which reveals that their actions are exerted through inflammatory cells recruitment and activation which might further result in increased VEGF expression.

AMD and cardiovascular diseases (like coronary heart disease) share common pathological features including deposition of lipoproteins and impair angiogenesis.[34] Smoking, age, hypertension, high blood cholesterol levels *etc* are known widespread causative agents for both diseases.[35] In this study we have shown that VEGF level was less in AMD patients without heart disease as compared to AMD patients with history of heart disease. Similarly, the homeostasis between hemorheologic factors (e.g. von Willebrand factor, fibrinogen, blood viscosity *etc*), VEGF levels and endothelial dysfunction were shown to be impaired in both AMD and hypertensive patients sharing common pathological changes including angiogenic (VEGF) and plasma factors.[36,37]

Some studies with Avastin treatment in AMD patients have reported decreased VEGF levels in vitreous as well as in systemic circulation.[32,33] In this study, AMD patients with anti-VEGF (Avastin) treated group did not show any significant reduction of systemic VEGF levels but it was found that there was a trend of its decrease in treated group. Our data did not show that VEGF was overexpressed in wet AMD as compared to dry AMD even though the role of VEGF is well defined in wet AMD progression.[32,33] Together these studies indicate a need to undertake Mendedial Randomization analysis of SNP-Biomarker involvement so that this complex disease can be successfully predicted and personalized medicine can be instituted appropriately. Levels of VEGF in this study is supporting and consistent with previous discoveries in the field that showing the increased VEGF levels in the serum, human choroids and RPE cells.[5,37,40]

Authorship contribution

Akshay Anand: Study PI and executed study plan, Manuscript editing; **Ramandeep Singh:** Provided samples for study, **Kaushal Sharma:** Manuscript writing, **Neel K Sharma:** Conducted experiment, Statistics analysis, Manuscript editing.

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References

1. Liegl R, Koenig S, Siedlecki J, et al. Temsirolimus inhibits proliferation and migration in retinal pigment epithelial and endothelial cells via mTOR inhibition and decreases VEGF and PDGF expression. PLoS One. 2014; 9(2): e88203.
2. Zehetner C, Kirchmair R, Neururer SB, et al. Systemic upregulation of PDGF-B in patients with neovascular AMD. Invest Ophthalmol Vis Sci. 2014; 55(1): 337–44.
3. Zeng J, Jiang D, Liu X et al. Matrix metalloproteinases expression in choroidal neovascular membranes. Yan Ke Xue Bao. 2004; 20(3): 191–3.
4. Anand A, Sharma K, Chen W, Sharma NK. Using current data to define new approach in age related macular degeneration: need to accelerate translational research. Curr Genomics. 2014 Aug; 15(4): 266–77.
5. Bhutto IA, McLeod DS, Hasegawa T, et al. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. Experimental Eye Research. 2006; 82(1): 99–110.
6. Vidro-Kotchan E, Yendluri BB, Le-Thai T, et al. NBHA reduces acrolein-induced changes in ARPE-19 cells: possible involvement of TGFβ. Curr Eye Res. 2011; 36(4): 370–8.
7. Zhang L, Lim SL, Du H, et al. High temperature requirement factor A1 (HTRA1) gene regulates angiogenesis through transforming growth factor-β family member growth differentiation factor 6. J Biol Chem. 2012; 287(2): 1520–6.
8. Nagineni CN1, Samuel W, Nagineni S, et al. Transforming growth factor-beta induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: involvement of mitogen-activated protein kinases. J Cell Physiol. 2003; 197(3): 453–62.
9. Sharma K, Anand A, Sharma NK. Why AMD is a disease of ageing and not of development: mechanisms and insights. Front Aging Neurosci. 2014; 6: 151.
10. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A. 2006; 103(7): 2328–33.
11. Jonas JB, Neumaier M. Vascular endothelial growth factor and basic fibroblast growth factor in exudative age-related macular degeneration and diffuse diabetic macular edema. Ophthalmic Res. 2007; 39(3): 139–42.
12. Lee SJ, Kim JH, Kim JH, et al. Human apolipoprotein E2 transgenic mice show lipid accumulation in retinal pigment epithelium and altered expression of VEGF and bFGF in the eyes. J Microbiol Biotechnol. 2007; 17(6): 1024–30.
13. Haines JL, Schnetz-Boutaud N, Schmidt S, et al. Functional candidate genes in age-related macular degeneration: signif- icant association with VEGF, VLDLR, and LRP6. Invest Ophthalmol Vis Sci. 2006; 47: 329–335.
14. Sharma NK, Sudesh Prabhakar S, Anand A. Age related macular degeneration – advances and trends. Annals of Neurosciences.2009; 2: 62–71.
15. Grisanti S, Zhu Q, Tatar O, et al. Differential expression of vascular endothelial growth factor-A isoforms in neovascular age related macular degeneration. Retina. 2014. [Epub ahead of print].
16. Suganthalakshmi B, Anand R, Kim R, et al. Association of VEGF and eNOS gene polymorphisms in type 2 diabetic retinopathy. Mol. Vis. 2006; 12: 336–341.
17. Watson CJ, Webb NJA, Bottomley MJ et al. Identification of polymorphisms within the vascular endothelial growth factor [VEGF] gene: correlation with variation in VEGF protein production. Cytokine. 2000; 12: 1232–1235.
18. Churchill AJ, Carter JG, Lovell HC, et al. VEGF polymorphisms are associated with neovascular age-related macular degeneration. Hum Mol Genet. 2006; 15: 2955–2961.
19. Grisanti S, Zhu Q, Tatar O, et al. Differential expression of vascular endothelial growth factor-A isoforms in neovascular age related macular degeneration. Retina. 2014. [Epub ahead of print].
20. Ablonczy Z, Dahrouj M, Marneros AG. Progressive dysfunction of the retinal pigment epithelium and retina due to increased VEGF-A levels. FASEB J. 2014; 28(5): 2369–79.
21. Marneros AG, Fan J, Yokoyama Y, et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. Am J Pathol. 2005; 167(5): 1451–9.
22. Sharma NK, Gupta A, Prabhakar S, et al. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Curr Neurovasc Res. 2012; 9(4): 256–65.
23. Sharma NK, Gupta A, Prabhakar S, et al. Serum levels and polymorphism investigation in CFH loci in age related macular degeneration. PLoS ONE 8(7): e70193.
24. Anand A, Sharma NK, Gupta A, et al. Superoxide Dismutase1 Levels in North Indian Population with Age-Related Macular Degeneration.

25. Anand A, Sharma NK, Singh R, et al. Does DcR1 (TNF-related apoptosis-inducing-ligand Receptor 3) have any role in human AMD pathogenesis? Sci Rep. 2014; 4: 4114.
26. Sharma NK, Sharma K, Gupta A, et al. Does Toll like receptor-3 have any role in Indian AMD phenotype? Molecular and Cellular Biochemistry. 2014; 393(1–2): 1–8.
27. Sharma NK, Sharma SK, Gupta A, et al. Predictive model for earlier diagnosis of suspected AMD patients. DNA and Cell Biology, 2013; 32(9): 549–555.
28. Reich SJ, Fosnot J, Kuroki A, et al. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis. 2003; 9: 210–6.
29. Cashman SM, Bowman L, Christofferson J, et al. Inhibition of Choroidal Neovascularization by Adenovirus-Mediated Delivery of Short Hairpin RNAs Targeting VEGF as a Potential Therapy for AMD. Invest. Ophthalmol. Vis. Sci. 2006; 47 (8): 83496–3504.
30. Nagineni CN, Kommineni VK, William A, et al. Regulation of VEGF expression in human retinal cells by cytokines: implications for the role of inflammation in age-related macular degeneration. J Cell Physiol. 2012; 227(1): 116–26.
31. Anand A, Sharma NK, Gupta A, et al. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PLoS One. 2012; 7(11): e49905.
32. Sharma NK, Gupta A, Prabhakar S, et al. CC chemokine receptor-3 as new target for age-related macular degeneration. Gene. 2013; 523(1): 106–11.
33. Sharma NK, Prabhakar S, Gupta A, et al. New biomarker for neovascular age-related macular degeneration: eotaxin-2. DNA Cell Biol. 2012; 31(11): 1618–27.
34. Snow KK, Seddon JM. Do age related macular degeneration and cardiocascular disease share common antecedents? Ophthalmic Epidemiol. 1999; 6(2): 125–43.
35. Hyman L, Schachat AP, He Q, et al. Hypertension, cardiovascular disease, and age-related macular degeneration. Age-Related Macular Degeneration Risk Factors Study Group. Arch Ophthalmol. 2000; 118(3): 351–8.
36. Felmeden DC, Spencer CG, Belgore FM, et al. Endothelial damage and angiogenesis in hypertensive patients: relationship to cardiovascular risk factors and risk factor management. Am J Hypertens. 2003; 16(1): 11–20.
37. Lip PL, Blann AD, Hope-Ross M, et al. Age related macular degeneration is associated with increased vascular endothelial growth factors, hemorheology and endothelial dysfunction. Ophthalmology. 2001; 108(4): 705–10.
38. Zehetner C, Kirchmair R, Huber S, et al. Plasma levels of vascular endothelial growth factor before and after intravitreal injection of bevacizumab, ranibizumab and pegaptanib in patients with age-related macular degeneration, and in patients with diabetic macular oedema. Br J Ophthalmol. 2013; 97(4): 454–9.
39. Carneiro AM, Costa R, Falcão MS, et al. Vascular endothelial growth factor plasma levels before and after treatment of neovascular age-related macular degeneration with bevacizumab or ranibizumab. Acta Ophthalmol. 2012; 90(1): e25–30.
40. Ohno-Matsui K, Morita I, Tombran-Tink J et al. Novel mechanism for age-related macular degeneration: An equilibrium shift between the angiogenesis factors VEGF and PEDF. J Cell Physio.2001; 189(3): 323–333.

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Does toll-like receptor-3 (TLR-3) have any role in Indian AMD phenotype?

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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

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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

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

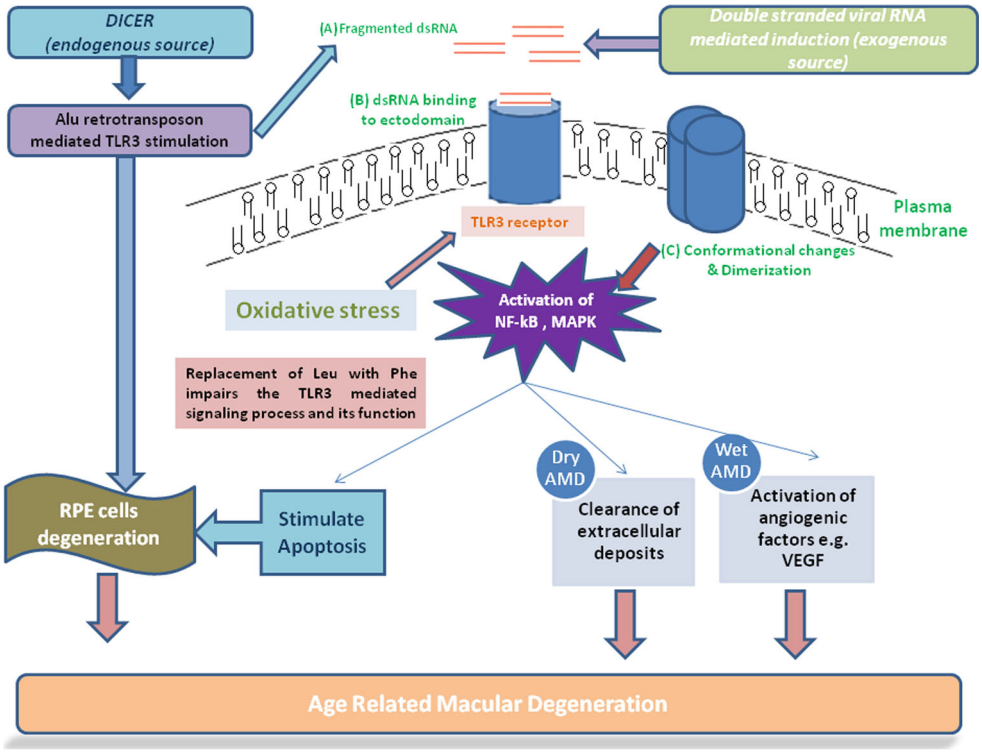

Fig. 1 Schematic representation of pathogenesis of AMD mediated by *TLR3* signaling process. **a** Fragmented dsRNA obtained from digestions of Alu retrotransposon and endogenous viral dsRNA genome. **b** Binding of fragmented dsRNA on ectodomain of *TLR3* rerceptor. **c** Conformational changes and 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

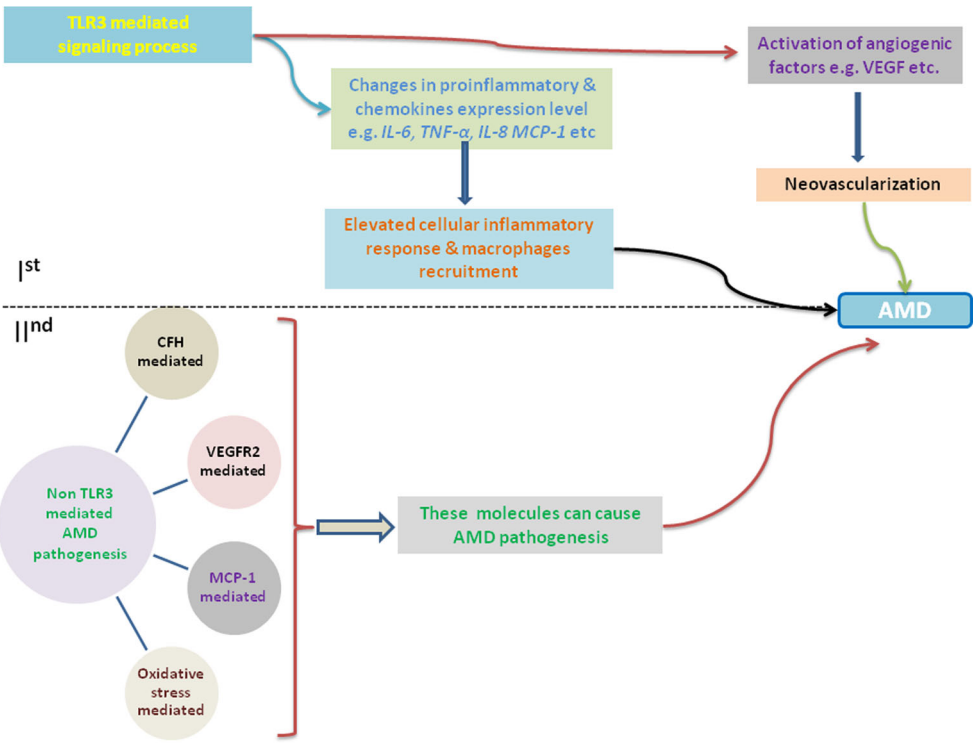

Fig. 2 AMD pathogenesis. Ist part showing the AMD pathogenesis mediated by *TLR3* by stimulating proinflammatory cytokines and angiogenic factors. IInd part conferring the role of other genetic factors can cause AMD pathology independent of *TLR3* mediated process

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.

References

1. De Jong PT (2006) Age-related macular degeneration. N Engl J Med 355:1474–1485

2. Gorin MB (2005) A new vision for age-related macular degeneration. Eur J Hum Genet 13:793–794
3. Kumar MV, Nagineni CN, Chin MS, Hooks JJ, Detrick B (2004) Innate immunity in the retina: toll-like receptor (TLR) signaling in human retinal pigment epithelial cells. J Neuroimmunol 153: 7–15
4. Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, Davies DR (2008) Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 320:379–381
5. Van B Jr, Buurman WA, Griffioen AW (2008) Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). Angiogenesis 11:91–99
6. Park DW, Baek K, Lee JG, Park YK, Kim JH, Kim JR, Baek SH (2007) Activation of toll-like receptor 4 modulates vascular endothelial growth factor synthesis through prostacyclin-IP signaling. Biochem Biophys Res Commun 362:1090–1095
7. Ambati J, Ambati BK, Yoo SH, Ianchulev S, Adamis AP (2003) Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. Surv Ophthalmol 48:257–293
8. Donoso LA, Kim D, Frost A, Callahan A, Hageman G (2006) The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 51:137–152
9. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11:373–384
10. Kleinman ME, Kaneko H, Cho WG, Dridi S, Fowler BJ, Blandford AD, Albuquerque RJ, Hirano Y, Terasaki H, Kondo M, Fujita T, Ambati BK, Tarallo V, Gelfand BD, Bogdanovich S, Baffi JZ, Ambati J (2012) Short-interfering RNAs induce retinal degeneration via TLR3 and IRF3. Mol Ther 20(1):101–108
11. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088
12. Zhu Y, Liang L, Qian D, Yu H, Yang P, Lei B, Peng H (2013) Increase in peripheral blood mononuclear cell Toll-like receptor 2/3 expression and reactivity to their ligands in a cohort of patients with wet age-related macular degeneration. Mol Vis 19:1826–1833
13. Maloney SC, Antecka E, Orellana ME, Fernandes BF, Odashiro AN, Eghtedari M, Burnier MN Jr (2010) Choroidal neovascular membranes express toll-like receptor 3. Ophthalmic Res 44(4): 237–241
14. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Karikó K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW, Ambati J (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 452(7187):591–597
15. Zareparsi S, Buraczynska M, Branham KE, Shah S, Eng D, Li M, Pawar H, Yashar BM, Moroi SE, Lichter PR, Petty HR, Richards JE, Abecasis GR, Elner VM, Swaroop A (2005) Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration. Hum Mol Genet 14:1449–1455
16. Yang Z, Stratton C, Francis PJ, Kleinman ME, Tan PL, Gibbs D, Tong Z, Chen H, Constantine R, Yang X, Chen Y, Zeng J, Davey L, Ma X, Hau VS, Wang C, Harmon J, Buehler J, Pearson E, Patel S, Kaminoh Y, Watkins S, Luo L, Zabriskie NA, Bernstein PS, Cho W, Schwager A, Hinton DR, Klein ML, Hamon SC, Simmons E, Yu B, Campochiaro B, Sunness JS, Campochiaro P, Jorde L, Parmigiani G, Zack DJ, Katsanis N, Ambati J, Zhang K (2008) Toll-like receptor 3 and geographic atrophy in age-related macular degeneration. N Engl J Med 359:1456–1463
17. Cho Y, Wang JJ, Chew EY, Ferris FL 3rd, Mitchell P, Chan CC, Tuo J (2009) Toll-like Receptor Polymorphisms and Age-Related Macular Degeneration: replication in Three Case-Control Samples. IOVS 12:5614–5618
18. Edwards AO, Chen D, Fridley BL, James KM, Wu Y, Abecasis G, Swaroop A, Othman M, Branham K, Iyengar SK, Sivakumaran TA, Klein R, Klein BE, Tosakulwong N (2008) Toll-like receptor polymorphisms and age-related macular degeneration. Invest Ophthalmol Vis Sci 49:1652–1659
19. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma S, Anand A (2012) Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Curr Neurovascul Res 9:256–265
20. McAllister CS, Lakhdari O, Pineton de Chambrun G, Gareau MG, Broquet A, Lee GH, Shenouda S, Eckmann L, Kagnoff MF (2013) TLR3, TRIF, and caspase 8 determine double-stranded RNA-induced epithelial cell death and survival in vivo. J Immunol 190(1):418–427
21. Gordon S (2002) Pattern recognition receptors: doubling up for the innate immune response. Cell 111:927–930
22. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D (2004) mRNA is an endogenous ligand for toll-like receptor 3. J Biol Chem 279:12542–12550
23. Bell JK, Botos I, Hall PR, Askins J, Shiloach J, Segal DM, Davies DR (2005) The molecular structure of the toll-like receptor 3 ligand-binding domain. Proc Natl Acad Sci USA 102: 10976–10980
24. Sun J, Duffy KE, Ranjith-Kumar CT, Xiong J, Lamb RJ, Santos J, Masarapu H, Cunningham M, Holzenburg A, Sarisky RT, Mbow ML, Kao C (2006) Structural and functional analyses of the human toll-like receptor 3. Role of glycosylation. J Biol Chem 281:11144–11151
25. Choe J, Kelker MS, Wilson IA (2005) Crystal structure of human toll-like receptor 3 (*TLR3*) ectodomain. Science 309:581–585
26. Ranjith-Kumar CT, Miller W, Xiong J, Russell WK, Lamb R, Santos J, Duffy KE, Cleveland L, Park M, Bhardwaj K, Wu Z, Russell DH, Sarisky RT, Mbow ML, Kao CC (2007) Biochemical and functional analyses of the human toll-like receptor 3 ectodomain. J Biol Chem 282:7668–7688
27. de Bouteiller O, Merck E, Hasan UA, Hubac S, Benguigui B, Trinchieri G, Bates EE, Caux C (2005) Recognition of double stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem 280:38133–38145
28. Cai Jiyang, Nelson KC, Wu Mei, Sternberg Paul Jr, Jones DP (2000) Oxidative damage and protection of the RPE. Prog Retin Eye Res 19(2):205–221
29. Scholl HP, Fleckenstein M, Fritsche LG, Schmitz-Valckenberg S, Göbel A, Adrion C, Herold C, Keilhauer CN, Mackensen F, Mössner A, Pauleikhoff D, Weinberger AW, Mansmann U, Holz FG, Becker T, Weber BH (2009) CFH, C3 and ARMS2 are significant risk loci for susceptibility but not for disease progression of geographic atrophy due to AMD. PLoS One 4(10): e7418A
30. Klein ML, Ferris FL, Francis PJ, Lindblad AS, Chew EY, Hamon SC, Ott J (2010) Progression of geographic atrophy and genotype in age-related macular degeneration. Ophthalmology 117(8): 1554–1559
31. Wörnle M, Merkle M, Wolf A, Ribeiro A, Himmelein S, Kernt M, Kampik A, Eibl-Lindner KH (2011) Inhibition of *TLR3*-mediated proinflammatory effects by alkylphosphocholines in human retinal pigment epithelial cells. IOVS 52(9):10–6993
32. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, Gupta PK (2012) Single nucleotide polymorphisms in MCP-1 and Its receptor are associated with the risk of age related macular degeneration. PLoS One 7(11):e49905
33. Klettner A, Koinzer S, Meyer T, Roider J (2013) Toll-like receptor 3 activation in retinal pigment epithelium cells-Mitogen-

activated protein kinase pathways of cell death and vascular endothelial growth factor secretion. Acta Ophthalmol 91(3): e211–e218
34. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, Anand A (2013) CC chemokine receptor-3 as new target for age-related macular degeneration. Gene 523:106–111
35. Sharma NK, Prabhakar S, Gupta A, Singh R, Gupta PK, Gupta PK, Anand A (2012) New Biomarker for Neovascular Age-Related Macular Degeneration: eotaxin-2. DNA Cell Biol 31: 1618–1627
36. Swaroop A, Chew EY, Rickman CB, Abecasis GR (2009) Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annu Rev Genomics Hum Genet 10:19–43
37. Klein R, Klein BE, Moss SE (1998) Relation of smoking to the incidence of age-related maculopathy. The beaver dam eye study. Am J Epidemiol 147:103–110
38. Seddon JM, Rosner B, Sperduto RD, Yannuzzi L, Haller JA, Blair NP, Willett W (2001) Dietary fat and risk for advanced age-related macular degeneration. Arch Ophthalmol 119:1191–1199
39. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, Baccarelli A (2009) Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 130:234–239
40. Tohgi H, Utsugisawa K, Nagane Y, Yoshimura M, Ukitsu M, Genda Y (1999) Decrease with age in methylcytosines in the promoter region of receptor for advanced glycated end products (RAGE) gene in autopsy human cortex. Brain Res Mol Brain Res 65:124–128

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Does DcR1 (TNF-related apoptosis-inducing-ligand Receptor 3) have any role in human AMD pathogenesis?

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It has been postulated that there is a link between age related degenerative diseases and cancer. The TNF-related apoptosis-inducing ligand (TRAIL) has been shown to selectively kill tumor cells by binding to pro-apoptotic and anti-apoptotic receptors. Our aim was to study the levels of anti-apoptotic receptor (DcR1) in age related macular degeneration (AMD) and controls. AMD patients (115) were classified into two groups: Dry and Wet AMD. Wet AMDs were further classified into occult, predominant classic and minimal classic. 61 healthy individuals were recruited as normal controls. After normalization with total protein, DcR1 levels were analyzed by ELISA. Mann Whitney U-statistic was used for analysis of DcR1 ELISA results. We have observed DcR1 levels in serum sample which were significantly lower in AMD patients as compared to controls ($p = 0.001$). On the other hand, we did not find difference in DcR1 levels between wet and dry AMD. The present study defines the plausible role of DcR1 in AMD pathology signifying a new therapeutic target for AMD.

Age is the most important risk factor for neurodegenerative disorders including age-related macular degeneration (AMD)[1]. It is a leading cause of irreversible vision loss in elderly in both the developed as well as developing world. The disease is broadly classified according to the severity of disease. It has been reported that about two million individuals are affected with AMD in United Kingdom[2] and statistics on AMD in India show frequency varies from 2.7% early AMD to 0.6% late AMD in South India to 4.7% in North India[3]. The major hallmark characteristic of early AMD is the presence of drusen. Drusen are tiny accumulations of extracellular material which accumulate between the retinal pigment epithelium and Bruch's membrane of the eye. The drusen consists of lipoproteins (especially Lipoprotein E), complement system components (Complement factor H, complement factor B and complement factor C etc.), apolipoproteins (apolipoproteins B48 and B100), clusterin and some exosome molecules [CD63 (Cluster of Differentiation 68), LAMP2 (Lysosome-associated membrane protein 2), and CD81(Cluster of Differentiation 81) etc)[4]. There are two types of AMD i.e. Dry AMD and Wet AMD. Dry AMD is marked by drusen or depigmentation caused by products of the photoreceptors and retinal pigment epithelium (RPE). Wet AMD is caused due to the growth of abnormal blood vessels below the retina and RPE, which results in subretinal bleeding and consequent scar formation occluding vision. This may affect any one or both of the eyes. Patients with dry AMD may progress into wet AMD. Several genetic and environmental factors have already been associated with AMD. Among the genetic factors, the genes which are involved in cell apoptosis and its regulation have not been much investigated in AMD. However, apoptosis plays a major role in pathology of AMD[5]. In this study, we measured the expression level of DcR1 in serum of AMD patients. Previously, many genetic studies have confirmed the role of various genes in AMD by use of whole blood from AMD patients of similar sample size[6–9]. The disease progression is characterized by impairment of regulatory processes like apoptosis, chronic inflammation, increase in cell numbers, invasiveness etc. Moreover, there are some common risk factors and underlying molecules for AMD and cancer like smoking, complementary factor H (CFH) with inflammation being main mediator in the progression of diseases

Table 1 | Demographic characteristics of Controls and AMD patients

Variables	AMD	Controls
Total	115	61
Wet AMD	84	----
Dry AMD	31	----
Sporadic Cases	105	----
Familial Cases	10	----
Duration of disease¥	23 ± 2.6 (M)	----
Smokers	50	11
Non Smokers	65	44
Alcoholic	37	17
Non-alcoholic	78	38
Vegetarian	61	31
Non-vegetarian	54	24
Age	64.97 ± 7.1	60.38 ± 13.2
Heart Disease	16	----
No Heart Disease	60	55
Male	75	40
Female	40	21

Demographic and clinical details of subjects. Age, Age of onset; M, Months; ¥ Duration of disease is the interval between appearance of first symptom of AMD and collection of sample. Values are mean ± SD.

pathology[10]. TNF-related apoptosis-inducing ligand (TRAIL) acts as antitumor agent, which induces apoptosis in cancer cells. TRAIL is a cytokine and can bind with four receptors i.e. Death receptor 4 (DR4) and Death receptor 5 (DR5) (pro-apoptotic) and with anti-apoptotic (DcRs), Death receptor 1 (DcR1) and Death receptor 2 (DcR2)[11]. By binding with DR4 or DR5 receptors TRAIL can induce apoptosis by caspase-8 dependent manner which further activates the effector caspases like caspases-3, 6,7 etc. DcR1 is a GPI-anchored member of the tumor necrosis factor receptor (TNFR) super family which is also known as CD263, TRID and TRAIL-R3. It is not expressed in all tumors. It includes a transmembrane domain and an extracellular TRAIL binding domain. It does not contain a functional death domain. It acts as TRAIL decoy receptor by reducing the apoptosis. The TRAIL's binding to DcR1 or DcR2 stimulate the NFk-β leading to activation of transcription genes antagonizing apoptotic mechanisms or promoting inflammation. Decoy receptors after binding to TRAIL inhibit the TRAIL-induced apoptosis by inhibiting binding to proapoptotic receptors i.e. DR4 and DR5.

In this study, we hypothesized that the lower levels of DcR1 in serum of AMD patients may provide an environment conducive for degenerative processes. TRAIL binding and therefore, DcR1 mediated anti-apoptotic process may not be active which may lead to photoreceptor degeneration leading to AMD.

Results

Summary statistics of main variables has been reported in Table 1. The serum concentration of DcR1 is shown in Table 2. DcR1 serum levels were skewed therefore, Kruskal-Wallis test was applied for statistical comparison. The serum concentration of DcR1 was found to be lower in AMD cases as compared to controls (Table 2, Figure 1A, $p = 0.0001$). Difference was not significant between serum concentration of DcR1 in dry and wet AMD (Table 2, Figure 1B, $p = 0.093$). The levels of dry and wet AMD were significantly lower as compared to normal controls (Figure 1B, $p = 0.001$ & 0.0001 respectively). To estimate the predictive value of DcR1, serum levels of DcR1 were again segregated into minimal classic, predominant classic and occult AMD. No significant difference was found between the wet AMD subgroups (Table 2). While adjusting the risk factors to AMD an independent analysis was carried out. Important risk factors like smoking, alcohol, food habits, diabetes and heart diseases were analyzed to examine their association with DcR1. However, no significant difference observed between AMD smokers versus AMD never smokers, alcohol consumers versus never alcohol consumers, vegetarian versus non vegetarian, diabetic versus non diabetic, AMD with heart disease versus AMD without heart disease and Male versus female (Table 2). When multivariate analysis was carried out with adjustment for age, smoking, alcohol, food habits, diabetes and heart disease, we found significant difference between AMD and controls (OR = 11.181, $p = 0.001$).

The association between levels of DcR1 in AMD and age have been computed using Chi square ($\chi^2 = 3.929$, $p = 0.141$), which shows that there is no significant association between age and DcR1 levels. However, the association between severity of AMD and levels of DcR1 was significant ($\chi^2 = 5.982$, $p = 0.014$). The prediction equation for AMD, by considering DcR1 levels as independent variable, shows that 74.4% of the cases have been correctly classified with

Table 2 | Comparison of serum DcR1 levels between control, AMD and their subtypes along with odds ratio (adjusted for covariates)

		Mann Whitney Statistic	Unadjusted p value	Multivariate analysis adjusted for, age smoking, alcohol, food habits, diabetes and heart disease		
Subjects	Mean Rank	Z -Value	p-value	Odd Ratio	CI (95%)	p- value
AMD	48.12	5.762	0.0001*	11.181	2.67–46.7	0.001
Control	86.32					
Dry	41.85					
Wet	33.29	1.681	0.093	0.296	0.049–1.790	0.182
Minimal Classic	17.00					
Predominant Classic	12.11	0.867	0.386	*	*	*
Occult	20.05	0.611	0.541	*	*	*
Alcoholic	38.38					
Non Alcoholic	35.56	0.538	0.591	0.286	0.021–3.892	0.347
Smokers	37.40					
Non Smokers	35.86	0.308	0.758	0.457	0.053–3.962	0.477
Vegetarian	38.19					
Non Vegetarian	34.71	0.704	0.481	0.964	0.165–5.638	0.968
Heart Disease	28.00					
No heart Disease	25.57	0.445	0.671	0.529	0.044–6.309	0.614
Diabetic	33.62					
No Diabetes	35.93	0.370	0.711	1.727	0.246–12.136	0.583
Male	37.09					
Female	35.52	0.308	0.758	0.182	0.011–2.99	0.233

*Odds ratio can not be computed (with reference category as minimal classic) because of the constant values.

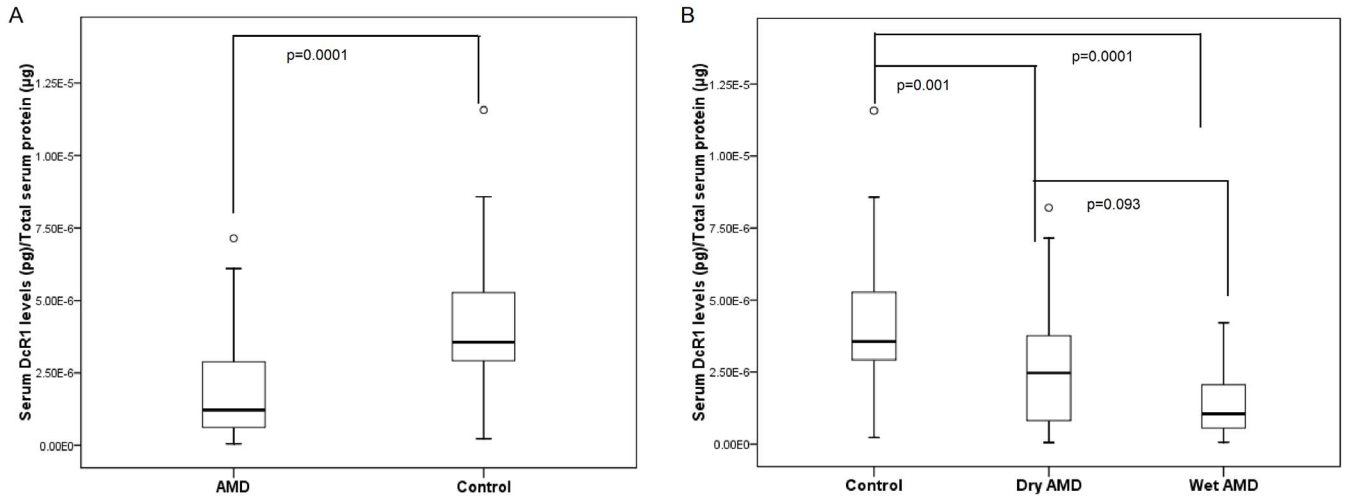

Figure 1 | (A) Serum levels of DcR1 in AMD and controls. (B) Serum levels of DcR1 in dry, wet and normal controls. pg, picogram; µg, microgram.

model authenticity of 74.4% and close confidence intervals for ROC curve. The area under ROC was 0.75 (p = .0001) with standard error of 0.044 and confidence interval of 0.660–0.834 (Figure 2).

Discussion

In a number of diseases, apoptosis plays a major role in development of disease pathology like AMD and retinitis pigmentosa. AMD causes a gradual loss of central vision due to the death of photoreceptors in the retina especially in central part of retina called the macula. The study was performed to determine whether the difference in the levels of serum DcR1 are associated with AMD. We showed that the DcR1 expression decreased significantly in patients of AMD as compared to controls irrespective of other environmental factors that indicates the noon redundant role of DcR1 mediated processes independent of these factors. Additionally, we also did not find the considerable difference in serum DcR1 levels in between dry AMD and wet AMD indicating apoptosis as a common phenomenon in both types of AMD.

In pathological processes of many degenerative diseases, apoptosis and necrosis play an important role. Histochemical and ultrastructural analysis shows that during AMD, RPE cells overlying drusen degenerate because of necrosis[12]. Initiation of necrosis in cells results in morphological modifications, membrane rupture which may lead to release of cytoplasmic contents from the cell. Necrotic material from these cells is believed to activate the complement, inducing inflammation in local tissue. Krabb et al has also shown that histones, which are released during necrosis, also constitute drusen[13] leading to RPE atrophy which further results in geographic atrophy. There are several studies which have simultaneously implicated the role of apoptotic processes in degeneration of photoreceptors, retinal pigment epithelium (RPE) and inner nuclear layer (INL) in AMD[14]. It has already been reported that reduced expression of DcR1 was associated with sensitivity to TRAIL in many cell lines of cancers[15]. In carcinomas, it has also been examined that lack of expression of the DcRs may provide them extra susceptibility to apoptosis induced by TRAIL[16].

However, apoptotic action mediated by TRAIL has been shown to be exerted by participation of p53 thus exacerbating anti-tumor activity by down regulating the DcR1 expression in colon cancer[17]. Moreover, it has been found to be overexpressed in the p53 mutated cell line. Recently, Ambati et al have also examined the role of p53 in retinal angiogenesis and have found disruption of p53 transcriptional network can abolish the anti-angiogenic activity of Nutlin3 (drugs commonly used in cancer). Even though the group did not show any relation with DcR1 or DcR2 receptors the role of DcR1 in apoptotic process could be examined in the context of p53 processes[18]. Several mouse models for retinal degeneration have been established by genetic manipulations in several retinal related genes and also created by light injury of retinal layers in which apoptosis contributes towards photoreceptor cell death[19–21]. Human retinitis pigmentosa, serous retinal detachment and pathologic myopia are characterized by similar pathologic photoreceptor apoptosis[22,23]. In glaucoma retinal ganglion cells also undergo apoptosis[24]. Additionally, it was also shown that the number of TUNEL-positive cells in the retinal pigment epithelium, choroid, outer nuclear layer, and inner nuclear layer are significantly more in AMD eyes as compared to control eyes, signifying that these cells may possibly die by apoptosis[12]. Therefore, these studies have revealed that main cause of photoreceptor death in retinal and other diseases presumably regulated by TRAIL signaling. Due to low levels of DcR1, TRAIL has increased affinity to bind with TRAILR1 or TRAILR2 which results in receptor oligomerization and initiation of apoptosis. Enhanced

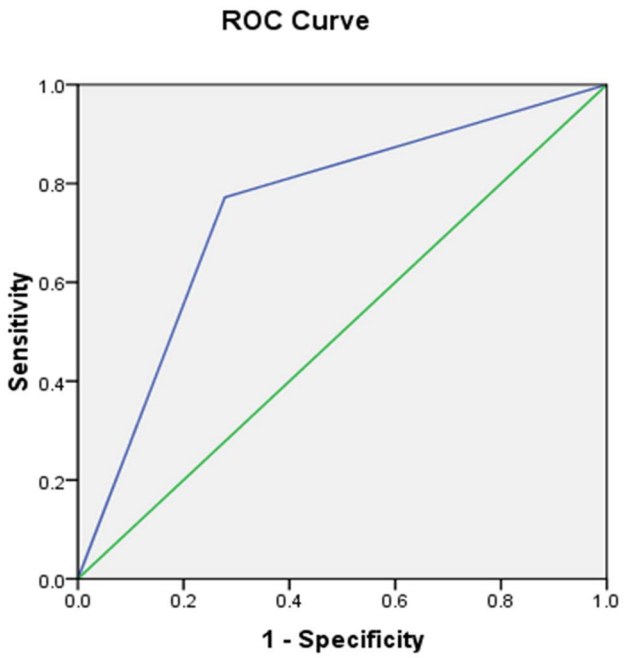

Figure 2 | **Receiver operating characteristic (ROC) obtained from binary logistic regression model which generates significant predictors of AMD.** Area under the curve is reported to be 75%.

apoptosis can result in tissue degeneration, a feature of AMD, while reduced apoptosis results in accumulation of immune cells. Therefore, cellular protection protocols engage a delicate balance in pro- apoptotic and anti-apoptotic factors. Apoptosis involves two major signaling pathways: the extrinsic death pathway and intrinsic death pathway. Extrinsic pathway is initiated through apoptotic signal transduction cascades mediated by members of TNFR while intrinsic death pathway is mediated by pro-apoptotic and anti-apoptotic Bcl2 family proteins at the mitochondria[25].

We did not find any difference in the levels of DcR1 for dry and wet AMD and the difference was not found to be significant for wet AMD subtypes. However, after categorizing DcR1 levels (below and above median) the association with severity of AMD was found to be significant but it was not significant with age. While analyzing the other risk factors to AMD, no association of DcR1 levels was observed. Death of photoreceptor cells is the main cause in the pathogenesis of AMD. We have attempted to predict AMD based on DcR1 levels (below and above median) using logistic regression, which showed 74.4% model predictivity and AUC is 0.75. The moderate value of AUC may be used to diagnose AMD patients with very less standard error. TNF-related apoptosis inducing ligand stimulates apoptosis and DcR1 is believed to act as dominant-negative receptor for TNF-related apoptosis sensitising ligand. Due to low levels of DcR1, the levels of TNF-related apoptosis inducing ligand may be enhanced resulting in apoptosis mediated AMD. The present study highlights that the lower levels of DcR1 may be associated with AMD however additional mechanistic studies can shed more light on the putative mechanism.

Conclusion

Conclusively, this study substantiates the role of apoptosis mediated by DcR1 receptor. A bigger study is imperative. An effective method of neuroprotection based on targeting of DcR1 could potentially supplement the current treatment strategies for this disorder.

Methods

Study population. A signed informed consent was taken from each participant. Individuals with AMD were recruited from advanced eye centre, PGIMER, Chandigarh, India. We included 176 cases which contained 115 AMD samples (75 male and 40 females) and 61 normal healthy controls (40 male and 21 females) after obtaining a signed informed consent as per inclusion exclusion criteria. The unrelated attendants like spouses who accompanied AMD patients to Eye clinic were recruited as controls. The samples were collected at the same site using same procedure by the same individual. Ethical clearance was taken from the Ethics Committee of the Institute vide letter No Micro/10/1411.

Inclusion and exclusion criteria. Inclusion criteria included the age of 50 years or older with the diagnosis of age related macular degeneration defined by choroidal neovascularization and/or dry AMD with at least five drusen in one eye. The controls were of 50 years or older without drusen and without any AMD diagnostic criteria. Exclusion criteria excluded the retinal diseases in the outer retinal layers and/or photoreceptors (other than AMD) like central serous retinopathy, high myopia, diabetic retinopathy, retinal dystrophies, uveitis, vein occlusion and other problems that precluded satisfactory stereo fundus photography. These situations contain occluded pupils due to ocular diseases like cataracts, opacities and synechiae[9].

Diagnosis of AMD. A retina consultant examined the patients for best corrected visual acuity (BCVA), intraocular pressure, slit lamp biomicroscopy (SLB) of anterior and posterior segment with 90D lens and indirect ophthalmoscopy for peripheral fundus examination. All patients underwent fluorescein fundus-angiography (FFA) and optical coherence-tomography (OCT). AMD diagnosis was based on ophthalmoscopic and FFA findings[10].

Demographic profile. The demographic detail was taken by a trained interviewer after getting signed consent form using a risk factor questionnaire[9]. The detail (age, sex, race, cigarette smoking, food habit, alcohol intake, diabetes and heart diseases) was self reported by participants. Smokers were districted as those having smoked at least three cigarettes per day or 54-boxes for at least 6 contiguous months and were separated further into never smokers and smokers. Non-vegetarian patients were distincted as those consuming meat, chicken,or fish for at least 6 contiguous months and alcohol consumer patients were defined as those having rum, whiskey, wine or homemade alcohol for at least 6 contiguous months. Heart diseases were determined based on the participant's answers to whether a physician had ever told them about this finding and whether they had ever taken medicine for this situation[10].

Serum separation. Blood samples were collected from all individuals for carrying out ELISA. About 4.0 ml of blood was collected in a serum separator tube (BD Biosciences, USA) and was left for 30 minutes at room temperature to allow it to clot and after centrifugation at 3000-rpm for 30 minutes, serum was separated. The samples were labeled, coded and stored to study the levels by established procedures described below.

Total protein estimation. Total serum proteins for normalization of DcR1 levels from ELISA was done by Bradford assay. The procedure was carried out according to manufacturer's recommendations. In double distilled water, serum samples were diluted upto 1500 times. The standard curve was generated by using protein Bovine Serum Albumin (BSA). Standard protein BSA and samples were mixed in ratio 4 : 1 with coomassie brilliant blue G – 250 dye (Bradford reagent) followed by incubation at room temperature for about 15 minutes on shaker. The absorbance was taken at 595 nm in 680XR model of Microplate reader (Biorad, Hercules, CA, USA). Standard curve was generated with quadratic fit or linear models[11].

ELISA. ELISA for DcR1 was performed according to the manufacturer's protocol (Abcam, Cat no ab46018). Sample serum assays were performed in duplicate. Standard DcR1 was assayed over a concentration range of 10000–312.5 pg/ml in duplicates for each experiment. The assay recognizes both natural and recombinant human DcR1 and the sensitivity of assay was $<$147 pg/ml. Appropriate blanks were also incorporated into experimental design. Normalization was done with total protein.

Statistical analysis. The assumption of normality was tested with the help of Normal Quantile plot (Q-Q plot) and it was observed that data was not normally distributed. Mann-Whitney U-test was, therefore, applied for comparing the two groups. For comparing more than two groups, Kruskal Wallis 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. All statistical analysis such as linear regression, quadratic fit and test of significance were performed with statistical product and service solutions (SPSS; IBM SPSS Statistics 20.0, Chicago, Illinois, USA) 20.0 software. Receiver operating characteristic (ROC) curve, which is defined as a plot of true positive rate (test sensitivity) versus false positive (1-specificity) rate for different cut-off points of DcR1 levels was drawn for predicted model. Area under the ROC curve is considered as an effective measure of inherent validity of a diagnostic test.

1. Brown, R. C., Lockwood, A. H. & Sonawane, B. R. Neurodegenerative Diseases, An Overview of Environmental Risk Factors. *Environ Health Perspect* **113**, 1250–1256 (2005).
2. Owen, C. G., Fletcher, A. E., Donoghue, M. & Rudnicka, A. R. How big is the burden of visual loss caused by age related macular degeneration in the United Kingdom? *Br J Ophthalmol.* **87**, 312–317 (2003).
3. Azad, R., Chandra, P. & Gupta, R. The economic implications of the use of anti-vascular endothelial growth factor drugs in age-related macular degeneration. *Indian Journal of Ophthalmology.* **55**, 441–443 (2007).
4. Wang, A. L. *et al.* Autophagy and exosomes in the aged retinal pigment epithelium, Possible relevance to drusen formation and age-related macular degeneration. *PLoS ONE* **4**, e4160 (2008).
5. Bhattacharya, S., Chaum, S., Johnson, D. A. & Johnson, L. R. Age-Related Susceptibility to Apoptosis in Human Retinal Pigment Epithelial Cells Is Triggered by Disruption of p53–Mdm2 Association. *Invest. Ophthalmol.* **53**, 8350–8366 (2012).
6. Sharma, N. K. *et al.* Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. *PLoS ONE* **8**, e70193 (2013).
7. Sharma, N. K. *et al.* Single Nucleotide Polymorphism and Serum Levels Of VEGFR2 are Associated With Age Related Macular Degeneration. *Curr Neurovasc Res.* **9**, 256–65 (2012).
8. Sharma, N. K. *et al.* New Biomarker for Neovascular Age Related Macular Degeneration, Eotaxin-2. *DNA Cell Biol.* **31**, 1618–1627 (2012).
9. Anand, A. *et al.* Single Nucleotide Polymorphisms in MCP-1 and Its Receptor Are Associated with the Risk of Age Related Macular Degeneration. *PLoS ONE* **7**, e49905 (2012).
10. Klein, R. *et al.* Risk alleles in CFH and ARMS2 and the long-term natural history of age-related macular degeneration, the Beaver Dam Eye Study. *JAMA Ophthalmol.* **131**, 383–92 (2013).
11. Johnstone, W. R., Frew, A. J. & Smyth, M. J. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nature* **8**, 782–798 (2008).
12. Hageman, G. S. *et al.* An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog. Retin. *Eye Res.* **20**, 705–732 (2001).
13. Crabb, J. W. *et al.* Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc. Natl. Acad. Sci.* **99**, 14682–14687 (2002).

14. Dunaief, J. L., Dentchev, T., Ying, G. & Milam, A. H. The Role of Apoptosis in Age-Related Macular Degeneration. *Arch Ophthalmol.* **120**,1435–1442 (2002).
15. Ozoren, N. & El-Deiry, W. S. Cell surface death receptor signaling in normal and cancer cells. *Semin Cancer Biol.* **13**, 135–147 (2003).
16. Yamanaka, T. *et al.* Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. *Hepatology* **32**, 482–490 (2000).
17. Toscano, F. *et al.* P53-mediated upregulation of DcR1 impairs oxaliplatin/TRAIL-induced synergistic anti-tumour potential in colon cancer cells. *Oncogene* **27**, 4161–71(2008).
18. Chavala, S. H. *et al.* Retinal angiogenesis suppression through small molecule activation of p53. *J Clin Invest.* **123**, 4170–4181 (2013).
19. Chang, B. *et al.* Retinal degeneration mutants in the mouse. *Vision Res.* **42**, 517–25 (2002).
20. Chang, B. *et al.* Age-related retinal degeneration (arrd2) in a novel mouse model due to a nonsense mutation in the Mdm1 gene. *Hum Mol Genet.* **17**, 3929–41 (2008).
21. Hawes, N. L. *et al.* Retinal degeneration 6 (rd6): a new mouse model for human retinitis punctata albescens. *Invest Ophthalmol Vis Sci.* **41**, 3149–57 (2000).
22. Li, Z. Y. & Milam, A. H. Apoptosis in retinitis pigmentosa. Anderson RELaVail MMHollyfield JGeds.Degenerative Diseases of the Retina. *New York, NY Plenum Press* 1–8 (1995).
23. Xu, G. Z., Li, W. W. & Tso, M. O. Apoptosis in human retinal degenerations. *Trans Am Ophthalmol Soc.* **94**, 411–430 (1996).
24. Kerrigan, M. S., Zack, D. J., Quigley, H. A., Smith, S. D. & Pease, M. E. TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch Ophthalmol.* **115**, 1031–1035 (1997).
25. Johnstone, R. W., Ruefli, A. A. & Lowe, S. W. Apoptosis, a link between cancer genetics and chemotherapy. *Cell* **108**, 153–164 (2002).

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Author contributions

A.A. conceptualized the study and framework. N.K.S. and A.A. conducted the study, data analysis interpreted the results and wrote the manuscript. S.K.S., A.K.B., N.J. and P.K.G. contributed to the data analysis and prepared some figures. A.G., S.P. and R.D.S. contributed to the interpretation and reviewed the manuscript. All authors reviewed the manuscript.

Additional information

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Short communication

CC chemokine receptor-3 as new target for age-related macular degeneration

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ABSTRACT

CC chemokine receptor-3 (CCR3) is involved in angiogenic processes. Recently, CCR3 was accounted to participate in choroidal neovascularization (CNV) and CCR3 targeting was reported to be superior to standard antivascular endothelial growth factor-A (VEGF-A) administration when tested in an artificially induced CNV in animals. As human CCR3 studies are lacking in age-related macular degeneration (AMD) patients we sought to determine if CCR3 has any association with inflammatory processes that occur in CNV. A total of 176 subjects were included on the basis of inclusion criteria. Real time PCR was used to analyze the single nucleotide polymorphism in CCR3 of AMD (115) and normal controls (n = 61). Genotype frequency was adjusted for possible confounders like cigarette smoking, alcohol, meat consumption and other risk factors. Chi-square test was used for analysis of polymorphism. The genotype distribution of CCR3 (rs3091250) polymorphism was significantly different in AMD patients in the Indian population. GT (heterozygous) and TT (homozygous) at the rs3091250 SNP increased risk of AMD as compared to the GG genotypes (OR = 4.8, CI 95% = 2.2–10.8 and OR = 4.1, CI 95% = 1.6–10.1 respectively). Subgroup analysis of AMD patients in wet and dry revealed no significant differences. There was no significant difference for rs3091312 in AMD and control group. A significant association between AMD and CCR3 (rs3091250) polymorphism localized on chromosome 3p21.3 was detected. The results suggest the possible contribution of rs3091250, a new predisposing allele in AMD.

1. Introduction

Age-related macular degeneration (AMD) is a primary cause of central vision loss in the aged in industrialized countries (Cook et al., 2008). Symptoms of AMD may appear in one or in both eyes. Early symptoms include metamorphopsia or blurring of central vision. AMD is characterized by the development of drusen in Bruch's membrane, the degeneration of the macular retinal pigment epithelium (RPE), geographic atrophy and neovascularization.

According to clinical age-related maculopathy grading system, age-related maculopathy grades are: without drusen, several minute drusen and no RPE changes, retinal RPE alteration but no drusen, both small drusen and RPE changes, several large and intermediate-size drusen, RPE detachment, geographic atrophy and choroidal neovascular membrane with disciform scarring (Seddon et al., 2006).

It is established that there is a complex participation of environmental and genetic factors in the pathogenesis of AMD. In complement system, microglial recruitment, inflammation, DNA repair, and neovascularization activation studies have identified numerous AMD-associated genes (Ding et al., 2009).

A lot of the earlier works done on genetic factors impacting AMD have been focused on single nucleotide polymorphisms (SNPs). Although extremely significant statistical relations between various single nucleotide polymorphisms and AMD have been discovered, they do not account for the whole genetic aspect of the disease. It is supposed that complement activation resulting from dysfunction of CC chemokines may contribute to inflammation. The infiltration of monocytes is accompanied by inflammatory chemokines as key mediators.

Studies recently indicated that inflammation plays a fundamental role in the development of CNV (Rohrer et al., 2009). Additionally, genetic evidence has identified variations in multiple genes (Sharma et al., 2009). Studies had also investigated the role of asthma with AMD and found that asthma could be a risk factor for AMD (Sun et al., 2012).

In senescent mice deficient in monocyte chemoattractant protein-1 (CCL2, also known as MCP-1) or its receptor we earlier described the

Abbreviations: AMD, age-related macular degeneration; CCR3, CC chemokine receptor-3; CEC, choroidal endothelial cell; CNV, choroidal neovascularization; FFA, fluorescein fundus angiography; GPCR, G-protein-coupled receptor; MCP1, monocyte chemoattractant protein-1; OCT, optical coherence tomography; RPE, retinal pigment epithelium; SDS, Sequence Detection System; SNPs, single nucleotide polymorphisms; VEGF-A, vascular endothelial growth factor-A.

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spontaneous development of CNV postulating its key role in AMD pathogenesis (Ambati et al., 2003). We hypothesized that CCR3 is also involved in similar processes and any polymorphism in this gene may consequence in chronic inflammation by continued activation of the complement system contributing to the pathogenesis of AMD.

Antivascular endothelial growth factor treatment is currently used for wet AMD patients (The CATT Research Group, 2011). Even though Eghøj and Sorensen (2012) showed that out of the 1076 eyes, a total of 20 (2%) eyes met the criteria for tachyphylaxis i.e. drug did not respond at the time of reactivation of CNV in AMD patients who had responded to the treatment initially anti-VEGF-A therapy produces dysfunction and damage to the outer and inner murine retina (Nishijima et al., 2007; Saint-Geniez et al., 2008), raising a question of recurrent CNV or potential retinal toxicity. In this context it is pertinent to review the report of Takeda et al. who reported that the G-protein-coupled receptor (GPCR), CCR3 is important in neovascular AMD showing that CCR3 neutralizing antibodies are more effective than VEGF-A neutralizing antibodies in inhibiting the CNV in mice model (Takeda et al., 2009). Furthermore, genetically engineered mice that were lacking in CCR3 or its ligands were also protected to some extent from the effect of laser injury on the choroidal vasculature (Takeda et al., 2009). Additional evidence also supports its role in CNV (Ahmad et al., 2011). However, a study recently showed that CCR3 was not significant in CNV development when using a Matrigel CNV model (Li et al., 2011). Therefore, further study regarding the potential role of CCR3 in AMD is needed.

CCR3 is a receptor for eotaxin found on the surface of a variety of cells, including white blood cells. It is most commonly related with mast cells and eosinophils that play a main role in allergic reactions (Pope et al., 2005) as well as angiogenesis (Takeda et al., 2009). CCR3 gene is located on the short arm of chromosome 3. AMD is a complex disease, which is influenced by genetic and environmental factors. The absence of any such genetic association studies of CCR3 and AMD prompted us to explore the role of this chemokine in these patients. We therefore wanted to determine the polymorphism of CCR3 in the human AMD patients.

2. Materials and methods

The study population included 176 subjects, which include 115 AMD patients and 61 normal healthy controls from the Advanced Eye Center, Post-Graduate Institute of Medical Education and Research, Chandigarh, India. 50 years or older AMD patients with the diagnosis of advanced AMD including geographic atrophy and/or choroidal neovascularization with drusen more than five in at least one eye were incorporated in the study. The controls in the study included those more than 50 years of age with the absence of other diagnostic criteria for AMD.

The exclusion criteria defined retinal diseases involving the photoreceptors and/or outer retinal layers other than AMD loss such as high myopia, central serous retinopathy, retinal dystrophies, diabetic retinopathy, vein occlusion, uveitis or similar outer retinal diseases that have been present before an age of 50 and opacities of the ocular media, limitations of pupillary dilation or other problems enough to preclude sufficient stereo fundus photography. These conditions include cataracts, opacities due to ocular diseases and occluded pupils due to synechiae. Ethical approval was taken for the study by the Institute Ethics Committee, Post-Graduate Institute of Medical Education and Research, Chandigarh, India vide letter No Micro/10/1411. Informed consent was taken in the approved format endorsed by the Institute Ethical Committee.

2.1. Ophthalmic examination

Patients underwent complete clinical 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.

2.2. Demographic characterization

All the subjects were informed of the purpose of the study and interviewed. A written informed consent was taken from individual participants. The risk factor questionnaire included information about demographic characteristics such as cigarette smoking, alcohol intake, food habit, comorbidity etc. 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 never smokers. Non-vegetarian patients were defined as those having chicken, meat or fish for at least 6 months and alcoholic patients were defined as those having whiskey, rum, wine or homemade alcohol for at least 6 months. Co-morbidity was determined based on the participant's responses to whether any physician had told them for diagnosis of stroke, migraine or any heart diseases. Subjects were also asked to report any prior diagnosis of any neurological, cardiovascular or metabolic disorders etc.

2.3. Selection of single-nucleotide polymorphisms

The SNPs used in our study have been previously examined for other allergic and inflammatory diseases like asthma, because like CNV, asthma is a multifactorial disorder with both genetic and environmental factors (Mizutani et al., 2009). Because some of the mechanisms of progression of both CNV and asthma are similar (Sun et al., 2012), we hypothesized that there is an association between these diseases. Previously several population-based studies have accounted asthma to be associated with elevated risk of developing CNV.

2.4. DNA isolation

DNA was extracted using DNA extraction kits (Qiagen) as per the instructions provided by the manufacturer. Extracted DNA was stored at 4 °C to further investigate the polymorphism in CCR3 gene.

2.5. Real time PCR

Real time PCR was used to analyze SNPs and was performed in the 48 well model StepOne™ (Applied Biosystems Inc., Foster city, CA) using published TaqMan® SNP Genotyping Assays. Real time PCR was carried out for 20.0 μl volume containing 10 μl master mix, 5 μl 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 the 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. Negative controls in the PCR mix did not contain DNA. PCR amplification and SNP estimation were done by StepOne TM v 2.0 software (Applied Biosystems Inc., Foster City, CA). 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.

2.6. Statistical analysis

Genotypes estimated by the real time PCR for each mutation were stratified for homozygosity and heterozygosity for the respective allelic variants. Between various groups association was analyzed by Pearson's chi-square test. Genotype distributions were analyzed by logistic regression, integrating adjustments. 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. SPSS 20.0 software was used to perform statistical analysis.

Table 1
Demographic characteristics of controls and AMD patients.

Variables	AMD	Controls
Total	115	61
Wet AMD	84 (73.04%)	–
Dry AMD	31 (26.96%)	–
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). AMD subjects were asked to provide all clinical and demographic details at the age of disease-onset.
[a] Duration of disease is the interval between appearance of first symptom of AMD and collection of sample.

3. Results

The summary statistics of the important variables have been reproduced in Table 1. In a case–control study of 176 subjects, common genetic variants in CCR3 were analyzed. Tables 2 and 3 show the SNPs of CCR3 in AMD and normal controls. While examining rs3091250 loci a significant difference was observed for the genotype and allele frequency. Both the heterozygous (GT) and homozygous (TT) genotypes at rs3091250 SNP were found to be significantly more frequent in AMD patients (Table 2, Fig. 1A, OR = 4.8, CI 95% = 2.2–10.8, p = 0.001 and OR = 4.1, CI 95% = 1.6–10.1, p = 0.002 respectively). While examining rs3091312 loci no significant difference was found for AMD and controls (Table 2, Fig. 1A). However, the T allele frequency was found to be significant among AMD patients for both SNPs (Table 3, Fig. 1C). We did not observe any significant difference in genotype and allele frequency for wet and dry AMD patients (Tables 2, 3, Figs. 1B and D). Logistic regression analysis in both SNPs for food habit, smoking, alcohol and comorbidity did not show any difference (Table 4, Fig. 2). The difference was also not significant when comparison was made between familial patients, number of eyes affected, gender and wet AMD patients including minimally classic, predominantly classic and occult (data not shown).

The association of genetic polymorphism and other risk factors was analyzed by using logistic regression. We analyzed age, gender and smoking as risk factors which are known to be associated with AMD previously. To account for gender, age and smoking effects, we did a logistic regression analysis with gender, age, smoking and genotypes as independent variables. The results confirmed the significant association between the GT and TT genotype of rs3091250 and AMD (Table 2).

4. Discussion

AMD is a serious progressive irreversible disease influenced by genetic and other environmental factors. Our previous studies showed that MCP1/Ccr2 deficient mice show features of AMD. Combined with recent studies establishing CCR3 as an emerging candidate for AMD pathogenesis the focus has shifted on its role in AMD. CCR3 is the major chemokine receptor on basophils and eosinophils (Heath et al., 1997) and its ligands are important elements for the basophils, chemotaxis and activation of eosinophils at the place of allergic inflammation (Grimaldi et al., 1999).

In the present study we examined the two CCR3 SNPs and discovered that the GT and TT genotypes from rs3091250 could be responsible for AMD. This is the first study showing the relationship of rs3091250 with AMD, importantly from the Indian subcontinent. Mutation can affect the effectiveness of gene transcription, which in turn can change levels of mRNA and, thus, protein levels in general. Mutations in CCR3 may alter the levels of CCR3 in blood. It has been earlier reported that activated CCR3 promotes choroidal endothelial cell (CEC) migration, thus causing Rac 1 and VEGFR2 activation resulting to neovascular AMD (Wang et al., 2011). The function of CCR3 is to activate and recruit eosinophils to the site of inflammation and stimulate macrophage activation. Activated eosinophils can release reactive oxygen species which contribute to host tissue damage during chronic inflammatory responses.

We assume these results have strong implications for developing new approaches for targeting CCR3 in AMD. These results indirectly support the results of Takeda et al. who demonstrated that CCR3 is a therapeutic agent as compared to others for AMD (Takeda et al., 2009), demonstrating that CCR3 may influence the pathogenesis of the disease. CCR3 blockade had been shown to be extra effective in reducing CNV development than VEGF-A neutralization. However, Li et al. (2011) showed that CCR3 was not involved in CNV induced by

Table 2
Effect of CCR3 rs3091250 and CCR3 rs3091312 variants on disease phenotype.

Genotype	Number (frequency)		Unadjusted p-value			Multivariate analysis, adjusted for age, gender and smoking		
			OR	95% CI	p-Value	OR	95% CI	p-Value
CCR3 rs3091250								
	AMD	Controls						
GG	35 (0.32)	41 (0.68)	Reference			Reference		
GT	46 (0.42)	11 (0.18)	4.8	2.2–10.8	0.001	6.180	2.25–16.93	0.0001
TT	28 (0.26)	8 (0.14)	4.1	1.6–10.1	0.002	0.118	0.031–0.452	0.002
	Wet AMD	Dry AMD						
GG	26 (0.33)	9 (0.31)	Reference			Reference		
GT	29 (0.36)	17 (0.59)	0.59	0.22–1.55	0.28	1.658	0.594–4.626	0.334
TT	25 (0.31)	3 (0.10)	2.88	0.699–11.90	0.14	2.085	0.582–7.463	0.259
CCR3 rs3091312								
	AMD	Controls						
AA	39 (0.36)	32 (0.52)	Reference			Reference		
AT	32 (0.30)	15 (0.25)	1.75	0.80–3.78	0.154	1.677	0.690–4.07	0.254
TT	36 (0.34)	14 (0.23)	2.10	0.97–4.57	0.059	0.633	0.255–1.56	0.323
	Wet AMD	Dry AMD						
AA	22 (0.28)	7 (0.25)	Reference			Reference		
AT	32 (0.40)	10 (0.36)	1.01	0.33–3.08	0.97	1.644	0.547–4.942	0.376
TT	25 (0.32)	11 (0.39)	0.72	0.23–2.18	0.56	.546	0.143–2.078	0.374

Table 3
Allele frequency of CCR3 rs3091250 and CCR3 rs3091312 in AMD and normal controls.

Allele	Number (frequency)		OR	95% CI	p-Value
CCR3 rs3091250					
	AMD	Controls			
G	116 (0.53)	93 (0.78)	Reference		
T	102 (0.47)	27 (0.22)	3.02	1.8–5.01	0.001
	Wet AMD	Dry AMD			
G	81 (0.51)	35 (0.60)	Reference		
T	79 (0.49)	23 (0.40)	1.48	0.80–2.73	0.20
CCR3 rs3091312					
	AMD	Controls			
A	110 (0.51)	79 (0.65)	Reference		
T	104 (0.49)	43 (0.35)	1.73	1.09–2.74	0.018
	Wet AMD	Dry AMD			
A	76 (0.48)	24 (0.43)	Reference		
T	82 (0.52)	32 (0.57)	0.80	0.43–1.49	0.499

gelatinous protein presumably because the model used did not employ aged mice, a known factor associated with AMD.

The mechanism of development of both AMD and asthma appears to share a common mechanism. During the last few years some population based studies accounted that high risk of developing CNV is associated with a history of asthma (Sun et al., 2012). However, other studies have not shown any association between CNV and asthma (Moorthy et al., 2011). Therefore, the association between these two diseases eludes us. After analyzing the asthma related SNPs in AMD patients, we speculate that the mechanism of development of both diseases is far from being dissimilar. Previously, whole-genome scans have also suggested that a gene cluster of CC chemokine receptors, including CCR3 on chromosome region 3p21–24 is linked to asthma (Ober et al., 2000).

Interestingly, rs3091250 mutation is also reported in aspirin-intolerant asthma patients (Kim et al., 2008). Mutation in CCR3 gene was associated with asthma in a British population (Fukunaga et al., 2001) and not in Japanese (Kim et al., 2008), Taiwanese (Wang et al., 2007), and Korean populations. Three intronic CCR3 SNPs (−22557G/A, −520T/G, −174C/T) were identified in a Korean population (Lee et al., 2007).

Our study showed the non-redundant role of CCR3 in pathogenesis of AMD and may encourage multi-ethnic studies to verify the evidence presented here.

It is pertinent to note that many of the important risk factors were investigated in the present study, for example, several epidemiological investigations have found an association between smoking, alcohol, comorbidity, other risk factors and increased risk of development of AMD (Brody et al., 2001; Pons and Marin-Castaño, 2011), but our results did not show any association with smoking, alcohol, food habit and comorbidity.

For early detection of CNV, CCR3 could represent a novel marker with capacity to be exploited as new therapeutic entity through future studies. Abundant levels of CCR3-specific binding molecule spotted in the retina could be suggestive of AMD. Early detection of AMD may allow treatment to be initiated early. Additional immunolocalization and molecular studies utilizing autopsy specimens are imperative in order to verify the claims held by this study. Besides, future studies can explore the association of CCR3 polymorphism in Caucasian populations.

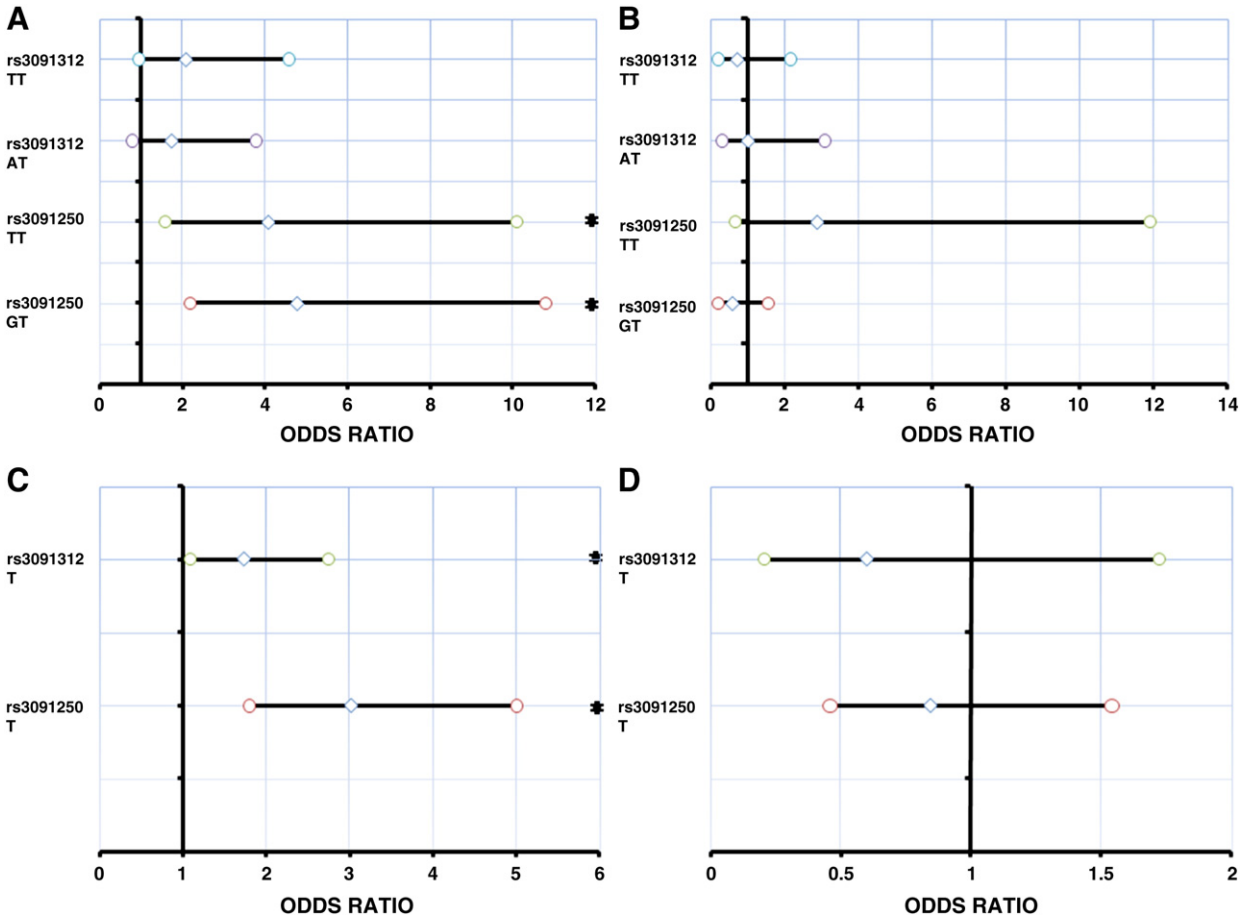

Fig. 1. A) Univariate logistic regression analysis in AMD/control as dependent variable and CCR3 rs3091250 and CCR3 rs3091312 polymorphism as independent variable. B) Univariate logistic regression analysis in wet/dry AMD patients as dependent variable and CCR3 rs3091250 and CCR3 rs3091312 polymorphism as independent variable. C) Univariate logistic regression analysis in AMD/Control as dependent variable and CCR3 rs3091250 and CCR3 rs3091312 allele frequency as independent variable. D) Univariate logistic regression analysis in wet/dry AMD patients as dependent variable and CCR3 rs3091250 and CCR3 rs3091312 allele frequency as independent variable.

Table 4
Logistic regression of the association of CCR3 rs3091250, CCR3 rs3091312 and progression of AMD stratified by comorbidity, food habits, smoking and alcohol.

Genotype	Number (frequency)		Unadjusted p-Value			Multivariate analysis, adjusted for age and gender		
			OR	95% CI	p-Value	OR	95% CI	p-Value
CCR3 rs3091250								
	AMD with comorbidity	AMD without comorbidity						
GG	23 (0.29)	11 (0.41)	Reference					
GT	33 (0.41)	12 (0.44)	1.31	0.49–3.49	0.582	0.771	0.288–2.063	0.605
TT	24 (0.30)	4 (0.15)	2.86	0.79–10.31	0.106	3.880	0.878–17.150	0.08
	Non-vegetarian AMD	Vegetarian AMD						
GG	16 (0.32)	19 (0.32)	Reference					
GT	20 (0.40)	26 (0.44)	0.91	0.37–2.21	0.841	0.997	0.408–2.439	0.994
TT	14 (0.28)	14 (0.24)	1.18	0.43–3.21	0.735	0.884	0.300–2.604	0.823
	Smokers' AMD	Non-smokers' AMD						
GG	17 (0.35)	18 (0.29)	Reference					
GT	17 (0.35)	29 (0.48)	0.62	0.25–1.51	0.295	1.631	0.594–4.482	0.343
TT	14 (0.30)	14 (0.23)	1.05	0.39–2.86	0.910	1.141	0.328–3.969	0.836
	Alcoholic	Non-alcoholic						
GG	11 (0.305)	24 (0.33)	Reference					
GT	14 (0.390)	32 (0.44)	0.954	0.36–2.46	0.923	0.781	0.249–2.455	0.672
TT	11 (0.305)	17 (0.23)	1.411	0.49–3.99	0.516	1.514	0.446–5.136	0.506
CCR3 rs3091312								
	AMD with comorbidity	AMD without comorbidity						
AA	26 (0.35)	12 (0.4)						
AT	22 (0.29)	9 (0.3)	1.12	0.40–3.17	0.819	0.767	0.258–2.280	0.633
TT	27 (0.36)	9 (0.0)	1.38	0.50–3.83	0.53	1.494	0.519–4.295	0.457
	Non-vegetarian AMD	Vegetarian AMD						
AA	19 (0.39)	20 (0.34)	Reference					
AT	13 (0.26)	19 (0.33)	0.72	0.28–1.85	0.49	0.705	0.273–1.821	0.471
TT	17 (0.35)	19 (0.33)	0.94	0.38–2.33	0.89	1.206	0.491–2.966	0.683
	Smokers' AMD	Non-smokers' AMD						
AA	16 (0.36)	23 (0.37)	Reference					
AT	14 (0.31)	18 (0.29)	1.11	0.43–2.87	0.817	0.812	0.281–2.346	0.700
TT	15 (0.33)	21 (0.34)	1.02	0.40–2.57	0.955	1.014	0.356–2.890	0.979
	Alcoholic	Non-alcoholic						
AA	13 (0.39)	26 (0.34)	Reference					
AT	8 (0.25)	24 (0.32)	0.66	0.23–1.88	0.445	1.255	0.379–4.155	0.709
TT	12 (0.36)	26 (0.34)	0.92	0.35–2.39	0.869	1.060	0.247–3.242	0.918

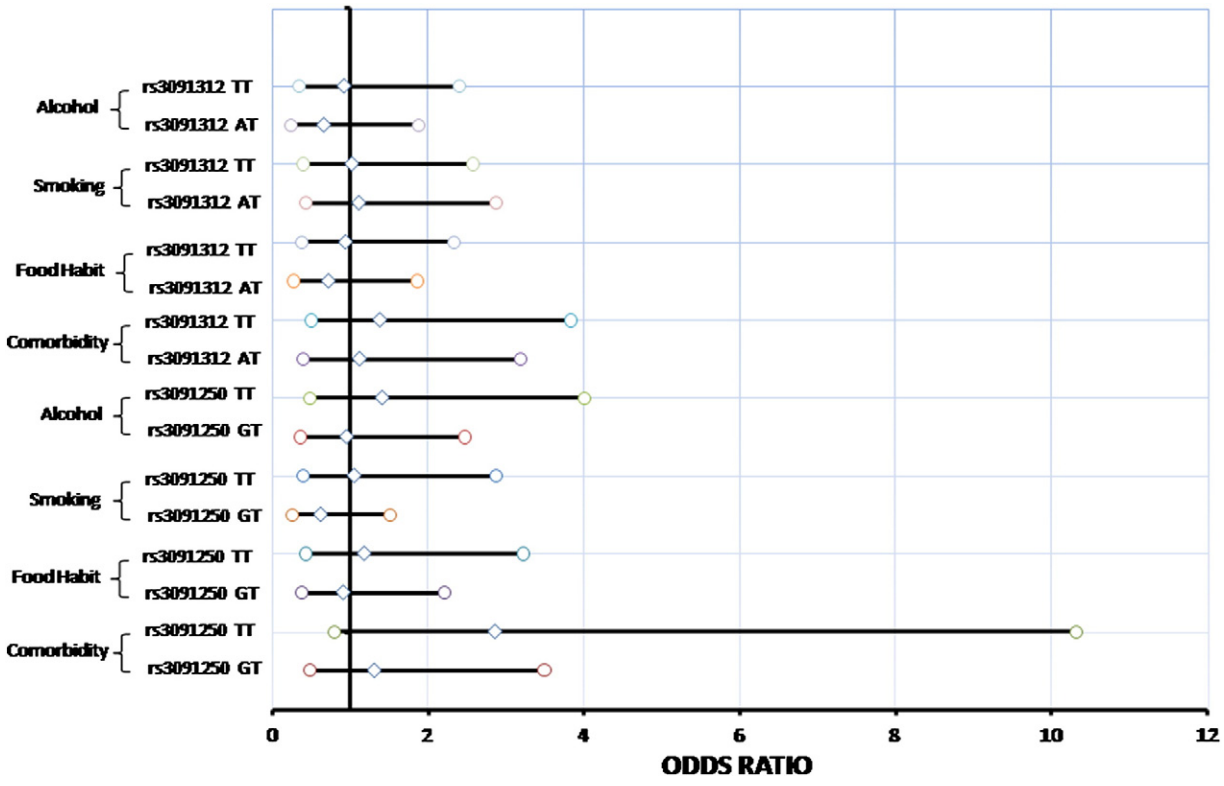

Fig. 2. Univariate logistic regression analysis in comorbidity, food habit, smoking and alcohol as dependent variable and CCR3 rs3091250 & CCR3 rs3091312 polymorphism as independent variable.

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

Acquisition of data and writing of manuscript: NKS. Grant co-PI and editing of manuscript: SP. Inclusion of patients and clinical scoring: AG RS. Statistical analysis: AKB. Interpretation and analysis of data, grant PI and editing of manuscript: AA.

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References

Ahmad, I., et al., 2011. Regulation of ocular angiogenesis by Notch signaling: implications in neovascular age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 52, 2868–2878.
Ambati, J., et al., 2003. An animal model of age-related macular degeneration in senescent Ccl-2 or Ccr-2-deficient mice. Nat. Med. 9, 1390–1397.
Brody, B.L., et al., 2001. Depression, visual acuity, comorbidity, and disability associated with age-related macular degeneration. Ophthalmology 10, 1893–1900.
Cook, H.L., Patel, P.J., Tufail, A., 2008. Age-related macular degeneration: diagnosis and management. Br. Med. Bull. 85, 127–149.
Ding, X., Patel, M., Chan, C.C., 2009. Molecular pathology of age-related macular degeneration. Prog. Retin. Eye Res. 28, 1–18.
Eghøj, M.S., Sorensen, T.L., 2012. Tachyphylaxis during treatment of exudative age-related macular degeneration with ranibizumab. Br. J. Ophthalmol. 96 (1), 21–23.
Fukunaga, K., et al., 2001. Genetic polymorphisms of CC chemokine receptor 3 in Japanese and British asthmatics. Eur. Respir. J. 17 (1), 59–63.
Grimaldi, J.C., et al., 1999. Depletion of eosinophils in mice through the use of antibodies specific for C–C chemokine receptor 3 (CCR3). J. Leukoc. Biol. 65, 846–853.
Heath, H., et al., 1997. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J. Clin. Invest. 99, 178–184.
Kim, S.H., et al., 2008. Association of four-locus gene interaction with aspirin-intolerant asthma in Korean asthmatics. J. Clin. Immunol. 28 (4), 336–342.
Lee, J.H., et al., 2007. Genetic effect of CCR3 and IL5RA gene polymorphisms on eosinophilia in asthmatic patients. J. Allergy Clin. Immunol. 120 (5), 1110–1117.
Li, Y., Huang, D., Xia, X., Wang, Z., Luo, L., Wen, R., 2011. CCR3 and choroidal neovascularization. PLoS One 6, e17106.
Mizutani, N., Nabe, T., Yoshino, S., 2009. Complement C3a regulates late asthmatic response and airway hyperresponsiveness in mice. J. Immunol. 183, 4039–4046.
Moorthy, S., Cheung, N., Klein, R., Shahar, E., Wong, T.Y., 2011. Are lung disease and function related to age-related macular degeneration? Am. J. Ophthalmol. 151, 375–379.
Nishijima, K., et al., 2007. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. Am. J. Pathol. 171, 53–67.
Ober, C., Tsalenko, A., Parry, R., Cox, N.J., 2000. A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. Am. J. Hum. Genet. 67, 1154–1162.
Pons, M., Marin-Castaño, M.E., 2011. Cigarette smoke-related hydroquinone dysregulates MCP-1, VEGF and PEDF expression in retinal pigment epithelium in vitro and in vivo. PLoS One 6 (2), e16722.
Pope, S.M., Zimmermann, N., Stringer, K.F., Karow, M.L., Rothenberg, M.E., 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J. Immunol. 175, 5341–5350.
Rohrer, B., et al., 2009. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 50, 3056–3064.
Saint-Geniez, M., et al., 2008. Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. PLoS One 3, e3554.
Seddon, J.M., Sharma, S., Adelman, R.A., 2006. Evaluation of the clinical age-related maculopathy staging system. Ophthalmology 113, 260–266.
Sharma, N.K., Prabhakar, S., Anand, A., 2009. Age related macular degeneration — advances and trends. Ann. Neurosci. 2, 62–71.
Sun, Y., et al., 2012. Is asthma related to choroidal neovascularization? PLoS One 7 (5), e35415.
Takeda, A., et al., 2009. CCR3 is a target for age-related macular degeneration diagnosis and therapy. Nature 460, 225–230.
The CATT Research Group, 2011. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N. Engl. J. Med. 364, 1897–1908.
Wang, T.N., et al., 2007. The polymorphisms of Eotaxin 1 and CCR3 genes influence on serum IgE, eotaxin levels and mild asthmatic children in Taiwan. Allergy 62 (10), 1125–1130.
Wang, H., Wittchen, E.S., Jiang, Y., Ambati, B., Grossniklaus, H.E., Hartnett, E., 2011. Upregulation of CCR3 by age-related stresses promotes choroidal endothelial cell migration via VEGF-dependent and -independent signaling. Retina 52, 8271–8277.

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Vascular endothelial growth factor and monocyte chemoattractant protein-1 levels unaltered in symptomatic atherosclerotic carotid plaque patients from North India

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We aimed to identify the role of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein (MCP-1) as a serum biomarker of symptomatic carotid atherosclerotic plaque in North Indian population. Individuals with symptomatic carotid atherosclerotic plaque have high risk of ischemic stroke. Previous studies from western countries have shown an association between VEGF and MCP-1 levels and the incidence of ischemic stroke. In this study, venous blood from 110 human subjects was collected, 57 blood samples of which were obtained from patients with carotid plaques, 38 neurological controls without carotid plaques, and another 15 healthy controls who had no history of serious illness. Serum VEGF and MCP-1 levels were measured using commercially available enzyme-linked immunosorbent assay. We also correlated the data clinically and carried out risk factor analysis based on the detailed questionnaire obtained from each patient. For risk factor analysis, a total of 70 symptomatic carotid plaque cases and equal number of age and sex matched healthy controls were analyzed. We found that serum VEGF levels in carotid plaque patients did not show any significant change when compared to either of the controls. Similarly, there was no significant upregulation of MCP-1 in the serum of these patients. The risk factor analysis revealed that hypertension, diabetes, and physical inactivity were the main correlates of carotid atherosclerosis ($p < 0.05$). Prevalence of patients was higher residing in urban areas as compared to rural region. We also found that patients coming from mountain region were relatively less vulnerable to cerebral atherosclerosis as compared to the ones residing at non mountain region. On the contrary, smoking, obesity, dyslipidemia, alcohol consumption, and tobacco chewing were not observed as the determinants of carotid atherosclerosis risk in North India ($p > 0.05$). We conclude that the pathogenesis of carotid plaques may progress independent of these inflammatory molecules. In parallel, risk factor analysis indicates hypertension, diabetes, and sedentary lifestyle as the most significant risk factors of ischemic stroke identified in North India. This could be helpful in early identification of subjects at risk for stroke and devising health care strategies.

Keywords: carotid atherosclerotic plaque, vascular endothelial growth factor, serum protein levels, monocyte chemoattractant protein, enzyme-linked immunosorbent assay

INTRODUCTION

Stroke continues to be the principal contributor of functional impairment and disability in adults and is the second leading cause of death worldwide (Feigin, 2005). It is characterized by a sudden reduction of blood flow in an area of the brain resulting in neurological deficits. Ischemia can produce a transient ischemic attack (TIA) (Kiely et al., 1993) which was defined as acute onset of focal neurological deficit lasting less than 24 h. Deposition of atherosclerotic lesions/plaque in carotid arteries may produce a high risk of ischemic stroke (Aydiner et al., 2007). Therefore, the identification of molecular biomarkers in serum of patients presenting with carotid plaque would assist in the early detection of patients at risk for the ischemic stroke.

Vascular endothelial growth factor (VEGF) has been strongly implicated in brain ischemia (Cobbs et al., 1998). It plays a variety of roles in the disease process, such as forming new and porous blood vessels through a process known as angiogenesis, which stimulates endothelial cells to proliferate and migrate to areas of the brain affected by ischemia (Ferrara et al., 1991; Dvorak et al., 1995; Nagy et al., 2002; Hoeben et al., 2004). In addition to its known role as an angiogenic growth factor, VEGF confers neuroprotection by reducing the neurological damage that occurs after ischemic insult (Yang et al., 2012). VEGF and its receptor VEGFR1/soluble form of full-length transmembrane receptor (sflt-1) are expressed at significantly higher levels in rat neurons after occlusion of the middle cerebral artery (Lennmyr et al., 1998). Analysis of human post-mortem brain tissue after an

ischemic stroke has shown that different isoforms of VEGF, including VEGF165 and VEGF189, as well as their soluble receptors, are expressed at higher levels than in samples from patients without ischemic stroke (Krupinski et al., 1999). Because VEGF plays a crucial role in physiological and pathophysiological angiogenesis, measurement of VEGF in serum is of diagnostic and prognostic value as a marker for atherothrombotic disease. Moreover, inflammatory cytokines that are induced by VEGF, such as monocyte chemoattractant protein (MCP-1) have been previously shown to be involved in the pathogenesis and progression of carotid atherosclerosis (Yamada et al., 2003). Therefore, to further elucidate the role of VEGF and MCP-1 as potential biomarkers in ischemic stroke, we sought to estimate their levels in the sera of stroke patients presenting with carotid atherosclerotic plaques in the North Indian population.

MATERIALS AND METHODS

The study was initiated after getting approval from the ethics committee of the Institute. The study was conducted in the Doppler laboratory, Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.

Following were the Inclusion criteria for enrollment:

1. Patients of ischemic stroke/TIA > 15 years of age
2. Stroke in the anterior circulation confirmed by neuroimaging-Cranial CT or MRI scan
3. Presence of extracranial atherosclerotic disease on cervical duplex ultrasound
4. Fully informed consent available

The Exclusion criteria were:

1. Patients of hemorrhagic stroke or venous strokes
2. Patients with cardioembolic strokes or family history of thrombotic predisposition
3. Patients with short neck, bony abnormality precluding a cervical duplex study
4. Patients with a high cervical bifurcation on duplex ultrasound
5. Pregnancy

SUBJECTS FOR VEGF AND MCP-1 ESTIMATION

We defined symptomatic carotid atherosclerotic plaque as aggregation of plasma lipids (especially cholesterol), cells (smooth muscle cells and monocytes/macrophages), and connective tissue matrix (proteoglycans) in carotid artery as detected by duplex ultrasound (Garcia and Khang-Loon, 1996). Symptomatic patients with age above 15 years; TIA; left or right hemiparesis underwent duplex ultrasound with carotid atherosclerotic plaque. Detected in anterior circulation were included in the study.

Patients who had symptoms of TIA but were not positive for carotid plaque as reported by Doppler ultrasound constituted the neurological controls. All volunteer asymptomatic family members and individuals who accompanied the patients with no history of serious illness constituted the healthy control group. Healthy controls were not subjected to Doppler examination. However, after informed consent, their blood samples were collected and further processed in the neuroscience research lab. The blood samples of all the patients as well as controls were collected randomly without considering any time limit. It should be noted that the patients who came for follow up a few months after they encountered stroke were also included in the study. **Table 1** shows the study population for protein estimation and the duration of disease for patients and neurological controls.

The severity of carotid atherosclerosis was graded according to the stenosis percentage. (1) Mild: with intima media thickness (IMT) >0.08 cm in CCA (common carotid artery) (2) Moderate: stenosis <50% in ICA (internal carotid artery) (3) Severe: stenosis >50% in ICA (internal carotid artery).

VEGF and MCP-1 estimation

Vascular endothelial growth factor and MCP-1 protein levels secreted in the serum of ischemic stroke patients and controls were quantified by enzyme-linked immune assay (Quantikine kits obtained from R&D Systems). All samples were analyzed in duplicate and subsequently used in all further statistical analysis. The assay sensitivity was 5.0 pg/ml for VEGF and 9.0 pg/ml for MCP-1.

Total protein and bovine serum albumin estimation

Total protein and albumin were estimated using Biorad protein assay kit. Five dilutions of a protein standard (representing protein solution to be tested) were prepared. Protein solutions were assayed in duplicate at linear range of 8.0 mg/ml to 80 mg/ml approximately. Standard and sample solution of 160 ml each were dispensed into different microtiter plate wells followed by addition of 40 ml of dye reagent concentrate. Multi channel pipet (to dispense the reagent) was used to mix sample and reagent thoroughly followed by incubation for 5 min at room temperature. Then absorbance was measured using microplate enzyme-linked immunosorbent assay (ELISA) reader at 595 nm and samples were analyzed for total protein. Levels of VEGF and MCP-1 were normalized to total protein and further subjected to statistical analysis.

RISK FACTOR ANALYSIS

The patients with carotid atherosclerotic disease and equal number of age and sex matched healthy controls were analyzed. All the IS patients were interviewed after the duplex ultrasound and the clinical and socio-demographic details including hypertension, diabetes, and lipid profile were tabulated in a pre-validated questionnaire. Socio-demographic details such as body mass index

Table 1 | Study Population for VEGF and MCP-1 estimation.

	Sample size (*n*)	Number of symptomatic patients and controls related to duration of disease			
		1–3 weeks	1–6 months	7–12 months	More than 12 months
Stroke (with carotid plaque)	57	7	30	5	8
Neurological controls (without carotid plaque)	38	6	19	4	9
Healthy controls	15				

(BMI), alcohol consumption, tobacco chewing, smoking, physical inactivity, geographic location (rural/urban), topographic region (mountaineer/plain) was also included. Similar details were collected from controls. Proper informed consent was taken from all the participants. Those who were unable to give their consent were recruited on the basis of an accompanying person's signed agreement. Illiterate people who were not capable to read the agreement were explained the content of the form verbally and were asked to place their thumbprints on the form.

MEASURES

Age and gender of the patients and controls was recorded in the clinical *pro forma*. Most of the variables are self explanatory or otherwise stated.

CLINICAL PARAMETERS

Hypertension

Patients were categorized as hypertensive if the blood pressure was more than 140/90 mmHg or there was a history of receiving anti-hypertensive medications.

Diabetes mellitus

Patients were interviewed whether they were diabetic or non-diabetic. They were confirmed diabetic if their fasting plasma glucose was more than 126 mg/dl as per their reports or if they were receiving any anti-diabetic medications.

Lipid profile

Levels of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides were noted from patients. Patients who did not have lipid test reports were asked to undergo the test and report the levels.

SOCIO-DEMOGRAPHIC PARAMETERS

Body mass index

Patients whose BMI was in the range 25–29.9 were considered overweight and whose BMI fell between 30 and 34.9 were obese.

Smoking

Smoking was classified into two categories: current smokers and non-smokers. Current smokers represented as reported when interviewed. Former smokers included those who smoked for a period of 10 years before stroke onset. Non-smokers were defined as those who had never smoked in their lifetime and the ones who had quit for more than 10 years before the onset of disease.

Physical inactivity

On the basis of low (only walking), moderate, or vigorous exercise, patients were classified as physically active and non-active. Low (only walking) activity was under physically inactive category while moderate and vigorous activity was grouped under physically active category.

Alcohol consumption

Patients were categorized into alcohol consumers and non-alcohol consumers on the basis of alcohol intake. Amount of alcohol was also recorded in the questionnaire from alcohol consumers.

STATISTICAL ANALYSIS

For VEGF and MCP-1 estimation, Mann–Whitney U test was used and $p < 0.05$ was considered statistically significant. Chi-square test (Pearson Uncorrected) was used as a test of significance for socio-demograhic analysis and p-value less than 0.05 was considered as significant. Whenever the values in any of the cells of the contingency table were below 10 Fisher's exact test was applied.

RESULTS

The study population comprised of 110 subjects of whom 57 symptomatic patients with carotid plaque, 38 symptomatic neurological controls without carotid plaque, and asymptomatic 15 healthy controls were enrolled for VEGF and MCP-1 estimation. Baseline characteristics of the study population for risk factor analysis are described in **Table 2**. Prevalence of IS in North India was found more in men than women (71.4% in men). The disease is more commonly seen in elderly people (51.4%). Our results revealed that there was no significant upregulation of VEGF in carotid plaque cases as compared to controls. We also studied MCP-1 levels but here also we did not observe any significant upregulation.

SERUM VEGF LEVELS IN CAROTID PLAQUE PATIENTS

The mean of the VEGF concentration in serum of carotid plaque patients versus healthy controls was 9.7 ± 0.798 pg/ml while the mean VEGF concentration in serum of carotid plaque patients versus neurological controls was 16.468 ± 1.48 pg/ml. VEGF levels were not found to be significantly elevated in patients as compared to either of the controls ($p > 0.05$) (**Figure 1**).

SERUM MCP-1 LEVELS IN CAROTID PLAQUE PATIENTS

The mean of the MCP-1 concentration in serum of carotid plaque patients versus healthy controls was 4.913 ± 0.22 pg/ml while the mean MCP-1 concentration in serum of carotid plaque patients versus neurological controls was 3.904 pg/ml. We did not observe any significant alteration in MCP-1 levels when compared to either of the controls ($p > 0.05$) (**Figure 2**).

GEOGRAPHICAL DISTRIBUTION, DEMOGRAPHY, AND RISK FACTOR ANALYSIS

Clinical details and socio-demographic characteristics of carotid plaque patients in North India are described in **Table 3**. The risk factor analysis revealed that hypertension, diabetes, and physical inactivity were the main correlates of carotid atherosclerosis

Table 2 | Baseline characteristics of study population for clinical/socio-demographic analysis.

Number of carotid plaque patients	70
Men	50 (71.4%)
Mean age (years)	59.3 ± 12.2 years
Range (years)	
30–39	2.9%
40–49	17.6%
50–59	27.9%
60 and above	51.4%

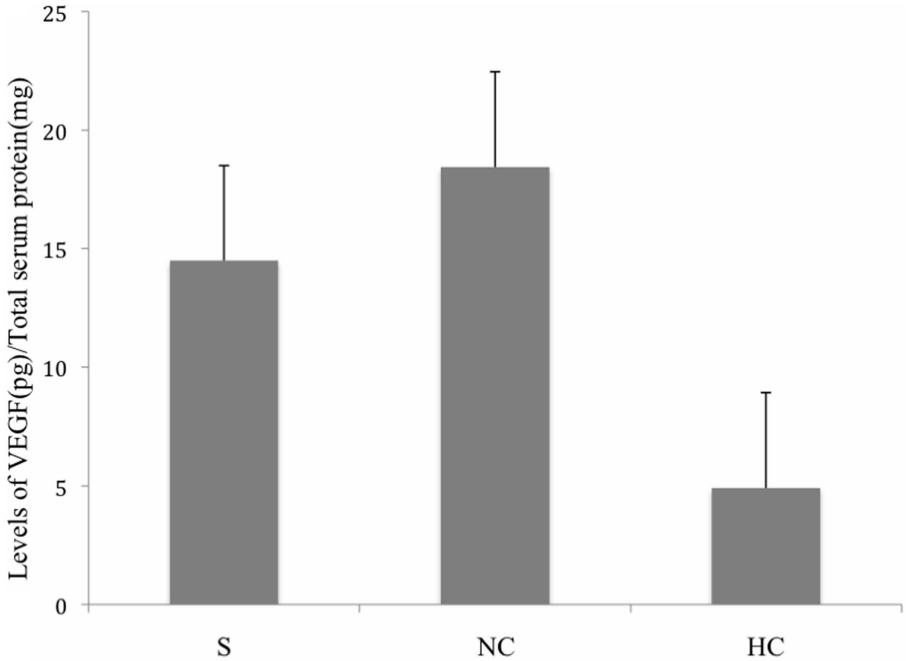

FIGURE 1 | Levels of VEGF in serum of stroke patients, healthy controls, and neurological controls. Group means were plotted ±SE. No significant difference was observed among the given conditions ($p > 0.05$). Data was analyzed by Mann–Whitney Test. Levels of VEGF were normalized to total serum protein. (S, Stroke Patients; NC, Neurological Controls; HC, Healthy Controls).

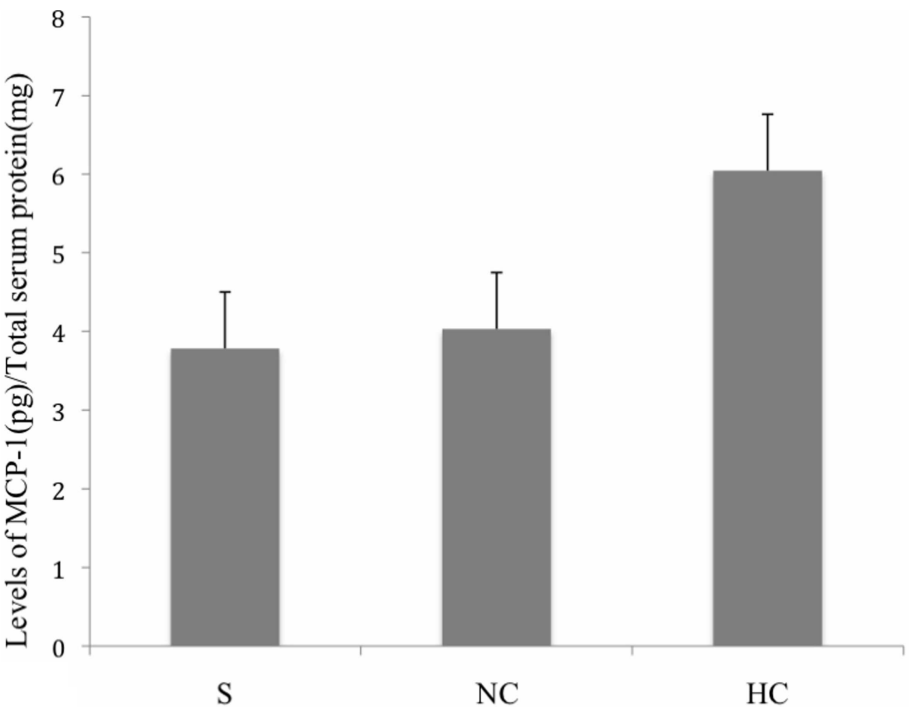

FIGURE 2 | Levels of MCP-1 in serum of stroke patients, neurological control, and healthy control subjects. Group means were plotted ±SE. No significant difference was observed among the given conditions ($p > 0.05$). Data was analyzed by Mann–Whitney Test. Levels of VEGF were normalized to total serum protein. (S, Stroke Patients; NC, Neurological Controls; HC, Healthy Controls).

Table 3 | Clinical details and socio-demographic characteristics of ischemic stroke patients in North India.

Clinical details	Prevalence (%)	Socio-demographic characteristics	Prevalence (%)
Hypertension	72.7	Smoking	20.5
Diabetes	28.7	Alcohol consumption	29.4
Total cholesterol		Physical inactivity	82.08
250–390 mg/dl (high)	5	Tobacco consumption	6
<250	97	Obesity	6
HDL cholesterol		Fish consumption	33.8
<40 mg/dl (high risk)	38.8	Non-vegetarian	56
>40 mg/dl	61.1	BMI; overweight subjects	28
Triglycerides		Topography (plain region)	88.05
>225 mg/dl (high risk)	8.5	Geographic location (urban)	70
<225 mg/dl	88.5		
LDL cholesterol			
>160 mg/dl (high risk)	4.8		
<160 mg/dl (protective)	95		

LDL, low-density lipoprotein; HDL, high-density lipoprotein; BMI, body mass index.

Table 4 | Risk factors of IS with odd ratios, relative risks at 95% CI and *p*-values; *p*-values < 0.05 significant.

Variables	Odds ratio (OR) 95% confidence interval	Relative risks (RR) 95% confidence interval	*p*-value
*Hypertension	12.06 (4.7–31.5)	3.06 (2.04–4.46)	<0.001
*Diabetes[#]	4.68 (1.50–15.5)	1.73 (1.19–2.13)	0.003
Alcohol consumption	1.26 (0.57–2.79)	1.11 (0.76–1.56)	0.519
Smoking	1.92 (0.72–5.19)	1.32 (0.85–1.78)	0.148
Obesity[#]	0.92 (0.19–4.18)	0.95 (0.32–1.73)	1.000
Geographic location	1.53 (0.70–3.34)	1.23 (0.84–1.89)	0.243
BMI (25–29.9 kg/m^2)	1.05 (0.46–2.38)	1.03 (0.64–1.52)	0.886
Tobacco consumption[#]	1.83 (0.27–15.0)	1.27 (0.45–1.85)	0.683
*Physical inactivity	4.73 (2.0–11.1)	1.95 (1.3–2.6)	<0.001

**Statistically significant variable.*

[#]Fisher's exact test.

($p < 0.05$). Prevalence of patients was higher residing in urban areas as compared to rural region. We also found that patients coming from mountaineer region were relatively less vulnerable to cerebral atherosclerosis as compared to the ones residing in non-mountainous region. On the contrary, smoking, obesity, dyslipidemia, alcohol consumption, and tobacco chewing were not observed as the determinants of carotid atherosclerosis risk in North India ($p > 0.05$). **Table 4** illustrates the odd ratios, relative risk at 95% confidence interval and the *p*-values of clinical and socio-demographic parameters.

DISCUSSION

Ischemic stroke has become a major health problem worldwide (Murray and Lopez, 1997), and therefore it is crucial to identify novel biomarkers and preventive strategies for the treatment of the disease. Brain ischemia accounts for a significant proportion of all strokes, and atherosclerosis is considered to be the major cause of most of the brain infarcts (Fuster et al., 1992; Ross, 1999). The atheromatous plaques represent a series of specific cellular and molecular responses that include lipoprotein, hematologic, and inflammatory components (Ross, 1999). Various reports have shown that inflammation may promote atherosclerosis and plaque formation by elevating serum levels of fibrinogen (Ernst and Koenig, 1997), leukocytes (Ernst et al., 1987), clotting factors (Juhan Vague et al., 1996), and cytokines (Dinerman et al., 1990) and by altering the metabolism of endothelial cells and monocyte/macrophages (Dinerman et al., 1990; Celletti et al., 2001). Viral and bacterial infections reflected in elevated levels of various acute-phase proteins (Mattila, 1989) may be partly responsible for the inflammatory processes which in turn may be associated with the occurrence of ischemic symptoms (Mattila et al., 1998). A study identified a particular protein in the sera of healthy subjects, which was initially absent in acute ischemic stroke patients and reappeared after treatment (Kashyap et al., 2006). This finding suggests that the protein may be useful as an important diagnostic marker. Moreover, several growth factors, such as basic fibroblast growth factor (bFGF), VEGF, and MCP-1 are known to play an essential role in mediating recovery in ischemic stroke patients.

The main objective of the current study was to improve our knowledge about the potential biomarkers in the sera of patients presenting with carotid plaque. Since carotid atherosclerotic lesions may develop ischemic stroke, a better understanding and identification of these biomarkers may lead to improved patient care and novel therapeutic approaches for treatment of ischemic stroke. Our study revealed no alteration in the levels of VEGF and MCP-1 after ELISA was conducted. We used various parameters such as socio-demographic variables based on certain risk factors for carotid plaque development such as advanced age, sex, hypertension, diabetes mellitus, and physical inactivity. The male/female sex ratio for stroke in India has been estimated to be 1.7:1 (Sethi, 2002). We noticed that elderly people above 60 years of age are the most affected individuals (51.4%) with men more likely to develop the disease compared to women (71.4% men). In relation to this we report a significant association of hypertension with increased risk of stroke [R 12.06 at 95% CI (4.7–31.5); $p < 0.05$]. Similarly, diabetes mellitus is identified as a putative risk factor for stroke in case-control studies. Our data showed that diabetic individuals are more vulnerable to the disease compared with the ones whose blood sugar level is normal. A significant association of diabetes with the risk of ischemic stroke identifies it to be an important risk factor in North Indian population, like previous studies [OR 4.68 at 95% CI (1.50–15.5); $p < 0.05$]. It is interesting to report there is a conflicting finding published as Dubbo study which did not report diabetes as a risk factor for stroke (Simons et al., 1998). Several lines of evidence suggest a link between physical inactivity and ischemic heart disease (Batty, 2002). Our data is consistent with the previous reports suggesting

physical inactivity to be significantly associated with stroke risk (Paffenbarger and Wing, 1967; Wannamethee and Shaper, 1992; Hu et al., 2000) [OR 4.73 at 95% CI (2.0–11.1); $p < 0.05$]. This finding is consistent with the previous reports (Lanska, 1993). On the other hand no significant association of obesity, smoking, dyslipidemia, alcohol, and tobacco consumption with the stroke risk was found. The result is consistent with the Dubbo study that revealed alcohol intake and smoking as a non-determinant of stroke and indirectly supports of previous studies in which high alcohol consumption has been associated to stroke risk in comparison to moderate consumption. (Gill et al., 1991; Jamrozik et al., 1994; Caicoya et al., 1999; Malarcher et al., 2001). We also did not find any significant linkage of dyslipidemia with the risk of stroke in this report. This may be due to the fact that most of the stroke patients were being followed up and were put on medications. The current finding reveals that the prevalence of stroke was greater among stroke patients residing in urban places (Brown et al., 1996; Sacco et al., 1998; Fang, 2012) than the ones from rural areas but the difference was not statistically significant. The causes to this observation have previously been pointed out to result from less active lifestyle of urban residents than their rural counterparts (Banerjee and Das, 2006; Joshi et al., 2006). Therefore, urbanization might play a crucial role in the pathogenesis of the disease.

This finding is not in agreement with those of many previous reports from outside India where serum VEGF level was found to be upregulated in patients of ischemic stroke. The probable reason for unaltered expression of VEGF in systemic blood flow is enigmatic. We ascribe this unusual finding to limited release of this growth factor localized in and around the damaged tissue than being secreted in circulating blood stream or partly a result of activated negative feedback system (with a speculation that increased VEGF expression in ischemic penumbra may produce a molecular mediator that may turn on an inhibitory feedback mechanism). Expectedly, MCP-1 being induced by the upregulation of VEGF, its level was also found to be equally unaltered (Marumo et al., 1999). Since Cooper et al. (1999) observed an elevated expression of VEGF in experimental diabetes many studies have estimated the VEGF levels in different diseases (Slevin et al., 2000; Andrew et al., 2002; Blann et al., 2002). Also an enhanced expression of MCP-1 in patients with ischemic stroke and myocardial infarction have been observed in some studies (Arakelyan et al., 2005). It has been suggested that lifestyle of an individual has great impact on risk factors associated with stroke (Welin et al., 1987). Individuals with hypertension and diabetes have twice to sixfold chances of having stroke as per published reports (Kannel and McGee, 1979; Wolf et al., 1992; Burchfiel et al., 1994). Similarly, the results of Framingham Heart Study revealed the effect of diabetes on cerebral and peripheral arteries (Benjamin et al., 1998). Individual studies in India have estimated the prevalence rate of the ischemic stroke from 21/100,000 for individuals in 20–40 years of age group to 625/100,000 above 60 years age and 27–34/100,000 in the 35–44 age group to 822–1116/100,000 above 75 years age (Bansal et al., 1973; Razdan et al., 1989; Dhamija et al., 2000; Dalal, 2008; Sridharan, 2009). Moderate physical exercise is reported to reduce the depression rate and prevents the occurrence of heart disease and stroke by lowering the blood pressure and raising the level of HDL cholesterol in blood (Shephard, 1997; Thompson et al., 2003) which are in support to a study done in Italian population which stated that moderate amount of exercise may help in reducing the risk of stroke (Menotti and Seccareccia, 1985). The current data shows that people residing at mountain region have low rates of stroke as compared to the ones residing at plain region. Another modifiable factor, cigarette smoking, has been reported to be associated with all types of stroke including ischemic stroke, intra-cerebral, and subarachnoid hemorrhage (Shinton and Beevers, 1989; Juvela et al., 1993; Kurth et al., 2003). In addition, risk of having stroke declines when smoking is discontinued (Feigenbaum, 1993). In view of these contradictory reports, alcohol consumption, dyslipidemia, and smoking as a stroke predictor remain controversial pending population-based prospective studies.

The present study had some limitations like risk factors in other subtypes of stroke, socio-demographic parameters such as depression or stress were not assessed as well as no mechanistic information regarding stroke prevention and rehabilitation was provided. Still there is a great promise in the search for serum biomarkers to help in the prognosis of atherosclerotic disease but many theoretical and practical challenges stand in the way. Further performing studies with a large patient cohort focusing on risk factors of stroke are however, necessary to examine additional biomarkers including VEGF and MCP-1 with reduced sample time after stroke onset to corroborate this preliminary data.

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AUTHOR NOTE

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.

REFERENCES

Andrew, D. B., Funmi, M. B., McCollum, C. N., Stanley, S., Peck, L. L., Gregory, Y. H. (2002). Vascular endothelial growth factor and its receptor, Flt-1, in the plasma of patients with coronary or peripheral atherosclerosis, or type II diabetes. *Clin. Sci.* 102, 187–194.

Arakelyan, A., Petrkova, J., Hermanova, Z., Boyajyan, A., Lukl, J., and Petrek, M. (2005). Serum levels of the MCP-1 chemokine in patients with ischemic stroke and myocardial infarction. *Mediators Inflamm.* 3, 175–179.

Aydiner, O., Boztosun, B., Sirvanci, M., Akçakoyun, M., Karaman, K., Aksoy, T., et al. (2007). Early and late outcomes of carotid artery stenting. *Anadolu Kardiyol. Derg.* 7, 152–157.

Banerjee, T., and Das, S. (2006). Epidemiology of stroke in India. *Neurol. Asia* 11, 1–4.

Bansal, B. C., Parkash, C., Jain, A. C., and Brahmanandan, K. R. V. (1973). Cerebrovascular disease in young individuals below the age of 40 years. *Neurol. India* 21, 11–18.

Batty, G. D. (2002). Physical activity and coronary heart disease in older adults – a review of epidemiological studies. *Eur. J. Public. Health* 12, 171–176.

Benjamin, E. J., Wolf, P. A., D'Agostino, R. B., Silbershatz, H., Kannel, W. B., and Levy, D. (1998). Impact of atrial fibrillation on the risk of death: the Framingham heart study. *Circulation* 98, 946–952.

Blann, A. D., Belgore, F. M., McCollum, C. N., Silverman, S., Lip, P. L., and Lip, G. Y. (2002). Vascular

endothelial growth factor and its receptor, Flt-1, in the plasma of patients with coronary or peripheral atherosclerosis, or Type II diabetes. *Clin. Sci. (Lond.).* 102, 187–194.

Brown, R. D., Whisnant, J. P., Sicks, J. D., O'Fallon, W. M., and Wiebers, D. O. (1996). Stroke incidence, prevalence, and survival: secular trends in Rochester, Minnesota, through 1989. *Stroke* 27, 373–380.

Burchfiel, C. M., Curb, J. D., Rodriguez, B. L., Abbott, R. D., Chiu, D., and Yano, K. (1994). Glucose intolerance and 22-year stroke incidence: the Honolulu heart program. *Stroke* 25, 951–957.

Caicoya, M., Rodriguez, T., Corrales, C., Cuello, R., and Lasheras, C. (1999). Alcohol and stroke. A community case control study in Asturias, Spain. *J. Clin. Epidemiol.* 52, 677–684.

Celletti, F. L., Waugh, J. M., Amabile, P. G., Brendolan, A., Hilfiker, P. R., and Dake, M. D. (2001). Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat. Med.* 7, 425–429.

Cobbs, C. S., Chen, J., Greenberg, D. A., and Graham, S. H. (1998). Vascular endothelial growth factor expression in transient focal cerebral ischemia in the rat. *Neurosci. Lett.* 249, 79–82.

Cooper, M. E., Vranes, D., Youssef, S., Stacker, S. A., Cox, A. J., Rizkalla, B., et al. (1999). Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* 48, 2229–2239.

Dalal, P. (2008). Population-bases stroke survey in Mumbai, India: incidence and 28-day case fatality. *Neuroepidemiology* 31, 254–261.

Dhamija, R. K., Mittal, S., and Bansal, B. C. (2000). Trends in clinico-epidemiological correlates of stroke in the community. *J. Indian Acad. Clin. Med.* 1, 27–31.

Dinerman, J. L., Mehta, J. L., Saldeen, T. G., Emerson, S., Wallin, R., Davda, R., et al. (1990). Increased neutrophil elastase release in unstable angina pectoris, and acute myocardial infarction. *J. Am. Coll. Cardiol.* 15, 1559–1563.

Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* 146, 1029–1039.

Ernst, E., Hammerschmidt, D. E., Bagge, U., Matrai, A., and Dormandy, J. A. (1987). Leukocytes and the risk of ischemic diseases. *JAMA* 257, 2318–2324.

Ernst, E., and Koenig, W. (1997). Fibrinogen and cardiovascular risk. *Vasc. Med.* 2, 115–125.

Fang, J. (2012). Prevalence of stroke – United States, 2006–2010. *MMWR* 61, 379–382.

Feigenbaum, L. Z. (1993). Smoking cessation and risk of stroke in women. *JAMA* 269, 2214–2214.

Feigin, V. L. (2005). Stroke epidemiology in the developing world. *Lancet* 365, 2160–2161.

Ferrara, N., Houck, K. A., Jakeman, L. B., Winer, J., and Leung, D. W. (1991). The vascular endothelial growth factor family of polypeptides. *J. Cell. Biochem.* 47, 211–218.

Fuster, V., Badimon, L., Badimon, J. J., and Chesebro, J. H. (1992). The pathogenesis of coronary artery disease and the acute coronary syndromes. *N. Engl. J. Med.* 326, 242–250.

Garcia, J. H., and Khang-Loon, H. (1996). Carotid atherosclerosis. Definition, pathogenesis, and clinical significance. *Neuroimaging Clin. N. Am.* 6, 801–810.

Gill, J. S., Shipley, M. J., Tsementzis, S. A., Hornby, R. S., Gill, S. K., Hitchcock, E. R., et al. (1991). Alcohol consumption: a risk factor for hemorrhagic and nonhemorrhagic stroke. *Am. J. Med.* 90, 489–497.

Hoeben, A., Landuyt, B., Highley, M. S., Wildiers, H., Van Oosterom, A. T., and De Bruijn, E. A. (2004). Vascular endothelial growth factor and angiogenesis. *Pharmacol. Rev.* 56, 549–580.

Hu, F. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., Rexrode, K. M., Willett, W. C. (2000). Physical activity and risk of stroke in women. *JAMA* 283, 2961–2967.

Jamrozik, K., Broadhurst, R. J., Anderson, C. S., Stewart-Wynne, E. G. (1994). The role of lifestyle factors in the etiology of stroke: a population-based case-control study in Perth, Western Australia. *Stroke* 25, 51–59.

Joshi, R., Cardona, M., Iyengar, S., Sukumar, A., Raju, C. R., Raju, K. R., et al. (2006). Chronic disease now a leading cause of death in rural India-mortality data from the Andhra Pradesh rural health initiative. *Int. J. Epidemiol.* 35, 1522–1529.

Juhan Vague, I., Pyke, S. D. M., Alsse, M. C., Jesperson, J., Haverkate, F., and Thompson, S. G. (1996). Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. *Circulation* 94, 2057–2063.

Juvela, S., Hillbom, M., Numminen, H., Koskinen, P. (1993). Cigarette smoking and alcohol consumption as risk factors for aneurysmal subarachnoid hemorrhage. *Stroke* 24, 639–646.

Kannel, W. B., and McGee, D. L. (1979). Diabetes and cardiovascular disease: the Framingham study. *JAMA* 241, 2035–2038.

Kashyap, R. S., Kabra, D. P., Nayak, A. R., Mishra, R. N., Deshpande, S. K., Karandikar, P. N., et al. (2006). Protein electrophoretogram in serum of acute ischemic stroke patients & its correlation with S-100ββ and neuron specific enolase level: a pilot study. *Ann. Neurosci.* 13, 36–40.

Kiely, D. K., Wolf, P. A., Cupples, L. A., Beiser, A. S., and Myers, R. H. (1993). Familial aggregation of stroke: the Framingham study. *Stroke* 24, 1366–1371.

Krupinski, J., Bujny, T., Kumar, S., Kaluza, P., and Kumar, P. (1999). Vascular endothelial growth factor and its receptor, KDR, in human brain tissue after ischaemic stroke. *Lab. Invest.* 79, 417–425.

Kurth, T., Kase, C. S., Berger, K., Gaziano, J. M., Cook, N. R., Buring, J. E., et al. (2003). Smoking and risk of hemorrhagic stroke in women. *Stroke* 34, 2792–2795.

Lanska, D. J. (1993). Geographic distribution of stroke mortality in the United States: 1939–1941 to 1979–1981. *Neurology* 43, 1839–1851.

Lennmyr, F., Ata, K. A., Funa, K., Olsson, Y., and Terent, A. (1998). Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. *J. Neuropathol. Exp. Neurol.* 57, 874–882.

Malarcher, A. M., Giles, W. H., Croft, J. B., Wozniak, M. A., Wityk, R. J., Stolley, P. D., et al. (2001). Alcohol intake, type of beverage, and the risk of cerebral infarction in young women. *Stroke* 32, 77–83.

Marumo, T., Schini-Kerth, V. B., and Busse, R. (1999). Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes* 48, 1131–1137.

Mattila, K. J. (1989). Viral and bacterial infections in patients with acute myocardial infraction. *J. Intern. Med.* 225, 293–296.

Mattila, K. J., Valtonen, V. V., Nieminen, M. S., and Asikainen, S. (1998). Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. *Clin. Infect. Dis.* 26, 719–734.

Menotti, A., and Seccareccia, F. (1985). Physical activity at work and job responsibility as risk factors for fatal coronary heart disease and other causes of death. *J. Epidemiol. Community. Health* 39, 325–329.

Murray, C. J., and Lopez, A. D. (1997). Mortality by cause for eight regions of the world: global burden of disease study. *Lancet* 349, 1269–1276.

Nagy, J. A., Vasile, E., Feng, D., Sundberg, C., Brown, L. F., Detmar, M. J., et al. (2002). Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J. Exp. Med.* 196, 1497–1506.

Paffenbarger, R. S., and Wing, A. L. (1967). Characteristics in youth predisposing to fatal stroke in later years. *Lancet* 1, 753–754.

Razdan, S., Kaul, R. L., Motta, A., and Kaul, S. (1989). Cerebrovascular disease in rural Kashmir, India. *Stroke* 20, 1691–1693.

Ross, R. (1999). Atherosclerosis: an inflammatory disease. *N. Engl. J. Med.* 340, 115–126.

Sacco, R. L., Boden-Albala, B., Gan, R., Chen, X., Kargman, D. E., Shea, S., et al. (1998). Stroke incidence among white, black, and Hispanic residents of an urban community: the Northern Manhattan stroke study. *Am. J. Epidemiol.* 147, 259–268.

Sethi, P. (2002). Stroke-incidence in India and management of ischaemic stroke. *Neurosciences* 4 , 139–141.

Shephard, R. J. (1997). Exercise and relaxation in health promotion. *Sports Med.* 23, 211–216.

Shinton, R., and Beevers, G. (1989). Meta-analysis of relation between cigarette smoking and stroke. *BMJ* 298, 789–794.

Simons, L. A., McCallum, J., Friedlander, Y., and Simons, J. (1998). Risk Factors for Ischemic Stroke Dubbo Study of the Elderly. *Stroke* 29, 1341–1346.

Slevin, M., Krupinski, J., Slowik, A., Kumar, P., Szczudlik, A., and Gaffney, J. (2000). Serial measurement of vascular endothelial growth factor and transforming growth factor β1 in serum of patients with acute ischemic stroke. *Stroke* 31, 1863–1870.

Sridharan, S. (2009). Incidence, types, risk factors and outcome of stroke in a developing country: the Trivadrum stroke registry. *Stroke* 40, 1212–1218.

Thompson, P. D., Buchner, D., Pina, I. L., Williams, M. A., Marcus, B. H., Berra, K., et al. (2003). American heart association scientific statement: exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. *Circulation* 107, 3109–3116.

Wannamethee, G., and Shaper, A. G. (1992). Physical activity and stroke in British middle-aged men. *BMJ* 304, 597–601.

Welin, L., Svardsudd, K., Wilhelmsen, L., Larsson, B., Tibblin, G. (1987). Analysis of risk factors for stroke in a cohort of men born in 1913. *N. Engl. J. Med.* 317, 521–526.

Wolf, P. A., D'Agostino, R. B., O'Neal, M. A., Sytkowski, P., Kase, C. S., Belanger, A. J., et al. (1992). Secular trends in stroke incidence and mortality: the Framingham study. *Stroke* 23, 1551–1555.

Yamada, M., Kim, S., Egashira, K., Takeya, M., Ikeda, T., and Mimura, O. (2003). Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. *Arterioscler. Thromb. Vasc. Biol.* 23, 1996–2001.

Yang, J., Guo, L., Liu, R., and Liu, H. (2012). Neuroprotective effects of VEGF administration after focal cerebral ischemia/reperfusion: dose response and time window. *Neurochem. Int.* 60, 592–596.

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New Biomarker for Neovascular Age-Related Macular Degeneration: Eotaxin-2

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Recently, eotaxin–CCR3 was reported to play an important role in choroidal neovascularization (CNV) development and was documented to be superior than vascular endothelial growth factor-A treatment when tested in CNV animals. As eotaxin studies are lacking in the human age-related macular degeneration (AMD) patients, we sought to determine whether eotaxin-2 (CCL24) has any association with inflammatory processes that occur in CNV. CCL24 levels were determined by enzyme linked immunosorbant assay (ELISA) after normalization to total serum protein and levels of ELISA were correlated to various risk factors in about 133 AMD patients and 80 healthy controls. The CCL24 levels were significantly higher in wet AMD patients as compared with dry AMD and normal controls. There was a significant difference when compared among wet AMD patients (i.e., minimally classic, predominantly classic, and occult). We also report significant difference in the CCL24 levels of Avastin-treated and untreated AMD patients. This study shows that CCL24 levels were found to be significantly increased in AMD patients despite Avastin treatment as compared with normal controls and those without Avastin, indicating that CCL24 may have an association with CNV and may be an important target to validate future therapeutic approaches in AMD in tandem with Avastin treatment.

Introduction

Age-related macular degeneration (AMD) is among the commonest causes of blindness across the world. There are about 30–50 million people affected globally in which 90% of vision loss is due to wet form of AMD (Ambati *et al*., 2003). It is estimated that by 2020 about 2.95 million people in the United States will have advanced AMD and data on AMD in India show that prevalence from 2.7% (early age related macular degeneration [ARMD]) to 0.6% (late ARMD) in South India to 4.7% in North India (Azad *et al*., 2007). The pathogenesis of AMD is complex and many risk factors, such as age, family history, and smoking, have earlier been implicated in the pathogenesis of AMD. The wet AMD is the severe form of disease causing blindness while the dry form is the milder variant characterized by geographic atrophy. Earlier studies in genetics, environment, and demography association have failed to conclusively establish any potential predictor of AMD. Besides, very few studies have been conducted in Indian subcontinent. Currently, there is no treatment for this disease and successful targeting of abnormal choroidal neovascularization (CNV) remains critical in reducing the burden of disease. One of the standard treatments in opthalmology practice includes the use of anti-vascular endothelial growth factor (anti-VEGF) therapy, such as Avastin (bevacizumab) or Lucentis (ranibizumab). Anti-VEGF that constitutes these drugs have, therefore, emerged as one of the treatments for patients with CNV but this does not completely treat AMD with only about one-third of patients are believed to benefit from it. Early diagnosis and better cure of CNV may increase the success rate of such intervention. There are many human and animal studies that have reported the role of various genes responsible for AMD. Many multiple genetic studies have established the role of inflammation in AMD. For example, CFH gene is involved in the complement pathway; the complement component 2 gene and the complement factor B gene have repetitively been associated with AMD (Maller *et al*., 2006). It is believed that complement activation resulting from dysfunction of these genes may contribute to inflammatory response. In the infiltration of monocytes for inflammation, CCL2 and CCR2 are the key mediators. We previously described the spontaneous development of CNV in senescent mice deficient in monocyte chemoattractant protein-1 (CCL2, also known as MCP-1) or its receptor postulating its key role in AMD pathogenesis (Ambati *et al*., 2003b). CCL2 thus plays an important role in regulating monocyte trafficking to sites of inflammation besides CCR1, CFH, and CCR5 whose association with eotaxin 2 (CCL24) has not been adequately studied. The eotaxins are a family of

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CC chemokines that direct the recruitment of inflammatory cells, in particular, eosinophils, to locations of allergic inflammation. CCL24 induced chemotaxis of eosinophils as well as basophils, with a typically bimodal concentration dependence, and the release of histamine and leukotriene C4 from basophils that had been primed with interleukin-3 (IL-3) (Forssmann *et al.*, 1997). CCL24 interacts with chemokine receptor CCR3 to induce chemotaxis in eosinophils (White *et al.*, 1997). It is most frequently associated with eosinophils and mast cells that play a major role in allergic reactions (Teixeira *et al.*, 1997; Humbles *et al.*, 2004; Pope *et al.*, 2005) as well as angiogenesis (Salcedo *et al.*, 2001) and have previously been analyzed in serum by ELISA (Kagami *et al.*, 2003; Jahnz *et al.*, 2005; Manousou *et al.*, 2010; Mo *et al.*, 2010).

We hypothesized that CCL24 is also involved in similar processes that result in chronic inflammation by sustained activation of complement system contributing to the pathogenesis of AMD. This CCL24 is located on human chromosome 7. Takeda *et al.* (2009) had recently shown that the CCL11 and CCL24 protein levels increased in wild-type mice after laser injury and the CCL11 and CCL24 neutralizing antibodies are more effective than VEGF-A neutralizing antibodies (68.63% vs. 57.64%) at inhibiting laser-induced CNV in mice, indicating their causal role in pathogenesis of AMD. They showed that eotaxin–CCR3 signaling has an active role in CNV: *in vitro*, eotaxin–CCR3 stimulates proliferation of human choroidal endothelial cells, and in a mouse model of CNV involving laser injury to the eye, intraocular administration of eotaxin and CCR3-specific neutralizing antibodies or a small inhibitor molecule reduces the laser-induced pathology. Further, genetically engineered mice that were deficient in eotaxin or its receptor CCR3 were also protected to some extent from the effect of laser injury on the choroidal vasculature. It was also reported that the mRNA for ligands of CCR3 (CCL11, CCL24, and CCL26) was increased in response to hydrogen peroxide challenge (Wang *et al.*, 2011). Another contradictory report sought to dispel any association of AMD with CCR3. Li *et al.* (2011) provided evidence from animals that VEGF-A neutralizing antibodies significantly suppressed CNV as compared with CCR3, thus playing no significant role in CNV development. We therefore estimated the expression of *CCL24* in the serum of human AMD patients which was previously shown as constitutively expressed eosinophil chemokine likely to be involved in homeostatic, allergen-induced, and IL-4-associated immune responses (Zimmermann *et al.*, 2000).

Materials and Methods

Ethical clearance

Ethical clearance was obtained for this study by the Institute Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, India (vide letter No. Micro/10/1411). Informed consent was obtained in the prescribed format endorsed by the Institute Ethical Committee.

Inclusion and exclusion criteria

Fifty years or older AMD patients with the diagnosis of advanced AMD as defined by geographic atrophy and/or CNV with drusen more than 5 in at least one eye were included in the study. 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 synechia, cataracts, and opacities due to ocular diseases.

Ophthalmic examination

All the patients were examined 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. The diagnosis of AMD was based on ophthalmoscopic and FFA findings. AMD patients were divided into wet and dry AMD patients. Wet AMD patients were further divided into minimally classic AMD, predominantly classic AMD, and occult AMD (Shah and Del Priore, 2009).

Serum separation

About 4.0 mL of blood sample was collected and left for 1 h at 37°C to allow it to clot and serum was subsequently separated in serum separator tube (BD Biosciences) after centrifugation at 3000 rpm for 30 min.

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 min. The absorbance was read at 595 nm in Microplate reader (680XR; BioRad). The standard curve of BSA was estimated with linear or quadratic fit models.

Eotaxin quantitation

The expression of CCL24 was analyzed using commercially available enzyme-linked immunosorbant assay (RayBio; Cat. No. ELH-Eotaxin2-001) as per manufacturer's protocol and absorbance was read at 450 nm using 680XR model of Microplate reader (BioRad). Sample assays were performed in duplicate. This assay recognizes recombinant and natural human CCL24 with detection range of 0.87–5.2 pg/mL. The linear regression analysis was used to generate the standard curve for CCL24 estimation in both patients and controls. All the values were normalized to total serum protein.

Demographic characterization

A trained staff interviewed all subjects 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 risk factor questionnaire included information about demographic characteristics, cigarette smoking, alcohol intake, and others. Smokers were defined as having smoked at least one cigarette per day for at least 6 months and segregated into smokers and nonsmokers. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg at examination, or diagnosed by a physician previously and self-reported by the participant's responses to whether a physician had ever informed them of this diagnosis and whether they had ever taken medications for this condition. Similar protocols have been used earlier in previous studies (Tin *et al.*, 2002). Subjects were also asked to report any prior diagnosis of stroke, use of antihypertensive medications, diabetes, migraine, and history of heart diseases.

Statistical analysis

The study was analyzed in three phases. In the first phase, 79 subjects were recruited during the year of 2008–2009 for preliminary study, which included 47 AMD patients (32 wet and 15 dry patients) and 32 controls. Later, the second phase of the study was initiated by including additional 134 subjects during the year of 2009–2011; this included 86 AMD patients (23 dry AMD and 63 wet AMD) and 48 healthy controls. Out of 86 AMD and 48 control samples, about 4 samples were destroyed due to unforeseen reasons, including handling or delayed refrigeration.

The statistical analysis for this study was executed at three stages. First stage or Phase I stage analysis was mainly confined to assess the significant variation of the eotaxin-2 levels between AMD and control subjects along with the role of hypertension in moderating eotaxin-2 levels. The data were thus analyzed using independent *t*-test and analysis of variance (ANOVA) with *post hoc* analysis at 5% level of significance under the pretext objectives, whereas descriptive statistics was represented by scatter diagram and table. Second stage or Phase II analysis was also conducted in a similar fashion. However, the final analysis was based on complete data of 213 observations that included 38 dry AMD, 95 wet AMD, and 80 healthy controls. The final analysis included 184 observations, because 29 observations had to be abandoned as one or more observations of particular variables were missing. Under this study, almost all probable risk factors of AMD were recorded for each patient/control, which has been included in the extended analysis. Descriptive study in the final analysis is presented using tabular and graphical methods using appropriate measures of central tendency/dispersion based on the nature of variables. To obtain the most significant factor that influenced the CCL24 levels, a linear model was applied using CCL24 as dependent variable and other factors as independent variables. All statistical analyses were performed in R version 2.13.0 (2011-04-13).

Results

Phase I study was performed to validate the hypothesis whether levels of CCL24 are associated with AMD pathogenesis in patients of Indian origin. The outcome of the study endorsed the hypothesis at 5% level of significance. Hence, the study was extended in Phase II and Phase III including additional patients and controls. Therefore, the study was conducted in three phases in order to validate the new findings and accord significance to the results. The demographic details of all AMD patients and controls are reproduced in Table 1.

Table 1. Demographic Characteristics of Controls and Age-Related Macular Degeneration Patients

Variables	*AMD*	*Controls*
Total	133	80
Wet AMD	95 (71.4%)	–
Dry AMD	38 (28.6%)	–
Duration of disease* (months)	23 ± 2.6	–
Age** (years)	66.56 ± 7.6	54.24 ± 7.01
CCL24 (pg/μg)	0.0216 ± 0.016	0.0130 ± 0.008
Men	88 (66.2%)	57 (71.2%)
Women	45 (33.8%)	23 (28.7%)

Age, age of onset; values are mean ± SD or (percentage). AMD subjects were asked to provide all clinical and demographic details at the age of disease onset.

*Duration of disease is the interval between appearance of first symptom of AMD and collection of sample.

**Unpaired, independent 2-tailed Student's *t*-test analysis showed that mean age differs significantly among the groups ($p = 0.02$).

AMD, age-related macular degeneration.

Levels of CCL24 in AMD and controls

The role of CCL24 in pathogenesis of AMD patients became apparent after completion of all phases of analysis. ANOVA followed by Fisher's least significant difference *post hoc* analysis showed that CCL24 levels were significantly elevated in wet AMD (0.0236 pg/μg) patients as compared with the dry AMD (0.0167 pg/μg) and controls (0.0130 pg/μg) (Table 2; Fig. 1A–C and Fig. 3; $p = 0.02$ and $p = 0.0001$, respectively). Parametric unpaired, independent 2-tailed Student's *t*-test revealed significant difference in CCL24 levels between AMD patients (0.0216 pg/μg) and controls (0.0130 pg/μg) (Table 2; Fig. 2; $p = 0.0001$). In all three phases, the levels of CCL24 were found to be increased in AMD patients. Further, the difference was significant when

Table 2. Comparison of CCL24 Levels Among Age-Related Macular Degeneration Patients and Normal Controls

Study	*Subjects*	n	p-*Value*
Phase I	AMD/Control	47/32	0.0001
	Dry/Control	15/32	0.07
	Wet/Control	32/32	0.02
	Dry/Wet	15/15	0.99
Phase II	AMD/Control	83/44	0.0001
	Dry/Control	22/44	0.50
	Wet/Control	61/44	0.0001
	Dry/Wet	22/61	0.02
Phase III (total)	AMD/Control	130/76	0.0001
	Dry/Control	37/76	0.34
	Wet/Control	93/76	0.0001
	Dry/Wet	37/93	0.02

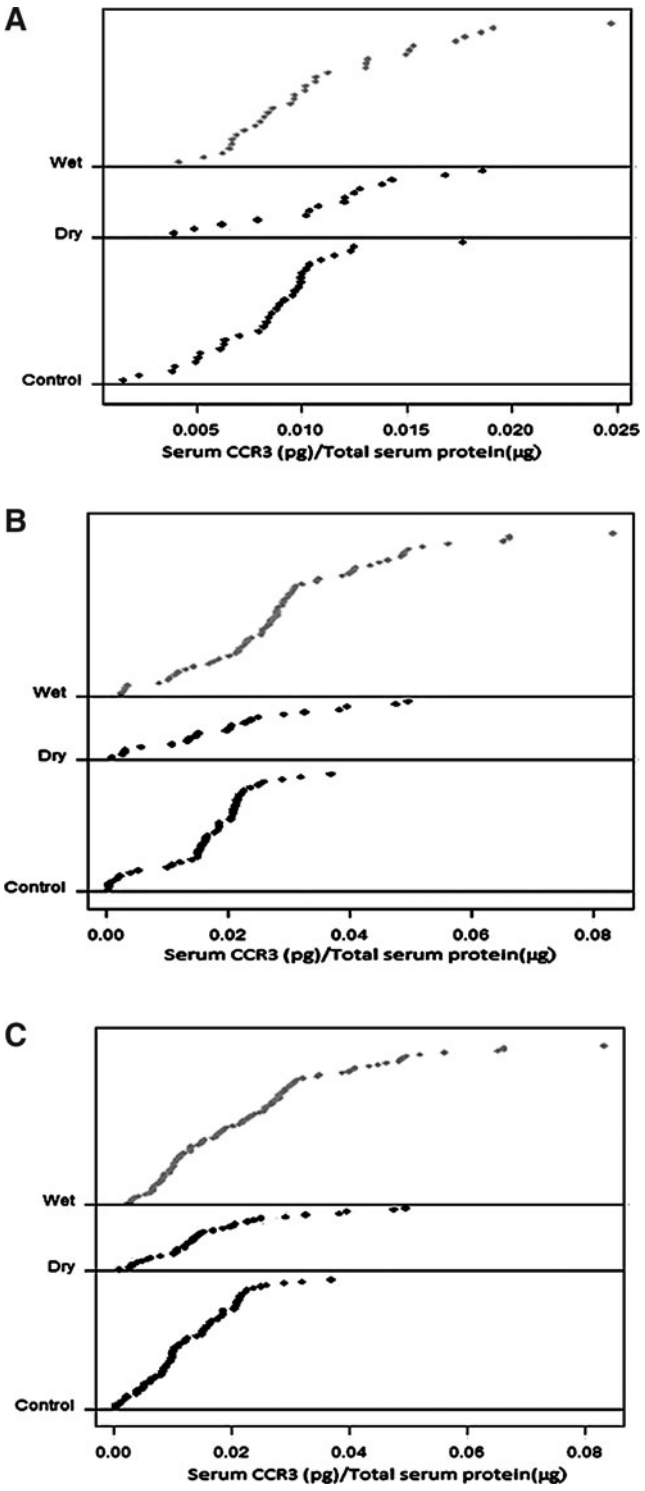

FIG. 1. **(A)** Diagnosis-wise distribution of CCL24 (Phase I). **(B)** Diagnosis-wise distribution of CCL24 (Phase II). **(C)** Diagnosis-wise distribution of CCL24 (Total).

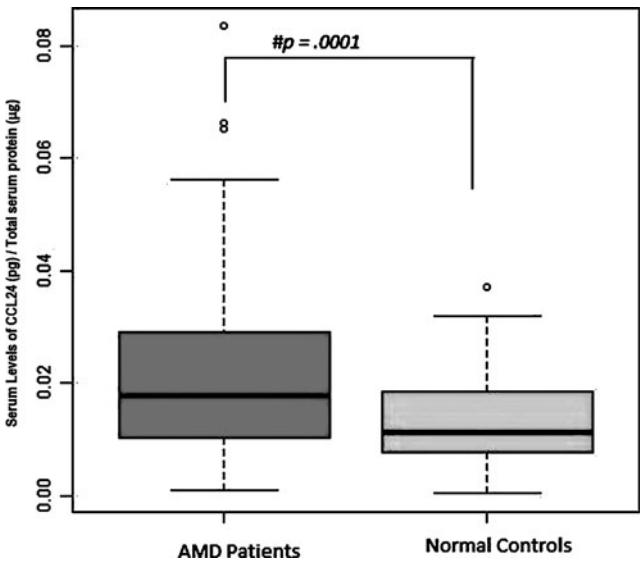

FIG. 2. Serum levels of CCL24 in age-related macular degeneration (AMD) and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by parametric unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). pg, pictogram; µg, microgram.

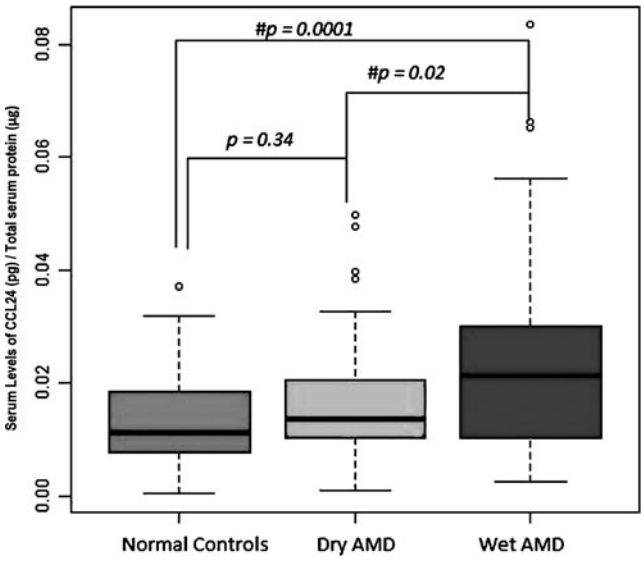

FIG. 3. Serum levels of CCL24 in dry AMD, wet AMD, and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* analysis among the groups. pg, pictogram; µg, microgram.

compared between the wet AMD patients. The levels were significantly elevated in the occult (0.026 pg/µg) and predominantly classic AMD (0.021 pg/µg) as compared with minimally classic AMD (0.009 pg/µg) (Fig. 4). CCL24 levels were significantly increased in the individuals with one eye

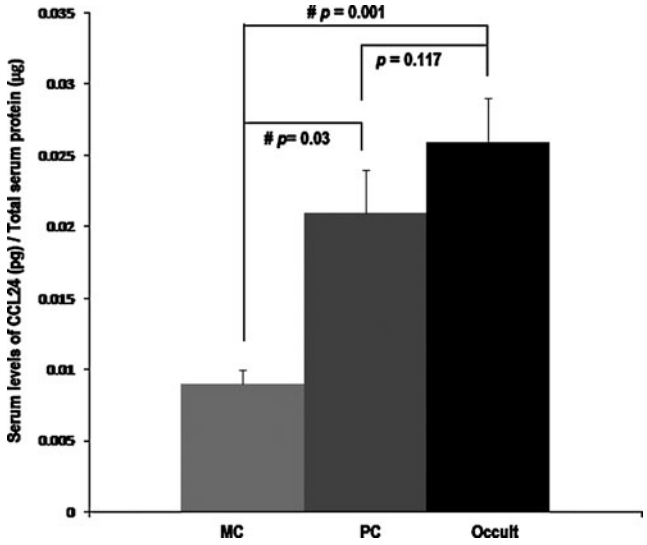

FIG. 4. Serum levels of CCL24 in minimally classic (MC), predominantly classic (PC), and occult AMD patients. Values are plotted as mean ± standard error in the bar diagram. Data were analyzed by unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). Levels of CCL24 were normalized to total protein.

(0.0201 pg/µg) or both eyes affected (0.0226 pg/µg) as compared with normal controls (Fig. 5).

Effect of Avastin treatment on CCL24

CCL24 levels were also compared between wet variant of AMD patients who received single Avastin treatment (dose

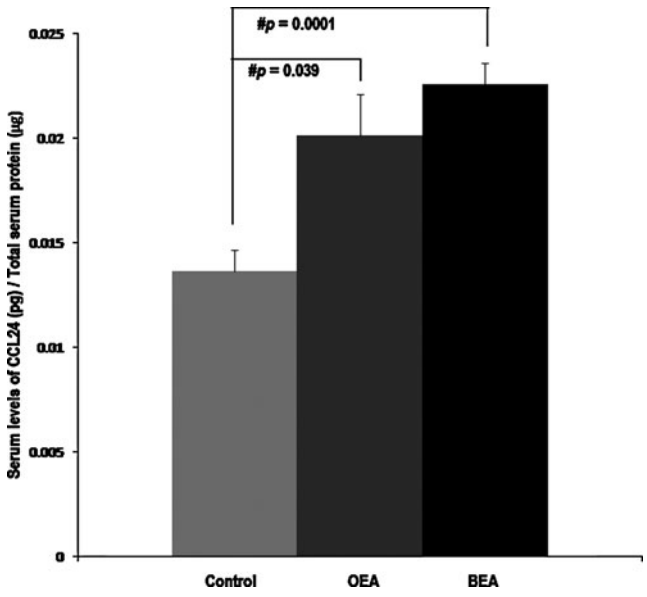

FIG. 5. Serum levels of CCL24 in AMD patients in one eye affected (OEA), both eyes affected (BEA), and normal controls. Values are plotted as mean ± standard error in the bar diagram. Data were analyzed by unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). Levels of CCL24 were normalized to total protein.

Table 3. Comparison of CCL24 Levels Among Avastin and Non-Avastin Wet Age-Related Macular Degeneration Patients

Study	*Subjects*	n	p-*Value*
Total	Wet AMD patients who received Avastin treatment	68	0.0001
	Wet AMD patients who did not receive Avastin treatment	25	

1.25 mg in 0.05 mL) and those who did not (Table 3) in a final phase analysis. CCL24 levels were significantly elevated in the wet AMD patients (0.0273 pg/µg) who received Avastin treatment as compared with those wet AMD patients who did not receive Avastin treatment (0.0134 pg/µg) (p-value = 0.0001 at $\alpha = 0.05$; Fig. 6).

Risk factors associated with CCL24

A separate analysis was carried out while adjusting the risk factors to AMD. Important risk factors that induced alterations in CCL24 levels included smoking, alcohol, and hypertension. The results shown in Table 4 were obtained by segregating AMD patients and controls based on hypertension status, revealing that CCL24 levels vary significantly within hypertensive and nonhypertensive AMD patients. The CCL24 levels were also found significant when it was compared between hypertensive AMD patients and

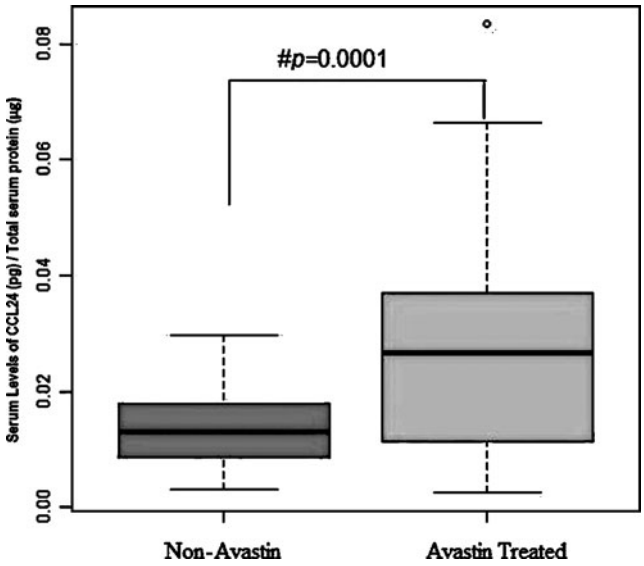

FIG. 6. Serum levels of CCL24 in Avastin-treated and not treated wet AMD. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by parametric unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). pg, pictogram; µg, microgram.

Table 4. Comparison of CCL24 Levels Among Hypertensive and Nonhypertensive Age-Related Macular Degeneration Patients and Normal Controls

Study	*Subjects*	n	p-*Value*
Phase I	Control HT/Control NHT	7/21	0.69
	AMD HT/AMD NHT	21/26	0.08
	Control HT/AMD HT	7/21	0.08
Phase II	Control HT/Control NHT	9/35	0.43
	AMD HT/AMD NHT	37/44	0.06
	Control HT/AMD HT	9/37	0.0001
Phase III (total)	Control HT/Control NHT	11/56	0.82
	AMD HT/AMD NHT	58/70	0.05
	Control HT/AMD HT	11/58	0.002

HT, hypertensive; NHT, nonhypertensive.

hypertensive healthy controls (Table 4; Fig. 7). We did not find any significant difference between smoker/nonsmoker and alcoholic/nonalcohlic AMD patients (Tables 5 and 6). However, when AMD smokers and control smokers were analyzed for CCL24 levels, it was found to be upregulated in the former; similar trends were seen when compared with alcohol consumer AMD and alcohol consumer controls (Tables 5 and 6; Figs. 8 and 9). But there was no significant

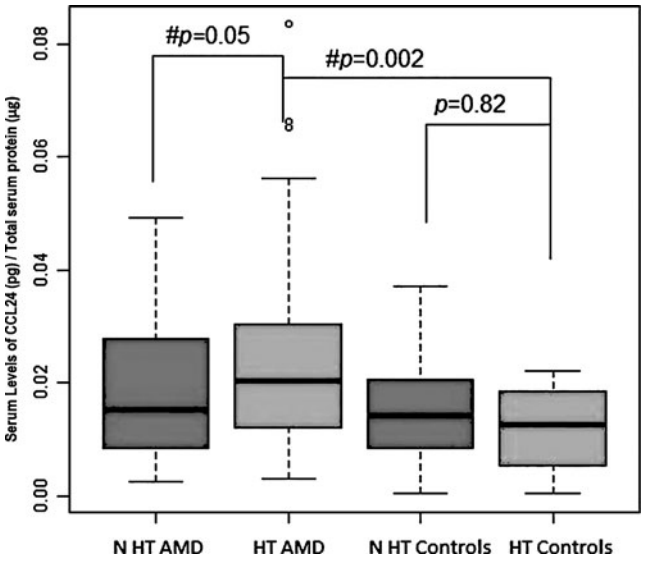

FIG. 7. Serum levels of CCL24 in hypertensive and nonhypertensive AMD and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by ANOVA followed by Fisher's LSD *post hoc* analysis among the groups. pg, pictogram; µg, microgram; N HT AMD, nonhypertensive AMD; HT AMD, hypertensive AMD; N HT Controls, nonhypertensive controls; HT Controls, hypertensive controls.

Table 5. Comparison of CCL24 Levels Among Age-Related Macular Degeneration Smokers and Nonsmokers and Normal Controls

Study	*Subjects*	n	p-*Value*
Phase I	Control Smk/Control N-Smk	7/21	0.09
	AMD Smk/AMD N-Smk	23/24	0.05
	Control Smk/AMD Smk	7/23	0.11
Phase II	Control Smk/Control N-Smk	10/34	0.34
	AMD Smk/AMD N-Smk	34/49	0.85
	Control Smk/AMD Smk	10/34	0.0001
Phase III (total)	Control Smk/Control N-Smk	12/55	0.93
	AMD Smk/AMD N-Smk	57/73	0.79
	Control Smk/AMD Smk	12/57	0.01

Smk, smoker; N-Smk, nonsmoker.

difference between AMD smokers versus AMD nonsmokers and alcohol consumers versus nonconsumers, thus enhancing reliability of end points assessed. The variable selection tool utilizing the linear model was applied to uncover the association between risk factors and altered CCL24 levels suggesting hypertension to have etiopathological association with AMD. The data remained unchanged whether it was analyzed from two categories [AMD and control; Akaike information criteria (AIC) = −1588.1] or three categories (dry AMD, wet AMD, and healthy control; AIC = −1590.6) (AIC: whose lowest value provides best model fit among all possibilities). Further, multivariate analysis was applied using CCL24 as a dependent variable and diagnosis and hypertension as an independent variable, which revealed similar outcomes, that is, significant difference at $\alpha = 0.05$. However, hypertension was found to be significant at $\alpha = 0.1$. Box plots shown in Figures 2, 3, and 7–9 depict the distribution of CCL24 levels varied by AMD and controls (Figs. 2 and 3) and the same has been stratified to see the impact of hypertension, smoking, and alcohol (Figs. 7–9). In addition to the significant changes in CCL24 levels, these box plots also depict a few outliers.

Discussion

AMD is one of the devastating disorders affecting central vision. Many studies have attempted to associate various

Table 6. Comparison of CCL24 Levels Among Alcohol Consumer and Nonalcoholic Age-Related Macular Degeneration Patients and Normal Controls

Study	*Subjects*	n	p-*Value*
Phase I	Control Al/Control N-Al	7/16	0.67
	AMD Al/AMD N-Al	18/29	0.41
	Control Al/AMD Al	7/18	0.04
Phase II	Control Al/Control N-Al	11/33	0.6
	AMD Al/AMD N-Al	22/61	0.3
	Control Al/AMD Al	11/22	0.01
Phase III (total)	Control Al/Control N-Al	18/49	0.56
	AMD Al/AMD N-Al	40/90	0.16
	Control Al/AMD Al	18/40	0.01

Al, alcoholic; N-Al, nonalcoholic.

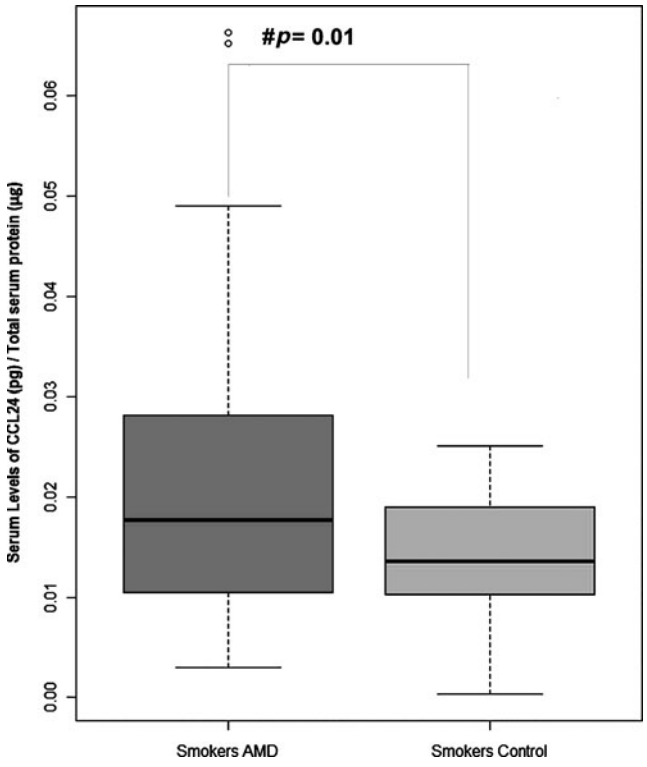

FIG. 8. Serum levels of CCL24 in AMD smokers and control smokers. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by parametric unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). pg, pictogram; µg, microgram.

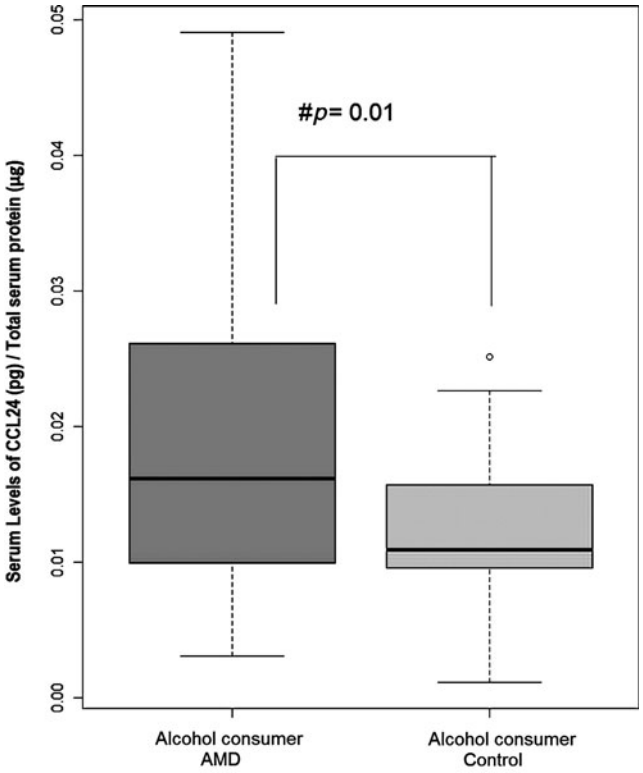

FIG. 9. Serum levels of CCL24 in alcohol consumer AMD and alcohol consumer controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bars refer to 10th and 90th percentile, respectively. The black horizontal line in the box represents median for each dataset. The symbol # indicates significant difference ($p < 0.05$) between the given conditions. Levels of CCL24 were normalized to total protein. Outliers are shown in circles. Data were analyzed by parametric unpaired, independent 2-tailed Student's *t*-test. The symbol # indicates significant difference among the groups ($p < 0.05$). pg, pictogram; µg, microgram.

biomarkers and candidate targets in the pathogenesis of AMD. Our earlier studies that showed that CCL2/CCR2-deficient mice exhibit features of AMD when combined with recent studies establishing CCL24 as an emerging candidate for AMD pathogenesis result in launching of CCL2 and CCL24 analysis in serum of AMD patients. The data presented here are limited to CCL24 analysis.

This study was conducted to determine whether differences in levels of serum CCL24 exist between patients with AMD and healthy controls. Our results indicate that the CCL24 expression level increased significantly in wet type of AMD patients as compared with dry type of AMD and normal controls. Within the wet AMD group, the levels were significantly higher in occult and predominantly classic AMD as compared with minimally classic AMD. This may be due to the increase in the inflammatory cells in occult and predominantly classic AMD because CCL24 is secreted by inflammatory cells such as macrophages and eosinophils. We believe these observations to have strong implications for evolving new strategies for targeting CCL24 in wet AMD. These results indirectly support the results of Takeda *et al.* (2009) who very recently showed that eotaxin and its receptor is a potential therapeutic agent as compared to others for treating wet AMD (Forssmann *et al.*, 1997), indicating that CCL24 may impact the pathogenesis of the disease. CCL24 blockade had been shown to be more effective at inhibiting CNV development than VEGF-A neutralization. As anti-VEGF drugs, such as Lucentis and Avastin, are one of the most effective tools currently available to combat CNV. Many argue that unlike VEGF-A blockade, CCL24 blockade could be less toxic to the retina and holds potential for AMD treatment (Forssmann *et al.*, 1997). The intravitreal injection of Avastin causes many problems, as it is associated with significant risk of acute intraocular inflammation that may result in significant visual loss (Wickremasinghe *et al.*, 2008; Georgopoulos *et al.*, 2009; Johnson *et al.*, 2010) and increase the intraocular pressure (Good *et al.*, 2011). For promoting inflammation, eotaxin–CCR3 interaction is likely to represent a major mechanism (Yawalkar *et al.*, 1999). Our report provides evidence directly from human samples showing that CCL24 levels increase substantially in the Avastin-treated patients as compared with the untreated AMD patients. With this background it is important to review our knowledge about eotaxins that are of three types, namely, eotaxin-1/CCL11 (Ponath *et al.*, 1996), eotaxin-2/CCL24 (Forssmann *et al.*,

1997), and eotaxin-3/CCL26 (Kitaura *et al.*, 1999). All of these activate the C-C chemokine receptor-3 and share (Daugherty *et al.*, 1996; Kitaura *et al.*, 1996) several functions like eosinophil chemoattraction and activation (Luster and Rothenberg, 1997; Rothenberg, 1999; Shahabuddin *et al.*, 2000). Eotaxin-2, also known as myeloid progenitor inhibitory factor-2, is a member of the CC chemokine subfamily and is designated as CCL24. Further, it is also known that lipopolysaccharide and Interleukin-4 (IL-4) differentially regulate the expression of eotaxin-2 in monocytes and macrophages. Functionally, eotaxin-2 is more closely related to eotaxin-1/CCL11 and eotaxin-3/CCL26. The three proteins share low sequence homology but have been shown to be potent eosinophil chemoattractants that bind and activate the chemokine receptor CCR3, a receptor that is highly expressed in eosinophils. CCL24 also has the ability to suppress myeloid cell proliferation, a biological function not shared by eotaxin (Petkovic *et al.*, 2004).

CCR3 has also recently been designated as CD193 (cluster of differentiation 193). The protein encoded by this gene is a receptor for C-C-type chemokines. It belongs to family 1 of the G protein–coupled receptors. This receptor binds and responds to a variety of chemokines, including eotaxin-2 (CCL24), eotaxin-3 (CCL26), MCP-3 (CCL7), MCP-4 (CCL13), and RANTES (CCL5). In addition, it has been shown that eotaxin–CCR3 is expressed on the surface of CNV vessels in humans but is absent from normal retinal vascular tissue. Our report uncovers the nonredundant role of CCL24 in pathogenesis of AMD and may stimulate multicenter and multiethnic studies to verify the evidence presented here. It is pertinent to note that many of the important molecules being investigated in the recent studies bear direct association with lifestyle risk factors; for instance, many epidemiology studies have found an association between hypertension, smoking, and increased risk of AMD (Hogg *et al.*, 2008; Kabasawa *et al.*, 2011). Our results also showed association of hypertension with levels of CCL24 in the AMD patients analyzed, but the difference between smoker/nonsmoker and alcoholic/nonalcohlic AMD patients was not significant. Hypertension may increase the oxidative stress known to be associated with AMD resulting in expression of CCL24 in choroidal endothelial cells and its ligands in RPE (Chong *et al.*, 2008). Notwithstanding AMD as an eye disorder, the analysis of serum is consistent with several previous reports in both retina and brain (Sharma *et al.*, 2009; Baas *et al.*, 2010; Vinish *et al.*, 2010). Therefore, CCL24 could represent a novel biomarker for early detection of CNV with potential to be targeted as a new therapeutic entity through future studies. Early analysis of CCL24 in patients presenting with CNV—prior even to the lesion entering the subretinal space, compared to other progressive stages of AMD—could shed light on whether CCL24 is causally related to AMD or is the consequence of CNV. Thus, at this point, we are unable to speculate whether CCL24 precedes CNV. A logistic longitudinal study is implicated to address such outstanding questions. Abundant quantity of CCL24-specific binding molecule either detected in the retina or choroid could be indicative of CNV. Early detection may allow for therapy to be initiated early, even before CNV takes place, thereby resulting in halting the disease. In addition, labeling of CCL24 molecules can be used to monitor the progress of therapy.

Our observations concluded that higher levels of serum CCL24 are associated with AMD patients despite Avastin treatment as compared with normal controls and those without Avastin, indicating that CCL24 may have an association with CNV and may be an important target to validate future therapeutic approaches in AMD in tandem with Avastin treatment but other ligands need to be studied before concluding that CCL24 is the only one among other ligands to be involved in the study. Additional immunohistochemical and biochemical investigations in autopsy specimens are needed to verify the claims held by this study. Besides, future studies can focus on the association of CCL24 levels in various populations with previously reported genetic risk factors.

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Disclosure Statement

The authors declare that they have no competing interests to disclose.

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References

Ambati, J., Ambati, B.K., Yoo, S.H., Ianchulev, S., and Adamis, A.P. (2003a). Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. Surv Ophthalmol **48,** 257–293.

Ambati, J., Anand, A., Fernandez, S., Sakurai, E., Lynn, B.C., Kuziel, W.A., Rollins, B.J., and Ambati, B.K. (2003b). An animal model of age-related macular degeneration in senescent Ccl-2 or Ccr-2-deficient mice. Nat Med **9,** 1390–1397.

Azad, R., Chandra, P., and Gupta, R. (2007). The economic implications of the use of anti-vascular endothelial growth factor drugs in age-related macular degeneration. Indian J Ophthalmol **55,** 441–443.

Baas, D.C., Ho, L., Ennis, S., Merriam, J.E., Tanck, M.W.T., Uitterlinden, A.G., de Jong, P.T., Cree, A.J., Griffiths, H.L., Rivadeneira, F., Hofman, A., van Duijn, C., Smith, R.T., Barile, G.R., Gorgels, T.G., Vingerling, J.R., Klaver, C.C., Lotery, A.J., Allikmets, R., and Bergen, A.A. (2010). The complement component 5 gene and age-related macular degeneration. Ophthalmology **117,** 500–511.

Chong, E.W., Kreis, A.J., Wong, T.Y., Simpson, J.A., and Guymer, R.H. (2008). Alcohol consumption and the risk of age-related macular degeneration: a systematic review and meta-analysis. Am J Ophthalmol **145,** 707–715.

Daugherty, B.L., Siciliano, S.J., DeMartino, J.A., Malkowitz, L., Sirotina, A., and Springer, M.S. (1996). Cloning, expression, and characterization of the human eosinophil eotaxin receptor. J Exp Med **183,** 2349–2354.

Forssmann, U., Uguccioni, M., Loetscher, P., Dahinden, C.A., Langen, H., Thelen, M., and Baggiolini, M. (1997). Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. J Exp Med **185,** 2171–2176.

Georgopoulos, M., Polak, K., Prager, F., Prunte, C., and Schmidt-Erfurth, U. (2009). Characteristics of severe intraocular inflammation following intravitreal injection of bevacizumab (Avastin). Br J Ophthalmol **93,** 457–462.

Good, T.J., Kimura, A.E., Mandava, N., and Kahook, M.Y. (2011). Sustained elevation of intraocular pressure after intravitreal injections of anti-VEGF agents. Br J Ophthalmol **95,** 1111–1114.

Hogg, R.E., Woodside, J.V., Gilchrist, S.E., Graydon, R., Fletcher, A.E., *et al.* (2008). Cardiovascular disease and hypertension are strong risk factors for choroidal neovascularization. Ophthalmology **115,** 1046–1052.

Humbles, A.A., Lloyd, C.M., McMillan, S.J., Friend, D.S., Xanthou, G., *et al.* (2004). A critical role for eosinophils in allergic airways remodeling. Science **305,** 1776–1779.

Jahnz-Rozyk, K., Targowski, T., Paluchowska, E., Owczarek, W., and Kucharczyk, A. (2005). Serum thymus and activation-regulated chemokine, macrophage-derived chemokine and eotaxin as markers of severity of atopic dermatitis. Allergy **60,** 685–688.

Johnson, D., Hollands, H., Hollands, S., and Sharma, S. (2010). Incidence and characteristics of acute intraocular inflammation after intravitreal injection of bevacizumab: a retrospective cohort study. Can J Ophthalmol **45,** 239–242.

Kabasawa, S., Mori, K., Horie-Inoue, K., Gehlbach, P.L., Inoue, S., Awata, T., Katayama, S., and Yoneya, S. (2011). Associations of cigarette smoking but not serum fatty acids with age-related macular degeneration in a Japanese population. Ophthalmology **18,** 1082–1088.

Kagami, S., Kakinuma, T., Saeki, H., Tsunemi, Y., Fujita, H., Nakamura, K., Takekoshi, T., Kishimoto, M., Mitsui, H., Torii, H., Komine, M., Asahina, A., and Tamaki, K. (2003). Significant elevation of serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, in patients with atopic dermatitis: serum eotaxin-3/CCL26 levels reflect the disease activity of atopic dermatitis. Clin Exp Immunol **134,** 309–313.

Kitaura, M., Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H.L., Murphy, P.M., and Yoshie, O. (1996). Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. J Biol Chem **271,** 7725–7730.

Kitaura, M., Suzuki, N., Imai, T., Takagi, S., Suzuki, R., Nakajima, T., Hirai, K., Nomiyama, H., and Yoshie, O. (1999). Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. J Biol Chem **274,** 27975–27980.

Li, Y., Huang, D., Xia, X., Wang, Z., Luo, L., and Wen, R. (2011). CCR3 and choroidal neovascularization. PLoS ONE **6,** e17106.

Luster, A.D., and Rothenberg, M.E. (1997). Role of the monocyte chemoattractant protein and eotaxin subfamily of chemokines in allergic inflammation. J Leukoc Biol **62,** 620–633.

Maller, J., George, S., Purcell, S., Fagerness, J., Altshuler, D., Daly, M.J., and Seddon, J.M. (2006). Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. Nat Genet **38,** 1055–1059.

Manousou, P., Kolios, G., Valatas, V., Drygiannakis, I., Bourikas, L., Pyrovolaki, K., Koutroubakis, I., Papadaki, H.A., and Kouroumalis, E. (2010). Increased expression of chemokine receptor CCR3 and its ligands in ulcerative colitis: the role of colonic epithelial cells in *in vitro* studies. Clin Exp Immunol **162,** 337–347.

Mo, F.M., Proia, A.D., Johnson, W.H., Cyr, D., and Lashkari, K. (2010). Interferon inducible protein-10 (IP-10) and eotaxin as biomarkers in age-related macular degeneration. Retina **51,** 4226–4236.

Petkovic, V., Moghini, C., Paoletti, S., Uguccioni, M., and Gerber, B. (2004). Eotaxin-3/CCL26 is a natural antagonist for CC chemokine receptors 1 and 5. J Biol Chem **22,** 23357–23367.

Ponath, P.D., Qin, S., Ringler, D.J., Clark-Lewis, I., Wang, J., Kassam, N., Smith, H., Shi, X., Gonzalo, J.A., Newman, W., Gutierrez-Ramos, J.C., and Mackay, C.R. (1996). Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. J Clin Invest **97,** 604–612.

Pope, S.M., Zimmermann, N., Stringer, K.F., Karow, M.L., and Rothenberg, M.E. (2005). The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol **175,** 5341–5350.

Rothenberg, M.E. (1999). Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. Am J Respir Cell Mol Biol **21,** 291–295.

Salcedo, R., Young, H.A., Ponce, M.L., Ward, J.M., Kleinman, H.K., Murphy, W.J., and Oppenheim, J.J. (2001). Eotaxin (CCL11) induces *in vivo* angiogenic responses by human CCR31 endothelial cells. J Immunol **166,** 7571–7578.

Shah, A.R., and Del Priore, L.V. (2009). Natural history of predominantly classic, minimally classic, and occult subgroups in exudative age-related macular degeneration. Ophthalmology **116,** 1901–1907.

Shahabuddin, S., Ponath, P., and Schleimer, R.P. (2000). Migration of eosinophils across endothelial cell monolayers: interactions among IL-5, endothelial-activating cytokines, and C-C chemokines. J Immunol **164,** 3847–3854.

Sharma, N.K., Prabhakar, S., and Anand, A. (2009). Age related macular degeneration—advances and trends. Ann Neurosci **2,** 62–71.

Takeda, A., Baffi, J.Z., Kleinman, M.E., Cho, W.G., Nozaki, M., Yamada, K., Kaneko, H., Albuquerque, R.J.C., Dridi, S., Saito, K., Raisler, B.J., Budd, S.J., Geisen, P., Munitz, A., Ambati, B.K., Green, M.G., Ishibashi, T., Wright, J.D., Humbles, A.A., Gerard, C.J., Ogura, Y., Pan, Y., Smith, J.R., Grisanti, S., Hartnett, M.E., Rothenberg, M.E., and Ambati, J. (2009). CCR3 is a target for age-related macular degeneration diagnosis and therapy. Nature **460,** 225–230.

Teixeira, M.M., Wells, T.N., Lukacs, N.W., Proudfoot, A.E., Kunkel, S.L., Williams, T.J., and Hellewell, P.G. (1997). Chemokine-induced eosinophil recruitment. Evidence of a role for endogenous eotaxin in an *in vivo* allergy model in mouse skin. J Clin Invest **100,** 1657–1666.

Tin, L.L., Beevers, D.G., and Lip, G.Y.H. (2002). Systolic vs. diastolic blood pressure and the burden of hypertension. J Hum Hypertens **16,** 147–150.

Vinish, M., Prabhakar, S., Khullar, M., Verma, I., and Anand, A. (2010). Genetic screening reveals high frequency of PARK2 mutations and reduced Parkin expression conferring risk for Parkinsonism in North West India. J Neurol Neurosurg Psychiatry **81,** 166–170.

Wang, H., Wittchen, E.S., Jiang, Y., Ambati, B., and Grossniklaus, H.E. (2011). Upregulation of CCR3 by age-related stresses promotes choroidal endothelial cell migration via VEGF-dependent and -independent signaling. Invest Ophthalmol Vis Sci **52,** 8271–8277.

White, J.R., Imburgia, C., Dul, E., Appelbaum, E., and O'Donnell, K. (1997). Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophils. J Leukoc Biol **62,** 667–675.

Wickremasinghe, S.S., Michalova, K., Gilhotra, J., Guymer, R.H., Harper, C.A., Wong, T.Y., and Qureshi, S. (2008). Acute intraocular inflammation after intravitreous injections of bevacizumab for treatment of neovascular age-related macular degeneration. Ophthalmology **115,** 1911–1915.

Yawalkar, N., Uguccioni, M., Schärer, J., Braunwalder, J., and Karlen, S. (1999). Enhanced expression of eotaxin and CCR3 in atopic dermatitis. J Invest Dermatol **113,** 43–48.

Zimmermann, N., Hogan, S.P., Mishra, A., Brandt, E.B., Bodette, T.R., *et al.* (2000). Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. J Immunol **165,** 5839–5846.

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SHORT COMMUNICATION

Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients

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Background and purpose: North Indian patients with amyotrophic lateral sclerosis (ALS) exhibit substantially extended survival time after onset of the disease as compared to their Western counterparts. Earlier, we found that vascular endothelial growth factor-A (VEGF-A) may be associated with increased survival of these patients. We now measured soluble vascular endothelial growth factor receptor-1 (sVEGFR1), an inhibitor receptor for VEGF-A, in these patients with ALS.

Methods: Patients with sporadic ALS (n = 36) attending the Neurology Outpatient at Post Graduate Institute of Medical Education and Research (PGIMER) at Chandigarh were included on the basis of El Escorial criteria. The sVEGFR1 levels were analyzed in serum of these patients using enzyme-linked immunosorbent assay (ELISA) and compared with normal controls (n = 36).

Results: Soluble vascular endothelial growth factor receptor-1 was found to be decreased significantly in serum of patients with ALS. Serum obtained from definite ALS revealed significantly lower sVEGFR1 as compared to probable ALS. However, there was no difference in serum sVEGFR1 levels between male and female patients with ALS.

Conclusions: Soluble vascular endothelial growth factor receptor-1 downregulation may result in increased serum VEGF-A reported previously in our patients with ALS and may indicate the activation of compensatory mechanism in response to neurodegeneration. The lower serum sVEGFR1 levels may have a possible clinicopathological association, if not causal, to the extended survival of North Indian patients with ALS; however, the result needs further investigations particularly in comparable Caucasian ALS population.

Introduction

Amyotrophic lateral sclerosis (ALS) is a motor neuron disorder with multifactorial pathogenesis. Existing reports suggest that delivery of angiogenic factor vascular endothelial growth factor-A (VEGF-A) activates PI3-K/Akt anti-apoptotic pathway and thus delays the onset and progression of ALS in superoxide dismutase-1-mutated transgenic mouse model [1,2]. On the other hand, soluble vascular endothelial growth factor receptor-1 (sVEGFR1) is believed to scavenge VEGF-A ligand effectively, and reducing VEGF-A-mediated angiogenesis and inhibiting the response of VEGF-A, [3]; however, there is no earlier attempt to investigate the role of sVEGFR1 in pathogenesis of ALS. sVEGFR1 is a 110-kDa truncated protein resulting from alternate splicing of VEGFR1 mRNA and to some extent by proteolytic cleavage of full length 180-kDa VEGFR1 protein [4]. We have recently hypothesized that increased levels of VEGF-A, by virtue of its angiogenic and neurotrophic nature, may account for significantly enhanced survival of North Indian patients with ALS [5]. Because sVEGFR1 is anti-angiogenic, sVEGFR1 was analyzed in serum of these patients to test whether decreased level of sVEGFR1 could be a possible factor in enhanced survival duration of these patients.

Subjects and methods

Thirty-six patients diagnosed with ALS were recruited after obtaining informed consent as per institute ethical committee guidelines. Ethical approval was obtained by the institute's ethical committee, PGIMER, 160012

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Chandigarh, India (No. 7055-PG-1Tg-05/4348-50). All patients were sporadic in nature and born in Northern India. Of the patients included, 17 patients fulfilled the 'El Escorial criteria' for definite ALS, 10 individuals fulfilled the criteria for 'probable ALS', and nine patients for 'possible ALS' at the time of sample collection. Based on revised ALS-Functional Rating Score (ALSFRS-r), seven patients were characterized by respiratory insufficiency along with orthopnea and dyspnea, although none of the patients needed respiratory support. ALSFRS-r further revealed that there were 23 ALS patients with moderate impairment and 13 presented with mild neurological impairment. Patients with history of stroke, pre-eclampsia, diabetic neuropathy, glaucoma or those on riluzole, anti-inflammatory drugs, or other treatments were excluded. The study also included 36 genetically unrelated healthy controls without any complaints of hypertension, diabetes, heart disease etc. for comparison. The clinical and biochemical details of the subjects have been shown in Table 1.

Serum preparation

In serum separator tube, 4.0 ml of blood was collected (SST) (BD Biosciences, Franklin Lakes, NJ, USA) and allowed to clot for 30 min at room temperature before centrifugation for 15 min at 280 ***g*** to separate serum. Serum was stored at −80°C within 1 h of sample collection until assayed.

sVEGFR1 quantitation

Serum sVEGFR1 was quantitated using sandwich enzyme-linked immunosorbent assay (ELISA; Cusabiotech, Wuhan-Hubei Province, China; Catalog no. CSB-E11885h) as per manufacturer's instructions, and absorbance was read at 450 nm using 680XR model of Microplate reader (Biorad, Hercules, CA, USA). ELISA assay kit detects natural unbound human sVEGFR1 with detection range 156–10 ng/ml. Standard curve was generated using linear regression to calculate sVEGFR1 values in patients and normal controls followed by normalization with total serum protein.

Statistical analysis

Because the data were normally distributed, parametric student *t*-test was used for statistical analysis. Normal distribution of the dataset was checked using quintile–quintile (Q–Q) plots. The values were represented as mean ± SE (standard error). The *P*-value was considered significant at ≤0.05. All statistical analysis was performed by statistical package and service solutions (SPSS; IBM corporation, Armonk, NY, USA) 16 software. Analysis was performed by two independent masked research workers.

Results

The ELISA analysis showed that the level of serum sVEGFR1 was decreased in the ALS samples when compared to controls (Fig. 1a; $P = 0.039$ and Figure S1A).

To evaluate prognostic value of sVEGFR1, serum levels of sVEGFR1 were further segregated amongst definite, probable, and possible ALS patients. We observed significantly reduced sVEGFR1 in definite ALS as compared to controls and probable ALS (Fig. 1b; $P = 0.002$ and $P = 0.048$, respectively, and Figure S1B); however, the difference was not significant between the probable and possible ALS when compared to controls (Fig. 1b; $P > 0.05$ and Figure S1B).

Because patients and controls are not gender matched, serum sVEGFR1 levels were compared between men and women (in controls and in patients). The levels were found to be comparable between male and female control group ($P = 0.889$). Similarly, the difference in serum sVEGFR1 levels was not significant between male and female patients with ALS ($P = 0.168$) (data not shown).

Discussion

The median survival duration of 114.83 ± 25.9 (SE) months has been earlier reported in 1153 Indian patients with ALS after disease onset. This duration for male Indian patients was 110 ± 27.4 (SE) months and

Table 1 Characteristics of subjects

Subjects	Age (years)[a]	M/F (*n*)	Age of onset (years)	Disease duration (months)[b]	B/L (*n*)	Total serum protein (g/l)[a]
ALS	47.5 ± 12.3	30/06	46.0 ± 12.7	18.9 ± 13.1	04/32	47.1 ± 24.2
Controls	42.6 ± 12.6	26/10				45.3 ± 28.4

Clinical and biochemical summary of subjects. *n*, Number; M, male; F, female; B, bulbar; L, limb; g, gram; l, litre; CSF, cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; Age, age of onset, duration of disease and total serum protein are indicated as mean ± standard deviation (SD). [a]Unpaired, independent 2-tailed student *t*-test showed that mean age and mean concentration of total protein in serum did not differ significantly between the groups ($P > 0.05$); [b]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.

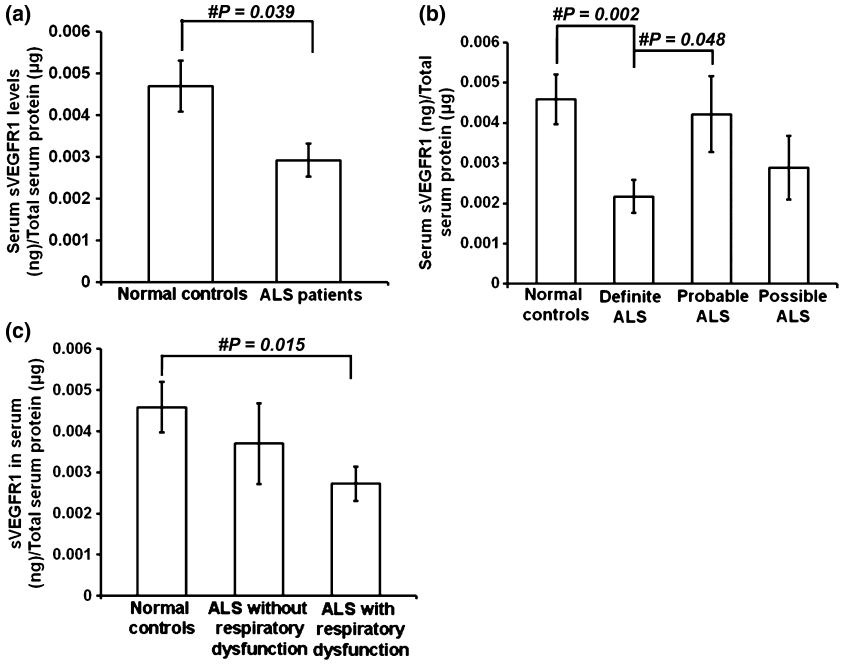

Figure 1 (a) Level of sVEGFR1 in serum of patients with ALS and normal controls. (b) Level of sVEGFR1 in serum of definite, probable, possible ALS patients and normal controls. (c) Serum sVEGFR1 in ALS patients with respiratory dysfunction and without respiratory dysfunction. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data were analyzed by unpaired, independent 2-tailed student *t*-test. #Significant difference between the groups ($P < 0.05$). Levels of sVEGFR1 were normalized to total serum protein. ALS, amyotrophic lateral sclerosis; sVEGFR1, soluble vascular endothelial growth factor receptor-1; ng, nanogram; µg, microgram. The same data have also been reproduced in nanogram of sVEGFR1/ml of serum and shown as Figure S1.

that for female patients was 118.9 ± 6.3 (SE) months [6]. This is in contrast to the patients from Western countries who survive upto 3–6 years after disease onset [7]. The analysis of sVEGFR1 in North Indian patients with ALS, thus, provides a maiden opportunity to understand the pathogenesis of ALS disease as these patients exhibit significantly extended survival duration after onset of disease.

The significantly reduced serum sVEGFR1 levels in our patients with ALS suggest the clinicopathophysiological relevance of serum in ALS pathogenesis, particularly in definite ALS. It must be pointed out that there is no earlier evidence to corroborate or contradict these findings. At this point, we are unable to speculate whether this is a response to or a consequence of respiratory problems, amyotrophy, genetic polymorphisms, or environmental factors such as diet, demography. Nevertheless, it is pertinent to note that our analysis did not show any significant reduction in sVEGFR1 in seven ALS patients with respiratory dysfunction, indicating that hypoxia may not be associated with altered sVEGFR1 (Fig. 1c; Table 2 and Figure S1C).

Because sVEGFR1 hampers angiogenesis by sequestering VEGF-A [3], its downregulation in serum samples is suggested to enhance the serum VEGF-A as also reported earlier in the same patients with ALS [5]. Whether the reduced serum sVEGFR1 increases the cerebrospinal fluid (CSF) VEGF-A to induce anti-apoptotic pathways and ameliorate glutamate excitotoxicity in the central nervous system (CNS) or not, the role of reduced serum sVEGFR1 (and increased serum VEGF-A) in enhancing the blood perfusion through blood collaterals to dying peripheral motor neurons and skeletal muscles cannot be ruled out [8]. It may further contribute toward maintenance of neuromuscular junctions by increasing sprouting of nerve endings as a possible compensatory response to the injury [9]. Regardless of the association of reduced sVEGFR1 with ALS, its direct role in prolonged survival of North Indian patients with ALS awaits a crosscultural and crossethnic comparison with comparable Caucasian patients with ALS.

This study also emphasizes the need of screening of regulatory sequences of sVEGFR1 and VEGF-A for subtle genetic and epigenetic changes in larger cohorts of Indian and Western ALS where patients could be categorized amongst short and long survivors, to examine if sVEGFR1 actually contributes in the

Table 2 Clinical details of 7 ALS patients with respiratory dysfunction

ALS	El Escorial criteria	ALSFRS-R	Impairment	Disease duration (months)[a]
Patient 1	Definite	27	Moderate	12
Patient 2	Definite	23	Severe	12
Patient 3	Definite	29	Moderate	12
Patient 4	Definite	29	Moderate	4
Patient 5	Definite	29	Moderate	9
Patient 6	Definite	31	Moderate	8
Patient 7	Definite	35	Moderate	24

ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating score-revised; Impairment was measured with ALSFRS-R. [a]Duration of disease is the interval between appearance of first symptom of ALS and collection of sample. Median disease duration in patients with respiratory dysfunction was 10.5 (4–24) months which was found to be significantly lower than median duration of disease in patients without respiratory dysfunction [18 (3–72) months] upon Mann–Whitney *U* analysis ($P = 0.029$).

enhanced survival and if so, the molecular mechanisms involved.

The finding that sVEGFR1 levels were significantly decreased in definite ALS and were not significantly different between probable and possible ALS as compared to controls (Fig. 1b) may indicate that sVEGFR1 finds importance in later stages of the disease only and may not be implicated in early stages of disease. This suggests that alteration of sVEGFR1 serum levels may be secondary to primary motor neuron degeneration, and not causally contribute to disease pathogenesis. It may nevertheless play a role during disease progression at later stages and supports the utility of sVEGFR1 as prognostic marker for ALS.

Although the direct association of serum sVEGFR1 with CNS pathology in the diseases like ALS has not been adequately substantiated, such studies provide significant information about disease pathogenesis. The present findings, therefore, can be validated in brain autopsies or CSF samples of patients with ALS. Earlier, serum has been investigated in many neurodegenerative disorders including Parkinson's disease [10].

A head-to-head comparison of sVEGFR1 between ALS and other neurological controls can further validate its utility as possible diagnostic and/or prognostic marker of ALS. A strategy to augment VEGF-A by inhibiting sVEGFR1 expression may be a useful therapeutic approach to ameliorate this devastating disorder and should be tested.

Conclusion

Although the study does not conclusively establish any causal relationship between reduced sVEGFR1 and increased survival of sporadic North Indian patients with ALS, a possible clinicopathological, etiological, or epidemiological association of reduction in this factor with survival warrants inter-cultural and inter-ethnic investigations.

Acknowledgements

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Disclosure of conflict of interest

The authors declare no financial or other conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Values plotted as mean ± SE (standard error). Data was analysed by 2-tailed student *t*-test. #Significant difference among the groups ($P < 0.05$).

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References

1. Azzouz M, Ralph GS, Storkebaum E, *et al*. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature* 2004; **429:** 413–417.
2. Storkebaum E, Lambrechts D, Dewerchin M, *et al*. Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. *Nat Neurosci* 2005; **8:** 85–92.
3. Gehlbach P, Demetriades AM, Yamamoto S, *et al*. Periocular gene transfer of sFlt-1 suppresses ocular neovascularization and vascular endothelial growth factor-induced breakdown of the blood retinal barrier. *Hum Gene Ther* 2003; **14:** 129–141.
4. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously

encoded soluble receptor. *Proc Natl Acad Sci U S A* 1993; **90:** 10705–10709.
5. Gupta PK, Prabhakar S, Sharma S, Anand A. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in Amyotrophic Lateral Sclerosis (ALS) patients. *J Neuroinflammation* 2011; **8:** 47.
6. Nalini A, Thennarasu K, Gourie-Devi M, Shenoy S, Kulshreshtha D. Clinical characteristics and survival pattern of 1,153 patients with amyotrophic lateral sclerosis: experience over 30 years from India. *J Neurol Sci* 2008; **272:** 60–70.
7. Sorenson EJ, Stalker AP, Kurland LT, Windebank AJ. Amyotrophic lateral sclerosis in Olmsted County, Minnesota, 1925 to 1998. *Neurology* 2002; **59:** 280–282.
8. Sun Y, Jin K, Xie L, *et al.* VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest* 2003; **111:** 1843–1851.
9. Zheng C, Sköld MK, Li J, Nennesmo I, Fadeel B, Henter JI. VEGF reduces astrogliosis and preserves neuromuscular junctions in ALS transgenic mice. *Biochem Biophys Res Commun* 2007; **363:** 989–993.
10. Vinish M, Prabhakar S, Khullar M, Verma I, Anand A. Genetic screening reveals high frequency of PARK2 mutations and reduced Parkin expression conferring risk for Parkinsonism in North West India. *J Neurol Neurosurg Psychiatry* 2010; **81:** 166–170.

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SHORT REPORT Open Access

Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in Amyotrophic Lateral Sclerosis (ALS) patients

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Abstract

Background: Vascular endothelial growth factor-A (VEGF-A) and chemokne ligand-2 (CCL2) levels have been examined in Amyotrophic Lateral Sclerosis (ALS) patients in Western countries. We measured these values in North Indian ALS patients, since these patients display considerably enhanced survival duration.

Methods: Sporadic ALS patients were included on the basis of El Escorial criteria. VEGF-A and CCL2 levels were analyzed in serum and cerebrospinal fluid (CSF) of 50 ALS patients using enzyme linked immunosorbent assay (ELISA) and compared with normal controls. Their levels were adjusted for possible confounders like cigarette smoking, alcohol and meat consumption.

Results: Contrary to previous studies, VEGF-A was found to be elevated significantly in serum and CSF in ALS patient population studied. We also found an increase in CCL2 levels in CSF of these ALS patients. Serum and CSF from definite ALS revealed higher VEGF-A as compared to probable and possible ALS. CCL2 was unaltered between definite, probable and possible ALS. Univariate and multivariate analysis revealed a lack of association of smoking, alcohol and meat consumption with VEGF-A and CCL2 levels.

Conclusions: VEGF-A upregulation may indicate an activation of compensatory responses in ALS which may reflect or in fact account for increased survival of North Indian ALS patients after disease onset. The intrathecal synthesis of CCL2 suggests the involvement of adult neural stem cells and microglial activation in ALS pathogenesis which needs further investigation.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder with genetic and clinical heterogeneity. Existing evidence suggests that Vascular endothelial growth factor-A (VEGF-A) delivery delays the onset and progression of ALS in superoxide dismutase-1 (SOD1) mutated transgenic mouse model by activating PI3-K/Akt anti apoptotic pathway [1,2]. VEGF-A also shown to be involved in proliferation and differentiation of adult mouse neural progenitor's cells [3]. On the other hand, chemokine ligand-2 (CCL2), a proinflammatory molecule, enhances microglial recruitment after injury to central nervous system (CNS) and exacerbates ALS [4]. It has been observed that CCL2 knockout mice have reduced involvement of immune cells and are resistant to stroke and autoimmune encephalomyelitis [5,6]. Reports suggest that VEGF-A_{165}, a major isoform of VEGF-A, and CCL2 may interact in synergistic manner to mount a response to disease [7]. We therefore hypothesized that VEGF-A-CCL2 axis plays a crucial role in ALS pathogenesis and could be a target for development of future therapy for ALS.

Subjects and methods

50 patients diagnosed with ALS were recruited after obtaining informed consent as per institute ethical committee guidelines (No. 7055-PG-1Tg-05/4348-50). All patients were born in Northern India. Of the patients examined, 25 patients fulfilled the "El Escorial criteria" for definite ALS, 15 individuals fulfilled for probable and 10 patients as possible ALS at the time of sample

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collection. Based on ALS-Functional Rating Score [8], 11 patients had respiratory insufficiencies alongwith orthopnea and dyspnea, although none of the patients needed respiratory support. There were 42 cases of limb and 8 cases of bulbar onset ALS. Patients with history of stroke, pre-eclampsia, diabetic neuropathy, glaucoma, diabetes, those who have been receiving riluzole, anti inflammatory drugs, antioxidants or other treatment were excluded. 50 genetically unrelated healthy normal controls without any complaints of hypertension, diabetes, heart disease etc were also included. Cerebrospinal fluid (CSF) from 42 subjects without any CNS disorders but undergoing routine spinal anesthesia for surgery was collected and considered control sample. Subjects were categorized as cigarette smokers and never smokers, alcohol consumers and nonalcoholics, vegetarian and non-vegetarian (or meat consumers) through a questionnaire [9]. The characteristics of subjects have been reproduced in Table 1.

Serum was separated from 4.0 ml blood collected in serum separator tube (BD Biosciences, USA). ~2.0 ml CSF was drawn in a sterilized container. Serum and CSF was stored at -80°C until assayed.

Serum VEGF-A, CCL2 and CSF CCL2 was measured using Quantikine sandwich enzyme linked immunosorbent assays (ELISA; R&D systems, USA) and read at 450 nm in 680XR microplate reader (Biorad, USA). CSF VEGF-A levels were quantitated with QuantiGlo chemiluminescent assay (R&D systems), and read as relative light units in luminometer (Biotek, USA). ELISA kits for VEGF-A measured unbound natural human VEGF-A_{165} splice variant.

Mann Whitney U test and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc analysis was applied to analyze skewed and normally distributed data respectively. As smoking, alcohol and meat consumption may affect VEGF-A and CCL2 levels, crude odds ratio (OR) of their association was evaluated by univariate logistic regression. Adjusted OR to investigate independent effect of these covariates was computed using multivariate logistic regression and χ^2 (chi square) test was performed to calculate p-value. p-value was considered significant at ≤ 0.05. Statistical analysis was performed by statistical package and service solutions 16.

Results

ELISA indicates elevated serum VEGF-A in ALS as compared to controls (Figure 1A; p = 0.046). Median CSF VEGF-A concentration was significantly higher in ALS patients than controls (Figure 1B; p = 0.0001). No difference in serum CCL2 was observed between ALS and controls (Figure 2A; p > 0.05). However, CSF CCL2 was found increased in ALS as compared to controls (Figure 2B; p = 0.003). There was elevated serum VEGF-A in definite ALS in comparison to controls, probable and possible ALS (Figure 3A; p = 0.015, p = 0.033 and p = 0.017 respectively). Similarly, CSF from definite ALS was reported to have statistically higher VEGF-A than controls, probable and possible ALS (Figure 3B; p = 0.0001, p = 0.018 and p = 0.017 respectively). However, serum and CSF CCL2 levels did not differ among definite, probable and possible ALS (Figure 4A-4B; p > 0.05).

No association of cigarette smoking, alcohol and meat consumption with VEGF-A (Table 2) and CCL2 (data not shown) levels in serum and CSF was observed upon univariate and multivariate analysis.

Discussion

Our study of North Indian ALS patients represents a unique opportunity to understand the disease from a distinct genetic perspective, given that these patients bring a peculiar spectrum of extended life expectancy in comparison to Western counterparts [10]. An earlier Indian study has reported the median survival duration of 114.83 ± 25.9(SE) months in 1153 ALS patients after disease onset. The mean survival duration of male patients was 110 ± 27.4(SE) months which was not significantly different from mean survival duration of

Table 1 Characteristics of subjects

Subjects	Age (y)†	M/F (n)	Age of onset (y)	Disease duration‡ (mo)	Smokers (n)	Alcohol consumers (n)	Non-vegetarian (n)	Total protein (g/l)†	
								CSF	Serum
ALS	47.4 ± 12.4	38/12	46.2 ± 12.8	19.0 ± 12.7	12	12	20	0.43 ± 0.2	48.2 ± 26.7
Controls (Serum)	40.0 ± 12.8	39/11			10	14	27		48.7 ± 28.7
Controls (CSF)	43.4 ± 17.1	35/07			08	09	10	0.42 ± 0.1	

Clinical and demographic summary of ALS cases and controls. n, Number; M, male; F, female; y, years; mo, months; g, gram; l, litre; CSF, cerebrospinal fluid; Age, age of onset, duration of disease, CSF and serum total protein are indicated as mean ± standard deviation (SD). † One way ANOVA followed by LSD post hoc analysis showed that mean age and, mean concentration of total protein in serum and CSF did not differ significantly among the groups (p > 0.05). ‡ 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.

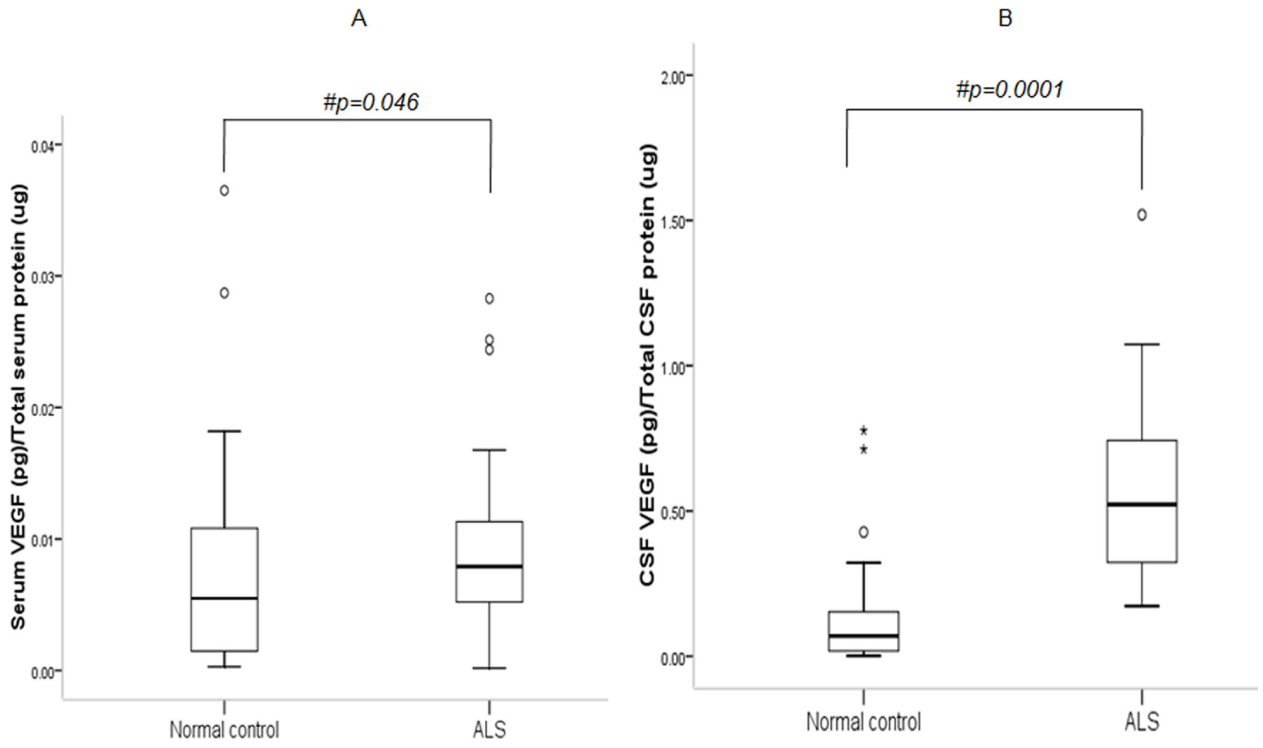

Figure 1 Level of VEGF-A in serum (A) and CSF (B) of ALS patients and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Levels of VEGF-A were normalized to total serum and CSF protein respectively. Outliers and extreme values are shown in circles and asterisk respectively. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. ALS, Amyotrophic Lateral Sclerosis; VEGF-A, vascular endothelial growth factor-A; pg, picogram; μg, microgram.

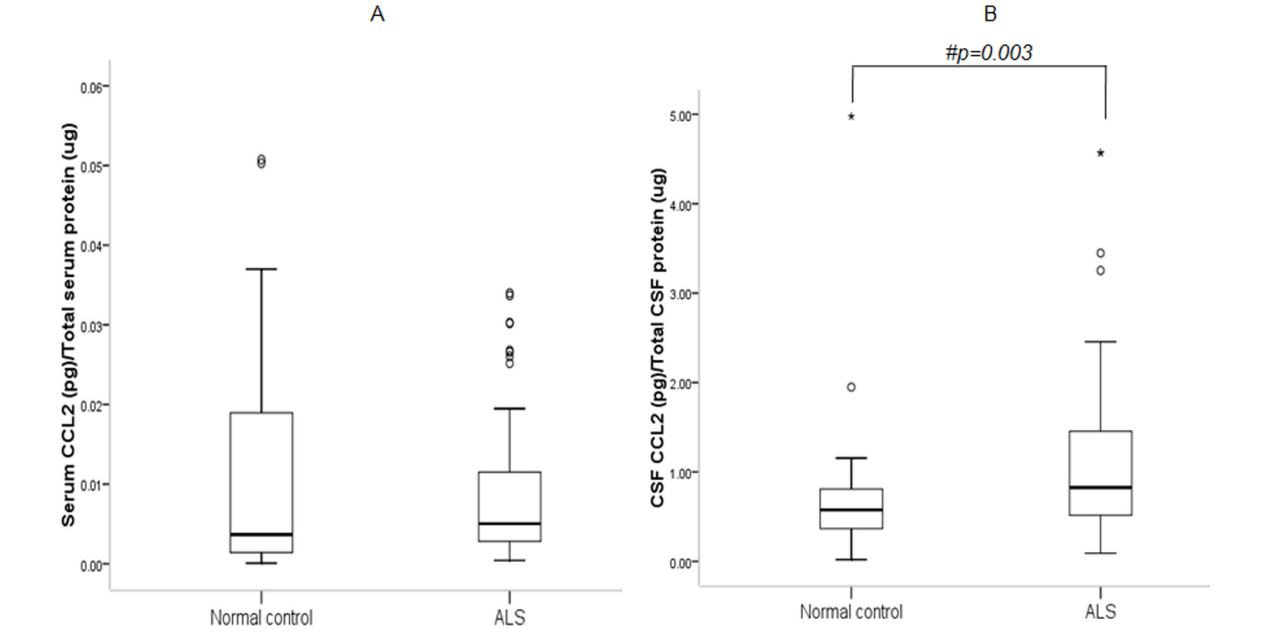

Figure 2 Level of CCL2 in serum (A) and CSF (B) of ALS patients and normal subjects. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Levels of CCL2 were normalized to total protein in the serum and CSF samples. Outliers and extreme values are shown in circles and asterisk respectively. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. ALS, Amyotrophic Lateral Sclerosis; CCL2, chemokine ligand 2; pg, picogram; μg, microgram.

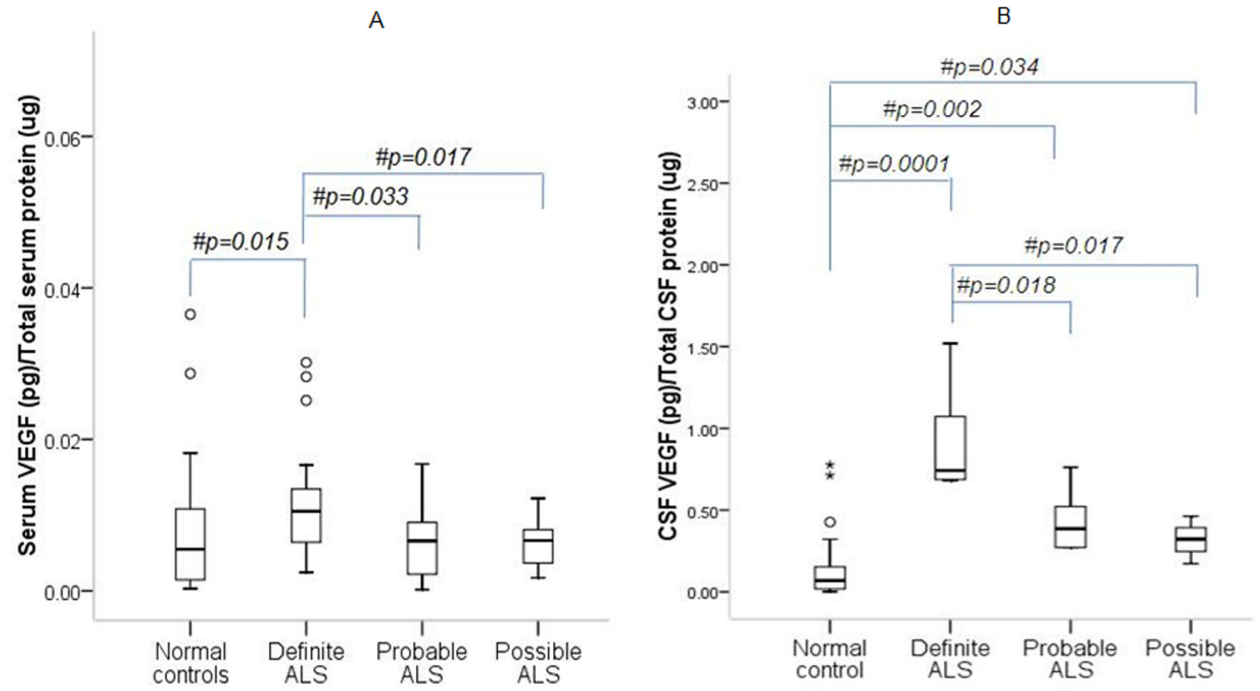

Figure 3 Level of VEGF-A in serum (A) and CSF (B) of definite ALS, probable ALS, possible ALS patients and normal subjects. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Levels of VEGF-A were normalized to total protein in the serum and CSF samples. Outliers and extreme values are shown in circles and asterisk respectively. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. ALS, Amyotrophic Lateral Sclerosis; VEGF-A, vascular endothelial growth factor-A; pg, picogram; μg, microgram.

female [118.9 ± 6.3(SE) months] patients [11], while, patients from USA and Europe make up 3-6 years of survival time [12,13].

The increased serum VEGF-A in our ALS patients is consistent with the existing report [14], and suggests that serum is a pathophysiologically relevant fluid in ALS, particularly in definite ALS, however, a few other studies have failed to observe significant difference in plasma and serum VEGF-A levels in ALS patients [15,16].

The elevated CSF VEGF-A in ALS indicates possible presymptomatic initiation of pathological events and intrathecal production from degenerating motor neurons, which is contrary to existing study where decreased VEGF-A_{165} dependent neuroprotection in ALS, due to reduced CSF VEGF-A, has been suggested [15]. Since the impaired VEGF-A expression in early ALS negatively influences the clinical outcome of the disease [15], its upregulation suggests a compensatory response, contributing to prolonged survival of our ALS patients. It is speculated that North Indian ALS patients generate increased VEGF-A or stimulate glutamate receptor-2 expression to ameliorate excitotoxicity [17,18]. We are unable to conclude whether this response is a consequence of amyotrophy, hypoxia, dietary or other environmental factors, or some subtle genetic differences [14,15].

Further analysis shows significantly increased VEGF-A in 11 ALS patients with respiratory dysfunction indicating a possible association with hypoxia (Figure 5A-5B; Table 3). Elevated VEGF-A in serum and CSF of definite ALS suggests extensive involvement of neuroaxis and relatively higher degree of neurodegeneration and regeneration than probable and possible variants. No significant difference in median disease duration between definite [14(4-36) months], probable [16(3-72) months] and possible ALS [14(9-24) months] cases was observed. It must be pointed out that while the disease duration was accurately documented, the actual survival time of patients after disease onset could not be ascertained.

Intrathecal secretion of CCL2 may offer neuroprotection against glutamate excitotoxicity either by reducing N-Methyl-D-aspartate (NMDA)-dependant release of glutamate and/or increasing astrocytes efficiency to clear synaptic cleft glutamate [19]. CCL2 promotes CCR2 and CCR5 expressing C17.2 neural progenitor cell migration and their differentiation into neuronal and glial phenotype [20,21]. CCL2 is also known to be angiogenic and participates in hypoxia inducible factor-1α induced VEGF-A expression [7]. Likewise, VEGF-A upregulates

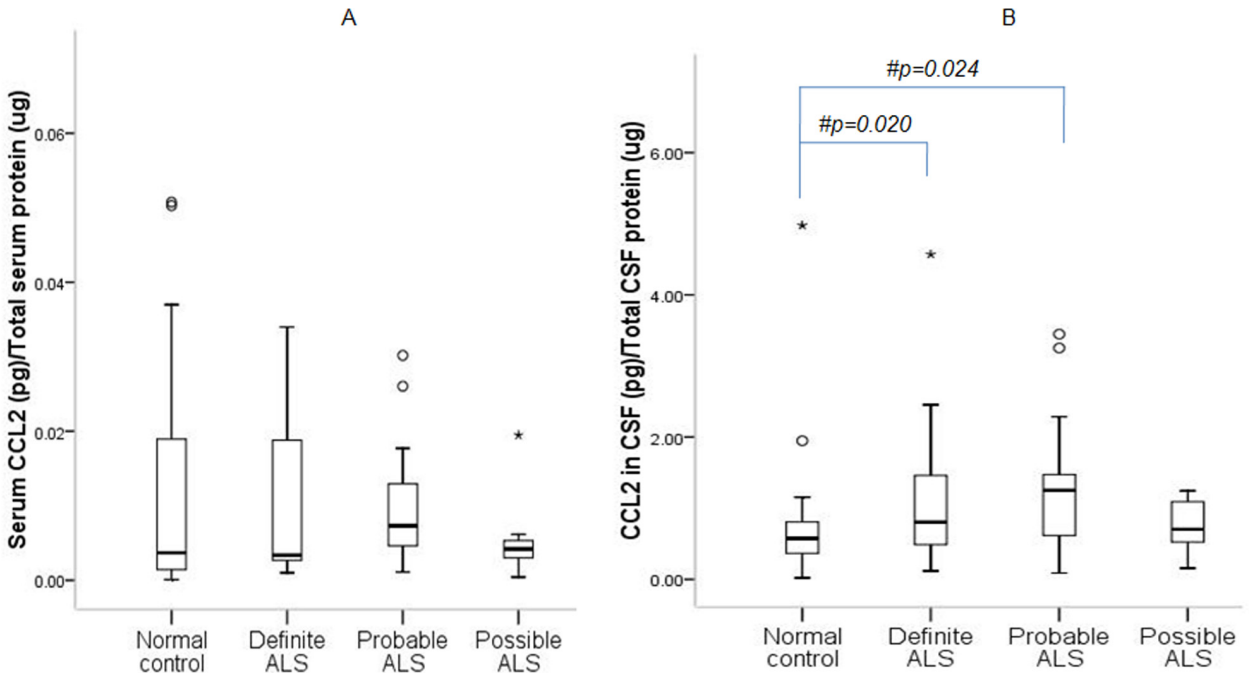

Figure 4 Level of CCL2 in serum (A) and CSF (B) of definite ALS, probable ALS, possible ALS patients and normal subjects. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Levels of CCL2 were normalized to total protein in the serum and CSF samples. Outliers and extreme values are shown in circles and asterisk respectively. No significant difference ($p > 0.05$) was found between the given conditions. Data was analyzed by Mann Whitney U Test. ALS, Amyotrophic Lateral Sclerosis; CCL2, chemokine ligand 2; pg, picogram; µg, microgram.

CCL2 expression by activating nuclear factor-kB via extracellular signal-regulated kinases (ERK) pathways in microglia [22]. Hence, it is possible that VEGF-A-CCL2 axis plays a crucial role in ALS pathogenesis.

The comparison between normal and neurological controls showed relatively pronounced increase in VEGF-A and CCL2 in the latter thus limiting the utility of these as biomarkers in ALS, however, further analysis of this data is being conducted by including homogeneous Parkinson's disease controls instead of the heterogeneous neurological controls used (data not shown).

Regardless of the reason, it is clear that elevated VEGF-A is associated with prolonged survival of Indian ALS patients. At this time, we have no explanation for the basis of this finding, but discuss its therapeutic potential. Increasing brain levels of VEGF-A by genetic engineering, direct infusion or stem cell transplantation may provide limited but significant prolonged life

Table 2 Crude and adjusted OR for VEGF-A levels in smokers, alcohol and meat consumers

	OR (95% CI)†	*p**	Adj. OR (95% CI) ‡	*p**
Serum VEGF-A				
Smoking	1.4 (0.4-4.5)	0.5	0.7 (0.2-2.5)	0.6
Alcohol consumption	0.7 (0.2-2.2)	0.6	0.9 (0.3-3.0)	0.9
Meat consumption	1.5 (0.5-3.9)	0.4	1.4 (0.5-4.0)	0.7
CSF VEGF-A				
Smoking	1.4 (0.4-4.5)	0.4	0.7 (0.2-2.5)	0.6
Alcohol consumption	0.7 (0.2-2.2)	0.4	0.9 (0.3-3.0)	0.8
Meat consumption	1.5 (0.5-3.9)	0.2	1.4 (0.5-4.0)	0.2
Never smoking/Nonalcoholic/Vegetarian**	1.0		1.0	

† Univariate logistic regression was used to evaluate crude OR. ‡ Multivariate logistic regression has been used to adjust the effect of smoking on VEGF-A levels with alcohol and meat consumption as covariates. Likewise, effect of alcohol is adjusted for smoking and meat consumption, and meat consumption is corrected for smoking and alcohol intake. * χ^2 (chi square test) was used to test the level of significance. ** Never smoking, nonalcoholic and vegetarian diet is considered as reference group. OR, odds ratio; CI, confidence interval; Adj, adjusted.

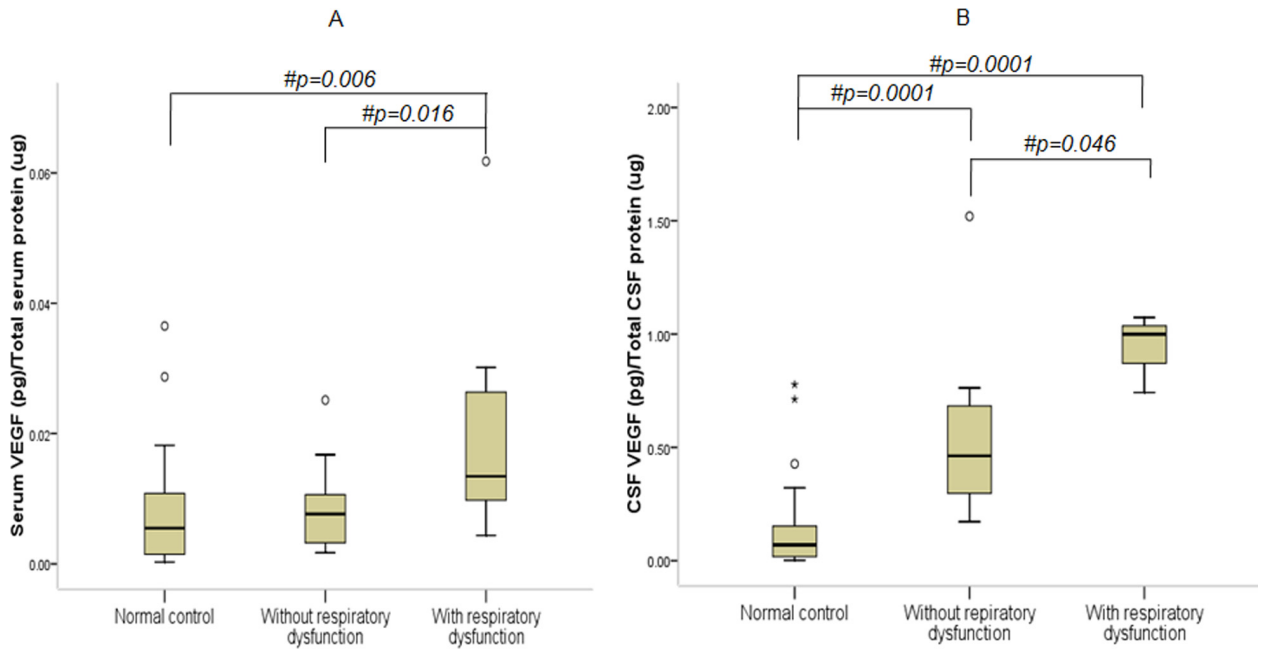

Figure 5 Level of VEGF-A in serum (A) and CSF (B) of ALS patients with respiratory dysfunction. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Levels of VEGF-A were normalized to total serum and CSF protein respectively. Outliers are shown in circles. # indicates significant difference ($p < 0.05$) between the given conditions. Data was analyzed by Mann Whitney U Test. ALS, Amyotrophic Lateral Sclerosis; VEGF-A, vascular endothelial growth factor-A; pg, picogram; μg, microgram.

expectancy of afflicted patients. Animal and culture studies have documented that aside from promoting revascularization, VEGF-A is a potent vasodilator, as well as attractant of marrow stromal cells and to some extent, hematopoietic stem cells [23]. Our results suggest that etiological factor VEGF-A needs to be augmented alongwith regulation of inflammation, as noted here by consistently increased CCL2, either by hematopoietic cell transplantation or by defining other neurotrophic and proinflammatory factors involved in ALS. Localized stem cell therapy designed to restore lost neural tissue presents subsequent therapeutic interventions [24]. Such a multifaceted approach will be helpful in presymptomatic identification of individuals who will develop ALS. These individual thus can be treated to prevent ALS from rapidly overwhelming the host defenses.

Table 3 Clinical summery of 11 ALS patients with respiratory dysfunction

ALS subjects	El Escorial criteria	ALSFRS-R	Impairment	Disease duration at sample collection (mo)‡
Patient 1	Definite	27	Moderate	12
Patient 2	Definite	23	Severe	12
Patient 3	Definite	18	Severe	24
Patient 4	Definite	16	Severe	18
Patient 5	Definite	29	Moderate	12
Patient 6	Definite	29	Moderate	04
Patient 7	Definite	29	Moderate	09
Patient 8	Definite	31	Moderate	08
Patient 9	Definite	35	Moderate	24
Patient 10	Probable	34	Moderate	12
Patient 11	Probable	35	Moderate	30

ALSFRS-R: ALS functional rating score-revised; Impairment was measured with ALSFRS-R. ‡ Duration of disease is the interval between appearance of first symptom of ALS and collection of sample. Median disease duration in patients with respiratory dysfunction is 12(4-30) months whereas median duration of disease was 17(3-72) months in patients without respiratory dysfunction, although the observed difference was not significant upon Mann-Whitney U analysis ($p > 0.05$).

The lack of association between smoking, alcohol and meat consumption with VEGF-A and CCL2 enhances the credibility of the results since these confounders increased the endpoints assessed but did so regardless of ALS status of the individual studied. The current report thus reliably indicates that ALS is associated with increased VEGF-A and CCL2 providing a foundation for subsequent studies to examine if this result is a factor in the enhanced survival of Indian ALS patients and if so, the mechanisms involved.

Conclusions

Our study supports that VEGF-A and CCL2 may be involved in enhancing the survival time of sporadic ALS patients, however, comprehensive understanding of growth factors network is required to unveil diagnostic and therapeutic efficacy of these molecules.

Ethical approval

Ethical approval was obtained by institute ethical committee, PGIMER, Chandigarh, India - 160012 (No. 7055-PG-1Tg-05/4348-50).

Abbreviations

ALS: amyotrophic lateral sclerosis; ANOVA: analysis of variance; CCL2: chemokine ligand-1; CCR2: chemokine receptor-2; CSF: cerebrospinal fluid; ELISA: enzyme liked immunosorbent assay; ERK: extracellular signal-regulated kinases; LSD: least significant difference; NMDA: N-Methyl-D-aspartate; SE: standard error; SOD1: superoxide dismutase 1; PI3-K: phosphatidylinositol 3-kinases; VEGF: vascular endothelial growth factor.

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Authors' contributions

PKG Acquisition of data and writing of manuscript; SP inclusion of patients, grant PI and clinical scoring; SS Statistical analysis; AA Interpretation and analysis of data, grant co PI 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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References

1. Zheng C, Nennesmo I, Fadeel B, Henter JI: **Vascular endothelial growth factor prolongs survival in a transgenic model of ALS.** *Ann Neurol* 2004, **56**:564-567.
2. Li B, Xu W, Luo C, Gozal D, Liu R: **VEGF-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death.** *Brain Res Mol Brain Res* 2003, **111**:155-164.
3. Meng H, Zhang Z, Zhang R, Liu X, Wang L, Robin AM, Chopp M: **Biphasic effects of exogenous VEGF on VEGF expression of adult neural progenitors.** *Neurosci Lett* 2006, **393**:97-101.
4. Henkel JS, Engelhardt JI, Siklos L, Simpson EP, Kim SH, Pan T, Goodman JC, Siddique T, Beers DR, Appel SH: **Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue.** *Ann Neurol* 2004, **55**:221-235.
5. Hughes PM, Allegrini PR, Rudin M, Perry VH, Mir AK, Wiessner C: **Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model.** *J Cereb Blood Flow Metab* 2002, **22**:308-317.
6. Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM: **Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis.** *J Exp Med* 2001, **193**:713-726.
7. Hong KH, Ryu J, Han KH: **Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A.** *Blood* 2005, **105**:1405-1407.
8. Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B, Nakanishi A: **The ALSFRS-R: a revised ALS functional rating scale that incorporates assessmentsof respiratory function. BDNF ALS Study Group (Phase III).** *J Neurol Sci* 1999, **169**:13-21.
9. Prabhakar S, Vinish M, Das CP, Anand A: **Occurrence of PARK2 Mutations in a Never-Smoker Population with Parkinson's Disease in North India.** *Neuroepidemiology* 2010, **35**:152-159.
10. Bradley WG: **Commentary on Professor Stephen Hawking's disability advice.** *Annals of Neurosciences* 2009, **16**:101-102.
11. Nalini A, Thennarasu K, Gourie-Devi M, Shenoy S, Kulshreshtha D: **Clinical characteristics and survival pattern of 1,153 patients with amyotrophic lateral sclerosis: experience over 30 years from India.** *J Neurol Sci* 2008, **272**:60-70.
12. Sorenson EJ, Stalker AP, Kurland LT, Windebank AJ: **Amyotrophic lateral sclerosis in Olmsted County, Minnesota, 1925 to 1998.** *Neurology* 2002, **59**:280-282.
13. Osuntokun BO, Adeuja AOG, Bademosi O: **The prognosis of motor neuron disease in Nigerian Africans. A prospective study of 92 patients.** *Brain* 1974, **97**:385-394.
14. Nygren I, Larsson A, Johansson A, Askmark H: **VEGF-A is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis.** *Neuroreport* 2002, **13**:2199-201.
15. Devos D, Moreau C, Lassalle P, Perez T, De Seze J, Brunaud-Danel V, Destée A, Tonnel AB, Just N: **Low levels of the vascular endothelial growth factor in CSF from early ALS patients.** *Neurology* 2004, **62**:2127-2129.
16. Cronin S, Greenway MJ, Ennis S, Kieran D, Green A, Prehn JH, Hardiman O: **Elevated serum angiogenin levels in ALS.** *Neurology* 2006, **67**:1833-1836.
17. McCloskey DP, Hintz TM, Scharfman HE: **Modulation of vascular endothelial growth factor (VEGF) expression in motor neurons and its electrophysiological effects.** *Brain Res Bull* 2008, **76**:36-44.
18. Bogaert E, Van Damme P, Poesen K, Dhondt J, Hersmus N, Kiraly D, Scheveneels W, Robberecht W, Van Den Bosch L: **VEGF protects motor neurons against excitotoxicity by upregulation of GluR2.** *Neurobiol Aging* 2008.
19. Madrigal JL, Leza JC, Polak P, Kalinin S, Feinstein DL: **Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline.** *J Neurosci* 2009, **29**:263-267.
20. Liu XS, Zhang ZG, Zhang RL, Gregg SR, Wang L, Yier T, Chopp M: **Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke.** *J Neurosci Res* 2007, **85**:2120-2125.
21. Magge SN, Malik SZ, Royo NC, Chen HI, Yu L, Snyder EY, O'Rourke DM, Watson DJ: **Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors.** *J Neurosci Res* 2009, **87**:1547-1555.
22. Marumo T, Schini-Kerth VB, Busse R: **Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells.** *Diabetes* 1999, **48**:1131-1137.
23. Schichor C, Birnbaum T, Etminan N, Schnell O, Grau S, Miebach S, Aboody K, Padovan C, Straube A, Tonn JC, Goldbrunner R: **Vascular**

endothelial growth factor A contributes to glioma-induced migration of human marrow stromal cells (hMSC). *Exp Neurol* 2006, **199**:301-310.

24. El-Badri NS, Hakki A, Saporta S, Liang X, Madhusodanan S, Willing AE, Sanberg CD, Sanberg PR: **Cord blood mesenchymal stem cells: Potential use in neurological disorders.** *Stem Cells Dev* 2006, **15**:497-506.

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Genetic markers

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Gene networks determine predisposition to AMD

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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.

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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

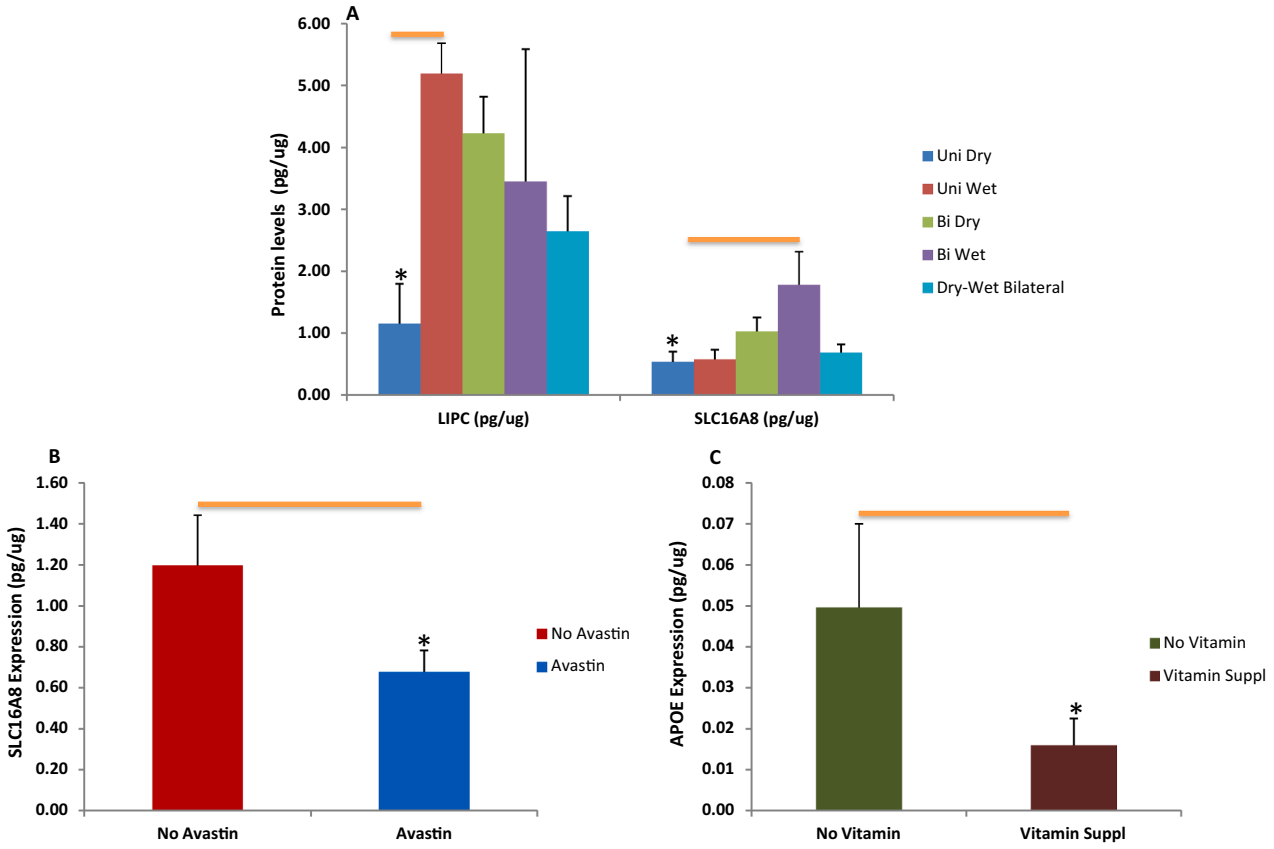


Fig. 1. (1A) Expression levels of LIPC and SLC16A8 along with error bars in unilateral-bilateral AMD pathology. (n) uni wet (63); uni dry (20); Bi wet (29); Bi dry (25); dry-wet (50). (1B–1C) Effect of treatment (including both anti- VEGF and vitamin supplementations) on the protein expressions (B) Alteration 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

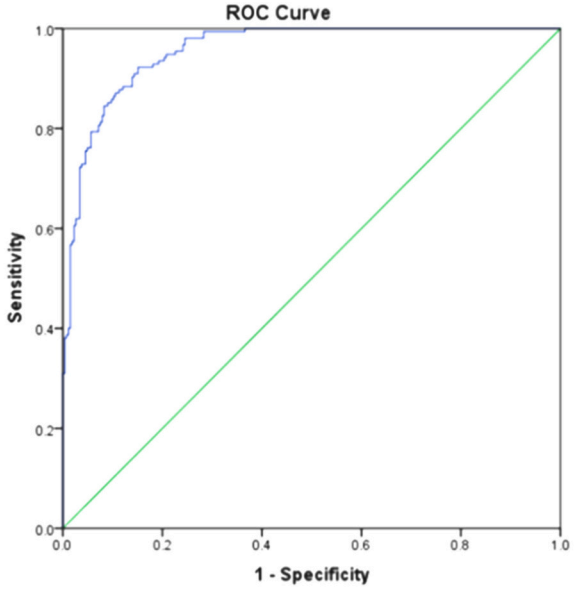
$$P(X) = \frac{1}{1 + e^{-\left(\beta_0 + \sum_{j=1}^{k} \beta_j X_j\right)}}$$

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.

References

[1] K. Sharma, N.K. Sharma, A. Anand, Why AMD is a disease of ageing and not of development: mechanisms and insights, Front. Aging Neurosci. 6 (2014) 151.
[2] L.G. Fritsche, W. Igl, J.N. Bailey, A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants, 48 (2016), pp. 134–143.
[3] A. Anand, K. Sharma, S.K. Sharma, R. Singh, N.K. Sharma, K. Prasad, AMD genetics in India: the missing links, Front. Aging Neurosci. 8 (2016) 115.
[4] T.E. Clemons, R.C. Milton, R. Klein, J.M. Seddon, F.L. Ferris, Risk factors for the incidence of advanced age-related macular degeneration in the age-related eye disease study (AREDS) AREDS report no. 19, Ophthalmology 112 (2005) 533–539.
[5] N.K. Sharma, A. Gupta, S. Prabhakar, et al., Association between CFH Y402H polymorphism and age related macular degeneration in north Indian cohort, PLoS One 8 (2013) e70193.
[6] L.G. Fritsche, W. Chen, M. Schu, et al., Seven new loci associated with age-related macular degeneration, Nat. Genet. 45 (433–9) (2013) (439e1-2).
[7] B. Rohrer, A. Frazer-Abel, A. Leonard, et al., Association of age-related macular degeneration with complement activation products, smoking, and single nucleotide polymorphisms in South Carolinians of European and African descent, Mol. Vis. 25 (2019) 79–92.
[8] N.K. Sharma, S. Prabhakar, A. Gupta, et al., New biomarker for neovascular age-related macular degeneration: eotaxin-2, DNA Cell Biol. 31 (2012) 1618–1627.
[9] K. Sharma, R. Tyagi, R. Singh, S.K. Sharma, A. Anand, Serum levels of TIMP-3, LIPC, IER3, and SLC16A8 in CFH-negative AMD cases, J. Cell. Biochem. 118 (2017) 2087–2095.
[10] V.M. Rajagopal, A.P. Rajkumar, K.S. Jacob, M. Jacob, Gene-gene interaction between DRD4 and COMT modulates clinical response to clozapine in treatment-resistant schizophrenia, Pharmacogenet. Genomics 28 (2018) 31–35.
[11] X. Sun, Q. Lu, S. Mukherjee, P.K. Crane, R. Elston, M.D. Ritchie, Analysis pipeline for the epistasis search - statistical versus biological filtering, Front. Genet. 5 (2014) 106.
[12] X. Wan, C. Yang, Q. Yang, et al., BOOST: a fast approach to detecting gene-gene interactions in genome-wide case-control studies, Am. J. Hum. Genet. 87 (2010) 325–340.
[13] N.K. Sharma, S.K. Sharma, A. Gupta, S. Prabhakar, R. Singh, A. Anand, Predictive model for earlier diagnosis of suspected age-related macular degeneration patients, DNA Cell Biol. 32 (2013) 549–555.
[14] N.K. Sharma, K. Sharma, R. Singh, S.K. Sharma, A. Anand, CCL2 single nucleotide polymorphism of rs1024611 implicates prominence of inflammatory cascade by univariate modeling in Indian AMD, 13 (2018) e0193423.

Article

The Role of *Dystrophin* Gene Mutations in Neuropsychological Domains of DMD Boys: A Longitudinal Study

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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 including poor performance in working memory,

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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.

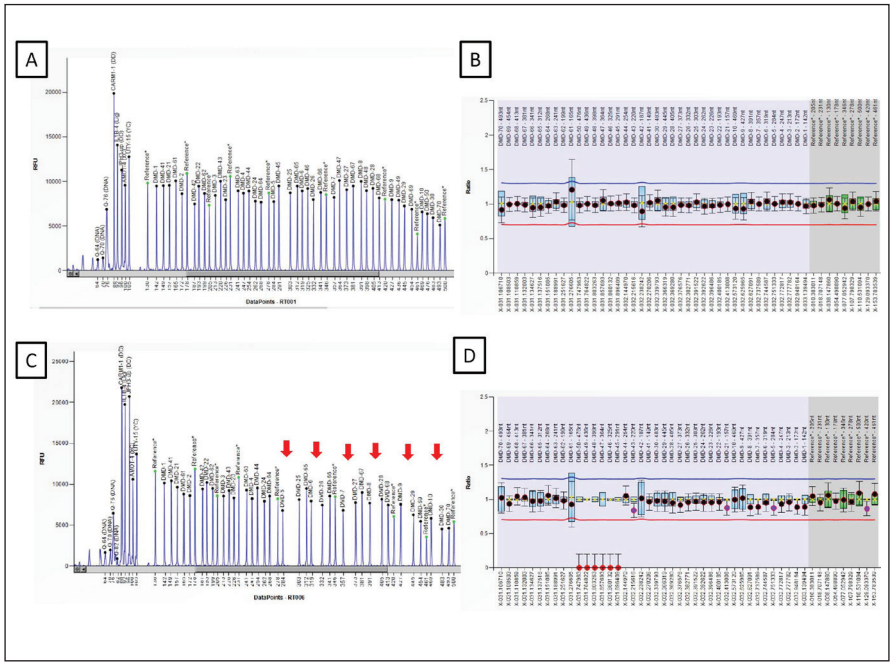

Figure 1. Electropherogram Obtained after Multiplex Ligation Dependent Probe Amplification (MLPA) PCR Followed by Capillary Electrophoresis of the Amplified Products. **(A & B)** Electropherogram and Ratio Chart Representing Profile of a Normal Control Sample. **(C & D)** Electropherogram and Ratio Chart 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.

Statement of Ethics

The study was approved by Institute Ethics Committee of PGIMER, Chandigarh vide no. INT/IEC/2015/732 dated 19 November 2015.

ICMJE Compliance

Manuscript complies with the guidelines outlined by ICMJE.

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.

References

1. Doorenweerd N, Mahfouz A, van Putten M, et al. Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. Sci Rep 2017; 7(1): 12575.
2. Allikian MJ, McNally EM. Processing and assembly of the dystrophin glycoprotein complex. Traffic 2007; 8(3): 177–183.
3. Constantin B. Dystrophin complex functions as a scaffold for signalling proteins. Biochim Biophys Acta 2014; 1838(2): 635–142.
4. Stark AE. Determinants of the incidence of Duchenne muscular dystrophy. Ann Transl Med 2015; 3(19): 287.
5. Perumal AR, Rajeswaran J, Nalini A. Neuropsychological profile of Duchenne muscular dystrophy. Appl Neuropsychol Child 2015; 4(1): 49–57.
6. Anand A, Tyagi R, Mohanty M, Goyal M, Silva KR, Wijekoon N. Dystrophin induced cognitive impairment: mechanisms, models and therapeutic strategies. Ann Neurosci 2015; 22(2): 108–118.
7. Wingeier K GE, Strozzi S, Kreis R, et al. Neuropsychological impairments and the impact of dystrophin mutations on general cognitive functioning of patients with Duchenne muscular dystrophy. J Clin Neurosci 2011; 18(1): 90–95.
8. Ricotti V, Mandy WP, Scoto M, et al. Neurodevelopmental, emotional, and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations. Dev Med Child Neurol 2016; 58(1): 77–84.
9. Chamova T, Guergueltcheva V, Raycheva M, et al. Association between loss of dp140 and cognitive impairment in Duchenne and Becker dystrophies. Balkan J Med Genet 2013; 16(1): 21–30.
10. Doorenweerd N, Straathof CS, Dumas EM, et al. Reduced cerebral gray matter and altered white matter in boys with Duchenne muscular dystrophy. Ann Neurol 2014; 76(3): 403–411.
11. Rae MG, O'Malley D. Cognitive dysfunction in Duchenne muscular dystrophy: a possible role for neuromodulatory immune molecules. J Neurophysiol 2016; 116(3): 1304–1315.
12. Baumann N, Pham-Dinh D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 2001; 81(2): 871–927.
13. Aranmolate A, Tse N, Colognato H. Myelination is delayed during postnatal brain development in the mdx mouse model of Duchenne muscular dystrophy. BMC Neurosci 2017; 18(1): 63.

14. Perronnet C, Vaillend C. Dystrophins, utrophins, and associated scaffolding complexes: role in mammalian brain and implications for therapeutic strategies. J Biomed Biotechnol 2010; 2010: 849426.
15. Cotton SM VN, Greenwood KM. Association between intellectual functioning and age in children and young adults with Duchenne muscular dystrophy: further results from a meta-analysis. Dev Med Child Neurol 2005; 47(4): 257–265.
16. Banihani R, Smile S, Yoon G, et al. Cognitive and Neurobehavioral Profile in Boys With Duchenne Muscular Dystrophy. J Child Neurol 2015; 30(11): 1472–1482.
17. Filippo TD, Parisi L, Roccella M. Psychological aspects in children affected by Duchenne de Boulogne muscular dystrophy. Ment Illn 2012; 4(1): e5.
18. Abi Daoud MS, Dooley JM, Gordon KE. Depression in parents of children with Duchenne muscular dystrophy. Pediatr Neurol 2004; 31(1): 16–19.
19. Tyagi R KS, Dalal A, Mohammed F, et al. Repurposing Pathogenic Variants of DMD Gene and its Isoforms for DMD Exon Skipping Intervention. Curr Genomics 2019; 20(1): 519–530.
20. Sharma K, Tyagi R, Singh R, Sharma SK, Anand A. Serum Levels of TIMP-3, LIPC, IER3, and SLC16A8 in CFH-Negative AMD Cases. J Cell Biochem 2017; 118(8): 2087–2095.
21. Cotton S VN, Greenwood KM. Intelligence and Duchenne muscular dystrophy: full-scale, verbal, and performance intelligence quotients. Dev Med Child Neurol 2001; 43(7): 497–501.
22. Taylor PJ, Betts GA, Maroulis S, et al. Dystrophin gene mutation location and the risk of cognitive impairment in Duchenne muscular dystrophy. PLoS One 2010; 5(1): e8803.
23. Anderson SW RD, Ionasescu VV. Serial position memory of boys with Duchenne muscular dystrophy. Dev Med Child Neurol 1988; 30(3): 328–333.
24. Homack S RC. A meta-analysis of the sensitivity and specificity of the Stroop Color and Word Test with children. Arch Clin Neuropsychol 2004; 19(6): 725–743.
25. Wicksell RK, Kihlgren M, Melin L, Eeg-Olofsson O. Specific cognitive deficits are common in children with Duchenne muscular dystrophy. Dev Med Child Neurol 2004; 46(3): 154–159.
26. Remmelink E, Aartsma-Rus A, Smit AB, Verhage M, Loos M, van Putten M. Cognitive flexibility deficits in a mouse model for the absence of full-length dystrophin. Genes Brain Behav 2016; 15(6): 558–567.
27. Bailey M, Miller N. DMD Open-access Variant Explorer (DOVE): A scalable, open-access, web-based tool to aid in clinical interpretation of genetic variants in the DMD gene. Mol Genet Genomic Med 2019; 7(1): e00510.
28. Landfeldt E, Lindgren P, Bell CF, et al. The burden of Duchenne muscular dystrophy: an international, cross-sectional study. Neurology 2014; 83(6): 529–536.
29. Ryder S, Leadley RM, Armstrong N, et al. The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet J Rare Dis 2017; 12(1): 79.

Are *COL1A1* gene polymorphisms associated with anterior cruciate ligament tear in the Indian population? Results of a preliminary case-control study

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Summary

***Introduction*: Despite the identification of various intrinsic and extrinsic risk factors associated with ACL tear, the exact etiopathogenesis of ACL tear is still incompletely understood. The evidence for association of COL1A1 gene polymorphisms with ACL tear has been somewhat conflicting.**
Objective of the study is: to determine if rs1800012 and rs1107946 polymorphisms of COL1A1 gene are associated with ACL tear in an Indian population study group.
***Methods*: Fifty clinically, radiologically and surgically-proven ACL deficient patients and 52 healthy controls were included in the study. After isolation of DNA from peripheral blood monocytes, real time PCR was carried out to genotype *COL1A1* rs1800012 and rs1107946 polymorphisms.**
***Results*: Both the groups were matched for age and sex. Hardy Weinberg equilibrium was maintained for both genotypes. There was no significant difference in the genotype or allele distribution between the case and control groups for both rs1800012 (p=0.516) and rs1107946 polymorphisms (p=0.971 for GT and p=0.823 for TT) in this preliminary study.**
***Conclusion*: The rs1800012 polymorphism of *COL1A1* gene remains the first and most extensively tested gene polymorphism for its association with ACL tear. Under-representation of the TT genotype has been observed in Swedish and South African Caucasian populations with ACL tear. This has not been noted in the Polish Caucasian population and in our study. More studies with larger study samples from different ethnic populations are needed before *COL1A1* gene polymorphism screening tests are incorporated into ACL tear prevention programs.**
Level of evidence: III b (case-control study).

KEY WORDS: anterior cruciate ligament (ACL), COL1A1 *gene, single nucleotide polymorphism, genotype frequency, allele frequency.*

Introduction

Anterior cruciate ligament (ACL) tear is one of the most common ligament injuries of the knee[1,2]. The etiology of ACL tear has been proven to be multifactorial[3, 4]. Although various intrinsic and extrinsic risk factors and their complex interaction have been identified and explored in great detail, the exact etiopathogenesis of ACL tears has not yet been entirely deciphered[3, 4].
Over the last decade, 33 different single nucleotide polymorphisms (SNPs) of various genes have been investigated for association with ACL tear[5-7]. *COL1A1* rs1800012 polymorphism was the first specific gene polymorphism to be positively associated with ACL tear[3, 4]. It is also the most extensively investigated SNP in the literature with various research teams having investigated the association between *COL1A1* SNPs and ACL tear in different Caucasian population subsets with mixed results[5-8]. However, till date, there is no data from the Asian continent on the association of *COL1A1* SNPs and ACL tear[7].
We conducted a genetic association, case-control study to investigate the association between SNPs of *COL1A1* gene and ACL tear in the Indian population.

Materials and methods

This study was carried out at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India after obtaining clearance from the institutional ethics committee. An informed, written consent was obtained from subjects of both case and control groups. The study has been conducted ethically according to accepted international standards and meets the ethical standards of the journal[9].

Recruitment of participants

Fifty patients with clinico-radiologically and surgically proven ACL tear were enlisted into the study group from July 2012 to December 2013. The inclusion criteria were: I) age between 18-40 years; II) no multiligament injuries or signs of knee osteoarthritis; III) no associated comorbidities. Fifty-two subjects with normal knees with no history of ligament/tendon injuries were recruited as control subjects. All participants underwent a thorough physical examination and completed a comprehensive proforma containing demographic details, mode of injury, relevant past, personal and family histories along with details of participation in sports. The participants who have only ACL injury were included in the study. The diagnosis was confirmed by both MRI and intra-operatively during diagnostic arthroscopy. Multi-ligament injuries were excluded from the study.

Experimentations pertaining to the study were performed in Neuroscience Research Lab (NRL), Department of Neurology, PGIMER, Chandigarh, India which follows good laboratory practices (GLP).

All experiments were performed using pre-calibrated instruments; quality assurance norms and established standard operating protocols (SOP) were adhered to. Records were maintained in Data Recording Sheets (DRS) by qualified research scholars; as prescribed by the OECD (Organisation for economic co-operation and development) guidelines, the investigators were blinded to the samples.

DNA extraction

Venous blood (5 ml) was drawn into an ethylenediaminetetraacetic acid (EDTA) vaccutainer and stored at room temperature for 2 hours to allow sedimentation of red blood cells. The upper layer was then aspirated and placed over the same amount of ficoll paque solution (*Amersham Biosciences, USA*) followed by centrifugation at 1800 rpm for 30 minutes. After centrifugation, the peripheral blood monocytes (PBMC) settle as a thin middle layer; the PBMC were separated and kept in a separate tube. The PBMC was now subjected to centrifugation at 5000 rpm for 5 minutes, washed with phosphate-buffered saline (PBS) and processed for DNA isolation. Next, the genomic DNA was isolated using commercially-available QIAGEN DNeasy kit. Ultraviolet-visible spectrophotometry (*Backman Coulter*) was used to quantify the DNA. Electrophoresis on 1% agarose gel *(Bio-rad)* was performed to validate the quality of DNA (Fig. 1).

COL1A1 ***genotyping***

RT-PCR (real-time polymerase chain reaction) was used to analyse SNPs and a 48-well StepOne™ (*Applied Biosystems Inc, Foster City, USA*) RT-PCR instrument was used for the same. RT-PCR was car-

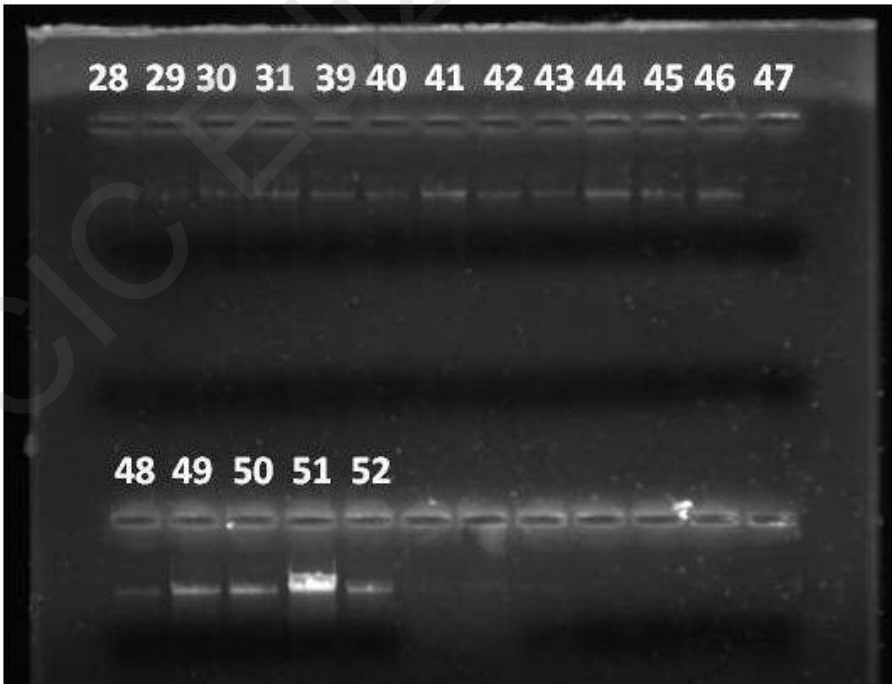

Figure 1. Genomic DNA isolated from PBMCs subjected to electrophoresis on 1% agarose gel for quality verification.

ried out for 20 µL solution (containing 10 µL master mix, 5 µL assay, 20 ng DNA and 5 µL molecular biology grade water). All these reactions were carried out using TaqMan®/SYBR™ Green pre-designed genotyping assays according to the manufacturers' recommendations. Both alleles in genotyping assay were tagged two reporter dyes namely VIC and FAM to detect the SNP changes at the particular locus. Software Step One™ v2.0 (Applied Biosystems, Foster City, USA) was utilised to perform amplification and to estimate SNPs followed by importing of the fluorescence (Rn) values recorded during the plate read using the Sequence Detection System (SDS) software. The DNA samples were genotyped for *COL1A1* rs1800012 and rs1107946 SNPs using PCR amplification.

Statistical analysis

The SPSS (Statistical Package for the Social Sciences) software (version 16.0) was used for data analysis. Normal quantile (Q-Q) plots were constructed to check for the normality of the data distribution. Unpaired Student's *t*-test was used to test the association between cases and controls. Before proceeding to genotyping analysis, Hardy-Weinberg equilibrium was tested; the genotypes for each SNP were stratified for homozygosity and heterozygosity, and of the respective allelic variant. Pearson's chi-square test and Cochran-Mantel-Haenszel equation were applied for the genotyping result analysis.

Results

***SNPs analysis of rs1800012 (Sp1 binding site) and rs1107946 of* COL1A1**

Frequency of both genotypes GT and TT of rs180 0012 locus haven't shown any significant association with ACL tear. Similarly, frequency of T alleles (p=0.5163) did not show any correlation with pathology. Conclusively, results of rs1800012 SNP analysis shown no statistically significant difference in both genotype as well as (Supplementary information Table I) (p=0.516) between ACL and control groups (Tab. I, Fig. 2). All groups were in Hardy Weinberg equilibrium (HWE value=0.11).

Moreover, genotype (GT and TT p=0.9711, p=0.8226, respectively) of rs1107946 SNP did not reveal any association with ACL pathology as compare to controls. Additionally, genotype ad allele frequency (sup-

Table I. Effect of genetic variants of rs1800012 on ACL tear phenotype

Genotype	Number	OR	95%CI	Z- statistics	Value
	ACL	Controls			
GG	1	0	Reference		
GT	7	7	*	*	* *
TT	39	43	*	*	* *

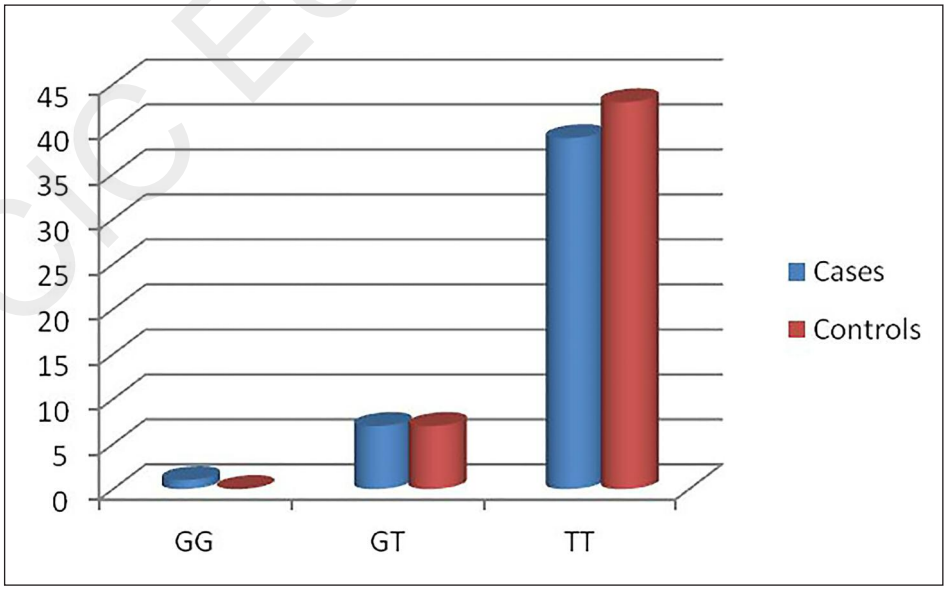

Figure 2. Graph depicting relative frequency of genotypes (GG, GT and TT) for rs18000012 polymorphism of *COL1A1* gene.

plementary information Tab. II) of the same locus has also not shown any statistical difference between ACL control groups (p=0.7665) (Tab. II, Fig. 3).

Discussion

ACL tear has a complex, multifactorial etiology; an interaction between predisposing genetic factors, environmental and intrinsic risk factors has been proposed by multiple research groups[3,4]. Overall, 20 genes (33 different genetic variants) have been investigated for their association with ACL tear; out of these, 10 gene polymorphisms (SNPs) and 8 haplotypes have been linked to ACL tear[5,6]. Till date, four different research groups have tested for the association of *COL1A1* polymorphisms with ACL tears (Tab. III)[10-13]. These studies linking gene polymorphisms to ACL tears are essential as these tests can be used to predict injury risk in athletes and alter clinical management protocols and training regimens in these "high-risk" individuals.

The ACL is predominantly made up of collagen protein (75%) which is encoded by different collagen genes; each type of collagen performs diverse functions[14]. The major structural collagen in ACL is type I collagen (comprising 70% to 80% of the dry mass of ACL). Type I collagen is a heterotrimer of two α1 and one α2 chains; these two chains are encoded by *COL1A1* and *COL1A2* genes respectively[15]. The *COL1A1* gene is located on the long arm of chromosome 17 (17q21.3-q22) whereas the *COL1A2* gene is located on the long arm of chromosome 7 (7q22.1). The *COL1A1* gene consists of 52 exons and is 18kb in size[16]. It has been found that the substitution of G to T at the binding site of Sp1 (rs1800012) consequently led to enhanced expression of proteins concomitant with the increased binding affinity of Sp1. (Fig. 4)[17].

The rs1107946 SNP is located in the proximal promoter region of *COL1A1* gene at position -1997 relative to the transcription start site[12]. This SNP has been associated with bone mineral density (BMD) and has a role in the *in vitro* regulation of osteoblasts[18,19]. It has been investigated for an association with ACL tear susceptibility by Ficek et al.[12] who reported no independent association.

Table II. Effect of genotypic variants of COL1A1 rs1107946 on ACL tear phenotype.

Genotype	Number		OR	95%CI	Z- statistics	p-value
	ACL	Control				
GG	7	8	*Reference*			
GT	17	19	1.023	0.306- 3.419	0.036	0.971
TT	23	23	1.143	0.356 - 3.673	0.224	0.823

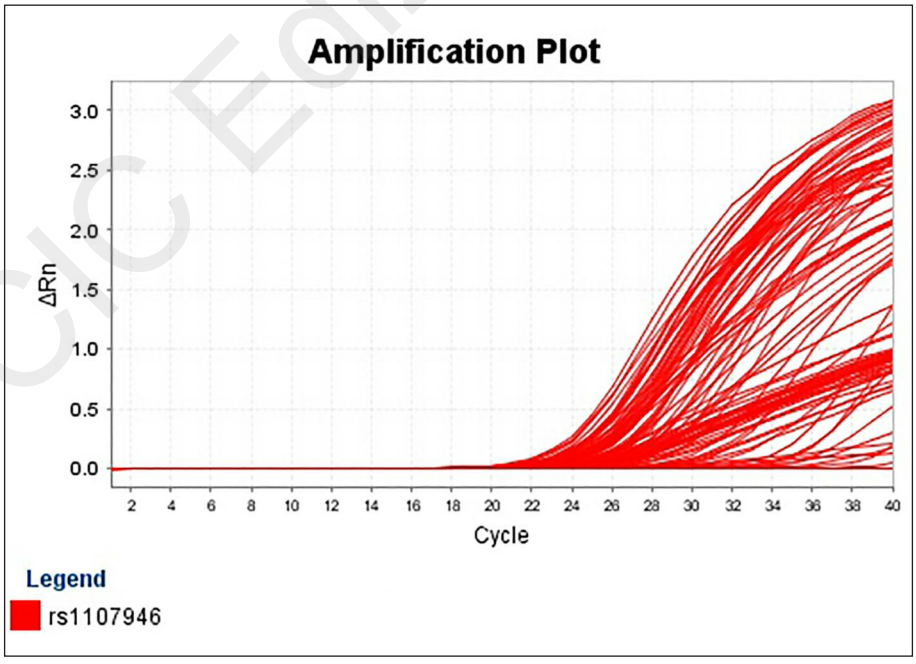

Figure 3. Representative amplification plot for ACL control and patient PBMCs from SNP genotyping analysis for rs1107946 variant.

Table III. Summary of all studies investigating association of COL1A1 SNPs and ACL tear.

Sno	Authors (Year)	Population	SNP analysed	Sample size	Salient Results of study
1	Khoschnau et al. (11) 2008	Sweden	rs1800012	558 (233)	TT genotype of the rs1800012 was shown to be significantly under-represented in participants with ACL tears (only 1 in 233 cases had this genotype). The presence of TT genotype was postulated to be protective against ACL tear.
2	Posthumus et al. (10) 2009	South Africa	rs1800012	247 (117)	Under-representation of TT genotype in ACL group (OR = 0.08 (95% CI, \0.01-1.46); p = 0.031)
3	Stepien-Slodkowska et al. (13) 2013	Poland	rs1800012	366 (183)	Risk was 1.43 times lower in carriers of a minor allele G as compared to carriers of the allele T (p=0.045). **No under-representation of the TT genotype noted.**
4	Ficek et al. (12) 2013	Poland	rs1800012 rs1107946	234 (91)	**No significant association of genotype/allele distribution with ACL tear. No under-representation of the TT genotype noted.**
5	This study	India	rs1800012 rs1107946	102 (50)	**No significant association of genotype/allele distribution with ACL tear No under-representation of the TT genotype noted.**

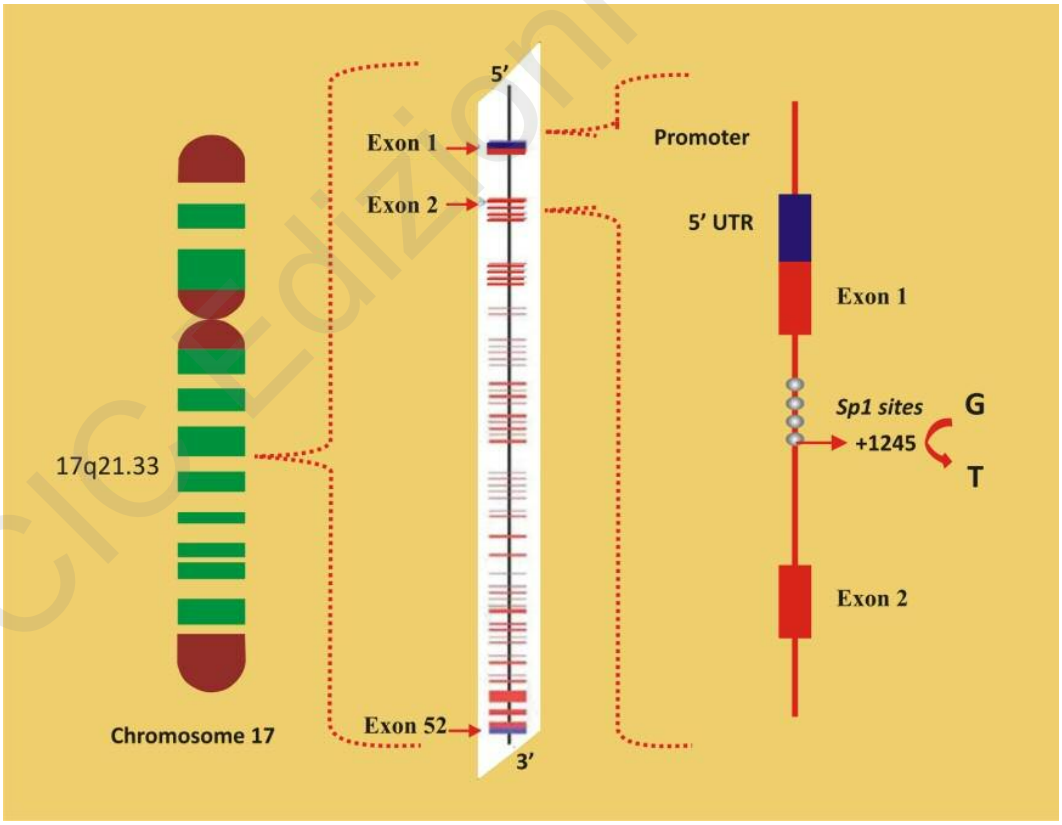

Figure 4. Diagrammatic representation of *COL1A1* gene locus on chromosome 17, and functional polymorphism at the Sp1 site from guanine (G) to thymidine (T).

Although a familial predisposition to ACL tear was postulated by Harner et al.[20] and Flynn et al.[1], Khoschnau et al.[11] were the first to investigate for a specific gene polymorphism linked to ACL tear. They reported that the TT genotype of the rs1800012 polymorphism was significantly under-represented in patients with cruciate ligament tears and shoulder dislocations[11]. Posthumus et al. have investigated the genetic association of rs1800012 (COL1A1) in 117 Caucasian ACL individuals (surgically diagnosed) and 113 controls. Results have demonstrated the lack of TT genotype in ACL participants (zero in 117 ACL individual) as compared to controls (6 in 113 individuals) (p=0.031). The results have suggested the imperative role of TT genotype in ACL pathology.

Stepien-Slodkowska et al.[13] tested 321 male skiers (138 cases and 183 controls) for the association of rs18000012 polymorphism with ACL tear. Results showed 1.43 times lower risk of ACL tear was observed in G allele as compared to T allele in the ACL individuals (p=0.045). However, they did not observe under-representation of the TT genotype in the ACL group unlike the previous two studies[10, 11].

Ficek et al.[12] tested both rs1800012 and rs1107946 SNPs for their association with ACL tear in 377 male Polish soccer players (234 cases and 143 controls). They observed that both these SNPs were not significantly associated with ACL tear (p=0.228 for rs1107946 and p=0.138 for rs1800012 polymorphism). However, on performing a further haplotype analysis of these two SNPs, they noted that a higher frequency of the *COL1A1* G-T (rs1800012 and rs1107946) haplotype expression was significantly associated with decreased risk of ACL tear (haplotype score, -1.98; p= 0.048)[12]. It is to be noted that both these Polish studies were exclusively limited to male populations and therefore it was not possible to ascertain gender-specific association unlike Posthumus et al.[10].

Due to a non-Mendelian inheritance pattern, the association between *COL1A1* SNPs and ACL tear is quite complex unlike the Mendelian-inherited genetic diseases of *COL1A1* gene (e.g.: osteogenesis imperfecta)[15,17,21]. Complex gene-gene and gene-environment interactions alter the severity of the phenotype in non-Mendelian pattern inherited conditions like ACL tear[7, 21]. Recently, Yao et al. linked the rs1107946 polymorphism to the geometric size of ACL in the Chinese population[22]. Bell et al.[23] have linked the rs1800012 polymorphism to increased knee joint laxity. Both geometric size of ACL and joint laxity are individual intrinsic risk factors for ACL tear; therefore, *COL1A1* SNPs may predispose a person to an ACL tear in more ways than one.

It is also interesting to note that, *COL1A1* SNPs have also been associated with other sports injuries like Achilles tendinopathy, tennis elbow and shoulder dislocations[11,17,23,25]. Wang et al. examined association of SNPs and sports-related ligament and tendon COL1A1 injuries (like ACL tear, Achilles tendinopathy, tennis elbow and should dislocation). They observed that the rare TT genotype may be protective against these sports-related ligament/tendon injuries[7]. Apart from sports injuries, the rs18000012 SNP of *COL1A1* gene has most notably been linked to osteoporosis; various other complex disorders of connective tissue, including, myocardial infarction, lumbar disc disease and stress urinary incontinence have also shown association with this SNP[26-29].

Our study showed that *COL1A1* rs1800012 and rs1107946 polymorphisms were present in the Indian population, follow the Hardy-Weinberg equilibrium but do not seem to be associated with ACL tear. This is similar to the results obtained by Ficek et al.[12] in the Polish population. There was no under-representation of the TT genotype in patients with ACL tear in the Indian population subset as noted by Posthumus et al.[10] and Khoschnau et al.[11] in South African and Swedish Caucasian populations respectively.

This is the first study from Asia to test the association between ACL tear and *COL1A1* gene polymorphisms. Malila et al.[30] have conducted the only other Asian genetic association study for ACL tear. They studied the association between matrix metalloproteinase (MMP) genes and ACL tears and observed no significant association[30]. It is to be noted that all other *COL1A1* genetic association studies have been performed on Caucasian study subjects[10-13]. There are, however, a few limitations of this study. Most of the patients are from the northern region of India and the results could vary significantly if patients from other parts of the country are tested. The sample size is relatively small compared to similar studies; however, this is a preliminary study of a larger ongoing research project. Also, the number of female participants in the study was less; however, the primary objective of the study was not to look for gender-specific association.

Conclusion

In conclusion, this study found that there is no significant association between rs1800012 and rs11079846 polymorphism frequencies with ACL tear in the Indian population unlike most other Caucasian studies. Genetic research studies and tests will pave the way for customised preventive measures and personalised nutrition, training regimens to help reduce the incidence of ACL tears[31]. However, we believe that larger studies in different ethnic populations are needed across the world to define the association of this specific sequence variant with ACL tear before incorporating these genetic screening tests in an ACL tear preventive model.

Author's contribution

SP: Principle investigator, co-conceptualization of study, editing of manuscript, and recruitment of participants; RJ: Recruitment of participants and writing of manuscript; MSD: Editing of manuscript and co-conceptualization of study; KS: Experimentations and editing of manuscript; SB: Samples process and experimentation of the study; AA: Principle investigator, co-conceptualization of study and editing of manuscript.

Conflict of interest

All Authors have expressed no conflict of interest persist in any part of the study.

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References

1. Flynn RK, Pedersen CL, Birmingham TB, Kirkley A, Jackowski D, Fowler PJ. The familial predisposition toward tearing the anterior cruciate ligament. Am J Sports Med. 2005;33(1):23-28.
2. John R, Dhillon MS, Syam K, Prabhakar S, Behera P, Singh H. Epidemiological profile of sports-related knee injuries in northern India: An observational study at a tertiary care centre. J Clin Orthop Trauma. 2016;7(3):207-211.
3. Smith HC, Vacek P, Johnson RJ, Slauterbeck JR, Hashemi J, Shultz S, et al. Risk factors for anterior cruciate ligament injury: a review of the literature ? Part 1: neuromuscular and anatomic risk factors, Sports Health. 2012;4(1):69-78.
4. Smith HC, Vacek P, Johnson RJ, Slauterbeck JR, Hashemi J, Shultz S, et al. Risk Factors for Anterior Cruciate Ligament Injury: A Review of the Literature - Part 2: Hormonal, Genetic, Cognitive Function, Previous Injury, and Extrinsic Risk Factors, Sports Health. 2012;4(2):155-161.
5. Kaynak M, Nijman F, van Meurs J, Reijman M, Meuffels DE. Genetic variants and anterior cruciate ligament rupture: A systematic review. Sports Med (Internet). 2017. Available from: http://link.springer.com/10.1007/s40279-017-0678-2
6. John R, Dhillon MS, Sharma S, Prabhakar S, Bhandari M. Is there a genetic predisposition to anterior cruciate ligament tear? A systematic review, Am J Sports Med. 2016;44(12):3262-3269.
7. Wang C, Li H, Chen K, Wu B, Liu H. Association of polymorphisms rs1800012 in COL1A1 with sports-related tendon and ligament injuries: a meta-analysis. Oncotarget (Internet) 2017. Available from: http://www.oncotarget.com/abstract/15271.
8. Maffulli N, Margiotti K, Longo UG, Loppini M, Fazio VM, Denaro V. The genetics of sports injuries and athletic performance. MLTJ. 2013;3(3):173-189.
9. Padulo J, Oliva F, Frizziero A, Maffulli N. Muscles, Ligaments and Tendons Journal - Basic principles and recommendations in clinical and field science research: 2016 update. MLTJ. 2016;6(1):1-5.
10. Posthumus M, September AV, Keegan M, O'Cuinneagain D, Van der Merwe W, Schwellnus MP, et al. Genetic risk factors for anterior cruciate ligament ruptures: COL1A1 gene variant, Br J Sports Med. 2009;43(5):352-356.
11. Khoschnau S, Melhus H, Jacobson A, Rahme H, Bengtsson H, Ribom E, et al. Type I collagen α 1 Sp1 polymorphism and the risk of cruciate ligament ruptures or shoulder dislocations, Am J Sports Med. 2008;36(12):2432-2436.
12. Ficek K, Cieszczyk P, Kaczmarczyk M, Maciejewska-Karłowska A, Sawczuk M, Cholewinski J, et al. Gene variants within the COL1A1 gene are associated with reduced anterior cruciate ligament injury in professional soccer players. J Sci Med Sport. 2013;16(5):396-400.
13. Stepien-Slodkowska M, Ficek K, Eider J, Leolska-Duniec A, Maciejewska-Karlowska A, Sawczuk M, et al. The +1245 G/T polymorphisms in the Collagen type I alpha 1 (COL1A1) gene in Polish skiers with anterior cruciate ligament injury. Biol Sport. 2013;30(1):57-60.
14. Frank CB. Ligament structure, physiology and function. J Musculoskelet Neuronal Interact. 2004;4(2):199-201.
15. Myllyharju J, Kivirikko KI. Collagens and collagen-related diseases. Ann Med. 2001;33(1):7-21.
16. Anjankar S, Poornima S, Raju S, Jaleel M, Bhiladvala D, Hasan Q. Degenerated intervertebral disc prolapse and its association of collagen I alpha 1 Spl gene polymorphism: A preliminary case control study of Indian population. Indian J Orthop. 2015;49(6):589.
17. Collins M, Posthumus M, Schwellnus MP. The COL1A1 gene and acute soft tissue ruptures. Br J Sports Med. 2010;44(14):1063-1064.
18. Yazdanpanah N, Rivadeneira F, van Meurs JBJ, Zillikens MC, Arp P, Hofman A, et al. The -1997 G/T and Sp1 polymorphisms in the collagen type I alpha1 (COLIA1) gene in relation to changes in femoral neck bone mineral density and the risk of fracture in the elderly: the Rotterdam study, Calcif Tissue Int. 2007;81(1):18-25.
19. García-Giralt N, Enjuanes A, Bustamante M, Mellibovsky L, Nogués X, Carreras R, et al. In vitro functional assay of alleles and haplotypes of two COL1A1-promoter SNPs, Bone. 2005;36(5):902-908.
20. Harner CD, Paulos LE, Greenwald AE, Rosenberg TD, Cooley VC. Detailed analysis of patients with bilateral anterior cruciate ligament injuries. Am J Sports Med. 1994;22(1):37-43.
21. van Heyningen V. Mechanisms of non-Mendelian inheritance in genetic disease. Hum Mol Genet. 2004;13(Supl 2):R225-233.
22. Yao C, Zhu B, Liu Y, Xu X, Yang X, Xu Z et al. Association of MMP3 COL5A1 and COL1A1 gene polymorphisms with ACL geometric size in Chinese population. Int J Clin Exp Pathol. 2017;10(2):1991-1997.
23. Bell RD, Shultz SJ, Wideman L, Henrich VC. Collagen gene variants previously associated with anterior cruciate ligament injury risk are also associated with joint laxity, Sports Health. 2012;4(4):312-318.
24. Erduran M, Altinisik J, Meric G, Ates O, Ulusal AE, Akseki D. Is Sp1 binding site polymorphism within COL1A1 gene associated with tennis elbow? Gene. 2014;537(2):308-311.
25. Posthumus M, September AV, Schwellnus MP, Collins M. Investigation of the Sp1-binding site polymorphism within the COL1A1 gene in participants with Achilles tendon injuries and controls. J Sci Med Sport. 2009;12(1):184-189.
26. Mann V, Hobson EE, Li B, Stewart TL, Grant SF, Robins SP, et al. A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. J Clin Invest. 2001;107(7):899-907.

27. Speer G, Szenthe P, Kósa JP, Tabák AG, Folhoffer A, Fuszek P, et al. Myocardial infarction is associated with Spl binding site polymorphism of collagen type 1A1 gene. Acta Cardiol. 2006;61(3):321-325.
28. Tilkeridis C, Bei T, Garantziotis S, Stratakis CA. Association of a COL1A1 polymorphism with lumbar disc disease in young military recruits. J Med Genet. 2005;42(7):e44.
29. Skorupski P, Król J, Starega J, Adamiak A, Jankiewicz K, Rechberger T. An alpha-1 chain of type I collagen Sp1-binding site polymorphism in women suffering from stress urinary incontinence. Am J Obstet Gynecol. 2006;194(2):346-350.
30. Malila S, Yuktanandana P, Saowaprut S, et al. Association between matrix metalloproteinase-3 polymorphism and anterior cruciate ligament ruptures, Genet Mol Res. 2011;10(4): 4158-4165.
31. Goodlin GT, Roos AK, Roos TR, Hawkins C, Beache S, Baur S, et al. Applying personal genetic data to injury risk assessment in athletes. PLOS ONE 2015;10(4):e0122676.

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RESEARCH ARTICLE

CCL2 single nucleotide polymorphism of rs1024611 implicates prominence of inflammatory cascade by univariate modeling in Indian AMD

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Abstract

Background

The role of chemotactic protein *CCL2*/MCP-1 has been widely explored in age related macular degeneration (AMD) patients as well as animal models through our previous studies.

Aim

Aim of the study was to examine the association of another variance of *CCL2*, rs1024611 in pathophysiology of AMD.

Methods

This particular SNP has been found to be involved in inflammatory processes in various diseases. Total 171 subjects were recruited in the study with all demographic details by administering a standard questionnaire. SNP analysis was performed with TaqMan assay. Linear univariate and ANCOVA modeling was performed to show the interaction of rs1024611 with another SNP variant of *CCL-2*/*CCR-2* (rs4586 and rs1799865) and impact of individual genotypes on *CCL-2* expression in the context of AMD pathology.

Results

Results showed that both heterozygous (AG, p = 0.01) and homozygous (GG, p = 0.0001) genotypes are associated with AMD pathology. Allele frequency analysis showed that 'G' allele is frequent in AMD patients as compared to controls (p = 0.0001). Moreover, AMD patients who smoke were found to be associated with 'AG' genotype (p = 0.0145). Although, we did not find any significant interaction between the SNP variants by linear univariate

analysis but results show the effect of 'CT' genotype on 'TT' genotype in rs4586 by considering rs1024611 as covariate.

Conclusion

Based on these results it is imperative that *CCL2* mediated pathology may be associated with AMD.

1. Introduction

AMD can be defined with several pathological conditions including drusen formation, macrophages infiltration, apoptosis of retinal cell layers and new blood vessels formation from the choroid. The inflammatory processes have been reported in AMD to result in drusen deposits (dry AMD) which can further provoke the wet AMD pathology. Consequently, these pathological conditions lead to impaired visual function. Chemokine (C-C motif) ligand-2 (*CCL2* or monocyte chemoattractant protein-1) plays an important role in recruitment of monocytes from peripheral blood [1, 2]. The cellular inflammatory processes have been implicated in several degenerative diseases (e.g. multiple sclerosis, Alzheimer disease, arthrosclerosis, rheumatoid arthritis etc.) including cancer.

We wanted to examine whether there is a human link to our previous study in which we showed that *CCL2* produced from mice RPE or choroids facilitates choroidal macrophage recruitment mediated by C5a and IgG as shown in *CCL2*-/- mice study. Therefore, impaired macrophages infiltration would be expected to show AMD features from accumulation of IgG and C5a and further activation of vascular endothelial growth factor (VEGF) [3]. We have also found that 'TT' genotype of both *CCL2* (rs4586; p = 0.003) and *CCR2* (rs1799865; p = 0.015) genes is significantly associated with AMD pathology. In case of multivariate analysis the 'TT' genotype for both genes i.e. *CCL2* (rs4586) and *CCR2* (rs1799865) were also significantly associated with AMD pathophysiology after adjusting for age (p = 0.005) and gender (p = 0.017) respectively. Moreover, elevated expression levels of *CCL2* and *CCR2* in serum and lymphocytes respectively, in AMD patients, as compared to controls, have also indicated the effect of chemokine ligands and receptors mediating cellular inflammatory processes in AMD pathophysiology [4]. Interestingly, Despriet *et al* did not find any correlation of major alleles in both *CCL2* and *CCR2* haplotypes with AMD patients, however, this study did not include Indian AMD patients. Instead, the minor allele of one haplotype was found to be significant (p = 0.03) with disease phenotypes but there was no effect on mRNA expression profile of these genes in Caucasian population including both Netherlands and USA populations raising the importance of genetic epidemiology in AMD[5].

2. Materials and methods

2.1 Participants

111 AMD and 60 controls were recruited from Advanced Eye Centre, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India to conduct the study. Participants were included only after obtaining written consent forms. The ethical clearance of the study was obtained from Institutional Ethical Committee (IEC), PGIMER, Chandigarh, India vide letter number Micro/10/1411.

2.2 AMD diagnosis

AMD patients were recruited on the basis of their disease phenotypes observed under fundus angiography (FA) and optical coherence tomography (OCT) by retinal specialists. Various ophthalmic parameters were also screened which included pupils dilation, best corrected visual acuity (BCVA), and opacity of lens by slit lamped microscopy.

2.3 Demographic information

Demographic details of the participants were obtained to correlate with the genetic outcome of the study of AMD patients and further compared with controls. The standard questionnaire which includes the set of queries related to food habits, smoking, and their associated co-morbidities (cardiovascular history, hypertension or diabetes etc) were collected (Table 1).

2.4 Inclusion and exclusion criteria

AMD patients were recruited after comprehensive ophthalmological examination by retinal specialist. >5 drusen with size of 125 microns in at least one eye were included as AMD patients. Other pathological features of AMD like leaky blood vessels (by FFA) and degeneration of macular photoreceptors (by OCT) were also included as AMD patients. The participants with less than 5 drusen with size of 60 microns were considered as control subjects. Any pathological conditions resembling AMD phenotypes (e.g. uveitis, retinal dystrophy, vein occlusion, neovascularization due to diabetic retinopathy *etc*) were excluded from the study. The age below 50 years were also excluded from the study.

2.5 PBMCs isolation

5ml blood was taken from all participants in EDTA vial and was kept at room temperature for separation of two layers. Supernatant of the samples layered on equal volume of histopaque

Table 1. Demographic characteristics of controls and AMD patients.

Variables	AMD	Controls
Total	111	60
Male	74	39
Female	37	21
Duration of disease¥	24.35 M	—
Dry	28	—
Wet	83	—
Smokers	48	11
Non Smokers	63	43
Vegetarian	59	31
Non Vegetarian	52	23
Comorbidity	81	10
No Comorbidity	28	44
Age	65±7	61±13

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.

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(SIGMA-ALDRICH, USA) and further centrifuged at 1800rpm for 30minutes. The middle buffy coat were washed with 1X PBS and stored at -80˚C for further use.

2.6 DNA isolation

Genomic DNA was extracted from PBMCs by commercially available genomic DNA kit (QIAGEN, Germany or INVITROGEN, USA) as per the manufacturer's instruction. Concentration and purity of genomic DNA were measured by UV spectrophotometer (BeckMan Coulter, USA). The extracted DNA was appropriately coded and stored for further use.

2.7 Genotyping assay

Single nucleotide polymorphism (SNP) analysis of *CCL2* rs1024611 was carried out with SNP genotyping TaqMan assay in StepOne real time PCR machine (Applied Biosysystems Inc., Foster city, CA). Reaction set up contained genomic DNA concentration of 20ng and 5μl TaqMan assay (Applied Biosystems). Final volume of the reaction was made up with master mix up to 20μl. Probes were tagged with FAM and VIC dyes to discriminate the allelic changes located at rs1024611 in the SNP assay which posses 5' nuclease activity. The negative control (without genomic DNA) was also put in reaction setup. The overall protocol for SNP analysis was followed as per the manufacturer's instruction. The SNP analysis and reaction amplification was done with StepOne V 2.0 software (Applied Biosysystems Inc., Foster city, CA). Fluorescence generated from the SNP discrimination reaction was analyzed by Sequence Detection Software (SDS). The analysis was done between fluorescence amount (Rn value) versus amplification of the products.

2.8 Statistical analysis

Genotyping data obtained from SNP analysis was categorized in homozygous and heterozygous variants. The association with SNP changes among various groups was analyzed by Pearson's Chi square test. Binary logistic regression model was used to get best line fit of distributed genotypes in the population. The correlation with SNP data and strength with disease phenotype (Odd's ratio or OR) with 95% confidence interval was calculated by logistic regression. All results in SNP correlation with disease pathology were considered significant when analysis p value were less than 0.05.

2.9 Linear univariate and ANCOVA analysis

To analyze the impact and/or interaction of rs1024611 (lies in promoter region) on previously published SNP rs4586 (lies in coding region) of *CCL-2* and its receptor rs1799865 (Anand *et al*., 2012) [4], we performed linear univariate modeling. Moreover, ANCOVA analysis was also carried out to test the main effect of rs10246 on other two SNPs and vice versa by assuming any one of them as covariate. We also derived the interaction model further to identify whether presence of one SNP aggravates the AMD pathology. Bonferroni correction analysis for multiple comparisons was done to exclude the false positive outcome of the results.

3. Results

3.1. Genotype analysis

The studied population was consisting of 111 AMD patients and 60 controls. The demographic details of the population are given in Table 1. The effect of particular genotype with reference to disease phenotypes has been shown in Table 2. The genotype analysis revealed both heterozygous AG and homozygous GG genotypes have their deleterious effects on AMD

Table 2. Effect of *CCL2* rs1024611 variants on disease phenotype.

Genotype	Number (frequency)		OR	95%CI	P Value
***CCL2* rs1024611**					
	AMD	**Controls**			
AA	40 (3.6)	41 (6.83)	Reference		
AG	40 (3.6)	16 (2.67)	2.56	1.24–5.29	0.01
GG	31 (2.8)	3 (0.5)	10.59	2.99–37.43	0.0001
	Wet AMD	**Dry AMD**			
AA	29 (34.9)	11 (3.93)	Reference		
AG	33 (39.8)	7 (2.50)	1.78	0.61–5.21	0.28
GG	21 (25.3)	10 (3.57)	0.8	0.28–2.21	0.66

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pathology as compared with controls (p = 0.01 & p = 0.0001 respectively). On the contrary, any of the genotypes including AA, GA and GG did not demonstrate any association with wet and dry form of AMD. Moreover, the allelic frequency data (Table 3) showed that 'G' allele, in comparison to 'A' allele in the A/G genotype, has shown significant association with progression of AMD pathology. Similarly, both A and G alleles did not show any effect on both forms of AMD. Moreover, we have also depicted the odd's ratio (OR) of both allele and genotype frequencies have also been plotted in **Fig 1** for AMD and controls.

The genotype data was further associated with socio-demographic and co-morbidity variables of the participants (Table 4). The logistic regression analysis demonstrated that heterozygous genotype 'AG' has found to be associated with smoking habits and progression of AMD pathology. But both AA and GG genotype haven't shown any correlation with smoking. None of the genotypes i.e. AA, GA and GG have demonstrated significant correlation with comorbidity. However, GG genotype may have association with comorbidity (p = 0.0625). Food habits of the participants were not found to bear significant association with any of the genotypes.

Using (i) chi-square value (ii) effect size (iii) degrees of freedom used in association (iv) level of significance, and (v) number of observations, the power of the study has been computed. All calculations were made in R software using *pwr.chisq.test* (w = effect size, N = number of observations, df = degrees of freedom, sig. level = 0.05, power = NULL). By specifying all other parameters, the power has been computed for various associations. In all associations, the power was found to be more than 80%".

3.2. Individual SNPs impact on AMD pathology

We have already reported exonic SNP variant of *CCL2* (rs4586) and its receptors (rs1799865) were found to be associated with AMD progression [4]. Promoter SNP variants of *CCL2* (rs1024611) and *CCL2* receptor (rs1799865) interaction was non-significant (F = 1.099;

Table 3. Allele frequency of *CCL2* in AMD and normal controls.

Allele	Number (frequency)		OR	95%CI	P Value
***CCL2* rs1024611**					
	AMD	Controls			
A	120	98	Reference		
G	102	22	3.7864	2.2232 -6.4485	0.0001
	Wet AMD	Dry AMD			
A	91	29	Reference		
G	75	17	1.4059	0.7178–2.753	0.3205

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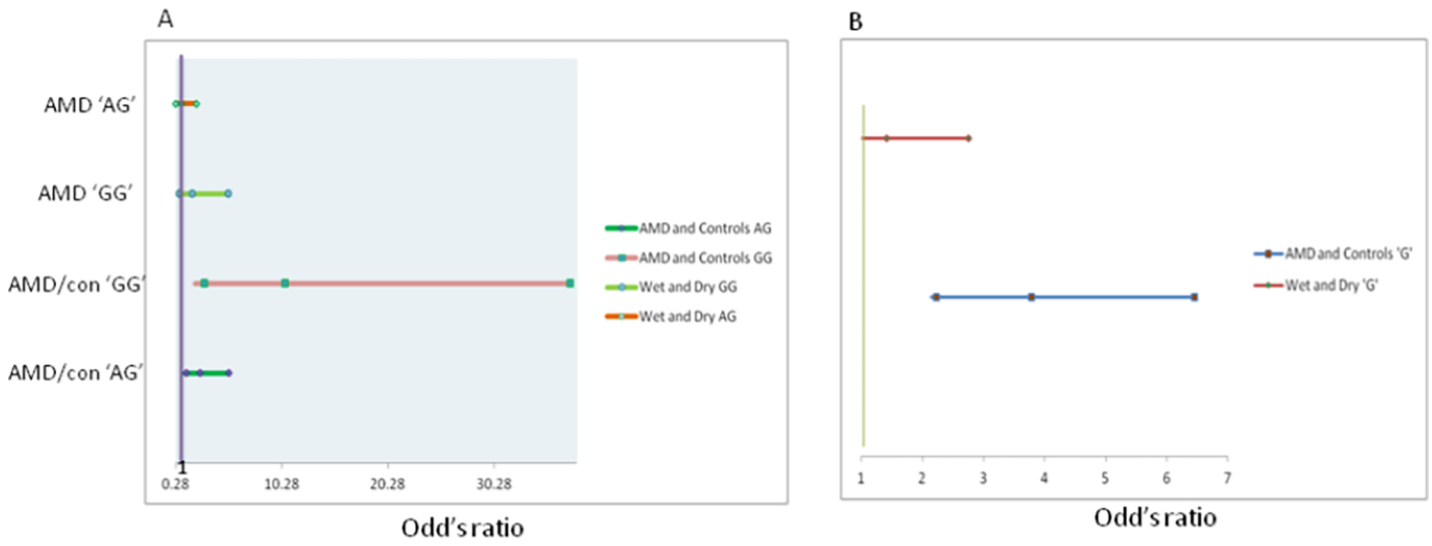

Fig 1. Schematic representation of odd's ratio (OR) for both genotypes and allele frequencies of rs1024611 locus. (A) Genotypes frequency (B) allele frequency.

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p = 0.359). Moreover, there is no interaction between both *CCL2* SNP variants i.e. promoter and exonic variants (rs1024611 and rs4586) (F = 1.824; p = 0.127) (**Fig 2A**). However, the linear univariate modeling shows that the interaction between both SNPs i.e. rs1799865 and rs4586 is non-significant (F = 0.254; p = 0.907) (**Fig 2B**). Although, Fig 2A shows slight interaction but it is statically non-significant. Therefore, we observed that all 3 SNPs (rs1024611, rs4586 and rs1799865) were impacting the AMD pathology individually.

3.3. ANCOVA analysis

Since all the interactions between factors and co-variates were found to be non-significant, therefore, analysis of co-variance was performed with expression levels of *CCL2* as dependent variable and other SNP variants as factors/covariates. For different factors and covariates, the results are presented in Table 5. By considering rs1024611 as covariate and three genotypes of *CCL2* rs4586 (TT = 2, CT = 1 and CC = 0) were compared by taking TT as reference. It was

Table 4. Logistic regression of the association of *CCL2* and progression of AMD.

Genotype	Number (frequency)		OR	95%CI	P-value
***CCL2* rs1024611**					
	Non Vegetarian AMD	**Vegetarian AMD**			
AA	16 (0.31)	24 (0.41)	**Reference**		
AG	23 (0.44)	17 (0.29)	2.0294	0.8329 to 4.9448	0.1193
GG	13 (0.25)	18 (0.30)	1.0833	0.4175 to 2.8109	0.8693
	Smokers AMD	**Non Smokers AMD**			
AA	12 (0.25)	28 (0.44)	**Reference**		
AG	23 (0.48)	17 (0.27)	3.1569	1.2554 to 7.9384	0.0145
GG	13 (0.27)	18 (0.29)	1.6852	0.6306 to 4.5035	0.2981
	AMD with Comorbidity	**AMD without Comorbidity**			
AA	27 (0.33)	13 (0.46)	**Reference**		
AG	27 (0.33)	11 (0.39)	1.1818	0.4507 to 3.0989	0.7341
GG	27 (0.33)	4 (0.14)	3.2500	0.9394 to 11.2437	0.0627

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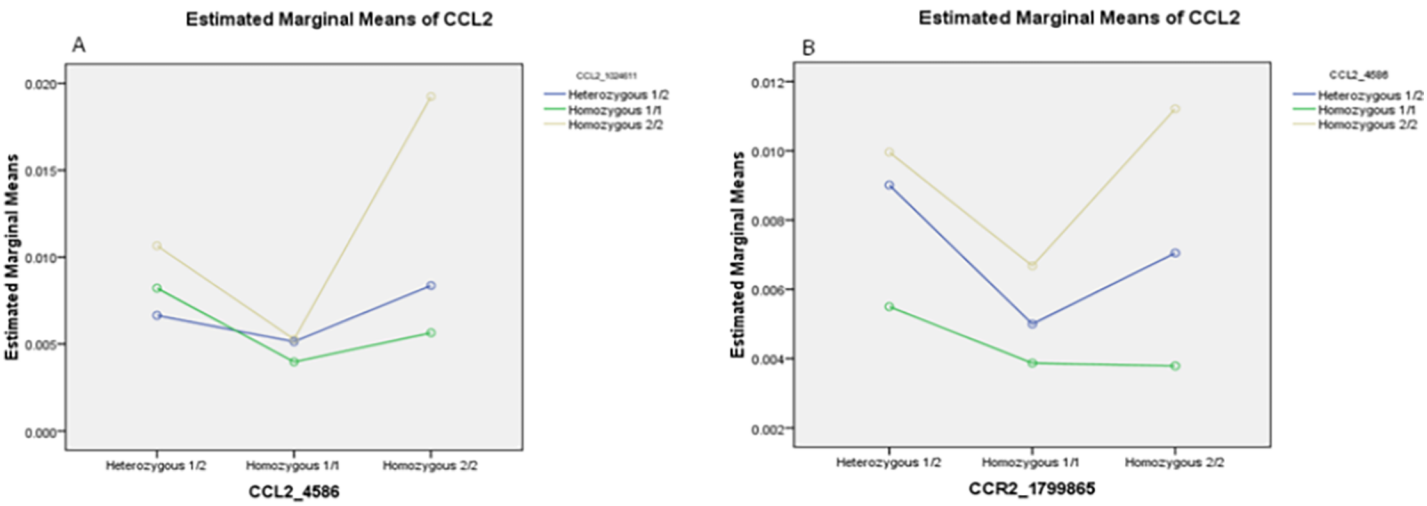

Fig 2. Linear univariate modeling analysis. The interaction shows between (A) rs1024611 and rs4586. Heterozygous 1/2 (AG); homozygous 1/1(AA); and homozygous 2/2(GG) of rs1024611; (B) between rs1799865 and rs4586 with levels of *CCL-2*. Heterozygous 1/2 (CT); homozygous 1/1(CC); and homozygous 2/2(TT) of rs4586.

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observed that for a fixed reference TT, CT was found to be significantly (p = 0.027) affecting the TT genotype. However, there was no effect on CC genotype (p = 0.356). Similarly, three genotypes of *CCR-2* rs1799865 SNP variant (CC = 2, CT = 1 and TT = 0) were also compared with rs1024611 *CCL2* variants considering as covariate and TT genotype as reference. Remaining both genotypes i.e. CT and CC were not found to be affecting TT genotype (p>0.05). Moreover, in case of *CCL-2* exonic variant as covariate and TT genotype of *CCR-2* variants as reference, analysis showed no significant effect of *CCR-2* rs1799865 genotypes CT and CC on TT genotype (p>0.05). Therefore, impact of genotypes of *CCL-2* (both promoter, the coefficient of rs1024611 is significant, as well as exonic SNP variants) and *CCR-2* genes on reference genotypes may lead to the predictive modeling which may support the experimental evidence

Table 5. ANCOVA analysis to determine the affect of genotypes on reference genotype and expression levels by considering one SNP as covariate.

	***CCL-2* levels as dependent variable**				
	Parameter	**B**	**Std. Error**	**t-value**	**p-value**
Covariate Factors	Intercept	0.007	0.002	4.459	0.000
	CCL2 rs1024611	0.003	0.001	2.832	0.005
	[*CCL2* rs4586 = CC]	-0.002	0.002	-0.925	0.356
	[*CCL2* rs4586 = CT]	-0.005	0.002	-2.228	0.027
	[*CCL2* rs4586 = TT]	Ref	.	.	
Covariate Factors	Intercept	0.004	0.002	2.410	0.017
	CCL2 rs1024611	0.004	0.001	3.338	0.001
	[*CCR2* rs1799865 = CC]	0.002	0.002	1.301	0.195
	[*CCR2* rs1799865 = CT]	-0.003	0.002	-1.283	0.201
	[*CCR2* rs1799865 = TT]	Ref	.	.	.
Covariate Factors	Intercept	0.008	0.002	4.725	0.000
	CCL2 rs4586	0.001	0.001	1.389	0.167
	[*CCR2* rs1799865 = CC]	0.000	0.002	-0.086	0.931
	[*CCR2* rs1799865 = CT]	-0.004	0.002	-1.558	0.121
	[*CCR2* rs1799865 = TT]	Ref	.	.	.

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of enhanced expression of *CCL-2* in AMD patients as compared to controls, and/or may also modify the binding affinity of *CCL-2* ligand with its receptor (*CCR-2*).

Therefore, we have proposed a univariate model in Eq 1:

$$y_{ij} = \mu + \alpha_i + \beta_j x_{ij} + e_{ij} \quad (1)$$

Where y_{ij}: represent *CCL-2* expression, and

μ: overall general effect

α_i: effect of i^{th} genotype

x_{ij}: covariate

e_{ij}: error with mean 0 and variance σ^2

To rule out the false positive outcome of the results obtained from SNPs interaction and the ANCOVA analysis, we applied the Bonferroni correction for multiple comparisons. It is evident from Table 6 that for *CCL2* rs1024611 the mean difference *CCL-2* levels while comparing genotypes AG versus GG and AA versus GG were significant ($p<0.05$) whereas AG versus AA were non-significant ($p>0.05$). However, for *CCL2* rs4586 and *CCR-2* rs1799865 all multiple comparisons were revealed non-significant results (Table 6).

4. Discussion

The role of chemokine receptors and their ligands in relation to inflammatory processes in AMD is well documented. Most macrophages or microglial cells express the receptors for chemokine ligands and show the chemotactic movements with chemokines gradient at inflammatory site. Both chemokine receptors CX3CR1 and *CCR-2* are expressed on inflammatory macrophages but non-inflammatory macrophages contain only CX3CR1 receptor [6]. Prolonged and persistent existence of macrophages in sub retinal space results in the release of various chemokines and angiogenic factors which consequently stimulate the accumulation of drusen at local inflammatory sites. CX3CR1 variant (M280) have shown the defective migration of macrophages at inflammatory site and found to have enhanced interaction with its ligands in retinal transmembrane [7]. The functional studies have revealed that these cascades of pathological changes in the retinal layers and surrounding microenvironment leads to prominent disease phenotypes i.e. formation of drusen, atrophy of photoreceptors and choroidal neovascularization (CNV), mediated by CX3CR1 signaling [7–10]. Similarly, we have previously investigated the abnormal deposition of C5 and IgG molecules in *CCL2*-/- and *CCR-2*-/- mice due to impaired macrophage recruitment at the site of deposition suggesting the imperative role of macrophages recruitment to clear debris in between retinal layers steered by *CCL2* and *CCR-2* signaling mechanism [3].

CCL2 genetic studies have not previously shown the association with AMD pathology. Genetic analysis, by considering univariate of both *CCR-2* and *CCL2*, along with TLR4 gene did not reveal any association between studied SNPs and AMD pathology. Even the haplotype analysis in case of *CCR-2* and TLR4 has not shown any correlation with pathology. However, the haplotype analysis of minor allele C35C has demonstrated pathological association ($p = 0.03$) with AMD pathophysiology in Netherlands and USA populations but mRNA expression did not show significant difference between AMD and control groups [5]. Our investigations have demonstrated that SNP variants of both *CCR-2* (rs1799865) and *CCL2* (rs4586) are associated with AMD pathology. Moreover, the expression of both chemo-attractant proteins was found to be elevated in AMD patients as compared to control groups [4]. Similarly, we have also observed the association of other chemo-attractant proteins including the CCR-3 variants [11] and expression levels of eotaxin-2[12] in AMD patients and further comparison with control groups. Both genes primarily regulate the inflammatory processes by

Table 6. Multiple comparison using Bonferroni correction analysis to adjust the p values for independent and/or dependent SNPs of rs4586, rs1024611 and rs1799865.

Benferroni Multiple Comparisons test						
Dependent Variable: *CCL2* levels						
***CCL2*_4586**		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Heterozygous CT	Homozygous CC	.003183	.002289	.383	-.002475	.008841
	Homozygous TT	-.002053	.001702	.485	-.006260	.002153
Homozygous CC	Homozygous TT	-.005236	.002271	.073	-.010850	.000377
Benferroni Multiple Comparisons test						
Dependent Variable: *CCL2* levels						
***CCL2*_1024611**		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Heterozygous AG	Homozygous AA	.000613	.001738	.940	-.003683	0.004909
	Homozygous GG	-.007647	.002226	**0.003**	-.013149	-0.002144
Homozygous AA	Homozygous GG	-.008260	.002144	**0.001**	-.013561	-0.002958
Benferroni Multiple Comparisons test						
Dependent Variable: *CCL2* levels						
***CCR-2*_1799865**		Mean Difference (I-J)	Std. Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Heterozygous CT	Homozygous CC	0.003228	.002232	.354	-.002289	.008745
	Homozygous TT	-0.000301	.001713	.985	-.004536	.003935
Homozygous CC	Homozygous TT	-0.003529	.002222	.286	-.009023	.001964

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recruiting the eosinophiles and T-lymphocytes mediated mechanisms. Above mentioned studies have suggested the role of cellular processes mediated by chemo-attractant proteins in order to regulate the inflammatory processes in AMD pathology. Interesting finding from Pham et al have demonstrated allelic variance at rs1024611 which leads to allelic expression imbalance (AIE) of *CCL2* which has been reported in various disease phenotypes including atherosclerosis, tuberculosis suggesting that the given allele expression is context dependent which could be influenced with interaction of various proteins [13] that is consistent with our previous findings [4]. Moreover, it has also been explored that *CCL2* expression could also regulate the angiogenic process by affecting VEGF and its associated molecules with the involvement of *Ets*-1 transcription factor [14]. Similarly, our findings with VEGF [15] and its receptor i.e. VEGFR2 have been found to be associated with AMD pathology and the expression levels of both proteins were significantly high in AMD patients as compared to age matched controls [16].

Pathological hallmarks of AMD are similar to age related changes like metabolic changes and apoptosis [17, 18] and enhanced inflammatory responses evident from various age related and inflammatory diseases including Alzheimer's disease, ischemia, and myocardial infarction [19–21]. In all these studies, the *CCL2* expression was found to be elevated suggesting that AMD pathological phenomenon are induced with inflammatory responses created by various cellular and protein responses. Additionally, we have recently demonstrated the elevated SOD1 levels in AMD as compared to controls, which also show the inflammatory response characterizing AMD [22].

Smoking has also been shown to have impact on various diseases and has been found to be associated with *CCL2* polymorphism and their levels in patients of myocardial infarction [21]. Smoking can also hamper the development of organs in offspring [23, 24]. However, the precise mechanism behind pathological changes induced by smoking in association with genetic markers is being debated. In our finding with *CCL2* it has been shown that heterozygous allele

AG is more frequent (p = 0.0145) in smoker AMD patients as compared to non-smoker AMD patients, suggesting a causative role of smoking in possible alteration of genetic allele which may lead to differential expression of *CCL2* protein in the AMD patients [21]. Similarly, logistic regression analysis has also demonstrated correlation of homozygous allele 'GG' with co-morbidity (p = .0625) in AMD patients even though it was not significant.

The studies have shown the SNP changes from A to G in enhancer region at -2578 position (rs1024611; A>G) lead to increase expression levels of *CCL2* in various bio-fluids [21, 25, 26] and facilitate the leukocytes recruitment in the tissues [27]. In our earlier observations we have found increased levels of *CCL2* in AMD patients as compared to controls but how rs1024611 influences the *CCL2* expression is still unclear. However, it has been demonstrated by various studies that rs1024611 polymorphism induces the transcriptional activity of *CCL2* gene [28, 29]. 'G' allele has found to be induced higher expression of *CCL2* protein in *in vitro* and *in vivo* as compared to 'A' allele. Similarly, leukocytes with 'GG' genotype as compared to 'AA' genotype have also induced increased production of *CCL2* protein. Therefore, these studies suggest the biological impact of the rs1024611 polymorphism in inflammation by recruitment monocytes [21, 26] and its pathological impact on various diseases. We have, however, not analysed the half life and affinity of receptor.

Conclusively, our finding suggests the genetic role of *CCL2* mediated processes in AMD pathology which may lead to infiltration of macrophages and other monocytes thus signifying the importance of inflammatory processes in AMD. It is possible that other environmental changes like smoking may be associated with AMD thus influencing *CCL2* genotype. However, additional studies of *CCL2* genes in South Indian population, which differs in dietary and environmental exposure, based on our current and previous finding with *CCL2*[4], are warranted.

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References

1. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011; 475(7355):222–5. Epub 2011/06/10. PubMed Central PMCID: PMCPmc3208506. https://doi.org/10.1038/nature10138 PMID: 21654748

2. Raoul W, Auvynet C, Camelo S, Guillonneau X, Feumi C, Combadiere C, et al. CCL2/CCR2 and CX3CL1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration. Journal of neuroinflammation. 2010; 7:87. Epub 2010/12/04. https://doi.org/10.1186/1742-2094-7-87 PMID: 21126357; PubMed Central PMCID: PMCPmc3003653.
3. Ambati J, Anand A, Fernandez S, Sakurai E, Lynn BC, Kuziel WA, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nature medicine. 2003; 9(11):1390–7. Epub 2003/10/21. https://doi.org/10.1038/nm950 PMID: 14566334.
4. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, et al. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PloS one. 2012; 7(11):e49905. Epub 2012/11/28. https://doi.org/10.1371/journal.pone.0049905 PMID: 23185481; PubMed Central PMCID: PMCPmc3503775.
5. Despriet DD, Bergen AA, Merriam JE, Zernant J, Barile GR, Smith RT, et al. Comprehensive analysis of the candidate genes CCL2, CCR2, and TLR4 in age-related macular degeneration. Investigative ophthalmology & visual science. 2008; 49(1):364–71. Epub 2008/01/04. https://doi.org/10.1167/iovs.07-0656 PMID: 18172114; PubMed Central PMCID: PMCPmc2754756.
6. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003; 19(1):71–82. Epub 2003/07/23. PMID: 12871640.
7. Daoudi M, Lavergne E, Garin A, Tarantino N, Debre P, Pincet F, et al. Enhanced adhesive capacities of the naturally occurring Ile249-Met280 variant of the chemokine receptor CX3CR1. The Journal of biological chemistry. 2004; 279(19):19649–57. Epub 2004/03/03. https://doi.org/10.1074/jbc.M313457200 PMID: 14990582.
8. Combadiere C, Feumi C, Raoul W, Keller N, Rodero M, Pezard A, et al. CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. The Journal of clinical investigation. 2007; 117(10):2920–8. Epub 2007/10/03. https://doi.org/10.1172/JCI31692 PMID: 17909628; PubMed Central PMCID: PMCPmc1994614.
9. Raoul W, Keller N, Rodero M, Behar-Cohen F, Sennlaub F, Combadiere C. Role of the chemokine receptor CX3CR1 in the mobilization of phagocytic retinal microglial cells. Journal of neuroimmunology. 2008; 198(1–2):56–61. Epub 2008/05/30. https://doi.org/10.1016/j.jneuroim.2008.04.014 PMID: 18508131.
10. Ma W, Zhao L, Fontainhas AM, Fariss RN, Wong WT. Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD. PloS one. 2009; 4(11):e7945. Epub 2009/11/26. https://doi.org/10.1371/journal.pone.0007945 PMID: 19936204; PubMed Central PMCID: PMCPmc2775955.
11. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, Anand A. CC chemokine receptor-3 as new target for age-related macular degeneration. Gene. 2013; 523(1):106–11. Epub 2013/04/10. https://doi.org/10.1016/j.gene.2013.03.052 PMID: 23566847.
12. Sharma NK, Prabhakar S, Gupta A, Singh R, Gupta PK, Gupta PK, et al. New biomarker for neovascular age-related macular degeneration: eotaxin-2. DNA and cell biology. 2012; 31(11):1618–27. Epub 2012/10/03. https://doi.org/10.1089/dna.2012.1786 PMID: 23025269.
13. Pham MH, Bonello GB, Castiblanco J, Le T, Sigala J, He W, et al. The rs1024611 regulatory region polymorphism is associated with CCL2 allelic expression imbalance. PloS one. 2012; 7(11):e49498. Epub 2012/11/21. https://doi.org/10.1371/journal.pone.0049498 PMID: 23166687; PubMed Central PMCID: PMCPmc3500309.
14. Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV. CCL2 regulates angiogenesis via activation of Ets-1 transcription factor. Journal of immunology (Baltimore, Md: 1950). 2006; 177(4):2651–61. Epub 2006/08/05. PMID: 16888027.
15. Sharma K, Sharma NK, Singh R, Anand A. Exploring the role of VEGF in Indian Age related macular degeneration. Annals of neurosciences. 2015; 22(4):232–7. Epub 2015/11/04. https://doi.org/10.5214/ans.0972.7531.220408 PMID: 26526736; PubMed Central PMCID: PMCPmc4627204.
16. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma S, Anand A. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Current neurovascular research. 2012; 9(4):256–65. Epub 2012/10/04. PMID: 23030506.
17. Sharma K, Sharma NK, Anand A. Why AMD is a disease of ageing and not of development: mechanisms and insights. Frontiers in aging neuroscience. 2014; 6:151. Epub 2014/07/30. https://doi.org/10.3389/fnagi.2014.00151 PMID: 25071560; PubMed Central PMCID: PMCPmc4091411.
18. Anand A, Sharma NK, Singh R, Gupta A, Prabhakar S, Jindal N, et al. Does DcR1 (TNF-related apoptosis-inducing-ligand Receptor 3) have any role in human AMD pathogenesis? Scientific reports. 2014; 4:4114. Epub 2014/02/19. https://doi.org/10.1038/srep04114 PMID: 24534820; PubMed Central PMCID: PMCPmc3927205.

19. Pola R, Flex A, Gaetani E, Proia AS, Papaleo P, Di Giorgio A, et al. Monocyte chemoattractant protein-1 (MCP-1) gene polymorphism and risk of Alzheimer's disease in Italians. Experimental gerontology. 2004; 39(8):1249–52. Epub 2004/08/04. https://doi.org/10.1016/j.exger.2004.05.001 PMID: 15288699.

20. Kim MP, Wahl LM, Yanek LR, Becker DM, Becker LC. A monocyte chemoattractant protein-1 gene polymorphism is associated with occult ischemia in a high-risk asymptomatic population. Atherosclerosis. 2007; 193(2):366–72. Epub 2006/08/29. https://doi.org/10.1016/j.atherosclerosis.2006.06.029 PMID: 16934270.

21. McDermott DH, Yang Q, Kathiresan S, Cupples LA, Massaro JM, Keaney JF Jr., et al. CCL2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the Framingham Heart Study. Circulation. 2005; 112(8):1113–20. Epub 2005/08/24. https://doi.org/10.1161/CIRCULATIONAHA.105.543579 PMID: 16116069.

22. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R. Superoxide dismutase1 levels in North Indian population with age-related macular degeneration. Oxidative medicine and cellular longevity. 2013; 2013:365046. Epub 2013/12/24. https://doi.org/10.1155/2013/365046 PMID: 24363822; PubMed Central PMCID: PMCPmc3864086.

23. Al-Odat I, Chen H, Chan YL, Amgad S, Wong MG, Gill A, et al. The impact of maternal cigarette smoke exposure in a rodent model on renal development in the offspring. PloS one. 2014; 9(7):e103443. Epub 2014/07/25. https://doi.org/10.1371/journal.pone.0103443 PMID: 25058584; PubMed Central PMCID: PMCPmc4110029.

24. Allina J, Grabowski J, Doherty-Lyons S, Fiel MI, Jackson CE, Zelikoff JT, et al. Maternal allergy acts synergistically with cigarette smoke exposure during pregnancy to induce hepatic fibrosis in adult male offspring. Journal of immunotoxicology. 2011; 8(4):258–64. Epub 2011/07/02. https://doi.org/10.3109/1547691X.2011.589412 PMID: 21718087.

25. Cho ML, Kim JY, Ko HJ, Kim YH, Kim WU, Cho CS, et al. The MCP-1 promoter -2518 polymorphism in Behcet's disease: correlation between allele types, MCP-1 production and clinical symptoms among Korean patients. Autoimmunity. 2004; 37(1):77–80. Epub 2004/04/30. PMID: 15115316.

26. Fenoglio C, Galimberti D, Lovati C, Guidi I, Gatti A, Fogliarino S, et al. MCP-1 in Alzheimer's disease patients: A-2518G polymorphism and serum levels. Neurobiology of aging. 2004; 25(9):1169–73. Epub 2004/08/18. https://doi.org/10.1016/j.neurobiolaging.2003.11.008 PMID: 15312962.

27. Gonzalez E, Rovin BH, Sen L, Cooke G, Dhanda R, Mummidi S, et al. HIV-1 infection and AIDS dementia are influenced by a mutant MCP-1 allele linked to increased monocyte infiltration of tissues and MCP-1 levels. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99(21):13795–800. Epub 2002/10/11. https://doi.org/10.1073/pnas.202357499 PMID: 12374865; PubMed Central PMCID: PMCPmc129777.

28. Rovin BH, Lu L, Saxena R. A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. Biochemical and biophysical research communications. 1999; 259(2):344–8. Epub 1999/06/11. https://doi.org/10.1006/bbrc.1999.0796 PMID: 10362511.

29. Jibiki T, Terai M, Shima M, Ogawa A, Hamada H, Kanazawa M, et al. Monocyte chemoattractant protein 1 gene regulatory region polymorphism and serum levels of monocyte chemoattractant protein 1 in Japanese patients with Kawasaki disease. Arthritis and rheumatism. 2001; 44(9):2211–2. Epub 2001/10/11. PMID: 11592387.

Association of ACL tears and single nucleotide polymorphisms in the collagen 12 A1 gene in the Indian population - a preliminary case-control study

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Summary

Background: **Genetic predisposition to ACL tears has received tremendous interest in the past few years with many SNPs of different genes being linked to ACL tear.**
Study Objectives: **To examine if specific sequence variants in COL12A1 gene are associated with ACL tears in Indian population.**
Study design**: Case-control study.**
Materials and methods**: 50 patients with surgically diagnosed ACL tear and 52 healthy, age-matched controls without any ligament/tendon injuries were genotyped for rs970547 and rs240736 SNPs using real time PCR method.**
Results: **The AG and GG genotypes were significantly under-represented in study group patients in rs970547 region (p=0.0361). However, there was no significant difference in genotype/allele frequencies in the rs240736 region.**
Conclusions: **The COL12A1 rs970547 SNP is associated with ACL tears in the Indian population. However, these results need to be validated further so that predisposed individuals can be screened in the future for counselling and intervention.**
Level of evidence**: III**

KEY WORDS: ACL tear, COL12A1, gene polymorphisms, genetic association study, single nucleotide polymorphism (SNP).

Introduction

Anterior Cruciate Ligament (ACL) tears are now understood to have a multi-factorial etiology[1,2]. Although trauma to the knee is an essential pre-requisite for an ACL tear, various risk factors (both extrinsic and intrinsic) have been identified, which predispose an individual to tearing his ACL[1, 2].
Over the past few years, familial predisposition and specific single nucleotide polymorphisms (SNP's) of various collagen and extracellular matrix protein genes have been linked to ACL tear in different population subsets[3 ,4]. However, most of these studies have focussed on Caucasian populations; till date, there are no published studies looking into the genetic risk factors of ACL tear from the Indian subcontinent[3, 4].
Collagen XII is one of the main structural components of ACL collagen along with collagen types I, III-VI, XIV and various proteoglycans and glycoproteins[5]. The COL12A1 gene (mapped to chromosome 6q12-q13) encodes the α1 chains of the long (XIIA) and short (XIIB) homo-trimeric isoforms of type XII collagen[6-10]. Database hosted by the National Center for Biotechnology and Information (NCBI), has identified 5 SNPs in COL12A1 exonic regions of which only 2 (rs970547 and rs240736) have been identified as non-synonymous SNPs (*i.e.* SNPs that change the amino acid sequence in the gene product)[11]. These 2 SNPs have been tested for association with ACL tears in South African and Polish populations with variable results [12,13].
The aim of our study was to determine if these 2 SNPs (rs970547 and rs240736) of the COL12A1 gene have any association with ACL tears in the Indian population.

Materials and methods

The study was conducted in the Department of Orthopaedics in collaboration with Neuroscience Research Laboratory at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Ethical clearance was obtained from the institute ethics committee prior to the commencement of the study. The study has been conducted ethically according to set international standards and meets the ethical standards of the journal [14].

Selection of patients

Fifty patients with clinico-radiological and surgically proven ACL tears were recruited into the study group from July 2012 to December 2013. The inclusion criteria were: (i) age between 18-40 years, (ii) no multiligament injuries or signs of osteoarthritis knee (iii) no associated co-morbidities. Fifty-two age-matched patients with normal knees and no history of ligament/tendon injuries were recruited as controls. All subjects underwent thorough clinical evaluation. Informed, written consent was obtained from cases and controls. All subjects completed a detailed questionnaire consisting of general details, mode of injury, relevant past, personal and family histories along with details of participation in sports.

All procedures were conducted in a good laboratory practice (GLP) compliant laboratory utilising pre-calibrated instruments, quality assurance norms and as per established standard operating protocols (SOP's); documentation was done in DRS (display resource file) format by trained research scholars who were blinded to samples as prescribed by OECD (Organisation for economic co-operation and development) guidelines. External audit of results was carried out by seeking expert opinion from an outside laboratory.

DNA isolation

Five ml of venous blood was drawn into an EDTA vacutainer which was kept for around 2 hours at room temperature. Once the RBC's settled down the upper layer was aspirated and laid over equal amount of ficoll-paque solution (Amersham Biosciences, USA). After centrifugation at 1800 rpm for 30 minutes, a thick buffy coat of lymphocytes (peripheral blood monocytes/PBMC) found as a thin middle layer was collected in a fresh tube. The PBMC was pelleted by centrifugation at 5000 rpm for 5 minutes. The PBMC was then washed with PBS and processed for DNA isolation.

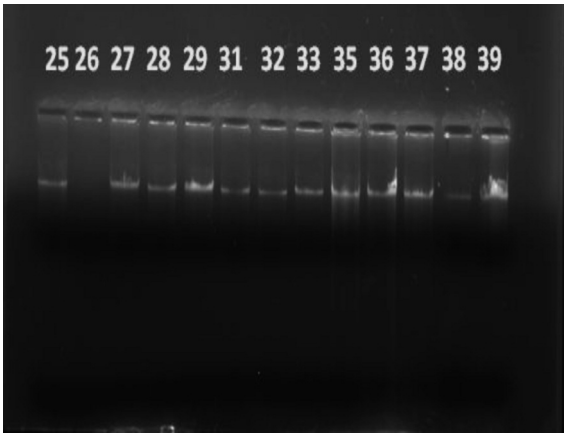

Figure 1. DNA isolated from PBMC electrophoresed on 1% agarose gel for quality verification.

Genomic DNA was isolated using QIAGEN DNeasy kit. The eluted DNA was quantified using UV spectrophotometer (Backman Coulter) and run on 1% agarose gel (Biorad) to verify the quality of DNA (Fig. 1).

COL12A1 genotyping

SNPs were analysed by using real-time PCR (polymerase chain reaction) performed in the 48 wells model Step one™ (Applied Biosystems Inc, Foster City, USA). Real time PCR was carried out for 20 μL containing 10 μL master mix, 5 μL assay, 20 ng DNA and molecular biology grade water was added to make the volume 20 μL. All reactions were carried out using TaqMan/SybrGreen SNP genotyping assays according to manufacturers' 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 Step One™ v2.0 (Applied Biosystems, Foster City, USA) was used to perform amplification and to estimate SNPs. 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.

Statistical analysis

Data was analysed using SPSS software version 16.0 (Statistical Package for the Social Sciences). Normal-Quantile (Q-Q) plots were constructed in order to examine whether the data was normally distributed or not. For comparison of the 2 groups, unpaired student-t test was applied. Hardy Weinberg equilibrium was established prior to genotyping analysis. In RT-PCR, the genotypes for each mutation were stratified for heterozygosity, and homozygosity of the respective allelic variant. Pearson's Chi-square test followed by Cochran-Mantel-Haenszel equation was applied for the analysis of genotyping results.

Results

SNP rs970547

The AG and GG genotypes were significantly underrepresented in study group patients (p=0.0361 and 0.0374 respectively; Tab. I, Figs. 2, 3). No significant difference between allele frequencies was observed (p=0.091). All groups were in Hardy Weinberg Equilibrium (HWE value=0.5).

SNP rs240736

There was no significant difference in the genotype (p=0.712) or allele frequencies (p=0.4882) between the 2 groups for the rs240736 region (Tab. II, Fig. 4). All groups were in Hardy Weinberg Equilibrium for this region (HWE value=0.02).

Table I. Effect of rs970547 variant on disease phenotype.

Genotype	Number		OR	95%CI	Z-statistics	p Value
	ACL	Controls				
AA	10	3	Reference	-	-	-
AG	19	26	0.2192	0.053-0.9064	2.096	0.0361
GG	15	21	0.2143	0.0502-0.9139	2.082	0.0374

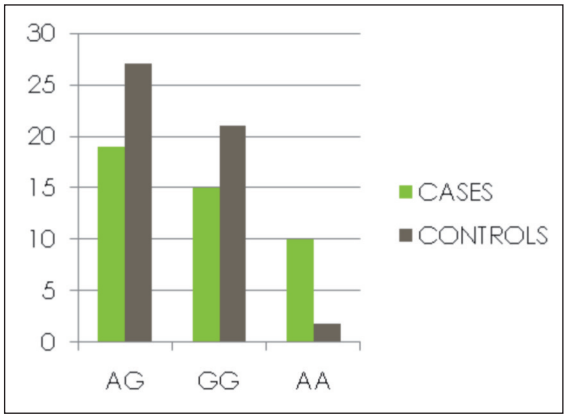

Figure 2. Bar diagram depicting relative frequency of genotypes of rs970547 in COL12A1 gene.

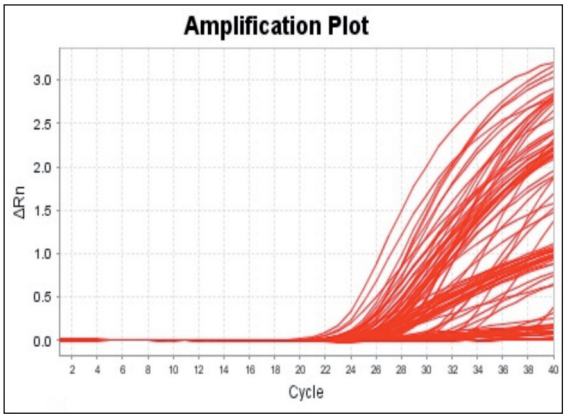

Figure 3. Amplification plot for ACL control and patient from SNP genotyping analysis for rs970547 variant.

Table II. Effect of rs240736 variant on disease phenotype.

Genotype	Number		OR	95%CI	Z-statistics	p Value
	ACL	Controls				
CC	28	28	Reference	-	-	-
CT	17	21	0.81	0.354-1.85	0.501	0.693
TT	0	1	*	*	*	*

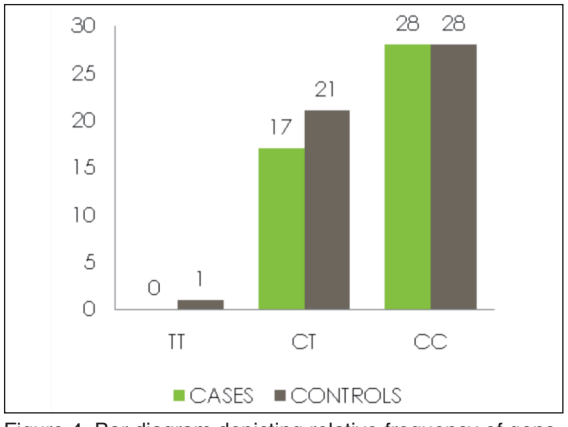

Figure 4. Bar diagram depicting relative frequency of genotypes of rs240736 in COL12A1 gene.

Discussion

Type XII collagen protein is associated with the surface of the collagen micro fibril and is a member of the Fibril Associated Collagens with Interrupted Triple helices (FACIT) sub-family. It has numerous functions: (i) involves in fibrillogenesis along with type XIV collagen, (ii) forms inter-fibrillar connections and mediates fibril interaction with other extracellular and cell surface molecules within ligaments there by influencing fibril and matrix density, (iii) regulates expression in response to mechanical loading, (iv) increases in content during healing along with type V and type III collagen, (v) regulates collagen fibril diameter (along with type V collagen) [5-10].

The association of COL12A1 SNP with ACL tear has been investigated previously in 2 Caucasian popula-

Table III. Summary of studies in literature investigating association of COL12A1 gene SNPs with ACL tears.

Sl no.	Authors	Year of publication	Study type	No of ACL cases	No of controls	Population	Results
1	**Posthumus et al.[11]**	2010	Case control study	129	216	**South Africa**	Over-representation of AA genotype of rs970547 in female patients with ACL tears. No association of rs240736 with ACL tear.
2	**Ficek et al.[12]**	2014	Case control study	91	143	**Poland**	No association of ACL tear with rs970547 SNP.
3	**Our study**	-	Case control study	50	52	**India**	Over-representation of AG & GG genotypes of rs970547 in ACL tear cases. No association of rs240736 with ACL tear.

tions[12,13] Posthumus et al.[12] conducted a case-control genetic association study in South African participants (129 ACL tear patients and 216 healthy controls) to look for association between COL12A1 gene and ACL tears. They reported an over-representation of AA genotype of rs970547 (AluI RFLP) in female patients with ACL tears (p=0.048) but no significant association of the AA genotype with ACL tear when females and males were analysed together (p=0.067) or when males were analysed separately (p>0.05). However, rs240736 (BsrI RFLP) did not have any association with ACL tear in the South African cohort[12]. The same research group had also noted the association of COL12A1 SNPs with Achilles tendon ruptures[11]. The rare CC and GG genotypes of the COL12A1 rs240736 (BsrI RFLP) and rs970547 (AluI RFLP), respectively, were absent in participants with Achilles tendon ruptures[11].

Ficek et al.[13] conducted a similar case-control genetic association study in the Polish population (91 male football players with ACL tears and 143 healthy, male football players all of Polish descent). They observed no statistically significant association of SNP rs970547 (A9285G polymorphism) with ACL tears[13].

Our study results demonstrate that the AG and GG genotypes of rs970547 of COL12A1 was significantly under-represented in the study group participants (p=0.0361 and 0.0374 respectively; Figs. 2, 3). This means that the presence of AG and GG genotypes of this SNP could be somehow protective towards an ACL tear in the Indian population. This is in contrast to the observations of Posthumus et al.[12] who noticed over-representation of AA genotype only in females and to the observations of Ficek et al.[13] who noted no association of this SNP to ACL tear. On the other hand, our finding that SNP rs240736 is not associated with ACL tear is consistent with the findings of Posthumus et al.[12] who also reported no significant association between ACL tears and polymorphisms in rs240736 (BsrI RFLP).

The under-representation of these genotypes somehow results in an altered type XII collagen protein, which may lead to an alteration of the biomechanical properties of the collagen fibrils, thus resulting in structurally weaker collagen fibres; this in turn may predispose an individual to an increased risk of ACL tear[12]. However, this hypothesis is speculative and needs further research before validation (Tab. III).

This is the first study from Asia looking into the role of COL12A1 gene polymorphisms in the etiology of ACL tear. We used real time PCR for genotyping similar to Ficek et al.[13] whereas the RFLP method of genotyping was used by the South African research group[12]. RT-PCR is technically easier to perform, allows rapid quantification and has a higher accuracy and reliability[15].

We acknowledge some limitations of our study. The sample size is relatively small; hence these findings must be interpreted with caution. Further research with a larger sample size is needed which will not only validate these findings but will also facilitate haplotype analysis. We could not comment on the differences in the genotype/allele distributions between male and female groups owing to lesser number of female participants. Gender-specific association, however, was not a primary objective of this study. Future studies should ideally include more number of female participants in both groups to discern any gender-specific associations between SNPs and ACL tear in the Indian population.

Conclusions

AG and GG genotypes in exon 65 of COL12A1 are associated with ACL tears in the Indian population. Thus, type XII collagen may be a good candidate for genetic screening of predisposed individuals, so that these individuals may be counselled, strategies may be employed to prevent ACL tear and in the future may be a potential target for gene therapy.

References

1. Smith HC, Vacek P, Johnson RJ, et al. Risk factors for anterior cruciate ligament injury: a review of the literature-part 1: neuromuscular and anatomic risk. Sports Health. 2012;4(1): 69-78.
2. Smith HC, Vacek P, Johnson RJ, et al. Risk factors for anterior cruciate ligament injury: a review of the literature—part 2: hormonal, genetic, cognitive, previous injury and extrinsic risk factors. Sports Health. 2012;4(2):155-161.
3. John R, Dhillon MS, Sharma S, et al. Is There a Genetic Predisposition to Anterior Cruciate Ligament Tear? A Systematic Review. Am J Sports Med. 2016.
4. Maffulli N, Margiotti K, Longo UG, Loppini M, Fazio VM, Denaro V. The genetics of sports injuries and athletic performance. MLTJ. 2013;3(3):173-189.
5. Frank CB. Ligament structure, physiology and function. J Musculoskelet Neuronal Interact. 2004;4(2):199-201.
6. Gordon MK, Gerecke DR, Olsen BR. Type XII collagen: distinct extracellular matrix component discovered by cDNA cloning. Proc Natl Acad.Sci. U. S. A. 1987;84:6040-6044.
7. Birk DE, Fitch JM, Babiarz JP, et al. Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci. 1990;95:649-657.
8. Olsen BR. New insights into the function of collagens from genetic analysis. Curr Opin Cell Biol. 1995;7:720-727.
9. Young BB, Zhang G, Koch M, et al. The roles of types XII and XIV collagen in fibrillogenesis and matrix assembly in the developing cornea. J Cell Biochem. 2002;87:208-220.
10. Dressler MR, Butler DL, Wenstrup R, et al. A potential mechanism for age-related declines in patellar tendon biomechanics. J Orthop Res. 2002;20:1315-1322.
11. September AV, Posthumus M, van der Merwe L, et al. The COL12A1 and COL14A1 genes and Achilles tendon injuries. Int J Sports Med. 2008;29:257-263.
12. Posthumus M, September AV, O'Cuinneagain et al. The association between the COL12A1 gene and anterior cruciate ligament ruptures. Br J Sports Med. 2010;44(16):1160-1165.
13. Ficek K, Stepien-Slodkowska M, Kaczmarczyk M, et al. Does the A9285G polymorphism in collagen type XII gene associated with the risk of anterior cruciate ligament ruptures? Balk J Med Gen. 2014;17(1):41-46.
14. Padulo J, Oliva F, Friziero A, Maffulli N. Muscles, Ligaments and Tendon Journal. Basic principles and recommendations in clinical field science research: 2016 Update. MLTJ. 2016;6(1):1-5.
15. Gormus U, Selvi N, Yaylim-Eraltan I. PCR-RFLP and Real-Time PCR Techniques in Molecular Cancer Investigations, Polymerase Chain Reaction, Dr Patricia Hernandez-Rodriguez (Ed.). 2012.

ASSOCIATION OF GENE POLYMORPHISMS IN COL1A1 AND COL12A1 WITH ACL TEARS- A STUDY IN THE INDIAN POPULATION

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Abstract

Context: Gene polymorphisms are increasingly being identified in white populations as an intrinsic risk factor predisposing an individual to an ACL tear. This is the first study in a non-Caucasian population to look into genetic risk factors in ACL tear as well as to study the protein expression profile of these polymorphisms.

Objective: 1)To evaluate if there are SNP's (single nucleotide polymorphisms) in *COL1A1* and *COL12A1* that result in an ACL tear phenotype. 2)To investigate any association between protein expression of *COL1A1* and*COL12A1* and the genetic polymorphisms identified in these loci.

Design: Case-control genetic association study.

Setting: Research laboratory and operation theatre.

Participants: Fifty patients with ACL tear taken up for arthroscopic reconstruction. Fifty-two patients with unilateral, closed fracture of upper limb served as controls (Age group: 18-45 years). Mean age of cases was 24.76+/-6.1 years whereas that of control group was 30.25+/-7.15 years. Females comprised 6% and 13.5% of the study and control populations respectively.

Interventions: Venous blood samples collected from cases and controls whereas tissue samples collected from cases only. Lymphocytes extracted from blood. ACL remnant tissue excised during arthroscopic intervention and stored for DNA extraction. DNA isolated from both lymphocytes and tissue using commercial kits. The eluted DNA was quantified using ultraviolet spectrophotometry and run on agarose gel to verify the quality. By using RT-PCR (real time-polymerase chain reaction) amplification, *COL1A1* and *COL12A1* genes were analyzed for SNPs using specific primers.rs970547 (AluI polymorphism), rs240736 (BsrI polymorphism) of COL12A1 and rs1800012 (Sp1 binding site polymorphism) and rs1107946 of COL1A1gene tested. The RT-PCR amplification products were imported by sequence detection software for the detection of SNP. ELISA(enzyme-linked immune sorbant assay) analysis of ACL tissue extracted from cases was carried out and the results normalized to corresponding total protein.

Results:1)AG, GG genotypes of rs970547 of *COL12A1* significantly under-represented in the study group (Odds ratio{OR}=0.219;Confidence interval{CI}=0.053-0.906;Z value=2.096;p value=0.036 for AG and OR=0.214;CI=0.05-0.914;Z=2.082;p=0.037 for GG).2)No significant difference in genotype and allele distributions in rs240736 of *COL12A1* gene (p=0.712), rs1800012 (p=0.516) and rs1107946 (p=0.971) of *COL1A1*gene.3)No significant association found in the protein expression pattern in the ELISA test. Gender-specific analysis of the genotype results was not possible due to the relatively small population of female patients in both groups.

Conclusions: We found that the AG and GG genotypes of rs970547(Alu1 polymorphism) of COL12A1 gene were significantly under-represented in ACL tear patients in the Indian population. No significant association of the rs240736 SNP of COL12A1 gene, rs1800012 and rs1107946 SNP's of COL1A1 gene were noted.

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Using Current Data to Define New Approach in Age Related Macular Degeneration: Need to Accelerate Translational Research

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Abstract: Age related macular degeneration (AMD) is one of the major retinal degenerative disease of ageing whose complex genetic basis remains undeciphered. The involvement of various other factors like mitochondrial genes, cytoskeletal proteins and the role of epigenetics has been described in this review. Several population based AMD genetic studies have been carried out worldwide. Despite the increased publication of reports, clinical translation still eludes this davastating disease. We suggest models to address roadblocks in clinical translation hoping that these would be beneficial to drive AMD research towards innovative biomarkers and therapeutics Therefore, addressing the need large autopsy studies and combining it with efficient use of bioinformatic tools, statistical modeling and probing SNP-biomarker association are key to time bound resolution of this disease.

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Keywords: Age related macular degeneration, Mitochondrial genes, Epigenetics, SNP, Biomarkers, Translational research, Bio-informatics.

INTRODUCTION

Age related macular degeneration is one of most common retinal degenerative disease among elderly individuals resulting in irreversible vision loss. Indeed, the prevalence of AMD is believed to be higher in western countries than Asian counterparts but it remains to be seen how the incidence pattern changes with fast ageing population of India, China and other countries. The pathology of AMD is distinguishable by the presence of characteristic drusen. The advanced forms of AMD have several pathological hallmarks including degeneration of retinal pigmentary cells (RPE) and formation of new vessels from underlying choroid. These outgrowths of choroidal vessels penetrate the Bruch's membrane (BM) and disrupt the integrity of RPE cell layer and their function. These pathological changes in retina impact vision and cause irreversible blindness.

Both environment and genetic factors contribute equally to the progression of AMD pathology. Several studies have defined the role of modifiable environment factors like smoking, BMI, omega-3, carotenoids, *trans*-unsaturated fat intake *etc* [1-5] which could modulate the combined effect of several genetic susceptibility factors functional in multiplicative or in additive manner [6-8]. The fundamental basis of modulation of these genetic factors may be explained by epigenetic changes in genes or on the regulatory sequences of these genes. By introducing these changes in genes or regulatory sequences it may or may not influence the functional nature of corresponding proteins. Such epigenetic changes are mostly not inheritable. On the contrary, several genetic factors alone have sufficient potential to initiate AMD pathology. Among these genetic factors, most of proteins are basically involved in metabolic processes in the cells, participate in modulating immunity of the body, especially innate immunity components, angiogenic processes, and regulatory proteins involved in apoptosis, miRNA processing (DICERs) and proteins of extracellular matrix. Gene association studies have thus been widely explored in several populations as well as by manipulating genetic makeup in animal models thus indirectly validating their association with AMD.

This review will not only shed light on different strategies underlying AMD research such as the role of mitochondrial mutations, cytoskeleton, epigenetics, lifestyle and environment that impact advancement of field but also provide insight about various roadblocks in translation of basic knowledge for clinical benefit. Why genetic association studies are not being translated for drug development shall remain the mainstay of the review. We suggest that the integrated role of bioinformatics and statistical approaches may result in unveiling the genetic complexity of AMD.

GENETICS OF AMD

Several family and twin studies have revealed the inheritability nature of and role of genetic influences on AMD pathogenesis [9-13]. The other genetic studies have shown that the first degree relatives of AMD patients have higher risk for occurrence of AMD even earlier in life as compared with first degree relatives of those without AMD [14-16]. Several population based genetic studies have demonstrated

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that several genetic susceptible loci are associated with AMD pathology. Various genetic loci which have been shown to be associated with different forms of AMD i.e. dry, wet and geographical atrophy have been listed (Table **1**). The components of innate immunity have been investigated and their role in regulation of AMD related inflammatory processes have been established. Several complement factors especially alternative complement pathway genes, including the CFH [17], component C3, Factor I, Factor B and C2, have been reported in pathogenesis of AMD. Among the dominant factors, like complement families, is the complement factor H (CFH) which regulates the alternative complement pathway. SNP analysis of CFH have shown it to be most important genetic risk factor for AMD pathogenesis. Several genetic studies on various populations have strongly shown the role of CFH in AMD pathogenesis. Most of the AMD studies related to CFH now focus on a single SNP in the coding region of CFH, Y402H –a Tyr402His substitution – as the causal variant even though the original GWAS studies analysed only the Caucasian populations. Some studies have shown that the CFH gene on chromosome 1q31 is the first major AMD susceptibility gene [18]. The association of increased risk of AMD is reported to result from the Y402H variant in exon 9 (rs1061170, T>C) [19]. The coding and non coding region of the CFH gene, which are associated with either decreased or increased risk of AMD, have also been recognized. CFH gene is known to be involved in maintaining homeostasis of complement system evident by the role of inflammation in AMD. Any disruption in this system either in the form of altered functions of CFH variants, or CFH expression is believed to induce the AMD pathology. Apart from the known Y402H variant, other CFH variants, and their haplotypes can also predispose an individual to either increased or reduced risk of AMD pathogenesis in which a major risk haplotype lie in coding region of Y402H SNP and two protective haplotypes lie in intronic variants [20]. Moreover, the association between environmental factors such as smoking and obesity, and CFH genotype have been also been examined in disease progression, including the effect of genotype on response to treatment [21]. It has even been demonstrated that monogenic inheritance of CFH variants results in deposition of drusen which could initiate RPE atrophy [22].

The 50-70% cases of AMD are influenced with complement factor genes like CFH, factor I, and C2. The SNP in these genes have been shown to be associated with progression of AMD [23]. Interestingly, very few of these studies have analysed CFH protein in serum or plasma, let alone examine SNP-protein associations or carried out SNP risk factor association analysis. Besides, majority of these studies are cross sectional, not longitudinal.

Regarding other complement factors, meta-analysis study by Thakkinstian *et al.* pooled data from 19 studies which took place between 2006 and 2011 for 4 SNPs: rs9332739 and rs547154 for C2 gene and rs4151667 and rs641153 for CFB gene, suggesting that these alleles contribute to lowering the risk of AMD pathogenesis in Caucasian population by 2.0% to 6.0% [30]. Recently, it has been reported that complement factor B polymorphism (R32Q) correlates with early AMD but have protective effect on late AMD as seen in Caucasian population [31]. On the contrary, it has been reported in INDEYE study that polymorphisms in ARMS2/HTRA1 locus are significantly associated with early and late AMD but instead of this locus the complement factor components like C2, CFH and CFB have not been found to be associated with AMD [32]. Additionally, we have reported several proteins involved in apoptosis and angiogenesis to be altered in AMD patients signifying the role of associated genetic loci and their expression in pathology. These include VEGFR2, eotaxin-2, CCR-3, DCR1, SOD1, CCL-2 *etc.* [27, 29, 33-35].

Table 1. Several non-GWAS gene loci and their association with various forms of AMD.

Dry AMD			
Gene	**Locus**	**p-value**	**Reference**
SELP	rs3917751	0.0029	24
Geographical Atropy			
Gene	**Locus**	**p-value**	**Reference**
TLR3 DICER	rs3775291 NA	P=0.005 NA	25 26
Wet AMD			
Gene	**Locus**	**p-value**	**Reference Number**
VEGFR2	rs1531289	0.047	27
All AMD Subtype			
VEGF CCR3	rs1413711 rs3091250	0.002 GT-0.001 TT-0.002	28 29

Apart from role of innate immunity in AMD pathology, genes related to metabolism, especially cholesterol metabolizing genes have also been widely explored in different populations throughout the world and found to be associated with AMD pathology. Holliday *et al.* conducted a GWAS meta-analysis of early AMD, reporting association of variants at the CFH, ARMS2 loci, and suggesting the polymorphisms of Apolipoprotein E (ApoE) to be associated with early AMD. This study suggested a weaker genetic effect on the risk of early AMD as compared to late AMD [36].

In 2010, a genome wide association study (GWAS) was conducted which reported an association of HDL with susceptibility with AMD. This study was conducted among 2157 cases and revealed strongest association between two genes namely LIPC and CETP with AMD [37]. However, in a recent case control study carried out in Chinese population having AMD (n=535) CETP was studied as one of the gene among 10 genes with different variants. No significant association of CETP was seen in AMD patients. It is possible that in case of genetic studies, different genes respond differently depending on the severity of disease [38] which may even vary between populations testifying to the role of gene-environment interactions.

AMD is a heterogeneous disease and the role of most of established genetic factors are inconclusive in AMD pathology, as suggested by analysis of SNP in different population based studies even though these are limited to Caucasian populations. Therefore, a common pathway for AMD disease pathology continues to elude us. Do these gene association approaches in AMD analysis provide sufficient ground for understanding AMD pathogenesis.

MITOCHONDRIA AND AMD

The burden of oxidative stress is a common feature of metabolically active tissues. Retina, being one of the metabolically active tissue in the body, remains susceptible for oxidative stress due to frequency of photochemical reactions. Photoreceptors contain large number of mitochondria resulting in increased reactive oxygen species (ROS). ROS can damage the integrity of macromolecules and organelles. Apart from this, increased ROS concentration can also damage the mitochondrial-DNA (mtDNA) which has been studied as major factor in pathogenesis of AMD [39, 40]. mtDNA damage has reduced capacity to cope with increased ROS burden as compared to nuclear DNA in the photoreceptor cells due to absence of repair mechanism except excision base and mismatch repair [40]. It has been shown that accumulation of mtDNA deletion and deficiency of cytochrome-c oxidase is often observed in photoreceptors, especially the fovea [41]. The number of deletions and rearrangements have been found to be higher in mtDNA of retina of AMD patients as compared to AMD control, suggesting that the impairment of mitochondrial DNA is directly related to AMD which is affected by microenvironment of retina [42]. However, these studies are again limited to Caucasian populations and such analyses need worldwide attention and validation in populous countries.

The Blue Mountain Eye study has revealed that haplotype H of mtDNA was more prevalent in European population and found to be protective for AMD [43]. Several SNPs of noncoding region of mtDNA at T16126C, T16126C + G13368A, A4917G +A73G, and T3197C+ A12308G was strongly associated with AMD. It is know that the SNP A4917G codes for NADH dehydrogenase, therefore, the changes in its SNP may hamper the function and consequently increase the burden of ROS inside retina [44, 45]. The extent to which these mechanisms disrupt retinal function has bearing on why AMD is not a developmental disorder.

EPIGENETICS AND AMD

The role of epigenetics have been widely explored in human cancer. The epigenetic changes serve to provide protection of genome by host restriction enzymes, regulation and activation of genes. These changes are known to be confined to CpG islands in most of eukaryotic cells. A series of CpG islands are present in our genome and regulatory sequences of genes. The epigenetic changes include: methylation, sumoylation, phosphorylation and acetylation. It has been established that environment factors and dietary factors introduce these epigenetic changes in the genome [46]. It has also been shown that the DNA methylation of genome was drastically decreased with age [46]. Recently, a monozygotic twins study carried out by L wei (unpublished data) have revealed that about 256 genes to be hypomethylated and 744 genes hypermethylated in AMD twins. The epigenetic changes could be inherited and introduced by several modulatory environmental factors especially smoking and deficiency of anti-oxidant in diets. Additional studies across populations are warranted that can examine the role of epigenetics in progression of AMD.

CYTOSKELETON AND AMD

The cytoskeleton plays crucial role in maintaining functional and intact retinal layers. Therefore, any changes in cytoskeleton coding genes are associated with pathological changes in these cells remains to be seen. The cytoskeleton and their associated proteins have already been implicated in development of neural retina and integrity of retinal cells layers. However, there are a few studies examining its biological effects in AMD models or SNP screening.

Apart from the well established genetic factors in AMD, the cytoskeleton and associated proteins could be involved in morphogenetic of choroidal neovascularization (CNV) in case of wet AMD through which proliferating endothelium cells of choroid remodel themselves in neovessels. It has been demonstrated that the defective cytoskeletons in cone photoreceptor results in macular degeneration [47]. Additionally, the initial inflammatory responses against accumulation of drusen, leakage of fluids after rupture of Bruch's membrane by recruiting macrophages cells, pericytes, or by activating complement cascade mechanism in sub-retinal space involve cytoskeleton remodeling. Moreover, changes in functionality of vascular endothelial growth factor (VEGF), along with transforming growth factor- β (TGF-β), and platelet-derived growth factor (PDGF) can activate the endothelial cells resulting in remodeling of the extracellular matrix (ECM) through activation of the components involved in structure and function. This, whole process can evoke the angiogenic cascade which leads to CNV formation. However, the precise mechanism behind cytoskeleton

mediated neovascularization and recruitment of cells is still not clear and requires multinational and interdisciplinary efforts.

EXTRACELLULAR MATRIX (ECM) PROTEINS AND AMD

In various human inflammatory diseases metalloproteinase regulation becomes disrupted. RPE is responsible for regulating the photoreceptors activity and extracellular matrix. The dysregulation in the dynamics between photoreceptors and choroid-RPE complex highlights the role of ECM. ECM is a key mediator in the pathogenesis of AMD. There are 28 Matrix metalloproteinases (MMP) [48] and 29 members in ADAM family out of which some encode proteolytically active proteins [49]. MMPs degrade different components of extracellular matrix and are regulated through TIMPs by inhibition of the function of these proteins. ECM proteins are chiefly involved in AMD. These include: TIMP3, COL8A1, COL10A1, ADAMTS9, HTRA1, TGFBR1, B3GALTL etc. However, practical functional application of majority of these genes is still not confirmed. A model depicting dynamics between ADAMTS, B3GALTL and AMD has been depicted in (Fig. **1**). ADAMTS which is on chromosome 3 has a key role in angiogenesis and cartilage degeneration [50]. Previously, it was demonstrated that ADAMTS9 is antiangiogenic metalloproteinases which is expressed in microvascular endothelial cells and inhibition of this gene results in angiogenesis and apoptosis [51]. ADAMTS9 regulates the Akt/mTOR pathway which promotes expression of genes related to glycolysis and glucose uptake by increasing the transcription factor HIF1a. The serine-threonine protein kinase Akt is a common mediator of cellular survival signals and failure of Akt-mediated signaling can cause apoptosis which can lead to photoreceptor degeneration, bruch membrane thickening, extracellular deposits, decreased permeability leading to RPE damage, causing AMD. Jomarv *et al*. demonstrated that in rd mouse, inactivation of Akt survival pathway results in photoreceptor cell death [52]. Akt signaling targets mTOR which promotes angiogenesis. Zhaw *et al*. showed that mTOR-mediated dedifferentiation of the RPE indicates photoreceptor degeneration in mice [53]. B3GALTL gene, which is also involved in glycosylation, contributes in the elongation of O-fucosylglycan on thrombospondin (TSP) type repeat (TSP are secreted ECM glycoproteins involved in the ECM and cellular interactions which is antiangiogenic). TSP-1 and TSP-2 are the known initial protein inhibitors of angiogenesis [54]. TSP-1 has been reported to be secreted by RPE which controls angiogenesis in the eye. Therefore, any impaired addition of sugar molecules to protein can disrupt the function of several proteins, which may lead to the abnormal angiogenesis resulting in AMD.

SNPs ANALYSIS AND SLOW PACE OF DRUG DEVELOPMENT IN AMD

Several genome wide association studies have been carried out to dissect the role of genes and their association with AMD pathogenesis (Table **2**). Even though the population based genetic studies in AMD are increasing every year, however, most of these SNPs have not been translated in development of human diagnostic, prognostic and pharmaceutical applications. The outcome of gene association studies appears over-rated in small sample size when compared to studies

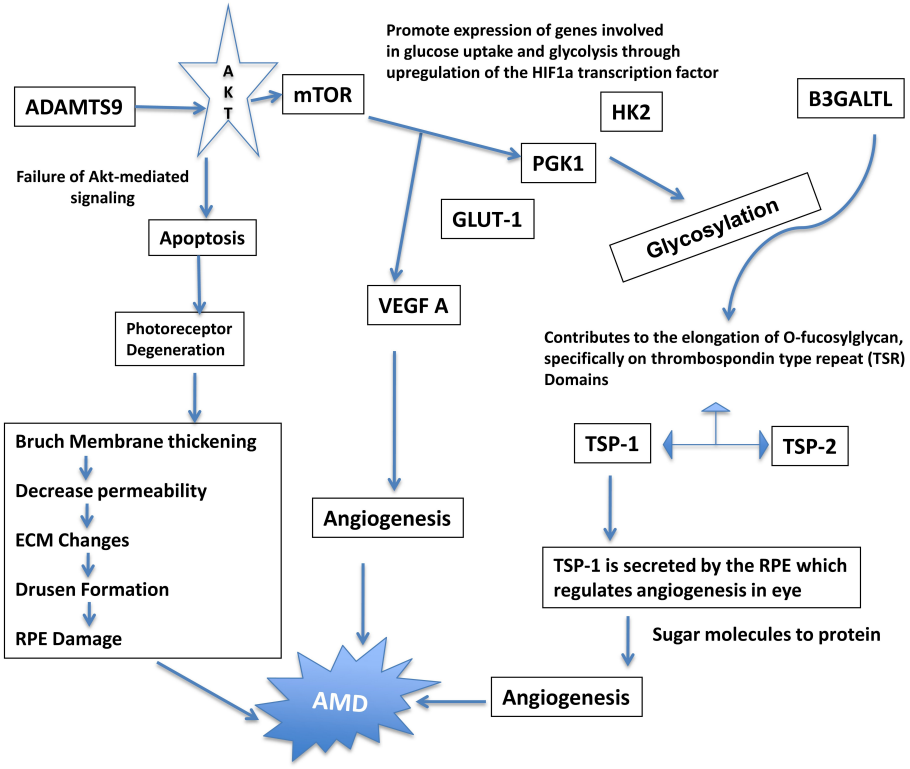

Fig. (1). Schematic representation of AMD pathogenesis mediated by the proteins responsible for integrity of extra-cellular matrix that required for function of RPE cells and imbalance in function of these proteins leads to pathology.

Table 2. Important GWAS studies throughout the world in field of AMD and their significance.

Studied Gene	Study Type	No. of Patients		p-value	Locus	Geographical Attribution	Reference Number
CFH *ARMS2* *RDBP*/*CFB*/*C2* *DNAJC27* OTOS *FSTL5*	Case Control Study	AMD Case 1207	Control 686	7.51×10^{-30} 1.94×10^{-23} 4.37×10^{-10} 6.58×10^{-6} 6.81×10^{-6} 2.70×10^{-6}	rs1831282 rs10490924 rs522162 rs713586 rs4854022 rs10857341	USA	58
CFI, C3, C9	Case- control study	1,676	745	2×10^{-8} 3.5×10^{-5} 2.4×10^{-5}	rs4698775 rs147859257 rs34882957	USA	59
Seven new loci *COL8A1-FILIP1L* *IER3-DDR1* *SLC16A8* *TGFBR1* *RAD51B* *ADAMTS9* *B3GALTL*	Case control	>17,100	>60,000	$4 X 10^{-13}$ $2X10^{-11}$ $2X10^{-11}$ $3X10^{-11}$ $9X10^{-11}$ $5X10^{-9}$ $2X10^{-8}$	rs13081855 rs3130783 rs8135665 rs334353 rs8017304 rs6795735 rs9542236	USA	60
CFH ARMS2 ApoE GLI3 GLI2 TYR	Case Control Study	4,089	20,453	1.5×10^{-31} 4.3×10^{-24} 1.1×10^{-6} 8.9×10^{-6} 6.5×10^{-6} 3.5×10^{-6}	rs2075650 rs2049622 rs6721654 rs621313	Australia	61
ARMS2/ HTRA1	Case Control Study	2594	4134	4.3×10^{-9} 7.4×10^{-14}	rs10490924	USA	62
ARMS2-HTRA1 CFH C2-CFB C3 CFI VEGFA LIPC	Case Control Study	893	2199	2.7×10^{-72} 2.3×10^{-47} 5.2×10^{-9} 2.2×10^{-3} 3.6×10^{-3} 1.2×10^{-3} 0.04	rs10490924/ rs2284665 rs10801555 rs541862 rs7690921 rs7690921 rs943080 rs10468017	UK	63
CFH ARMS2 TNFRSF10A-LOC389641 REST-C4orf14-POLR2B-IGFBP7	Case Control Study	1,536	18,894	4.23×10^{-15} 8.67×10^{-29} 1.03×10^{-12} 2.34×10^{-8}	rs800292 rs3750847 rs13278062 rs1713985	Japan	64
FRK/COL10A1 VEGFA ARMS2/HTRA1 CFH CFB C3 C2 CFI LIPC TIMP3 CETP	Case Control Study	2594 (replication-5640)	4134 (replication-52174)	1.1×10^{-8} 8.7×10^{-9} 1.2×10^{-144} 5.6×10^{-138} 2.1×10^{-134} 2.9×10^{-22} 1.4×10^{-18} 4.3×10^{-12} 2.4×10^{-11} 4.6×10^{-5} 3.7×10^{-4} 1.2×10^{-4}	rs1999930 rs4711751 rs10490924 rs1061170/ rs1410996 rs641153 rs2230199 rs9332739 rs10033900 rs10468017 rs9621532 rs3764261	USA	65

(Table 2) contd….

Studied Gene	Study Type	No. of Patients		p-value	Locus	Geographical Attribution	Reference Number
RIMS3	Case Control Study	444	300	0.23	rs11208590	USA	66
ABCA4				0.66	rs2297634		
CFHR4				0.2	rs1853883		
YOD1				0.91	rs2054780		
OBSCN				0.29	rs287614		
OBSCN				0.2	rs1150910		
ROBO1				0.51	rs2055451		
SKIV2L				0.38	rs1447338		
ADCYAP1R1				0.07	rs13129209		
ARMS2				0.48	rs429608		
TEX9				0.96	rs2267742		
CTRB1				0.45	rs10275700		
METT10D				0.25	rs10274362		
DCC				0.53	rs988426		
DCC				0.17	rs2014307		
DCC				0.04	rs2713935		
FBXO15				0.58	rs6493856		
C3				0.45	rs8056814		
SEZ6L				0.65	rs4268798		
GRID2				0.37	rs9950970		
OR1Q1				0.3	rs1367634		
				0.7	rs12954274		
				0.84	rs8086078		
				0.64	rs2230199		
				0.43	rs6137194		
				0.41	rs9608466		
					rs6532378		
					rs972925		
					rs2838378		

analysing the same polymorphism in a high number sample size. The primary outcome of biased false-positive results from scientific reports can hamper or mislead the advancement of scientific knowledge and decision making in clinical and drug development settings. Despite rapid increase in SNP association studies in AMD (regardless of claims that this will stop, personal communication: Anand Swaroop), there seems to be no hope emerging from such investigations. Therefore, there is need to define the criteria for evaluation of the significance of these results. It has been observed that majority of high impact journals and editors only accept the manuscripts which present positive results. It is necessary that reviewers and editor of journals critically evaluate results, parameters involved in these studies, application of statistical expertise without remaining influenced by positive results of the reports [55-57]. In order to provide high quality of results in basic investigations of AMD, especially with regards to genetic association studies we need to address the following issues in clinical translation:

i) Scientific knowledge gap between clinicians and basic research scientist.

ii) Absence of longitudinal studies in AMD.

iii) Lack of autopsy studies to assess the gene-pathophysiology correlation.

iv) Lack of Meta-analysis in preclinical studies in various Asian, western counterparts.

v) Studies to assess both biomarker level along with their SNP analysis.

vi) Selection of statistical tools to analyse the results of these population based studies by statisticians.

vii) Uniformity in GLP practices in results analysis, selection of standard operating protocol (SOP), and randomization in experiments.

Knowledge Gap Between Practising Clinicians and Basic Scientists

This is very unfortunate drawback in education system of both developing and developed countries that the fundamental knowledge in basic research is virtually deficient among practising clinicians and the basic knowledge of human anatomy and physiology is virtually lacking among basic scientists. In order to bridge this chasm between clinicians and basic scientists, in 2003, an effort was made by National Institutes of Health (NIH) who developed a framework of priorities to optimize and equalize entire medical research portfolio [67]. It is important to implement the guidelines, training in research and analysis, revamp of education system by inclusion of both MD-PhD and PhD-MD education

systems, cultivating additional medical research scientists in both pre-clinical and clinical settings [68, 69] in order to streamline the translational outcome i.e. development of diagnostic and prognostic markers as well as therapeutic drugs [70-72]. Moreover, it is also more important that, apart from implementing research education and training programs for clinicians, it is necessary to share clinical data and findings of AMD patients with research scientists in a real time manner. This will help to evolve an understanding of AMD pathology in a precise manner and also facilitate basic and translational research. This tie up between basic research scientists and clinicians has several benefits: development of new drugs and improving the understanding of the scientific basis of AMD pathology, developing information system driven from patients care and the results to become more reliable for translation.

Association Studies Between SNP and Biomarkers

In most of the gene association studies in AMD, it has been observed that the investigators have examined either SNPs or level of a particular protein in serum or plasma. The results obtained from such reports provide only a partial understanding of the genetics of AMD. By examining both SNP as well as protein levels in serum or plasma it is possible to examine whether SNPs changes are associated with expression level of corresponding protein. Such mendelian randomization approach (an approach to rule out the biased interpretation of confounders in genetic variation which seems to have role in disease pathology) analysis can give an insight into the nature of SNP in the population and facilitate discovery of the diagnostic biomarkers for that population making the data more creditable and reliable for drug discovery programs. Mendelian randomization approach is employed in population and epidemiological studies to examine the correlation between the genetic variation and environmental factors. By this approach biased interpretation can be avoided and confounder factors used in such genetic research may increase the robustness of the data [73].

Absence of Longitudinal Studies in AMD

Most of data from AMD research has been reported from single time point analysis. The results obtained from such investigation have several drawbacks in drawing conclusions. First, it is difficult to estimate the effect of ongoing interventions on disease progression, second; protein levels may change at different time points and, finally: several experimental errors may occur in one time analysis. Therefore, the association of these protein levels with progression of disease can enable in deciphering the causative and/or prognostic potential of these biomarkers. The longitudinal follow up of these patients at a defined time interval, will enable in determining the causative effect of protein with progression of disease accurately. These results, with longitudinal follow up can facilitate the quality of research outcome from such association studies making them more reliable for clinical translational.

Meta-analysis in Preclinical Studies in Various Populations

Preclinical studies define the safety and efficacy of administering drugs before applying them on patients. Therefore, preclinical trials have their own importance in drug discovery program. The preclinical data should not be biased and influenced with false-positive results since these studies act as raw material for development of diagnostics and prognostic markers, besides providing the substrate for validation in drug pharmacokinetics. Preclinical trials of bevacizumab, ranibizumab and pegaptanib on cynomolgus monkey, rabbits, and rats have been carried out in order to evaluate the pharmacokinetics, serum bioavailability and dose toxicity after single drug is known to vary from 2.88 days to 3 days for ranibizumab, and bioavailability of these all drugs, at higher concentration, are confined in to vitreous fluid [74, 75]. Toxicity was not observed at even higher concentration of all three drugs when tested in NMDA induced retinal degeneration model of rat [76]. This data will provide the basic information about amount of dose, based on body mass index of the patient, and the suitability of route of drug administration. Therefore, meta-analysis of preclinical studies among animals, which are not even phylogenetically related to each other would be beneficial to evaluate significance of the drug effectiveness as well as the reliability of drug to avoid false-positive outcome.

Use of Statistical Tools in Genetic Association Studies

The implication of appropriate statistical tools and analysis of obtained results is one of the important points in experimental setup (both pre clinical and clinical studies). Most of degenerative diseases have equal role of confounders along with genetic factors in disease progression. Unfortunately, most of these studies do not include the significance of these confounders in disease pathology in association with genetic data. At the preclinical setup, the statistical methods should be incorporated with the active participation of collaborator in the field. Therefore, multivariate analysis of disease pathology with genetic as well as other environmental factors should be defined in the experimental procedure. Moreover, in case of animal studies, proper experimental setup, defined groups, mortality rate, other side effects of the drugs should be spelled out properly in order to reduce the biased interpretation of results.

Autopsy Studies in AMD

Another important reason argued as a cause of failed trials in AMD is the lack of autopsy studies, because it is not possible to look in to the precise mechanism in AMD pathogenesis because most of animal models are not able to recapitulate all cardinal features of AMD, besides, these models are not phylogenetically similar to humans especially in lacking macula. Additionally, the cell culture based studies are not able to reproduce the exact mechanism or the role of these genes in AMD pathology due to improper physiological complexity of tissue in relation to three dimensional human eye. Therefore, it is very challenging to examine the precise mechanism even though this is an accepted model of analysis. Hence, it is very important to expand study design by dissecting the actual role and localization of these genes in AMD pathogenesis with the hope that these studies will provide the new vistas for the discovery of diagnostic and prognostic biomarkers.

Lack of Uniformity in GLP Practice with Respect to Result Analysis, Selection of Standard Operating Protocol (SOP), and Randomization in Experiments

To avoid false-positive and biased reporting of experiments, preclinical and clinical data, it is desirable to perform all experiments under good lab practice (GLP) regulations especially when bench workers come from varying nationalities and working environments. The GLP compliance, including the stringent documentation requirements for personnel training, periodic study monitoring and quality assurance of data and resources is the hallmark of universal acceptance of data critical for translation [77, 78]. The data from various scientific reports is expected to have variations in results due to use of different methods and procedures of experiments and different techniques to conduct a given experiment. Even the tools used in data analysis of these gene association studies vary between various research groups. The variation of results may be due to wrong interpretation of results or by applying inappropriate analytical tools. Hence, it is very important to devote quality time to analyse data from research in an appropriate manner which can be applied to build basic knowledge in a particular field. It is for this reason that compliance to GLP can ensure the research results to become more valid and reproducible. These can be used as supportive studies in translational applications.

USE OF GENETIC INFORMATION IN CLINICAL TRIALS FOR AMD

Numerous clinical trials have been conducted all over the world to facilitate drug developments and clinical research. Meanwhile, DNA information is routinely collected during the trials under informed consent. However, there is a considerable gap between the genetic studies and clinical trials. The data needs to be synthesized together to better understand disease biology. For example, the National Eye Institute (NEI), of the U.S. National Institutes of Health (NIH) launched a multi-center and randomized clinical trial named the Age-Related Eye Disease Study (AREDS) in 2001 [79, 80]. The trial is designed to study if nutrients (Antioxidant Vitamins and Zinc) can help AMD patients. Over 3,600 people with different stages of AMD participated in this trial with a median follow-up period 5 years. The research group showed that high levels of antioxidants and zinc could reduce the risk of advanced AMD and its progression. Later in 2006, NEI launched another trial (AREDS2) to study if a modified nutrient formulation can reduce the risk of AMD and its progression in a cohort of over 4,000 participants [81]. In addition to the clinical and epidemiological studies, most participants in AREDS1 and AREDS2 have consented for genetic research and the DNA was collected and stored. The samples have been genotyped using commercial whole-genome genotyping platforms for multiple purposes. Merging the genetic data with the comprehensive clinical phenotypes can help researchers reach the following goals:

1) Identify genes that affect AMD progression. Clinical trials typically follow patients for a long period with multiple visits. The collected longitudinal phenotypes (e.g. AMD scale of severity) provide the necessary information to identify genes that can increase/reduce the time to advanced AMD if the genome-wide genetic data is available. The type of survival analysis can be performed to include censoring data points, which is treated as missing data in a typical case-control study. A genome-wide scan on progression time will further our understanding of AMD on top of the great success of GWAS from previous case-control studies.
2) Improve statistical models for AMD risk and progression. After we obtain a list of variants associated with AMD, we can build statistical methods for predicting the risk of AMD based on top variants together with other demographic and environmental variables. Previous literature has shown that the risk and progression of AMD can be accurately predicted [82-84]. We can further improve the prediction accuracy based on a more completed list of variants that are discovered in 1. Furthermore, we can model two eyes separately with the consideration of within-person correlation and predict the eye-level risk using clinical information from each eye.
3) Identify genes that affect individual response to treatment. In pharmacogenomics, individual response to drugs/treatment can be affected by genetic differences through biological pathways. A second generation of genome-wide scan on drug responses or pharmacological treatments will facilitate phenotypic screening in addition to drug discovery based on targeted pathways.
4) Identify the disease subgroups that can benefit from certain drugs/treatment. The effect of drugs/treatments may vary from person to person. Individual genetic profiles provide potential for researchers to identify a subgroup of patients that have better drug responses or less side effects than general populations. Therefore, we can improve the effectiveness of certain drugs/treatment.

Moreover, given the successes of genetic studies in AMD, we can apply findings to directly facilitate the design of clinical trials of AMD. For example, Hu *et al.* proposed a statistical method to reduce the cost of prevention trials by merging genetic risk scores calculated from GWAS findings with demographic factors [85]. The rationale is to enroll a subgroup of individuals with increased incidence of AMD for early stage screening, thus saving cost for patient follow-up. Their results show that using both genetic and clinical factors can reduce trial cost by 33%.

In summary, combining genetic information with longitudinal phenotypic data from clinical trials for AMD will greatly enhance our knowledge about etiology and pathology of AMD, improve existing statistical models for predicting AMD progression, provide clues for drug developments towards personalized medicine, and save cost for future clinical trials. We anticipate that the integrative analysis of genetics, genomics, and environmental factors will have a great impact on clinical practice.

BIOINFORMATICS APPROACH

The population based gene association studies have greater significance in drug discovery programs based on outcome of SNP analysis in a population. Bio-informatics approach plays an imperative role in SNP based drug discovery settings. Several bioinformatic approaches are being adopted to signify and confirm the nature of SNPs of these

synonymous and nonsynonymous SNP (nsSNP) changes in the genome. The results of bioinformatic analysis will provide a biological annotation of nsSNP in the candidate genes thus enabling prediction of the impact of variation in structure and function of proteins. Disease risk can be predicted based on effect of nsSNPs and by analyzing its role in functional protein in the early age of the AMD patients who will be likely to develop AMD in the later stage of their life.

The amino acid sequence of a protein provides valuable information with regard to its biochemical features. The primary structure of the proteins holds the key for its higher order of conformation which will ultimately bring about its function. A number of similar sub-structures, known as domains, occur in many functionally related or unrelated proteins. Super secondary structures, also known as motifs or folds, are mostly stable arrangements of numerous elements of secondary structure. The particular order of amino acid sequence of a protein can provide the knowledge of 3-dimensional structure and function of the protein. The nature of protein, whether it is secretary, membranous or targeted for various intracellular organelles, depends on their tagged signal sequences. Mainly, these signal sequences are present on N-terminal of the peptides. Several post-translational modifications also occur in these proteins by addition and removal of several functional groups such as phosphate, acetate, various lipids and carbohydrates. It is important to map nsSNPs onto these features of proteins.

Further, It may be interesting to study the conservative/non-conservative nsSNP across classes of amino acids (I- R, K, H; II- D, E; III- A, G, I, L, M, N, P, Q, S, T, V; IV- Y, F, W) and changes from any amino acid residues (R, K, H, D, E, A, G, I, L, M, N, P, Q, S, T, V, Y, F and W) to C and vice versa. Importantly, analysis of these nsSNPs will have significant impact predicting the changes in secondary and tertiary structure of the corresponding protein using available tools. Additionally, the holistic approach to correlate these different disciplines could provide better treatment strategy to deal with such complex diseases. Recently, several studies have recommended the role of 'omics' sciences with system biology in order to provide personalized medicine to combat complex disease phenotypes [86-88]. Bowler *et al.* have combined the 'omics' approach with system biology to discover biomarkers in emphysema. They have found lower plasma and mRNA levels in PBMCs of emphysema patients that were associated with body mass index and age by using multivariate approach and have concluded the 'omics' analysis coupled with systems biology can be useful in advancement of personalized medicine [89].

The results of systems biology and bioinformatic analysis will therefore provide a biological annotation of nsSNP in the candidate genes. Thus, it will provide an insight how the changes at genomic level will reflect at the protein level, affecting protein function and progression of AMD. The study of nsSNPs in genetically associated genes will provide better understanding of the phenotype variation in AMD patients across various continents.

CONCLUSION

AMD remains a devastating and complex genetic eye disease due to the lack of effective treatments. The current treatment strategy targets the VEGF to prevent the choroidal angiogenesis which provides the symptomatic relief from the disease only in one third of the AMD cases. Some other treatment strategies are also in used in AMD based on blocking of sphingosine-1phosphate signaling process to alleviate angiogenesis [90], a variant of which already exists in cancer treatment. The review provides a critical update with attending barriers of translation in AMD research (Fig. **2**). The review recommends that systemic and 'omics' science should be merged to improve the personalized medicine approach to accelerate translation research in the field.

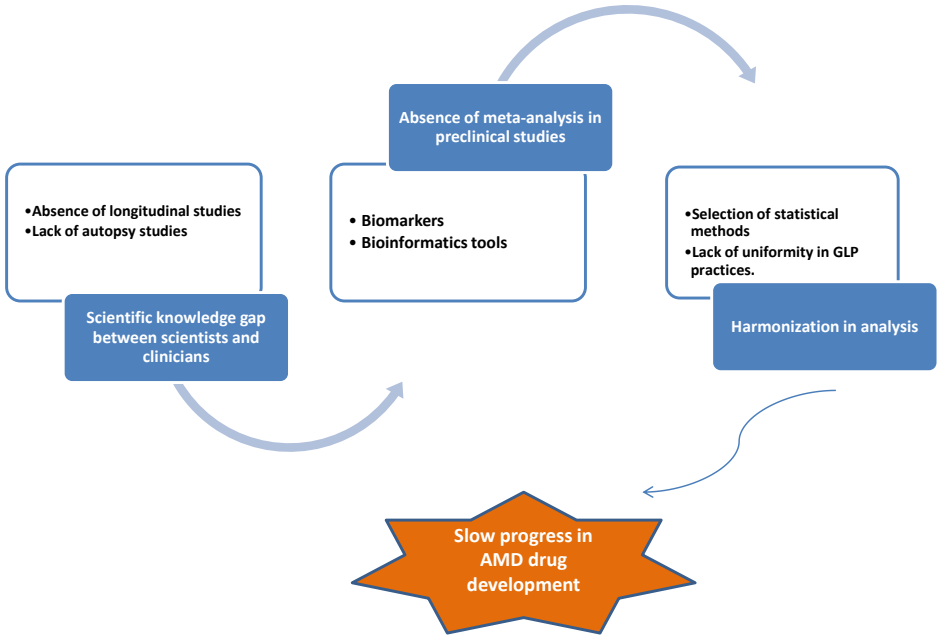

Fig. (2). Schematic representation of roadblocks in AMD translation research.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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AUTHOR'S CONTRIBUTIONS

Compiling information and initial drafting of manuscript: KS, AA, NS. Editing of manuscript: AA, WC. Concept of the manuscript: AA.

REFERENCES

[1] Chakravarthy, U.; Wong, T.Y.; Fletcher, A.; Piault, E.; Evans, C.; Zlateva, G.; Buggage, R.; Pleil, A.; Mitchell, P. Clinical risk factors for age related macular degeneration: a systematic review and meta-analysis. *BMC Ophthalmol.*, **2010**, *10*, 31.

[2] Chong, E.W.; Robman, L.D.; Simpson, J.A.; Hodge, A.M.; Aung, K.Z.; Dolphin, T.K.; English, D.R.; Giles, G.G.; Guymer, R.H. Fat consumption and its association with age-related macular degeneration. *Arch. Ophthalmol.*, **2009**, *127*, 674–680.

[3] Seddon, J.M.; George, S.; Rosner, B. Cigarette smoking, fish consumption, omega-3 fatty acid intake, and associations with age-related macular degeneration: the US Twin Study of Age-related Macular Degeneration. *Arch. Ophthalmol.*, **2006**, *124*, 995–1001.

[4] Delcourt, C.; Carrière, I.; Delage, M.; Barberger-Gateau, P.; Schalch, W. POLA Study Group. Plasma lutein and zeaxanthin and other carotenoids as modifiable risk factors for agerelated maculopathy and cataract: the POLA Study. *Invest. Ophthalmol. Vis. Sci.*, **2006**, *47*, 2329–2335.

[5] Age-Related Eye Disease Study Research Group; San Giovanni, J.P.; Chew, E.Y.; Clemons, T.E.; Ferris, F.L.; Gensler, G.; Lindblad, A.S.; Milton, R.C.; Seddon, J.M.; Sperduto, R.D. The relationship of dietary carotenoid and vitamin A, E, and C intake with age-related macular degeneration in a case-control study: AREDS Report No 22. *Arch. Ophthalmol.*, **2007**, *125*, 1225–1232.

[6] Munoz, B.; Klein, R.; Rodriguez, J.; Snyder, R.; West, S.K. Prevalence of age-related macular degeneration in a population-based sample of Hispanic people in Arizona: Proyecto VER. *Arch. Ophthalmol*., **2005**, *123*(11), 1575–1580.

[7] Thornton J, Edwards R, Mitchell P, Harrison RA, Buchan, I.; Kelly, S.P. Smoking and age related macular degeneration: A review of association. *Eye* (*Lond*), **2005**, *19*(9), 935–944.

[8] Klein, R. Overview of progress in the epidemiology of age-related macular degeneration. *Ophthalmic Epidemiol*., **2007**, *14*(4), 184–187.

[9] Melrose, M.A.; Magargal, L.E.; Lucier, A.C. Identical twins with subretinal neovascularization complicating senile macular degeneration. *Ophthalmic Surg*., **1985**, *16*, 648-51.

[10] Meyers, S.M.; Zachary, A.A. Monozygotic twins with age-related macular degeneration. *Arch. Ophthalmol.*, **1988**, *106*, 651-3.

[11] Dosso, A.A.; Bovet, J. Monozygotic twin brothers with age-related macular degeneration. *Ophthalmologica*, **1992**, *205*, 24-8.

[12] Piguet, B.; Wells, J.A.; Palmvang, I.B.; Wormald, R.; Chisholm, I.H.; Bird, A.C. Age related Bruch's membrane change: a clinical study of the relative role of heredity and environment. *Br. J. Ophthalmol.*, **1993**, *77*, 400-3.

[13] Klein, M.L.; Mauldin, W.M.; Stoumbos, V.D. Heredity and age-related macular degeneration: observations in monozygotic twins. *Arch. Ophthalmol.*, **1994**, *112*, 932-7.

[14] Seddon, J.M.; Ajani, U.A.; Mitchell, B.D. Familial aggregation of age-related maculopathy. *Am. J. Ophthalmol.*, **1997**, *123*, 199-206.

[15] Sharma, N.K.; Gupta, A.; Prabhakar, S.; Singh, R.; Sharma, S.K.; Chen, W.; Anand, A. Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. *PLoS One*, **2013**, *8*(7), e70193.

[16] Klaver, C.C.; Wolfs, R.C.; Assink, J.J.; van Duijn, C.M.; Hofman, A.; de Jong, P.T. Genetic risk of age-related maculopathy: population- based familial aggregation study. *Arch. Ophthalmol.*, **1998**, *116*, 1646-51.

[17] Assink, J.J.; Klaver, C.C.; Houwing-Duistermaat, J.J.; Wolfs, R.C.; van Duijn, C.M.; Hofman, A.; de Jong, P.T. Heterogeneity of the genetic risk in age-related macular disease: a population- based familial risk study. *Ophthalmology*, **2005**, *112*, 482-7.

[18] Zareparsi, S.; Branham, K.E.; Li, M.; Shah S, Klein R.J.; Ott, J,; Hoh, J.; Abecasis, G.R.; Swaroop, A. Strong association of the Y402H variant in complement factor H at 1q32 with susceptibility to age-related macular degeneration. *Am. J. Hum. Genet*., **2005**, *77*, 149-153.

[19] Sepp, T.; Khan, J.C.; Thurlby, D.A.; Shahid, H.; Clayton, D.G.; Moore, A.T.; Bird, A.C.; Yates, J.R. Complement factor H variant Y402H is a major risk determinant for geographic atrophy and choroidal neovascularization in smokers and nonsmokers. *Invest. Ophthalmol. Vis. Sci.*, **2006**, *47*, 536-540.

[20] Hageman, G.S.; Anderson, D.H.; Johnson, L.V.; Hancox, L.S.; Taiber, A.J.; Hardisty, L.I.; Hageman, J.L.; Stockman, H.A.; Borchardt, J.D.; Gehrs, K.M.; Smith, R.J.; Silvestri, G.;Russell, S.R.; Klaver, C.C.; Barbazetto, I.; Chang, S.; Yannuzzi, L.A.; Barile, G.R.; Merriam, J.C.; Smith, R.T.; Olsh, A.K.; Bergeron, J.; Zernant, J.; Merriam, J.E.; Gold, B.; Dean, M.; Allikmets, R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc. Natl. Acad. Sci. USA*, **2005**, *102*, 7227–32.

[21] Gehrs, K.M.; Jackson, J.R.; Brown, E.N.; Allikmets, R.; Hageman, G.S. Complement, age-related macular degeneration and a vision of the future. *Arch. Ophthalmol.*, **2010**, *128*, 349–58.

[22] Boon, C.J.; Klevering, B.J.; Hoyng, C.B.; Zonneveld-Vrieling, M.N.; Nabuurs, S.B.; Blokland, E.; Cremers, F.P.; den Hollander, A.I. Basal laminar drusen caused by compound heterozygous variants in the CFH gene. *Am. J. Hum. Genet.*, **2008**, *82*, 516–23.

[23] de Jong, P.T. Age-related macular degeneration. *N. Engl. J. Med*., **2006**, *355*(14),1474-85.

[24] Mullins, R.F.; Skeie, J.M.; Folk, J.C.; Solivan-Timpe, F.M.; Oetting, T.A.; Huang, J.; Wang, K.; Stone, E.M.; Fingert, J.H. Evaluation of variants in the selectin genes in age related macular degeneration. *BMC Med. Genet.*, **2011,** *12*, 58.

[25] Yang, Z.; Stratton, C.; Francis, P.J.; Kleinman, M.E.; Tan, P.L.; Gibbs, D.; Tong, Z,; Chen, H.; Constantine, R.; Yang, X.; Chen, Y.; Zeng, J.; Davey, L.; Ma, X.; Hau, V.S.; Wang, C.; Harmon, J.; Buehler, J.; Pearson, E.; Patel, S.; Kaminoh, Y.; Watkins, S.; Luo, L.; Zabriskie, N.A.; Bernstein, P.S.; Cho, W.; Schwager, A.; Hinton, D.R.; Klein, M.L.; Hamon, S.C.; Simmons, E.; Yu, B.; Campochiaro, B.; Sunness, J.S.; Campochiaro, P.; Jorde, L.; Parmigiani, G.; Zack, D,J.; Katsanis, N.; Ambati, J.; Zhang, K. Toll-like receptor 3 and geographic atrophy in age-related macular degeneration. *N. Engl. J. Med*., **2008**, *359*, 1456–1463.

[26] Kaneko, H.; Dridi, S.; Tarallo, V.; Gelfand, B.D.; Fowler, B.J.; Cho, W.G.; Kleinman, M.E.; Ponicsan, S.L.; Hauswirth, W.W.; Chiodo, V.A.; Karikó, K.; Yoo, J.W.; Lee, D.K.; Hadziahmetovic, M.; Song, Y.; Misra, S.; Chaudhuri, G.; Buaas, F.W.;Braun, R.E.; Hinton, D.R.; Zhang, Q.; Grossniklaus, H.E.; Provis, J.M.; Madigan, M.C.; Milam, A.H.; Justice, N.L.; Albuquerque, R.J.; Blandford, A.D.; Bogdanovich, S.; Hirano, Y.; Witta, J.; Fuchs, E.; Littman, D.R.; Ambati, B.K.;, Rudin, C.M.; Chong, M.M.; Provost, P.; Kugel, J.F.; Goodrich, J.A.; Dunaief, J.L.; Baffi, J.Z.; Ambati, J. DICER1 deficit induces *Alu* RNA toxicity in age-related macular degeneration. *Nature*, **2011**, *471*(7338), 325-30.

[27] Sharma, N.K.; Gupta, A.; Prabhakar, S.; Singh, R.; Sharma, S.; Anand, A. Single Nucleotide Polymorphism and Serum Levels of VEGFR2 are Associated with Age Related Macular Degeneration. *Curr. Neurovasc. Res.*, **2012**, *9*, 256-265.

[28] Almeida, L.N.; Melilo-Carolino, R.; Veloso, C.E.; Pereira, P.A.; Miranda, D.M.; De Marco, L.A.; Nehemy, M.B. Homozygosity for the +674C>T polymorphism on VEGF gene is associated with age-related macular degeneration in a Brazilian cohort. *Graefes Arch. Clin. Exp. Ophthalmol.*, **2012**, *250*(2), 185-189.

[29] Sharma, N.K.; Gupta, A.; Prabhakar, S.; Singh, R.; Bhatt, A.K.; Anand, A. CC chemokine receptor-3 as new target for age-related macular degeneration. *Gene*, **2013**, *523*, 106–111.

[30] Thakkinstian, A.; McEvoy, M.; Chakravarthy, U.; Chakrabarti, S.; McKay, G.J.; Ryu, E.; Silvestri, G.; Kaur, I.; Francis, P.; Iwata, T.; Akahori, M.; Arning, A.; Edwards, A.O.; Seddon, J.M.; Attia, J. The Association between Complement Component 2/Complement

Factor B Polymorphisms and Age-related Macular Degeneration: A HuGE Review and Meta-Analysis. *Am. J. Epidemiol.*, **2012**, *176*(5), 361-372.

[31] Mantel, I.; Ambresin, A.; Moetteli, L.; Droz, I,; Roduit, R.; Munier, F.L.; Schorderet, D.F. Complement Factor B Polymorphism and the Phenotype of Early Age-related Macular Degeneration. *Ophthalmic Genet.*, **2014**, *35*(1), 12-17.

[32] Sundaresan, P.; Vashist, P.; Ravindran, R.D.; Shanker, A.; Nitsch, D.; Nonyane, B.A.; Smeeth, L.; Chakravarthy, U.; Fletcher, A.E. Polymorphisms in ARMS2/HTRA1 and complement genes and age-related macular degeneration in India: findings from the INDEYE study. *Invest. Ophthalmol. Vis. Sci.*, **2012**, *53*(12), 7492-7.

[33] Sharma, N.K.; Prabhakar, S.; Gupta, A.; Singh, R.; Gupta, P.K.; Gupta, P.K.; Anand, A. New biomarker for neovascular age-related macular degeneration: eotaxin-2. *DNA Cell Biol.*, **2012**, *31*(11),1618-27.

[34] Anand, A.; Sharma, N.K.; Singh, R.; Gupta, A.;, Prabhakar, S.; Jindal. N.;, Bhatt, A.K.;, Sharma, S.K.;, Gupta, P.K. Does DcR1 (TNF-related apoptosis-inducing-ligand Receptor 3) have any role in human AMD pathogenesis? *Sci. Rep.*, **2014**, *4*, 4114.

[35] Anand, A.; Sharma, N.K.; Gupta, A.; Prabhakar, S.; Sharma, S.K.; Singh, R.; Gupta, P.K. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. *PLoS One*, **2012**, *7*(11), e49905.

[36] Holliday, E.G.; Smith, A.V.; Cornes, B.K.; Buitendijk, GH.; Jensen, R.A.; Sim, X.; Aspelund, T.; Aung, T.; Baird, P.N.; Boerwinkle, E.; Cheng, C.Y.; van Duijn, C.M.; Eiriksdottir, G.; Gudnason, V.; Harris, T.; Hewitt, A.W.; Inouye, M.; Jonasson, F.; Klein, B.E.; Launer, L, Li, X.; Liew, G.; Lumley, T.; McElduff, P.; McKnight, B.; Mitchell, P.; Psaty, B.M.; Rochtchina, E.; Rotter, J.I.; Scott, R.; Tay, W.; Taylor, K.; Teo, Y.Y.; Uitterlinden, A.G.; Viswanathan, A.; Xie, S.; Wang, J.J. Insights into the Genetic Architecture of Early Stage Age-Related Macular Degeneration: A Genome-Wide Association Study Meta-Analysis. *PLoS One*, **2013**, *8*(1), e53830.

[37] Chen, W.; Stambolian, D.; Edwards, A.O.; Branham, K.E.; Othman, M.; Jakobsdottir, J.; Tosakulwong, N.; Pericak-Vance, M.A.; Campochiaro, P.A.; Klein, M.L.; Tan, P.L.; Conley, Y.P.; Kanda, A.; Kopplin, L.; Li, Y.; Zack, D.J.; Chew, E.Y.; Heckenlively, J.R.; Yoshimura, N.; Iyengar, S.K.; Francis, P.J.;, Katsanis, N.; Seddon, J.M.; Haines, J.L.; Gorin, M.B.; Abecasis, G.R.; Swaroop, A. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc. Natl. Acad. Sci. USA*, **2010**, *107*(16), 7401-6.

[38] Tian, J.; Yu, W.; Qin, X.. Association of genetic polymorphisms and age-related macular degeneration in Chinese population. *Invest. Ophthalmol. Vis. Sci.*, **2012**, *53*, 4262-4269.

[39] Jarrett, S.G.; Lin, H.; Godley, B.F.; Boulton, M.E. Mitochondrial DNA damage and its potential role in retinal degeneration. *Prog. Retin. Eye Res.* **2008**, *27*, 596–607.

[40] Gredilla, R.; Bohr, V.A.; Stevnsner, T. Mitochondrial DNA repair and association with aging–an update. *Exp. Gerontol.*, **2010**, *45*, 478–488.

[41] Barron, M.J.; Johnson, M.A.; Andrews, R.M.; Clarke, M.P.; Griffiths, P.G.; Bristow, E.; He, L.P.; Durham, S.;, Turnbull, D.M. Mitochondrial abnormalities in ageing macular photoreceptors. *Invest. Ophthalmol. Vis. Sci.*, **2001**, *42*, 3016–3022.

[42] Kenney, M.C.; Atilano, S.R.; Boyer, D.; Chwa, M.; Chak, G.; Chinichian, S.; Coskun, P.; Wallace, D.C.; Nesburn, A.B.; Udar, N.S. Characterization of retinal and blood mitochondrial DNA from age-related macular degeneration patients. *Invest. Ophthalmol. Vis. Sci.*, **2010**, *51*, 4289–4297.

[43] Jones, M.M.; Manwaring, N.; Wang, J.J.; Rochtchina, E.; Mitchell, P.; Sue, C.M. Mitochondrial DNA haplogroups and age-related maculopathy. *Arch. Ophthalmol.*, **2007**, *125*, 1235–1240.

[44] Canter, J.A.; Olson, L.M.; Spencer, K.; Schnetz-Boutaud, N. Mitochondrial DNA polymorphism A4917G is independently associated with age-related macular degeneration. *PLoS One*, **2008**, *3*, e2091.

[45] SanGiovanni, J.P,; Arking, D.E.; Iyengar, S.K. Mitochondrial DNA variants of respiratory complex I that uniquely characterize haplogroup T2 are associated with increased risk of age-related macular degeneration. *PLoS One*, **2009**, *4*, e5508.

[46] Bollati, V.; Schwartz, J.; Wright, R. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech. Ageing Dev.*, **2009**, *130*, 234–239.

[47] Eckmiller, M.S. Defective cone photoreceptor cytoskeleton, alignment, feedback, and energetics can lead to energy depletion in macular degeneration. *Prog. Retin. Eye Res.*, **2004**, *23*(5), 495 522.

[48] Marchenko, G.N.; Strongin, A.Y. MMP-28, a new human matrixmetalloproteinase with an unusual cysteine-switch sequence is widely expressed in tumors. *Gene*, **2001**, *265(1–2*), 87–93.

[49] Primakoff, P.; Myles, D.G. The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet.*, **2000**, *16*(2), 83–87.

[50] Wight, T.N. The ADAMTS Proteases, Extracellular Matrix, and Vascular Disease. *Arterioscler. Thromb. Vasc. Biol.*, **2005**, *25*, 12-14.

[51] Du, W.; Wang, S.; Zhou, Q. ADAM TS9 is a functional tumor suppressor through inhibiting AKT/mTOR pathway and associated with poor survival in gastric cancer. *Oncogene*, **2013**, *32*, 3319–3328.

[52] Jomary, C.; Cullen, J.; Jones, S.E. Inactivation of the Akt Survival Pathway during Photoreceptor Apoptosis in the Retinal Degeneration Mouse. *IOVS*, **2006**, *47*, 41620-1629.

[53] Zhao, C.; Yasumura, D.; Li, X. mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. *J. Clin. Invest.*, **2011**, *121*(1), 369–383.

[54] Bornstein, P. Thrombospondins function as regulators of angiogenesis. *JCCS*, **2009**, *3*(3-4), 189-200.

[55] Dickersin, K.; Min, Y.I.; Meinert, C.L. Factors influencing publication of research results. Follow-up of applications submitted to two institutional review boards. *JAMA*, **1992**, *267*, 374–8.

[56] Easterbrook, P.J.; Berlin, J.A.; Gopalan, R.; Matthews, D.R. Publication bias in clinical research. *Lancet*, **1991**, *337*, 867–72.

[57] Begg, C.B.; Berlin, J.A. Publication bias: a problem in interpreting medical data. *J. R. Stat. SOC A.*, **1988**, *151*, 419-63.

[58] Naj, A.C.; Scott, W.K.; Courtenay, M.D.; Cade, W.H. Genetic factors in nonsmokers with age-related macular degeneration revealed through genome-wide gene-environment interaction analysis. *Ann. Hum. Genet.*, **2013**, *77*(3), 215-31.

[59] Seddon, J.M.; Yu, Y.; Miller, E.C.; Reynolds, R.; Tan, P.L.; Gowrisankar, S.; Goldstein, J.I.; Triebwasser, M.; Anderson, H.E.; Zerbib, J.; Kavanagh, D.; Souied, E.; Katsanis, N.; Daly, M.J.; Atkinson, J.P.; Raychaudhuri, S. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat. Genet.*, **2013**, *45*(11), 1366-70.

[60] Fritsche, L.G.; Chen, W.; Schu, M.; Yaspan, B.L.; Yu, Y.; Thorleifsson, G.; Zack, D.J.; Arakawa, S.; Cipriani, V.; Ripke, S.; Igo, R.P.; Jr, Buitendijk G.H.; Sim X, Weeks, D.; Guymer, R.H.; Merriam, J.E.; Francis, P.J.; Hannum, G.; Farrer, L.A.; Heid, I.M.; Abecasis, R. Seven new loci associated with age-related macular degeneration. *Nat. Genet.*, **2013**, *45*(4), 433-9.

[61] Holliday, E.G.; Smith, A.V.; Cornes, B.K. Insights intothe genetic architecture of early stage agerelated macular degeneration: a genome wide association study meta-analysis. *PLoS One*, **2013**, *8*(1), e53830.

[62] Sobrin, L.; Ripke, S.; Yu, Y. Heritability and genomewide association study to assess genetic differences between advanced age related macular degeneration subtypes. *Ophthalmology*, **2012**, *119*(9), 1874-85.

[63] Cipriani, V.; Leung, H.; Plagnol. V.; Bunce C. Genome-wide association study of age-related macular degeneration identifies associated variants in the TNXB–FKBPL–NOTCH4 region of chromosome 6p21.3. *Hum. Mol. Genet.*, **2012**, *21*(18), 4138-50.

[64] Arakawa, S.; Takahashi, A.; Ashikawa, K. Genome-wide association study identifies two susceptibility loci for exudative age-related macular degeneration in the Japanese population. *Nat. Genet.*, **2011**, *43*, 1001–1004.

[65] Yu, Y.; Bhangale, T.R.; Fagerness, J. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. *Hum. Mol. Genet.*, **2011**, *20*(18), 3699-09.

[66] Ryu, E.; Fridley, B.L.; Tosakulwong, N.; Bailey, K.R.; Edwards, A.O. Genome-wide association analyses of genetic, phenotypic, and environmental risks in the age-related eye disease study. *Mol. Vis.*, **2010**, *16*, 2811-21.

[67] Zerhouni, E. The NIH roadmap. *Science*, **2003**, *72*(302), 63-64.

[68] AAMC: Task Force II on Clinical Research. Promoting transla-

tional and clinical science: the critical role of medical schools and teaching hospitals. Washington, DC. Association of American Medical Colleges, **2006.**

[69] Murillo, H.; Reece, E.A.; Snyderman, R.; Sung, N.S. Meeting the challenges facing clinical research: solutions proposed by leaders of medical specialty and clinical research societies. *Acad. Med.*, **2006**, *81*(2), 107-112.

[70] Institute of Medicine: Crossing the Quality Chasm a New Health System for the 21st Century. Washington DC: National Academy Press, **2001**.

[71] Tunis, S.R.; Stryer, D.B.; Clancy, C.M. Pratical clinical trials: increasing the value of clinical research for decision making in clinical and health policy. *JAMA*, **2003**, *290*, 1624-1632.

[72] Macpherson, H. Pragmatic clinical trials. *Complement Ther. Med.*, **2004**, *12*(2-3), 136-140.

[73] Thomas, D.C.; Conti, D.V. Commentary: the concept of 'Mendelian Randomization. *Int. J. Epidemiol.*, **2004**, *33*, 21–25

[74] Gaudreault, J.; Fei, D.; Rusit, J.; Suboc, P.; Shiu, V. Preclinical Pharmacokinetics of Ranibizumab (rhuFabV2) after a Single Intravitreal Administration. *Invest. Ophthalmol. Vis. Sci.*, **2005**, *46*(2), 726-733.

[75] Gaudreault, J.; Fei, D.; Beyer, J.C. Pharmacokinetics and retinal distribution of ranibizumab, a humanized antibody fragment directed against VEGF-A, following intravitreal administration in rabbits. *Retina*, **2007**, *27*(9), 1260-6.

[76] Thaler, S.; Fiedorowicz, M.; Choragiewicz, T.J. Toxicity testing of the VEGF inhibitors bevacizumab, ranibizumab and pegaptanib in rats both with and without prior retinal ganglion cell damage. *Acta Ophthalmol.*, **2010**, *88*(5), e170-6.

[77] Adamo, J.E.; Bauer, G.; Berro, M. A Roadmap for Academic Health Centers to Establish Good Laboratory Practice-Compliant Infrastructure. *Acad. Med.*, **2012**, *87*(3), 279–284.

[78] Clarke, J.J.; Lawlor, T.E.; Madraymootoo, W.; Pant, K.; Young, R.R.; Wagner, V.O., Aardema, M.J. Summary of *in vitro* genetic toxicology assay results: expected and unexpected effects of recent study design modifications. *Environ. Mol. Mutagen*, **2012**, *53*(8), 631-5.

[79] Age-Related Eye Disease Study Research Group. A randomized, placebo controlled, clinical trial of highdose supplementation with vitamins C and E, beta-caroten, and zinc for agerelated macular degeneration and vision loss: AREDS report no. 8. *Arch. Ophthalmol.*, **2001**, *119*(10), 1417-36.

[80] Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of highdose supplementation with vitamins C and E and betacarotene for agerelated cataract and vision loss: AREDS report no. 9. *Arch. Ophthalmol.*, **2001**, *119*(10), 1439-52.

[81] Age-Related Eye Disease Study 2 Research Group. Lutein+zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye DiseaseStudy 2 (AREDS2) randomized clinical trial. *JAMA*, **2013**, *309*(19), 2005-15.

[82] Seddon, J.M.; Reynolds, R.; Maller, J.; Fagerness, J.A.; Daly, M.J.; Rosner, B. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. *Invest. Ophthalmol. Vis. Sci.*, **2009**, *50*(5), 2044-53.

[83] Klein, M.L.; Francis, P.J.; Ferris, F.L. 3rd.; Hamon, S.C.; Clemons, T.E. Risk assessment model for development of advanced age-related macular degeneration. *Arch. Ophthalmol.*, **2011**, *129*(12), 1543-50.

[84] Seddon, J.M.; Reynolds, R.; Yu, Y.; Rosner, B. Validation of a prediction algorithm for progression to advanced macular degeneration subtypes. *JAMA Ophthalmol.*, **2013**, *131*(4), 448-55.

[85] Hu, Y.; Li, L.; Ehm, M.G.; Bing, N.; Song, K.; Nelson, M.The benefits of using genetic information to design prevention trials. *Am. J. Hum. Genet.*, **2013**, *92*(4), 547-57.

[86] Güngör, K.; Hotez, P.J.; Ozdemir, V.; Aynacıoğlu, S. Glaucomics: A Call for Systems Diagnostics for 21(st) Century Ophthalmology and Personalized Visual Health. *OMICS*, **2014**, *18*(5), 275-9.

[87] Elrakaiby, M.; Dutilh, B.E.; Rizkallah, M.R.; Boleij, A.; Cole, J.N.; Aziz, R.K. Pharmacomicrobiomics:The Impact of Human Microbiome Variations on Systems Pharmacology and Personalized Therapeutics. *OMICS*, **2014** [in press].

[88] Ozdemir, V.; Endrenyi, L.; Aynacıoğlu, S. Bernard Lerer: recipient of the **2014** inaugural Werner Kalow Responsible Innovation Prize in Global Omics and Personalized Medicine (Pacific Rim Association for Clinical Pharmacogenetics). *OMICS*, **2014**, *18*(4), 211-21.

[89] Bowler, R.P.; Bahr, T.M.; Hughes, G.; Lutz, S.; Kim, Y.I.; Coldren, C.D.; Reisdorph, N.; Kechris, K.J. Integrative omics approach identifies interleukin-16 as a biomarker of emphysema. *OMICS*, **2013**, *17*(12), 619-26.

[90] Sabbadini, R.A. Sphingosine-1-phosphate antibodies as potential agents in the treatment of cancer and age-related macular degeneration. *Br. J. Pharmacol.*, **2011**, *162*(6), 1225-38.

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Association between CFH Y402H Polymorphism and Age Related Macular Degeneration in North Indian Cohort

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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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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

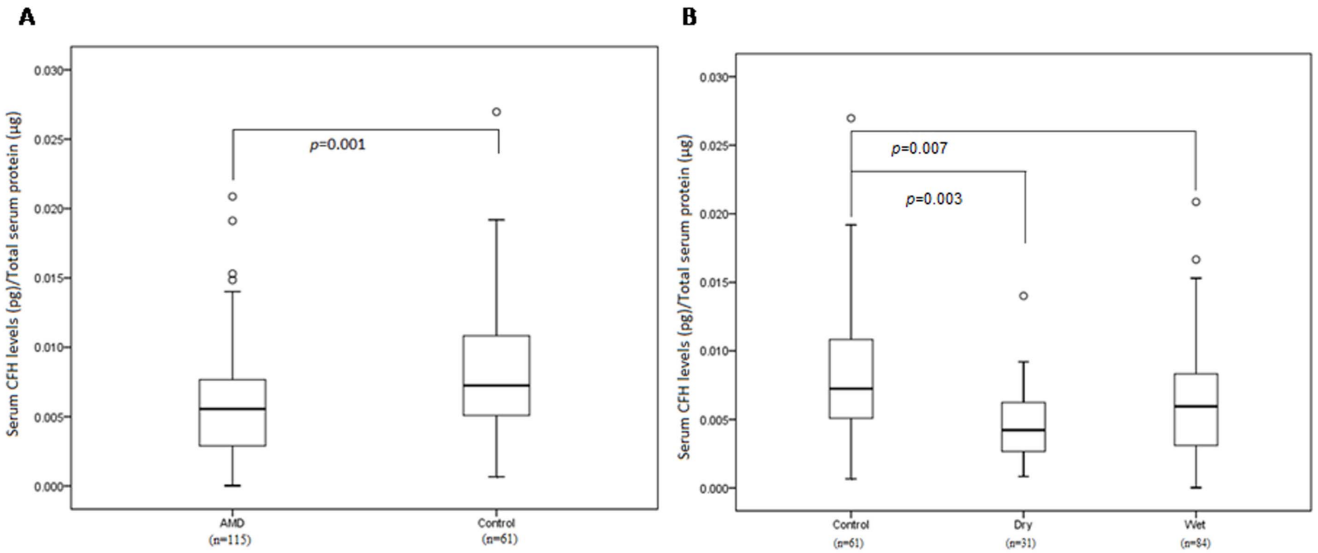

Figure 1. A) Serum levels of CFH in AMD and normal controls. B) Serum levels of CFH in Controls, Dry and Wet AMD. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Outliers and extreme values are shown in circles and asterisk respectively. Levels of CFH were 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-

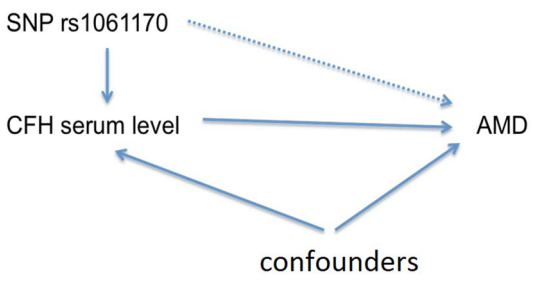

Figure 2. Mendelian randomization approach.
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Table 2. Genotype and allele frequency of CFH rs1061170 by Logistic Regression analysis.

			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.

References

1. Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, et al. (2004) Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 122: 564–72.
2. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, et al. (2013) CC chemokine receptor-3 as new target for age-related macular degeneration. Gene 523: 106–111.
3. VanNewkirk MR, Nanjan MB, Wang JJ, Mitchell P, Taylor HR, et al. (2000) The prevalence of age-related maculopathy: the visual impairment project. Ophthalmology 107: 1593–1600.
4. Nirmalan PK, Katz J, Robin AL, Tielsch JM, Namperumalsamy P, et al. (2004) Prevalence of vitreoretinal disorders in a rural population of Southern India: The Aravind Comprehensive Eye Survey. Arch Ophthalmol 122: 581–586.
5. Zareparsi S, Branham KE, Li M, Shah S, Klein RJ, et al. (2005) Strong association of the Y402H variant in complement factor H at 1q32 with susceptibility to age-related macular degeneration. Am J Hum Genet 77: 149–53.
6. Sepp T, Khan JC, Thurlby DA, Shahid H, Clayton DG, et al. (2006) Complement factor H variant Y402H is a major risk determinant for geographic atrophy and choroidal neovascularization in smokers and nonsmokers. Invest Ophthalmol Vis Sci 47: 536–40.
7. Goverdhan SV, Hannan S, Newsom RB, Luff AJ, Griffiths H, et al. (2008). An analysis of the CFH Y402H genotype in AMD patients and controls from the UK, and response to PDT treatment. Eye 22: 849–854.
8. Kaur I, Hussain A, Hussain N, Das T, Pathangay A, et al. (2006) Analysis of CFH, TLR4, and APOE Polymorphism in India Suggests the Tyr402His Variant of CFH to be a Global Marker for Age-Related Macular Degeneration. IOVS 47: 3729–3735.
9. Mandal MN, Ayyagari R (2006) Complement factor H: spatial and temporal expression and localization in the eye. Invest Ophthalmol Vis Sci 47: 4091–4097.
10. Skerka C, Lauer N, Weinberger AA, Keilhauer CN, Suhnel J, et al. (2007) Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol Immunol 44: 3398–3406.
11. Hageman GS, Hancox LS, Taiber AJ, Gehrs KM, Anderson DH, et al. (2006) Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Ann Med 38(8): 592–604.
12. Nozaki M, Raisler BJ, Sakurai E, Sarma JV, Barnum SR, et al. (2006) Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci USA 103: 2328–2333.
13. Heinen S, Hartmann A, Lauer A, Wiehl U, Dahse HM, et al. (2009) Factor H–related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. Blood 114: 2439–2447.
14. Abdelsalam A, Del Priore L, Zarbin MA (1999) Drusen in age-related macular degeneration: pathogenesis, natural course, and laser photocoagulation-induced regression. Surv Ophthalmol 44: 1–29.
15. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma S, et al. (2012) Single Nucleotide Polymorphism and Serum Levels Of VEGFR2 are Associated With Age Related Macular Degeneration. Curr Neurovasc Res 9(4): 256–65.
16. Sharma NK, Prabhakar S, Gupta A, Singh R, Gupta PK, et al. (2012) New Biomarker for Neovascular Age Related Macular Degeneration: Eotaxin-2. DNA Cell Biol 31: 1618–1627.
17. Anand A, Sharma NK, Prabhakar S, Sharma S, Gupta A, et al. (2012) Single Nucleotide Polymorphisms in MCP-1 and its Receptor are associated with the risk of Age Related Macular Degeneration. PLoS ONE 7(11): e49905.
18. Palmer TM, Sterne JAC, Harbord RM, Lawlor DA, Sheehan NA, et al. (2011) Instrumental Variable Estimation of Causal Risk Ratios and Causal Odds Ratios in Mendelian Randomization Analyses. American Journal of Epidemiology 173: 1392–1403.
19. Smith GD, Timpson N, Ebrahim S (2008) Strengthening causal inference in cardiovascular epidemiology through Mendelian randomization. Annals of Medicine 40: 524–541.
20. Fisher SA, Rivera A, Fritsche LG, Babadjanova G, Petrov S, et al. (2007) Assessment of the contribution of CFH and chromosome 10q26 AMD susceptibility loci in a Russian population isolate. Br J Ophthalmol 91: 576–8.
21. Thakkinstian A, Han P, McEvoy M, Smith W, Hoh J, et al. (2006) Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. Hum Mol Genet 15: 2784–2790.
22. Simonelli F, Frisso G, Testa F, di Fiore R, Vitale DF, et al. (2006) Polymorphism p.402Y>H in the complement factor H protein is a risk factor for age related macular degeneration in an Italian population. Br J Ophthalmol 90: 1142–5.
23. Lau LI, Chen SJ, Cheng CY, Yen MY, Lee FL, et al. (2006) Association of the Y402H Polymorphism in Complement Factor H Gene and Neovascular Age-Related Macular Degeneration in Chinese Patients. Invest Ophthalmol Vis Sci 47: 3242–6.
24. Fuse N, Miyazawa A, Mengkegale M, Yoshida M, Wakusawa R, et al. (2006) Polymorphisms in Complement Factor H and Hemicentin-1 genes in a Japanese population with drytype age-related macular degeneration. Am J Ophthalmol 142: 1074–6.
25. Uka J, Tamura H, Kobayashi T, Yamane K, Kawakami H, et al. (2006) No association of complement factor H gene polymorphism and age-related macular degeneration in the Japanese population. Retina 26: 985–7.
26. Dhillon B, Wright AF, Tufail A, Pappworth I, Hayward C, et al. (2010) Complement factor h autoantibodies and age-related macular degeneration. Invest Ophthalmol Vis Sci 51(11): 5858–63.
27. Greenfield JR, Samaras K, Jenkins AB, Kelly PJ, Spector TD, et al. (2004) Do gene-environment interactions influence fasting plasma lipids? A study of twins. Eur J Clin Invest 34: 590–8.
28. Wener MH, Daum PR, Mcquillan GM (2000) The influence of age, sex, and race on the upper reference limit of serum C-reactive protein concentration. J Rheumatol 27: 2351–9.
29. Silva AS, Teixeira AG, Bavia L, Lin F, Velletri R, et al. (2012) Plasma levels of complement proteins from the alternative pathway in patients with age-related macular degeneration are independent of Complement Factor H Tyr402His polymorphism. Molecular Vision 18: 2288–2299.
30. Johnson PT, Betts KE, Radeke MJ, Hageman GS, Anderson DH, et al. (2006) Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. Proc Natl Acad Sci U S A 103: 17456–61.
31. Conley YP, Thalamuthu A, Jakobsdottir J, Weeks DE, Mah T, et al. (2005) Candidate gene analysis suggests a role for fatty acid biosynthesis and regulation of the complement system in the aetiology of age-related maculopathy. Hum Mol Genet 14: 1991–2002.
32. Baird PN, Islam FM, Richardson AJ, Cain M, Hunt N, et al. (2006) Analysis of the Y402H variant of the complement factor H gene in age-related macular degeneration. Invest Ophthalmol Vis Sci 47: 4194–8.
33. Scott WK, Schmidt S, Hauser MA, Gallins P, Schnetz-Boutaud N, et al. (2007) Independent effects of complement factor H Y402H polymorphism and cigarette smoking on risk of age-related macular Degeneration. Ophthalmology 114: 1151–6.
34. Postel EA, Agarwal A, Caldwell J, Gallins P, Toth C, et al. (2006) Complement factor H increases risk for atrophic age-related macular degeneration. Ophthalmology 113: 1504–7.
35. Shuler RK, Hauser MA, Caldwell J, Gallins P, Schmidt S, et al. (2007) Neovascular age-related macular degeneration and its association with LOC387715 and complement factor H polymorphism. Arch Ophthalmol 125: 63–7.

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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.

36. Despriet DD, Klaver CC, Witteman JC, Bergen AA, Kardys I, et al. (2006) Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. JAMA 296: 301–9.
37. Hu CC, Ho JD, Lin HC (2010) Neovascular Age-Related Macular Degeneration and the Risk of Stroke : A 5 Year Population-Based Follow-Up Study. Stroke 41: 613–617.
38. Brody BL, Gamst AC, Williams RA, Smith AR, Lau PW, et al. (2011) Depression, visual acuity, comorbidity, and disability associated with age-related macular degeneration. American Academy of Ophthalmology 108: 1893–1900.
39. Kornsteiner M, Singer I, Elmadfa I (2008) Very low n-3 long-chain polyunsaturated fatty acid status in Austrian vegetarians and vegans. Ann Nutr Metab 52: 37–47.
40. Chong EW, Kreis AJ, Wong TY, Simpson JA, Guymer RH (2008) Dietary omega-3 fatty acid and fish intake in the primary prevention of age-related macular degeneration: a systematic review and meta-analysis. Arch Ophthalmol 126: 826–33.
41. Seddon JM, George S, Rosner B (2006) Cigarette smoking, fish consumption, omega-3 fatty acid Intake, and associations with age-related macular degeneration. Arch Ophthalmol 124(7): 995.
42. SanGiovanni JP, Chew EY, Agron E, Clemons TE, Ferris FL, et al (2008) The Relationship of Dietary ω-3 long-chain polyunsaturated fatty acid Intake with incident age-related macular degeneration. Arch Ophthalmol 126(9): 1274–1279.
43. Hodge WG, Schachter HM, Barnes D, Pan Y, Lowcock EC, et al (2006) Efficacy of omega-3 fatty acids in preventing age-related macular degeneration: a systematic review. Ophthalmology 113(7): 1165–1172.
44. Chong EW, Kreis AJ, Wong TY, Simpson JA, Guymer RH (2008) Dietary omega-3 fatty acid and fish intake in the primary prevention of age-related macular degeneration: a systematic review and meta-analysis. Arch Ophthalmol 126(6): 826–833.

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Single Nucleotide Polymorphisms in MCP-1 and Its Receptor Are Associated with the Risk of Age Related Macular Degeneration

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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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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

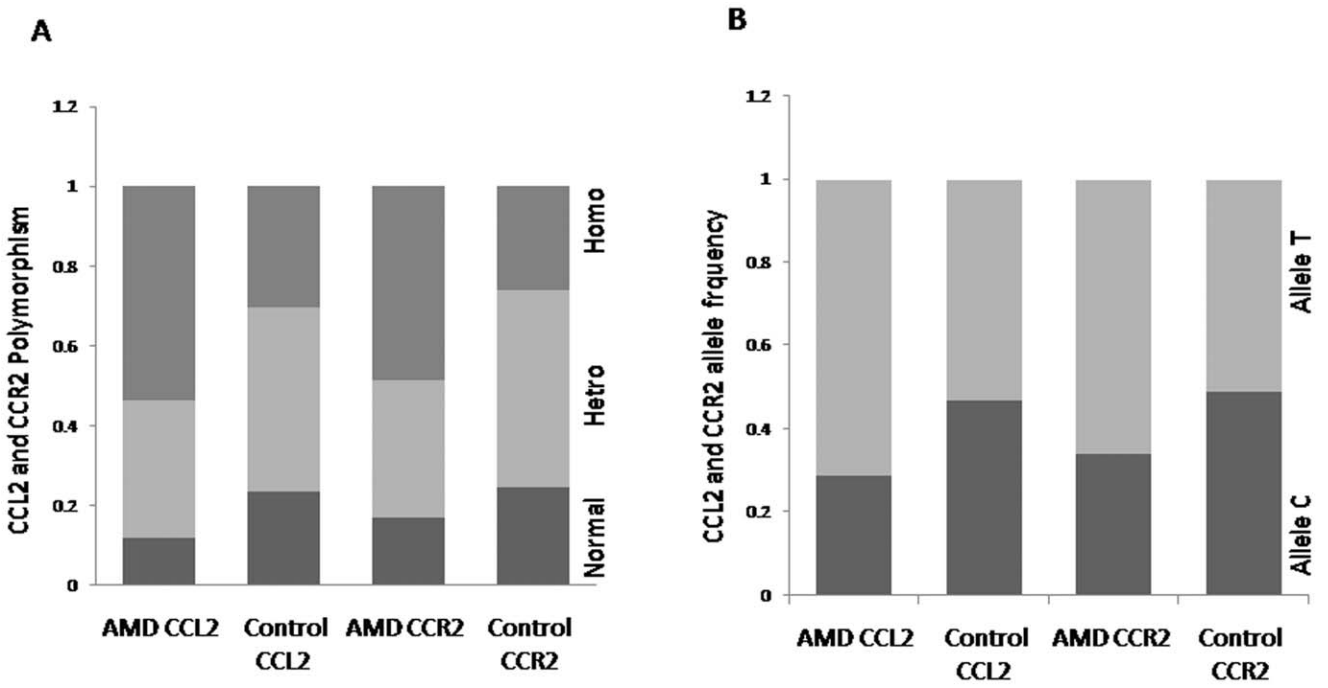

Figure 1. A) Genotype distribution (y-axis) of CCL2 and CCR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages. B) Allele frequency (y-axis) of CCL2 and CCR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages.
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to 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

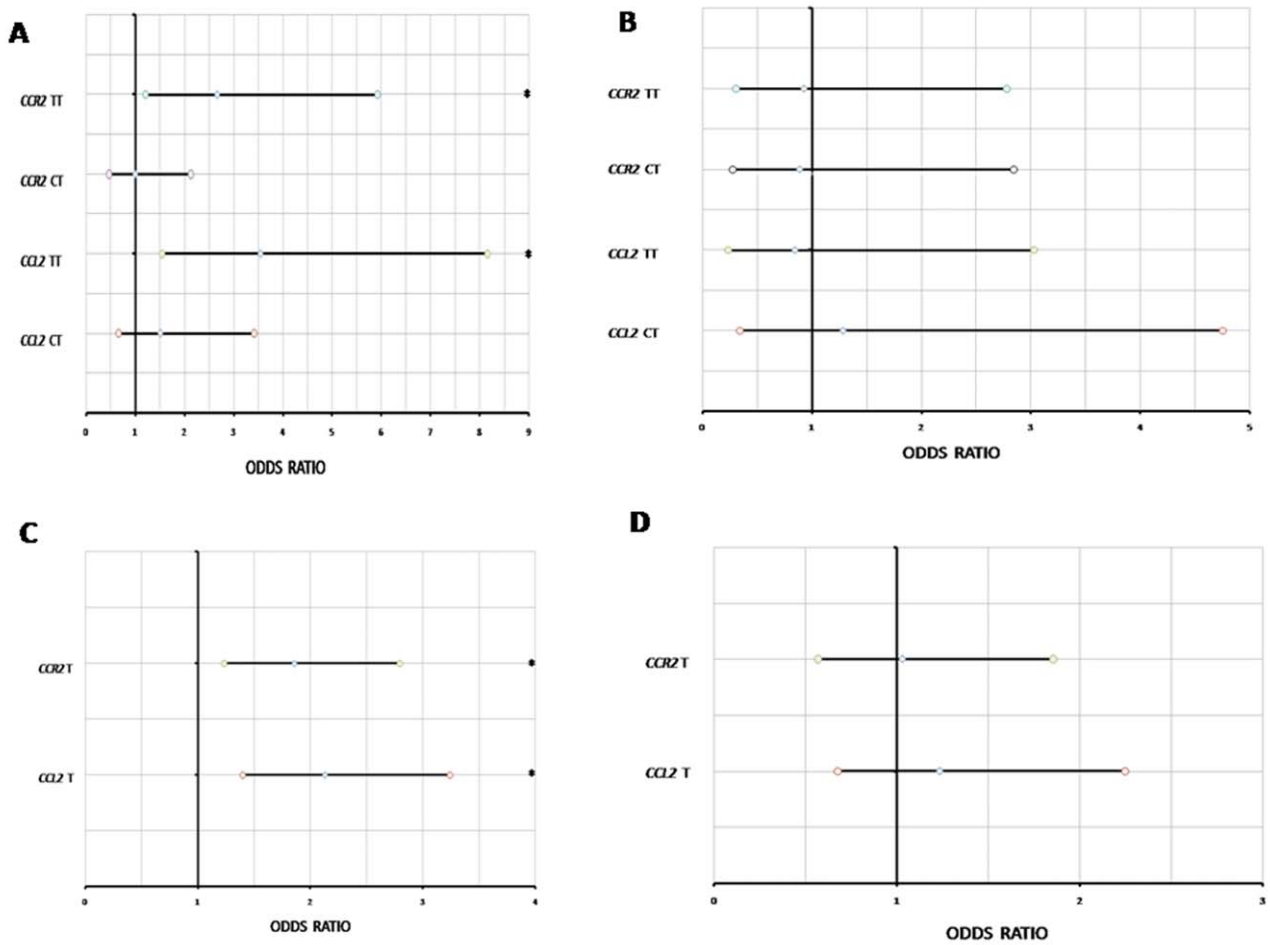

Figure 2. A) Univariate logistic regression analysis in AMD patients with CCL2 and CCR2 polymorphisms as independent and normal controls as a dependent variable. B) Univariate logistic regression analysis in Wet AMD patients with CCL2 and CCR2 polymorphisms as independent and Dry AMD as a dependent variable. 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.
doi:10.1371/journal.pone.0049905.t003

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

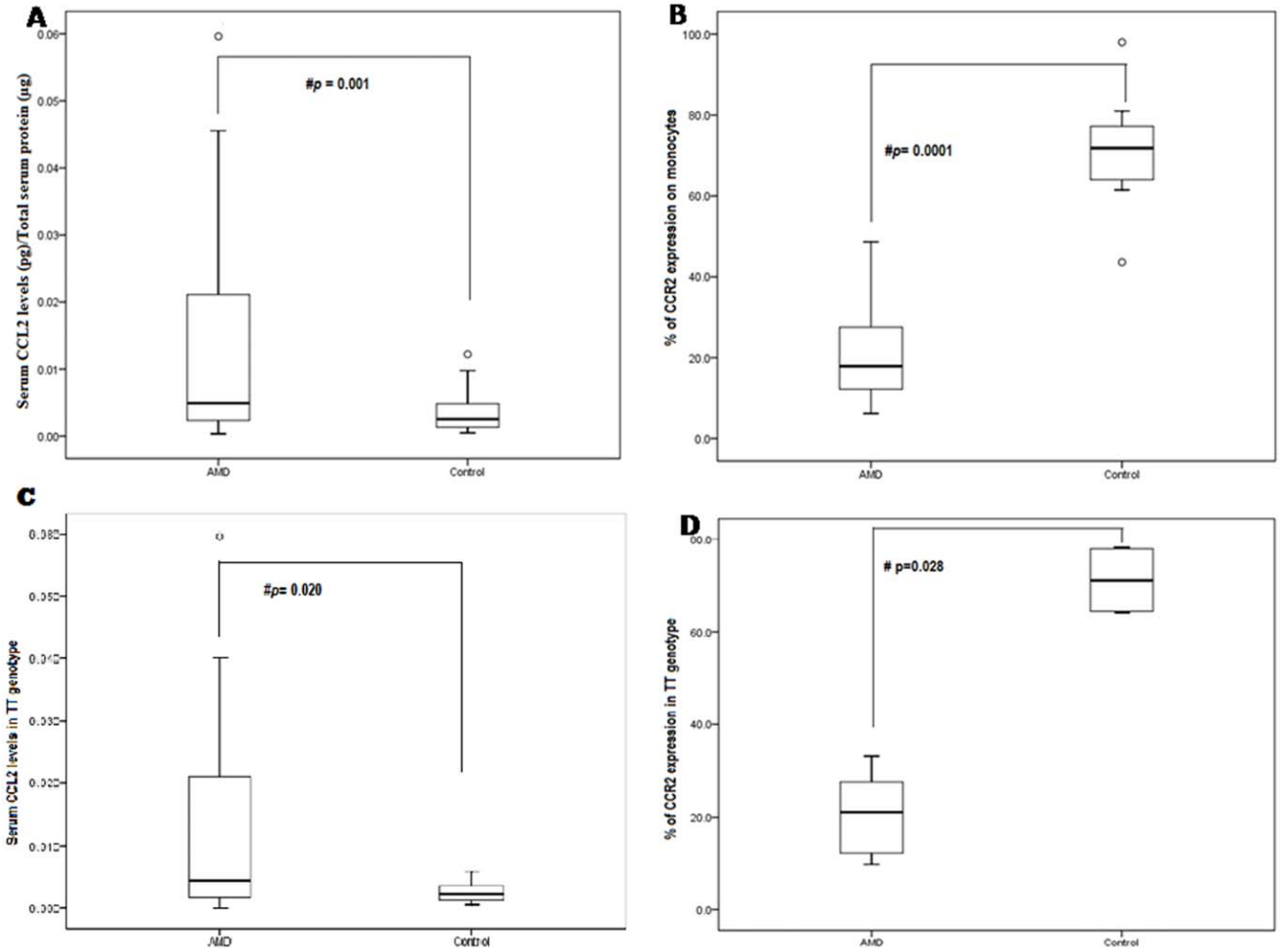

Figure 3. A) Serum levels of CCL2 in AMD and normal controls. B) Percentage (%) of PBMCs expressing CCR2 protein in AMD patients and Normal controls. C) Serum levels of CCL2 in TT genotype of AMD and normal controls. D) Percentage (%) of PBMCs expressing CCR2 protein in TT genotype of AMD patients and 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.
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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-

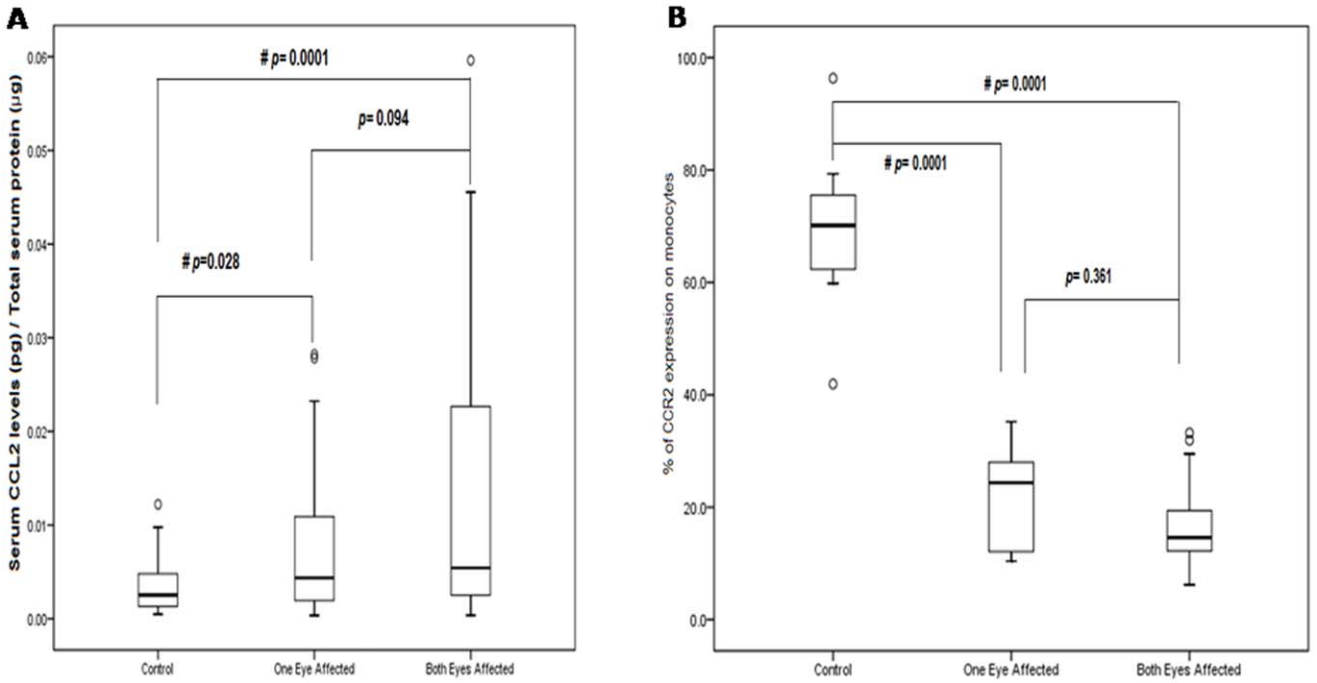

Figure 4. A) Serum levels of CCL2 in normal controls, AMD patients affected in one eye and AMD patients affected in both eyes. B) Percentage (%) of PBMCs expressing CCR2 protein in normal controls, AMD patients affected in one eye and AMD patients affected in both eyes. Boxes include values from first 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

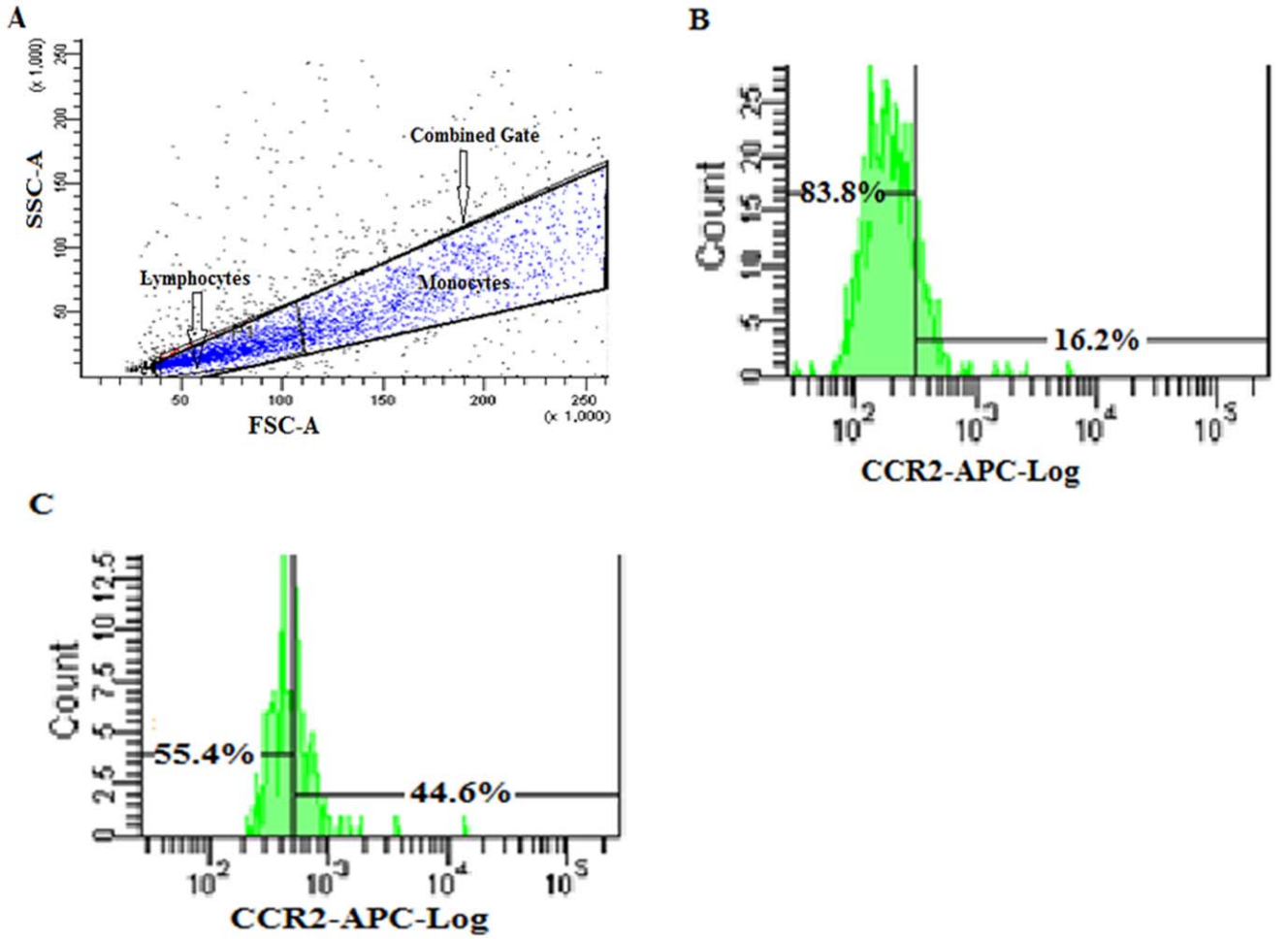

Figure 5. Percentage (%) of CCR2 + PBMCs in AMD patients and normal control subjects as measured by Flow Cytometry. (A) Dot plot showing side and forward scatter analysis of purified unlabeled PBMCs (large combined gate) from a AMD patient. PBMCs consists of two distinct populations namely lymphocytes 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)

Acknowledgments

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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.

References

1. Haas P, Steindl K, Aggermann T, Schmid-Kubista K, Krugluger W, et al. (2011) Serum VEGF and CFH in Exudative Age-Related Macular Degeneration. Current Eye Research 36(2): 143–148.
2. Simonelli F, Frisso G, Testa F, di Fiore R, Vitale DF, et al. (2006) Polymorphism p.402Y.H in the complement factor H protein is a risk factor for age related macular degeneration in an Italian population. Br J Ophthalmol 90: 1142–1145.
3. Ormsby RJ, Ranganathan S, Tong JC, Griggs KM, Dimasi DP, et al. (2008) Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. Invest Ophthalmol Vis Sci 49: 1763–1770.
4. Ambati J, Anand A, Fernandez S, Sakurai E, Lynn BC, et al. (2003) An animal model of age related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nat Med 9(11): 1390–1397.
5. Patel M, Chan CC (2008) Immunopathological aspects of age-related macular degeneration. Semin Immunopathol 30: 97–110.
6. Lommatzsch A, Hermans P, Muller KD, Bornfeld N, Bird AC, et al. (2008) Are low inflammatory reactions involved in exudative age-related macular degeneration? Morphological and immunhistochemical analysis of AMD associated with basal deposits. Graefes Arch Clin Exp Ophthalmol 246: 803–810.
7. Tuo J, Bojanowski CM, Zhou M, Shen D, Ross RJ, et al. (2007) Murine ccl2/cx3cr1 deficiency results in retinal lesions mimicking human age-related macular degeneration. Invest Ophthalmol Vis Sci 48: 3827–3836.
8. Luster AD (1998) Chemokines–chemotactic cytokines that mediate inflammation. N Engl J Med 338: 436–445.
9. Despriet DD, Bergen AA, Merriam JE, Zernant J, Barile GR, et al. (2008) Comprehensive Analysis of the Candidate Genes CCL2, CCR2, and TLR4 in Age-Related Macular Degeneration. Investigative Ophthalmology & Visual Science 49: 364–371.
10. Jonas JB, Tao Y, Neumaier M, Findeisen P (2010) Monocyte Chemoattractant Protein 1, Intercellular Adhesion Molecule 1, and Vascular Cell Adhesion Molecule 1 in Exudative Age-Related Macular Degeneration. Arch Ophthalmol. 128(10): 1281–1286.
11. Scholl HPN, Issa PC, Walier M, Janzer S, Pollok-Kopp B, et al. (2008) Systemic Complement Activation in Age-Related Macular Degeneration. PLoS ONE 3(7): e2593.
12. Kaur I, Hussain A, Hussain A, Das T, Pathangay A, et al. (2006) Analysis of CFH, TLR4, and APOE Polymorphism in India Suggests the Tyr402His Variant of CFH to be a Global Marker for Age-Related Macular Degeneration. Invest. Ophthalmol. Vis. Sci.47: 9 3729–3735.
13. Feng WX, Mokrousov I, Wang BB, Nelson H, Jiao WW, et al. (2011) Tag SNP Polymorphism of CCL2 and its Role in Clinical Tuberculosis in Han Chinese Pediatric Population. PLoS ONE 6(2): e14652.
14. Harmon BT, Orkunoglu-Suer EF, Adham K, Larkin JS, Dressman HG, et al. (2010) CCL2 and CCR2 variants are associated with skeletal muscle strength and change in strength with resistance training. J Appl Physiol 109: 1779–1785.
15. McDermott DH, Yang Q, Kathiresan S, Cupples LA, Massaro JM, et al. (2005) CCL2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the Framingham Heart Study. Circulation 112: 1113–1120.
16. Yamada K, Sakurai E, Itaya M, Yamasaki S, Ogura Y (2007) Inhibition of laser induced choroidal neovascularization by atorvastatin by downregulation of monocyte chemotactic protein-1 synthesis in mice. Invest Ophthalmol Vis Sci 48: 1839–1843.
17. Sharma NK, Prabhakar S, Anand A (2009) Age related macular degeneration – advances and trends. Annals of Neurosciences 2: 62–71.
18. Nakazawa T, Hisatomi T, Nakazawa C, Noda K, Maruyama K, et al. (2007) Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. Proc Natl Acad Sci USA 104: 2425–2430.
19. Higgins GT, Wang JH, Dockery P, Cleary PE, Redmond HP (2003) Induction of angiogenic cytokine expression in cultured RPE by ingestion of oxidized photoreceptor outer segments. Invest Ophthalmol Vis Sci 44: 1775–1782.

20. Ma W, Zhao L, Fontainhas AM, Fariss RN, Wong WT (2009) Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD. PLoS One 4: e7945.
21. Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, et al. (1994) Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. Proc Natl Acad Sci USA 91: 2752–2756.
22. Luhmann UF, Robbie S, Munro PM, Barker SE, Duran Y, et al. (2009) The drusenlike phenotype in aging Ccl2-knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages. Invest Ophthalmol Vis Sci 50: 5934–5943.
23. Jonas JB, Tao Y, Neumaier M, Findeisen P (2010) Monocyte chemoattractant protein 1, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 in exudative age-related macular degeneration. Arch Ophthalmol 128: 1281–1286.
24. Chen M, Forrester JV, Xu H (2011) Dysregulation in Retinal Para-Inflammation and Age Related Retinal Degeneration in CCL2 or CCR2 Deficient Mice. PLoS ONE 6(8): e22818.
25. Kim MP, Wahl LM, Yanek LR, Becker DM, Becker LC (2007) A monocyte chemoattractant protein-1 gene polymorphism is associated with occult ischemia in a high-risk asymptomatic population. Atherosclerosis 193: 366–372.
26. Jemaa R, Rojbani H, Kallel A, Ben Ali S, Feki M, et al. (2008) Association between the –2518G/A polymorphism in the monocyte chemoattractant protein-1 (MCP-1) gene and myocardial infarction in Tunisian patients. Clin Chim Acta 390: 22–125.
27. Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, et al. (2008) Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. Circulation 117: 1649–1657.
28. Charo IF, Taubman MB (2004) Chemokines in the pathogenesis of vascular disease. Circ Res 95(9): 858–866.
29. Tuo J, Bojanowski CM, Zhou1 M, Shen D, Ross1 RJ, et al. (2007) Murine Ccl2/Cx3cr1 Deficiency Results in Retinal Lesions Mimicking Human Age-Related Macular Degeneration. Invest Ophthalmol Vis Sci 48(8): 3827–3836.
30. Raoul W, Auvynet C, Camelo S, Guillonneau X, Feumi C, et al. (2010) CCL2/CCR2 and CX3CL1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration. Journal Of Neuroinflammation 7: 87.

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Single Nucleotide Polymorphism and Serum Levels of VEGFR2 are Associated With Age Related Macular Degeneration

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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

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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

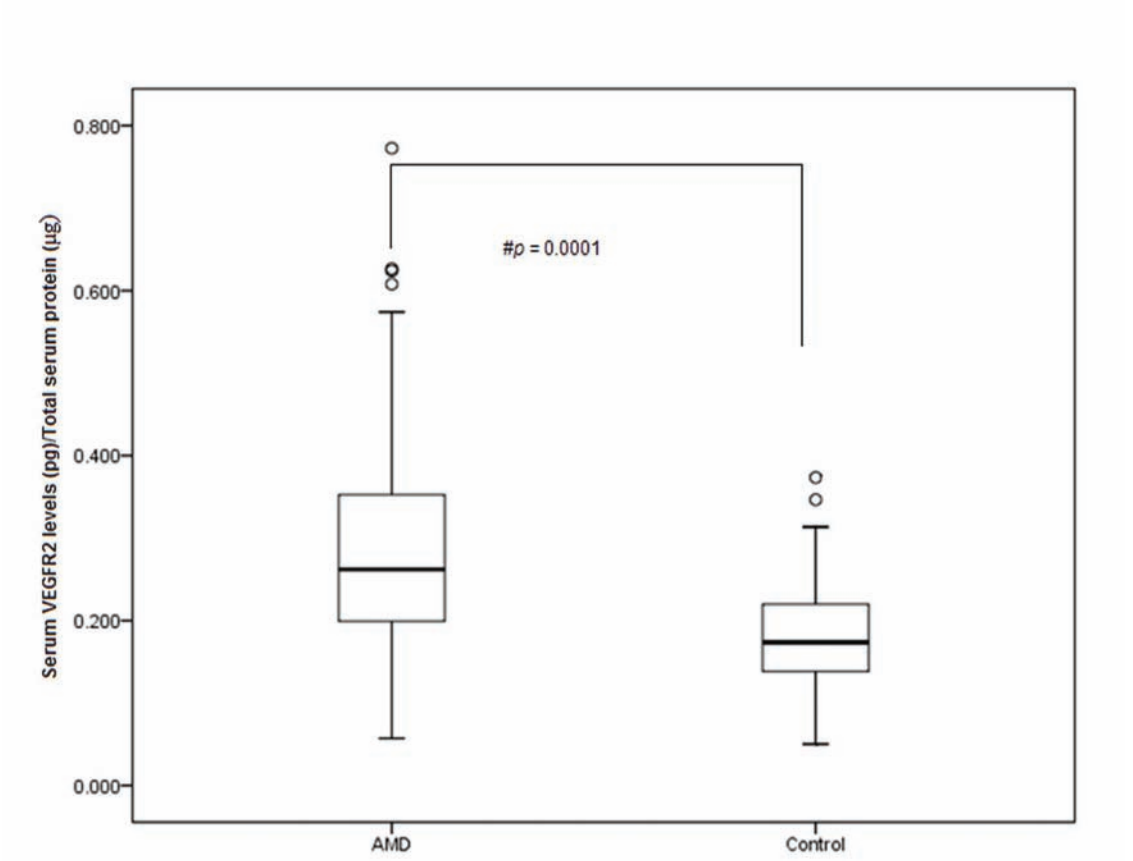

Fig. (1). Serum levels of VEGFR2 in AMD and normal controls. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk respectively. 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

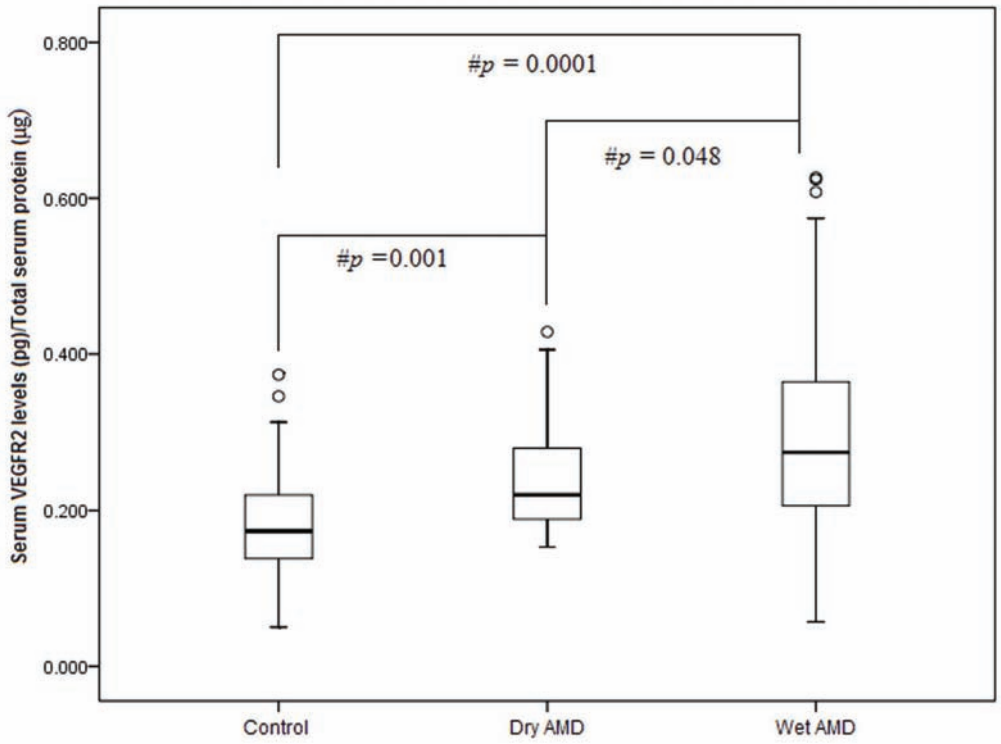

Fig. (2). Serum levels of VEGFR2 in normal controls, Dry AMD and Wet AMD. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk 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.

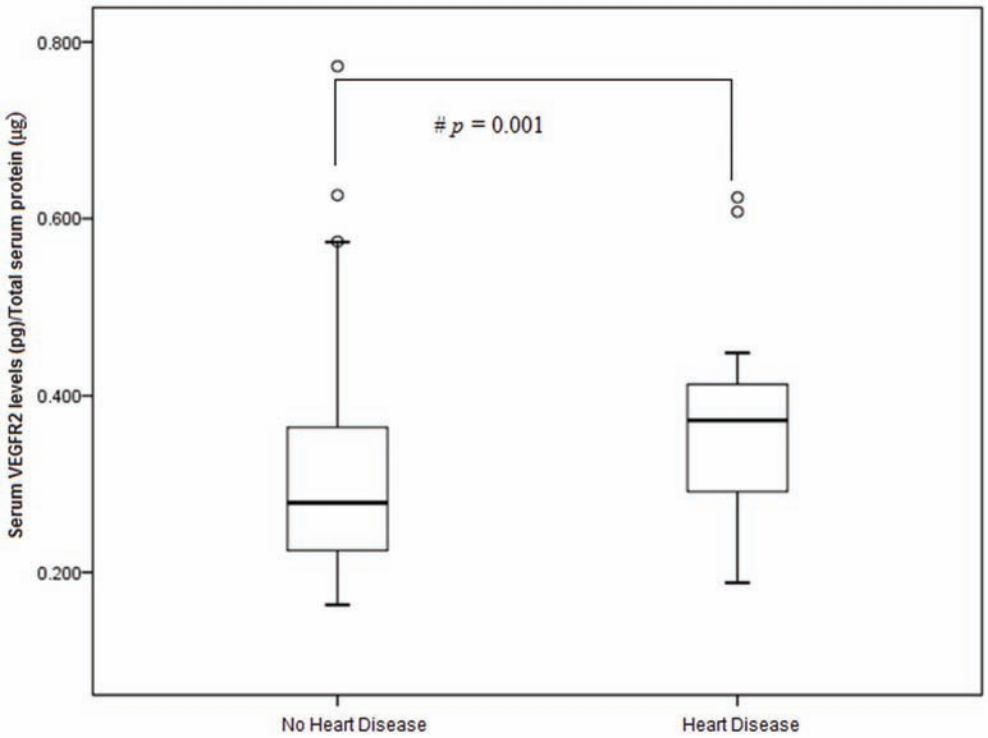

Fig. (3). Serum levels of VEGFR2 in AMD patients with heart disease and AMD patients without heart disease. Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values 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

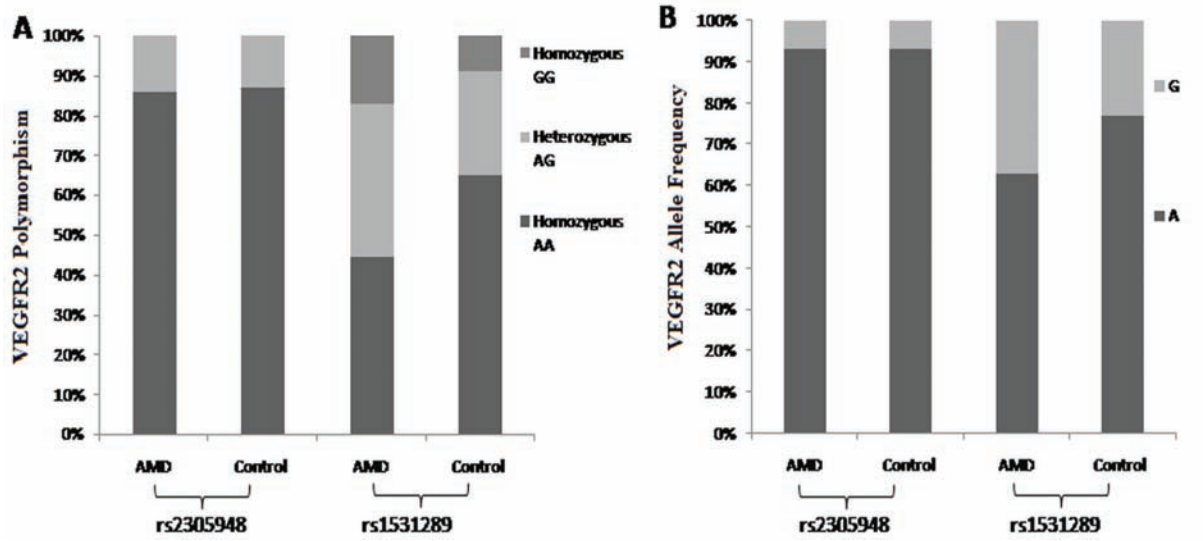

Fig. (4A). Genotype distribution (y-axis) of VEGFR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages (**B**) Allele frequency (y-axis) of VEGFR2 polymorphism in the AMD patients compared to the control group (x-axis) in percentages.

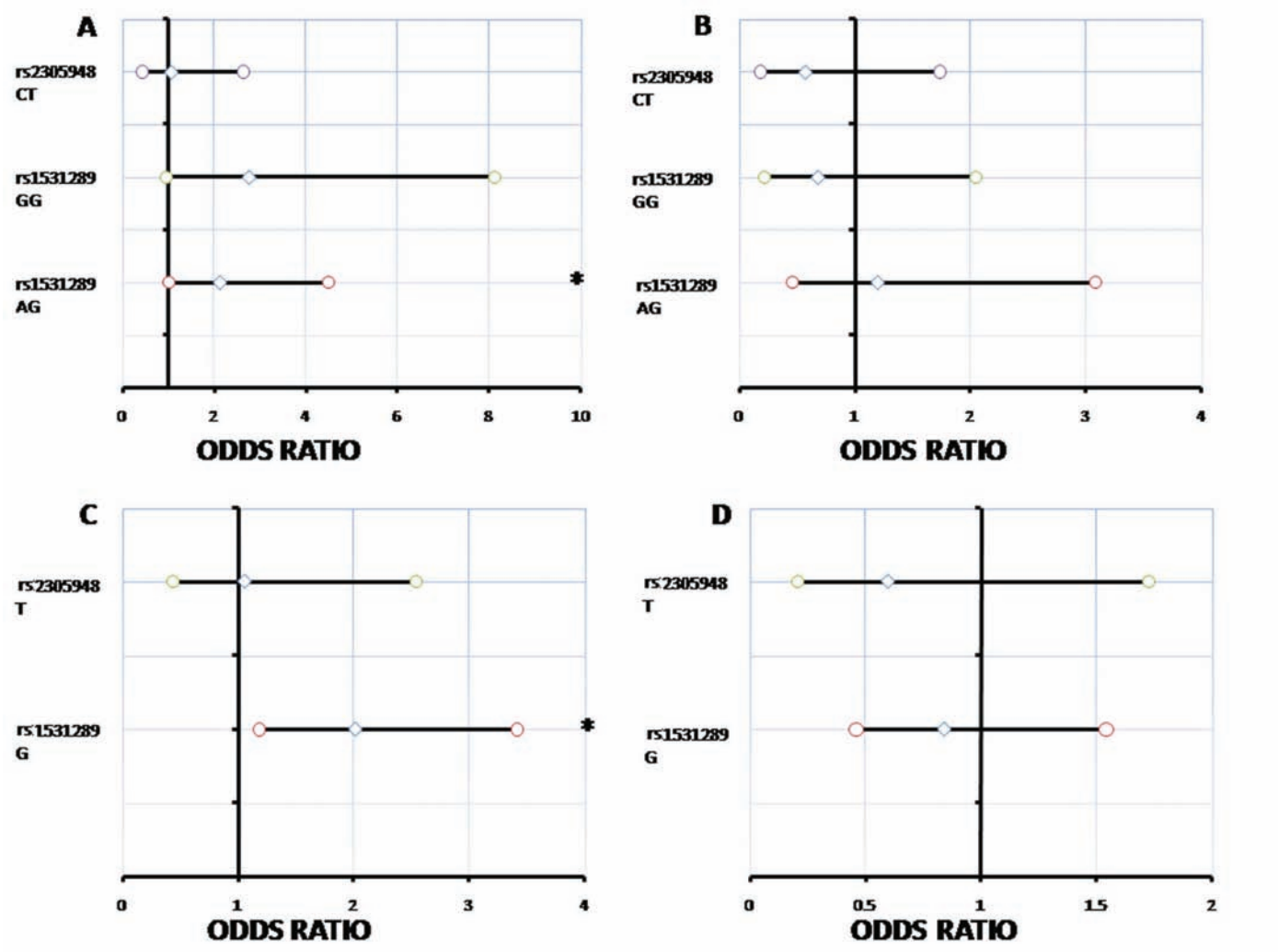

Fig. (5A). Univariate logistic regression analysis in AMD/Control as dependent variable and VEGFR2 polymorphism as independent variable. **B**) Univariate logistic regression analysis in Wet/Dry as dependent variable and VEGFR2 polymorphism as independent variable. **C**) Univariate logistic regression 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

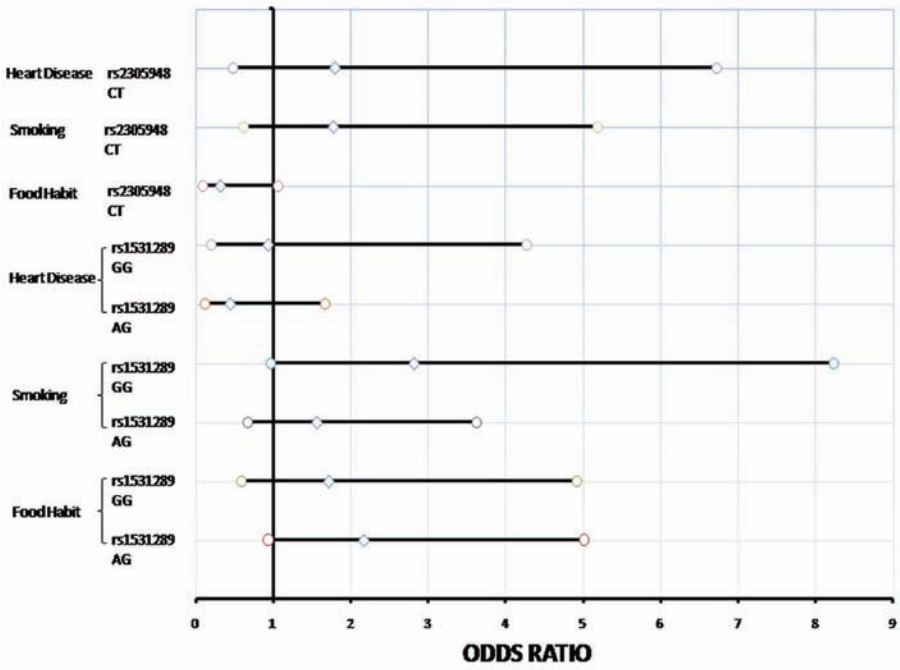

Fig. (6). Univariate logistic regression analysis in non-vegetarian, smokers and patients with comorbidity as dependent variable and VEGFR2 polymorphisms as independent variable.

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.

REFERENCES

[1] Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology. 1992; 99: 933-943.

[2] Seo MS, Kwak N, Ozaki H, *et al*. 1999.Dramatic inhibition of retinal and choroidal neovascularization by oral administration of a kinase inhibitor. Am. J. Pathol. 154,1743e1753.

[3] Chapman JA, Beckey C, Chapman. Pegaptanib: a novel approach to ocular neo- vascularization. Ann. Pharmacother. 2006; 40: 1322-1326.

[4] Edwards AO. Genetic testing for age-related macular degeneration. Ophthalmology 2006; 113: 509-510.

[5] Seddon JM, George S, Rosner B, Klein ML. CFH gene variant, Y402H, and smoking, body mass index, environmental associations with advanced age-related macular degeneration. Hum Hered. 2006; 61: 157-165.

[6] Haddad S, Chen CA, Santangelo SL, Seddon JM. The genetics of age-related macular degeneration: a review of progress to date. Surv Ophthalmol 2006; 51: 316-363.

[7] Carneiro AM, Costa R, Falcao MS, *et al.* Vascular endothelial growth factor plasma levels before and after treatment of neovascular age-related macular degeneration with bevacizumab or ranibizumab. Acta Ophthalmol. 2011; 29: 1755-3768.

[8] Philipp W, Speicher L, Humpel C. Expression of vascular endothelial growth factor and its receptors in inflamed and vascularized human corneas. Invest Ophthalmol Vis Sci. 2000; 41: 2514-2522.

[9] Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. ProcNatlAcadSci USA. 1998; 95: 9349-9354.

[10] Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 2000; 60: 203-12.

[11] Olsson AK, Dimberg A, Kreuger J and Claesson-Welsh L. VEGF receptor signalling — in control of vascular function. Molecular Cell Biology. 2006; 7: 359-371.

[12] Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. Prog Retin Eye Res. 2008; 27:372-90.

[13] Penn JS, Madan A, Caldwell RB, Bartoli M, Caldwell RW, Hartnett ME. Vascular endothelial growth factor in eye disease. Prog Retin Eye Res. 2008; 27:331-71.

[14] Boekhoorn SS, Isaacs A, Uitterlinden AG, *et al.* Polymorphisms in the vascular endothelial growth factor gene and risk of age-related macular degeneration: the Rotterdam Study. Ophthalmology. 2008; 115: 1899-903.

[15] Lin JM, Wan L, Tsai YY, et al. Vascular endothelial growth factor gene polymorphisms in age-related macular degeneration. Am J Ophthalmol. 2008; 145: 1045-51.

[16] Churchill AJ, Carter JG, Lovell HC, *et al*. VEGF polymorphisms are associated with neovascular age-related macular degeneration. Hum Mol Genet. 2006; 15: 2955-61.

[17] Fang AM, Lee AY, Kulkarni M, Osborn MP, Brantley MA. Polymorphisms in the VEGFA and VEGFR-2 genes and neovascular age-related macular degeneration. Molecular Vision 2009; 15: 2710-2719.

[18] Sharma NK, Prabhakar S, Anand A. Age related macular degeneration - advances and trends. Annals of Neurosciences. 2009; 2: 62-71.

[19] Ramrattan RS, Schaft TL, Mooy CM, de Bruyn CM, Mulder PGH, de Jong PTVM. Morphometric analysis of Bruch's membrane, the choriocapillaris and the choroid in aging. Invest Ophthalmol Vis Sci 1994; 35: 2857-64.

[20] Pabst S, Karpushova A, Dìaz-Lacava A, *et al.* VEGF gene haplotypes are associated with sarcoidosis. Chest 2010; 137: 156-163.

[21] Snow KK and Seddon JM. Do age-related macular degeneration and cardiovascular disease share common antecedents? Ophthalmic Epedemiology 1999; 6: 125-143.

[22] Carrillo de Santa Pau E, Arias FC, Caso Peláez E, *et al*. Prognostic significance of the expression of vascular endothelial growth factors A, B, C, and D and their receptors R1, R2, and R3 in patients with nonsmall cell lung cancer. Cancer. 2009; 115: 1701-12.

[23] Dougher M & Terman BI. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene. 1999; 18: 1619-1627.

[24] Rosenfeld PJ, Heier JS, Hantsbarger G, Shams N. Tolerabil-ity and efficacy of multiple escalating doses of ranibizumab [Lucentis] for neovascular age-related macular degeneration. Ophthalmology 2006; 113: 623-632.

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JOURNAL OF NEUROINFLAMMATION

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

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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

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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.

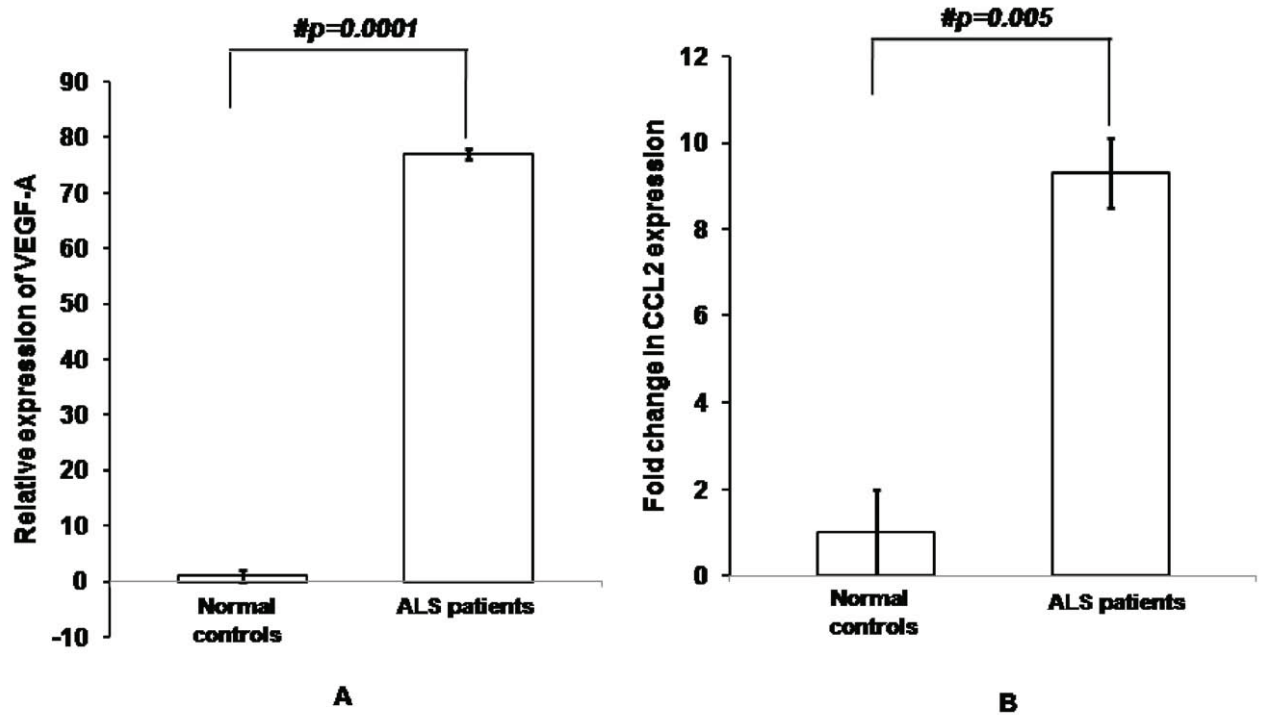

Figure 1 Relative mRNA expression of VEGF-A (A) and CCL2 (B) in PBMCs of ALS patients. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by unpaired, independent 2-tailed student *t* test. # indicates significant difference among the groups ($p < 0.05$). Expression 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

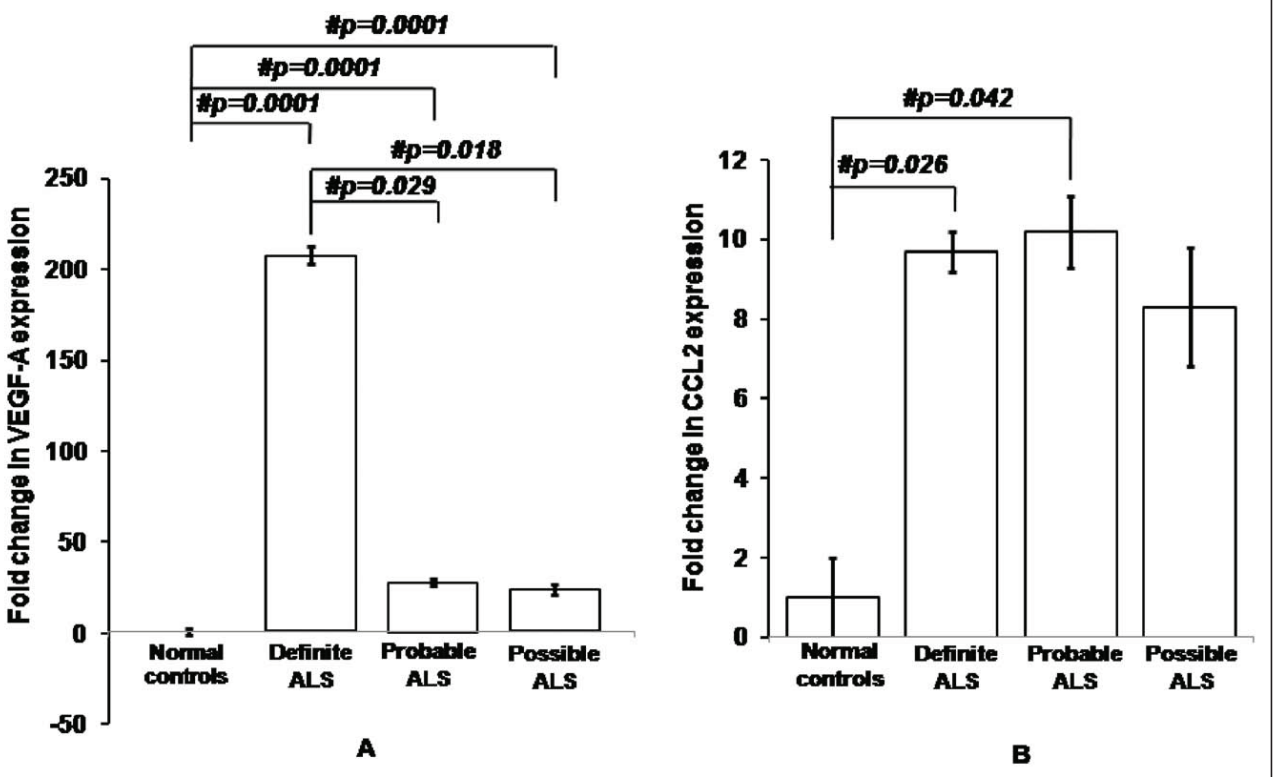

Figure 2 Relative mRNA expression of VEGF-A (A) and CCL2 (B) in PBMCs of definite, probable and possible ALS patients. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *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.

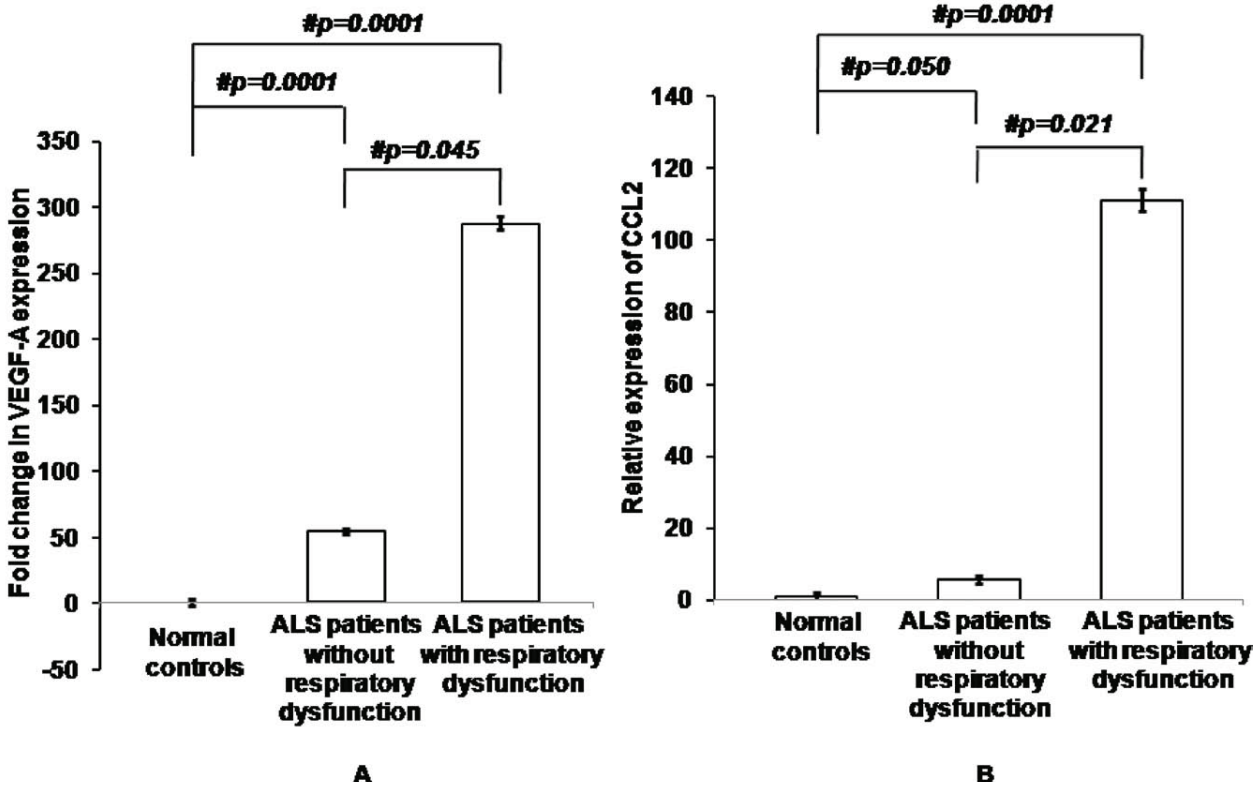

Figure 3 Fold change in expression of VEGF-A (A) and CCL2 (B) in PBMCs of ALS patients with respiratory dysfunction. Values are plotted as mean ± SE (Standard error) in the bar diagram. Data was analyzed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post 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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References

1. Carmeliet P: **Mechanisms of angiogenesis and arteriogenesis.** *Nat Med* 2000, **6**:389-395.
2. Storkebaum E, Lambrechts D, Carmeliet P: **VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection.** *Bioessays* 2004, **26**:943-954.
3. Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, Acker T, Vleminckx V, Van Den Bosch L, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D, Carmeliet P: **Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration.** *Nat Genet* 2001, **28**:131-138.
4. Hwang DH, Lee HJ, Park IH, Seok JI, Kim BG, Joo IS, Kim SU: **Intrathecal transplantation of human neural stem cells overexpressing VEGF provide behavioral improvement, disease onset delay and survival extension in transgenic ALS mice.** *Gene Ther* 2009, **10**:1234-1244.
5. Madrigal JL, Leza JC, Polak P, Kalinin S, Feinstein DL: **Astrocyte-derived MCP-1 mediates neuroprotective effects of noradrenaline.** *J Neurosci* 2009, **29**:263-267.
6. Nalini A, Thennarasu K, Gourie-Devi M, Shenoy S, Kulshreshtha D: **Clinical characteristics and survival pattern of 1,153 patients with amyotrophic lateral sclerosis: experience over 30 years from India.** *J Neurol Sci* 2008, **272**:60-70.
7. Bradley WG: **Commentary on Professor Stephen Hawking's disability advice.** *Annals of Neurosciences* 2009, **16**:101-102.
8. Sorenson EJ, Stalker AP, Kurland LT, Windebank AJ: **Amyotrophic lateral sclerosis in Olmsted County, Minnesota, 1925 to 1998.** *Neurology* 2002, **59**:280-282.
9. Gupta PK, Prabhakar S, Sharma S, Anand A: **Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in Amyotrophic Lateral Sclerosis (ALS) patients.** *Journal of Neuroinflammation* 2011, **8**:47.
10. Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B, Nakanishi A: **The ALSFRS-R: a revised ALS functional rating scale that incorporates assessmentsof respiratory function. BDNF ALS Study Group (Phase III).** *J Neurol Sci* 1999, **169**:13-21.
11. Prabhakar S, Vinish M, Das CP, Anand A: **Occurrence of PARK2 Mutations in a Never-Smoker Population with Parkinson's Disease in North India.** *Neuroepidemiology* 2010, **35**:152-159.
12. Meister B, Grünebach F, Bautz F, Brugger W, Fink FM, Kanz L, Möhle R: **Expression of vascular endothelial growth factor (VEGF) and its receptors in human neuroblastoma.** *Eur J Cancer* 1999, **35**:445-449.
13. Ebihara N, Yamagami S, Yokoo S, Amano S, Murakami A: **Involvement of C-C chemokine ligand 2-CCR2 interaction in monocyte-lineage cell recruitment of normal human corneal stroma.** *J Immunol* 2007, **178**:3288-3292.
14. Henkel JS, Engelhardt JI, Siklos L, Simpson EP, Kim SH, Pan T, Goodman JC, Siddique T, Beers DR, Appel SH: **Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue.** *Ann Neurol* 2004, **55**:221-235.
15. Lu L, Zheng L, Viera L, Suswam E, Li Y, Li X, Estévez AG, King PH: **Mutant Cu/Zn-superoxide dismutase associated with amyotrophic lateral sclerosis destabilizes vascular endothelial growth factor mRNA and downregulates its expression.** *J Neurosci* 2007, **30**:7929-38.
16. Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, Thijs V, Andersson J, van Marion I, Al-Chalabi A, Bornes S, Musson R, Hansen V, Beckman L, Adolfsson R, Pall HS, Prats H, Vermeire S, Rutgeerts P, Katayama S, Awata T, Leigh N, Lang-Lazdunski L, Dewerchin M, Shaw C, Moons L, Vlietinck R, Morrison KE, Robberecht W, Van Broeckhoven C, Collen D, Andersen PM, Carmeliet P: **VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death.** *Nat Genet* 2003, **34**:383-394.
17. Devos D, Moreau C, Lassalle P, Perez T, De Seze J, Brunaud-Danel V, Destée A, Tonnel AB, Just N: **Low levels of the vascular endothelial growth factor in CSF from early ALS patients.** *Neurology* 2004, **62**:2127-2129.
18. Nygren I, Larsson A, Johansson A, Askmark H: **VEGF-A is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis.** *Neuroreport* 2002, **13**:2199-2201.
19. Iłzecka J: **Cerebrospinal fluid vascular endothelial growth factor in patients with amyotrophic lateral sclerosis.** *Clin Neurol Neurosurg* 2004, **106**:289-93.
20. Cronin S, Greenway MJ, Ennis S, Kieran D, Green A, Prehn JH, Hardiman O: **Elevated serum angiogenin levels in ALS.** *Neurology* 2006, **67**:1833-1836.
21. Nagata T, Nagano I, Shiote M, Narai H, Murakami T, Hayashi T, Shoji M, Abe K: **Elevation of MCP-1 and MCP-1/VEGF ratio in cerebrospinal fluid of amyotrophic lateral sclerosis patients.** *Neurol Res* 2007, **29**:772-776.
22. Manzano R, Toivonen JM, Oliván S, Calvo AC, Moreno-Igoa M, Muñoz MJ, Zaragoza P, García-Redondo A, Osta R: **Altered Expression of Myogenic Regulatory Factors in the Mouse Model of Amyotrophic Lateral Sclerosis.** *Neurodegener Dis* 2011, **8**:386-396.
23. Bogaert E, Van Damme P, Poesen K, Dhondt J, Hersmus N, Kiraly D, Scheveneels W, Robberecht W, Van Den Bosch L: **VEGF protects motor neurons against excitotoxicity by upregulation of GluR2.** *Neurobiol Aging* 2010, **31**:2185-2191.
24. Meng H, Zhang Z, Zhang R, Liu X, Wang L, Robin AM, Chopp M: **Biphasic effects of exogenous VEGF on VEGF expression of adult neural progenitors.** *Neurosci Lett* 2006, **393**:97-101.
25. Liu XS, Zhang ZG, Zhang RL, Gregg SR, Wang L, Yier T, Chopp M: **Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke.** *J Neurosci Res* 2007, **85**:2120-2125.
26. Zhao W, Xie W, Le W, Beers DR, He Y, Henkel JS, Simpson EP, Yen AA, Xiao Q, Appel SH: **Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism.** *J Neuropathol Exp Neurol* 2004, **63**:964-977.
27. Bartholdi D, Rubin BP, Schwab ME: **VEGF mRNA induction correlates with changes in the vascular architecture upon spinal cord damage in the rat.** *Eur J Neurosci* 1997, **9**:2549-2560.
28. Rosenstein JM, Krum JM: **New roles for VEGF in nervous tissue–beyond blood vessels.** *Exp Neurol* 2004, **187**:246-253.
29. Zheng C, Sköld MK, Li J, Nennesmo I, Fadeel B, Henter JI: **VEGF reduces astrogliosis and preserves neuromuscular junctions in ALS transgenic mice.** *Biochem Biophys Res Commun* 2007, **363**:989-993.
30. Cui LY, Liu MS, Tang XF: **Single fiber electromyography in 78 patients with amyotrophic lateral sclerosis.** *Chin Med J (Engl)* 2004, **117**:1830-1833.

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Clinical Parameters and sociodemographic factors

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ORIGINAL RESEARCH

Genotyping of Clinical Parameters in Age-Related Macular Degeneration

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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	

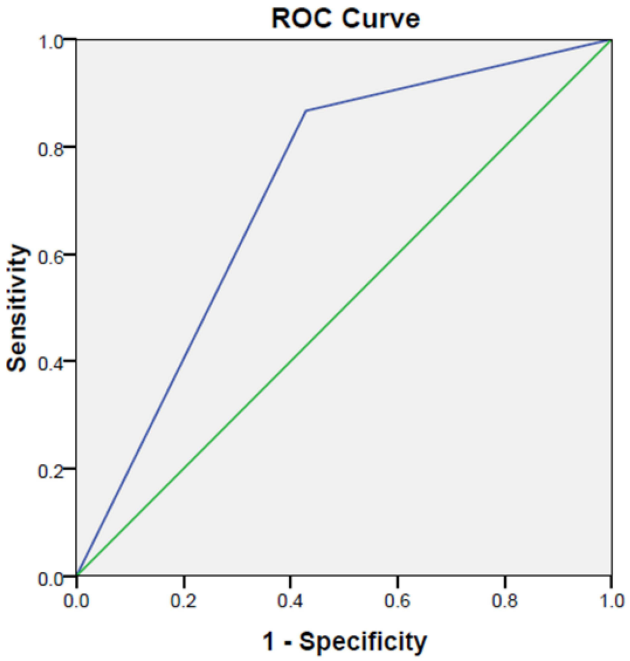
Abbreviations: AMD, age-related macular degeneration; SRF, subretinal fluid; IRF, intraretinal fluid; RPED, retinal pigment epithelium detachment; RPE Irr, retinal pigment epithelium irregularity.

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.

References

1. Randolph SA. Age-related macular degeneration. *Workplace Health Saf*. 2014;62(8):352. doi:10.1177/216507991406200807
2. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health*. 2014;2(2):e106–e116. doi:10.1016/S2214-109X(13)70145-1
3. Zając-Pytrus HM, Pilecka A, Turno-Kręcicka A, et al. The dry form of Age-Related Macular Degeneration (AMD): the current concepts of pathogenesis and prospects for treatment. *Adv Clin Exp Med*. 2015;24(6):1099–1104. doi:10.17219/acem/27093
4. Baumal CR. Clinical applications of optical coherence tomography. *Curr Opin Ophthalmol*. 1999;10(3):182–188. doi:10.1097/00055735-199906000-00006
5. Voo I, Mavrofrides EC, Puliafito CA. Clinical applications of optical coherence tomography for the diagnosis and management of macular diseases. *Ophthalmol Clin North Am*. 2004;17(1):21–31. doi:10.1016/j.ohc.2003.12.002
6. Schmidt-Erfurth UM, Richard G, Augustin A, et al. Guidance for the treatment of neovascular age-related macular degeneration. *Acta Ophthalmol Scand*. 2007;85(5):486–494. doi:10.1111/j.1755-3768.2007.00979.x
7. Spaide RF. Enhanced depth imaging optical coherence tomography of retinal pigment epithelial detachment in age-related macular degeneration. *Am J Ophthalmol*. 2009;147(4):644–652. doi:10.1016/j.ajo.2008.10.005
8. Penha FM, Rosenfeld PJ, Gregori G, et al. Quantitative imaging of retinal pigment epithelial detachments using spectral-domain optical coherence tomography. *Am J Ophthalmol*. 2012;153(3):515–523. doi:10.1016/j.ajo.2011.08.031
9. Nowak JZ. Age-related macular degeneration (AMD): pathogenesis and therapy. *Pharmacol Rep*. 2006;58(3):353.

10. Chakravarthy U, Wong TY, Fletcher A, et al. Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. *BMC Ophthalmol*. 2010;10(1):31. doi:10.1186/1471-2415-10-31
11. Tomany SC, Wang JJ, van Leeuwen R, et al. Risk factors for incident age-related macular degeneration: pooled findings from 3 continents. *Ophthalmology*. 2004;111(7):1280–1287. doi:10.1016/j.ophtha.2003.11.010
12. Zinkernagel MS, Zysset-Burri DC, Keller I, et al. Association of the intestinal microbiome with the development of neovascular age-related macular degeneration. *Sci Rep*. 2017;7(1):40826. doi:10.1038/srep40826
13. Zisimopoulos A, Klavdianou O, Theodossiadis P, et al. The role of the microbiome in age-related macular degeneration: a review of the literature. *Ophthalmologica*. 2021;244(3):173–178. doi:10.1159/000515026
14. Rudnicka AR, Kapetanakis VV, Jarrar Z, et al. Incidence of late-stage age-related macular degeneration in American whites: systematic review and meta-analysis. *Am J Ophthalmol*. 2015;160(1):85–93. e3. doi:10.1016/j.ajo.2015.04.003
15. Sharma K, Singh R, Sharma SK, et al. Sleeping pattern and activities of daily living modulate protein expression in AMD. *PLoS One*. 2021;16(6):e0248523. doi:10.1371/journal.pone.0248523
16. Sharma K, Sharma NK, Singh R, et al. Exploring the role of VEGF in Indian age related macular degeneration. *Ann Neurosci*. 2015;22(4):232–237. doi:10.5214/ans.0972.7531.220408
17. Sharma NK, Sharma K, Singh R, et al. CCL2 single nucleotide polymorphism of rs1024611 implicates prominence of inflammatory cascade by univariate modeling in Indian AMD. *PLoS One*. 2018;13(4):e0193423. doi:10.1371/journal.pone.0193423
18. Sharma K, Sharma NK, Singh R, et al. Gene networks determine predisposition to AMD. *Genomics*. 2021;113(1):514–522. doi:10.1016/j.ygeno.2020.09.044
19. Sharma NK, Sharma K, Gupta A, et al. Does toll-like receptor-3 (TLR-3) have any role in Indian AMD phenotype? *Mol Cell Biochem*. 2014;393(1–2):1–8. doi:10.1007/s11010-014-2040-4
20. Sharma K, Tyagi R, Singh R, et al. Serum levels of TIMP-3, LIPC, IER3, and SLC16A8 in CFH-negative AMD cases. *J Cell Biochem*. 2017;118(8):2087–2095. doi:10.1002/jcb.25837
21. Rajendran A, Dhoble P, Sundaresan P, et al. Genetic risk factors for late age-related macular degeneration in India. *Br J Ophthalmol*. 2018;102(9):1213–1217. doi:10.1136/bjophthalmol-2017-311384
22. Kan M, Weng X, Wang T, et al. No evidence of association between variant rs2075650 in lipid metabolism-related locus APOE/TOMM40 and advanced age-related macular degeneration in Han Chinese population. *Exp Biol Med*. 2015;240(2):230–234. doi:10.1177/1535370214553770
23. Wang Y-F, Han Y, Zhang R, Qin L, Wang MX, Ma L. CETP/LPL/LIPC gene polymorphisms and susceptibility to age-related macular degeneration. *Sci Rep*. 2015;5(1):1–13.
24. Wang Y, Wang M, Zhang X, et al. The association between LIPC rs493258 polymorphism and the susceptibility to age-related macular degeneration. *Int J Environ Res Public Health*. 2016;13(10):1022. doi:10.3390/ijerph13101022
25. Qureshi IZ, Ambreen F. Serum APOE, leptin, CFH and HTRA1 levels in Pakistani age related macular degeneration patients. *J Pak Med Assoc*. 2017;67(6):852–857.
26. Battu P, Sharma K, Rain M, et al. Serum levels of ARMS2, COL8A1, RAD51B, and VEGF and their correlations in age-related macular degeneration. *Curr Neurovasc Res*. 2021;18(2):181–188. doi:10.2174/1567202618666210531130711
27. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology*. 2000;107(12):2224–2232. doi:10.1016/s0161-6420(00)00409-7
28. Ritter M, Elledge J, Simader C, et al. Evaluation of optical coherence tomography findings in age-related macular degeneration: a reproducibility study of two independent reading centres. *Br J Ophthalmol*. 2011;95(3):381–385. doi:10.1136/bjo.2009.175976
29. Sharma NK, Gupta A, Prabhakar S, et al. CC chemokine receptor-3 as new target for age-related macular degeneration. *Gene*. 2013;523(1):106–111. doi:10.1016/j.gene.2013.03.052
30. Anand A, Gupta PK, Sharma NK, et al. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. *Eur J Neurol*. 2012;19(5):788–792. doi:10.1111/j.1468-1331.2011.03548.x
31. Anand A, Sharma NK, Gupta A, et al. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. *PLoS One*. 2012;7(11):e49905. doi:10.1371/journal.pone.0049905
32. Gupta PK, Prabhakar S, Abburi C, Sharma NK, Anand A. Vascular endothelial growth factor-a and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients. *J Neuroinflammation*. 2011;8(1):1–6. doi:10.1186/1742-2094-8-1
33. Sharma NK, Gupta A, Prabhakar S, et al. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. *Curr Neurovasc Res*. 2012;9(4):256–265. doi:10.2174/156720212803530681
34. Sharma NK, Gupta A, Prabhakar S, et al. Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. *PLoS One*. 2013;8(7):e70193. doi:10.1371/journal.pone.0070193
35. Sharma NK, Prabhakar S, Gupta A, et al. New biomarker for neovascular age-related macular degeneration: eotaxin-2. *DNA Cell Biol*. 2012;31(11):1618–1627. doi:10.1089/dna.2012.1786
36. Vinish M, Prabhakar S, Khullar M, et al. Genetic screening reveals high frequency of PARK2 mutations and reduced Parkin expression conferring risk for Parkinsonism in North West India. *J Neurol Neurosurg Psychiatry*. 2010;81(2):166–170. doi:10.1136/jnnp.2008.157255
37. Anand A, Saraf MK, Prabhakar S. Sustained inhibition of brotizolam induced anterograde amnesia by norharmane and retrograde amnesia by l-glutamic acid in mice. *Behav Brain Res*. 2007;182(1):12–20. doi:10.1016/j.bbr.2007.04.022
38. Singh T, Prabhakar S, Gupta A, et al. Recruitment of stem cells into the injured retina after laser injury. *Stem Cells Dev*. 2012;21(3):448–454. doi:10.1089/scd.2011.0002
39. Anand A, Saraf MK, Prabhakar S. Antiamnesic effect of B. monniera on L-NNA induced amnesia involves calmodulin. *Neurochem Res*. 2010;35(8):1172–1181. doi:10.1007/s11064-010-0171-x
40. Yasuda M, Kiyohara Y, Hata Y, et al. Nine-year incidence and risk factors for age-related macular degeneration in a defined Japanese population the Hisayama study. *Ophthalmology*. 2009;116(11):2135–2140. doi:10.1016/j.ophtha.2009.04.017
41. Horie-Inoue K, Inoue S. Genomic aspects of age-related macular degeneration. *Biochem Biophys Res Commun*. 2014;452(2):263–275. doi:10.1016/j.bbrc.2014.08.013

42. Seddon JM, Reynolds R, Rosner B. Associations of smoking, body mass index, dietary lutein, and the LIPC gene variant rs10468017 with advanced age-related macular degeneration. *Mol Vis*. 2010;16:2412.
43. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intravitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. *Am J Ophthalmol*. 2007;143(4):566–583. e2. doi:10.1016/j.ajo.2007.01.028
44. Rosenfeld PJ. Optical coherence tomography and the development of antiangiogenic therapies in neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2016;57(9):OCT14–OCT26. doi:10.1167/iovs.16-19969
45. Karacorlu M, Sayman Muslubas I, Arf S, et al. Membrane patterns in eyes with choroidal neovascularization on optical coherence tomography angiography. *Eye*. 2019;33(8):1280–1289. doi:10.1038/s41433-019-0415-1
46. Cascella R, Strafella C, Caputo V, et al. Towards the application of precision medicine in age-related macular degeneration. *Prog Retin Eye Res*. 2018;63:132–146. doi:10.1016/j.preteyeres.2017.11.004
47. Myers CE, Klein BEK, Gangnon R, et al. Cigarette smoking and the natural history of age-related macular degeneration: the Beaver Dam Eye Study. *Ophthalmology*. 2014;121(10):1949–1955. doi:10.1016/j.ophtha.2014.04.040
48. Rasoulinejad SA, Zarghami A, Hosseini SR, et al. Prevalence of age-related macular degeneration among the elderly. *Casp J Intern Med*. 2015;6 (3):141.
49. Rim TH, Cheng C-Y, Kim DW, et al. A nationwide cohort study of cigarette smoking and risk of neovascular age-related macular degeneration in East Asian men. *Br J Ophthalmol*. 2017;101(10):1367–1373. doi:10.1136/bjophthalmol-2016-309952
50. Vassilev ZP, Ruigomez A, Soriano-Gabarro M, et al. Diabetes, cardiovascular morbidity, and risk of age-related macular degeneration in a primary care population. *Invest Ophthalmol Vis Sci*. 2015;56(3):1585–1592. doi:10.1167/iovs.14-16271
51. Rohrer B, Frazer-Abel A, Leonard A, et al. Association of age-related macular degeneration with complement activation products, smoking, and single nucleotide polymorphisms in South Carolinians of European and African descent. *Mol Vis*. 2019;25:79–92.
52. Lee CS, Tyring AJ, Wu Y, et al. Generating retinal flow maps from structural optical coherence tomography with artificial intelligence. *Sci Rep*. 2019;9(1):5694. doi:10.1038/s41598-019-42042-y
53. Sharma K, Battu P, Singh R, Sharma SK, Anand A. Modulated anti-VEGF therapy under the influence of lipid metabolizing proteins in Age related macular degeneration: a pilot study. *Sci Rep*. 2022;12(1):714. doi:10.1038/s41598-021-04269-6

PLOS ONE

RESEARCH ARTICLE

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

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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.

https://doi.org/10.1371/journal.pone.0248523.t001

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.

https://doi.org/10.1371/journal.pone.0248523.t002

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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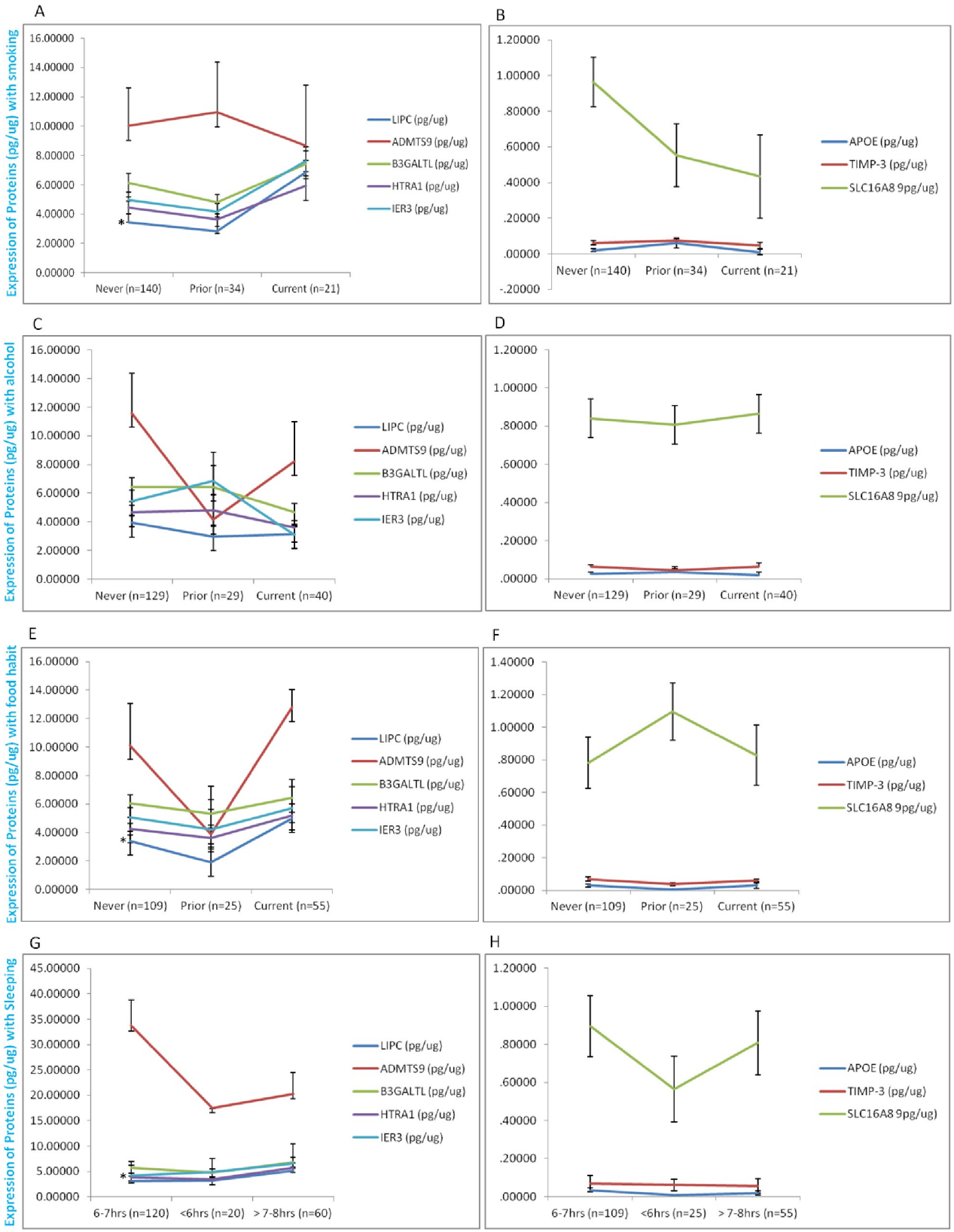

Fig 1. Expression of protein under ADL. Expression of lipid metabolizing (LIPC and APOE), proagniogenic (TIMP3 and ADAMTS9), regulatory (HTRA1, B3GALTL, and IER3) and momocarboxylic acid transporter (SLC16A8) proteins under the influence of ADL. LIPC (pg/ug) levels were

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.

https://doi.org/10.1371/journal.pone.0248523.t006

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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References

1. Sharma K, Sharma NK, Anand A. Why AMD is a disease of ageing and not of development: mechanisms and insights. Front Aging Neurosci. 2014; 6:151. Epub 2014/07/30. https://doi.org/10.3389/fnagi.2014.00151 PMID: 25071560; PubMed Central PMCID: PMC4091411.
2. Anand A, Sharma K, Sharma SK, Singh R, Sharma NK, Prasad K. AMD Genetics in India: The Missing Links. Front Aging Neurosci. 2016; 8:115. Epub 2016/06/03. https://doi.org/10.3389/fnagi.2016.00115 PMID: 27252648; PubMed Central PMCID: PMC4876307.
3. Fritsche LG, Chen W, Schu M, Yaspan BL, Yu Y, Thorleifsson G, et al. Seven new loci associated with age-related macular degeneration. Nat Genet. 2013; 45(4):433–9, 9e1-2. Epub 2013/03/05. https://doi.org/10.1038/ng.2578 PMID: 23455636; PubMed Central PMCID: PMC3739472.
4. Takeuchi A, Takeuchi M, Oikawa K, Sonoda KH, Usui Y, Okunuki Y, et al. Effects of dioxin on vascular endothelial growth factor (VEGF) production in the retina associated with choroidal neovascularization. Invest Ophthalmol Vis Sci. 2009; 50(7):3410–6. Epub 2009/02/03. https://doi.org/10.1167/iovs.08-2299 PMID: 19182260.
5. Wills NK, Ramanujam VM, Chang J, Kalariya N, Lewis JR, Weng TX, et al. Cadmium accumulation in the human retina: effects of age, gender, and cellular toxicity. Exp Eye Res. 2008; 86(1):41–51. Epub 2007/10/31. https://doi.org/10.1016/j.exer.2007.09.005 PMID: 17967453.
6. Velilla S, García-Medina JJ, García-Layana A, Dolz-Marco R, Pons-Vázquez S, Pinazo-Durán MD, et al. Smoking and age-related macular degeneration: review and update. J Ophthalmol. 2013; 2013:895147. Epub 2013/12/26. https://doi.org/10.1155/2013/895147 PMID: 24368940; PubMed Central PMCID: PMC3866712.
7. Kunchithapautham K, Atkinson C, Rohrer B. Smoke exposure causes endoplasmic reticulum stress and lipid accumulation in retinal pigment epithelium through oxidative stress and complement activation. J Biol Chem. 2014; 289(21):14534–46. Epub 2014/04/09. https://doi.org/10.1074/jbc.M114.564674 PMID: 24711457; PubMed Central PMCID: PMC4031511.
8. Venza I, Visalli M, Oteri R, Teti D, Venza M. Combined effects of cigarette smoking and alcohol consumption on antioxidant/oxidant balance in age-related macular degeneration. Aging Clin Exp Res. 2012; 24(5):530–6. Epub 2012/06/27. https://doi.org/10.3275/8477 PMID: 22732472.
9. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R. Superoxide dismutase1 levels in North Indian population with age-related macular degeneration. Oxid Med Cell Longev. 2013; 2013:365046. Epub 2013/12/24. https://doi.org/10.1155/2013/365046 PMID: 24363822; PubMed Central PMCID: PMC3864086.
10. Sharma K, Sharma NK, Singh R, Anand A. Exploring the role of VEGF in Indian Age related macular degeneration. Ann Neurosci. 2015; 22(4):232–7. Epub 2015/11/04. https://doi.org/10.5214/ans.0972.7531.220408 PMID: 26526736; PubMed Central PMCID: PMC4627204.
11. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma S, Anand A. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Curr Neurovasc Res. 2012; 9(4):256–65. Epub 2012/10/04. https://doi.org/10.2174/156720212803530681 PMID: 23030506.
12. Sharma NK, Sharma K, Singh R, Sharma SK, Anand A. CCL2 single nucleotide polymorphism of rs1024611 implicates prominence of inflammatory cascade by univariate modeling in Indian AMD. PLoS One. 2018; 13(4):e0193423. Epub 2018/04/18. https://doi.org/10.1371/journal.pone.0193423 PMID: 29664944; PubMed Central PMCID: PMC5903598.
13. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, Anand A. CC chemokine receptor-3 as new target for age-related macular degeneration. Gene. 2013; 523(1):106–11. Epub 2013/04/10. https://doi.org/10.1016/j.gene.2013.03.052 PMID: 23566847.
14. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, et al. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PLoS One. 2012; 7(11):e49905. Epub 2012/11/28. https://doi.org/10.1371/journal.pone.0049905 PMID: 23185481; PubMed Central PMCID: PMC3503775.
15. Anand A, Gupta PK, Sharma NK, Prabhakar S. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. Eur J Neurol. 2012; 19(5):788–92. Epub 2011/10/08. https://doi.org/10.1111/j.1468-1331.2011.03548.x PMID: 21978169.

16. Gupta PK, Prabhakar S, Abburi C, Sharma NK, Anand A. Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients. J Neuroinflammation. 2011; 8:114. Epub 2011/09/13. https://doi.org/10.1186/1742-2094-8-114 PMID: 21906274; PubMed Central PMCID: PMC3177897.

17. Anand A, Thakur K, Gupta PK. ALS and oxidative stress: the neurovascular scenario. Oxid Med Cell Longev. 2013; 2013:635831. Epub 2013/12/25. https://doi.org/10.1155/2013/635831 PMID: 24367722; PubMed Central PMCID: PMC3866720.

18. Anand A, Banik A, Thakur K, Masters CL. The animal models of dementia and Alzheimer's disease for pre-clinical testing and clinical translation. Curr Alzheimer Res. 2012; 9(9):1010–29. Epub 2012/06/16. https://doi.org/10.2174/156720512803569055 PMID: 22698073.

19. Sharma K, Sharma NK, Singh R, Sharma SK, Anand A. Gene networks determine predisposition to AMD. Genomics. 2020. Epub 2020/09/27. https://doi.org/10.1016/j.ygeno.2020.09.044 PMID: 32979492.

20. Gemenetzi M, Lotery AJ. Epigenetics in age-related macular degeneration: new discoveries and future perspectives. Cell Mol Life Sci. 2020; 77(5):807–18. Epub 2020/01/04. https://doi.org/10.1007/s00018-019-03421-w PMID: 31897542; PubMed Central PMCID: PMC7058675.

21. Liu MM, Chan CC, Tuo J. Genetic mechanisms and age-related macular degeneration: common variants, rare variants, copy number variations, epigenetics, and mitochondrial genetics. Hum Genomics. 2012; 6(1):13. Epub 2012/12/19. https://doi.org/10.1186/1479-7364-6-13 PMID: 23244519; PubMed Central PMCID: PMC3500238.

22. Gemenetzi M, Lotery AJ. The role of epigenetics in age-related macular degeneration. Eye (Lond). 2014; 28(12):1407–17. Epub 2014/09/23. https://doi.org/10.1038/eye.2014.225 PMID: 25233816; PubMed Central PMCID: PMC4268465.

23. Baird PN, Wei L. Age-related macular degeneration and DNA methylation. Epigenomics. 2013; 5 (3):239–41. Epub 2013/06/12. https://doi.org/10.2217/epi.13.19 PMID: 23750638.

24. Khurana RN, Porco TC, Claman DM, Boldrey EE, Palmer JD, Wieland MR. INCREASING SLEEP DURATION IS ASSOCIATED WITH GEOGRAPHIC ATROPHY AND AGE-RELATED MACULAR DEGENERATION. Retina. 2016; 36(2):255–8. Epub 2016/01/28. https://doi.org/10.1097/IAE.0000000000000706 PMID: 26815930.

25. Pérez-Canales JL, Rico-Sergado L, Pérez-Santonja JJ. Self-Reported Sleep Duration in Patients with Neovascular Age-Related Macular Degeneration. Ophthalmic Epidemiol. 2016; 23(1):20–6. Epub 2016/01/21. https://doi.org/10.3109/09286586.2015.1119288 PMID: 26786476.

26. Luu J, Palczewski K. Human aging and disease: Lessons from age-related macular degeneration. Proc Natl Acad Sci U S A. 2018; 115(12):2866–72. Epub 2018/02/28. https://doi.org/10.1073/pnas.1721033115 PMID: 29483257; PubMed Central PMCID: PMC5866596.

27. Cascella R, Strafella C, Caputo V, Errichiello V, Zampatti S, Milano F, et al. Towards the application of precision medicine in Age-Related Macular Degeneration. Prog Retin Eye Res. 2018; 63:132–46. Epub 2017/12/05. https://doi.org/10.1016/j.preteyeres.2017.11.004 PMID: 29197628.

28. Cascella R, Strafella C, Longo G, Ragazzo M, Manzo L, De Felici C, et al. Uncovering genetic and non-genetic biomarkers specific for exudative age-related macular degeneration: significant association of twelve variants. Oncotarget. 2018; 9(8):7812–21. Epub 2018/03/01. https://doi.org/10.18632/oncotarget.23241 PMID: 29487693; PubMed Central PMCID: PMC5814260.

29. Winkler TW, Brandl C, Grassmann F, Gorski M, Stark K, Loss J, et al. Investigating the modulation of genetic effects on late AMD by age and sex: Lessons learned and two additional loci. PLoS One. 2018; 13(3):e0194321. Epub 2018/03/13. https://doi.org/10.1371/journal.pone.0194321 PMID: 29529059; PubMed Central PMCID: PMC5846797.

30. Maugeri A, Barchitta M, Mazzone MG, Giuliano F, Agodi A. Complement System and Age-Related Macular Degeneration: Implications of Gene-Environment Interaction for Preventive and Personalized Medicine. Biomed Res Int. 2018; 2018:7532507. Epub 2018/09/19. https://doi.org/10.1155/2018/7532507 PMID: 30225264; PubMed Central PMCID: PMC6129329.

31. Chen Y, Zeng J, Zhao C, Wang K, Trood E, Buehler J, et al. Assessing susceptibility to age-related macular degeneration with genetic markers and environmental factors. Arch Ophthalmol. 2011; 129 (3):344–51. Epub 2011/03/16. https://doi.org/10.1001/archophthalmol.2011.10 PMID: 21402993; PubMed Central PMCID: PMC4134685.

32. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma SK, Chen W, et al. Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. PLoS One. 2013; 8 (7):e70193. Epub 2013/08/08. https://doi.org/10.1371/journal.pone.0070193 PMID: 23922956; PubMed Central PMCID: PMC3726372.

33. Sharma NK, Sharma K, Gupta A, Prabhakar S, Singh R, Gupta PK, et al. Does toll-like receptor-3 (TLR-3) have any role in Indian AMD phenotype? Mol Cell Biochem. 2014; 393(1–2):1–8. Epub 2014/04/01. https://doi.org/10.1007/s11010-014-2040-4 PMID: 24682730.

34. Sharma K, Tyagi R, Singh R, Sharma SK, Anand A. Serum Levels of TIMP-3, LIPC, IER3, and SLC16A8 in CFH-Negative AMD Cases. J Cell Biochem. 2017; 118(8):2087–95. Epub 2016/12/15. https://doi.org/10.1002/jcb.25837 PMID: 27966779.

35. Sharma NK, Prabhakar S, Gupta A, Singh R, Gupta PK, Gupta PK, et al. New biomarker for neovascular age-related macular degeneration: eotaxin-2. DNA Cell Biol. 2012; 31(11):1618–27. Epub 2012/10/03. https://doi.org/10.1089/dna.2012.1786 PMID: 23025269.

36. Sharma NK, Sharma SK, Gupta A, Prabhakar S, Singh R, Anand A. Predictive model for earlier diagnosis of suspected age-related macular degeneration patients. DNA Cell Biol. 2013; 32(9):549–55. Epub 2013/07/16. https://doi.org/10.1089/dna.2013.2072 PMID: 23848218.

37. Maugeri A, Barchitta M, Fallico M, Castellino N, Reibaldi M, Agodi A. Characterization of SIRT1/DNMTs Functions and LINE-1 Methylation in Patients with Age-Related Macular Degeneration. J Clin Med. 2019; 8(2). Epub 2019/02/06. https://doi.org/10.3390/jcm8020159 PMID: 30717113; PubMed Central PMCID: PMC6406755.

38. Porter LF, Saptarshi N, Fang Y, Rathi S, den Hollander AI, de Jong EK, et al. Whole-genome methylation profiling of the retinal pigment epithelium of individuals with age-related macular degeneration reveals differential methylation of the SKI, GTF2H4, and TNXB genes. Clin Epigenetics. 2019; 11(1):6. Epub 2019/01/16. https://doi.org/10.1186/s13148-019-0608-2 PMID: 30642396; PubMed Central PMCID: PMC6332695 the tenets of the Declaration of Helsinki. This project was approved by the University of Manchester and University of Liverpool's institutional ethical review boards (University of Manchester Research Ethics Committee 3, ref. 11,305; University of Liverpool Central University Research Ethics Committee for Physical Interventions, ref. 2326) and the University of Pennsylvania Institutional Review Board (IRB). Informed written consent was obtained for the eye tissue to be used for research and held by the respective Eye Banks. Guidelines established in the Human Tissue Act of 2008 (UK) were followed. CONSENT FOR PUBLICATION: N/A COMPETING INTERESTS: The authors declare they have no competing interests. PUBLISHER'S NOTE: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

39. Wei L, Liu B, Tuo J, Shen D, Chen P, Li Z, et al. Hypomethylation of the IL17RC promoter associates with age-related macular degeneration. Cell Rep. 2012; 2(5):1151–8. Epub 2012/11/28. https://doi.org/10.1016/j.celrep.2012.10.013 PMID: 23177625; PubMed Central PMCID: PMC3513594.

40. Fernandez-Robredo P, Recalde S, Hernandez M, Zarranz-Ventura J, Molins B, Casaroli-Marano RP, et al. Novel Association of High C-Reactive Protein Levels and A69S at Risk Alleles in Wet Age-Related Macular Degeneration Women. Front Immunol. 2018; 9:1862. Epub 2018/08/30. https://doi.org/10.3389/fimmu.2018.01862 PMID: 30154790; PubMed Central PMCID: PMC6102554.

41. Zhou J, McCandlish DM. Minimum epistasis interpolation for sequence-function relationships. Nat Commun. 2020; 11(1):1782. Epub 2020/04/15. https://doi.org/10.1038/s41467-020-15512-5 PMID: 32286265; PubMed Central PMCID: PMC7156698.

42. Wang Y, Liu X, Robbins K, Rekaya R. AntEpiSeeker: detecting epistatic interactions for case-control studies using a two-stage ant colony optimization algorithm. BMC Res Notes. 2010; 3:117. Epub 2010/04/30. https://doi.org/10.1186/1756-0500-3-117 PMID: 20426808; PubMed Central PMCID: PMC2880958.

43. Anand A, Saraf MK, Prabhakar S. Sustained inhibition of brotizolam induced anterograde amnesia by norharmane and retrograde amnesia by L-glutamic acid in mice. Behav Brain Res. 2007; 182(1):12–20. Epub 2007/06/15. https://doi.org/10.1016/j.bbr.2007.04.022 PMID: 17561282.

44. Anand A, Saraf MK, Prabhakar S. Antiamnesic effect of B. monniera on L-NNA induced amnesia involves calmodulin. Neurochem Res. 2010; 35(8):1172–81. Epub 2010/05/01. https://doi.org/10.1007/s11064-010-0171-x PMID: 20431943.

45. Mathur D, Goyal K, Koul V, Anand A. The Molecular Links of Re-Emerging Therapy: A Review of Evidence of Brahmi (Bacopa monniera). Front Pharmacol. 2016; 7:44. Epub 2016/03/15. https://doi.org/10.3389/fphar.2016.00044 PMID: 26973531; PubMed Central PMCID: PMC4778428.

46. Singh T, Prabhakar S, Gupta A, Anand A. Recruitment of stem cells into the injured retina after laser injury. Stem Cells Dev. 2012; 21(3):448–54. Epub 2011/05/13. https://doi.org/10.1089/scd.2011.0002 PMID: 21561324.

47. English D, Sharma NK, Sharma K, Anand A. Neural stem cells-trends and advances. J Cell Biochem. 2013; 114(4):764–72. Epub 2012/12/12. https://doi.org/10.1002/jcb.24436 PMID: 23225161.

48. Anand A, Sharma K, Chen W, Sharma NK. Using current data to define new approach in age related macular degeneration: need to accelerate translational research. Curr Genomics. 2014; 15(4):266–77. Epub 2014/08/19. https://doi.org/10.2174/1389202915666140516204512 PMID: 25132797; PubMed Central PMCID: PMC4133950.

49. Banik A, Brown RE, Bamburg J, Lahiri DK, Khurana D, Friedland RP, et al. Translation of Pre-Clinical Studies into Successful Clinical Trials for Alzheimer's Disease: What are the Roadblocks and How Can They Be Overcome? J Alzheimers Dis. 2015; 47(4):815–43. Epub 2015/09/25. https://doi.org/10.3233/JAD-150136 PMID: 26401762.

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Analysis of smoking and LPO in ALS

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ABSTRACT

Smoking has been suggested as one of the risk factor for amyotrophic lateral sclerosis (ALS) development. In order to investigate whether adverse effects of cigarette smoke in ALS have any association with increase in oxidative stress, disease severity, lipid hydroperoxides (LPO) and superoxide dismutase-1 (SOD1) levels were measured in biofluids of smoker and never smoker ALS patients and clinically correlated. Serum and CSF from sporadic ALS patients ($n = 50$) diagnosed with El Escorial criteria were collected in the study. Serum ($n = 50$) and CSF ($n = 42$) were also collected from normal healthy controls. The LPO levels were estimated using commercially available kits. Enzyme-linked immunosorbent assays (ELISAs) were used to quantitate SOD1. Their levels were further analyzed among smoker and never smoker subjects. Significantly elevated LPO in sera and CSF of ALS patients were observed ($p < 0.05$). There was considerably increased LPO in sera and CSF of smoker ALS subjects matched with disease severity as compared to never smoker ALS ($p < 0.05$). ALS group did not show any alteration in SOD1 when compared to controls ($p > 0.05$). In addition, no change has been observed in SOD1 levels in ALS subjects who smoke ($p > 0.05$). Increased LPO and unaltered SOD1 in ALS patients may suggest the neuro-pathological association of LPO with ALS disease independent of SOD1. With current findings, it may be proposed that LPO levels might constitute as probable biomarker for smoker ALS patients, however, it cannot be concluded without larger gender matched studies. Additional investigations are needed to determine whether LPO upregulation is primary or secondary to motor neuron degeneration in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurological disorder which is characterized by selective loss of motor neurons. In demographic centric studies it was shown that risk of ALS development is 1.4–3-fold higher in current cigarette smokers (Weisskopf et al., 2004; Wang et al., 2011) as compared to never smokers. Oxidative stress is the major key player in various degenerative disease such as AD, PD and ALS. Smoke is known to induce oxidative stress as evidenced by observation of increased lipid peroxidation in biofluids of normal smokers (Morrow et al., 1995). Reduced expression of antioxidant enzyme superoxide dismutase-1 (SOD1) has also been observed in human SH-SY5Y neuroblastoma cells upon cigarette smoke condensate exposure (Russo et al., 2011). In addition, cigarette smoke is known to impair hypoxia-inducible factor-1α (HIF-1α) induced vascular endothelial growth factor (VEGF) expression predisposing individuals to ALS (Michaud et al., 2003). Based on existing literature, we hypothesized that cigarette smoke may be an important risk factor for motor neuron degeneration in ALS and may exert its neurotoxic effects primarily by enhancing oxidative stress. Therefore, in the current study, we wanted to test the oxidative stress by measuring lipid hydroperoxides (LPO) and SOD1 levels in serum and cerebrospinal fluid (CSF) from smoker Asian Indian ALS subjects.

Lipid peroxidation is an important indicator of oxidative stress in pathophysiological conditions. The modifications of lipids, both saturated and unsaturated, initially generate highly unstable LPOs which upon decomposition produce stable α,β-unsaturatedaldehydic byproducts such as 4-hydroxy-2,3-trans-nonenal (4-HNE) and malondialdehyde (MDA). Therefore, the customary assays for measuring lipid peroxidation consist of 4-HNE and MDA. However, estimation of 4-HNE and MDA may lead to gross under or over-estimation of lipid peroxidation. It is known that 4-HNE is produced by denaturation of only omega-6 polyunsaturated fatty acids such as arachidonic and linoleic acids etc., and damage of

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cholesterol and mono unsaturated fatty acids like oleic acid does not generate 4-HNE (Benedetti et al., 1980). In addition, MDA may be produced by thromboxane synthase, an enzyme in platelets, during blood clotting (Panse et al., 1985) and may result in over-estimation of lipid peroxidation in serum samples. Because of these problems, authors used a kit which can directly measure unstable and reactive intermediate LPO in serum and CSF samples from Indian ALS patients which show longer survival time after onset of disease as compared to western ALS population (Nalini et al., 2008). Earlier studies have reported increased oxidative burden in blood, CSF and different regions of central nervous system (CNS) in ALS (Shaw et al., 1995; Oteiza et al., 1997; Tohgi et al., 1999; Siciliano et al., 2002; Simpson et al., 2002; Babu et al., 2008), however, such studies neither attempted to compare oxidative stress among smoker and never smoker ALS patients nor with disease severity. Hence, in current study we tried to explore the LPO levels as a potential biomarker for ALS.

2. Materials and methods

2.1. Subjects

Peripheral venous blood and CSF was collected from 50 sporadic ALS patients, diagnosed by "El Escorial" criteria. The patients were recruited from a outpatient Department of Neurology in Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India only after obtaining informed consent as per research protocol approved by the Institute ethical committee guidelines (No. 7055-PG-1Tg-05/4348–50). There were $n = 50$ ALS patients with 42 limb and 8 bulbar onset. None of the patients were on respiratory support system, however, 11 patients presented with respiratory insufficiencies with breathlessness while walking and lying based on amended ALS-functional rating score (ALSFRS-R) (Cedarbaum et al., 1999). ALSFRS scoring showed that 15 ALS patients presented with mild impairments [ALSFRS-$R_{mean} = 40 \pm 0.5$(SE)], 30 patients presented with moderate neurological impairments [ALSFRS-$R_{mean} = 32.5 \pm 0.4$(SE)] and 5 patients presented with severe clinical manifestations [ALSFRS-$R_{mean} = 18.5 \pm 1.5$(SE)]. ALS subjects on anti-inflammatory drugs (e.g. motrin, celebrex, naproxen, aspirin, ibuprofen etc.)/antioxidants and/or receiving any other treatment, patients with written history of cognitive impairments, immunogenic reaction for pre-eclampsia, stroke, glaucoma, riluzole, pulmonary hypertension, diabetic neuropathy, diabetes and those with uncorrected hyper or hypothyroidism were excluded from the study. For comparison, blood ($n = 50$) and CSF ($n = 42$) was also obtained from age matched, genetically unrelated normal controls which represent apparently healthy population without any complaints of hypertension, diabetes, heart problems and other diseases. Control and test subjects were divided under a category as smoker and never smokers on the basis of a criteria used in our previous study (Prabhakar et al., 2010). Smoking details in case of ALS subjects were obtained at the age of disease onset. Patients with smoking status included those that had smoked for at least 6 months or more. However, we did not obtain the details of smoking history such as total amount and duration of smoking. All the smokers in ALS and control groups were found to be males. The earlier published details of ALS subjects (Gupta et al., 2011) have been reproduced here in Table 1. Clinical summary of smoker ALS patients and never smoker ALS patients has been mentioned in Tables 2 and 4.

2.2. Serum preparation

Serum separation was done using serum separator tube (SST; BD Vacutainer®, USA) according to manufacturer's recommendations. Briefly, 4.0 ml blood was collected in SST and inverted gently ~5 times to mix the blood with clot activator. Blood was allowed to clot for 30 mins at room temperature followed by centrifugation at 2000 rpm for 15.0 mins. Serum samples were stored at −80 °C until assayed.

2.3. CSF preparation

1.0 ml–2.0 ml CSF was obtained in sterilized eppendorfs. CSF samples were stored in crude form without any processing at −80 °C until assayed within 1 h of collection. CSF samples contaminated with blood were not included in the study.

2.4. LPO estimation

LPO levels were measured in CSF and serum of ALS patients using commercially available LPO assay kits (Catalog No. 437639; Calbiochem®, USA). Briefly, this kit directly utilizes unstable lipid hydroperoxides for redox reaction with ferrous ions that ultimately produce ferric ions. Later, thiocyanate ions were used to detect resulting ferric ions at 500 nm, using spectrophotometer (Beckman Coulter, USA). The results were obtained from linear regression and standard curve which was plotted after normalization with total protein. Since in serum, a large proportion of LPO is lost with the plasminogen during whole blood clot and CSF may have undetectable LPO levels, both serum and CSF samples were extracted using chloroform to concentrate the LPO so that they could be in a detectable range. Moreover, serum and CSF may contain hydrogen peroxide that ineracts with ferrous ion readily in the sample and leads to over-estimation of LPO. The extraction of samples in chloroform circumvents these problems.

2.5. SOD1 quantitation

Serum and CSF SOD1 levels were assayed using colorimetric sandwich enzyme-linked immunosorbent assays (ELISA; Catalog

Table 1
Characteristics of ALS patients and controls.

Subjects	Age (y)[a]	M/F (n)	Smoker (n)	Age of onset (y)	Disease duration[c] (mo)	El Escorial criteria	ALSFRS-R	Total protein (g/l)[b]	
								CSF	Serum
ALS patients	47.4 ± 12.4	38/12	12	46.2 ± 12.8	14 (3–72)	25 Definite 15 Probable 10 Possible	34.3 ± 6.1	0.43 ± 0.2	48.2 ± 26.7
Controls (serum)	40.0 ± 12.8	39/11	10					48.7 ± 28.7	
Controls (CSF)	43.4 ± 17.1	35/07	08					0.42 ± 0.1	

Characteristics of subjects. ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating score-revised; CSF; cerebrospinal fluid; F, female; g, gram; l, liter; M, male; mo, months; n, number of subjects; y, years. Age, age of onset, CSF and serum total protein is shown as mean ± standard deviation (SD).

[a] One way ANOVA followed by Fisher's least significant difference (LSD) post hoc analysis showed that mean age did not differ significantly among the groups ($p > 0.05$).

[b] For CSF and serum total protein, group means were compared using unpaired, independent 2- tailed *student t-test* with equal variance ($p > 0.05$).

[c] Disease duration is the interval between appearance of first symptom of ALS disease and collection of sample and indicated as median (range).

Table 2
Clinical summary of 12 ALS smoker patients.

ALS subjects	Age (y)	Gender	Age of onset (y)	Disease duration[a] (mo)	El Escorial criteria	ALSFRS-R	Impairment	B/L onset
Patient 1	47	M	46	12	Definite	36	Moderate	L
Patient 2	43	M	42	12	Definite	30	Moderate	B
Patient 3	44	M	42	24	Definite	35	Moderate	L
Patient 4	60	M	59	12	Definite	29	Moderate	L
Patient 5	41	M	40	12	Definite	34	Moderate	L
Patient 6	68	M	65	36	Definite	35	Moderate	L
Patient 7	47	M	46	12	Definite	29	Moderate	L
Patient 8	46	M	45	12	Definite	31	Moderate	L
Patient 9	52	M	50	24	Definite	35	Moderate	B
Patient 10	50	M	48	24	Definite	24	Moderate	L
Patient 11	46	M	42	48	Definite	36	Moderate	L
Patient 12	50	M	49	12	Definite	36	Moderate	L

Clinical summary of smoker ALS patients: ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating score-revised; B, bulbar; L, limb; mo: months; y, years. Impairment was measured using ALSFRS-R.

[a] Duration of disease is the interval between appearance of first symptom of ALS and collection of sample. Median disease duration in smoker patients is 12 (12–48) months whereas median duration of disease was reported to be 14 (3–72) months in never smoker patients, although the observed difference was not significant upon nonparametric Mann–Whitney *U* analysis ($p > 0.05$). Mean age of onset in smoker ALS patients was 47.8 ± 7.4(SD) years whereas it was 45.0 ± 14.2(SD) in never smoker patients. Mean ALSFRS-R score of smoker patients was 32.5 ± 3.8(SD) and mean ALSFRS-R of neversmoker patients was 35.0 ± 6.6(SD). The difference of mean age of onset and mean ALSFRS-R between smoker and neversmoker ALS subjects was not stastically significant upon unpaired, independent 2-tailed *student t-test* with unequal variance ($p > 0.05$).

Table 3
Crude and adjusted OR for LPO levels in ALS patients.

Subjects	Crude OR (95% CI)[a]	p^*	Adj. OR (95% CI)[b]	p^*
ALS				
Serum LPO	0.63 (0.20–1.9)	0.42	0.64 (0.24-1.7)	0.72
CSF LPO	0.79 (0.30–2.1)	0.20	0.41 (0.13–1.2)	0.12
Normal controls	1.0		1.0	

[a] Univariate logistic regression was used to evaluate crude OR.

[b] Multivariate logistic regression has been used to adjust the effect of gender on LPO levels with smoking as covariates.

[*] χ^2 (chi square test) was used to test the level of significance. Normal control group is considered as reference group. ALS, amyotrophic lateral sclerosis; LPO, lipid hydroperoxides; OR, odds ratio; CI, confidence interval; Adj, adjusted.

No. QIA 97; Calbiochem®, USA) as per instructions provided by manufacturer and consequently read at 450 nm in the Microplate reader (model 680XR, Biorad, USA). The standard curve was generated and results obtained through linear or quadratic regression after normalization with total protein. The ELISA assay is specific to unbound and free SOD1 and since the assay is highly sensitive (40 pg/ml detection limit), SOD1 could be measured even though it was present in very low concentration in the extracellular fluid. As epitope screening was not done by the supplier while manufacturing SOD1 ELISA kit, it is not possible to comment whether it recognizes only wild type SOD1 and not mutated SOD1.

2.6. Statistical analysis

The non-parametric Kruskal–Wallis *H* test and Mann Whitney *U* test was carried out for statistical comparisons among the groups if the distribution of data was skewed. Parametric unpaired independent 2-tailed student *t*-test with equal or unequal variance, and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test with equal variances was employed for comparison when data was normally distributed. Skewed and normally distributed data was represented as median (10th percentile-90th percentile) and mean ± standard error (SE) respectively. Distribution of data was elucidated using quantile–quantile (q–q) plots. Variance among the groups was compared using Levene's test for equality of variances. Since gender may affect LPO levels, crude odds ratio (OR) using univariate logistic regression was calculated to find its association with LPO. Further, multivariate logistic regression was used to compute adjusted OR with smoking as covariate to analyze independent relation of gender with LPO levels. Level of significance for this association was measured by performing χ^2 (chi square) test. *p*-Values were considered significant at $\leqslant 0.05$. All statistical analysis was done in statistical package and service solutions (SPSS) 16 software.

3. Results

LPO in CSF and serum of ALS patients was measured as an indicator of oxidative stress. Serum LPO was found to be higher in ALS patients than normal controls (Fig. 1A; $p = 0.014$). Upon segregation, serum LPO was marginally but significantly increased in ALS patients who smoke as compared to never smoker ALS patients (Fig. 1B; $p = 0.042$). Moreover, serum LPO was marginally higher in smoker ALS subjects as compared with smoker and never smoker controls (Fig. 1B; $p = 0.029$ and $p = 0.006$ respectively). Although there is a trend towards increased serum LPO in never smoker ALS than smoker and never smoker controls, it is not significant (Fig. 1B; $p > 0.05$). No change in serum LPO was seen among smoker and never smoker controls (Fig. 1B; $p > 0.05$). CSF from ALS patients was also found to have significantly enhanced LPO when compared with normal controls (Fig. 1C; $p = 0.016$). A profound increase in LPO was observed in CSF of smoker ALS patients in comparison to never smoker ALS patients, smoker and never smoker controls (Fig. 1D; $p = 0.0001$, $p = 0.0001$ and $p = 0.0001$ respectively). Even never smoker ALS patients had higher levels of LPO in CSF than never smoker normal controls (Fig. 1D; $p = 0.043$). Levels of CSF LPO were comparable between never smoker ALS and smoker controls (Fig. 1D; $p > 0.05$), and never smoker and smoker controls (Fig. 1D; $p > 0.05$).

Level of antioxidant enzyme SOD1 was also estimated in ALS subjects and no difference was observed in serum SOD1 between controls and ALS (Fig. 2A; $p > 0.05$). Values of serum SOD1 were comparable between never smoker controls, smoker controls, never smoker ALS and smoker ALS subjects (Fig. 2B; $p > 0.05$). Similarly, no difference was observed between levels of SOD1 in CSF from given groups (Fig. 2C and D; $p > 0.05$). Association of LPO level with age of onset was also analyzed, but no significant value was obtained (Pearson Correlation, $p = 0.156$). Association of smoker/never-smoker ALS patients was also analyzed with disease severity. The risk estimate for smoker ALS patients was compared to corresponding never smokers which was 1.923 (with 95% confidence limits as 1.320, 2.803, Table 5). The results indicate that risk of severity among ALS smokers is almost double as compared to never smokers. In order to analyze the impact of disease duration

Table 4
Summary of ALS never smoker patients having details of disease duration.

ALS subjects	Age	Gender	Age of onset (Y)	Disease duration (M)	ALSFRS	El Escorial criteria	Impairment	B/L onset
Patient 1	26	F	–	–				L
Patient 2	48	M	–	–				L
Patient 3	32	F	30	24				L
Patient 4	50	M	48	24		Definite		L
Patient 5	22	M	21	12		Definite		L
Patient 6	55	F	54	12	37	Possible	Mild	L
Patient 7	73	F	72	12		Definite		L
Patient 8	50	F	49	12	37	Definite	Mild	L
Patient 9	18	M	14	48	41	Probable	Mild	L
Patient 10	29	F	26	36	30	Definite	Moderate	L
Patient 11	75	M	74	14	39	Probable	Mild	L
Patient 12	65	M	35	24	40	Possible	Mild	L
Patient 13	37	M	26	18	34	Definite	Moderate	L
Patient 14	28	M	39	12	36	Definite	Moderate	L
Patient 15	40	F	69	12	32	Definite	Moderate	L
Patient 16	70	M	42	36	27	Definite	Moderate	L
Patient 17	45	F	40	12	31	Definite	Moderate	L
Patient 18	41	M	49	12	34	Probable	Moderate	L
Patient 19	50	M	49	12	31	Definite	Moderate	L
Patient 20	50	F	57	10	36	Probable	Moderate	L
Patient 21	58	M	48	18	43	Possible	Mild	L
Patient 22	49	F	45	8	16	Possible	Severe	B
Patient 23	45	M	39	18	40	Probable	Mild	L
Patient 24	41	M	26	24	31	Possible	Moderate	L
Patient 25	28	M	50	12	39	Possible	Mild	L
Patient 26	51	M	59	18	34	Definite	Moderate	L
Patient 27	60	M	39	12		Possible		B
Patient 28	40	F	53	18	34	Possible	Moderate	L
Patient 29	55	M	55	3	35	Definite	Moderate	L
Patient 30	55	M	45	12	42	Probable	Mild	L
Patient 31	46	F	36	16	30	Definite	Moderate	B
Patient 32	38	M	54	24	34	Definite	Moderate	L
Patient 33	56	F	58	18	38	Definite	Mild	L
Patient 34	58	M	33	9	38	Probable	Mild	L
Patient 35	33	M	54	24	44	Possible	Mild	L
Patient 36	56	M	14	72	35	Probable	Moderate	L
Patient 37	20	M			42	Probable	Mild	L

on the levels of LPO ALS smokers and ALS never-smokers it was found to be comparable ($p = 0.826$, 0.820). It appears that smoking neither affects the age of onset nor enhances the risk of ALS but affects LPO level regardless of severity of disease.

As gender could be a confounding factor the levels of LPO were adjusted with gender with smoking as covariates (Table 3). However, LPO levels of smoker ALS males with those of corresponding sex matched control groups revealed no significant difference ($p = 0.958$, 0.962; $p = 0.598$, 0.551; $p = 0.222$, 0.307) suggesting the gender associated protection in females.

4. Discussion

Oxidative stress is known to be a major contributor for development degenerative diseases. Studies have shown the potential of oxidative burden in worsening the cellular degeneration. Certain molecules such as HNE, MDA, lsoprostanes and TBARS are well studied as biomarkers for increase of oxidative stress through LPO (Pedersen et al., 1998; Rukhsana et al., 2013). The elevated levels of serum LPO in ALS patients included in this report are consistent with an existing report where Simpson et al., 2002 observed significantly increased 4-HNE in sera of ALS patients. Similarly, elevated LPO in blood of ALS patients has been reported in a few other studies (Oteiza et al., 1997; Siciliano et al., 2002). Apart from ALS, LPO can diffuse passively through intact lipid biomembrane, it is, therefore, possible that LPO may have been produced intrathecally and subsequently leaked into serum (Simpson et al., 2002) since we have also found significantly higher LPO levels in CSF of our ALS patients. Increased CSF LPO, 4-HNE and MDA, because of their intrathecal production, has previously been reported in many neurological diseases including ALS (Arlt et al., 2002; Simpson et al., 2002; Boll et al., 2008). In addition, there are studies reporting huge increase in oxidative burden in different regions of CNS in ALS patients (Shaw et al., 1995; Ferrante et al., 1997). Ryberg et al. (2004), however, did not observe any significant difference in amount of 3-nitrotyrosine, a marker for protein oxidative damage, in CSF from ALS patients and control individuals and argued that the different protocol used for 3-nitrotyrosine measurement in CSF samples could be a probable cause for neutral results. However, most of these studies did not analyze the profile of biomarkers between smoker and never smoker patients. Apart from diffusion from CSF, increased serum LPO raises the possibility of activation of some different cascade outside the CNS enabling LPO to be directly secreted in blood, like generation of free radicals in hypoxic skeletal muscles (Lundby et al., 2003). However, currently there is no study involving ALS subjects which has reported enhanced oxidative damage in peripheral organs. Biopsy studies can reveal the presence of lipid peroxidation markers in ALS, if any. Above data indicates that LPO could be a potential biomarker for ALS smokers. Although the exact mechanism of LPO, smoking and ALS is unclear in these patients further preclinical studies are required to understand the crosstalk between LPO and other oxidative stress markers such as SOD1, CCL2, CCR2 (Anand et al., 2012a,b; Gupta et al., 2012a,b). Our study, therefore, opens a new window to examine the association of LPO as a risk factor of ALS especially in larger smoker groups.

Levels of antioxidant enzyme SOD1 were also studied in patients of ALS and found to be unaltered. It is possible that at the time of sample collection, there was no extra leakage of

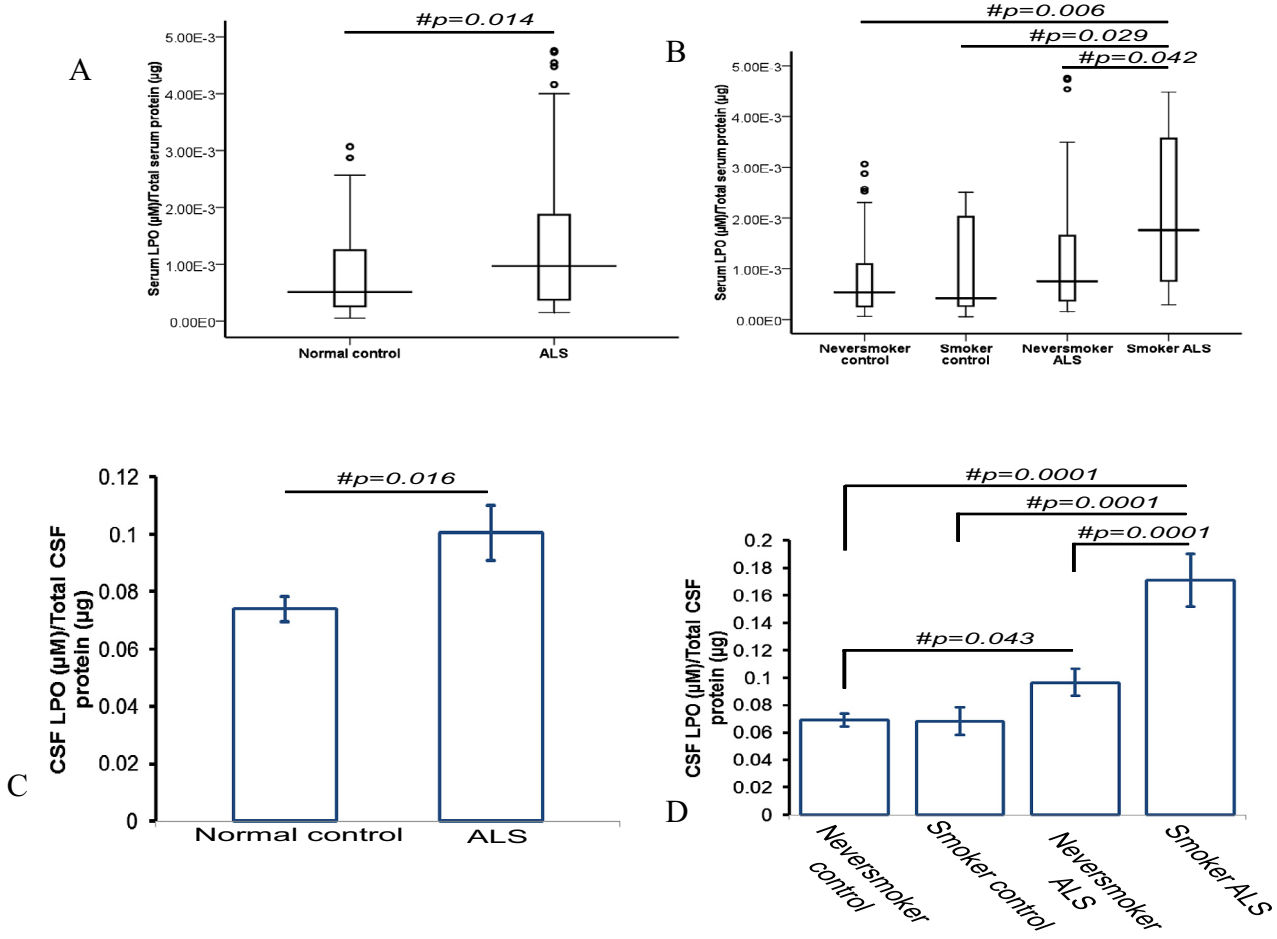

Fig. 1. Levels of LPO in ALS and control subjects. (A) Box and Whisker diagram represents serum LPO levels in ALS patients and normal controls. (B) Serum LPO in smoker ALS subjects. (A, B) Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The thick horizontal line in the box represents median for each dataset. Outliers are shown in circles. Data is analyzed by nonparametric Kruskal–Wallis *H* test followed by asymptotic Mann–Whitney *U* test. Levels of serum LPO were normalized to total serum protein. (C) LPO levels in CSF of ALS patients and normal subjects. Values are plotted as mean ± standard error (SE) in the bar diagram. Data was analyzed by parametric unpaired, independent 2-tailed student *t*-test with unequal variances. CSF LPO was normalized to total CSF protein. (D) LPO in CSF from smoker ALS subjects. Values are plotted as mean ± standard error (SE). Data was analyzed by parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test with equal variances. Levels of CSF LPO were normalized to total CSF protein. #, indicates significant difference among the given conditions ($p \leqslant 0.05$); μM, micromolar; μg, microgram; ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; LPO, lipid hydroperoxides.

SOD1 in CSF owing to intact architecture of bio-membrane of degenerating motor neurons in these patients, which is further be supported by unchanged levels of serum and CSF total protein (Table 1). Regardless of various conflicting reports, the unaltered SOD1 levels may indicate that there may not be any genetic change in the regulatory regions such as promoter and terminators of the SOD1 gene, however, mutations in functional regions such as exon/intron domain cannot be ruled out and should be screened in sporadic Indian ALS patients. Although SOD1 levels remain intact, possible aberrations in functional regions may result in production of mutated and misfolded SOD1 molecules with harmful disrupted activity. Earlier, altered SOD1 activity and mutations in SOD1 gene have been reported in ALS patients (Burr et al., 1987; Ihara et al., 1995; Przedborski et al., 1996; Corrado et al., 2006; Liu et al., 2009; Luigetti et al., 2009; vanEs et al., 2010). Hence, these results may not discredit the hypothesis of neurotoxicity caused by extracellular misfolded SOD1 and is consistent with existing reports where authors observed unaltered SOD1 levels in muscles biopsies and CSF of sporadic and familial ALS patients (Jacobsson et al., 2001; Frutiger et al., 2008). Similarly, no difference in CSF SOD1 was observed in ALS patients when compared with neurological controls (Zetterström et al., 2011). However, markedly decreased SOD1 expression was previously observed in lymphocytes and serum from sporadic ALS patients (Mokuno et al., 1996; Cova et al., 2006). Hence from above discussion it appears that monitoring the oxidative stress levels, such as SOD 1 and LPO in addition to other stress markers may prove useful in studying an association of two mechanisms i.e. oxidation and antioxidation in relation to each other. Based on additional cohort studies the level of different oxidants and antioxidant markers could be predicted using a statistical approach of predictive modeling for diagnosis and prognosis of diseases in its milder form (Gupta et al., 2012a,b).

We also did not find any change in SOD1 in ALS patients who smoke, although a recent study demonstrated significantly decreased SOD1 expression in SH-SY5Y human neuroblastoma cells upon exposure of cigarette smoke condensate (Russo et al., 2011). Hence, altered activity and/or downregulated expression of other antioxidant enzymes such as catalase and glutathione peroxidase is suggested in smoker ALS patients, however, such analysis has not been conducted. Therefore, we plan to analyze additional number of ALS patients which will include detailed smoking history as well as other markers of stress.

It is not possible to explain why there is elevated LPO in smoker ALS subjects and not in smoker normal controls, but it may indi-

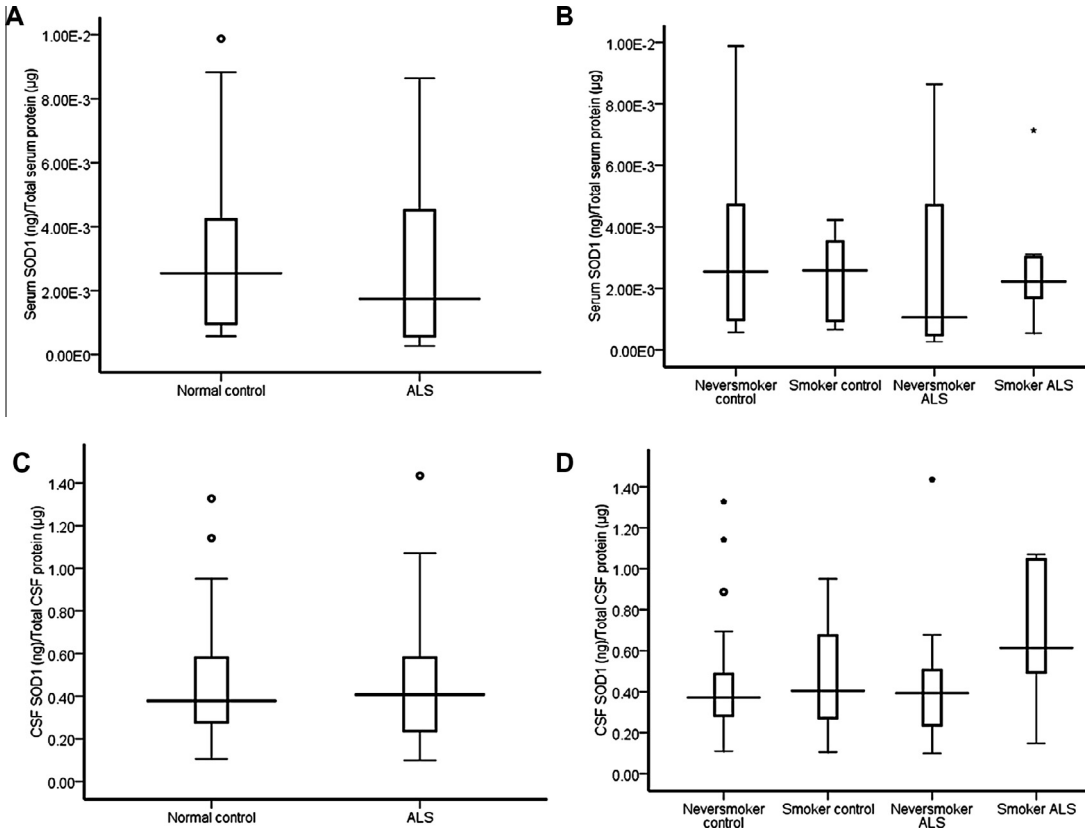

Fig. 2. SOD1 levels in ALS patients and normal controls. (A) Levels of SOD1 in serum in ALS patients and normal controls. (B) Levels of SOD1 in serum in smoker ALS patients. (C) SOD1 in CSF of ALS patients. (D) Levels of SOD1 in CSF of smoker ALS patients. (A–D) Boxes include values from first quartile (25th percentile) to third quartile (75th percentile). Lower and upper error bar refers to 10th and 90th percentile respectively. The black horizontal line in the box represents median for each dataset. No significant difference ($p > 0.05$) was observed between the given conditions. Data was analyzed by nonparametric Kruskal–Wallis *H* test followed by asymptotic Mann–Whitney *U* test. Extreme values and outliers are shown in asterisk and circles respectively. Levels of serum and CSF SOD1 were normalized to their respective total protein. ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; SOD1, superoxide dismutase 1; ng, nanogram; µg, microgram.

Table 5
Risk estimate for ALS male smoker vs. ALS male never-smoker (1.923 with 95% confidence limits as 1.320, 2.803). Chi square test was used to test the level of significance ($\chi^2 = 5.376$, $p = 0.020$). Total numbers of cases were 32 including two groups of ALS smoker and ALS never-smoker differentiated on the bases of disease severity.

Subjects	Risk estimate	Pearson chi square (95% CI)[†]	p^*
ALS male smokers vs. male never smokers	1.923	5.376	0.020
Number of valid subjects	32		

[†] Multivariate regression analysis was used to evaluate risk estimate.
[*] Chi square test was used to test the level of significance.

cate the relatively high vulnerability of diseased motor neurons in ALS to oxidative stress than those of healthy motor neurons in normal smokers. It is interesting to note that when LPO levels of male ALS smokers and never smokers were compared for disease duration and age of onset, no association was observed even though significant LPO levels were recorded in ALS smokers when compared with ALS never smokers. These results are consistent with previous studies which show that women have comparatively reduced LPO levels than males (Bayir et al., 2004; Mahboob et al., 2005; Sandra et al., 2008). The limited number of smoker ALS patients did not allow for analysis of a direct association of smoking with risk of ALS using univariate and multivariate statistical analysis; however, based on existing literature (Weisskopf et al., 2004; Gallo et al., 2009; Wang et al., 2011), it is clear that smoking is an important risk factor for ALS, and LPO may be associated with ALS smokers, but how it contributes as a risk factor for ALS is yet to be elucidated. It is also pertinent to point out that ALS smokers are twice at risk, as compared to never smoker ALS patients, for developing disease severity but the power of the study is not sufficiently convincing. Certain studies in which smoking has been shown to be associated with the risk of ALS have argued it to provoke cascade of events leading to increased susceptibility of body to oxidative damage (Gallo et al., 2009; Kamel et al., 1999; Sonja et al., 2012). Elevated LPO may activate neurotoxic cascades such as the induction of apoptotic enzymes, damage of genomic DNA, damage to protein and change in their conformation, the release of cytochrome C from mitochondria and hence may lead to degeneration of motor neurons (Niki et al., 2005, BBRC). Dysregulated LPO is also known to cause damage through enhanced recruitment of innate and acquired immune cells and upregulation of proinflammatorychemokines such as chemokine ligand-2 (CCL2) (Curzio et al., 1987; Simpson et al., 2002). Hence, it is possible that the increased lipid peroxidation in present patients may result in enhanced secretion of CCL2 chemokine in the same set of ALS patients as reported previously (Gupta et al., 2011) and it appears that a causative or indirect linear correlation exists between inflammation and oxidative stress (Fig. 3A and B). However, additional multi-centric, correlational and dose–response studies with a larger number of ALS smokers adjusted to antioxidant diet or supplements are required to examine whether ever smokers show higher levels of LPO as compared to former smokers who quit smoking and if this is positively associated with the progress of ALS and whether the risk of ALS increases with increasing smoking

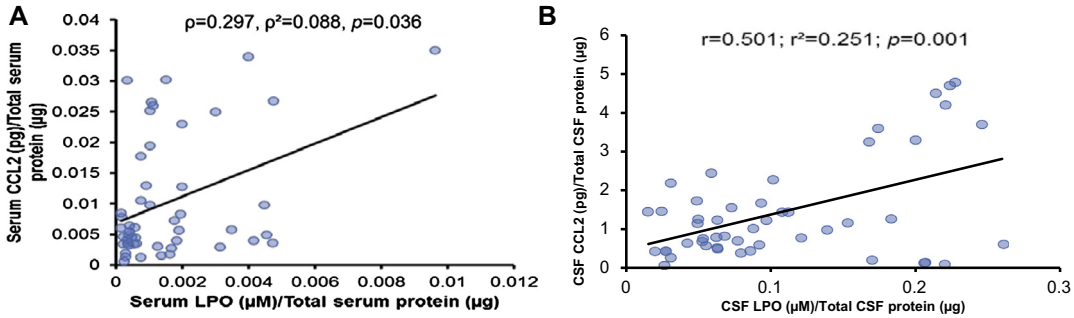

Fig. 3. Correlation of oxidative stress as measured by LPO and inflammation in terms of CCL2 levels in ALS patients. (A) Correlation in serum from ALS patients. Since the data was skewed, nonparametric 2-tailed Spearman rank correlation coefficient (ρ) was used to find correlation among the given variables ($\rho = 0.297$, $\rho^2 = 0.088$). A significant and positive correlation has been observed between LPO and CCL2 in serum samples ($p = 0.036$). (B) Correlation of LPO and CCL2 in CSF from ALS patients. Parametric 2-tailed Pearson product-moment correlation coefficient (r) was used to find level of significance of correlation among the given variables as data was normally distributed ($r = 0.501$, $r^2 = 0.251$). A significant and positive correlation has also been observed between LPO and CCL2 in CSF of patients ($p = 0.0001$). In both (A) and (B), level of significance was measured using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test. ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; CCL2, chemokine ligand-2; LPO, lipid hydroperoxides; pg, pictogram; µM, micromolar; µg, microgram.

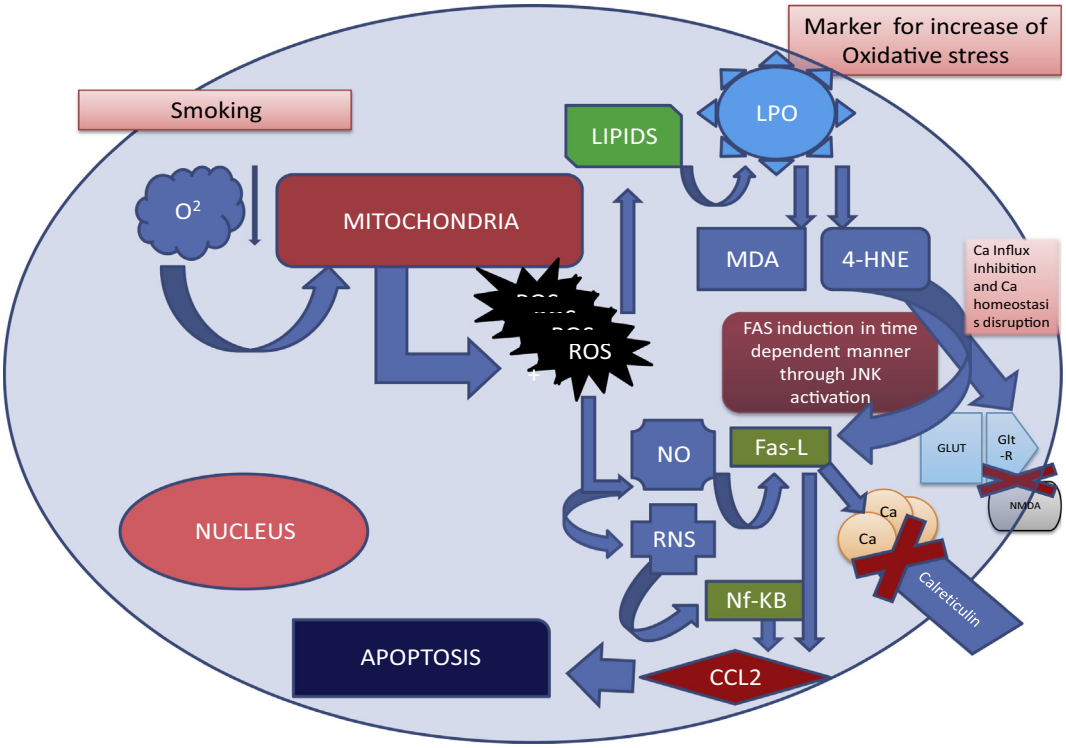

Fig. 4. Alteration in mitochondrial metabolism resulting from oxygen deficits may generate ROS and subsequently LPO which may further impair Glutamate receptor responsible for Ca homeostasis and induce FAS L through activation of JNK pathway. ROS may also activate FAS L and RNS thus activating NFkB induced CCL2 production responsible for apoptosis.

intensity, which would result in increased LPO among ever smokers.

We propose that alteration in mitochondrial metabolism resulting from oxygen deficits may generate ROS and subsequently LPO which may further impair Glutamate receptor responsible for Ca homeostasis and induce FAS L through activation of JNK pathway (Fig. 4). ROS may also activate FAS L and RNS thus activating NFkB induced CCL2 production responsible for apoptosis (Kwiecien et al., 2002; Blanc et al., 1997; Anand et al., 2012a,b; Li et al., 2006; Duplan et al., 2010; Khorooshi et al., 2008; Selam et al., 2006). At this time this remains speculative, at best.

Clinically, the hypothesis directly relating cigarette smoke-induced oxidative stress to the progression of ALS does not find analogy in other neurodegenerative diseases associated with increased oxidative stress such as Parkinson's disease (PD), where smoking improves the clinical outcome. Evidently, mechanisms more specific than general peroxidation of cell membrane lipids could account for specific progression in case of ALS.

5. Conclusion

Even though disease controls have not been included, our preliminary report may suggest a clear pathological non redundant association of LPO with smoker ALS patients. Although not conclusive, our results suggest that cigarette smoke may enhancing LPO levels, however, subsequent larger studies should be undertaken to validate present findings and to unravel the mechanisms by

which smoking increases LPO in Indian ALS patients. Therapies directed at alleviating LPO either by supplementing antioxidants which can particularly target lipid peroxidation or preventing the damaging effects of smoking could be tested in future animal and in vitro studies.

Author contributions

A.A. and P.K.G. contributed equally to this work and are first co-author; S.P. inclusion of patients, grant PI and clinical scoring; P.K.G. data acquisition, statistical analysis, data interpretation and writing of manuscript; A.A. writing of manuscript conception, design of experiment, interpretation of data, grant co PI and editing of manuscript.

Competing interests

Authors have no competing interests.

Financial disclosure

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Ethical approval

Ethical approval was obtained by institute ethical committee, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh 160012, India (No. 7055-PG-1Tg-05/4348-50).

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References

Anand, A., Banik, A., Thakur, K., Masters, C., 2012a. The animal models of dementia and Alzheimer's disease for preclinical testing and translation. Curr. Alzheimer Res. 9, 1010–1029.
Anand, A., Gupta, P.K., Sharma, N.K., Prabhakar, S., 2012b. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. Eur. J. Neurol. 19, 788–792.
Arlt, S., Kontush, A., Zerr, I., et al., 2002. Increased lipid peroxidation in cerebrospinal fluid and plasma from patients with Creutzfeldt–Jakob disease. Neurobiol. Dis. 10, 150–156.
Babu, G.N., Kumar, A., Chandra, R., et al., 2008. Oxidant-antioxidant imbalance in the erythrocytes of sporadic amyotrophic lateral sclerosis patients correlates with the progression of disease. Neurochem. Int. 52, 1284–1289.
Bayir, H., Marion, D.W., Puccio, A.M., et al., 2004. Marked gender effect on lipid peroxidation after severe traumatic brain injury in adult patients. J. Neurotrauma 21, 1–8.
Benedetti, A., Comporti, M., Esterbauer, H., 1980. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochim. Biophys. Acta 620, 281–296.
Blanc, E.M., Kelly, J.F., Mark, R.J., et al., 1997. 4-Hydroxynonenal, an aldehydic product of lipid peroxidation, impairs signal transduction associated with muscarinic acetylcholine and metabotropic glutamate receptors: possible action on G alpha(q/11). J. Neurochem. 69, 570–580.
Boll, M.C., Alcaraz-Zubeldia, M., Montes, S., et al., 2008. Free copper, ferroxidase and SOD1 activities, lipid peroxidation and NO(x) content in the CSF. A different marker profile in four neurodegenerative diseases. Neurochem. Res. 33, 1717–1723.
Burr, I.M., Asayama, K., Fenichel, G.M., 1987. Superoxide dismutases, glutathione peroxidase, and catalase in neuromuscular disease. Muscle Nerve 10, 150–154.
Cedarbaum, J.M., Stambler, N., Malta, E., et al., 1999. The ALSFRS-R, a revised ALS functional rating scale that incorporates assessments of respiratory function. BDNF ALS Study Group (Phase III). J. Neurol. Sci. 169, 13–21.
Corrado, L., D'Alfonso, S., Bergamaschi, L., et al., 2006. SOD1 gene mutations in Italian patients with Sporadic amyotrophic lateral sclerosis (ALS). Neuromuscul. Disord. 16, 800–804.
Cova, E., Cereda, C., Galli, A., et al., 2006. Modified expression of Bcl-2 and SOD1 proteins in lymphocytes from sporadic ALS patients. Neurosci. Lett. 399, 186–190.
Curzio, M., Esterbauer, H., Poli, G., et al., 1987. Possible role of aldehydic lipid peroxidation products as chemoattractants. Int. J. Tissue React. 9, 295–306.
Duplan, L., Bernard, N., Casseron, W., et al., 2010. Collapsin response mediator protein 4a (CRMP4a) is upregulated in motoneurons of mutant SOD1 mice and can trigger motoneuron axonal degeneration and cell death. J. Neurosci. 30, 785–796.
Ferrante, R.J., Browne, S.E., Shinobu, L.A., et al., 1997. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J. Neurochem. 69, 2064–2074.
Frutiger, K., Lukas, T.J., Gorrie, G., et al., 2008. Gender difference in levels of Cu/Zn superoxide dismutase (SOD1) in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. 9, 184–187.
Gallo, V., Bueno-De-Mesquita, H.B., Vermeulen, R., et al., 2009. Smoking and risk for amyotrophic lateral sclerosis, analysis of the EPIC cohort. Ann. Neurol. 65, 378–385.
Gupta, P.K., Prabhakar, S., Sharma, S., et al., 2011. Vascular endothelial growth factor-A (VEGF-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients. J. Neuroinflamm. 8, 47–55.
Gupta, P.K., Prabhakar, S., Sharma, N.K., Anand, A., 2012a. Possible association between expression of chemokine receptor-2 (CCR2) and amyotrophic lateral sclerosis (ALS) patients of North India. PLoS One 7, e38382.
Gupta, P.K., Prabhakar, S., Sharma, S., Anand, A., 2012b. A predictive model for amyotrophic lateral sclerosis (ALS) diagnosis. J. Neurol. Sci. 312, 68–72.
Ihara, Y., Mori, A., Hayabara, T., et al., 1995. Superoxide dismutase and free radicals in sporadic amyotrophic lateral sclerosis, relationship to clinical data. J Neurol. Sci. 134, 51–56.
Jacobsson, J., Jonsson, P.A., Andersen, P.M., et al., 2001. Superoxide dismutase in CSF from amyotrophic lateral sclerosis patients with and without CuZn-superoxide dismutase mutations. Brain 124, 1461–1466.
Kamel, F., Umbach, D.M., Munsat, T.L., et al., 1999. Association of cigarette smoking with amyotrophic lateral sclerosis. Neuroepidemiology. 18, 194–202.
Khorooshi, R., Babcock, A.A., Owens, T., 2008. NF-kappaB-driven STAT2 and CCL2 expression in astrocytes in response to brain injury. J. Immunol. 181, 7284–7291.
Kwiecien, S., Brzozowski, T., Konturek, P.Ch., et al., 2002. The role of reactive oxygen species in action of nitric oxide-donors on stress-induced gastric mucosal lesions. J. Physiol. Pharmacol. 53, 761–773.
Li, J., Sharma, R., Patrick, B., et al., 2006. Regulation of CD95 (Fas) expression and Fas-mediated apoptotic signalling in HLE B-3 cells by 4-hydroxynonenal. Biochemistry 45, 12253–12264.
Liu, H.N., Sanelli, T., Horne, P., et al., 2009. Lack of evidence of monomer/misfolded superoxide dismutase-1 in sporadic amyotrophic lateral sclerosis. Ann. Neurol. 66, 75–80.
Luigetti, M., Conte, A., Madia, F., et al., 2009. Heterozygous SOD1 D90A mutation presenting as slowly progressive predominant upper motor neuron amyotrophic lateral sclerosis. Neurol. Sci. 30, 517–520.
Lundby, C., Pilegaard, H., van Hall, G., et al., 2003. Oxidative DNA damage and repair in skeletal muscle of humans exposed to high-altitude hypoxia. Toxicology 192, 229–236.
Mahboob, M., Rahman, M.F., Grover, P., 2005. Serum lipid peroxidation and antioxidant enzyme levels in male and female diabetic patients. Singapore Med. J. 46, 322–324.
Michaud, S.E., Ménard, C., Guy, L.G., et al., 2003. Inhibition of hypoxia-induced angiogenesis by cigarette smoke exposure, impairment of the HIF-1alpha/VEGF pathway. FASEB J. 17, 1150–1152.
Mokuno, K., Kiyosawa, K., Honda, H., et al., 1996. Elevated serum levels of manganese superoxide dismutase in polymyositis and dermatomyositis. Neurology 46, 1445–1447.
Morrow, J.D., Frei, B., Longmire, A.W., et al., 1995. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N. Engl. J. Med. 332, 1198–11203.
Nalini, A., Thennarasu, K., Gourie-Devi, M., et al., 2008. Clinical characteristics and survival pattern of 1153 patients with amyotrophic lateral sclerosis, experience over 30 years from India. J. Neurol. Sci. 272, 60–70.
Niki, E., Yoshida, Y., Saito, Y., et al., 2005. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun. 338, 668–676.
Oteiza, P.I., Uchitel, O.D., Carrasquedo, F., et al., 1997. Evaluation of antioxidants, protein, and lipid oxidation products in blood from sporadic amyotrophic lateral sclerosis patients. Neurochem. Res. 22, 535–539.
Panse, M., Block, H.U., Förster, W., et al., 1985. An improved malondialdehyde assay for estimation of thromboxane synthase activity in washed human blood platelets. Prostaglandins 30, 1031–1040.
Pedersen, W.A., Fu, W., Keller, J.N., Markesbery, W.R., Appel, S., Smith, R.G., Kasarskis, E., Mattson, M.P., 1998. Protein modification by the lipid peroxidation product 4-hydroxynonenal in the spinal cords of amyotrophic lateral sclerosis patients. Ann. Neurol. 44, 819–824.
Prabhakar, S., Vinish, M., Das, C.P., et al., 2010. Occurrence of PARK2 mutations in a never-smoker population with Parkinson's disease in North India. Neuroepidemiology 35, 152–159.
Przedborski, S., Donaldson, D.M., Murphy, P.L., et al., 1996. Blood superoxide dismutase, catalase and glutathione peroxidase activities in familial and sporadic amyotrophic lateral sclerosis. Neurodegeneration 5, 57–64.

Rukhsana, S., Giovanna, C., Butterfield, D.A., 2013. Biomarkers of oxidative stress in neurodegenerative diseases. In: Villamena, Frederick A. (Ed.), Molecular Basis of Oxidative Stress: Chemistry, Mechanisms, and Disease Pathogenesis. John Wiley & Sons Inc.

Russo, M., Cocco, S., Secondo, A., et al., 2011. Cigarette smoke condensate causes a decrease of the gene expression of Cu–Zn superoxide dismutase, Mn superoxide dismutase, glutathione peroxidase, catalase, and free radical-induced cell injury in SH-SY5Y human neuroblastoma cells. Neurotox. Res. 19, 49–54.

Ryberg, H., Söderling, A.S., Davidsson, P., et al., 2004. Cerebrospinal fluid levels of free 3-nitrotyrosine are not elevated in the majority of patients with amyotrophic lateral sclerosis or Alzheimer's disease. Neurochem. Int. 45 (1), 57–62.

Sandra, S., Tihomir, B., Borka, K., et al., 2008. Differential response to lipid peroxidation in male and female mice with age: correlation of antioxidant enzymes matters. Biogerontology 9 (5), 335–343.

Selam, B., Kayisli, U.A., Akbas, G.E., et al., 2006. Regulation of FAS ligand expression by chemokine ligand 2 in human endometrial cells. Biol. Reprod. 75, 203–209.

Shaw, P.J., Ince, P.G., Falkous, G., et al., 1995. Oxidative damage to protein in sporadic motor neuron disease spinal cord. Ann. Neurol. 38, 691–695.

Siciliano, G., D'Avino, C., Del Corona, A., et al., 2002. Impaired oxidative metabolism and lipid peroxidation in exercising muscle from ALS patients. Amyotroph. Lateral Scler. Other Motor Neuron Disord. 3, 57–62.

Simpson, E.P., Henry, Y.K., Henkel, J.S., et al., 2002. Increased lipid peroxidation in sera of ALS patients, a potential biomarker of disease burden. Neurology 62, 1758–1765.

Sonja, W.D.J., Mark, H.B.H., Nadia, A.S., Anneke, J.V.D.K., Marianne, D.V., Helenius, J.S., et al., 2012. Smoking, alcohol consumption, and the risk of amyotrophic lateral sclerosis: a population-based study. Am. J. Epidemiol. 176, 233–239.

Tohgi, H., Abe, T., Yamazaki, K., et al., 1999. Increase in oxidized NO productsandreduction in oxidizedglutathione in cerebrospinal fluid frompatientswithsporadic form ofamyotrophic lateral sclerosis. Neurosci. Lett. 260, 204–206.

vanEs, M.A., Dahlberg, C., Birve, A., et al., 2010. Large-scale SOD1 mutation screening provides evidence for genetic heterogeneity in amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 81, 562–566.

Wang, H., O'Reilly, É.J., Weisskopf, M.G., et al., 2011. Smoking and risk of amyotrophic lateral sclerosis, a pooled analysis of 5 prospective cohorts. Arch. Neurol. 68, 207–213.

Weisskopf, M.G., McCullough, M.L., Calle, E.E., et al., 2004. Prospective study of cigarette smoking and amyotrophic lateral sclerosis. Am. J. Epidemiol. 160, 26–33.

Zetterström, P., Andersen, P.M., Brännström, T., et al., 2011. Misfolded superoxide dismutase-1 in CSF from amyotrophic lateral sclerosis patients. J. Neurochem. 117, 91–99.

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REVIEW ARTICLE

Role of iron in ischemia-induced neurodegeneration: mechanisms and insights

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Abstract Iron is an important micronutrient for neuronal function and survival. It plays an essential role in DNA and protein synthesis, neurotransmission and electron transport chain due to its dual redox states. On the contrary, iron also catalyses the production of free radicals and hence, causes oxidative stress. Therefore, maintenance of iron homeostasis is very crucial and it involves a number of proteins in iron metabolism and transport that maintain the balance. In ischemic conditions large amount of iron is released and this free iron catalyzes production of more free radicals and hence, causing more damage. In this review we have focused on the iron transport and maintenance of iron homeostasis at large and also the effect of imbalance in iron homeostasis on retinal and brain tissue under ischemic conditions. The understanding of the proteins involved in the homeostasis imbalance will help in developing therapeutic strategies for cerebral as well retinal ischemia.

Keywords Iron metabolism · Iron transport · Cerebral ischemia · Retinal ischemia · Dietary iron · Iron chelators

Introduction

Iron is an indispensible component of cellular metabolism as it is a part of various enzyme systems of biological processes. Iron is found in two forms—heme and non-heme. Heme iron is found in hemoglobin, myoglobin and enzymes, where as non-heme iron is found bound to storage and carrier proteins (Conrad and Umbreit 2000). Iron taken in diet is usually excreted by body in the form of sweat, skin shedding or menstruation in case of females. However if excess iron is not actively excreted by the body, it starts accumulating in various tissues (Loh et al. 2009) and because of high potential of free ion to undergo redox reactions it generates highly toxic free radical species. These adverse effects of iron accumulation need to be properly regulated to maintain normal cellular biological machinery and for this reason iron metabolism is tightly regulated and monitored by a variety of proteins.

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Iron transport, metabolism and homeostasis

Iron plays a dual role in eukaryotic physiology. It is known to play an important role in oxygen transport; it acts as a co-factor for many enzymes and is involved in electron transfer. On the contrary, iron is also associated with normal ageing. The iron content increases with age (Martin et al. 1998; Zecca et al. 2004). Iron is an important micronutrient required by retinal tissue as it is involved in cellular metabolism due to its involvement as a cofactor in various enzymes. Retina is an active tissue with photoreceptors discs shedding continuously. To compensate for this shedding, desaturase, an iron containing enzyme, facilitates the replenishment of membrane by extensive biogenesis or membrane formation. Even the second messenger cGMP involved in phototransduction cascade is dependent on iron conjugated enzyme guanylate cyclase for its synthesis (Yefimova et al. 2000, 2002).

The level of iron in the body has to be maintained for its utilization in positive way. Among the two states of iron, ferrous iron is more toxic compared to the ferric iron and in the presence of hydrogen peroxide or molecular oxygen can generate hydroxyl and superoxide free radicals. Consequently,

iron metabolism is analysed at various levels and there are many regulatory proteins that handle the transport and metabolism of iron, such as transferrin, ferritin, and many other similar iron-handling proteins (De Silva et al. 1996).

Different proteins are involved in transport and storage of iron and hence in maintaining homeostasis. Iron transport is maintained by protein transferrin which transports iron from storage sites, such as the liver to the site of utilization. Transferrin protein is an iron transporter that binds to ferric iron and helps in its circulation. It cannot cross the blood retinal barrier; instead it is internalized by receptor mediated phagocytosis. Blood-retinal barrier, therefore, limit the transport of excess iron into the retinal tissue (Thaler et al. 2010). Ferritin is the protein involved in storage of iron. Another protein involved in movement of iron is ferroxidase ceruloplasmin (Cp). Transferrin binds to only oxidized i.e. ferric form of iron and ceruloplasmin promotes the binding of iron to transferrin by converting ferrous form of iron to the ferric form through oxidation (Osaki et al. 1966). These proteins reduce the iron load by transporting the iron from site of accumulation (Patel et al. 2002) (Fig. 1).

Role of iron in mechanisms of ischemic injury

Iron overload has been shown to have adverse effects and plays an important role in ischemic brain damage as well as other neurological disorders, such as, Parkinson's, Alzheimer's and Huntington's diseases (Davalos et al. 2000; Ke and Ming 2003; Millan et al. 2007; Riederer et al. 1989). Apart from being an essential nutrient; iron also has two problems, i.e. it has low solubility and due to its redox nature it can act as a potential agent for toxicity as iron can lead to formation of reactive oxygen species (ROS). As iron is a transition metal with two oxidation states—ferrous (Fe^{2+}) and ferric (Fe^{3+}) the redox property of iron is useful for biological systems as it works in catalysis; but this property is also hazardous. Therefore, iron is always found in reversibly bound state. In normal physiology, the stable form of iron is Fe^{3+}. Brain is highly susceptible to damage caused by oxidative stress due to high lipid content, high oxygen demand, increased ROS production and higher levels of iron (Evans 1993; Hallgren and Sourander 1958; Saeed et al. 2007). An organism has transcriptional and post-transcriptional mechanisms that regulate iron homeostasis (Eisenstein 2000). There are iron-regulatory RNA-binding proteins and contain iron-regulatory elements (IREs) (Muckenthaler et al. 1998). The iron-regulatory proteins (IRP) respond directly to the levels of oxygen and its metabolites. The IRPs contain iron–sulphur clusters and maintain iron homeostasis by regulating the synthesis of proteins that are involved in iron metabolism. These proteins have been shown to have binding for IREs in ferritin mRNA and hence, blocks the translation. In case of transferrin mRNA, the binding of IRP controls its degradation (Klausner et al. 1993). These proteins are mostly involved in regulation of transferring receptors (Donovan et al. 2000; McKie et al. 2000). The exposure of cultured cells to hydrogen peroxide in a study has led to decrease in ferritin levels and increase in transferrin levels (Martins et al. 1995; Pantopoulos and Hentze 1995). The hydrogen peroxide acts by removing the iron–sulphur clusters in IRPs and further enhances the RNA binding capacity (Pantopoulos et al. 1997; Pantopoulos and Hentze 1998).

Earlier the studies on mechanism of ischemia were related to energy failure due to disrupted blood supply to the brain.

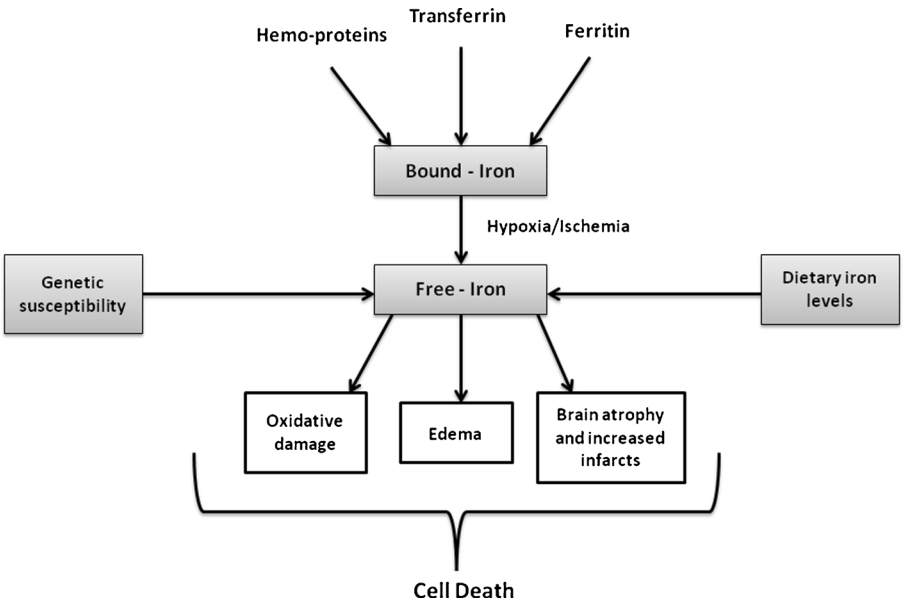

Fig. 1 Schematic depicting various proteins involved in iron metabolism and the cascade to cell death by ischemia. Different proteins that are involved in transport and metabolism of iron and how the increased availability of free-iron after ischemia-reperfusion leads to cell death through oxidative damage, edema and increased infarcts

But the ischemic injury involves many more factors and interactions. The mechanism is complex and multi-factorial. Oxidative stress regulates the inflammation caused after stroke. Increase in oxygen radicals leads to increase in gene expression of pro-inflammatory markers through various transcription factors that are redox sensitive transcription factors such as NF-kappa β (Eltzschig and Carmeliet 2011). There are three known routes of neuronal death after ischemia/reperfusion—excitoxicity, oxidative stress and apoptosis. The oxidative stress caused due to generation of free radicals leads to delayed neuronal death (Won et al. 2002). Mitochondria in the neurons are the site for ATP generation through electron transport chain. Any free electron produced has a potential to form superoxide radicals (Burtke and Sandstrom 1994; Morel and Barouki 1999). In normal physiology, these are removed by various anti-oxidant enzymes (MeGowan et al. 1996). Oxidative stress is the condition caused by the imbalance between the production of free radicals and their timely removal. Thus, the most significant factor involved in pathophysiology of ischemia-reperfusion injury is the reactive oxygen species (ROS) (Dixon and Stockwell 2014). The reactive oxygen species are derived from molecular oxygen with less than four electrons, for example superoxide anion, hydrogen peroxide, hydroxyl radical (Grisham and McCord 1986; Halliwell and Gutteridge 1989). The free-radicals are produced through the following reactions, such as mitochondrial oxidation, phospholipid metabolism and proteolytic pathways (Chen et al. 2011; Sims and Muyderman 2010). These reactions leading to formation of reactive oxygen species is a spontaneous but slow reaction. But the presence of transition metals can act as catalyst and thus, can speed up these reactions (Grisham 1993). The support for the involvement of reactive oxygen species in ischemia comes from various previous studies which have shown their presence in ischemic tissues using technique of electron spin resonance (ESR) (Bolli et al. 1989; Kramer et al. 1987). In other studies, use of ROS scavengers or anti-oxidants attenuates the injury caused by ischemia-reperfusion (Downey 1990; Korthuis and Granger 1986). The ROS generation can modify and degrade biomolecules through DNA nicking, membrane lipid peroxidation, protein degradation and thus, alter cellular function (Grisham 1993; Halliwell and Gutteridge 1989) (Fig. 2). This directly affects the membrane stability by decreasing the fluidity (Kaplan et al. 1995). The ROS generated can also act as or form chemoattractants for neutrophils (Zimmerman et al. 1990). Studies have proposed that ROS act like mediators of apoptosis and initiate the death signaling pathway (Dixon and Stockwell 2014; Sugawara and Chan 2003; Kannan and Jain 2000). Ryu et al. showed apoptosis like features such as condensed nuclear chromatin, TUNEL positive neurons in cultured neuronal cells on exposure to Fe^{2+} (Ryu et al. 1998). The main target of ROS is mitochondrial DNA which encodes enzymes important for electron transport chain and hence, ATP generation. The shutdown of the energy centre of the cell leads to ATP depletion and hence, cell death (Anderson et al. 1981; Ott et al. 2007). Another proposed mechanism behind neuronal cell death caused through ROS generation is through p53 expression, which further activates the Bax and caspase-dependent apoptotic pathway through cytochrome c (Macip et al. 2003). Abnormal balance between the oxidative and protective mechanisms in clinical cases can be a factor in aggravating ischemic injury. SOD levels and vitamin E levels were less in stroke cases and also the stroke cases had higher MDA levels (Kaur et al. 2011).

In ischemic stroke it has been shown that the levels of low molecular weight iron increases during global ischemia caused by elevation of intracranial pressure in canine model. Iron which is tightly bound to transport proteins but during ischemia as the pH decreases leading to release of iron from transferrin and other carrier proteins (Biemond et al. 1988). This low molecular weight free iron acts as a catalyst in free radical reactions (Lipscomb et al. 1998). The iron released is in ferrous state and upon reperfusion is oxidized to ferric state and makes the tissue susceptible to damage (Halliwell and Gutteridge 1989). Thus, metabolite accumulation and decrease in pH plays an important role in release of iron and hence, toxicity. Ischemic stroke reduces the flow of blood to the brain and disrupts the blood–brain barrier. Due to this disturbance in blood flow the iron stored in ferritin and free iron in endothelial cells of capillaries accumulates in region of damage. These elevated levels of iron further support the oxidative stress and aggravate the damage caused (Carbonell and Rama 2007). Millerot-Serrurot et al. also studied the changes in free iron levels after cerebral ischemia with time. The authors subjected rats to photothrombotic ischemic stroke and checked the levels of free iron and levels of ferritin mRNA. The study showed a transient increase in iron levels with time and also that the early treatment with iron chelators results in decreased ischemic damage as compared to later treatment (Millerot-Serrurot et al. 2008). The earlier rescue strategies with respect to ischemia involved early restoration of blood supply to the brain thereby decreasing the extent of injury caused due to ischemia. However the restoration of blood supply or reperfusion is now known to initiate damage secondary to the ischemic injury but more severe than the ischemia itself. The restoration of blood supply exposes the ischemic tissue to the molecular oxygen and thus, the oxidative stress (Bagenholm et al. 1998; McCord 1985). In case of hemorrhagic stroke, erythrocyte lysis in the brain leads to the release of free heme or iron protoporphyrin IX (Fujii et al. 1994). The free heme in the presence of the enzyme heme oxygenase breaks down into ferrous iron and biliverdin along with carbon monoxide. This iron overload caused leads to microglia activation, brain edema, oxidative stress and increase in cytokine production (Gong et al. 2000; Xi et al. 2006).

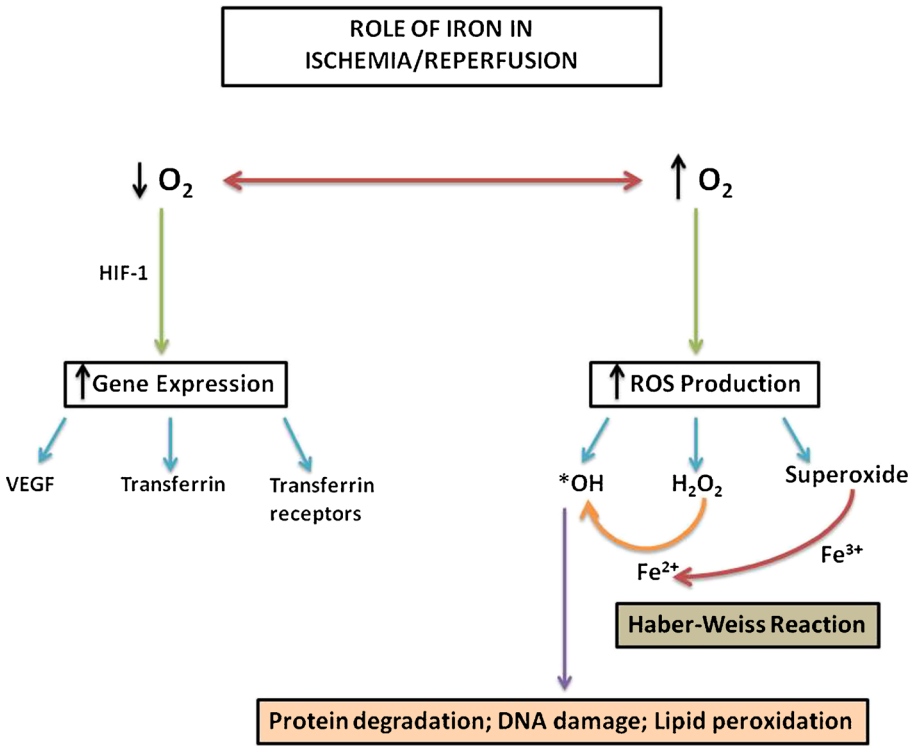

Fig. 2 The role of iron in ischemia/reperfusion injury. The reperfusion or restoration of blood supply after ischemia causes pronounced iron-mediated damage. The available free-iron catalyzes production of reactive-oxygen species through Haber-Weiss reaction, which leads to damage to biomolecules such as DNA, proteins and lipids resulting in cell death

The dietary iron levels are equally important for the structure and function of brain. The optimum intake of iron has been linked with many diseases including neurodegenerative diseases but not many studies have been done to correlate between iron uptake and the risk of stroke. Patt et al. in 1990 showed that the gerbils deprived of iron in their diets were less susceptible to cerebral damage when subjected to ischemia/reperfusion injury through temporary unilateral carotid artery ligation as compared to the respective controls. Perinatal iron deficiency has also been studied as a risk of ischemia and shown to increase vulnerability to ischemic injury. Maternal dietary iron deprivation during gestation in rats has shown greater loss of neuronal activity when exposed to ischemia through carotid artery ligation (Rao et al. 1999). In another study by Castellanos et al. (2002) the authors have shown in the permanent MCAO model in rats that the increase in dietary iron intake increases the infarct volume in iron-fed animals as compared to the control animals by 66 %. As it has also been investigated in various studies the link between sickle-cell anaemia and the risk of occurrence of stroke (Tantawy et al. 2013). In a case report, iron-deficiency anemia was found to be associated with stroke in a pre-menopausal woman. The iron-deficiency results in haemoglobin deficiency which generates hypoxic condition in the brain due to low oxygen supply leading to secondary stroke (Mehta et al. 2012). Most of the young women in India have iron-deficiency anemia and thus, prospective studies seeking to analyze the role of iron as a risk factor for stroke in such women can be investigated. The increase in stroke risk with increase in iron uptake questions the iron supplements being used world-wide to fight anemia. Thus, the maintenance of iron levels is crucial. The iron homeostasis shows a very narrow acceptable range; high levels of free iron available leads to damage through catalysis of ROS production and the deficiency of iron leads to damage by causing hypoxia.

Iron toxicity in retina

Iron dysregulation results in retinal degeneration in several neurodegenerative diseases. These neurodegenerative disorders include aceruloplasminemia, traumatic siderosis, Friedreich's ataxia. Similarly, in various mouse models, retinal degeneration is found to be associated with retinal iron homeostasis disturbances (Yefimova et al. 2000, 2002). Due to presence of blood retinal barrier the free circulation of iron in retina is restricted. The intake of iron into the retinal tissue is primarily through the transferrin receptors expressed by retinal pigment cells by the process of receptor mediated phagocytosis (Song and Dunaief 2013). Hadziahmetovic et al. (2011a) have suggested vascular endothelial cells as another possible route for iron entry into the retina. Unsaturated bonds are more liable to free radicals attack thereby propagating a chain of reactions leading to the formation of cytotoxic lipid peroxides. Levels of unsaturated fatty acids are high in retina and thus it is more prone to oxidative stress injury. Increase in level of iron causes increase in lipid peroxidation (Rodríguez Diez et al. 2012). Iron ions produce hydroxyl free radicals, via

Haber Weiss and Fenton reactions, which are toxic to cells. Association of iron load with retinal degeneration has been reported. Double knockout mouse with ferroxide ceruloplasmin and hephaestin knocked out showed excessive iron accumulation in retina resulting in oxidative stress leading to retinal degeneration (Hadziahmetovic et al. 2008). Retinal ischemia resulting in activation of oxidative enzymes and thereby causing oxidative stress is a major risk factor in various optic neuropathies.

In-vitro studies on mixed retinal cells have proved the fact that exposure of neuronal cells to ferrous or ferric chloride results in death of neurons due to oxidative stress. The study further demonstrated that neuronal cells are more susceptible to iron than photoreceptor or glial cells (Song and Yoon 1998). In the retina, iron stress has been correlated with age-related macular degeneration (Wong et al. 2007). Elevated levels of iron with age indicates role of iron with other age-related retinal disorders (Hahn et al. 2006). Further iron-related proteins have also been observed to increase in glaucomatous eyes (Farkas et al. 2004).

Hemochromatosis is a disease related with excessive accumulation of iron in various systemic organs (Pietrangelo 2010). In general retina is been thought to be resistant to changes in iron levels, implying that in hemochromatosis retina is not affected (Martin et al. 2006). However studies has shown that retina is not completely immune-privileged as elevated iron level due to Hfe mutation or cytomegalovirus (CMV) infection in the retina has been observed (Gnana-Prakasam et al. 2011).

Decrease in iron-load as potential therapeutic target

The restoration of oxygen levels when the blood supply to the brain improves leads to further damage again through the formation of free-radical species. As iron is involved in the production of hydroxyl radicals, any method for iron-chelation could work out as a good therapeutic approach to prevent reperfusion injury caused after stroke. Chelating agents are compounds that are used to scavenge metal.

One such chelating agent that has been tested in various animal models of stroke is deferoxamine. This compound binds to free iron in the blood and leads to its removal. It is ferric iron chelator and is in routine use in patients to relieve the iron-overload (Selim et al. 2011). Deferoxamine forms a hexadentate complex with iron and further prevents reactions and damage. Deferoxamine is a known activator of hypoxia-inducible factor-1 (HIF-1) (Wang and Semenza 1993). HIF-1 is a transcriptional factor activated in response to hypoxia (Semenza 2000). Deferoxamine also has been shown to block cell cycle and prevent neuronal cell death (Farinelli and Greene 1996; Katchanov et al. 2001). HIF-1 activation also increases the expression of genes involved in adaptation to hypoxic conditions (Zaman et al. 1999) (Fig. 3). Not all studies support the notion that the use of iron chelators such as deferoxamine lead to a decrease in the extent of damage caused by free-radicals after ischemic injury. 2,2-dipyridyl, another iron chelators has shown contrary results. Caliaperumal et al. have reported in a rat model of intracerebral hemorrhage, that the administration of dipyridyl had no effect on histological as well as behavioural recovery (Caliaperumal et al. 2013). Deferoxamine has indeed shown decrease in levels of iron in the brain after the injury, but the effect on the injury itself and the functional outcome are still doubtful and thus, questioning the clinical translation of the use of this therapy in patients (Auriat et al. 2012).

Iron chelators have also been studied extensively to screen their role as an inducer of ischemic tolerance and prevention of retinal injury. A variety of iron chelators have been reported to rescue from the damage caused by iron in ischemic injury. Iron chelators bind to the free iron and make it unavailable to cause any sort of injury. A possible mechanism behind this may be scavenging of iron from the intravascular space and thereby preventing the damage caused to blood retinal barrier (BRB) by limiting free radical generation by iron. Disruption of BRB would otherwise result in extravagation of intravascular injury mediators which in turn would damage the neural elements. Another mechanism possible may involve extraction of iron from surrounding tissue creating an intravascular iron sink and thus preventing formation of free radicals (Gehlbach and Purple 1994).

Preconditioning results in protection from ischemic damage. It involves the transcriptional activation as well as post translational processing of survival-promoting genes, regulating the pathways involved in metabolism, inflammation, oxidative injury and apoptosis (Gidday 2006). Cytokines, metabolic inhibitors and a variety of pharmacologic treatments can be used as preconditioning agents. Preconditioning in case of ischemic disease will require agents that will ultimately lead to activation of endogenous pathways for survival in that tissue. Administration of deferroxamine (DFX), an iron chelator as a preconditioning stimulus, has shown to provide retinal protection against ischemia (Zhu et al. 2008). Deferroxamine has also shown protective effect against light (Michon et al. 1999) induced as well as ischemia induced retinal degeneration in rodents (Zhu et al. 2008).

Another well studied iron chelator is Deferiprone (DFP). It is found to have ability to cross the much restricted blood–brain barrier and can be easily orally absorbed (Boddaert et al. 2007). Its toxicity in humans has been studied in clinical trials for diabetic nephropathy and primary glomerulonephritis. It was found to be well tolerated by humans and has very less toxic effects (Shah and Rajapurkar 2009). Deferiprone has been reported to reduce the iron load in retinal tissue of mouse and its non-toxicity to retina was also confirmed in animal study (Hadziahmetovic et al. 2011b). Mice exposed to bright

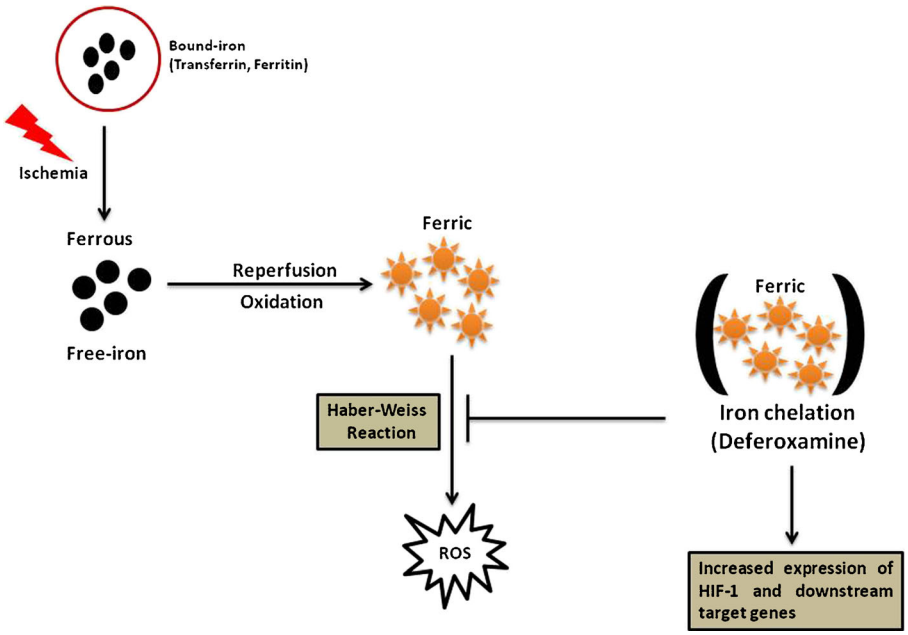

Fig. 3 The schematic shows the neuroprotective action of iron chelators (e.g. Deferoxamine) after ischemia-reperfusion injury. The increased level of free iron after ischemia and oxidation of ferrous to ferric state on reperfusion leads to neuronal injury. Deferoxamine prevents formation of ROS by binding to free iron and also by increasing the expression of HIF-1 as adaptive mechanism after ischemia

light results in oxidative stress in retina and this is confirmed by the increased RNA levels of ceruplasmin and hemeoxygenase1 genes. Both genes are marker for oxidative stress and there upregulation in light exposed mice indicates the generation of reactive oxygen species in retinal tissue. DFP treated mice showed diminished level of these oxidative stress related genes (Song et al. 2012). Iron chelators bind to the free iron and make it unavailable for the catalyzing activity thereby breaking the vicious cycle of cell death (Lukinova et al. 2009; Reif 1992; Chevion et al. 1993; Rouault and Cooperman 2006).

In vitro studies on ARPE-19 human cell line has proved salicylaldehyde isonicotinoyl hydrazone (SIH) to protect the RPE of retina from death induced by various insults such as hydrogen peroxide, anti-Fas, staurosporine, and exposure to A2E plus blue light by chelating labile iron (Lukinova et al. 2009). RPE cells treated with high levels of iron had shown elevated intracellular iron load. The iron overload results in production of ROS which is directly proportional to time as well as concentration of iron. Further the decreased levels of antioxidant enzymes such as GSH has shown that iron overload in RPE cells lead to imbalance between the reactive species generation and antioxidative potential of cells (Volobоueva et al. 2007).

As ferrous form is more toxic, Cp prevents the oxidative damage caused by the ferrous form by oxidizing the ferrous state of iron into comparatively less harmful ferric form (Gutteridge 1992). Various cells in retina, brain and choroid plexus have been found to express ceruloplasmin to protect the tissue from iron toxicity (Klomp and Gitlin 1996; Patel and David 1997; Patel et al. 2000). Ceruplasmin secreted by hepatocytes is unable to cross the blood–brain barrier and to overcome this problem astrocytes in brain express a different form of Ceruplasmin which is glycosylphosphatidylinositol (GPI)-anchored (Patel and David 1997; Patel et al. 2000). The elevated levels of iron in retinal tissue of Ceruplasmin knockout (Cp−/−) mice confirmed the regulatory role of ceruplasmin in iron homeostasis (Patel et al. 2002).

Selim and Ratan (2004) have reviewed the protective role of tirilazad mesylate and bipyridyl against the ischemic damage caused by iron. While, tirilazad mesylate inhibits the iron dependent lipid peroxidation, bipyridyl on the other hand act as an iron chelator rendering free iron unavailable for lipid peroxidation. Chao et al. (2008) found that animals treated with ferrous sulphate showed dose dependent decrease in a and b wave amplitude of electroretinogram. They suggested that this decrease may be due to altered biochemical processes such as influx of calcium into the retinal cells or •OH production leading to oxidative stress. Further the authors found that Ferulic acid (FA) treatment reverses the effect of ferrous sulphate and improves the electroretinogram signals (Chao et al. 2008). Thus, ferulic acid is another alternative to treat the iron induced retinal disorders.

Future perspectives

Metal ions play important role in neurotoxicity, ischemia and neurodegeneration. Brain and retina have the highest metabolic activity and hence the source of high amount of iron. The maintenance of iron transport, storage and metabolism is therefore critical for tissues of central nervous system. Both iron surplus and deficiency have been found to be associated with neurodegenerative disorders. In ischemia/reperfusion

injury in brain or retina, free iron is released which catalyzes the production of free-radicals and increases the oxidative stress. These free-radicals cause damage to proteins, DNA resulting in cell death.

A better understanding of the molecular mechanisms can provide deeper insight into iron homeostasis and processes of neurodegenerative processes underlying ischemia. New therapeutic strategies against ischemia as well as other neurodegenerative disorders can be screened on the basis of this analysis.

References

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, Colbourne F (2012) Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 234(1):136–143

Bagenholm R, Nilsson UA, Gotborg CW, Kjellme I (1998) Free radicals are formed in the brain of fetal sheep during reperfusion after cerebral ischemia. Pediatr Res 43:271–275

Biemond P, Swaak AJ, van Eijk HG, Koster JF (1988) Superoxide dependent iron release from ferritin in inflammatory diseases. Free Radic Biol Med 4:185–198

Boddaert N, Le QS, Rotig A, Leroy-Willig A, Gallet S, Brunelle F, Sidi D, Thalabard JC, Munnich A, Cabantchik ZI (2007) Selective iron chelation in Friedreich ataxia: biologic and clinical implications. Blood 110:401–408

Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK et al (1989) Direct evidence that oxygen-derived free radicals contribute to post ischemic myocardial dysfunction in the intact dog. Proc Natl Acad Sci U S A 86:4695–4699

Burtke TM, Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. Immunol Today 15:7–10

Caliaperumal J, Wowk S, Jones S, Ma Y, Colbourne F (2013) 2,2 dipyridyl, An iron chelator, does not reduce intracerebral iron toxicity or improve outcome after intracerebral hemorrhagic stroke in rats. Stroke 44, ATP108

Carbonell T, Rama R (2007) Iron, oxidative stress and early neurological deterioration in ischemic stroke. Curr Med Chem 14:857–874

Castellanos M, Puig N, Carbonell Teresa T, Castillo J, Martinez JM, Rama R, Davalos A (2002) Iron intake increases infarct volume after permanent middle cerebral artery occlusion in rats. Brain Res 952:1–6

Chao HM, Chen YH, Liu JH, Lee SM, Lee FL, Chang Y, Yeh PH, Pan WHT, Chi CW, Liu TY, Lui WY, Ho LT, Kuo CD, Lin DE, Chan CC, Yang DM, Lin AMY, Chao FP (2008) Iron-generated hydroxyl radicals kill retinal cells *in vivo*: effect of ferulic acid. Hum Exp Toxicol 27:327–339

Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, Maier CM, Narasimhan P, Goeders CE, Chan PH (2011) Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection. Antioxid Redox Signal 14: 1505–1517

Chevion M, Jiang Y, Har-El R, Berenshtein E, Uretzky G, Kitrossky N (1993) Copper and iron are mobilized following myocardial ischemia: possible predictive criteria for tissue injury. Proc Natl Acad Sci U S A 90:1102–1106

Conrad ME, Umbreit JN (2000) Iron absorption and transport: an update. Am J Hematol 64:287–298

Davalos A, Castillo J, Marrugat J, Fernandez-Real JM, Armengou A, Cacabelos P, Rama R (2000) Body iron stores and early neurologic deterioration in acute cerebral infarction. Neurology 54:1568–1574

de Silva DM, Askwith CC, Kaplan J (1996) Molecular mechanisms of iron uptake in eukaryotes. Physiol Rev 76(1):31–47

Dixon SJ, Stockwell BR (2014) The role of iron and reactive oxygen species in cell death. Nat Chem Biol 10:9–17

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ et al (2000) Positional cloning of zebrafish ferroportin 1 identifies a conserved vertebrate iron transporter. Nature 403:776–781

Downey JM (1990) Free radicals and their involvement during long-term myocardial ischemia and reperfusion. Annu Rev Physiol 52:487–504

Eisenstein RS (2000) Iron regulatory proteins and the molecular control of mammalian iron metabolism. Annu Rev Nutr 20:627–662

Eltzschig HK, Carmeliet P (2011) Hypoxia and inflammation. N Engl J Med 364:656–665

Evans PH (1993) Free radicals in brain metabolism and pathology. Br Med Bull 49(3):577–587

Farinelli SE, Greene LA (1996) Cell cycle blockers mimosine, ciclopirox, and deferoxamine prevent the death of PC12 cells and postmitotic sympathetic neurons after removal of trophic support. J Neurosci 16: 1150–1162

Farkas RH, Chowers I, Hackam AS, Kageyama M, Nickells RW, Otteson DC, Duh EJ, Wang C, Valenta DF, Gunatilaka TL, Pease ME, Quigley HA, Zack DJ (2004) Increased expression of iron-regulating genes in monkey and human glaucoma. Invest Ophthalmol Vis Sci 45:1410–1417

Fujii Y, Tanaka R, Takeuchi S, Koike T, Minakawa T, Sasaki O (1994) Hematoma enlargement in spontaneous intracerebral hemorrhage. J Neurosurg 80:51–57

Gehlbach P, Purple RL (1994) Enhancement of retinal recovery by conjugated deferoxamine after ischemia-reperfusion. Invest Ophthalmol Vis Sci 35:669–676

Gidday JM (2006) Cerebral preconditioning and ischemic tolerance. Nat Rev Neurosci 7:437–448

Gnana-Prakasam JP, Ananth S, Prasad PD, Zhang M, Atherton SS, Martin PM, Smith SB, Ganapathy V (2011) Expression and iron-dependent regulation of succinate receptor GPR91 in retinal pigment epithelium. Invest Ophthalmol Vis Sci 52:3751–3758

Gong C, Hoff JT, Keep RF (2000) Acute inflammatory reaction following experimental intracerebral hemorrhage in rat. Brain Res 871:57–65

Grisham MB (1993) Reactive metabolites of oxygen and nitrogen in biology and medicine. Landes, Texas

Grisham MB, McCord JM (1986) Chemistry and cytotoxicity of reactive oxygen metabolites. In: Taylor AE, Matalon S, Ward PA (eds) Physiology of oxygen radicals. Am Physiol Soc, Bethesda, pp 1–18

Gutteridge JM (1992) Iron and oxygen radicals in brain. Ann Neurol 32: S16–S21

Hadziahmetovic M, Dentchev T, Song Y, Haddad N, He X, Hahn P, Pratico D, Wen R, Harris JL, Lambris JD, Beard J, Dunaief JL (2008) Ceruloplasmin/hephaestin knockout mice model morphologic and molecular features of AMD. Invest Opthalmol Vis Sci 49: 2728–2736

Hadziahmetovic M, Song Y, Wolkow N, Iacovelli J, Kautz L, Roth M (2011a) Bmp6 regulates retinal iron homeostasis and has altered expression in age-related macular degeneration. Am J Pathol 179: 335–348

Hadziahmetovic M, Song Y, Wolkow N, Iacovelli J, Grieco S, Lee J, Lyubarsky A, Pratico D, Connelly J, Spino M, Harris ZL, Dunaief JL (2011b) The oral iron chelator deferiprone protects against iron overload-induced retinal degeneration. Invest Ophthalmol Vis Sci 52:959–968

Hahn P, Ying GS, Beard J, Dunaief JL (2006) Iron levels in human retina: sex difference and increase with age. Neuroreport 17:1803–1806
Hallgren B, Sourander P (1958) The effect of age on the non-haemin iron in the human brain. J Neurochem 3:41–51
Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. Clarendon, Oxford
Kannan K, Jain SK (2000) Oxidative stress and apoptosis. Pathophysiology 7(27):153–163
Kaplan P, Racay P, Lehotsky J, Mezesova V (1995) Change in fluidity of brain endoplasmic reticulum membranes by oxygen free radicals: a protective effect of stobadine, α-tocopherol acetate, and butylated hydroxytoluene. Neurochem Res 20(7):815–820
Katchanov J, Harms C, Gertz K, Hauck L, Waeber C, Hirt L, Priller J, von Harsdorf R, Bruck W, Hortnagl H, Dirnagl U, Bhide PG, Endres M (2001) Mild cerebral ischemia induces loss of cyclindependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci 21:5045–5053
Kaur J, Arora S, Singh B, Thakur LC, Gambhir J, Prabhu KM (2011) Role of oxidative stress in pathophysiology of transient ischemic attack and stroke. Int J Biol Med Res 2(3):611–615
Ke Y, Ming QZ (2003) Iron misregulation in the brain: a primary cause of neurodegenerative disorders. Lancet Neurol 2:246–253
Klausner RD, Rouault TA, Harford JB (1993) Regulating the fate of mRNA: the control of cellular iron metabolism. Cell 72:19–28
Klomp LW, Gitlin JD (1996) Expression of the ceruloplasmin gene in the human retina and brain: implications for a pathogenic model in aceruloplasminemia. Hum Mol Genet 5:1989–1996
Korthuis RJ, Granger DN (1986) Ischemia-reperfusion injury: role of oxygen derived free radicals. In: Taylor AE, Matalon S, Ward PA (eds) Physiology of oxygen radicals. Am Physiol Soc, Bethesda, pp 217–250
Kramer JH, Arroyo CM, Dickens BF, Weglicki WB (1987) Spin-trapping evidence that graded myocardial ischemia alters post-ischemic superoxide production. Free Radic Biol Med 3:153–159
Lipscomb DC, Gorman LG, Traystman RJ, Hurn P (1998) Low molecular weight iron in cerebral ischemic acidosis *in vivo*. Stroke 29: 487–493
Loh A, Hadziahmetovic M, Dunaief JL (2009) Iron homeostasis and eye disease. Biochim Biophys Acta 1790:637–649
Lukinova N, Iacovelli J, Dentchev T, Wolkow N, Hunter A, Amado D, Ying GS, Sparrow JR, Dunaief JL (2009) Iron chelation protects the retinal pigment epithelial cell line ARPE-19 against cell death triggered by diverse stimuli. Invest Ophthalmol Vis Sci 50:1440–1447
Macip S, Igarashi M, Berggren P, Yu J, Lee SW, Aaronson SA (2003) Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol Cell Biol 23:8576–8585
Martin WR, Ye FQ, Allen PS (1998) Increasing striatal iron content associated with normal aging. Mov Disord 13(2):281–286
Martin PM, Gnana Prakasam JP, Roon P, Smith RG, Smith SB, Ganapathy V (2006) Expression and polarized localization of the hemochromatosis gene product HFE in retinal pigment epithelium. Invest Ophthalmol Vis Sci 47:4238–4244
Martins EAL, Robalino RL, Meneghini R (1995) Oxidative stress induces activation of a cytosolic protein responsible for control of iron uptake. Arch Biochem Biophys 316:128–134
McCord JM (1985) Oxygen-derived free radicals in post-ischemic tissue injury. N Engl J Med 312:159–163
McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K et al (2000) A novel duodenal specific iron-regulated transporter, IREG1, implicated in basolateral transfer of iron to the circulation. Mol Cells 5:299–309
MeGowan AJ, Fernandes RS, Samali A, Cotter TG (1996) Antioxidants and apoptosis. Biochem Soc Trans 24:229–267
Mehta PJ, Chapman S, Jayam-Trouth A, Kurukumbi M (2012) Acute ischemic stroke secondary to iron deficiency anemia: a case report. Case Rep Neurol Med 2012:487080
Michon JJ, Li ZL, Shioura N, Anderson RJ, Tso MO (1999) A comparative study of methods of photoreceptor morphometry. Invest Ophthalmol Vis Sci 32:280–284
Millan M, Sobrino T, Castellanos M, Nombela F, Arenillas JF, Riva E, Cristobo I, Garcia MM, Vivancos J, Serena J, Moro MA, Castillo J, Davalos A (2007) Increased body iron stores are associated with poor outcome after thrombolytic treatment in acute stroke. Stroke 38:90–95
Millerot-Serrurot E, Bertrand N, Mossiat C, Faure P, Prigent-Tessier A, Garnier P, Bejot Y, Giroud M, Beley A, Marie C (2008) Temporal changes in free iron levels after brain ischemia: relevance to the timing of iron chelation therapy in stroke. Neurochem Int 52:1442–1448
Morel Y, Barouki R (1999) Repression of gene expression by oxidative stress. Biochem J 342:481–496
Muckenthaler M, Gray NK, Hentze MW (1998) IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4f. Mol Cells 2:383–388
Osaki S, Johnson DA, Frieden E (1966) The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem 241:2746–2751
Ott M, Gogvadze V, Orrenius S, Zhivotovsky B (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12:913–922
Pantopoulos K, Hentze MW (1995) Rapid responses to oxidative stress mediated by iron regulatory protein. EMBO J 14:2917–2924
Pantopoulos K, Hentze MW (1998) Activation of iron regulatory protein-1 by oxidative stress in vitro. Proc Natl Acad Sci U S A 95:10559–10563
Pantopoulos K, Mueller S, Atzberger A, Ansorge W, Stremmel W et al (1997) Differences in the regulation of iron regulatory protein-1 (IRP-1) by extra- and intracellular oxidative stress. J Biol Chem 272:9802–9808
Patel BN, David S (1997) A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. J Biol Chem 272:20185–20190
Patel BN, Dunn RJ, David S (2000) Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. J Biol Chem 275:4305–4310
Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP, David S (2002) Ceruloplasmin regulates iron levels in the cns and prevents free radical injury. J Neurosci 22(15):6578–6586
Patt A, Horesh IR, Berger EM, Harken AH, Repine JE (1990) Iron depletion or chelation reduces ischaemia/reperfusion-induced edema in gerbil brains. J Pediatr Surg 25:224–228
Pietrangelo A (2010) Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. Gastroenterology 139:393–408
Rao R, de Ungria M, Sullivan D, Wu P, Wobken JD, Nelson CA, Georgieff MK (1999) Perinatal brain iron deficiency increases the vulnerability of rat hippocampus to hypoxic ischemic insult. J Nutr 129:199–206
Reif DW (1992) Ferritin as a source of iron for oxidative damage. Free Radic Biol Med 12:417–427
Riederer P, Sofic E, Rausch WD, Schmidt B, Reynolds GP, Jellinger K, Youdim MB (1989) Transition metals, ferritin, glutathione, and ascorbic acid in Parkinsonian brains. J Neurochem 52:515–520
Rodríguez Diez G, Uranga RM, Mateos MV, Giusto NM, Salvador GA (2012) Differential participation of phospholipase A2 isoforms during iron-induced retinal toxicity. Implications for age-related macular degeneration. Neurochem Int 61:749–758
Rouault TA, Cooperman S (2006) Brain iron metabolism. Semin Pediatr Neurol 13:142–148
Ryu B, Kim EY, Gwag BJ (1998) Oxidative stress is not required for neuronal apoptosis in cortical cell cultures, presented at 28th Annual Meeting of Society for Neuroscience, LA
Saeed SA, Shad KF, Saleem T, Javed F, Khan MU (2007) Some new prospects in the understanding of the molecular basis of the pathogenesis of stroke. Exp Brain Res 182:1–10

Selim MH, Ratan RR (2004) The role of iron neurotoxicity in ischemic stroke. Ageing Res Rev 3:345–353

Selim M, Yeatts S, Goldstein JN, Gomes J, Greenberg S, Morgenstern LB, Schlaug G, Torbey M, Waldman B, Xi G, Palesch Y (2011) Safety and tolerability of deferoxamine mesylate in patients with acute intracerebral hemorrhage. Stroke 42:3067–3074

Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88:1474–1480

Shah SV, Rajapurkar MM (2009) The role of labile iron in kidney disease and treatment with chelation. Hemoglobin 33:378–385

Sims NR, Muyderman H (2010) Mitochondria, oxidative metabolism and cell death in stroke. Biochim Biophys Acta 1802:80–91

Song D, Dunaief JF (2013) Retinal iron homeostasis in health and disease. Front Aging Neurosci 5:1–13

Song J, Yoon YH (1998) Iron induced cytotoxicity in cultured rat retinal neurons. Korean J Ophthalmol 12:77–84

Song D, Song Y, Hadziahmetovic M, Zhong Y, Dunaief JL (2012) Systemic administration of the iron chelator deferiprone protects against light induced photo receptor degeneration in the mouse retina. Free Radic Biol Med 53:64–71

Sugawara T, Chan PH (2003) Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal 5: 597–607

Tantawy AG, Andrawes NG, Adly AM, El-Kady BA, Shalash AS (2013) Retinal changes in children and adolescents with sickle cell disease attending a paediatric hospital in Cairo, Egypt: risk factors and relation to ophthalmic and cerebral blood flow. Trans R Soc Trop Med Hyg 107:205–211

Thaler S, Fiedorowicz M, Rejdak R, Choragiewicz TJ, Sulejczak D, Stopa P, Zarnowski T, Zrenner E, Grieb P, Schuettauf F (2010) Neuroprotective effects of tempol on retinal ganglion cells in a partial optic nerve crush rat model with and without iron load. Exp Eye Res 90:254–260

Volobouevа LA, Killilea DW, Atamna H, Ames BN (2007) N-tert-butyl hydroxylamine, a mitochondrial antioxidant, protects human retinal pigment epithelial cells from iron overload: relevance to macular degeneration. FASEB J 21:4077–4086

Wang GL, Semenza GL (1993) Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood 82:3610–3615

Won SJ, Kim DY, Gwag BJ (2002) Cellular and molecular pathways of ischemic neuronal death. J Biochem Mol Biol 35(1):67–86

Wong RW, Richa DC, Hahn P, Green WR, Dunaief JL (2007) Iron toxicity as a potential factor in AMD. Retina 27:997–1003

Xi G, Keep RF, Hoff JT (2006) Mechanisms of brain injury after intracerebral haemorrhage. Lancet Neurol 5:53–63

Yefimova MG, Jeanny JC, Guillonneau X, Keller N, Nguyen-Legros J, Sergeant C, Guillou F, Courtois Y (2000) Iron, ferritin, transferrin, and transferrin receptor in the adult rat retina. Invest Ophthalmol Vis Sci 41:2343–2351

Yefimova MG, Jeanny JC, Keller N, Sergeant C, Guillonneau X, Beaumont C, Courtois Y (2002) Impaired retinal iron homeostasis associated with defective phagocytosis in Royal College of Surgeons rats. Invest Ophthalmol Vis Sci 43:537–545

Zaman K, Ryu H, Hall D, O'Donovan K, Lin KI, Miller MP, Marquis JC, Baraban JM, Semenza GL, Ratan RR (1999) Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, $p21^{waf1/cip1}$, and erythropoietin. J Neurosci 19(22):9821–9830

Zecca L, Youdim MBH, Riederer P, Connor JR, Crichton RR (2004) Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci 5: 863–873

Zhu Y, Zhang L, Gidday JM (2008) Deferroxamine preconditioning promotes long-lasting retinal ischemic tolerance. J Ocul Pharmacol Ther 24:527–535

Zimmerman BJ, Grisham MB, Granger ON (1990) Role of oxidants in ischemia/reperfusion-induced granulocyte infiltration. Am J Physiol 258:G185–G190

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Review Article

ALS and Oxidative Stress: The Neurovascular Scenario

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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 [*TGFβ's*], and TGF*β* receptors, *αvβ*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

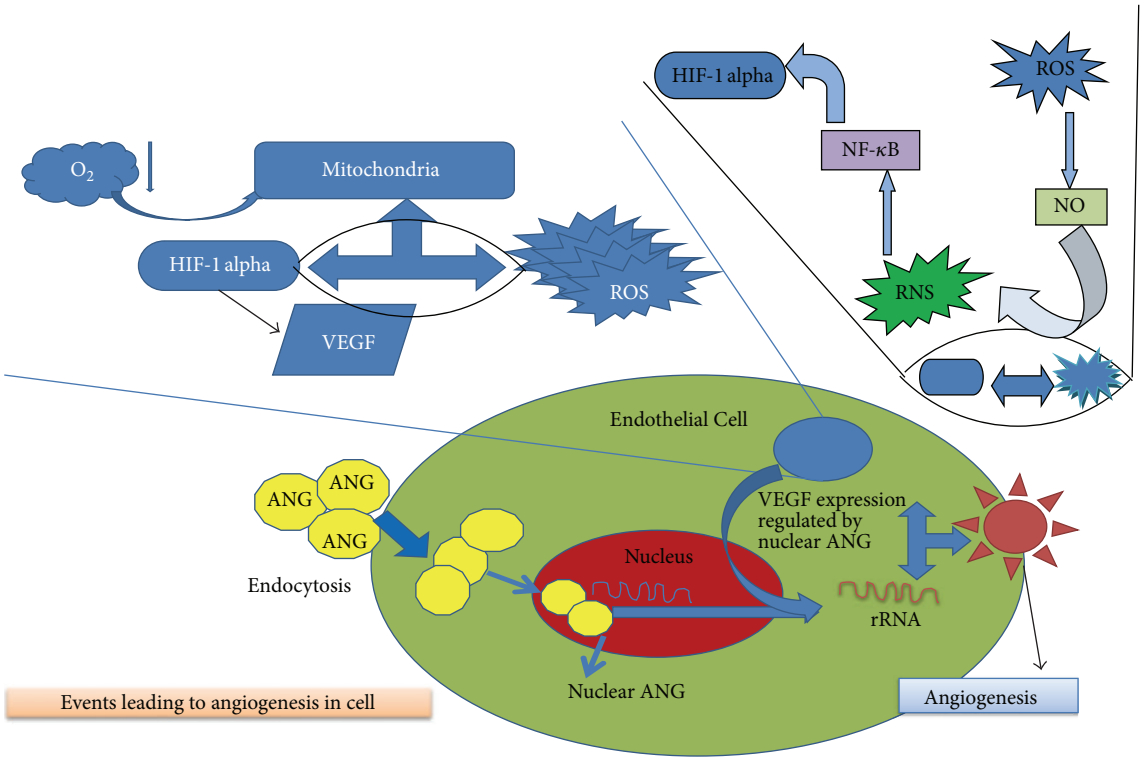

Figure 1: The role of hypoxia in stimulating the *VEGF* through an activation of HIF-1 alpha element. HIF-1 alpha gets activated in deficiency of oxygen in mitochondria leading to creation of oxidative stress. This involves the formation of reactive oxygen species which on reaction with free nitrogen 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

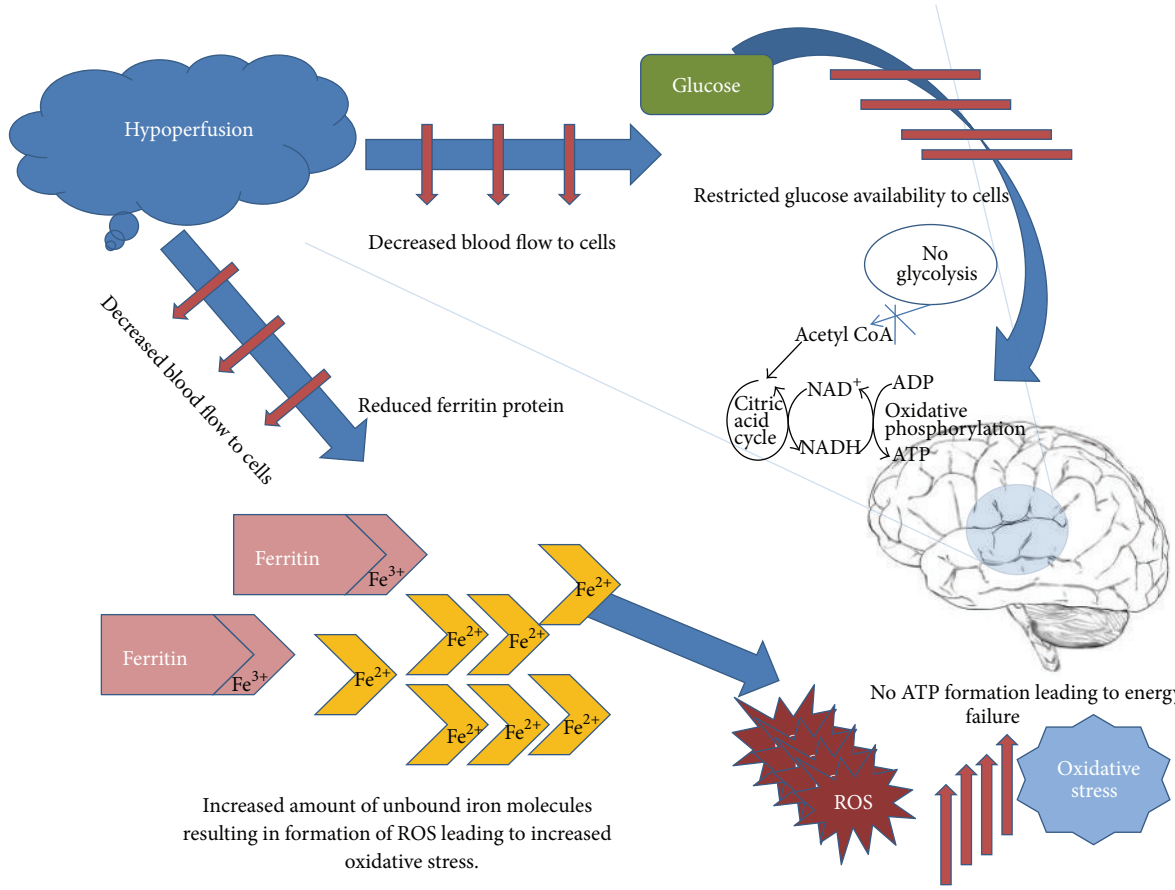

FIGURE 2: Role of hypoperfusion in elevation of oxidative stress and energy failure. As hypoperfusion reduces blood flow towards cells resulting in reduced ferritin Fe^{3+} protein, it releases unbound iron Fe^{2+} molecules resulting in formation of ROS thus increasing the oxidative stress. Hypoperfusion 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*)

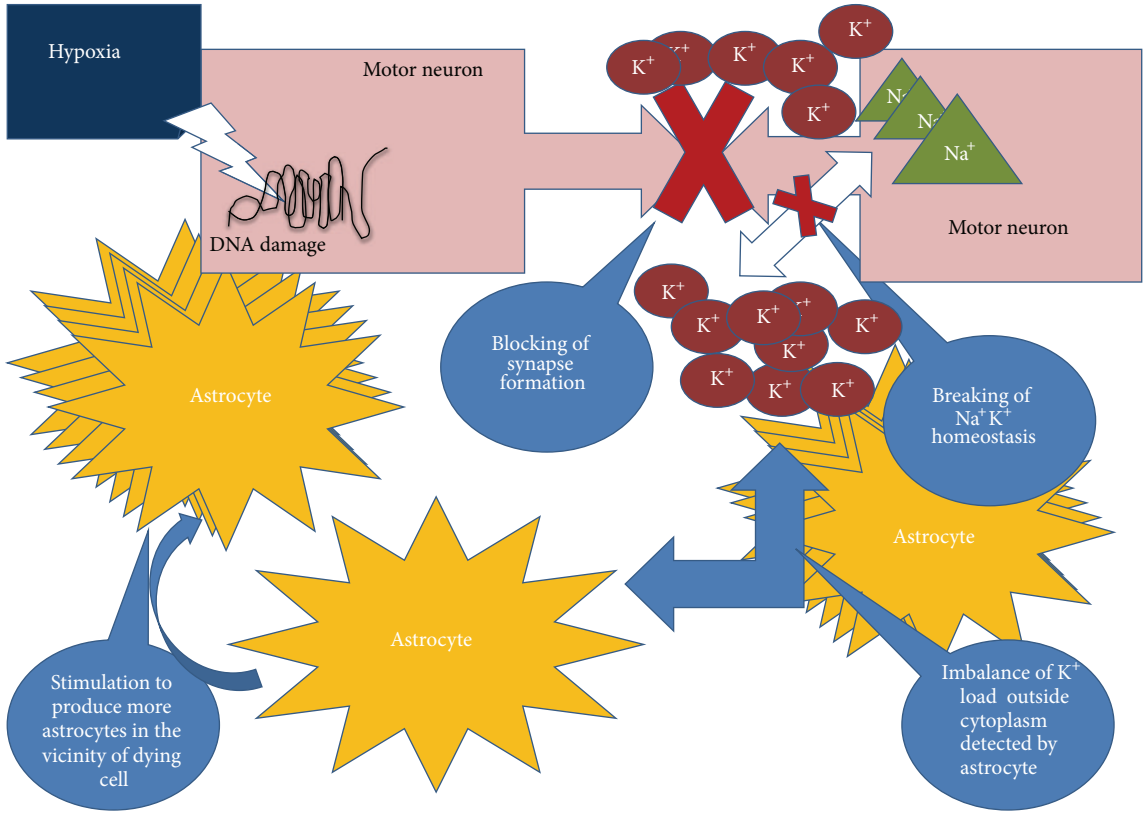

FIGURE 3: Hypoxia induced mobilisation of astrocytes. Astrogliosis is the result of aggressive increase of astrocytes number in the vicinity of damaged neuron cell. Synapse formation is hampered when there is neuronal damage thus leading to breakdown of Na^+K^+ homeostasis. This K^+ concentration is 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)

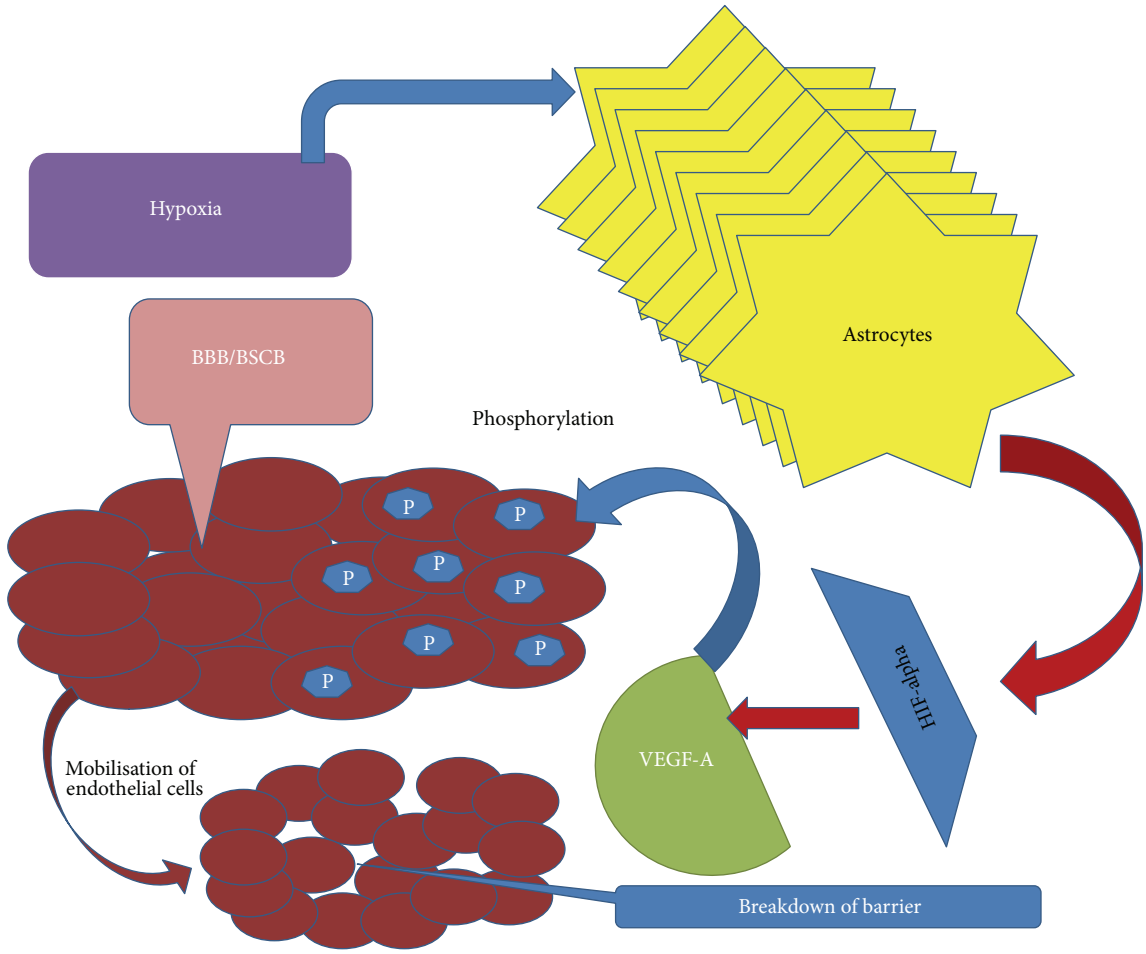

Figure 4: *VEGF* and permeability of blood brain barrier. Astrocytic expression of HIF-alpha and *VEGFA* leads to downregulation of claudins CLN-5 and their regulatory protein OCLN. *VEGFA*, by the virtue of tyrosine phosphorylation, downregulates the expression of CLN ultimately resulting in disruption 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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References

[1] J. M. James, C. Gewolb, and V. L. Bautch, "Neurovascular development uses *VEGF*-A signaling to regulate blood vessel ingression into the neural tube," *Development*, vol. 136, no. 5, pp. 833–841, 2009.

[2] V. L. Bautch and J. M. James, "Neurovascular development: the beginning of a beautiful friendship," *Cell Adhesion and Migration*, vol. 3, no. 2, pp. 199–204, 2009.

[3] J. H. McCarty, "Integrin-mediated regulation of neurovascular development, physiology and disease," *Cell Adhesion and Migration*, vol. 3, no. 2, pp. 211–215, 2009.

[4] D. Lambrechts and P. Carmeliet, "*VEGF* at the neurovascular interface: therapeutic implications for motor neuron disease," *Biochimica et Biophysica Acta*, vol. 1762, no. 11-12, pp. 1109–1121, 2006.

[5] D. C. Dugdale, D. B. Hoch, and D. Zieve, *Amyotrophic Lateral Sclerosis*, A.D.A.M. Medical Encyclopedia, 2010.

[6] B. Oosthuyse, L. Moons, E. Storkebaum et al., "Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration," *Nature Genetics*, vol. 28, no. 2, pp. 131–138, 2001.

[7] K. R. Mills, "Characteristics of fasciculations in amyotrophic lateral sclerosis and the benign fasciculation syndrome," *Brain*, vol. 133, no. 11, pp. 3458–3469, 2010.

[8] G. C. Román, "Neuroepidemiology of amyotrophic lateral sclerosis: clues to aetiology and pathogenesis," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 61, no. 2, pp. 131–137, 1996.

[9] D. R. Rosen, T. Siddique, D. Patterson et al., "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis," *Nature*, vol. 362, no. 6415, pp. 59–62, 1993.

[10] L. E. Cox, L. Ferraiuolo, E. F. Goodall et al., "Mutations in CHMP2B in lower motor neuron predominant amyotrophic lateral sclerosis (ALS)," *PLoS ONE*, vol. 5, no. 3, article e9872, 2010.

[11] P. F. Chance, B. A. Rabin, S. G. Ryan et al., "Linkage of the gene for an autosomal dominant form of juvenile amyotrophic lateral sclerosis to chromosome 9q34," *The American Journal of Human Genetics*, vol. 62, no. 3, pp. 633–640, 1998.

[12] Y. Z. Chen, C. L. Bennett, H. M. Huynh et al., "DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4)," *The American Journal of Human Genetics*, vol. 74, no. 6, pp. 1128–1135, 2004.

[13] A. L. Nishimura, M. Mitne-Neto, H. C. A. Silva et al., "A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis," *The American Journal of Human Genetics*, vol. 75, no. 5, pp. 822–831, 2004.

[14] I. Puls, C. Jonnakuty, B. H. LaMonte et al., "Mutant dynactin in motor neuron disease," *Nature Genetics*, vol. 33, no. 4, pp. 455–456, 2003.

[15] R. Fernández-Santiago, S. Hoenig, P. Lichtner et al., "Identification of novel Angiogenin (ANG) gene missense variants in German patients with amyotrophic lateral sclerosis," *Journal of Neurology*, vol. 256, no. 8, pp. 1337–1342, 2009.

[16] S. Cronin, M. J. Greenway, S. Ennis et al., "Elevated serum angiogenin levels in ALS," *Neurology*, vol. 67, no. 10, pp. 1833–1836, 2006.

[17] K. Kishimoto, S. Yoshida, S. Ibaragi et al., "Hypoxia-induced upregulation of angiogenin, besides *VEGF*, is related to progression of oral cancer," *Oral Oncology*, vol. 48, no. 11, pp. 1120–1127, 2012.

[18] D. Lambrechts, P. Lafuste, P. Carmeliet, and E. M. Conway, "Another angiogenic gene linked to amyotrophic lateral sclerosis," *Trends in Molecular Medicine*, vol. 12, no. 8, pp. 345–347, 2006.

[19] L. H. Barbeito, M. Pehar, P. Cassina et al., "A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis," *Brain Research Reviews*, vol. 47, no. 1–3, pp. 263–274, 2004.

[20] A. Plaitakis and J. T. Caroscio, "Abnormal glutamate metabolism in amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 22, no. 5, pp. 575–579, 1987.

[21] B. K. Kaspar, J. Lladó, N. Sherkat, J. D. Rothstein, and F. H. Gage, "Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model," *Science*, vol. 301, article 5634, pp. 839–842, 2003.

[22] I. Niebroj-Dobosz, Z. Jamrozik, P. Janik, I. Hausmanowa-Petrusewicz, and H. Kwieciński, "Anti-neural antibodies in serum and cerebrospinal fluid of amyotrophic lateral sclerosis (ALS) patients," *Acta Neurologica Scandinavica*, vol. 100, no. 4, pp. 238–243, 1999.

[23] S. J. Murch, P. A. Cox, S. A. Banack, J. C. Steele, and O. W. Sacks, "Occurrence of β-methylamino-L-alanine (BMAA) in. ALS/PDC patients from Guam," *Acta Neurologica Scandinavica*, vol. 110, no. 4, pp. 267–269, 2004.

[24] P. J. Shaw and C. J. Eggett, "Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis," *Journal of Neurology*, vol. 247, supplement 1, pp. I17–I27, 2000.

[25] G. Bensimon, L. Lacomblez, and V. Meininger, "A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group," *The New England Journal of Medicine*, vol. 330, no. 9, pp. 585–591, 1994.

[26] L. Lacomblez, G. Bensimon, P. N. Leigh, P. Guillet, and V. Meininger, "Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II," *The Lancet*, vol. 347, no. 9013, pp. 1425–1431, 1996.

[27] M. H. Yoo, H. J. Hyun, J. Y. Koh, and Y. H. Yoon, "Riluzole inhibits *VEGF*-induced endothelial cell proliferation *in vitro* and hyperoxia-induced abnormal vessel formation *in vivo*," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 12, pp. 4780–4787, 2005.

[28] T. P. Obrenovitch, "Amyotrophic lateral sclerosis, excitotoxicity and riluzole," *Trends in Pharmacological Sciences*, vol. 19, no. 1, pp. 9–11, 1998.

[29] H. Takahashi and M. Shibuya, "The vascular endothelial growth factor (*VEGF*)/*VEGF* receptor system and its role under physiological and pathological conditions," *Clinical Science*, vol. 109, no. 3, pp. 227–241, 2005.

[30] F. Shalaby, J. Rossant, T. P. Yamaguchi et al., "Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice," *Nature*, vol. 376, no. 6535, pp. 62–66, 1995.

[31] S. Hiratsuka, O. Minowa, J. Kuno, T. Noda, and M. Shibuya, "Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9349–9354, 1998.

[32] H. Fujisawa, "Roles of *neuropilin*neuropilin and plexin in the development of nervous system," *Tanpakushitsu kakusan koso*, vol. 42, no. 3, supplement 1, pp. 584–588, 1997.

[33] G. Neufeld, T. Cohen, N. Shraga, T. Lange, O. Kessler, and Y. Herzog, "The neuropilins: multifunctional semaphorin and *VEGF* receptors that modulate axon guidance and angiogenesis," *Trends in Cardiovascular Medicine*, vol. 12, no. 1, pp. 13–19, 2002.

[34] A.-K. Olsson, A. Dimberg, J. Kreuger, and L. Claesson-Welsh, "*VEGF* receptor signalling—in control of vascular function," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 5, pp. 359–371, 2006.

[35] S. Raab and K. H. Plate, "Different networks, common growth factors: shared growth factors and receptors of the vascular and the nervous system," *Acta Neuropathologica*, vol. 113, no. 6, pp. 607–626, 2007.

[36] F. Lennmyr, K. A. Ata, K. Funa, Y. Olsson, and A. Terént, "Expression of vascular endothelial growth factor (*VEGF*) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat," *Journal of Neuropathology and Experimental Neurology*, vol. 57, no. 9, pp. 874–882, 1998.

[37] X. Yang and C. L. Cepko, "Flk-1, a receptor for vascular endothelial growth factor (*VEGF*), is expressed by retinal progenitor cells," *Journal of Neuroscience*, vol. 16, no. 19, pp. 6089–6099, 1996.

[38] J. Iłzecka, "Cerebrospinal fluid vascular endothelial growth factor in patients with amyotrophic lateral sclerosis," *Clinical Neurology and Neurosurgery*, vol. 106, no. 4, pp. 289–293, 2004.

[39] D. P. McCloskey, T. M. Hintz, and H. E. Scharfman, "Modulation of vascular endothelial growth factor (*VEGF*) expression in motor neurons and its electrophysiological effects," *Brain Research Bulletin*, vol. 76, no. 1-2, pp. 36–44, 2008.

[40] E. Bogaert, P. van Damme, K. Poesen et al., "*VEGF* protects motor neurons against excitotoxicity by upregulation of GluR2," *Neurobiology of Aging*, vol. 31, no. 12, pp. 2185–2191, 2010.

[41] D. Devos, C. Moreau, P. Lassalle et al., "Low levels of the vascular endothelial growth factor in CSF from early ALS patients," *Neurology*, vol. 62, no. 11, pp. 2127–2129, 2004.

[42] C. Moreau, D. Devos, V. Brunaud-Danel et al., "Paradoxical response of *VEGF* expression to hypoxia in CSF of patients with ALS," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 77, no. 2, pp. 255–257, 2006.

[43] T. Nagata, I. Nagano, M. Shiote et al., "Elevation of MCP-1 and MCP-1/*VEGF* ratio in cerebrospinal fluid of amyotrophic lateral sclerosis patients," *Neurological Research*, vol. 29, no. 8, pp. 772–776, 2007.

[44] M. Suzuki, T. Watanabe, H. Mikami et al., "Immunohistochemical studies of vascular endothelial growth factor in skin of patients with amyotrophic lateral sclerosis," *Journal of the Neurological Sciences*, vol. 285, no. 1-2, pp. 125–129, 2009.

[45] P. K. Gupta, S. Prabhakar, C. Abburi, N. K. Sharma, and A. Anand, "Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients," *Journal of Neuroinflammation*, vol. 8, article 114, 2011.

[46] P. K. Gupta, S. Prabhakar, S. Sharma, and A. Anand, "Vascular endothelial growth factor-A (*VEGF*-A) and chemokine ligand-2 (CCL2) in amyotrophic lateral sclerosis (ALS) patients," *Journal of Neuroinflammation*, vol. 8, article 97, 2011.

[47] P. K. Gupta, S. Prabhakar, S. Sharma, and A. Anand, "A predictive model for amyotrophic lateral sclerosis (ALS) diagnosis," *Journal of the Neurological Sciences*, vol. 312, no. 1-2, pp. 68–72, 2012.

[48] A. Nalini, K. Thennarasu, M. Gourie-Devi, S. Shenoy, and D. Kulshreshtha, "Clinical characteristics and survival pattern of 1,153 patients with amyotrophic lateral sclerosis: experience over 30 years from India," *Journal of the Neurological Sciences*, vol. 272, no. 1-2, pp. 60–70, 2008.

[49] W. G. Bradley, "Commentary on Professor Stephen Hawking's disability advice," *Annals of Neurosciences*, vol. 16, pp. 101–102, 2009.

[50] A. Anand, P. K. Gupta, N. K. Sharma, and S. Prabhakar, "Soluble VEGF*R*1 (sVEGF*R*1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients," *The European Journal of Neurology*, vol. 19, no. 5, pp. 788–792, 2012.

[51] J. H. P. Skene and D. W. Cleveland, "Hypoxia and lou gehrig," *Nature Genetics*, vol. 28, no. 2, pp. 107–108, 2001.

[52] E. Storkebaum, D. Lambrechts, and P. Carmeliet, "*VEGF*: once regarded as a specific angiogenic factor, now implicated in neuroprotection," *BioEssays*, vol. 26, no. 9, pp. 943–954, 2004.

[53] G. Aliev, M. A. Smith, M. E. Obrenovich, J. C. de la Torre, and G. Perry, "Role of vascular hypoperfusion-induced oxidative stress and mitochondria failure in the pathogenesis of Alzheimer disease," *Neurotoxicity Research*, vol. 5, no. 7, pp. 491–504, 2003.

[54] K. Orino, L. Lehman, Y. Tsuji, H. Ayaki, S. V. Torti, and F. M. Torti, "Ferritin and the response to oxidative stress," *Biochemical Journal*, vol. 357, no. 1, pp. 241–247, 2001.

[55] J. Harned, J. Ferrell, M. M. Lall et al., "Altered ferritin subunit composition: change in iron metabolism in lens epithelial cells and downstream effects on glutathione levels and *VEGF* secretion," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 9, pp. 4437–4446, 2010.

[56] J. S. Henkel, J. I. Engelhardt, S. L. Siklós et al., "Presence of dendritic cells, MCP-1, and activated Microglia/Macrophages in amyotrophic Lateral sclerosis spinal cord tissue," *Annals of Neurology*, vol. 55, no. 2, pp. 221–235, 2004.

[57] C. Maihöfner, S. Probst-Cousin, M. Bergmann, W. Neuhuber, B. Neundörfer, and D. Heuss, "Expression and localization of cyclooxygenase-1 and -2 in human sporadic amyotrophic lateral sclerosis," *The European Journal of Neuroscience*, vol. 18, no. 6, pp. 1527–1534, 2003.

[58] W. G. M. Spliet, E. Aronica, M. Ramkema, J. Aten, and D. Troost, "Increased expression of connective tissue growth factor in amyotrophic lateral sclerosis human spinal cord," *Acta Neuropathologica*, vol. 106, no. 5, pp. 449–457, 2003.

[59] W. G. M. Spliet, E. Aronica, M. Ramkema et al., "Immunohistochemical localization of vascular endothelial growth factor receptors-1, -2 and -3 in human spinal cord: altered expression in amyotrophic lateral sclerosis," *Neuropathology and Applied Neurobiology*, vol. 30, no. 4, pp. 351–359, 2004.

[60] S. Malessa, P. N. Leigh, O. Bertel, E. Sluga, and O. Hornykiewicz, "Amyotrophic lateral sclerosis: glutamate dehydrogenase and transmitter amino acids in the spinal cord," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 54, no. 11, pp. 984–988, 1991.

[61] M. V. Sofroniew, "Molecular dissection of reactive astrogliosis and glial scar formation," *Trends in Neurosciences*, vol. 32, no. 12, pp. 638–647, 2009.

[62] B. A. Barres, "The mystery and magic of Glia: a perspective on their roles in health and disease," *Neuron*, vol. 60, no. 3, pp. 430–440, 2008.

[63] M. V. Sofroniew, "Reactive astrocytes in neural repair and protection," *Neuroscientist*, vol. 11, no. 5, pp. 400–407, 2005.

[64] M. Martineau, G. Baux, and J.-P. Mothet, "Gliotransmission at central glutamatergic synapses: D-serine on stage," *Journal of Physiology*, vol. 1, pp. 211–217, 2006.

[65] Q. Zhang and P. G. Haydon, "Roles for gliotransmission in the nervous system," *Journal of Neural Transmission*, vol. 112, no. 1, pp. 121–125, 2005.

[66] P. Li and M. Zhuo, "Cholinergic, noradrenergic, and serotonergic inhibition of fast synaptic transmission in spinal lumbar dorsal horn of rat," *Brain Research Bulletin*, vol. 54, no. 6, pp. 639–647, 2001.

[67] G. Karpati, G. Klassen, and P. Tanser, "The effects of partial chronic denervation on forearm metabolism," *Canadian Journal of Neurological Sciences*, vol. 6, no. 2, pp. 105–112, 1979.

[68] T. T. Rissanen, I. Vajanto, M. O. Hiltunen et al., "Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Fik-1) in ischemic skeletal muscle and its regeneration," *The American Journal of Pathology*, vol. 160, no. 4, pp. 1393–1403, 2002.

[69] I. Nygren, A. Larsson, A. Johansson, and H. Askmark, "*VEGF* is increased in serum but not in spinal cord from patients with amyotrophic lateral sclerosis," *NeuroReport*, vol. 13, no. 17, pp. 2199–2201, 2002.

[70] K. Vijayalakshmi, P. A. Alladi, T. N. Sathyaprabha, J. R. Subramaniam, A. Nalini, and T. R. Raju, "Cerebrospinal fluid from sporadic amyotrophic lateral sclerosis patients induces degeneration of a cultured motor neuron cell line," *Brain Research*, vol. 1263, pp. 122–133, 2009.

[71] V. Crugnola, C. Lamperti, V. Lucchini et al., "Mitochondrial respiratory chain dysfunction in muscle from patients with amyotrophic lateral sclerosis," *Archives of Neurology*, vol. 67, no. 7, pp. 849–854, 2010.

[72] S. Vielhaber, D. Kunz, K. Winkler et al., "Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis," *Brain*, vol. 123, no. 7, pp. 1339–1348, 2000.

[73] A. Krasnianski, M. Deschauer, S. Neudecker et al., "Mitochondrial changes in skeletal muscle in amyotrophic lateral sclerosis and other neurogenic atrophies," *Brain*, vol. 128, no. 8, pp. 1870–1876, 2005.

[74] B. M. Küst, J. C. W. M. Copray, N. Brouwer, D. Troost, and H. W. G. M. Boddeke, "Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis," *Experimental Neurology*, vol. 177, no. 2, pp. 419–427, 2002.

[75] A. Valavanidis, T. Vlachogianni, and C. Fiotakis, "8-hydroxy-2′ -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis," *Journal of Environmental Science and Health C*, vol. 27, no. 2, pp. 120–139, 2009.

[76] S.-K. Chen, W. A. Hsieh, M.-H. Tsai et al., "Age-associated decrease of oxidative repair enzymes, human 8-oxoguanine DNA glycosylases (*hOGG*1), in human aging," *Journal of Radiation Research*, vol. 44, no. 1, pp. 31–35, 2003.

[77] F. Coppedè, M. Mancuso, A. L. Gerfo et al., "Association of the *hOGG*1 Ser326Cys polymorphism with sporadic amyotrophic lateral sclerosis," *Neuroscience Letters*, vol. 420, no. 2, pp. 163–168, 2007.

[78] A. Brockington, J. Kirby, D. Eggitt et al., "Screening of the regulatory and coding regions of vascular endothelial growth factor in amyotrophic lateral sclerosis," *Neurogenetics*, vol. 6, no. 2, pp. 101–104, 2005.

[79] F. Gros-Louis, S. Laurent, A. A. S. Lopes et al., "Absence of mutations in the hypoxia response element of *VEGF* in ALS," *Muscle and Nerve*, vol. 28, no. 6, pp. 774–775, 2003.

[80] W. Chen, M. Saeed, H. Mao et al., "Lack of association of *VEGF* promoter polymorphisms with sporadic ALS," *Neurology*, vol. 67, no. 3, pp. 508–510, 2006.

[81] P. W. J. van Vught, N. A. Sutedja, J. H. Veldink et al., "Lack of association between *VEGF* polymorphisms and ALS in a Dutch population," *Neurology*, vol. 65, no. 10, pp. 1643–1645, 2005.

[82] Y. Zhang, H. Zhang, Y. Fu et al., "*VEGF* C2578A polymorphism does not contribute to amyotrophic lateral sclerosis susceptibility in sporadic Chinese patients," *Amyotrophic Lateral Sclerosis*, vol. 7, no. 2, pp. 119–122, 2006.

[83] D. Lambrechts, K. Poesen, R. Fernández-Santiago et al., "Meta-analysis of vascular endothelial growth factor variations in amyotrophic lateral sclerosis: increased susceptibility in male carriers of the -2578AA genotype," *Journal of Medical Genetics*, vol. 46, no. 12, pp. 840–846, 2009.

[84] R. Fernández-Santiago, M. Sharma, J. C. Mueller et al., "Possible gender-dependent association of vascular endothelial growth factor (*VEGF*) gene and ALS," *Neurology*, vol. 66, no. 12, pp. 1929–1931, 2006.

[85] N. Oates and R. Pamphlett, "An epigenetic analysis of SOD1 and *VEGF* in ALS," *Amyotrophic Lateral Sclerosis*, vol. 8, no. 2, pp. 83–86, 2007.

[86] A. Brockington, B. Wokke, H. Nixon, J. A. Hartley, and P. J. Shaw, "Screening of the transcriptional regulatory regions of vascular endothelial growth factor receptor 2 (*VEGF*R2) in amyotrophic lateral sclerosis," *BMC Medical Genetics*, vol. 8, article 23, 2007.

[87] L. A. Zil'ber, Z. L. Bajdakova, A. N. Gardašjan, N. V. Konovalov, T. L. Bunina, and E. M. Barabadze, "Study of the etiology of amyotrophic lateral sclerosis," *Bulletin of the World Health Organization*, vol. 29, no. 4, pp. 449–456, 1963.

[88] R. A. Smith, F. M. Balis, K. H. Ott, D. D. Elsberry, M. R. Sherman, and M. G. P. Saifer, "Pharmacokinetics and tolerability of ventricularly administered superoxide dismutase in monkeys and preliminary clinical observations in familial ALS," *Journal of the Neurological Sciences*, vol. 129, supplement 1, pp. 13–18, 1995.

[89] E. Storkebaum, D. Lambrechts, M. Dewerchin et al., "Treatment of motoneuron degeneration by intracerebroventricular delivery of *VEGF* in a rat model of ALS," *Nature Neuroscience*, vol. 8, no. 1, pp. 85–92, 2005.

[90] D. H. Hwang, H. J. Lee, I. H. Park et al., "Intrathecal transplantation of human neural stem cells overexpressing *VEGF* provide behavioral improvement, disease onset delay and survival extension in transgenic ALS mice," *Gene Therapy*, vol. 16, no. 10, pp. 1234–1244, 2009.

[91] D. Lambrechts, E. Storkebaum, M. Morimoto et al., "*VEGF* is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death," *Nature Genetics*, vol. 34, no. 4, pp. 383–394, 2003.

[92] Y. Sun, K. Jin, L. Xie et al., "*VEGF*-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia," *Journal of Clinical Investigation*, vol. 111, no. 12, pp. 1843–1851, 2003.

[93] C. Zheng, I. Nennesmo, B. Fadeel, and J. I. Henter, "Vascular endothelial growth factor prolongs survival in a transgenic mouse model of ALS," *Annals of Neurology*, vol. 56, no. 4, pp. 564–567, 2004.

[94] J. Widenfalk, A. Lipson, M. Jubran et al., "Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury," *Neuroscience*, vol. 120, no. 4, pp. 951–960, 2003.

[95] M. A. Kliem, B. L. Heeke, C. K. Franz et al., "Intramuscular administration of a *VEGF* zinc finger transcription factor activator (*VEGF*-ZFP-TF) improves functional outcomes in SOD1 rats," *Amyotrophic Lateral Sclerosis*, vol. 12, no. 5, pp. 331–339, 2011.

[96] D. F. Silva, D. L. Porto, I. G. A. Araújo et al., "Endothelium-derived nitric oxide is involved in the hypotensive and vasorelaxant effects induced by discretamine in rats," *Pharmazie*, vol. 64, no. 5, pp. 327–331, 2009.

[97] M. Kobari, K. Obara, S. Watanabe, T. Dembo, and Y. Fukuuchi, "Local cerebral blood flow in motor neuron disease: correlation with clinical findings," *Journal of the Neurological Sciences*, vol. 144, no. 1-2, pp. 64–69, 1996.

[98] A. G. Barbeito, L. Martinez-Palma, M. R. Vargas et al., "Lead exposure stimulates *VEGF* expression in the spinal cord and extends survival in a mouse model of ALS," *Neurobiology of Disease*, vol. 37, no. 3, pp. 574–580, 2010.

[99] M. Azzouz, G. S. Ralph, E. Storkebaum et al., "*VEGF* delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model," *Nature*, vol. 429, article 6990, pp. 413–417, 2004.

[100] I. Ay, J. W. Francis, and R. H. Brown Jr., "*VEGF* increases blood-brain barrier permeability to Evans blue dye and tetanus toxin fragment C but not adeno-associated virus in ALS mice," *Brain Research*, vol. 1234, pp. 198–205, 2008.

[101] Z. Zhong, R. Deane, Z. Ali et al., "ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration," *Nature Neuroscience*, vol. 11, no. 4, pp. 420–422, 2008.

[102] V. Silani, M. Braga, A. Ciammola, V. Cardin, and G. Scarlato, "Motor neurones in culture as a model to study ALS," *Journal of Neurology*, vol. 247, supplement 1, pp. I28–I36, 2000.

[103] L. Lu, L. Zheng, L. Viera et al., "Mutant Cu/Zn-superoxide dismutase associated with amyotrophic lateral sclerosis destabilizes vascular endothelial growth factor mRNA and downregulates its expression," *Journal of Neuroscience*, vol. 27, no. 30, pp. 7929–7938, 2007.

[104] A. B. Scandurro and B. S. Beckman, "Common proteins bind mRNAs encoding erythropoietin, tyrosine hydroxylase, and vascular endothelial growth factor," *Biochemical and Biophysical Research Communications*, vol. 246, no. 2, pp. 436–440, 1998.

[105] B. Li, W. Xu, C. Luo, D. Gozal, and R. Liu, "*VEGF*-induced activation of the PI3-K/Akt pathway reduces mutant SOD1-mediated motor neuron cell death," *Molecular Brain Research*, vol. 111, no. 1-2, pp. 155–164, 2003.

[106] J. S. Lunn, S. A. Sakowski, B. Kim, A. A. Rosenberg, and E. L. Feldman, "Vascular endothelial growth factor prevents G93A-SOD1-induced motor neuron degeneration," *Developmental Neurobiology*, vol. 69, no. 13, pp. 871–884, 2009.

[107] M. J. During and L. Cao, "*VEGF*, a mediator of the effect of experience on hippocampal neurogenesis," *Current Alzheimer Research*, vol. 3, no. 1, pp. 29–33, 2006.

[108] H. Meng, Z. Zhang, R. Zhang et al., "Biphasic effects of exogenous *VEGF* on *VEGF* expression of adult neural progenitors," *Neuroscience Letters*, vol. 393, no. 2-3, pp. 97–101, 2006.

[109] A. A. Rizvanov, A. P. Kiyasov, I. M. Gaziziov et al., "Human umbilical cord blood cells transfected with *VEGF* and L1CAM do not differentiate into neurons but transform into vascular endothelial cells and secrete neuro-trophic factors to support neuro-genesis-a novel approach in stem cell therapy," *Neurochemistry International*, vol. 53, no. 6–8, pp. 389–394, 2008.

[110] K. Poesen, D. Lambrechts, P. van Damme et al., "Novel role for vascular endothelial growth factor (*VEGF*) receptor-1 and its ligand *VEGF*-B in motor neuron degeneration," *Journal of Neuroscience*, vol. 28, no. 42, pp. 10451–10459, 2008.

[111] Y. Persidsky, S. H. Ramirez, J. Haorah, and G. D. Kanmogne, "Blood-brain barrier: structural components and function under physiologic and pathologic conditions," *Journal of Neuroimmune Pharmacology*, vol. 1, no. 3, pp. 223–236, 2006.

[112] H. J. Schluesener, R. A. Sobel, C. Linington, and H. L. Weiner, "A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease," *Journal of Immunology*, vol. 139, no. 12, pp. 4016–4021, 1987.

[113] A. Germanò, M. Caffo, F. F. Angileri et al., "NMDA receptor antagonist felbamate reduces behavioral deficits and blood-brain barrier permeability changes after experimental subarachnoid hemorrhage in the rat," *Journal of Neurotrauma*, vol. 24, no. 4, pp. 732–744, 2007.

[114] D. Graesser, A. Solowiej, M. Bruckner et al., "Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice," *Journal of Clinical Investigation*, vol. 109, no. 3, pp. 383–392, 2002.

[115] V. Bartanusz, D. Jezova, B. Alajajian, and M. Digicaylioglu, "The blood-spinal cord barrier: morphology and clinical implications," *Annals of Neurology*, vol. 70, no. 2, pp. 194–206, 2011.

[116] S. Garbuzova-Davis, S. Saporta, E. Haller et al., "Evidence of compromised blood-spinal cord barrier in early and late symptomatic SOD1 mice modeling ALS," *PLoS ONE*, vol. 2, no. 11, article e1205, 2007.

[117] H. Wolburg, K. Wolburg-Buchholz, J. Kraus et al., "Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme," *Acta Neuropathologica*, vol. 105, no. 6, pp. 586–592, 2003.

[118] E. Ambrosini, M. E. Remoli, E. Giacomini et al., "Astrocytes produce dendritic cell-attracting chemokines *in vitro* and in multiple sclerosis lesions," *Journal of Neuropathology and Experimental Neurology*, vol. 64, no. 8, pp. 706–715, 2005.

[119] A. T. Argaw, L. Asp, J. Zhang et al., "Astrocyte derived *VEGF*-A drives blood brain barrier disruption in CNS inflammatory disease," *Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2454–2468, 2012.

[120] S. Esser, M. G. Lampugnani, M. Corada, E. Dejana, and W. Risau, "Vascular endothelial growth factor induces VE-cadherin tyrosine," *Journal of Cell Science*, vol. 111, no. 13, pp. 1853–1865, 1998.

[121] K. Zhang, J. Lu, T. Mori et al., "Baicalin increases *VEGF* expression and angiogenesis by activating the ERRα/PGC-1α pathway," *Cardiovascular Research*, vol. 89, no. 2, pp. 426–435, 2011.

[122] R. B. Vega, J. M. Huss, and D. P. Kelly, "The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes," *Molecular and Cellular Biology*, vol. 20, no. 5, pp. 1868–1876, 2000.

[123] Z. Arany, "PGC-1 coactivators and skeletal muscle adaptations in health and disease," *Current Opinion in Genetics and Development*, vol. 18, no. 5, pp. 426–434, 2008.

[124] Z. Arany, S. Y. Foo, Y. Ma et al., "HIF-independent regulation of *VEGF* and angiogenesis by the transcriptional coactivator PGC-1α," *Nature*, vol. 451, article 7181, pp. 1008–1012, 2008.

[125] R. P. Singh, A. K. Tyagi, S. Dhanalakshmi, R. Agarwal, and C. N. Agarwal, "Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3," *International Journal of Cancer*, vol. 108, no. 5, pp. 733–740, 2004.

[126] M. Kaur, R. P. Singh, M. Gu, R. Agarwal, and C. Agarwal, "Grape seed extract inhibits *in vitro* and *in vivo* growth of human colorectal carcinoma cells," *Clinical Cancer Research*, vol. 12, no. 20, pp. 6194–6202, 2006.

[127] M. Arii, "Chemopreventive effect of grape seed extract on intestinal carcinogenesis in the APCMin mouse," *Proceedings of the American Association for Cancer Research*, vol. 39, article 20, 1998.

[128] J. Lu, K. Zhang, S. Chen, and W. Wen, "Grape seed extract inhibits *VEGF* expression via reducing HIF-1α protein expression," *Carcinogenesis*, vol. 30, no. 4, pp. 636–644, 2009.

[129] C. Desnuelle, M. Dib, C. Garrel, and A. Favier, "A double-blind, placeho-controlled randomized clinical trial of α-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol Study Group," *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, vol. 2, no. 1, pp. 9–18, 2001.

[130] M. Graf, D. Ecker, R. Horowski et al., "High dose vitamin E therapy in amyotrophic lateral sclerosis as add-on therapy to riluzole: results of a placebo-controlled double-blind study," *Journal of Neural Transmission*, vol. 112, no. 5, pp. 649–660, 2005.

[131] R. W. Orrell, R. J. M. Lane, and M. Ross, "A systematic review of antioxidant treatment for amyotrophic lateral sclerosis/motor neuron disease," *Amyotrophic Lateral Sclerosis*, vol. 9, no. 4, pp. 195–211, 2008.

[132] P. Klivenyi, R. J. Ferrante, R. T. Matthews et al., "Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis," *Nature Medicine*, vol. 5, no. 3, pp. 347–350, 1999.

[133] G. J. Groeneveld, J. H. Veldink, I. van der Tweel et al., "A randomized sequential trial of creatine in amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 53, no. 4, pp. 437–445, 2003.

[134] C. Raman, S. D. McAllister, G. Rizvi, S. G. Patel, D. H. Moore, and M. E. Abood, "Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid," *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, vol. 5, no. 1, pp. 33–39, 2004.

[135] P. Weydt, S. Hong, A. Witting, T. Möller, N. Stella, and M. Kliot, "Cannabinol delays symptom onset in SOD1 (G93A) transgenic mice without affecting survival," *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, vol. 6, no. 3, pp. 182–184, 2005.

Predictive Model Proposed

PLOS ONE

RESEARCH ARTICLE

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

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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

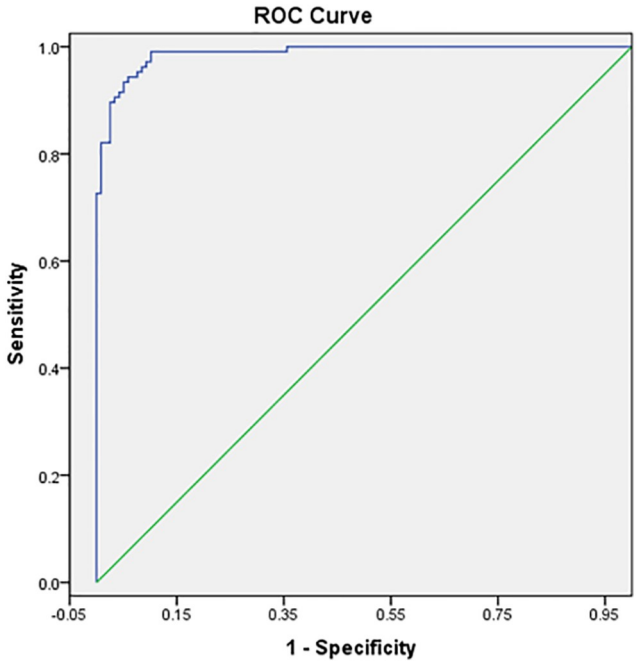

Fig 1. Receiver operating characteristic (ROC) for the forward stepwise binary logistic regression model for predicting ALS risk.

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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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References

1. Talbott E, Malek A, Lacomis D. The epidemiology of amyotrophic lateral sclerosis. In: Handbook of clinical neurology. Vol 138. Elsevier; 2016:225–238.
2. Manjaly ZR, Scott KM, Abhinav K, et al. The sex ratio in amyotrophic lateral sclerosis: a population based study. Amyotrophic Lateral Sclerosis. 2010; 11(5):439–442. https://doi.org/10.3109/17482961003610853 PMID: 20225930
3. Borras-Blasco J, Plaza-Macías I, Navarro-Ruiz A, Peris-Marti J, Anton-Cano A. Riluzole as a treatment for amyotrophic lateral sclerosis. Revista de neurologia. 1998; 27(160):1021–1027. PMID: 9951030
4. Su XW, Simmons Z, Mitchell RM, Kong L, Stephens HE, Connor JR. Biomarker-based predictive models for prognosis in amyotrophic lateral sclerosis. JAMA neurology. 2013 Dec 1; 70(12):1505–11. https://doi.org/10.1001/jamaneurol.2013.4646 PMID: 24145899
5. Magnus T, Beck M, Giess R, Puls I, Naumann M, Toyka KV. Disease progression in amyotrophic lateral sclerosis: predictors of survival. Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine. 2002 May; 25(5):709–14. https://doi.org/10.1002/mus.10090 PMID: 11994965
6. Taylor AA, Fournier C, Polak M, Wang L, Zach N, Keymer M, et al. Pooled Resource Open-Access ALS Clinical Trials Consortium. Predicting disease progression in amyotrophic lateral sclerosis. Annals of Clinical and Translational Neurology. 2016 Nov; 3(11):866–75. https://doi.org/10.1002/acn3.348 PMID: 27844032
7. Ong ML, Tan PF, Holbrook JD. Predicting functional decline and survival in amyotrophic lateral sclerosis. PLoS One. 2017 Apr 13; 12(4):e0174925. https://doi.org/10.1371/journal.pone.0174925 PMID: 28406915
8. Westeneng HJ, Debray TP, Visser AE, van Eijk RP, Rooney JP, Calvo A, et al. Prognosis for patients with amyotrophic lateral sclerosis: development and validation of a personalised prediction model. The Lancet Neurology. 2018 May 1; 17(5):423–33. https://doi.org/10.1016/S1474-4422(18)30089-9 PMID: 29598923
9. Modgil S, Khosla R, Tiwari A, Sharma K, Anand A. Association of Plasma biomarkers for Angiogenesis and Proteinopathy in Indian Amyotrophic Lateral Sclerosis Patients. Journal of Neurosciences in Rural Practice. 2020 Aug 20. https://doi.org/10.1055/s-0040-1714314 PMID: 33144793
10. Khosla R, Rain M, Chawathey S, Modgil S, Tyagi R, Thakur K, et al. Identifying putative cerebrospinal fluid biomarkers of amyotrophic lateral sclerosis in a north Indian population. Muscle & Nerve. 2020 Oct; 62(4):528–33.
11. Gupta PK, Prabhakar S, Sharma S, Anand A. A predictive model for amyotrophic lateral sclerosis (ALS) diagnosis. Journal of the neurological sciences. 2012 Jan 15; 312(1–2):68–72. https://doi.org/10.1016/j.jns.2011.08.021 PMID: 21907354
12. Brooks BR,Miller RG, Swash M, Munsat TL; World Federation of Neurology Research Group onMotor Neuron Diseases. El Escorial revisited:revised criteria for the diagnosis of amyotrophiclateral sclerosis.

Amyotroph Lateral Scler Other Motor Neuron Disord. 2000; 1(5):293–299. https://doi.org/10.1080/146608200300079536 PMID: 11464847

13. Agosta F, Al-Chalabi A, Filippi M, et al. The El Escorial criteria: strengths and weaknesses. Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration. 2015; 16(1–2):1–7. https://doi.org/10.3109/21678421.2014.964258 PMID: 25482030

14. Corcia P, Pradat PF, Salachas F, et al. Causes of death in a post-mortem series of ALS patients. Amyotrophic Lateral Sclerosis. 2008; 9(1):59–62. https://doi.org/10.1080/17482960701656940 PMID: 17924236

15. Renton AE, Chiò A, Traynor BJ. State of play in amyotrophic lateral sclerosis genetics. Nature neuroscience. 2014; 17(1):17. https://doi.org/10.1038/nn.3584 PMID: 24369373

16. Oosthuyse B, Moons L, Storkebaum E, et al. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nature genetics. 2001; 28 (2):131. https://doi.org/10.1038/88842 PMID: 11381259

17. Lambrechts D, Storkebaum E, Morimoto M, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nature genetics. 2003; 34(4):383 https://doi.org/10.1038/ng1211 PMID: 12847526

18. Gupta PK, Prabhakar S, Abburi C, Sharma NK, Anand A. Vascular endothelial growth factor-A and chemokine ligand (CCL2) genes are upregulated in peripheral blood mononuclear cells in Indian amyotrophic lateral sclerosis patients. Journal of neuroinflammation. 2011 Dec 1; 8(1):114. https://doi.org/10.1186/1742-2094-8-114 PMID: 21906274

19. Brockington A, Wharton SB, Fernando M, et al. Expression of vascular endothelial growth factor and its receptors in the central nervous system in amyotrophic lateral sclerosis. Journal of Neuropathology & Experimental Neurology. 2006; 65(1):26–36. https://doi.org/10.1097/01.jnen.0000196134.51217.74 PMID: 16410746

20. Vijayalakshmi K, Ostwal P, Sumitha R, Shruthi S, Varghese AM, Mishra P, et al. Role of VEGF and VEGFR2 receptor in reversal of ALS-CSF induced degeneration of NSC-34 motor neuron cell line. Molecular neurobiology. 2015 Jun 1; 51(3):995–1007. https://doi.org/10.1007/s12035-014-8757-y PMID: 24880751

21. Crivello M, O'riordan SL, Woods I, Cannon S, Halang L, Coughlan KS, et al. Pleiotropic activity of systemically delivered angiogenin in the SOD1G93A mouse model. Neuropharmacology. 2018; 133:503–11. https://doi.org/10.1016/j.neuropharm.2018.02.022 PMID: 29486168

22. Povysheva T, Shmarov M, Logunov D, Naroditsky B, Shulman I, Ogurcov S, et al. Post-spinal cord injury astrocyte-mediated functional recovery in rats after intraspinal injection of the recombinant adenoviral vectors Ad5-VEGF and Ad5-ANG. Journal of neurosurgery Spine. 2017; 27(1):105–15. https://doi.org/10.3171/2016.9.SPINE15959 PMID: 28452633

23. Anand A, Thakur K, Gupta PK. ALS and oxidative stress: the neurovascular scenario. Oxidative medicine and cellular longevity. 2013 Oct; 2013. https://doi.org/10.1155/2013/635831 PMID: 24367722

24. Pollari E, Goldsteins G, Bart G, Koistinaho J, Giniatullin R. The role of oxidative stress in degeneration of the neuromuscular junction in amyotrophic lateral sclerosis. Frontiers in cellular neuroscience. 2014 May 13; 8:131. https://doi.org/10.3389/fncel.2014.00131 PMID: 24860432

25. Akizuki M, Yamashita H, Uemura K, Maruyama H, Kawakami H, Ito H, et al. Optineurin suppression causes neuronal cell death via NF-κB pathway. Journal of neurochemistry. 2013; 126(6):699–704. https://doi.org/10.1111/jnc.12326 PMID: 23721573

26. Winton MJ, Igaz LM, Wong MM, Kwong LK, Trojanowski JQ, Lee VM-Y. Disturbance of nuclear and cytoplasmic Tar DNA binding protein (TDP-43) induces disease-like redistribution, sequestration and aggregate formation. Journal of Biological Chemistry. 2008. https://doi.org/10.1074/jbc.M800342200 PMID: 18305110

27. Nagata T, Nagano I, Shiote M, Narai H, Murakami T, Hayashi T, et al. Elevation of MCP-1 and MCP-1/VEGF ratio in cerebrospinal fluid of amyotrophic lateral sclerosis patients. Neurological research. 2007; 29(8):772–6. https://doi.org/10.1179/016164107X229795 PMID: 17672928

28. Henkel JS, Beers DR, Siklós L, Appel SH. The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS. Molecular and Cellular Neuroscience. 2006; 31(3):427–37. https://doi.org/10.1016/j.mcn.2005.10.016 PMID: 16337133

29. Anand A, Sharma NK, Gupta A, Prabhakar S, Sharma SK, Singh R, et al. Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PloS one. 2012 Nov 21; 7(11):e49905. https://doi.org/10.1371/journal.pone.0049905 PMID: 23185481

30. Sharma NK, Prabhakar S, Gupta A, Singh R, Gupta PK, Gupta PK, et al. New biomarker for neovascular age-related macular degeneration: eotaxin-2. DNA and cell biology. 2012 Nov 1; 31(11):1618–27. https://doi.org/10.1089/dna.2012.1786 PMID: 23025269

31. Anand A, Gupta PK, Sharma NK, Prabhakar S. Soluble VEGFR1 (sVEGFR1) as a novel marker of amyotrophic lateral sclerosis (ALS) in the North Indian ALS patients. European Journal of Neurology. 2012 May; 19(5):788–92. https://doi.org/10.1111/j.1468-1331.2011.03548.x PMID: 21978169

32. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, Anand A. CC chemokine receptor-3 as new target for age-related macular degeneration. Gene. 2013 Jul 1; 523(1):106–11. https://doi.org/10.1016/j.gene.2013.03.052 PMID: 23566847

33. Kamal Sharma N, Gupta A, Prabhakar S, Singh R, Sharma S, Anand A. Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Current neurovascular research. 2012 Nov 1; 9(4):256–65. https://doi.org/10.2174/156720212803530681 PMID: 23030506

34. Sharma NK, Gupta A, Prabhakar S, Singh R, Sharma SK, Chen W, et al. Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. PloS one. 2013 Jul 29; 8(7):e70193. https://doi.org/10.1371/journal.pone.0070193 PMID: 23922956

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Predictive Model for Earlier Diagnosis of Suspected Age-Related Macular Degeneration Patients

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The primary goal of tailored medicine is to presymptomatically identify individuals at high risk for disease using information of each individual's genetic profile and collection of environmental risk factors. Recently, algorithms were given the strong recognition of several replicated risk factors for age-related macular degeneration (AMD), this distant goal is beginning to seem less mysterious. The purpose of the study was to develop a statistical model for AMD. This study includes total 106 subjects. To identify the risk of earlier diagnosis of suspected AMD patients, 22 independent variables were included in the study. Forward stepwise (likelihood ratio) binary logistic regression has been used to find significant variables associated with the risk of AMD. Prediction equation, based on significant risk factors, and model authenticity have been developed. Hosmer–Lemeshow goodness of fit statistic ($\chi^2 = 0.143$, df = 8, $p = 1.0$), which is nonsignificant, indicates the appropriateness of the logistic regression model to predict AMD. After going through stepwise logistic regression, only 6 variables out of the 22 independent variables, namely, serum complement factor H (*CFH*), serum chemokine (C-C motif) ligand 2 (*CCL2*), serum superoxide dismutase 1 (*SOD1*), polymorphism in *CCL2* (rs4586), stress, and comorbidity were found to be significant ($p < 0.05$). The binary logistic regression model is an appropriate tool to predict AMD in the presence of serum *CFH*, serum *CCL2*, serum *SOD1*, polymorphism in CCL2 (rs4586), stress, and comorbidity with high specificity and sensitivity. The area under the receiver operating characteristic curve (0.909, $p = 0.001$) with less standard error of 0.034 and close 95% confidence intervals (0.842–0.976) further validates the model.

Introduction

Many diseases of aging characterized by complex inheritance patterns are progressive; the individual may be asymptomatic in the early stages. One of these diseases, age-related macular degeneration (AMD), is the mainly reason of visual impairment and the most important reason of blindness in old people.

AMD prevalence increases with advancing age in all populations studied. Moreover, it is expected that the cases of early AMD will rise to 17.8 million by 2050 and, if not treated, the cases of late AMD will rise to 3.8 million (Rein *et al.*, 2009). It has been determined that vision loss from AMD decreases the quality of life by 60%, similar to the experience of dealing with a stroke that requires intensive nursing care (Brown *et al.*, 2006). The clinical presentation and natural course of AMD are highly variable.

The clinical symptoms of AMD range from no visual disturbances in early disease to profound loss of central vision in the advanced late stages of the disease. Some patients never progress beyond early AMD; however, in about 6% of Caucasian patients with early stage disease, the condition progresses to an exudative neovascular (or wet form) or geographic atrophic (or dry form) AMD, which threatens vision (Edwards *et al.*, 2005; Hageman *et al.*, 2005; Dewan *et al.*, 2006; DeAngelis *et al.*, 2008). The phenotype is characterized by the development of subretinal choroidal neovascular complexes, hemorrhage, and fibrosis and is typically associated with severe central vision loss (Klein *et al.*, 2004).

In recent years, the information about epidemiologic and genetic associations with AMD has grown widely. There are some modifiable factors like smoking (Francis *et al.*, 2007), omega-3 fatty acid intake (AREDS, 2007a), and nutritional (AREDS, 2007b) antioxidants in addition to age, family history, and ethnicity. Numerous genetic variants with consistent and strong associations with AMD have been identified (Li *et al.*, 2006; Yang *et al.*, 2006; Maller *et al.*, 2007; Seddon *et al.*, 2007; Anand *et al.*, 2012).

Yet, it remains mysterious whether these environmental and genetic factors act jointly or independently and to what

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degree they can predict the incident of AMD or development to advanced AMD from initial stages. Such predictions may be helpful for screening the persons who are at a higher threat because of family history or those having any signs of disease. Some AMD-associated loci have been reported recently, which appear to be moderately associated with AMD risk and replication in additional groups will probably be required to establish their role in AMD pathogenesis (Chen *et al.*, 2010; Neale *et al.*, 2010).

In this study, we assessed the accuracy of a panel of *CFH* (rs1061170) which helps in regulation of complement system, *CCL2* (rs4586, rs1024611), *CCR2* (rs1799865), *CCR3* (rs3091250, rs3091312), single-nucleotide polymorphisms (SNPs), *CCL2*, *CFH*, *SOD1*, and eotaxin2 protein levels with other risk factors to predict the risk of developing AMD. Genes selected in the study were either studied previously in different ethnic populations for involvement in AMD or in other inflammatory diseases or selected due to their apparent functional significance (Seddon *et al.*, 2007; Jia *et al.*, 2010; Anand *et al.*, 2012; Sharma *et al.*, 2012a).

Detection in the early stages might decrease the rising societal load due to AMD by emphasizing and targeting modifiable habits and suggesting more frequent examinations for those who are extremely susceptible. To evaluate and develop such models, we evaluated the comparative involvement of genetic and other risk factors in a well-characterized AMD study population. Hence, effective and early diagnosis of AMD for improved managing of AMD patients needs the improvement of a statistical model.

Materials and Methods

Subjects

One hundred and six case–control samples were recruited from the Advanced Eye Center, PGIMER, Chandigarh, India in the study as per the Institute ethics guidelines (No. Micro/10/1411). The clinical information of participants has been listed in Table 1. A retina surgeon examined all patients and controls for visual acuity measurement and dilated fundus examination. All AMD patients had undergone fluoresce in fundus angiography. The diagnosis of AMD was based on ophthalmoscopic and fluorescein angiographic findings.

Demographic information

Subjects were asked to provide a written informed consent form (Sharma *et al.*, 2012a, 2012b) signed by each participant, containing the written risk factor questionnaire. The information such as age, gender, alcohol consumption, cigarette smoking status, food habit, physical activity, hypertension, diabetes, migraine, comorbidity, body mass index (BMI), and stress as reported by subjects were entered in the data base for investigation. Those who smoked at least 3 cigarettes per day or 54 boxes for at least 6 months were defined as smokers. Those who had chicken, meat, or fish for at least 6 months were defined as nonvegetarian. Information about alcohol use for at least 6 months was also collected. Comorbidities were determined based on the participant's answers to whether a physician had ever told them for diagnosis of any main neurological, cardiovascular, or metabolic illness. Hypertension was defined as diastolic blood pressure ≥ 90 mmHg and systolic blood pressure ≥ 140 mmHg at the time of examination, or detected by a physician previously and whether they had ever taken medications for this condition. Similar practices have been used in earlier studies (Sharma *et al.*, 2012b). Height (in meters) and weight (in kilogram) was measured for each subject to determine the BMI. Based on the value of BMI, subjects were categorized into four classes: (1) under weight: BMI below 18.5, (2) normal: BMI 18.6–24.9, (3) over weight: BMI 25–29.9, and (4) obese: BMI above 30.0. The demography of participants has been reproduced in Table 2. Subjects were also inquired to report any prior diagnosis of diabetes, migraine, and comorbidity. Comorbidity was determined based on the individual's response to whether a physician had ever informed them for finding of any main metabolic, neurological, or cardiovascular diseases.

DNA and serum separation

Eight milliliters of venous blood was collected from all subjects. Four milliliters was added in a serum separator tube (BD Biosciences) for separation of serum. Genomic DNA was extracted from the peripheral venous blood using a commercial kit (Qiagen and Invitrogen) according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay

The quantitative detection of serum for *CCL2*, *CFH*, eotaxin2, and *SOD1* was done by using enzyme-linked immunosorbent assay (ELISA) (Cusabiotech, Catalog no. CSB-E08931h; RayBio, Catalog no. ELH-MCP1-001; RayBio, Catalog no. ELH-Eotaxin2-001; AB Frontier, Catalog no. LF-EK0101) as per the manufacturer's protocol using 680XR Microplate reader (Biorad). All the values were normalized to total serum protein and linear regression analysis was done for standard curve formation.

Real-time PCR

Real-time PCR was used to check the SNP in DNA and was carried out in the 48-well version of Step One™ (Applied Biosystems, Inc.).

Statistical analysis

In binary logistic regression, the dependent variable is dichotomous and independent variables may have

Table 1. Clinical Details of Subjects

Subjects	*Age of onset (years)*	*M/F*	*Disease duration (months)*	*Wet/dry*	*Sporadic/familial*
AMD	64.32 ± 6.9	48/25	25.81 ± 2.6	53/20	67/6
Controls	58.79 ± 14.02	22/11			

Values are mean ± SD, disease duration is the interval between appearance of first symptom of AMD and collection of sample.
AMD, age-related macular degeneration.

Table 2. Demographic Characteristics of Subjects

Subjects	% of vegetarian/ nonvegetarian	% of smokers/ never smoker	% of alcoholic/ nonalcoholic	% of physical activity/no physical activity	% of stress/ no stress	% of diabetes/no diabetes	% of hypertension/ no hypertension	% of migraine/no migraine	% of comorbidity/ no comorbidity
AMD	53.04/46.96	43.48/56.52	32.17/67.83	56.52/43.48	33.33/66.67	18.58/81.42	46.02/53.98	18.26/81.74	73.45/26.55
Controls	56.36/43.64	20/80	30.91/69.09	83.33/16.67	12.73/87.27	5.45/94.55	18.18/81.82	1.19/98.18	18.52/81.48

combinations of continuous and categorical variables. The predicted probability in logistic regression always lies between 0 and 1, which is never the case when we model the probabilities with an ordinary linear regression model. The sudden increase in predicted probability gives an indication that when the threshold point is reached, the risk may increase to a very high level.

Twenty-two independent demographic and biochemical risk factors, which were believed to be associated with AMD, were included in the logistic regression model (Table 3). To predict the risk of AMD, a forward stepwise (likelihood ratio) was carried out in logistic regression. A numerical code (Table 3) was assigned to each qualitative risk factor. The analysis was carried out using statistical product and service solutions (SPSS) version 20.0 software. For analysis purpose, 22 risk factors were named from $X_1,\ldots,X_{22,}$ where X_1: serum *CCL2*; X_2: serum *CFH*; X_3: serum *SOD1*; X_4: serum eotaxin2; X_5: age; X_6: gender; X_7: smoking; X_8: alcohol; X_9: food habit; X_{10}: physical activity; X_{11}: stress; X_{12}: hypertension; X_{13}: diabetes; X_{14}: migraine; X_{15}: comorbidity; X_{16}: BMI; X_{17}: *CCL2*_rs4586; X_{18}: *CCL2*_rs1024611; X_{19}: *CCR2*_rs1799865; X_{20}: *CCR3*_rs3091250; X_{21}: *CCR3*_rs3091312; and X_{22}: *CFH*_rs1061170.

Results

Logistic regression model development

The predicted risk of AMD can be obtained from the equation:

$$P = \frac{1}{1+e^{-Y}}, \quad \text{where } Y = \beta_0 + \beta_1 X_1 + \ldots\ldots\ldots + \beta_{22} X_{22}.$$

The unknown parameter of the logistic regression model, that is, $\beta_0, \beta_1, \ldots, \beta_{22}$ has been estimated using the iterative maximum likelihood method.

We express the logistic regression model in terms of link function in which the model can act as a generalized linear model. This is achieved by taking log odds of the predicted risk, that is,

$$Y = Log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 X_1 + \ldots\ldots\ldots + \beta_{22} X_{22}.$$

Thus, the log odds have a linear relationship.

Table 3. Risk Factor Statistics

	AMD	*Controls*	p-*Value*
Quantitative variables			
Serum *CCL2* (pg/μg)	0.0115 ± 0.0128	0.0033 ± 0.0027	0.0001
Serum *CFH* (pg/μg)	0.0060 ± 0.0040	0.0084 ± 0.0049	0.001
Serum *SOD1* (pg/μg)	5.0568 ± 2.5315	2.6360 ± 1.4853	0.0001
Serum eotaxin2 (pg/μg)	0.0221 ± 0.0158	0.0136 ± 0.0078	0.0001
Age (years)	64.97 ± 7.145	60.38 ± 13.24	0.001
Numerical codes for qualitative variables			
Gender	Female = 1	Male = 0	
Smoking	Smokers = 1	Nonsmokers = 0	
Alcohol	Alcoholic = 1	Nonalcoholic = 0	
Food habit	Vegetarian = 1	Nonvegetarian = 0	
Physical activity	Exercise = 1	No exercise = 0	
Stress	Yes = 1	N0 = 0	
Hypertension	Yes = 1	No = 0	
Diabetes	Yes = 1	No = 0	
Migraine	Yes = 1	No = 0	
Comorbidity	Yes = 1	No = 0	
BMI	Underweight = 0	Normal = 1, overweight = 2	Obese = 3
*CCL2*_rs4586	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2
*CCL2*_rs1024611	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2
*CCR2*_rs1799865	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2
*CCR3*_rs3091250	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2
*CCR3*_rs3091312	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2
*CFH*_rs1061170	Heterozygous = 0	Homozygous_minor = 1	Homozygous_major = 2

BMI, body mass index.

Out of $X_1, \ldots, X_{22}$ independent variables, only six variables, namely, serum *CCL2*, serum *CFH*, serum *SOD1*, polymorphism in *CCL2* (rs4586), stress, and comorbidity were found to be significant ($p < 0.05$).The predicted risk (P) of AMD can be computed with the help of the following equation:

$$P = \frac{1}{1+e^{-Y}}, \quad \text{where } Y = \beta_0 + \beta_1 X_1 + \beta_1 X_2 + \beta_1 X_3 + \beta_1 X_{11} + \beta_1 X_{15} + \beta_1 X_{17} \quad (1)$$

Null hypothesis (H_0): the given data fits well to the logistic model against the alternate hypothesis (H_1): the given data does not fit well to the model that has been tested using the Hosmer–Lemeshow goodness of fit statistic, which indicates a good fit if the significance value is >0.05. For our data, $\chi^2 = 0.143$, df = 8, $p = 1.0$, indicates the appropriateness of the logistic regression model to predict AMD. Since p-value is >0.05, therefore the null hypothesis that given data fits well to the logistic model is accepted. Moreover, according to Omnibus tests of model coefficients, $\chi^2 = 5.692$ and $p = 0.017$, suggest that the forward stepwise (likelihood) procedure of logistic regression is appropriate. The Wald test clearly shows that there are six significant variables (or predictors), which can predict the AMD risk and following logistic regression equation, were obtained for the same (Table 4). The value of Y can be written as

$$Y = 10.266 - 2.27(CCL2\text{rs}4586) - 405.879(\text{serum}CCL2) + 664.16(\text{serum } CFH) - 2.821(\text{serum } SODI) - 3.437(\text{stress}) - 2.605(\text{comorbidity}) \quad (2)$$

The six variables chosen by the model show significant changes in the −2 log-likelihood method as shown in Table 5, which further supports the adequacy of the model. Coefficient of determination (R^2) was computed to check the association of variables in the current model. It is evident from Nagelkerke's $R^2 = 0.879$ value that there is a strong association of selected independent variables with dependent variables (Nagelkerke, 1991). The predicted risk (P) can be obtained from Equation (1), by substituting the value from Equation (2) after specifying the values of six predictors.

Model validation

In a binary logistic model, 94.3% of the original cases have been correctly classified (Table 6). The off diagonal subjects (i.e., four for control and two for AMD) in Table 6 are misclassified cases as predicted by the model. The model yielded 97.3% sensitivity and 87.9% specificity, which indicated the proportion of actual positives, which are correctly identified as positives, and actual negatives, which are correctly identified as negatives. Figure 1 offers an excellent visual performance of the model, based on six predictors. The receiver operating characteristic (ROC) curve and the area under the curve (AUC = 90.9%) suggest that the model works very well with minimum standard error of 0.034 and with a close range of 95% confidence intervals (CI 0.842–0.976).

Table 4. Special Features and Level of Significance of Selected Independent Variables by Maximum Likelihood Method for Logistic Regression Equation

Variable	*β*	*SE*	*Wald*	*df*	p-*Value*
*CCL2*_4586	−2.279	0.899	6.422	1	0.011
CCL2	−405.879	219.345	3.424	1	0.049
CFH	664.168	249.871	7.065	1	0.008
SOD1	−2.821	0.955	8.736	1	0.003
Stress	−3.437	1.740	3.904	1	0.048
Comorbidity	−2.605	1.081	5.806	1	0.016
Constant	10.266	3.363	9.320	1	0.002

SE, standard error.

Table 5. Change in −2 Log-Likelihood

Variable	*Model log-likelihood*	*Change in −2 log-likelihood*	*Df*	*Significance of the change*
*CCL2*_4586	−19.153	10.778	1	0.001
CCL2	−21.093	14.658	1	0.000
CFH	−20.363	13.197	1	0.000
SOD1	−37.347	47.166	1	0.000
Stress	−16.610	5.692	1	0.017
Comorbidity	−17.361	7.194	1	0.007

Df, degree of freedom.

Discussion

Based on a genetic risk score, six variants out of 22 risk factors for AMD were found to be independently associated with AMD, with adjustment for the genetic variants. With this, we expand earlier efforts to guess the genetic risk for AMD (Seddon *et al.*, 2009; Gibson *et al.*, 2010).

Seddon *et al.* (2009) explained a risk score model in four loci of the six genetic variants, also including other factors like smoking, BMI, diet, and age. Likewise, a study from Gibson *et al.* (2010) accounted 0.83 AUC (95% CI 0.81–0.86) using two environmental factors and six SNPs. This study consisted of a case–control study with 106 cases.

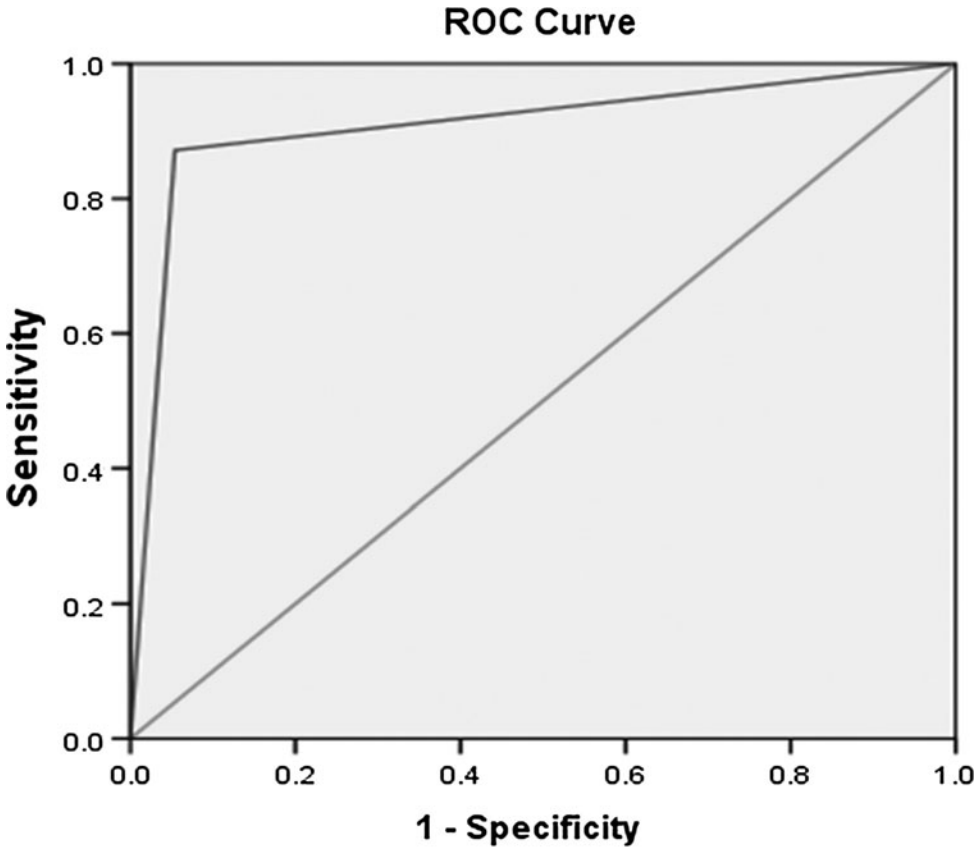
If the area under the ROC curve approaches unity, it means the efficacy of the diagnostic test is very high. In our case, the area under the ROC curve is 0.909, indicating that one can differentiate AMD and control with high accuracy, based on the six predictors, identified by the logistic regression. Moreover, the predicted risk of AMD can be evaluated by specifying the values of six independent significant factors.

Based on six independent variables, we have attempted to present a binary logistic regression equation for those at risk of AMD progression, including either demographic (stress, comorbidity) or molecular (serum *CCL2*, *CCL2*

Table 6. Classification Table

	Predicted		
Observed	*AMD (1)*		*Control (0)*
AMD (1)	71		2
Control (0)	4		29
Overall %		94.3%	

Classification table showing overall % for correct prediction of AMD. Normal control individuals are given an Arabic numeral code "0" and AMD patients were represented as "1."



Area	Std. Error	p value	Asymptotic 95% Confidence Interval
0.909	0.034	0.0001	0.842-0.976

FIG. 1. Receiver operating characteristics obtained from the binary logistic regression model, which generates significant predictors of AMD. The area under the curve is reported to be 90.9% and suggests that the current model is good enough to differentiate AMD individuals from normal controls. AMD, age-related macular degeneration.

polymorphism, serum *CFH*, and serum *SOD1*) predictors, which may be obtained easily by personal consultations or by way of noninvasive assessment of *CFH*, *CCL2*, and *SOD1* using established methods.

Previous molecular studies of AMD are in agreement with our predictions. Low *CFH* may have analogous proinflammatory effects in activation of the innate immune response in AMD (Zipfel *et al.*, 2010). Likewise, the serum *SOD* activity was found significantly higher in AMD patients as compared to controls (Jia *et al.*, 2010). Depressive disorder and comorbidity are significant problem for the elderly afflicted with advanced macular degeneration (Brody *et al.*, 2001).

Both the genetic and demographic risk factors were independently linked with AMD, when measured at the same time.

Risk factors identified by the logistic regression model not only shows their contribution for predicting AMD diagnosis (94.3%) with good specificity and excellent sensitivity as exposed by area under the ROC curve (Fig. 1). For bed side utility and clinical applicability of the model to predict suspected AMD patients, predictive correctness of the present model has to cross authenticated bigger populations, where apart from confirmed AMD patients, persons with different eye disorders should also be measured.

So the development of AMD problems can be slowed by treatment. If the disease development to advanced form is noticed in the beginning in high-risk patients, instant intervention might be necessary to maintain full vision for a more extended time.

Therefore, the individuals at high risk may be recommended to seek clinical follow-ups regularly and might also profit from dietary suggestions, including the intake of omega-3 fatty acids or antioxidants (ARED, 2007a). Recognition of persons at more risk for developing AMD may also facilitate to include defined persons in clinical trials, which may permit a better evaluation of therapeutic effects.

Efficacy of AMD genetic testing will shift forward if the outcome of a predictive test explains in to actionable information for the physician. This study highlights the need to carry on to explore the biology of AMD, to advance our understanding of the genetics linked with the disease and expand these findings in upcoming studies to estimate clinical performance metrics in the acute clinical population diagnosed with early AMD. The genetic test-identifying persons at risk of increasing AMD grab the promise for advance detection through risk-based observation protocols and better outcomes arising from more timely intervention.

In conclusion, the study presents a genetic risk score for AMD from a well identified case–control study underlining huge proportion of the disease elucidated by genetic markers mainly for younger subjects. However, large sample studies are still required to validate model authenticity, which may also include subjects with other eye disorders and AMD-like

symptoms. To some extent, screening would recognize high-risk subjects who would be expected to chase a healthy lifestyle by taking fish and vegetables, not smoking, getting exercise, maintaining weight, and taking antioxidant supplements. These factors are identified to influence the immune and inflammatory pathways, which are known to be involved in the pathogenesis of AMD (Nussenblatt and Ferris 2007). The binary logistic regression model seems to be an appropriate model to predict AMD in the presence of serum *CCL2*, serum *CFH*, serum *SOD1*, polymorphism in *CCL2* (rs4586), stress, and comorbidity with high specificity and sensitivity. The area under the ROC curve (0.909, $p = 0.001$) with less standard error of 0.034 and close 95% (CI 0.842–0.976) further validates the model. As the model was based on six predictors with correct classification of more than 94%, no overfitting or cross validation is required.

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Authors' Contributions

Involved in the design of study (A.A.); conduct of study (A.A., N.K.S.); collection of the data (N.K.S.); management of the data (A.A., N.K.S., A.G., S.P., R.S.); analysis and interpretation of the data (A.A., N.K.S., S.K.S.); preparation of the manuscript (A.A., N.K.S., S.K.S.); and the review and approval of the manuscript (A.A., N.K.S., A.G., S.P., R.S., S.K.S.).

Disclosure Statement

The authors declare that they have no competing interests.

References

Age-Related Eye Disease Study (AREDS) Research Group, SanGiovanni, J.P., Chew, E.Y., Clemons, T.E., Ferris, F.L., Gensler, G., Lindblad, A.S., Milton, R.C., Seddon, J.M., and Sperduto, R.D. (2007a). The relationship of dietary carotenoid and vitamin A, E, and C intake with age related macular degeneration in a case-control study. AREDS Report No. 22. Arch Ophthalmol **125,** 1225–1232.

Age-Related Eye Disease Study (AREDS) Group. (2007b). The relationship of dietary lipid intake and age-related macular degeneration in a case-control study. AREDS Report No. 20. Arch Ophthalmol **125,** 671–679.

Anand, A., Sharma, N.K., Prabhakar, S., Sharma, S., Gupta, A., Singh, R., and Gupta, P.K. (2012). Single nucleotide polymorphisms in MCP-1 and its receptor are associated with the risk of age related macular degeneration. PLoS One **7,** e49905.

Brody, B.L., Gamst, A.C., Williams, R.A., Smith, A.R., Lau, P.W., Dolnak, D., Rapaport, M.H., Kaplan, R.M., and Brown, S.I. (2001). Depression, visual acuity, comorbidity, and disability associated with age-related macular degeneration. Ophthalmology **108,** 1893–900.

Brown, G.C., Brown, M.M., Sharma, S. Joshua, D., Stein, J.D., Roth, Z., Campanella, J., and Beauchamp, G.R. (2006). The burden of age-related macular degeneration, a value-based medicine analysis. Trans Am Ophthalmol Soc **103,** 173–186.

Chen, W., Stambolian, D., Edwards, A.O., *et al.* (2010). Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. Proc Natl Acad Sci U S A **107,** 7401–7406.

DeAngelis, M.M., Ji, F., Adams, S. Morrison, M.A., Harring, A.J., Sweeney, M.O., Capone, A., Jr., Miller, J.W., Dryja, T.P., Ott, J., and Kim, I.K. (2008). Alleles in the HtrA serine peptidase 1 gene alter the risk of neovascular age-related macular degeneration. Ophthalmology **115,** 1209–1215.e7.

Dewan, A., Liu, M., Hartman, S. Zhang, S.S., Liu, D.T., Zhao, C., Tam, P.O., Chan, W.M., Lam, D.S., Snyder, M., Barnstable, C., Pang, C.P., and Hoh, J. (2006). HTRA1 promoter polymorphism in wet age-related macular degeneration. Science **314,** 989–992.

Edwards, A.O., Ritter, R., 3rd, Abel, K.J. Manning, A., Panhuysen, C., and Farrer, L.A. (2005). Complement factor H polymorphism and age-related macular degeneration. Science **308,** 421–424.

Francis, P.J., George, S., Schultz, D.W., Rosner, B., Hamon, S., Ott, J., Weleber, R.G., Klein, M.L., and Seddon, J.M. (2007). The LOC387715 gene, smoking, body mass index, environmental associations with advanced age-related macular degeneration. Hum Hered **63,** 212–218.

Gibson, J., Cree, A., Collins, A., Lotery, A., and Ennis, S. (2010). Determination of a gene and environment risk model for age-related macular degeneration. Br J Ophthalmol **94,** 1382–1387.

Hageman, G.S., Anderson, D.H., Johnson, L.V. Hancox, L.S., Taiber, A.J., Hardisty, L.I., Hageman, J.L., Stockman, H.A., Borchardt, J.D., Gehrs, K.M., Smith, R.J., Silvestri, G., Russell, S.R., Klaver, C.C., Barbazetto, I., Chang, S., Yannuzzi, L.A., Barile, G.R., Merriam, J.C., Smith, R.T., Olsh, A.K., Bergeron, J., Zernant, J., Merriam, J.E., Gold, B., Dean, M., and Allikmets, R. (2005). A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci U S A **102,** 7227–7232.

Jia, L., Dong, Y., Yang, H., Pan, X., Fan, R., and Zhai, L. (2010). Serum superoxide dismutase and malondialdehyde levels in a group of chines patients with age related macular degeneration. Aging Clin Exp Res **23,** 264–267.

Klein, R., Peto, T., Bird, A., and Vannewkirk, M.R. (2004). The epidemiology of age-related macular degeneration. Am J Ophthalmol **137,** 486–495.

Li, M., Atmaca-Sonmez, P., Othman, M., Branham, K.E.H., Khanna, R., Wade, M.S., Li, Y., Liang, L., Zareparsi, S., Swaroop, S., and Gonçalo, R., and Abecasis, G.R. (2006). CFH haplotypes without the Y402H coding variant show strong association with suscepti bility to age-related macular degeneration. Nat Genet **38,** 1049–1054.

Maller, J.B., Fagerness, J.A., Reynolds, R.C., Neale, B.M., Daly, M.J., and Seddon, J.M. (2007). Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat Genet **39,** 1200–1201.

Nagelkerke, N.J. (1991). A note on a general definition of the coefficient of determination. Biometrika **78,** 691–692.

Neale, B.M., Fagerness, J., Reynolds, R. Sobrin, L., Parker, M., Raychaudhuri, S., Tan, P.L., Oh, E.C., Merriam, J.E., Souied, E., Bernstein, P.S., Li, B., Frederick, J.M., Zhang, K., Brantley, M.A., Jr., Lee, A.Y., Zack, D.J., Campochiaro, B., Campochiaro, P., Ripke, S., Smith, R.T., Barile, G.R., Katsanis, N., Allikmets, R., Daly, M.J., and Seddon, J.M. (2010). Genomewide association study of advanced age-related macular degeneration

identifies a role of the hepatic lipase gene (LIPC). Proc Natl Acad Sci U S A **107,** 7395–7400.

Nussenblatt, R.B., and Ferris, F. (2007). Age-related macular degeneration and the immune response, implications for therapy. Am J Ophthalmol **144,** 618–626.

Rein, D.B., Wittenborn, J.S., Zhang, X., Honeycutt, A.A., Lesesne, S.B., and Saaddine, J., Vision Health Cost-Effectiveness Study Group. (2009). Forecasting age-related macular degeneration through the year 2050: the potential impact of new treatment. Arch Ophthalmol **127,** 533–540.

Seddon, J.M., Francis, P.J., George, S., Schultz, D.W., Rosner, B., and Klein, M.L. (2007). Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. JAMA **297,** 1793–1800.

Seddon, J.M., Reynolds, R., Maller, J., Fagerness, J.A., Daly, M.J., and Rosner, B. (2009). Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. Invest Ophthalmol Vis Sci **50,** 2044–2053.

Sharma, N.K., Gupta, A., Prabhakar, S., Singh, R., Sharma, S., and Anand, A. (2012a). Single nucleotide polymorphism and serum levels of VEGFR2 are associated with age related macular degeneration. Curr Neurovasc Res **9,** 256–265.

Sharma, N.K., Prabhakar, S., Gupta, A., Singh, R., Gupta, P.K., Gupta, P.K., and Anand, A. (2012b). New biomarker for neovascular age related macular degeneration: eotaxin-2. DNA Cell Biol **31,** 1618–1628.

Yang, Z., Camp, N.J., Sun, H., Tong, Z., Gibbs, D., Cameron, D.J., Chen, H., Zhao, Y., Pearson, E., Li, X., Chien, J., Dewan, A., Harmon, J., Bernstein, P.S., Shridhar, V., Zabriskie, N.A., Hoh, J., Howes, K., and Zhang, K. (2006). A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. Science **314,** 992–993.

Zipfel, P.F., Lauer, N., and Skerka, C. (2010). The role of complement in AMD. Adv Exp Med Biol **703,** 9–24.

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