Biochemical Analysis and Genetic Polymorphism of Angiogenic Factors and Inflammatory Markers in Pakistani Patients with Age-Related Macular Degeneration

By

FAREEHA AMBREEN

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad, Pakistan

2016

Biochemical Analysis and Genetic Polymorphism of Angiogenic Factors and Inflammatory Markers in Pakistani Patients with Age-Related Macular Degeneration

A thesis submitted in the partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Physiology

By

FAREEHA AMBREEN

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad, Pakistan

2016

DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree. I have tried my best to avoid plagiarism. I understand that I may be held responsible in case faulty, non-authentic or plagiarized results found in the dissertation.

FAREEHA AMBREEN

CERTIFICATE

The Department of Animal Sciences, Quaid-i-Azam University Islamabad accepts this thesis submitted by Miss Fareeha Ambreen in its present form, as satisfying the thesis requirement for the Degree of Doctor of Philosophy (PhD) in Physiology.

Supervisor: _____

(Prof. Dr. Irfan Zia Qureshi)

External Examiner: _____

External Examiner: _____

Chairperson: _____

Dated:

DEDICATED TO THE LITTLE SOLDIERS, WHO FACED THE CALAMITY OF TERRORISM IN THE SORROWFUL ARMY PUBLIC SCHOOL PESHAWAR MASSACRE ON 16TH DECEMBER, 2014

ACKNOWLEDGEMENTS

*All praise is to **Allah**, the Sovereign Lord and Creator of the universe, Who prospered my thoughts and flourished my ambitions in the form of this write up. All respect for the Holy Prophet Hazrat Mohammad ﷺ who enlightened our conscience with the essence of faith in Allah.*

I feel great honour to express my heartiest gratitude to my supervisor Prof. Dr. Irfan Zia Qureshi, Professor of Physiology and Chairman, Department of Animal Sciences, Quaid-i-Azam University, for his immense encouragement, guidance, constructive criticism and sincere facilitation during the whole period of my research work and dissertation from write up. I express my humble thanks to Prof. Dr. Wajid Ali Khan, Prof. Dr. Nadeem Qureshi, Dr. Nadeem Ishaq, Dr. Naveed Qureshi, Dr. Mohammad Ashraf, Dr. Amtul Aziz, Col. Shafiq Ullah, Dr. Sarah Zafar, Dr. Shama, Dr. Farah Islam, Rashid Natt, Sohail Ahmad, Mohammad Faisal and all the residents and staff of Al-Shifa Trust Eye hospital for their appreciable cooperation during the screening of patients. I am obliged to the patients and control subjects who participated in the study. I am grateful to Mrs. Shahnaz Murtaza, Mohammad Ali, Nasreen, Kausar and Khalid from Nuclear Oncology and Radiotherapy Institute, Islamabad (NORI) for extending laboratory facilities for my research work.

I sincerely thank to Higher Education Commission (HEC), Islamabad, Pakistan for providing financial support for my research work. I offer heartiest appreciation to Dr. Mohammad Ismail, Qaisar Mansoor and Amara Javed from Institute of Biotechnology and Genetic Engineering (IBGE), Islamabad for their cooperation for genetic analyses. I am also grateful to Dr. Fayyaz and Dr. Kiran Afshan, Quaid-i-Azam University, Islamabad, for their considerate guidance in the statistical analyses of my data. I would like to express my gratitude to all of my lab fellows and colleagues for their cooperation, encouragement and help during my research work. Special thanks to my very close friends Faiza and Shazia for their moral support during my doctorate studies.

Above all, I am grateful and indebted to my loving father Prof. Muhammad Yasin (late) and compassionate mother, brother, sisters, nieces and nephews for their prayers, love and care that have brought me to this stage. May Allah bless us all and bestow upon us secret of real knowledge to serve the nation sincerely (Amin).

FAREEHA AMBREEN

TABLE OF CONTENTS

CHAPTER # 4

Analyses of Gene Polymorphism

LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AMD	Age Related Macular Degeneration
Ang 1	Angiopoietin 1
ApoE	Apolipoprotein E
ARMS2	Age-Related Maculopathy Susceptibility 2
Blam D	Basal Laminar Deposits
Blin D	Basal Linear Deposits
BM	Bruch's Membrane
CD46	Complementary Domain 46
CFH	Complement Factor H
CFHRs	Complement Factor H Related Genes
CI	Confidence Interval
CNV	Choroidal Neovascularization
CRP	C-Reactive Protein
DHA	Docosahexaenoic Acid
ELISA	Enzyme Linked Immuniosorbent Assay
FB	Factor B
FFA	Fluorescent Fundus Angiography
FGF2	Fibroblast Growth Factor-2
FHL	Factor H Like Protein
GA	Geographic Atrophy
HDL	High Density Lipoproteins
HGF	Hepatocyte Growth Factor
HO-1	Heme Oxygenase-1
HRP	Horse Reddish Peroxidase
hsCRP	High Sensitivity C-Reactive Protein
HTRA1	High Temperature Required Factor A1
HuGE	Human Genome Epidemiology
ICAM-1	Intercellular Adhesion Molecule-1
IGF-1	Insulin Like Growth Factor-1
IL-6	Interleukin 6
IL-8	Interleukin 8
IOP	Intraocular Pressure
KDR	Kinase Insert Domain Receptor
LDL	Low Density Lipoproteins
logMAR	Log of Minimum Angle Resolution
MAC	Membrane Attack Complex
NIH	National Institute of Health
NSAIDs	Non-Steroid Anti-Inflammatory Drugs
OCT	Ocular Coherent Tomography
OPD	Out Patient Department
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PDT	Photodynamic Therapy
PEDF	Pigment Epithelium-Derived Factor
POS	Photoreceptors Outer Segments

PUFA	Polyunsaturated Fatty Acids
RFLP	Restriction Fragment Length Polymorphism
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
RPM	Revolutions Per Minute
SAP	Serum Amyloid P
SE	Standard Error
SNP	Single Nucleotide Polymorphism
STE	Saline Tris EDTA
TG	Triglycerides
TLR3	Toll-Like Receptor 3
TMB	Tetramethylbenzidine
TNF α	Tumour Necrosis Factor Alpha
TNFRSA	Tumor Necrosis Factor Receptor Superfamily, Member A
TSP	Thrombospondin
UAVA	Unaided Visual Acuity
UCSC	University of California Santa Cruz
USA	United States Of America
VAC	Visual Acuity Corrected
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization
χ^2	Chi-Square

List of Figures

List of Tables

ABSTRACT

ABSTRACT

Age related macular degeneration (AMD) is an ophthalmic disease with complex aetiologyand is considered to be one of the major reasons of blindness in aged population. Inflammatory processes are suggested to play an important role in AMD pathogenesis and its progression.

The present cross sectional and case-control study was carried out to investigate the role of inflammatory markers and angiogenic growth factors in AMD pathogenesis. Diagnosis was done through slit lamp examination, OCT and FFA. Normotensive and non-diabetic subjects aged ≥50 years diagnosed with AMD were selected. Age matched healthy individuals with no symptoms of AMD were selected as controls. Serum lipids, apolipoprotein E (ApoE), leptin, HTRA1, interleukin 6 (IL-6), IL-8, vascular endothelial growth factor (VEGF), C-reactive protein (CRP) and complimentary factor H (CFH) were determined in patients and controls. Genotype analyses of single nucleotide polymorphisms (SNPs) in IL-6 gene (rs1800795; rs1800796; rs1800797); IL-8 gene (rs4073; rs2227306; rs2227543); VEGF gene (rs3025039; rs699947) and CRP gene (rs1205; rs1130864) were done through restriction fragment length polymorphism.

Data were computed through SPSS version 18.0 (Chicago, Illinois, USA) and Graph Pad Prism (GraphPad Software, San Diego, California, USA). Data sets were compared between control and AMD patients through student's t-test. Genotype and allele frequencies were compared through χ^2. Significance level was $p<0.05$. Since the data were obtained from a sample population, to reduce heterogeneity of error, Box-Cox transformation algorithm was applied. Multivariate analysis of variance (M-ANOVA) was applied on the transformed data to investigate association of serum levels of IL-6, IL-8, VEGF and CRP with AMD.

Transformed data showed elevated serum levels of IL-8 ($p<0.015$) and VEGF ($p<0.0108$) and reduced ApoE ($p<0.032$) in AMD patients compared to control subjects. Serum VEGF levels were significantly raised ($p<0.0001$) in wet AMD patients compared to the dry AMD patients.

The IL-6 levels were significantly high in patients with genotype GG for rs4073($p<0.0001$). IL-8 levels were significantly high in patients with genotype GG for rs2227543($p<0.002$). Significantly high VEGF levels were observed in patients

with genotype TT for rs3025039($p<0.038$). CRP levels did not change significantly with respect to genotype forrs1205 and rs1130864.

The study concludes that inflammatory markers and angiogenic growth factors are significantly altered in AMD pathogenesis. Significant findings of the present study which are pertinent to mention is that altered levels of inflammatory markers and angiogenic factors are related to the genetic polymorphism for IL-6, IL-8 and VEGF genes. However, SNPs in relation to CRP were not related to serum CRP levelsin AMD patients.

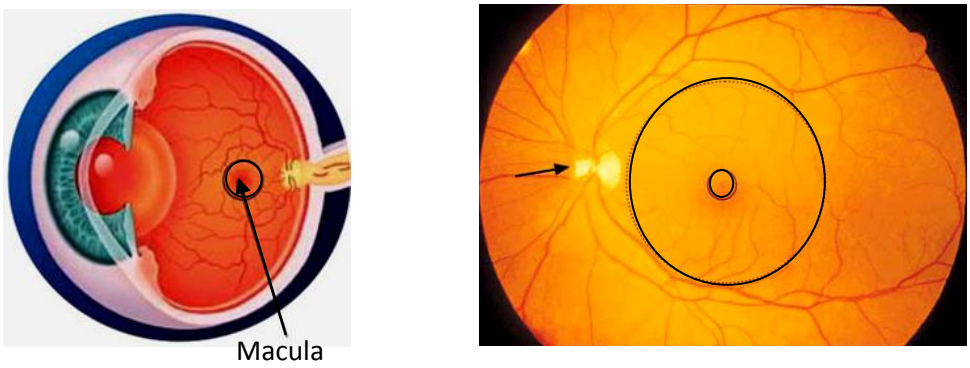
CHAPTER # 1
GENERAL INTRODUCTION

GENERAL INTRODUCTION

Age related macular degeneration (AMD) is one of the pathological states of macula that adversely affects vision in aged persons (Mullins et al., 2000). It leads to significant, irreversible and unmanageable vision loss due to damage to the central portion of retina called the macula (Fine et al., 2000). The disease was first reported in 1874 in medical literature as "choroido-retinal symmetrical central disease in senile persons" (Hutchinson and Tay, 1874). AMD has also been recognized as disciform senile macular degeneration. It was later designated as age related maculopathy and is currently known as "age related macular degeneration" (de Jong, 2006).

1.1 Normal Architecture of Macula

Macula is the portion present in the posterior centralretina (Fig. 1.1a). It is horizontally oval, 5 mm in diameter and contains the highest number of photoreceptors and functions for high resolution and visual acuity (Fig. 1.1b) (Fine et al., 2000). The foveola, which forms the central floor of macula, is the thinnest part of retina with a diameter of 0.35 mm. Its entire thickness consists only of cone photoreceptors and it functions for the most acute vision (Applegate, 2000).



(A) (B)

Fig 1.1 (A) The central portion of retina i.e. macula (Rao, 2012).
(B) Normal macula: Arrow shows optic Nerve, outer circle shows the macula, inner small circle shows avascular zone of the fovea (Fine et al., 2000)

Posterior to photoreceptors is present retinal pigment epithelium (RPE) which forms part of the barrier between eye and blood vessels. This blood-ocular barrier performs several functions like: phagocytosis of photoreceptors, transport of nutrients

and secretion of cytokines (Jager et al., 2008). Posterior to RPE, a semi-permeable barrier called Bruch's membrane is present that separates choroid from the RPE. Choroid supplies blood to the outer retinal layers (Fig. 1.2) (Sung and Chuang, 2010).

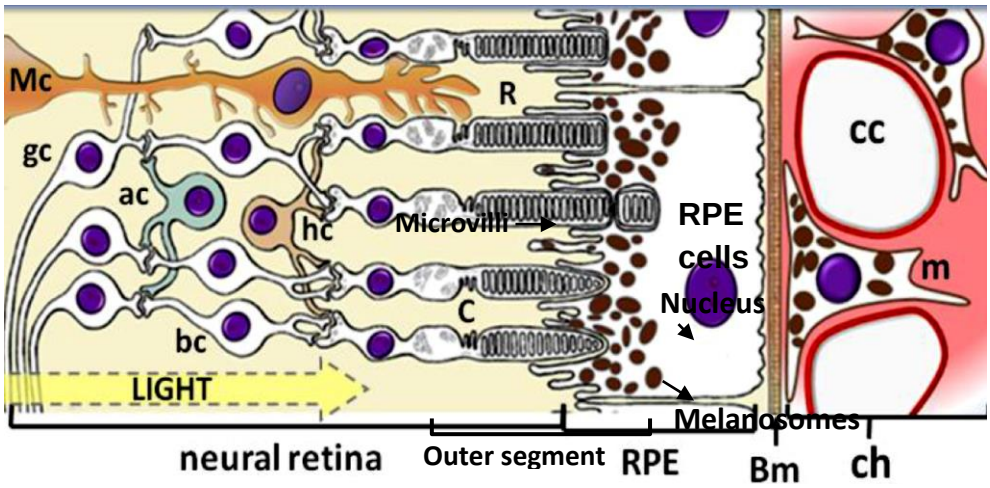


Fig. 1.2 Anatomy of retina. the neural retina has different types of cells responsible for phototransduction. rod cells (R), cone cells (C), bipolar cells (bc), ganglion cells (gc), horizontal cells (hc), amacrine cells (ac) and muller cells (Mc). Heavily pigmented single cell layer of pigment epithelium (RPE) is present between the vascularized choroid and neural retina. This highly vascularized choroid (ch) is separated from the RPE by Bruch's membrane (Bm). Choroid is nourished by the choriocapillary (CC) network and is highly pigmented due to choroidal monocytes (m) (modified from Sung and Chuang, 2010; Reinisalo, 2012).

1.2 Role of RPE and Photoreceptors in Visual System

Photoreceptors and RPE are separated by thin subretinal space filled with inter photoreceptor matrix. These layers are strongly interacted as each RPE cell is faced by nearly 20 to 40 photoreceptors (Marmorstein et al., 1998; Futter, 2006). The normal density of RPE at the fovea is about 4000 cells/mm^2 that decreases near the periphery (Panda-Jonas et al., 1996). The outer edges of photoreceptor outer segments (POS) face the apical RPE microvilli in sub-retinal space. Although post mitotic and non-dividing, the cells of the RPE are metabolically very active (Forrester et al., 1996). The renewal of POS is carried out by the phagocytosis which leads to shedding of POS into the adjacent RPE microvilli (Kevany and Palczewski, 2010). The RPE is

responsible for the functioning and renewal of photoreceptors by carrying out nutrient transport, recycling of retinoids, providing retinal blood barrier and enabling the daily phagocytosis of POS. The apical side of RPE is highly pigmented due to the presence of melanosomes thereby providing protection against the sunlight. RPE melanosomes also act as powerful free radical scavengers (Fig 1.2) (Reinisalo, 2012).

1.3 Retinal Changes with Aging

The vision and physiology of the eye are readily affected by the process of aging whereby the vision tends to decrease with advancing age (Beatty et al., 2000). Many structural changes take place in the RPE as a result of aging including: decreased number of melanosomes, lipofuscin accumulation, elevated density of residual bodies, basal deposit accumulation on or within Bruch's membrane(BM), drusen formation between inner collagenous layer of BM and the basal lamina of RPE, thickening of BM, microvilli atrophy and disorganized basal infoldings. However, the exact mechanisms involved in these age related changes are poorly understood (Bonilha, 2008).

1.4 Prevalence of AMD

Worldwide estimates show that nearly 20 to 25 million people show symptoms of AMD while the figure is expected to ascend three times in the next 30-40 years as the aged population is going to increase. AMD is the majorreason of visual loss in the Western world. Beaver Dam Eye Study from the United States has shownthat the AMD occurrence was 0.1% in 43-54 years aged population which increased further to 7.1% among 75 years or older subjects (Klein et al., 1992). In 2000, AMD was the leading cause of blindness among white Americans, accounting for 54% of all cases of blindness. In population-based studies of European populations, AMD was found to be the chief cause of blindness (The Eye Diseases Prevalence Research Group, 2004). Data from Caucasian population have shown that late AMD is evident in 10.9% of the subjects aged 80 years or more (Erke et al., 2012).

One of the systematic reviews estimated that nearly 0.3 million people in the United Kingdom are completely blind or have compromised vision due AMD (Chopdar et al., 2003). According to the statistics of World Health Organization (WHO), 8 million people have severe blindness due to AMD. However, this figure does not include countries where data are limited; which also includes Pakistan. In

Pakistan for example, the occurrence of AMD in Rawalpindi District is ~ 6.74 % which is less than that reported for the White races (Zafar and Afghani, 2009).

The demographic studies predict a major shift of population to older age groups having more than 65 years of age, in the developing countries that will further enhance the prevalence of AMD. At present the treatment of AMD is very expensive and with no satisfactory results, therefore health education and promotion are the tools to be focused for primary prevention of the disease (Nazimul et al., 2008).

1.5 Classification of AMD

A number of systems exist for the classification of AMD but the most authentic is the one that has been proposed by the Age-related eye disease study (Age-Related Eye Disease Study Research Group, 2000). International classification and grading system for age-related maculopathy and AMD have been modified by Beatty et al. (2000) to avoid confusing terminology proposed by Bird et al (1995).

AMDis classified into "early" and "late" stages. Minimal visual impairment is associated with early stagecharacterized by the presence of usually less than 20 medium-size drusen or abnormalities in retinal pigment layer. Visual loss in early AMD is mild and is therefore usually asymptomatic but blurred vision with visual scotomas, abnormal dark adaptation and decreased contrast sensitivity are most commonly observed symptoms. Characteristic findings of early AMD are therefore soft drusen associated with choroidal or outer retinal hyperpigmentation (Jager et al., 2008).

The intermediate stage of AMD is identified by the presence of at least one large druse, many medium size drusen or the geographic atrophy (GA) but the GA does not extend to central macula. In advanced stage of disease, drusen and GA extend to the central macula while peripheral or central visual scotomas with severe visual loss that gradually become severeas the time passes over months to years (Jumper et al., 2006). Late AMD may be atrophic or neovascular (Jager et al., 2008).

Most of the people who have spent 50 years of their life have at least one small druse (< 63 μm) in one or both eyes (Klein et al., 1992). The eyes having medium (63 to 124 μm) or large size drusen (>124 μm) are at a greater risk to developlate stage AMD (Klein et al., 1997). Depending on the appearance of their margins drusen can be hard or soft. Discrete margins are found in hard drusen

whereas, soft drusen are normally large with indistinct edges and are confluent (Fig. 1.3-A) (Bird et al., 1995).

1.5.1 AMD Types

Two types of late AMD are recognized: the atrophic or dry type and the neovascular exudative or wet type.

Atrophic or Dry AMD

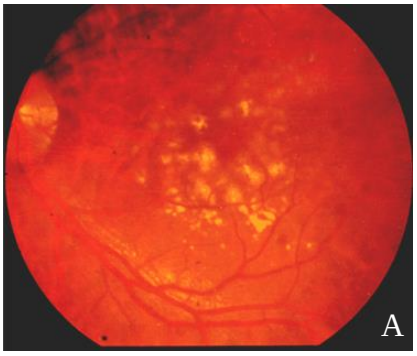
The atrophic form typically involves choriocapillaries, RPE and photoreceptor cells, the rods and cones. As this type of AMD does not involve exudation of blood or serum and leakage, therefore it is known as dry AMD (Sunness et al., 1997). In the later stage, there appears a sharply demarcated oval or round area with geographic atrophy (GA) of RPE. This can exceed upto 175 μm in size having visible choroidal vessels while no neovascularization is seen (van Leeuwen et al., 2003). The area with GA is identified by irregular, diffuse patches of increased autofluorescence (Holz et al., 2001) indicating that RPE lipofuscin, which is a fundus fluorophore, is associated with the pathogenesis of GA (Ambati et al., 2003). Late atrophic AMD refers to any sharply demarcated area of hypopigmentation, depigmentation or apparently absence of RPE in the macular area (Fig. 1.3 A and B) (Amin et al., 2006).

Patients with atrophic or dry AMD sometimes have better central vision (6/24 or better) but they may have considerable functional restrictions like fluctuating vision. In addition, lesser area of good vision causes difficulty in reading and the patients have impaired vision at night or under reduced light conditions (Steinmetz et al., 1993). The non-exudative AMD leads to severe visual loss in 20% of all AMD patients. As time passes, the areas having GA slowly enlarge associated with absolute scotoma and blindness (Mauschitz et al., 2012).

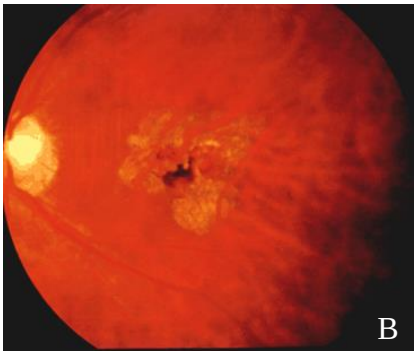
Exudative or Wet AMD

Exudative AMD is characterized by serous or haemorrhagic detachment of RPE with or without subretinal neovascular membrane leading to choroidal neovascularization (CNV). Since subretinal hemorrhage might occur with peripheral fibrous scar, this form of AMD is called wet AMD (Fig. 1.3 C and D) (van Leeuwen, 2003).

A) Multiple large soft drusen in the left macular region

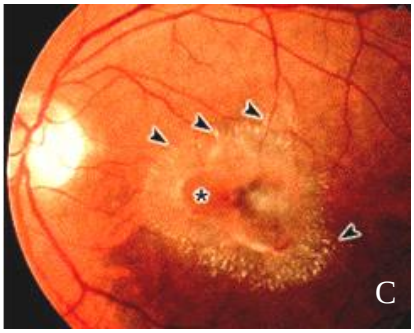


B) Geographic atrophy involving the left macular region



C) Exudative AMD

*Subretinal neovascularization surrounded by hard exudates (arrowheads)



D) Fluoresceinangiography, the CNV is shown stained by fluorescein (black arrow)

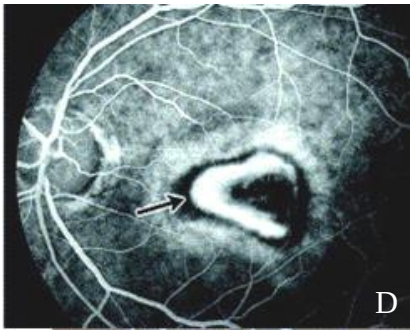


Fig 1.3 Types of AMD. (A-B from Amin et al., 2006, C-D from Witmer et al., 2003)

Neovascular AMD in late stages usually shows RPE detachment, CNV and scarred tissue that can be epiretinal, intraretinal or sub-RPE and macular hard exudates unrelated to any other retinal vascular diseases (Bird, 1995). Although there is no visual acuity component related to this classification system, early AMD is typically associated with a Snellen acuity of 6/18 or better, whereas late AMD has a profound negative impact on central vision (Beatty et al., 2000).

1.6 Pathophysiology of AMD

The risk of developing AMD increases with age because slow but persistent and cumulativeinjury to retina mediated by oxidative stress is aggravated by the process of aging. Retinal pigment epithelial cells are neuroectodermal in origin and support photoreceptor metabolism. The reduced physiological function in the aging RPE cells contributes to the formation of drusen deposits in AMD retina. In addition, age related damage to mitochondrial DNA appears to be another factor involved in the pathogenesis of AMD. As AMD is a degenerative disorder it involves the outer neural retina, BM, RPE and the choroid but the location of primary damage is yet not confirmed (Green, 1999).

Excessive drusen formation leads to RPE damage and a chronic anomalous inflammatory response that either causes GA or the secretion of angiogenic cytokines such as vascular endothelial growth factor (VEGF) or both. Additionally, abnormalities in elastin or collagen in BM, outer retina or choroid predispose some patients to neovascularization. The new vessels break through the BM which leads to subretinal hemorrhage, lipid deposition, fluid exudation and RPE detachment from the choroid, thus forming fibrotic scar or a combination of these abnormalities (Jager et al., 2008).

1.6.1 Pathophysiology of Drusen in AMD

Non-exudative AMD is characterized by decreased density of choroidal vessels with reduced lumen diameter (Ambati et al., 2003). Pathologically, changes in AMD include the emergence of basal linear deposits (BlinD) and basal laminar deposits (BlamD) (Green and Enger, 1993). The BlamD are formed by the granular material of the membrane and collagen deposits in patches between basal lamina of the RPE and the plasma membrane. BlinD deposits are formed by the vesicular material found in the inner collagenous zone of BM. The amount of BlamD increases

with the process of degeneration, which therefore can serve as a marker for progression of the disease (Curcio and Millican, 1999).

In the later stages of AMD soft drusen appear that represent the localized detachment of BlamD or BlinD (Green and Enger, 1993; Bressler et al., 1994). As these deposits show faint pattern in fluorescein angiogram, they might not be easily visible on fundus examination but they affect the vision adversely. Therefore, other biochemical analyses are required for the evaluation of all phenotypes of soft and hard drusen. These analyses involve immunohistochemisty of molecular constituents like apolipoprotein (Apo) E and B (Anderson et al., 2001; Malek et al., 2003), immunoglobulins, amyloid P component, factor X, complement C5 and C5b-9 terminal complex (Johnson et al., 2000; Johnson et al., 2001), vitronectin and fibrinogen (Hageman and Mullins, 1999; Hageman et al., 1999; Mullins et al., 2000).

Importantly, most of the above mentioned factors are part of cellular or humoral immunity which suggests that inflammatory and immunological processes are involved in drusen pathology. Morphologically distinct choroidal cells show the characteristics of dendritic, antigen-presenting cells which further increases the likelihood of involvement of immunological processes in AMD pathogenesis (Hageman et al., 2001).

1.6.2 Pathophysiology of Non-exudative AMD

Advanced stage of non-exudative AMD is characterized by geographic atrophy (GA). It is identified by atophic areas that enlarge slowly and correspond to the development of scotoma. The pathophysiological mechanisms that lead to GA are not yet fully understood (Mauschitz et al., 2012).It progresses slowly as compared to CNV and involves the fovea at later stages of disease leading ultimately to severe visual loss (Sunness et al., 1999). The reason for relatively slow progress of atrophy is unclear, although one of the views is that preferential vulnerability of rods to atrophy than the cone system leads to slow progress of atrophy towards the fovea which is an area rich in cones (Owsley et al., 2000). Others have however argued that the restricted choroidal blood supply to fovea is a protective measure against atrophy (Xu et al., 2010). Furthermore, slow progression of GA towards fovea has been suggested to be due to high density of luteal pigments including lutein and zeaxanthin at the neurosensory retina (Beatty et al., 2001).

Geographic atrophy leads to severe visual loss in 20% of affected eyes (Biarnés et al., 2011). On autopsy, about 37% of AMD eyes show the presence of geographic atrophy (GA) that overlies the photoreceptor layer (Green and Enger, 1993). Surprisingly, 34% of eyes with GA have CNV or disciform scarring. Geographic atrophy is associated with photoreceptor loss and absence of RPE. There may be a range of changes in RPE in different stages of AMD including atrophy, hypertrophy, hyperplasia, attenuation or clumping of pigment in sub-retinal space (Green and Enger, 1993; Bressler et al., 1994).

The fundus autofluorescence (FAF) is a tool that allows imaging of the topography of lipofuscin distribution in RPE cells monolayer. The absence of lipofuscin in RPE in GA shows severely reduced signal. These areas with GA show a high contrast due to hypofluorescence compared to non-atrophic areas (Mauschitz et al., 2012).

1.6.3 Pathophysiology of Exudative AMD

Neovascular or wet AMD is identified by the growth of increased blood vessels from choriocapillaries through BM that penetrate into and around photoreceptor layer and the RPE. The resultant haemorrhaging and exudation causes severe loss of vision (Ambati et al., 2003). The new vessels grow into a plane between BlamD and BM (Green and Enger, 1993) and sometimes extend through the subretinal space into RPE. The vessels grow as capillary like structures with multiple sites of origin and proceed to venules (Green and Key, 1997; Schneider et al., 1998). Retinal detachment is the outcome of CNV that may lead to pigment remodelling, RPE tear and leakage of lipids. The collagenous laminae of Bruch's membrane in the foveal area become thin as result of CNV (Hageman, 2002).

At the end stage of disease, disciform scar is seen which is usually vascularized having vascular contribution from choroid but sometimes from the retina with both sub-RPE and subretinal components. The size of scar is proportional to the degeneration of photoreceptors and the RPE. Neurosensory retinal detachment is the outcome in 10% of autopsied eyes having AMD which also can have sanguineous or serous components (Green and Enger, 1993). Histological preparation of eyes with disciform scars and CNV shows macrophage presence in the area surrounding the thinned Bruch's membrane and its ruptured sites (Killingsworth et al., 1990; van der Schaft et al., 1993)

1.7 Choroidal Neovascularization (CNV) in AMD

Choroid neovascularization is a characteristic feature of exudative AMD and is referred usually to the development of capillaries from choroid that grows through the Bruch's membrane. This generally accompanies subretinal haemorrhage, exudation and RPE detachment that ultimately leads to severe and sudden loss of vision which is incurable (Roth et al., 2004). Two-component model for the pathogenesis of CNV is proposed, according to which the CNV involves both angiogenesis and inflammatory processes. It is composed of pericytes and precursors of endothelial cells along with the vascular component. The inflammatory cells including lymphocytes, macrophages, granulocytes and foreign body giant cells are present as extravascular component (Spaide, 2006).

The classification of neovascular AMD depends upon the appearance of neovascularization observed through fluorescein angiography and as such CNV may be occult or classic type of lesion. Direct loss of photoreceptor cells in classic type of CNV leads to more aggressive type of AMD causing significant vision loss. On the other hand, vision is maintained unless RPE is decompensated in occult type of lesions (Schmidt-Erfurth et al., 2007).

Generally, there are three patterns of CNV growth in AMD that is, Sub RPE; subretinal or combined having both subretinal and subRPE pattern (Lee et al., 1996; Grossniklaus et al., 1998; Grossniklaus and Gass, 1998). Severe visual loss in AMD is due to bleeding, exudation and disciform scarring in more than 80% cases, all regarded as the consequence of CNV (Ferris et al., 1984). The diagnosis of the CNV pattern is carried out by indocyanine green (ICG) or fundus fluorescein angiography (FFA) (Fig. 1.3C and D) (Guyer et al., 1994; Watzke et al., 2000).

Clinically, the classification of CNV in AMD is according to the definitions of treatment of AMD with photodynamic therapy (TAP) and Visudyne in photodynamic therapy (VIP) studies. There are four subtypes characterized by different patterns observed in FFA: first is the classic type which is identified by the area demarcated with uniform hyperfluorescence having hypofluorescent margins in early phase and obscured boundaries due to the leakage of dye in middle and late stages. Second is the predominant classic CNV where >50% of fundus is occupied by the neovascular lesion and the fluorescence blocking constituents. Third is the minimally classic CNV in which classic CNV occupies <50% of the neovascular complex. Forth is the occult

CNV where no classic component is present, it has two types further: one is the fibrovascular RPE detachment that appears as spotted hypofluorescence with irregular elevation of RPE. The other type is identified with late phase dye leakage. It has undefined area as the leakage area does not correspond to classic CNV or fibrovascular RPE detachment as observed through FFA in early and middle stages (Fig. 1.4) (Bressler, 1999; Arnold et al., 2001; Bressler et al., 2005; Chakravarthy et al., 2006).

Depending upon the duration, CNV is divided into three stages. The initial stage is identified when the endothelial cells derived from the chorioapillaries multiply andmove towards retina through the Bruch's membrane; the noevascular complex grows further in the active stage and in the final and involutional stage a clear disciform scar appears and CNV becomes fibrotic (Gass, 1997).

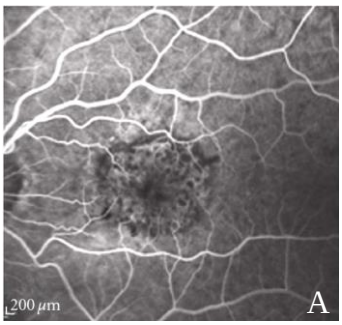
1.8 Risk factors for AMD

A characteristic that increases the chances of occurrence of the disease is referred to as risk factor. These factors are of two types i.e., modifiable and non-modifiable risk factors. Modifiable risk factors are those factors which can be changed like smoking, light exposure, diet, obesity, diabetes, and hypertension. Non-modifiable risk factors are those which cannot be changed by any kind of intervention, treatment or life style. These actors include age, race, gender and family history.

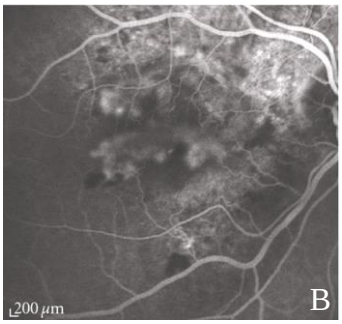
Cross-sectional, prospective cohort and case-control studies have identified several risk factors for AMD that include environmental, demographic and genetic factors (Fine et al., 2000). Studies have shown that age, race, diet, gender and history of cardiovascular disease are contributing risk factors for AMD (Coleman et al., 2008; Jager et al., 2008), for example, wet type of AMD is more prevalent in White race (Sommer et al., 1991; Klein et al., 1995).

Additionally, positive family history for AMD (Seddon, 1997), cigarette smoking (Seddon et al., 1996; Klein, 1998), diet poor in zinc and antioxidant vitamins (Yannuzz, et al., 1992; VandenLangenberg et al., 1998) also increase the risk of developing AMD. Lighter color of iris and increased exposure to sunlight also elevate the risk (Hyman et al., 1983; Goldberg et al., 1988; Cruickshanks, 1993).

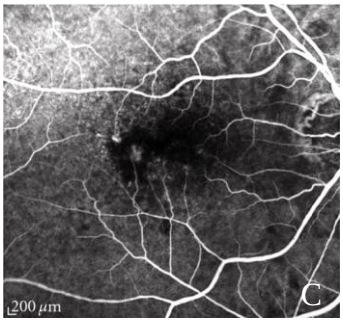
A) Classic CNV: The lesion is identified by a well demarcated area having uniform hyperfluorescence



B) Predominantly classic CNV: The lesion has a mixture of angiographic features with neovascular complex



C) Minimally classic CNV: The lesion has a mixture of the angiographic features of the occult and classic type



D) Occult CNV: Identified by the areas of irregular RPE elevation that present spotted hyperfluorescence.

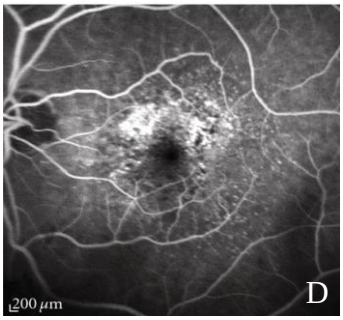


Fig. 1.4 Fluorescein angiography showing different types of CNV (Campa et al., 2010).

1.8.1 Demographic Factors

All forms of AMD have been reported to increase with advancing age. The incidence, prevalence and progression of the disease increase with the increasing age (Smith et al., 2001; Klein et al., 2002; Mitchell et al., 2002).

Smoking and AMD development have been studied to be statistically associatedthrough numerous cohort, case-control and cross-sectionalstudies (Klein, 2007). Smoking mediates the risk of AMD possibly by the impairment of generation of antioxidants like plasma carotenoids and vitamin C. This in turn leads to generation of reactive oxygen species (ROS), hypoxia and modification of blood flow in the choroid. Moreover, smoking enhances the risk of developing AMD as the nicotine is a stimulator for neovascularization inducing proliferation of endothelial cells, enhances fibrovascular growth (Heeschen et al., 2001) and also affects the immune system (Tsoumakidou et al., 2008).

In addition, excessive intake of fatty diet has also been correlated to increased risk of AMD (Seddon et al., 2003). Some studies have shown that nuts, fish, antioxidants and omega-3 polysaccharide unsaturated fatty acids have protective effects against AMD (Smith et al., 2000; AREDS, 2001; Cho et al., 2001).

Taking gender, females are at greater risk to develop AMD; women in general have longer life spans and therefore they show greater prevalence of the disease after the age of 75 years (Evans, 2001). Blue Mountains Eye Study has proposed that the frequency of neovascular AMD among women was doubled than that of men over a period of five years (Mitchell et al., 2002). Females show greater expression of vascular endothelial growth factor (VEGF) and the kinase insert domain receptor (KDR) in retinal capillary endothelium in response to 17β-estradiol (Tanemura et al., 2001). Contrary to this, data from the Beaver Dam Eye Study and the Rotterdam Study showed no gender difference in AMD patients (Smith et al., 2001).

The White population appears to be at greater risk to develop all types of AMD than the darkly pigmented races (Friedman et al., 1999; Klein et al., 2003) suggesting that melanin plays some kind of a protective role against the development of CNV. The melanin is also believed to protect against RPE lipofuscin formation. Lipofuscin is a senility marker and promoter of oxidative damage (Sundelin et al.,

2001; Kayatz et al., 2001). Few studies have however found no difference in melanin pigment density of the AMD and non AMD eyes (Berendschot et al., 2002).

1.8.2 Cardiovascular Factors

The similarity between molecular composition of atherosclerotic plaques and drusen provides a strong clue for the link between AMD and atherosclerosis (Vingerling et al., 1995; Friedman, 2000; Mullins et al., 2000). Although systemic hypertension and neovascular AMD are associated (Age-Related Eye Disease Study Research Group, 2000; Hyman et al., 2000) but epidemiological data do not support this link (Klein et al., 1995; Hyman et al., 2000).

1.8.3 Exposure to Sunlight

Prolonged exposure to sunlight is another factor for retinal damage because the development of AMD involves the generation of reactive oxygen intermediates (ROI), produced by photoxidation. The mechanism that has been suggested is that protoporphyrin is activated upon exposure to sunlight and leads to the generation of ROI in choroid and the outer retina (Cruickshanks et al., 1993). This in turn may lead to lipid peroxidation in the photoreceptor cells outer segment(POS) (Ambati et al., 2003).

The difficulty however lies in the fact that total light exposure cannot be quantified for the life span of a subject (Ambati et al., 2003). There have been conflicts in different studies as regards the effect of visible or ultraviolet light on AMD (Yannuzzi, et al., 1992; Cruickshanks et al., 1993; Darzins et al., 1997; Mitchell et al., 2002).

1.8.4 Dietary and Medication Factors

The role of diet in AMD development is supported by the fact that it is a highly modified factor. A prospective study carried out on more than 70,000 participants of both genders revealed a positive correlation between AMD and total fat intake (Cho et al., 2001). Moreover, particularly high consumption of linolenic acid increases the risk of developing AMD upto 49% whereas high levels of docosahexaenoic acid (DHA) in food reduces this risk upto 30% (Smith et al., 2000; Cho et al., 2001). In contrast, in another study, no association was found between low linoleinic acid intake and AMD in the Third National Health and Nutrition Examination Survey (Heuberger et al., 2001).

Several studies show association of high cholesterol intake with AMD (Mares-Perlman et al., 1995; Smith et al., 2000). Obesity is another factor that is linked to the greater risk of AMD development (Age-Related Eye Disease Study Research Group, 2000; Schaumberg et al., 2001). The consumption of more than 30 grams of alcohol in a day in females increases the risk of developing AMD slightly however, no association of caffeine or coffee to AMD was reported (Tomany et al., 2001).

Higher concentration of anti-oxidant vitamins and some minerals in retina and carotenoids in the macula after micronutrient supplementation provides aprotection against AMDdevelopment but no association has been reported between higher levels of serum carotenoids and lower AMD risk (Yannuzzi, et al., 1992; Sperduto, 1993). It has been studied that very low serum lycopene (the most abundant serum caroteinoid) levels reduce the chances of developing AMD although lutein and zeaxanthin, the macular pigments, do not show association with AMD (Mares-Perlman et al., 1995).

An inverse relationship has been reported between AMD and vitamin E, carotenoids and zinc intake (Vanden et al., 1998). Although a randomized study has also suggested positive role of zinc supplementation (Newsome et al., 1988) but Cho et al. (2001) found that moderate zinc intake through diet or supplement could not defend the subjects from developing AMD in 8-10 yearsof duration. Similar were the findings of a randomized trial for vitamin E supplementation in which no difference was found in the occurrence of AMD between supplemented and non-supplemented subjects (Taylor et al., 2002). Even though higher serum vitamin E levels have shown to be protective against AMD (West et al., 1994), convincing evidence supporting its supplementation is however lacking. This suggests that this difference is perhaps due to coexisting lower levels of serum lipids (Mares-Perlman et al., 1995) and the variation of micronutrient levels in diet (Ambati et al., 2003).

Higher serum zinc levels promote aggregation of amyloid fibrils and may also be involved in RPE cell apoptosis (Wood and Osborne, 2001). Furthermore, the use of medications that reducecholesterol levels and angiotensin converting enzyme (ACE) inhibitors increase the risk of developing AMD (McCarty et al., 2001). Contrary to this, a cross sectional study in the United Kingdom has proposed reduced risk of AMD in statin users than non-users (Hall et al., 2001). The cholesterol lowering medicines not only decrease linear deposits in the Bruch's membrane but also have

anti-inflammatory action thereby reducing the risk of AMD development (Dithmar et al., 2000; Sparrow et al., 2001).

1.9 Role of Growth Factors in AMD

The growth factors are a diverse group of proteins that share some biochemical features. These are secreted proteins which have the ability to act in an autocrine, paracrine or endocrine manner usually leading to the cell proliferation (Kvanta, 2006). The balance between angiogenic and anti-angiogenic growth factors prevents growth of abnormal blood vessels but CNV occursas a result of an imbalance (Bhutto et al., 2006). Investigations on experimentally induced CNV animals and surgically removed CNV membranes show the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) (Amin et al., 1994; Kvanta et al., 1996; Ishibashi et al., 1997).

Although, the growth of new vessels from already existing network is essential during embryonic development and tissue repair after birth but neovascularization can be dangerous in ocular pathologies like AMD. Neovascularization is a complex process that includes the interaction of many molecules in a sequence (Fig. 1.5) (Ambati et al., 2003). Many antiangiogenic growth factors like pigment epithelium-derived factor (PEDF), thrombospondin (TSP) and endostatin prevent new vessel growth in choroid to maintain a balanced situation (Bhutto et al., 2006). Likewise, insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), erythropoietin and angiopoietin play a positive role in retinal angiogenesis (Kvanata, 2006).

1.9.1 Vascular Endothelial Growth Factor (VEGF)

It is a strong candidate for angiogenesis promotion and increased vascular permeability and has therefore been extensively studied in ocular angiogenesis (Kvanta, 2006). It is a glycoprotein with angiogenic characteristics (Ferrara et al., 2003), whose gene is stimulated under hypoxic conditions (Shweiki et al., 1992). VEGF functions through tyrosine kinase surface receptors VEGFR 1 and VEGFR 2, expressed by endothelial cell surface. VEGF increases the survival of immature vessels and brings the pericytes around vessels (Ferrara et al., 2003).

Several cell types including the choroidal fibroblasts, RPE cells and infiltrating neutrophils produce VEGF (Adamis et al., 1993; Kvanta 1995; Zhou et al., 2005). The inflammation leads to elevated VEGF levels that in turn cause angiogenesis (Kvanta,

1995; Zhou et al., 2005). Hypoxia also stimulates VEGF secretion leading toneovascularization (Vinores et al., 2006). Reduced oxygen levels in the retina stimulate VEGF production in many cell types like endothelial cells in retina, Muller cells, RPE cells and pericytes (Aiello et al., 1995). Elevated VEGF levels in the retina lead to neovascularization (Pierce et al., 1995) while its inhibition down regulates CNV (Robinson et al., 1996). Animal models with induced CNV show VEGF expression (Ishibashi et al., 1997) whereas, treatment that inhibits VEGF (Krzystolik et al., 2002; Reich et al., 2003) or VEGFR 2 (Takeda et al., 2003; Kinose et al., 2005) reduces CNV formation. Studies by Kliffen et al. (1997) and Bhutto et al. (2006) show that VEGF levels are elevated in posterior segment before CNV formation occurs in AMD eyes but the exact mechanism of VEGF stimulation in CNV is still unclear.

The post mortem examination of AMD eyes at various stages shows higher expression of the VEGF (Kliffen et al., 1997). Similar is the finding in surgically excised CNV membranes where VEGF was found to be in higher concentration in RPE cells and choroidal fibroblasts (Frank et al., 1996; Kvanta et al., 1996; Lopez et al., 1996). Once secreted, VEGF induces mitosis in vascular endothelial cells and provokes tissue-type plasminogen activators and urokinase expression along with metalloproteinase interstitial collagenase. This induction in turn enhances local degradation of extracellular matrix to facilitate migration of endothelial cells. Vascular hyperpermeability results into the leakage of plasma proteins that play role as substrate for endothelium derived growth factor (Fig 1.5) (Ambati et al., 2003).

1.9.2 Pigment Epithelium Derived Growth Factor (PEDF)

It is a 50-kDa protein found in different eye compartments (Becerra, 1997; Dawson et al., 1999; Chader, 2001). It has dual function; at one end it enhances the survival of multiple types of neuronal cells including photoreceptors (Cayouette et al., 1999; Jablonski et al., 2000; Cao et al., 2001); while on the other it directly acts on endothelial cells and inhibit angiogenesis by inducing cell death (Stellmach et al., 2001).

PEDF is a member of the serine protease inhibitor (serpin) family and possess neurotrophic, neuroprotective and antiangiogenic properties (Tombran-Tink et al., 1991; Dawson et al., 1999).

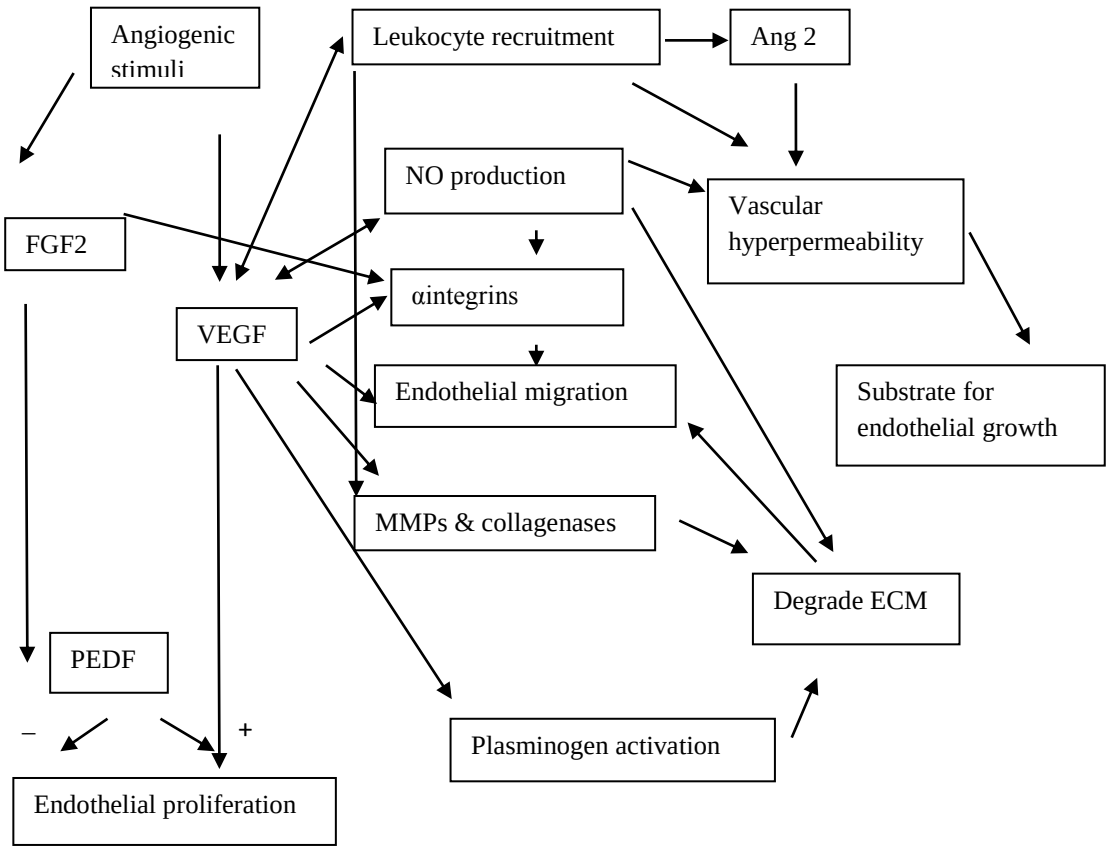


Fig. 1.5 Schematic figure of Angiogenesis in CNV (Ambati et al., 2003)

It plays a negative role in experimentally induced CNV although its presence in human choroid is still not confirmed (Ogata et al., 2002). Being anti-angiogenic factor, PEDF has effects opposite to that of VEGF thus over expression of PEDF or inhibition of VEGF leads to suppression of CNV (Krzystolik et al., 2002; Mori et al., 2002). Reduced levels of PEDF in vitreous humor and choroid-BM-RPE complex are related to CNV in AMD cases (Holekamp et al., 2002; Bhutto et al., 2006).

1.9.3 Insulin-Like Growth Factor-1 (IGF-1)

It is one of the most important growth factors present in the somatic cells, essential for growth hormone action. The specific receptor for IGF-1 is insulin-like growth factor 1 receptor (IGF1-R) located on cell surface of many tissues through which it plays its widespread functions. The binding of IGF-1 to its receptor initiates a receptor tyrosine kinase, intracellular signalling pathway. IGF-1 is one of the natural activators of AKT signalling pathway which stimulates cell growth and proliferation and inhibits apoptosis (Yilmaz et al., 1999).

IGF-1 also plays a role in angiogenesis (Kvanta, 2006). VEGF action is mediated via IGF-1 receptor through p44/42 mitogen-activated protein kinase activation (Smith et al., 1999). Studies on animal models have shown that low levels of IGF-1 in the serum slow down the angiogenesis (Smith et al., 1997) whereas intravitreally induced IGF-1 promotes this process in the retina (Danis and Bingaman, 1997). Mice models deficient in IGF-1 lack normal retinal vasculature and develop hypoxia that in turn leads to neovascularization (Hellstrom et al., 2001).

1.9.4 Hepatocyte Growth Factor (HGF)

HGF serves as angiogenesis stimulator in the retina, acting through tyrosine kinase receptor and c-met in retinal vascular endothelial cells. HGF promotes cell migration and proliferation (Cai et al., 2000). Elevated subretinal and intravitreal levels of HGF have been demonstrated in proliferative diabetic retinopathy and retinopathy at prematurity during neovascularization in choroidal tissue (Shinoda et al., 1999; Lashkari et al., 2000). Later, a study by Hernándezet al. (2004) however, could not find any correlation of HGF with angiogenic progression in diabetics.

1.9.5 Angiopoietin

Angiopoietins, the Ang-1 and Ang-2 stimulate angiogenesis at later stagesthrough vascular maturation and remodelling (Yancopoulos et al., 2000). Ang-1 stimulates Tie-2 tyrosine kinase receptor expressed by the vascular endothelium. On the contrary, inhibition of Tie-2 tyrosine kinase receptor inhibits angiogenesis (Hangai et al., 2001; Takagi et al., 2003). The same Tie-2 receptor is also used by Ang-2 which functions as a natural antagonist to Ang-1 (Oshima et al., 2005).

In patients having proliferative diabetic retinopathy, Ang-1, 2 and their receptors were found to be localized in the retinal epithelial membrane. It has been found that the inhibition of both Tie-2 and VEGF receptor more potently restrict retinal angiogenesis as compared to either of the receptors alone (Takagi et al., 2003). These findings suggest that Ang-1-Tie-2 system act separately and are not linked with VEGF system suggesting an additional target for antiangiogenic therapy in retinopathies (Kvanta, 2006).

1.9.6 Platelet-Derived Growth Factor (PDGF)

Unless surrounded by the pericytes, the regression of newly developed vessels takes place in PDGF presence that proves it to be an important growth factor for

angiogenesis. The pericytes are acquired by the vessels to increase their stability and permit them to branch further (Betsholtz et al., 2005), while a reduction in the number of pericytes due to low levels of PDGF may lead to the formation of microaneurysms and CNV (Enge et al., 2002). It has been shown in rats that inhibition of PDGF leads to reduced pericyte count and thus induces retinal angiogenesis as a result of hypoxia (Wilkinson-Berka et al., 2004). In patients with diabetic retinopathy, PDGF has been reported to be present on epiretinal membranes and in vitreous samples. This suggests that PDGF may be a target for therapy to inhibit abnormal retinal angiogenesis (Freyberger et al., 2000).

1.10 Role of Oxidative Stress in AMD

Insufficient antioxidants in diet increase the chances of developing AMD (Seddon et al., 1994). The eye is continuously exposed to atmospheric oxygen, radiations and chemicals present in the environment (Ohia et al., 2005). Such oxidative stresses may lead to ocular pathologies like the glaucoma, uvetitis, cataract and AMD. Many degenerative disorders of the eye have been studied through lipid peroxidation markers, antioxidant enzyme activity and estimation of low molecular weight antioxidants (Halliwell and Gutteridge, 1989).

Retina is at a greater risk due to oxidative damage as it has relatively higher oxygen concentration for consumption and higher polyunsaturated fatty acids than other parts of the body (Beatty et al., 2000). Therefore, retina is more prone to lipid peroxidation (De La Paz et al., 1992; Ito et al., 1995), a feature that increases with advancing age (Yildirim et al., 2011). Retina has most suitable conditions for the production of reactive oxygen species (ROS) generated as by-products of cellular metabolism or after photochemical reactions (Dargel, 1992; Kukreja and Hess, 1992). An antioxidant enzyme, heme oxygenase-1 (HO-1), induces apoptosis of the photoreceptors after exposure to blue light (Wu et al., 1999) while the administration of synthetic antioxidant drugs reduces this photoreceptor loss. Such findings provide a strong evidence of oxidative stress involvement in retinopathies like AMD (Organisciak et al., 1998).

Lipid bilayer of rod outer segment consists of 50% polyunsaturated fatty acids (PUFA) while the remaining half consists of proteins, of which docosohexanoic acid (DHA) is the most abundant (Stone et al., 1979). DHA and α-linoleic acid, a precursor of DHA are purely acquired from diet. Membrane PUFAs are peroxidized resulting

into the loss of membrane structure and function (Arstila, 1972). The macular and peripheral retina lipid peroxidation is age dependent as studied in cadaveric eyes. Moreover, the posterior pole of retina is more prone to lipid peroxidation with increasing age but such relationship is not evident for peripheral retina (De la Paz and Anderson, 1992). In addition, DHA has been found to be in lower concentration in the macular region than in the peripheral retina, thus this central region is at greater risk to face the oxidative stress than the peripheral tissue (van Kuijk and Buck, 1992). Therefore, the defence against oxidative damage in the macular region declines with the advancing age (Beatty et al., 2000).

1.11 Role of Inflammation in AMD

Multiple factors like aging, genetic tendency, oxidative stress and inflammatory factors all contribute to AMD (Fig 1.6) (Klein et al., 2007; Coleman et al., 2008; Klein et al., 2010). Single nucleotide polymorphism (SNP) of many inflammatory factors has been studied in AMD subjects suggesting that the occurrence of AMD is the result of inflammation (Hageman et al., 2001). These include Toll-like receptor 3 (TLR3), complement factor H (CFH), CX3CR1 and interleukin 8 (IL-8) (Ding et al., 2009).

Studies have shown that drusen contain certain complement system components like membrane attack complex (MAC), CD46, C3, C5 and C5b-9. Some additional factors including C2, C3 and complement factor B (FB) are also shown to increase the risk of AMD occurrence (Patel and Chan, 2008). The activation of C3 leads to the progression of AMD independent of CFH polymorphism (Sivaprasad et al., 2007). Inflammatory cells have been shown to take part in AMD progression and pathogenesis (Fig 1.6) (Penfold et al., 2001; Coleman et al., 2008).

Macrophages and giant cells are shown to be present near the drusen, CNV membrane and the Bruch's membrane. Additionally, cytokines derived from macrophages like IL-1 and tumour narcosis factor α (TNF-α) have been shown to increase the intercellular adhesion molecule-1 (ICAM-1) levels in vascular endothelial cells and RPE. These cytokines therefore provoke proliferation and migration of the vascular endothelial cells to induce CNV through the process of angiogenesis (Apte, 2010). In AMD, outer retina along with subretinal regions contain activated microglial cells which are stellate shaped cells that reach the retina during embryonic development (Langmann, 2007) and upon injury or degeneration are

transformed into the amoeboid appearance and migrate to outer retina. These cells play role in the inflammatory process and support the phagocytosis of debris by secreting many chemokines and cytokines to create neurotoxic milieu resulting in the progression of disease (Gupta et al., 2003).

An intermediate stage between basal and inflammatory states is known as para-inflammation. The tissue adapts to the malfunction and lethal conditions during this phase (Xu et al., 2009). This process is usually helpful to repair and restore tissue function. Para-inflammation involves innate immunity reactions in the aging retina but the process of tissue repair is interrupted in AMD (Wang et al., 2011).

The complement pathway is deregulated in AMD that leads to activation of inflammatory pathways and release of activation products (Donoso et al., 2006). Several regulators of the complement system, activation products and inflammatory proteins have been expressed in drusen. These include C3, C3d, terminal products C5, C6, C7, C8 and C9, terminal complement regulators clusterin and vitronectin, ApoE, ApoA1, AapoA4 along with serum amyloid P Component A (SAP-A), SAP-P and thrombospondin (Anderson et al., 2002; Crabb et al., 2002; Li et al., 2006). Activation of the complement system results to the progression of AMD which then leads to drusen formation, RPE cell damage and ultimate vision loss (Hageman et al., 2001).

The complement system is an important component of innate immunity during microbial infection (Rodriguez de Cordoba et al., 2004). It has three activation pathways: classical, alternative and lectin pathways. When activated, these pathways lead to unstable protease complexes named C3-convertases. They cleave C3 to generate C3b. The deposition of this protein enhances phagocytosis of the structure upon which it is deposited. This complex also cleaves C5 to initiate the assemblage of membrane attack complex named C5b-9 leading to the complement mediated cell lysis (Fig. 1.6). Immunohistochemical studies have demonstrated that many of these factors are expressed by the RPE and choroid indicating the presence of local inflammation in AMD pathogenesis (Zeiss, 2010).

C-reactive protein (CRP) is an inflammatory marker and a risk factor for cardiovascular and peripheral arterial disease (Nagoka et al., 2008; Thiruvagounder et al., 2010; Simundic, 2011). It is a 125 kDa pentraxin protein, a family member with five identical subunits. It is also a pathogenic factor that leads to endothelial

dysfunction in cell culture (Mueck et al. 2001). In addition, raised levels of CRP have been associated with elevated risk of hypertension (Sesso, et al., 2003) and diabetes mellitus (Chase et al. 2004) as these systemic diseases are related to major risk of retinal vascular disorders, therefore the association of these factors with inflammation and endothelial dysfunction is suggested in humans with retinopathy (Colak, et al., 2012). CRP is expressed in Bruch's membrane and drusen as studied by immunohistochemistry (Anderson et al., 2002; Laine et al., 2007; Skerka et al., 2007).

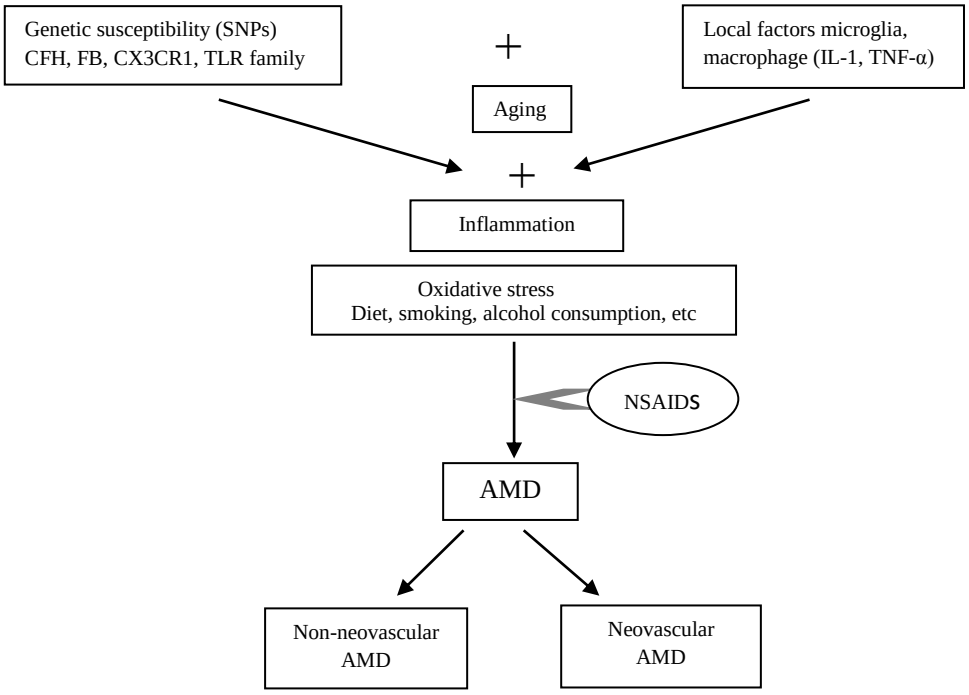


Fig. 1.6 Schematic diagram demonstrating the risk factors in AMD (adapted and modified from Wang et al., 2011).

1.12 Role of Lipids and Lipoproteins in AMD

The drusen found in AMD show a number of molecular constituents also found in the atherosclerotic plaques. These include lipids and some proteins like vitronectin, apolipoprotein E (ApoE), calcium and complement components demonstrating ultimate relevance between advanced AMD and carotid artery atherosclerosis. Furthermore, both of these pathologies are formed adjacent to an elastic layer, in the surrounding area of vascular endothelial cells and pericytes (Mullins et al., 2000). Lipids have been detected in the drusen through histochemical

techniques that include cholesterol in both unesterified and esterified form that accumulate in the Bruch's membrane throughout the adulthood. The RPE secretes lipoproteins that accumulate in the BM. The lipoproteins having hepatic or intestinal origin transported through plasma also contribute to the lipoproteins accumulated in the BM (Wang et al., 2010).

Controversies however exist as regards the association of the AMD with serum lipid profile. Abalain et al (2002) found no difference in total cholesterol, phospholipids, high and low density lipoprotein and triglycerides concentration between AMD patients and controls in a study. Likewise, in another study no significant difference could be found in the serum lipoprotein concentration between AMD patients and normal subjects (Nowak et al., 2005). Some correlations between serum lipid and AMD have however been shown in cases where the focus was high density lipoproteins. Two major studies done by van Leeuwen et al. (2004) and Delcourt et al. (2001) demonstrated that AMD was associated with elevated HDL levels. Contrary to this, Reynolds et al. (2010) found that high levels of HDL were inversely correlated with advanced AMD indicating HDL to reduce the risk of wet type AMD.

Leptin is an adipose-derived peptide that appears as hormonal signal between lipids and the peripheral adipose tissues. The leptin concentration in the serum reflects total amount of lipids in the body that directly inhibits intracellular lipid accumulation by the reduction of fatty acids and triglyceride synthesis. Leptin has a direct metabolic effect on lipoprotein metabolism or lipase activity. In case of leptin deficiency, fatty acid synthesis increases as result of inhibition of fatty acids uptake and oxidation by mitochondria resulting to elevated levels of fatty acid and triglycerides in the cells. The intracellular lipid accumulation in RPE leads to AMD that appears in the form of deposits on Bruch's membrane and drusen. Therefore, reduced leptin levels are expected to be a causative factor in AMD pathology acting through lipid peroxidation modulation (Everekliogl u, 2003).

1.13 AMD as a Genetic Disorder

Information as regards the genetic predisposition of AMD is less well defined; firstly, because the disease is age related and its onset is late in life therefore parents of the affected person are usually deceased and a family pedigree cannot be established, secondly, it is a complex pathology with undefined aetiology and affected

by various environmental factors (Haddad et al., 2006). Some data however exist in support of the involvement of genes in AMD pathology. Single gene or polygenic patterns with variable effects also contribute to the disease. An extensive data exist to provide evidence in favour of the family inheritance of AMD. Twin and sibling studies provide additional support for genetic basis of AMD. Moreover, family members of AMD patients are at greater risk to develop the disease in the late stages of life (Smith et al., 2001; Seddon et al., 2003). Studies on twin analyses (Hammond et al., 2002; Seddon et al., 2005), familial aggregation and general population show that AMD is an inherited disorder and that late AMD in most of the cases has specific genetic basis (Seddon et al., 1997). Human genome mapping project 2005 included genome-wide linkage studies that identified two main loci suspected to be responsible to enhance the risk of disease in about 50% of AMD patients (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005; Rivera et al., 2005).

Several studies show that most replicated signals for linkage and association are present on chromosome loci 1q25–31 and 10q26 (Majewski et al., 2003; Fisher et al., 2005; Jakobsdottir et al., 2005). The region on human chromosome 1q31 carries the cluster of complement factor H (CFH) gene while 10q26 chromosomal region has two genes located closely; one for age-related maculopathy susceptibility 2 (ARMS2) and the other is high temperature required factor A1 (HTRA1) (Dewan et al., 2006; Yang et al., 2006). The studies that identify the vulnerable genes playing role in AMD pathogenesis, are expected to provide more information about the biological processes involved in the progression of AMD, thus, the interaction between AMD phenotypes and genotypes is likely to be discovered (Haddad et al., 2006).

The AMD Gene Consortium has reported genetic association of macular degeneration that involved 18 international research groups and a large set of controls and cases. The study showed that susceptibility alleles exhibit different association across ethnic groups and subtypes of disease. It has been reported that ARMS2 risk alleles are associated with neovascularization while geographic atrophy was related to CFH risk alleles. In addition, variants near CFH exhibit stronger evidence for association among Europeans and Asians, variants near tumor necrosis factor receptor superfamily, member A (TNFRSFA)are associated with the disease. The strongest interaction was observed for rs10737680 located near CFH gene and rs429608 near C2/CFH. The subjects with these risk alleles at both loci were at more risk of

developing the disease. Differences in linkage disequilibrium between populations and environment and diagnostic factors contribute to modify genetic effects (Fritsche et al., 2013).

1.13.1 Complement Factor H (CFH)

The gene for CFH is located on human chromosome region 1q31 along with five complement factor H related genes (CFHRs); from CFHR1 upto CFHR5 (Rodriguez de Cordoba et al., 2004). Several single nucleotide polymorphisms (SNPs) have been identified in this region including promoter, coding as well as non-coding intronic regions (Li et al., 2006). One of the important SNP is located in exon 9 region causing a change in amino acid sequence of the protein by exchanging tyrosine with histidine at position 402 hence the variant is called Y402H. This variation enhancesAMD risk by two to four folds in heterozygous subjects and three to seven fold in homozygous subjects. Several studies in different populations confirm the association of Y402 polymorphism in diverse racial groups (Souied et al., 2005; Okamoto et al., 2006; Seitsonen et al., 2006; Simonelli et al., 2006).

Furthermore, some additional SNPs have also been identified that increase the risk of exudative AMD. They occur in the promoter region of CFH gene at 257 position in exon 2; it leads to isoleucine (I) residue 62 to be changed by valine (V) thus mutation I62V occurs. Additionally, more SNPs have been identified in exon 7, 15 and 16 (Chen et al., 2006). Contrary to this, a mutation that involves deletion of a fragment having 84 Kbp size in the region downstream to the CFH gene shows a protective effect against AMD. This region includes genes coding for CFHR1 and CFHR3 (Hughes et al., 2006; Schmid-Kubista et al., 2009).

A familiarSNP in C3 gene (rs2230199) encoded by the chromosome 19p13 has shown to be strongly recommended to be associated with AMD (Yates et al., 2007; Spencer et al., 2008). It causes change in the amino acid sequence at protein level whereby arginine is replaced by glycine at 102 position (R102G) thus enhancing the AMD risk factor approximately twice in heterozygous subjects and thrice in homozygous individuals. This association of SNPs in genes of the complement system further supports the idea that this inflammatory system is involved in the AMD pathology (Fig 1.7) (Zipfel et al., 2010).

1.13.2 C - Reactive Protein (CRP)

The genetic make-up of CRP is related to its plasma levels in healthy individuals as reported in young White population and aged African Blacks (Pankow et al., 2001; Vickers et al., 2002). CRP gene haplotype strongly influence plasma CRP levels in an individual (Carlson, et al., 2008; Szalai, et al., 2005). Therefore, it is logical that CRP might be involved in AMD and that alleles associated with elevated plasma CRP levels would confer increased risk of AMD.

1.13.3 Age-Related Maculopathy Susceptibility 2 (ARMS2)

Another locus associated with AMD is positioned on 10q26 chromosome that has two separate genes. They have two distinct products that are HTRA1 and ARMS2. The SNPs in these genes show strong linkage disequilibrium although effects of these mutations are not distinguished statistically (Jakobsdottir et al., 2005). The SNP associated with AMD is on exon 1 of the gene. The SNP is rs10490924 that increases the risk to 2.6 for heterozygous subjects and 7.0 for homozygous individuals (Rivera et al., 2005). This mutation leads to the non-synonymous A69S shift in the amino acid sequence of the protein. This variation is studied to be related to AMD (Fritsche et al., 2008).

1.13.4 High Temperature Required Factor A1 (HTRA1)

The other SNP in human chromosome 10q26 lies in the HTRA1 gene. It has a promoter variant for HTRA1 which is a heat shock serine protease (DeWan et al., 2006; Yang et al., 2006). A study in China by Zhang et al. (2012) has identified that A >G SNP in AMD patients is associated with increased HTRA1 expression. In addition, HTRA1 knockout mice (htra1 (-/-) were shown to have reduced vascularization in retina.

Multiple variants of the HTRA1 gene that increase the risk of AMD are known, hence multiple functional consequences are shown with respect to the haplotypes that present vulnerability to complex traits (Yang et al., 2010). The linkage of HTRA1 rs11200638 G/A systematically pooled by Tong et al. (2010) reported in Human Genome Epidemiology (HuGE) systemic review that an association between HTRA1 G/A polymorphism (rs11200638), LOC387715/ARMS2 G/T (rs10490924) polymorphism and AMD is present. An SNP in HTRA1 gene labelled as rs11200638 has been reported to be associated with CNV development. Homozygous individuals

for this variant show enhanced protein expression by factor 1.7 in comparison with the protective variant in donor eyes (Dewan et al., 2006; Yang et al., 2006).

1.13.5 Apolipoprotein E (ApoE)

It is protein with polymorphic gene having three allelic variants located on human chromosome 19. Three variants; E2, E3 and E4 are reportedly related to AMD pathogenesis. The frequency of E3 is higher in the White population. On the other hand, E4 allele is reported to reduce the risk of AMD or can delay the disease onset, whereas E2 allele appears to be involved in causing AMD at a relatively younger age (Baird et al., 2004). Controversy however exists as regards the involvement of ApoE to cause AMD. A study on French population by Asensio- Sanchez et al. (2006) has reported elevated risk of AMD as a result of E4 allele having an odds ratio 5.6. Contrary to this, no association of any apoE allele and AMD was reported in Norwegian population (Utheim et al., 2008). Similar were the findings in Hungarian population where no allele associated with AMD could be detected (Losonczy et al., 2011).

1.13.6 Interleukins

The variation in cytokine production at individual level is influenced by genetic polymorphism in the genes of several cytokines. It is important therefore to investigate these variations in AMD patients to determine the role of cytokines in this pathology. A study in Taiwan Chinese population examined 14 candidate SNPs of five interleukin genes. It was reported that rs2227306 T allele had significant association with wet type AMD (Tsai et al., 2008).

IL-10 is another cytokine that is involved in cell-mediated cytotoxic inflammatory responses (Wu et al., 2002). However, no SNP of IL-10 was found associated with AMD in a previous study where three variants of IL-10 promoter region i.e., 592, 819, and 1082, were found to have no specific role in AMD. Similarly, in case of IL-6, no specific genetic polymorphism has been identified in AMD subjects (Tsai et al., 2008). Thus, AMD seems to be the results of linkage between many different loci vulnerable to cause the disease and this might not be considered a single gene disorder. Moreover, it is the degree of interference and number of loci that vary a lot and the situation still remains unclear (Tong et al., 2010).

1.14 Effect of Reported SNPs on Protein Function

The genes of various proteins having SNPs as reported in the literature affect the function of protein products hence lead to AMD.

1.14.1 Complement Factor H and Other Complement Proteins

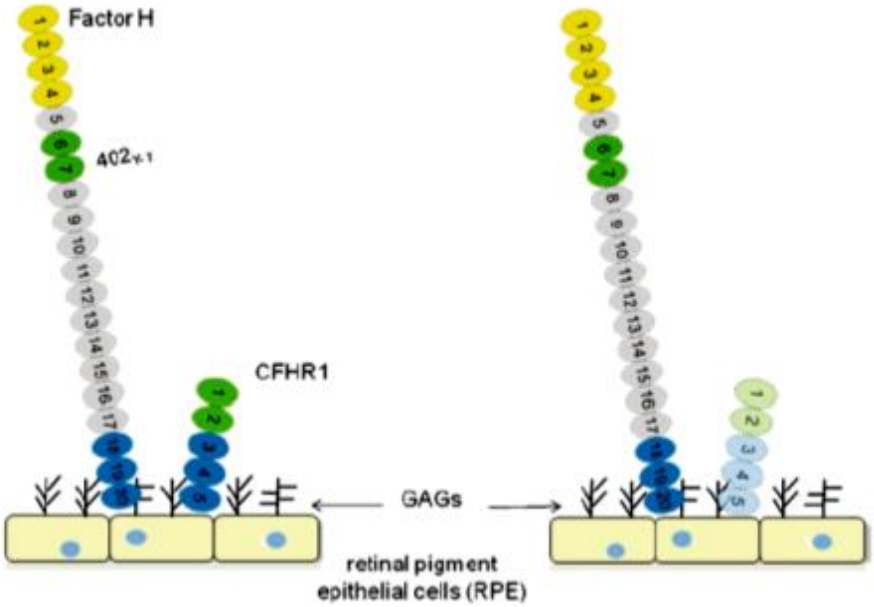
The gene mutations mentioned above show that complement factor H (CFH), factor H like protein 1 (FHL1), CFHR1, CFHR3, factor B and C3 are involved in AMD pathology. It is obvious that these changes in the gene sequence lead to an alteration in the functional status of the protein encoded by this gene. Therefore, a balance between complement system inhibition and activation is essential to evade inflammatory diseases like the AMD (Zipfel and Skerka, 2009).

Factor H and FHL1

Factor H and FHL1 are encoded by the human factor H gene (Jozsi and Zipfel, 2008). Alternative splicing of the gene product leads to FHL1, a secreted protein having seven N-terminal domains identical to factor H, this region also has codon 402 for which the Y402H SNP has been mentioned earlier. The protein has four amino acids extension at the C-terminus (Misasi et al., 1989). These two proteins regulate complement system at C3 convertase level and have binding sites for C3b, CRP and heparin in addition to biological and cell surface binding sites (Jarva et al., 1999; Giannakis et al., 2003). The binding affinity of CFH and FHL1 is affected by exchange of tyrosine to histidine at position 402 which is associated with the AMD (Laine et al., 2007; Sjoberg et al., 2007; Ormsby et al., 2008). The variationin the binding affinity because of the change in positively charged amino acids essential for binding to heparin properly over short consensus repeat 7 (Prosser et al., 2007; Ormsby et al., 2008). This decline in binding affinity related to risk variant of the protein reduces binding to cell surface in RPE cells that leads to insufficient regulation of complement system (Skerka et al., 2007). This SNP also affects binding with CRP, an inflammatory marker. The homozygotes for H402 have been reported to show 2.5 folds higher CRP immunoreactivity in extracellular deposits in Bruch's membrane and the choroid (Johnson et al., 2006). On the other hand, individuals with Y402 have lowered CRP levels in drusen, choroidal stroma and basal deposits. Therefore, it is suggested that factor H mediated CRP function is affected by the Y402H polymorphism. This leads to the local inflammation resulting from complement activation due to cellular injury of RPE cells (Zipfel et al., 2010).

Complement Factor H Related Proteins

The AMD developmentis protected by deletion of fragment of 84 kbp size present downstream to factor H cluster. In homozygous individuals, the deletion of this fragment leads to complete absence of two proteins in plasma, the CFHR1 and CFHR3 (Zipfel et al., 2007). CFHR1 and the factor H have almost the same C-termini. This terminal is important for surface recognition and cell binding, therefore CFHR1 and factor H can bind to cell surface simultaneously (Fig 1.7), thus in case of deficiency of CFHR1 more binding sites are accessible for factor H (Heinen et al., 2009).



(A) (B)

Fig. 1.7 (A) Factor H has 20 SCR while CFHR1 has 5 domains. Three amino acids in Factor H and CFHR1 are identical at C- terminus.

(B) Deletion in CFHR1 gene leads to loss of CFHR1 in vitreous humour or the plasma which is a defensive mechanism for AMD. Therefore, more sites for factor H binding are available providing a better local protection (Zipfel et al., 2010)

1.14.2 Factor B, C2, and C3: Other AMD Associated Complement Proteins

Another protective polymorphism related with protection against AMD is in the complement C2 and Factor B (Gold et al., 2006). These two proteins are present in the choroid, neural retina and RPE. Studies show that the protective effect is most probably mediated via factor B mutation rather than C2 gene variation. The C2

variants associated with AMD are either intronic or show conservative change (Lokki and Koskimies, 1991).

In addition, a single amino acid mutation in L9H factor B affects the protein secretion and processing. This causes change in activation of complement pathway and ultimately leads to complement degradation. The C3 polymorphism associated with AMD includes replacement of arginine (R) to glycine (G) at 102 amino acid position of sequence (Yates et al., 2007). It leads to change in protein mobility leading to "fast" C3F and "slow variant" C3S (Botto et al., 1990). Positively charged R102 is substituted by neutral G102 in C3b weakening the link between thioester activity and the charged surfaces. Thus, the risk of AMD is enhanced due to G102 variant that results into the formation of C3b-factor H complex leading to inefficient inactivation of C3b on retinal cell surface (Johnson et al., 2001; Nozaki et al., 2006).

1.14.3 Chromosome 10q26 Gene Products: HTRA1 and ARMS-2

A newly discovered 12 kDa protein with unknown biological function is encoded by the ARMS gene. The ARMS2 gene SNPs are more strongly associated with AMD compared to the HTRA1 gene (Kanda et al., 2007). The polyadenylation signal loss in ARMS2 mRNA resulted by the insertion-deletion polymorphism is shown to be associated with AMD. ARMS2 protein is reported to be associated with cytoskeleton and outer membrane of mitochondria. S69 variant of ARMS2 leads to absence of protein resulting to AMD progression (Zipfel et al., 2010).

A serine protease HTRA1 is encoded by the gene on human chromosome 10q26. Human and murine RPE cells express this protein (Oka et al., 2004), which regulate extracellular matrix proteoglycan degradation facilitating the access of other matrix degrading enzymes including matrix metalloproteinases and collagenases to their substrate (Grau et al., 2006). The elevated levels of HTRA1 change Bruch's membrane integrity and in turn facilitate choroidal capillary invasion across the extracellular matrix in wet AMD. This hypothesis provides an alternate mechanism for the AMD pathogenesis which does not involve complement-mediated progression of disease (Zipfel et al., 2010).

1.14.4 Interleukins

The IL-8 gene is located on human chromosome 4, q13-q21. The gene has three introns and four exons (Mukaida et al., 1989). IL-8 is a member of the

chemokine family and is multifunctional in nature which is involved in chronic and acute inflammation (Heinzmann et al., 2004). It also shows proangiogenic properties and is involved in CNV formation in AMD. The CNV of AMD patients have been reported to show VEGF and IL-8 expression (Kalayoglu et al., 2005). In fact, neovascularization in choroid is a complex process and involves many stimulators and inhibitors (Tsai et al., 2008). The role of IL-8 in AMD pathology is yet not confirmed.

1.15 Studies on AMD in Pakistan

A nationwide survey for eye diseases in Pakistan was carried out long ago in 1987-90 by National Committee for Prevention of Blindness but an authentic data for prevalence of AMD in Pakistan is still lacking (Woo et al., 2009). The survey reported 1.8% prevalence of blindness in Pakistan, of which cataract was the most common disease with 66.7% prevalence (Memon 1992). With the improvement in surgical and diagnostic facilities for cataract, the demography of eye diseases has shifted to the retinal pathologies in the Indian Subcontinent. Jadoon et al. (2006) updated the blindness data in Pakistan and reported that blindness is defined as visual acuity <3/60 in better eye. The cross-sectional data at national level showed that 2.7% adults of above 30 years of age are blind. Later, the Pakistan National Blindness and Visual Impairment Survey reported that macular degeneration was 2.1% of all the blindness pathologies; it did not address age-related macular degeneration separately (Dineen et al., 2007). Prevalence of the macular degeneration with moderate visual impairment was similar in all the four provinces of the country; Khyber Pakhtunkhwa (KPK) 1.2%, Balochistan (1.0%), Punjab (1.0%) and Sindh (0.8%). When compared, the urban and rural populations showed a clear difference with 1.8% and 0.6%, respectively although the reporters did not mention if the difference was significant or not. The same survey reported that 35,000 adults above 30 years of age had macular degeneration and the figure is expected to rise to 72,000 by 2020 (Woo et al., 2009).

AMD is one of the emerging challenges for public health and eye care professionals in Asia (Eong, 2006). Unfortunately, in Pakistan, the literature on AMD is scarce. The data on the involvement of inflammatory markers and angiogenic mediators in AMD is limited and with no defined consequences. The present study was therefore designed to investigate the association of selected angiogenic factors and markers of inflammation including IL-6, IL-8, ApoE, leptin, CFH, CRP, VEGF and HTRA1 with AMD and compare the serum levels of these factors in AMD

patients to that in normal age related controls. The single nucleotide polymorphism in the genes of angiogenic factors and inflammatory markers was also the focus of the study to highlight the gene loci involved in the pathology especially in Pakistani population.

1.16 Aim

The aim of the present study was to investigate the association of serum levels of inflammatory mediators and angiogenic growth factors with the genetic polymorphism in AMD patients selected from local Pakistani population.

1.17 Objectives

The focus of the study was to:

1. Highlight anthropomorphic differences between AMD patients and healthy subjects.
2. Determine serum cholesterol, triglycerides, LDL and HDL in AMD patients and healthy subjects.
3. Estimate the serum levels of IL-6, IL-8, VEGF, CRP, ApoE, HTRA1, leptin, and CFH in AMD patients and age related healthy subjects.
4. Identify risk related SNPs in gene loci of inflammatory markers and angiogenic factors including IL-6, IL-8, VEGF and CRPin Pakistani AMD patients.

CHAPTER # 2

DEMOGRAPHIC DATA AND CLINICAL DIAGNOSIS

Introduction

Age related macular degeneration (AMD) is a heterogeneous group of disorders that affects the macula of aging individuals. AMD is reported late in life usually in the fifth decade and later. It is one of the major eye related health problems in the developed countries and affects people of 55 years of age or older (Hollyfield, et al., 2007). Although several risk factors are recognized, of which age is the strongest. Systemic risk factors include smoking; hypertension and positive family history also contribute to enhance the risk of AMD. Soft drusen and pigmentary changes along with choroidal neovascularization (CNV) are ocular features related to AMD. As a person's age increases, retinal pigment epithelial (RPE) cells become less efficient in their function as they do not receive proper nourishment and accumulate waste material that leads to drusen. RPE cells degenerate and atrophy that leads to loss of central vision. Geographic atrophy progresses slowly over many years as it takes time from 5-10 years for a person suffering with AMD to be legally blind i.e. visual acuity ≤3/60 (Chopdar et al., 2003).

2.1 Clinical Appearance

Clinical symptoms of AMD include drusen, retinal pigment epithelium (RPE) changes, RPE atrophy (both geographic and non-geographic) and choroidal neovascularization (CNV). Although appearance of drusen is the first clinically visible abnormality in AMD patients, this may not be considered as the first detectable change. The patients with early stage AMD complain poor light adaptation although central vision remains normal. Abnormalities in dark adaptation precede clinically detectable macular changes. It suggests that dysfunction of rod photoreceptor in the retina is an early indicator of AMD (AREDS, 2005). Most commonly the patients of AMD complain with blurring of central vision, reduced visual acuity and metamorphosia. It can ultimately lead to the loss of central visual field and central scotomas. The distorted squares with wavy lines on a graph paper are seen by a person suffering with AMD.

AMD is usually classified by the presence or absence of abnormal neovascularization. It can be exudative or wet and non-exudative or dry AMD. The ophthalmoscopic examination shows chororetinal atrophy in case of dry AMD and macular oedema in wet AMD which is usually related with retinal haemorrhage and

lipid exudates around the macula (Chopdar et al., 2003).

AMD is usually reported in patients aged above 50 years and is characterized by changes in fundus that include drusen and lipofuscin deposits, degeneration and pigmentary atrophy and exudative elevation of outer retinal complex in macular area. Significant irreversible loss of central visual functions due to fibrous scarring or diffused geographic atrophy of macula is also associated with AMD. Moreover, extrafoveal lesions affect the vision if superimposed on the foveal region (Bressler et al., 1999).

2.2 Diagnostic Techniques

The evaluation of patient with AMD may include the diagnostic techniques including detailed patient history with clinical examination. The Optometric Clinical Practice Guideline for Care of the Patient with AMD (1997) provides guidelines for proper ocular examination and treatment procedures to help reduce severe vision loss and identify patients with high risk characteristics. Diagnosis of AMD is primarily carried out through various techniques of ocular imaging that have advanced considerably in recent years. It has also facilitated the development of evidence-based classification system of AMD including early and late AMD. Moreover, wet and dry types of AMD are also identified on the basis of this diagnosis (Rickman et al., 2013).

The awareness regarding etiopathogenesis of AMD has led to better diagnostic techniques and treatment options. The basic technique for diagnosis is the assessment of visual acuity along with ophthalmological examination. Mostly patients with severe loss of vision have exudative AMD while in dry AMD usually vision loss is gradual but significant. Therefore, patients with both types need continuous monitoring.

Routine examination includesfundus fluorescein angiography which is carried out to investigate wet or exudative AMD. In this invasive technique intravenous fluorescein (yellow dye) is injected and then sequential photographs are taken and recorded for assessment of choroidal and retinal blood flow. In CNV, leakage of dye into surrounding tissues leads to hyperfluorescence. Especially designed fundus cameras are used for angiographic imaging with appropriate filter combination. The detail of retinal vascular architecture can be acquired and recorded. Moreover, digital angiography and tomographic techniques have facilitated better delineation of CNV

for monitoring of disease progression and efficacy of treatment. In classical CNV the lesion penetrates RPE while in occult CNV; the lesion is present under RPE (Lim et al., 2012).

In classic leakage, hyperfluorescence has well-defined borders while in late phase obstruction of boundaries is seen in the angiogram. On the other hand, during occult phase, CNV is poorly defined that appears in late phase of dye. Eyes with CNV display different properties of classic and occult leakage (Chopdar, 1996).

Ocular Coherence Tomography (OCT) is a non-invasive examination technique that provides cross-sectional image of retina applied for the evaluation of stage of AMD. It is helpful to identify the extent to which retinal layers have been distorted and to examine the efficacy of treatment in terms of increase or decrease in retinal inflammation after treatment. OCT provides volumetric and cross sectional views of retina that facilitates measurement, visualization and monitoring of the layers in retina (Leuschen et al., 2013). Through OCT, ophthalmologists are able to identify alterations in RPE and secondary retinal changes in addition to identify drusen. However, structures like basal laminar deposits cannot be identified with this method. Classical CNV is evident in OCT but occult choroidal neovascular membranes appear less delineable structure with OCT. Therefore, OCT does not provide any additional information in patients with non-exudative AMD. However, OCT is able to characterize the relation of membrane to RPE and imaging may be possible through haemorrhage in patients with wet AMD. Optical coherence tomograms require further studies to be conducted including clinicopathologic correlation (Spraul et al., 1998).

The current study was carried out to estimate the prevalence of AMD patients having no other disease. The diagnosis was carried out through estimation of visual acuity, FFA and OCT. General and clinical history of the patients was also recorded.

Materials and Methods

2.3 Study Design and Duration

The present was a cross-sectional and case-control study. The sampling of patients was carried out done between February, 2010 and February 2011.

2.4 Approval Form Ethical Committees

The study was approved by Bio Ethical Committee, (BEC) Quaid-i-Azam University, Islamabad, Pakistan, (Protocol # BEC-FBS-QAU-06) and ethics committee of Al Shifa Trust Eye Hospital and Pakistan Medical and Research Council, Islamabad. Written informed consents were taken from each patient and control subjects after explaining the study procedures to each one of them.

2.5 Anthropomorphic History

A total of 3911 patients were screened for AMD in the Out-Patient Department (OPD) of Retina Clinic at Al Shifa Trust Eye Hospital, Rawalpindi. Healthy subjects, considered as controls, were recruited from the general population and hospital staff. They were also screened for any aliments that could bias the results. After the chief complaints were noted, a detailed history of the presenting illness was recorded for AMD patients.

2.5.1 Personal History

History of smoking was recorded. Anyone who quit smoking within one year was also considered as smoker. History of chewing or sniffing tobacco, alcohol consumption, huqqah and betal leaves (pan) was recorded.

Complete history of systemic diseases like hypertension, diabetes, cardiovascular diseases and arthritis along with the prescribed medications was also recorded.

2.5.2 Ocular History

Sudden vision loss without any pain was considered in the reported cases. History of distorted images, metamorphopsia or flashes of light and floaters was noted. History of near or farsightedness was recorded.

2.5.3 Family History

Family history of the subjects for any genetic or systemic disorder was registered.

2.6 Inclusion Criteria

Patients above 50 years of age, both genders, normotensive, non-diabetic, having no family history of such diseases or complications of posterior ocular chamber other than the AMD was included in the study. Control subjects were also screened with the same inclusion criteria and in addition they had no complication of posterior chamber including the AMD.

AMD patients presenting any of the followings features were included: drusens; geographic atrophy; retinal pigment epithelium (RPE) atrophy; sub retinal membrane; exudation; hemorrhages; edema and sub retinal neovascularization (CNV).

2.7 Exclusion Criteria

Patients below 50 years of age; have any inflammatory disease like arthritis; on medications like chloroquine, anti-psychotic drugs etc were excluded from the study.

2.8 Clinical Examination

2.8.1 Systemic Examination

Blood pressure of all the patients was checked using sphygmomanometer (MP570, MEK, USA).

2.8.2 Visual Acuity

The Snellen chart and illiterate E charts were used to estimate the visual acuity. All the patients were refracted by a trained optometrist. Unaided visual acuity was taken for distance and near and the best corrected visual acuity was also tested.

The log of minimum angle resolution (logMAR) was calculated using the following formula:

$$\text{LogMAR VA} = 0.1 + \text{LogMAR of the best line read by the subject}$$

The relationship between Snellen chart reading and logMAR is given in table 2.1.

Table 2.1 Relationship between Snellen chart reading, minimum angle of resolution (MAR) and the logarithm of minimum angle of resolution (logMAR) (Carlson and Kurtz, 2003).

Meters on Snellen Chart	MAR	logMAR
6/60	10	1.0
6/48	8.0	0.9
6/38	6.3	0.8
6/30	5.0	0.7
6/24	4.0	0.6
6/19	3.2	0.5
6/15	2.5	0.4
6/12	2.0	0.3
6/9	1.6	0.2
6/7.5	1.25	0.1
6/6	1.00	0.0
6/4.8	0.80	-0.1
6/3.8	0.63	-0.2
6/3.0	0.50	-0.3

Spherical equivalent refraction (SER) was calculated by the formula

$$\text{SER} = \text{sph.} + \frac{1}{2} \times \text{cyl.}$$

2.8.3 Examination through Slit Lamp

The slit lamp is a microscope to view the eye ball at a higher magnification. This instrument is known as slit lamp as it projects a narrow slit of light over the eyeball structures. This narrow slit allows the ophthalmologist to view an optical cross-section of multiple layers within the eye.

The slit lamp (C-153 TOPCON, SL 3D, USA) was used to observe the anterior chamber cells and flare to rule out any intraocular inflammation, in the anterior and posterior segment. The normal color of the iris was recorded. Lenticular cause of decreased vision was recorded.

2.8.4 Intraocular Pressure (IOP)

IOP is the pressure of fluid inside the eye. IOP is measured through tonometry method by eye care professionals. Mostly tonometers are standardized to measure pressure in millimetres of mercury (mm Hg). Intraocular pressure was measured by trained ophthalmologists. The eye was anesthetized using local anesthetic drops containing 0.5% proparacaine (Alcaine by Alcone Couvreur, Belgium) and then stained by fluorescein strips.

Currently, IOP was measured through Goldmann applantation tonometry. For this purpose, a special disinfected prism was placed against the cornea after mounting on the tonometer head. Then a cobalt blue filter was used by the examiner to view two green semi circles. Then the force applied to the tonometer head was attuned through a dial connected to a variable tension spring until the inner edges of the green semicircles in the viewfinder met. When an area of 3.06 mm was flattened, the opposing forces of the tear film and corneal rigidity were approximated. Thus the pressure inside the eye was determined from the applied force. The diameter of the circular zone of applanation was 3.06 mm.

2.8.5 Fundus Examination

Fundus was examined by an experienced retina specialist. Ophthalmoscopy was done with a fully dilated pupil. To better view the inside of the eye, temporary dilation of the pupil was achieved by the application of 1% Cyclopentolate-HCl (Eethical Laboratories, Pakistan)

The fundus was examined thoroughly using 90-diopter lens and 78-diopter lens with the slit lamp and indirect ophthalmoscope using 20D lens. The vitreous was

examined to rule out any inflammation. The shape, size and colour margin of the optic disc were noted. The macula was examined for the type of drusen, retinal pigment epithelium (RPE) atrophy, geographic atrophy, hemorrhages, oedema, sub retinal neovascularization or disciform scar.

2.8.6 Ocular Coherent Tomography (OCT)

It is one of the non-invasive techniques used for retinal imaging. It is used to view images of cross-section of the retina to detect and treat eye conditions such as macular holes, membranes, optic nerveand macular damage. It is comparable to the CT scan of internal organs as it rapidly scans the eye using the optical backscattering of light and display a pixel representation of the retinal anatomy. All of the ten layers can be differentiated and their thickness can be measured separately. OCT was done with OCT machine (Zeiss, Germany) after dilating the pupils, by an expert OCT technician.

2.8.7 Fluorescent Fundus Angiography (FFA)

Fluorescein angiogram provides information about the blood vessels of retina. It was performed by an expert vitreo-retina specialist. Fluorescein sodium based dye (Sipic Fluor 25%, Sipic International, USA) solution in vial was diluted in 1:1 ratio with sterilized injectable distilled water. About 4 ml of the diluted solution was injected intravenously. This dye appeared in the blood vessels of the retina in around 10-15 seconds. As it reached blood vessels in the retina, an ophthalmic photographer saved the pictures of retina with a specialized retinal camera. FFA was done with FFA machine (Topcon, USA, TRC50EX, retinal camera, Image Mat, 2000).

2.9 Patient Consent

After complete screening and confirmation of AMD, the patients were informed and explained about the study protocol and written informed consent was signed by each of the patient and control subject on the prescribed proforma (Annexure I).

2.10 Statistical Analyses

All statistical analyses were performed on "IBM Statistical Product and Service Solutions (SPSS) Statistics" (version 18.0, Chicago, Illinois, USA) and Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). All data were presented as mean ± standard error of mmean (SEM).

Significance level of $P < 0.05$ was used for all the comparisons. The non-parametric factors were compared between control and AMD patients by chi-square (χ^2) analyses.

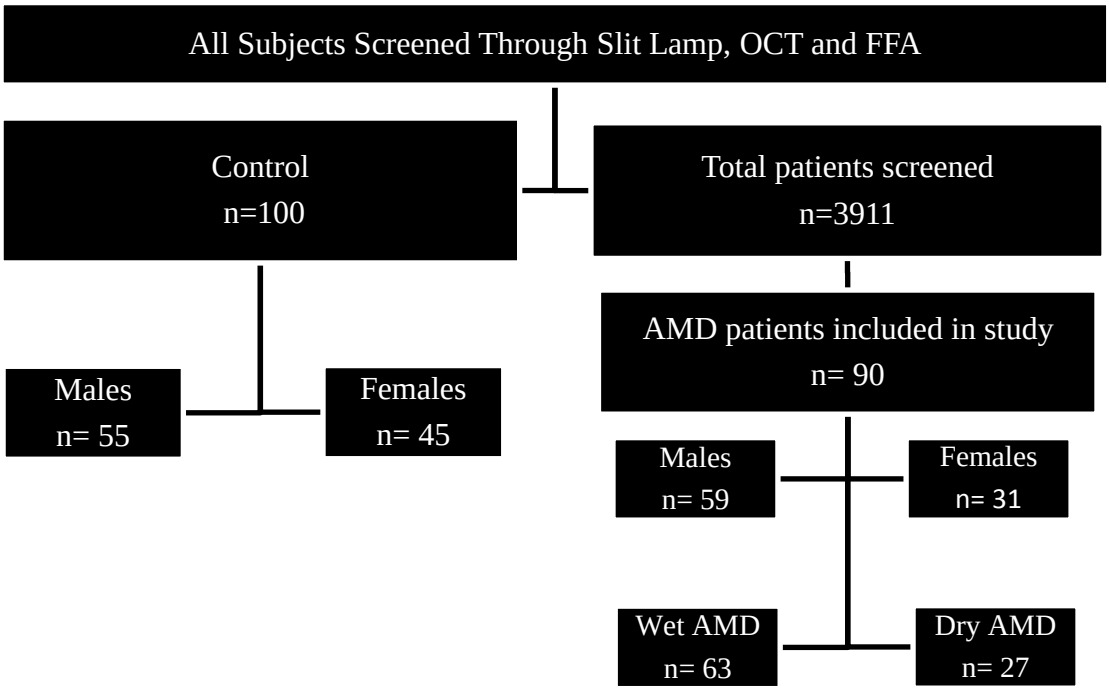


Fig. 2.1 Flow chart showing the number of subjects in control and AMD patients and the gender wise comparison of the two groups

Results

2.11 Patients and Controls Recruitment and Demography

A total of 3911 patients (2621 males; 1290 females) visiting the Vitreo-retina Clinic, Al-Shifa Trust Eye Hospital, Rawalpindi, Pakistan, for ophthalmic complications, were screened for AMD. Only 90 of these fulfilled the inclusion criteria (2.3%). Control healthy subjects (n=100) were recruited from the hospital's healthy staff and general public.

Among control subjects, male to female ratio was 1: 0.81 (55♂; 45♀). In AMD patients, male to female ratio was 1: 0.52 (59 ♂; 31 ♀) (Fig. 3.1). Chi-square ($\chi 2$) value calculated for control group was 0.640 ($p< 0.424$), whereas in AMD patients it was 8.711 ($p< 0.003$). This revealed that significantly greater number of males was affected with AMD (Fig. 2.2).

For control subjects, age range was 50-80 years, with a mean of 63.57 ± 0.84 (SEM) years. Relatively greater number of subjects was in the age range of 50-59 years. The age of AMD patients ranged between 50-100 years while mean age was 71.26 ± 1.07 years (Table 2.2). Only two patients had ages between 90-100 years whereas a single patient was above 100 years of age (Fig. 2.3; Table 2.2). The age of patients in AMD group was significantly greater than the control subjects ($p < 0.0001$) (Table 2.4).

2.12 Province-wise Distribution of Patients

Patients from far flung areas of all provinces visit Al-Shifa Trust Eye Hospital as it is country's one of the major and well equipped tertiary care hospitals with expert ophthalmologists and technicians. Due to travel expenses, distance, lack of awareness and for other obvious reasons the number of AMD patients who visited the hospital is not the same from the four provinces of Pakistan. The catchment area of the hospital covers patients from districts of the Punjab province and areas falling within the Federal capital of Islamabad. Province-wise distribution of control subjects and AMD patients included in the study is given in Table 2.3.

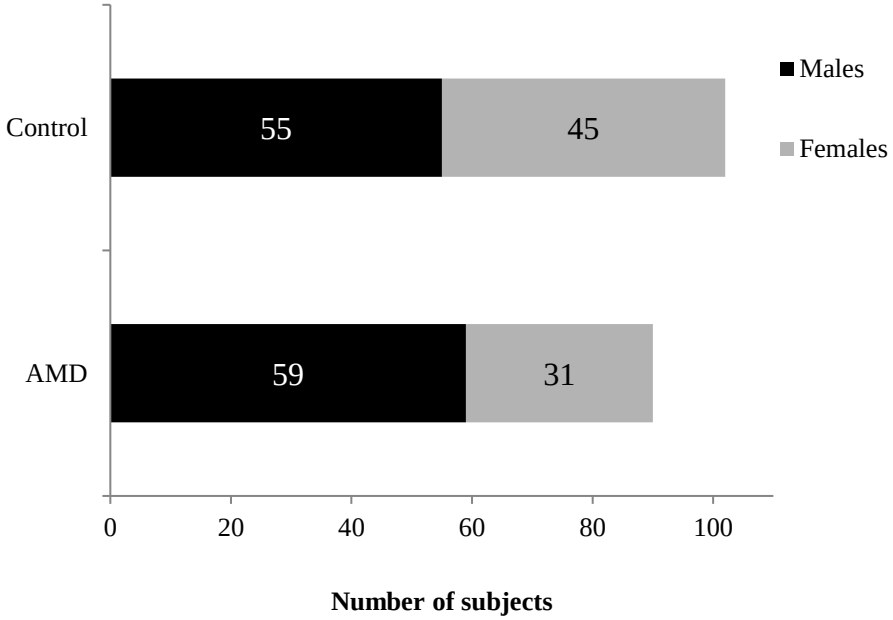


Fig. 2.2 Gender-wise distribution of control and AMD patients

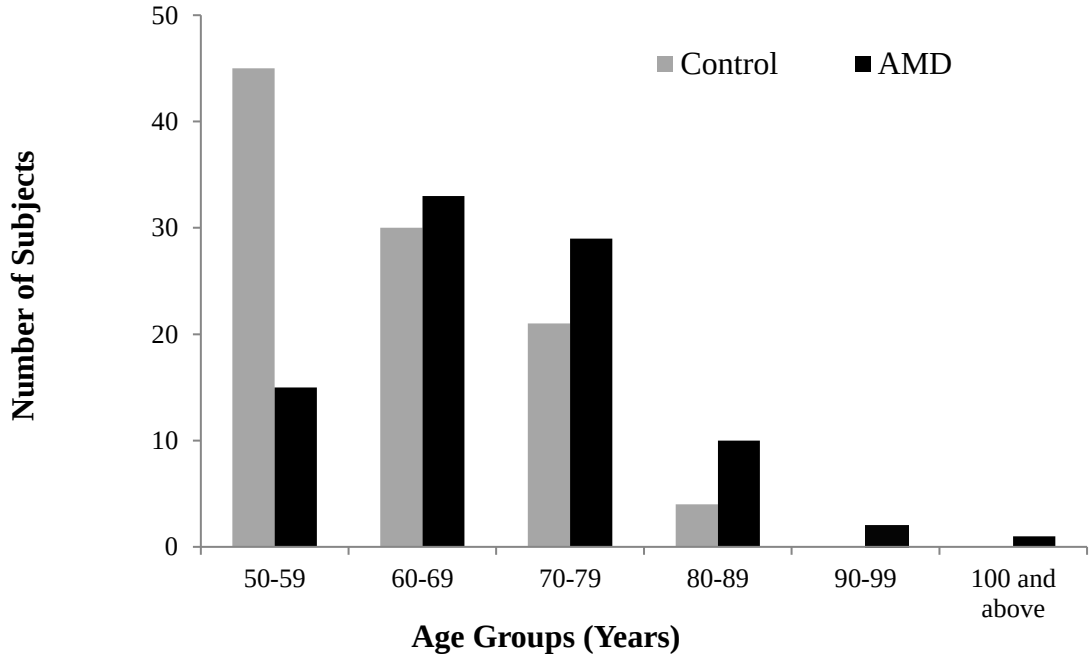


Fig. 2.3 Age-wise distribution of AMD and control groups

Table 2.2 Age groups of control subjects and AMD patients

Age groups	Control n=100	AMD n=90	Wet n=63	Dry n=27
50-59	45	15	8	7
60-69	30	33	26	7
70-79	21	29	19	10
80-89	4	10	9	1
90-99	0	2	0	2
100 and above	0	1	1	0

Table 2.3 Province-wise distribution of control subjects and AMD patients

Province	Control n=100	AMD Patients n=90
Islamabad	04	06
Azad Jammu and Kashmir	12	07
FATA	01	02
Punjab	74	65
Khyber Pakhtunkhwa	08	10
Balochistan	01	00
Sindh	00	00

2.13 History of Systemic Diseases

Neither control nor AMD patients were diagnosed with any of the systemic diseases like diabetes and hypertension.

2.14 History of Addiction

Among control subjects 40 were addicts, of whom 03 were huqqah smokers, 06 were taking niswar (powdered tobacco sniff), and 31 were cigarette smokers of which 16 were current smokers while15 had left smoking a few months ago.

In AMD patients, 23 patients were found to be addicted. Of these, a single patient was using huqqah. Three were taking niswar and 19 were cigarette smokers. Out of these 13 had left smoking and only 6 were current smokers.

No significant difference was observed between the number of smokers in control subjects and AMD patients ($p < 0.057$). The χ^2 value for control group was 4.0 ($p < 0.046$) whereas in the AMD group it was 21.51 ($p < 0.0001$), depicting that number of non-addicts was significantly greater than the addicts in both groups.

2.15 History of Disease

In the control group, 11 subjects had history of disease, out of which 5 had asthma, 3 had nephropathy, 1 had Bell's palsy, 1 had pollen allergy and 1 had gastric ulcer.

In the AMD group 11 (12.2%) patients showed history for disease. They included 5 with history of nephropathy, 3 were asthmatic, 2 with history of paralysis and one patient each of tuberculosis and cholecystectomy.

2.16 Parental Relationship

In controls, parents of 78 subjects were cousins and parents of 22 subjects were non-cousins.

In AMD patients, parents of 70 (77.8%) had consanguineous marriage while parents of 16 (17.8%) were not cousins, in addition, 4 (4.4%) patients were not confirmed about relationship of their parents as they died early in the childhood of the patient.

2.17 Family History for AMD

None of the control subjects had history of AMD. In AMD patients, 74.4% (n=67) had no family history of AMD while 12.2% (n=11) patients had positive

family history of AMD. Whereas 12 patients had unknown family history, as their parents had died earlier than the expected age of AMD onset.

The χ^2 value in AMD group was 40.20 ($p< 0.0001$) showing that the number of patients having no family history was significantly greater than the patients with positive family history for AMD.

2.18 Intraocular Pressure

In control subjects, the mean intraocular pressures (IOP) in the right and left eyes were 12.83 ± 0.34 mmHg and 12.61 ± 0.44 mmHg respectively. In AMD patients, the mean IOP of right eye was 14.53 ± 0.30 and in left eye 14.28 ± 0.26 mmHg. The IOP of all the control subjects and AMD patients was in normal range (10-20 mm Hg) (Table 2.4).

2.19 Visual Acuity

The mean value of spherical equivalent of refraction in the right eye of control group was -0.57 ± 0.41 and -0.31 ± 0.44 in left eye. Mean of unaided visual acuity in control subjects was 6/36 in both eyes (logMAR 0.70) and aided visual acuity was 6/24 (logMAR 0.60) in both eyes (Table 2.4).

The mean of spherical equivalent of refraction in AMD patients was -0.11 ± 0.30 in right eye and in left eye -0.23 ± 0.26. Average unaided visual acuity in AMD patients was 6/60 (logMAR 1.0) in both right and left eyes. Aided vision was 6/36 (logMAR 0.7) in right eye and 6/60 (logMAR 1.0) in the left eye (Table 3.3). The details of visual acuity with number of subjects in control subjects and AMD patients are given in Table 2.5.

Table 2.4 Parameters determined in control subjects vs AMD patients (mean ± Standard error of mean)

Parameter	**Control (n=100)**	**AMD (n=90)**	**p value**
Age (years)	63.57 ± 0.84	71.26 ± 1.07	0.0001***
IOP RE (mm Hg)	12.83 ± 0.34	14.53 ± 0.30	0.004**
IOP LE (mm Hg)	12.61 ± 0.44	14.28 ± 0.26	0.004**
Spherical equivalent of refraction RE(D)	-0.57 ± 0.41	-0.11 ± 0.30	0.354
Spherical equivalent of refraction LE(D)	-0.31± 0.44	-0.23± 0.26	0.847
Visual acuity unaided RE (LogMAR)	0.70 ± 0.01	1.0 ± 0.13	0.012*
Visual acuity corrected RE(LogMAR)	0.60 ± 0.05	0.7 ± 0.10	0.0001***
Visual acuity unaided LE(LogMAR)	0.70 ± 0.05	1.0 ± 0.16	0.0001***
Visual acuity corrected LE(LogMAR)	0.60 ± 0.05	1.0 ± 0.13	0.0001***
Visual acuity unaided RE	6/36	6/60	0.022*
Visual acuity corrected RE	6/24	6/36	0.002**
Visual acuity unaided LE	6/36	6/60	0.001**
Visual acuity corrected LE	6/24	6/60	0.0001***

IOP: Intraocular pressure; RE: right eye; LE: left eye; D: Diopter

*** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$

Table 2.5 Details of number of subjects regarding aided and unaided visual acuity in control and AMD patients

	VA RE		VAC RE		VA LE		VAC LE	
	Control	**AMD**	**Control**	**AMD**	**Control**	**AMD**	**Control**	**AMD**
6/6	1	0	2	6	0	0	1	8
6/9	4	11	13	18	6	9	11	9
6/12	4	5	11	5	6	7	7	8
6/18	10	12	6	21	6	14	6	18
6/24	10	10	13	9	6	11	12	4
6/36	15	13	10	11	12	9	7	15
6/60	10	12	11	6	9	13	12	6
1/60	18	8	24	2	17	12	27	10
CF	25	7	7	3	26	7	11	5
HM	1	8	1	7	8	5	3	5
PL	0	3	1	2	4	2	3	2
NPL	2	1	1	0	0	1	0	0

VA: Visual acuity; VAC: Visual acuity corrected; RE: Right eye; LE: Left eye;
CF: Counting finger; HM: Hand movement; PL: Perception of light;
NPL: No perception of light

2.20 Duration of AMD

The duration of disease in AMD patients was highly varied and ranged between 15 days to 96 months (8 years). The details of number of patients with duration of disease are given in Fig. 2.4.

2.21 AMD Type

Among AMD patients, 70% (n=63) had wet type AMD while 30% (n=27) had dry type of AMD. The χ^2 value was 14.40 ($p < 0.046$) revealing that the number of patients with wet AMD was significantly greater than the patients with dry AMD.

2.22 AMD Pattern

Exudative pattern was observed in 44.5% (n=40), maculopathy in 35.5% (n=32) and atrophic pattern in 20% (n=18) AMD patients. A χ^2 value of 8.267 ($p < 0.016$) in the AMD group showed that the number of patients with exudative pattern was significantly greater than both maculopathic and atrophic pattern of AMD.

2.23 Fundus Examination

No drusen, haemorrhage or scarring were observed in the fundus of any of the control subjects (Fig. 2.5). In contrast, in all AMD patients, small drusen were seen on fundus. Large drusen were observed in significantly ($\chi^2 = 54.44$; $p < 0.0001$) larger number of AMD patients (88.9%). RPE changes were observed in significantly greater number ($\chi^2 = 60.84$; $p < 0.0001$) of AMD patients (91.1%). CNV was observed in 72.2% patients ($\chi^2 = 17.78$; $p < 0.0001$) while geographic atrophy was seen in 36.7% patients ($\chi^2 = 6.40$; $p < 0.011$).

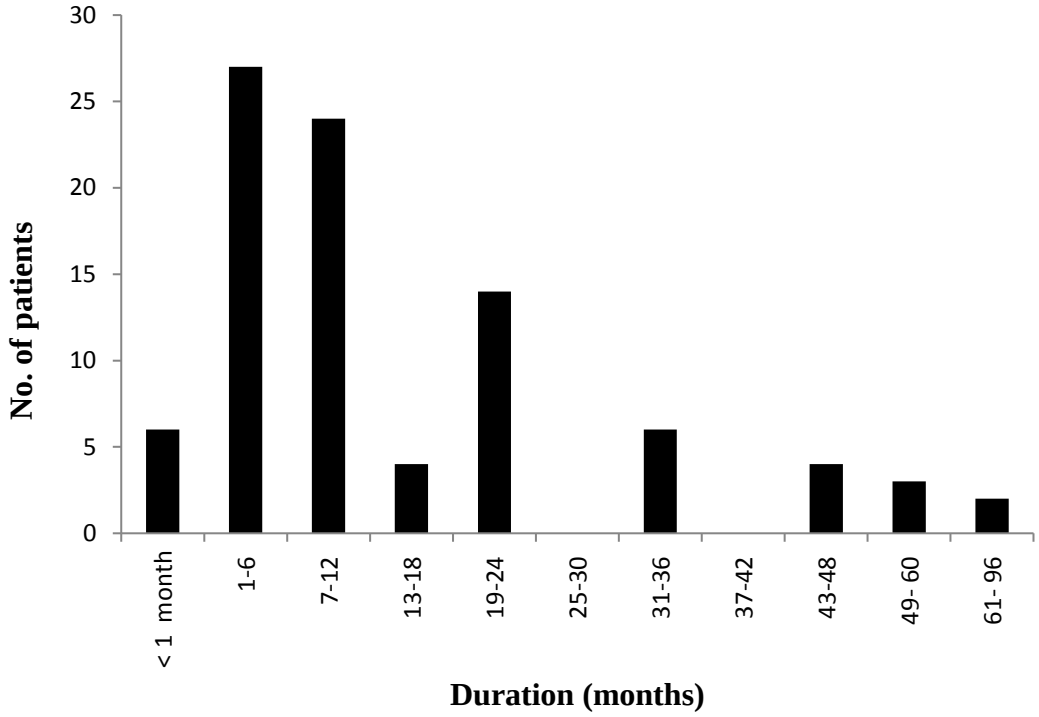
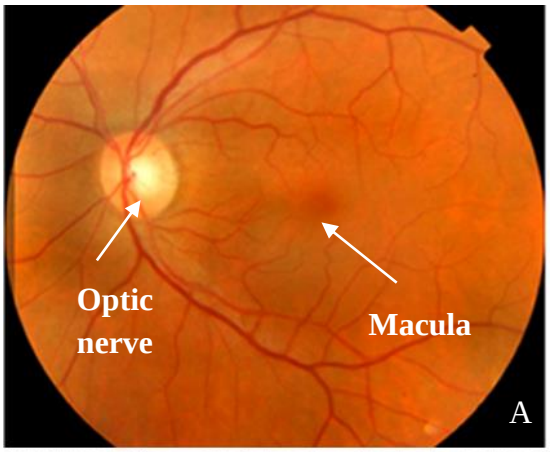


Fig. 2.4 Details of number of patients with varied duration of disease.

A) Fundus from a normal subject showing normal retina



B) Fluorescent fundus angiogram of retina from a normal subject

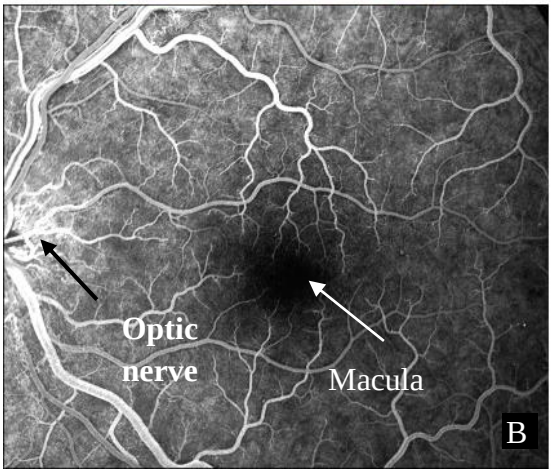


Fig. 2.5 Fundus photograph showing details of healthy retina of a control subject.

2.24 Diagnosis on the Basis of Type of AMD

2.24.1 Age range

The median age of patients with wet AMD was 72.0 ± 1.25 years while in patients with dry AMD median age was 70.37 ± 2.14 years. The detail of number of wet and dry AMD patients in each age group is given in Table 2.2.

2.24.2 Gender Distribution

Among patients with wet AMD, 65% (n=41) were males and 35% (n=22) were females. The number of males was significantly greater than the females (χ^2 = 5.20; $p < 0.015$). Among patients with dry AMD, 66.6% (n=18) were males and 33.3% (n=9) were females. Number of males was non-significantly greater than the females (χ^2= 3.0; $p < 0.083$) (Table 2.6).

2.24.3 AMD Pattern

The exudative pattern was observed in significantly greater number of patients with wet type AMD (63.49%) as compared to maculopathic and atrophic pattern (χ^2 =32.94; $p < 0.0001$). In patients with dry type of AMD none of the pattern was reported in significantly greater ratio (χ^2 = 0.038; $p < 0.847$) (Table 2.6; Fig. 2.6).

2.24.4 Small Drusen

In all AMD patients, occurrence of small drusen was readily noticeable (Table2.6; Fig. 2.6; Fig. 2.7).

2.24.5 Large Drusen

Large drusen were observed in significantly larger number (χ^2 = 43.10; $p < 0.0001$) of patients with wet AMD (93.5%). Likewise, large drusen were also observed in significantly larger number (78.57%) of patients with dry AMD (χ^2 = 8.33; $p < 0.004$) (Table 2.6; Fig. 2.6; Fig. 2.7).

2.24.6 RPE Changes

RPE changes were observed in 91.93% patients with wet AMD (χ^2= 46.62; $p < 0.0001$) whereas in patients with dry AMD 85% subjects had RPE changes (χ^2 =9.846; $p < 0.002$) (Table 2.6; Fig. 2.7; Fig. 2.7).

2.24.7 CNV

CNV was evident in significantly greater number of patients (87.09%) with wet AMD ($\chi 2 = 30.41$; $p < 0.0001$) whereas none of the patients with dry AMD, had CNV (Table 2.6; Fig. 2.6; Fig. 2.8).

2.24.8 Geographic Atrophy

Geographic atrophy was observed in only 25.80% patients with wet AMD therefore significantly larger number of patients did not show this symptom in patients with wet (χ^2 15.51; $p < 0.0001$). In case of patients with dry AMD, geographic trophy was observed in 39.3% patients that was not a significantly larger number ($\chi^2 = 0.93$; $p < 0.336$) (Table 2.6; Fig. 2.6; Fig 2.9).

The detail of interrelationship of different non parametric variables in AMD patients is given in Table 2.7.

Table 2.6 Diagnostic findings in wet and dry AMD patient group

Parameters	Wet AMD		Dry AMD (%)	
	χ^2 value	p value	χ^2 value	p value
Gender	5.92	0.015*	3.0	0.083
AMD pattern	32.94	0.0001***	0.04	0.847
Large Drusen	43.10	0.0001***	8.33	0.004***
RPE changes	46.62	0.0001***	9.85	0.002***
CNV	30.41	0.0001***	NA	NA
GA	15.51	0.0001***	0.93	0.336

NA: not available. χ^2could not be calculated for small drusen as all the patients showed this basic diagnostic symptom for AMD;

*** $p < 0.0001$, ** $P < 0.001$, * $P < 0.05$

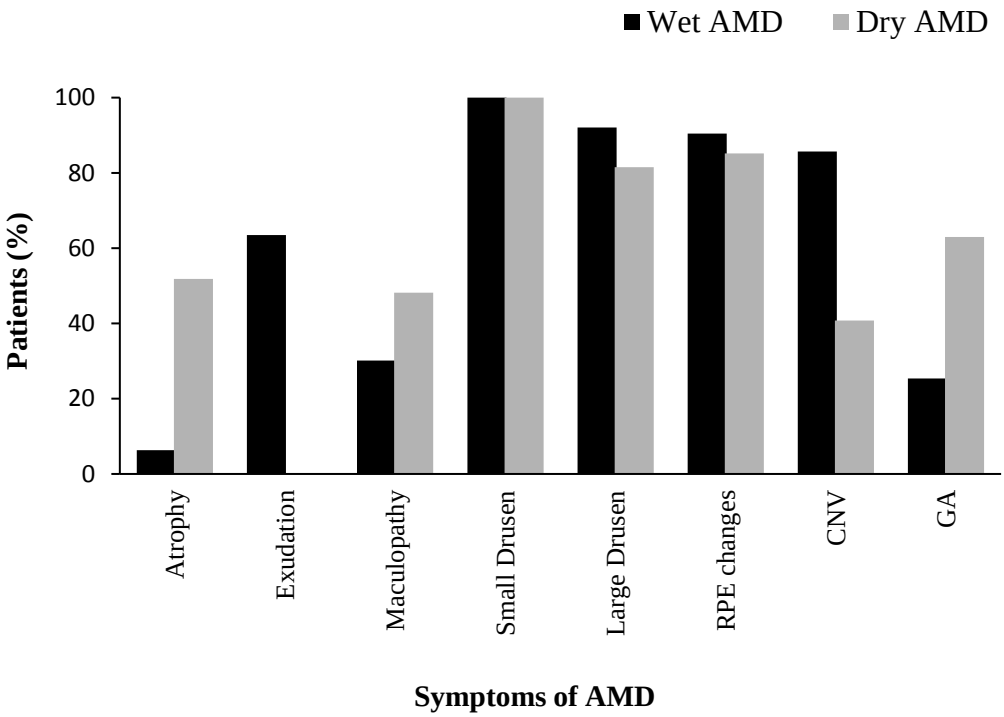


Fig. 2.6 Symptom-wise distribution of patients with wet and dry type AMD

A) Left fundus showing drusen, macular scarring and sub retinal hemorrhage in advanced Wet AMD

B) Left eye showing macular fibrosis

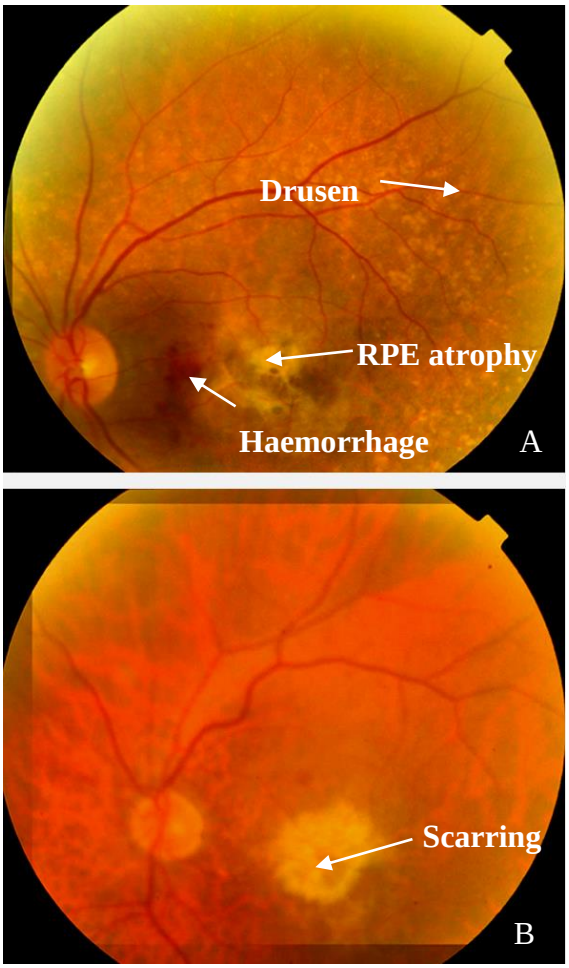


Fig. 2.7 Fundoscopic appearance of retina from a patient with wet type AMD

A) Left eye showing atrophy around disc and macula. There is atrophy of RPE with sub-macular fibrosis and pigmentation.

B) An arterio-venous phase angiogram showing hyperfluorescent lesions at macula

C) OCT showing intra retinal edema with choroidal neovascular membrane (CNVM)

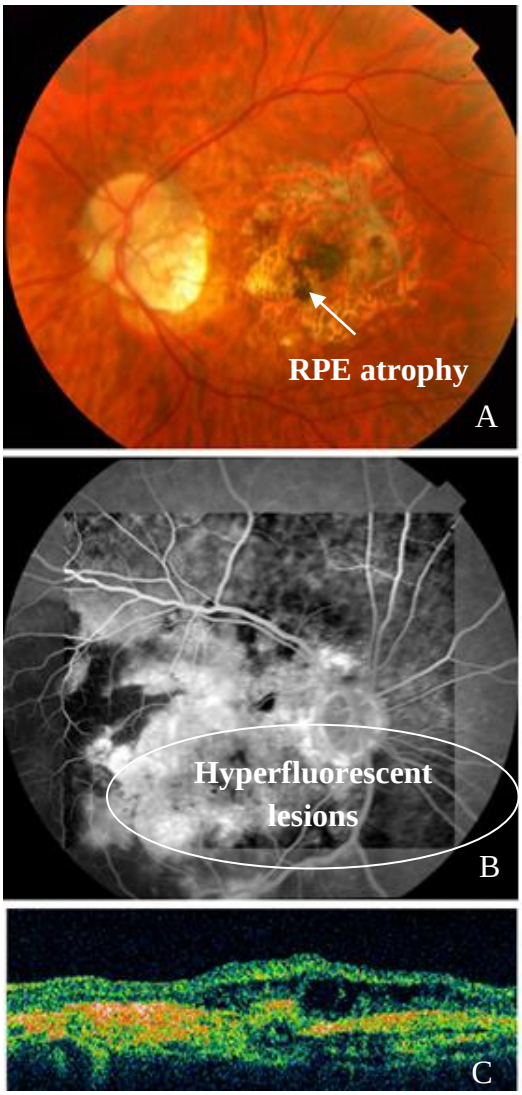
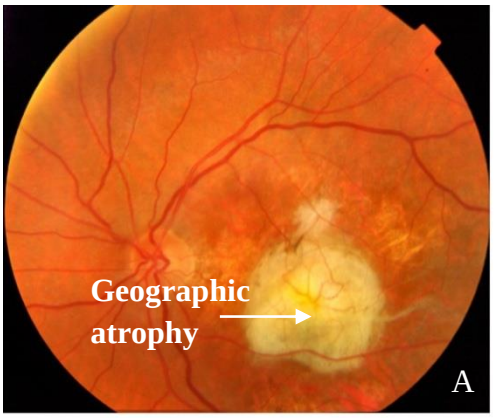
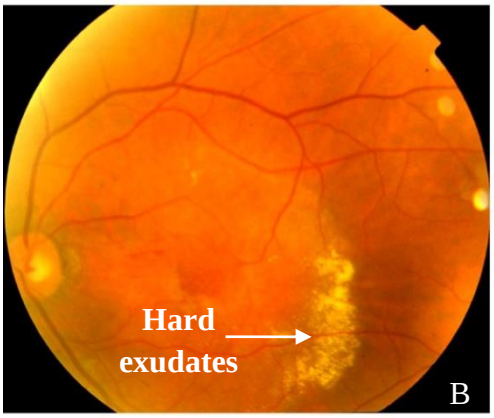


Fig. 2.8 Fundoscopic appearance of retina of a patient with dry type AMD

A) Left eye showing macular scarring and RPE atrophy with prominent large choroidal vessels



B) Left eye showing sub-macular haemorrhage and exudates



C) Right eye sub-retinal hard exudates due to chronic leakage

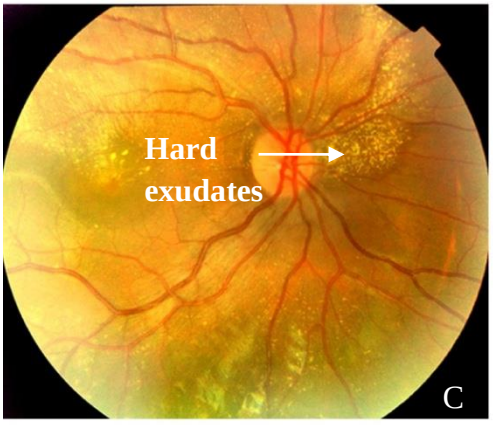


Fig. 2.9 Fundus photograph of retina from a patient with advanced AMD

Table 2.7 Inter-relation of different non parametric variables through cross tabulation in AMD group

Gender	• Addiction (χ^2 =3.979; $p < 0.046^*$) • Family history (χ^2 =3.935; $p < 0.164$) • AMD type ($\chi^2 = 0.021$; $p < 0.885$) • AMD pattern (χ^2 =1.553; $p < 0.460$) • Large drusen (χ^2 =0.154; $p < 0.695$) • RPE changes (χ^2 =0.347; $p < 0.556$) • CNV ($\chi^2 = 4.725$; $p < 0.030^*$) • Geographic Atrophy (χ^2 =0.085; $p < 0.771$)
Addiction	• Family history (χ^2 = 2.070; $p < 0.150$) • AMD type ($\chi^2 = 1.225$; $p < 0.268$) • AMD pattern (χ^2 =0.252; $p < 0.324$) • Large drusen ($\chi^2 = 1.234$; $p < 0.267$) • RPE changes (χ^2 =0.658; $p < 0.417$) • CNV ($\chi^2 = 1.985$; $p < 0.159$) • Geographic Atrophy ($\chi^2 = 4.943$; $p < 0.026^*$)
Family History	• AMD type ($\chi^2 = 0.188$; $p < 0.664$) • AMD pattern (χ^2 =0.356; $p < 0.168$) • Large drusen ($\chi^2 = 0.554$; $p < 0.457$) • RPE changes (χ^2 =1.329; $p < 0.249$) • CNV ($\chi^2 = 4.966$; $p < 0.026^*$) • Geographic Atrophy ($\chi^2 = 2.975$ $p < 0.008$**)
AMD type	• AMD pattern ($\chi^2 = 38.429$; $p < 0.0001^{***}$) • Large drusen ($\chi^2 = 4.821$; $p < 0.028^*$) • RPE changes (χ^2 =4.416; $p < 0.036^*$) • CNV ($\chi^2 = 19.055$; $p < 0.0001^{***}$) • Geographic Atrophy ($\chi^2 = 8.478$ $p < 0.004^*$)

*** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$

Continued-----

Table 2.7 Continued -------

AMD pattern	• Large drusen ($\chi^2 = 2.876$; $p < 0.237$) • RPE changes ($\chi^2 = 1.715$; $p < 0.424$) • CNV ($\chi^2 = 25.324$; $p < 0.0001^{***}$) • Geographic Atrophy ($\chi^2 = 10.108$; $p < 0.006^{*}$)
Large drusen	• RPE changes ($\chi^2 = 6.191$; $p < 0.013^{*}$) • CNV ($\chi^2 = 15.293$; $p < 0.0001^{***}$) • Geographic Atrophy ($\chi^2 = 0.215$; $p < 0.643$)
RPE changes	• CNV ($\chi^2 = 0.414$; $p < 0.520$) • Geographic Atrophy ($\chi^2 = 0.003$; $p < 0.959$)
CNV	• Geographic Atrophy ($\chi^2 = 8.116$; $p < 0.004^{*}$)

*** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$

Discussion

Age related macular degeneration (AMD) is one of the major causes of visual impairment in aged population. Accurate diagnosis of AMD is accomplished through detailed ocular examination that may involve techniques like retinal photography, fundus angiography and optical coherence tomography (Lim et al., 2012). Although, AMD is classified as wet or dry type, severe loss of central vision occurs in both types. Unfortunately, no therapeutic option is available to treat the disease in later stages although anti-vascular endothelial growth factor (anti-VEGF) therapy provides better results in early stages of wet AMD (Rickman et al., 2013).

In the current study subjects aged above 50 years with no systemic or ocular complication other than AMD were selected. Such stringent inclusion criteria resulted to exclusion of AMD patients for the study who were diagnosed for any other ocular or systemic disease along with AMD. Therefore, the figure in the current set of data present only the number of AMD patients who were having no other complication and not all the AMD patients in the studied population. Only 2.3 % of total reported subjectsfulfilled the selection criteria for the study. A study conducted in Pakistan showed high prevalence of AMD but it is usually observed with other ocular complications like pseudophakia, cataract and vitreous degeneration (Naghza et al., 2013).

Important to mention is the fact that very few population-based studies have actually been carried out to estimate the prevalence of AMD in Asia as compared to those in white population. A meta-analysis has suggested the prevalence estimates of 6.8% for early AMD and 0.56% for late AMD in pooled Asian population aged 40-79 years. However, racial and ethnic differences within Asian population cause a great range of diversity in AMD prevalence. A report from Chinese population claims prevalence upto 1.6% in Beijing Eye Study and 2.5% in Multi-ethnic Study of atherosclerosis but it is still unclear whether the difference is real or due to difference in methodology (Cheung, et al., 2012).

No significant difference was observed in the gender ratio between control subject and AMD patient group. Females are at greater risk to develop AMD than the males as suggested by previous large scale population based studies (Smith et al., 1997). One of the limitations of the current study was the lower sample size. The

findings may be confirmed when study is repeated with larger number of subjects with diversified ethnic back ground.

Average age of AMD patients group was about seventy years. Most of the patients (69%) were in the age range from 60 -80 years. Increasing age is a known risk factor for AMD. Smith et al. (2001) showed that in a pooled data of three studies AMD prevalence increased from 0.2% to 133% with advancing age from 55-64 to subjects more than 85 years old. A meta-analysis has shown that prevalence of late stage AMD was similar between Asian and European population in people aged 40-79 years but early stage AMD was more prevalent in Europeans than the Asians (Wong et al., 2014).

In the current study, addiction was non-significantly related to AMD. Although Khan et al. (2006) has demonstrated association of GA and CNV with pack years of cigarette smoking. In the foveal region of retina, yellow pigmentation is composed of zeaxanthin and leutin. Studies have shown that the concentration of these carotenoids decreases in smokers (Hammond et al., 1996). Thornton et al. (2005) in a review of previous studies demonstrated no relationship between AMD and smoking. In the current study, under reporting of smoking might be a factor that led to insignificant findings regarding association of smoking with AMD. The amount of tobacco consumption may also interfere in the association of AMD and smoking in our study. Therefore, further studies to investigate this association are required.

Visual acuity of AMD patients was significantly poor as compared to that of control patients. Progressive low vision is one of the symptoms that appear in aged subjects with early stage of AMD. Visual loss is associated with drusen present between Bruch's membrane and RPE. These drusen induce RPE degeneration that ultimately leads to the visual loss (Johnson et al., 2003). Currently, drusen were observed in all the AMD patients. A number of molecular changes occur in photoreceptors that overly drusen. These include protein expression, protein distribution and morphology. These changes are present in the macular and extramacular area of retina. With the increase in drusen size, these abnormalities also intensify leading to the loss of outer segment and reduced thickness of outer nuclear layer (ONL) in addition to changes in synaptic cytoarchitecture over large drusen.

Drusen protrude into the retina and laterally spread beneath RPE that affect severity of all the above mentioned changes (Johnson et al., 2003).

The number of wet AMD patients as recorded presently was significantly higher than patients with dry AMD. Likewise, significantly larger number of patients was diagnosed with exudative pattern. RPE changes were observed in most of the eyes with AMD. Exudation with CNV was the main outcome in eyes of the patients with wet AMD while geographic atrophy was observed in dry AMD eyes. AMD with CNV i.e., wet type AMD has already been suggested to be more common as compared to dry type related to geographic atrophy. Wet AMD progresses rapidly leading to acute visual loss therefore reported urgently to the clinician. Contrarily, dry AMD progresses slowly and patients perceive this visual loss as age related phenomena and report less frequently. The historic view narrates that nearly four fifths of all AMD cases have dry AMD, where dry AMD includes early stage without exudation or scarring of retinal tissue (Owen et al., 2003). If randomized prevalence studies are carried out on general population, then more patients with early stage of dry AMD are expected.

The current study suggests that prevalence of AMD in Pakistani population is similar to that in other Asian countries. Drusen were observed in all the AMD patients. Number of patients with exudative wet type AMD was higher as compared to non-exudative dry type AMD.

CHAPTER # 3

Estimation of Serum Angiogenic and Inflammatory Factors

Introduction

Keeping in view the tremendous rise in the number of AMD patients it has been included in the action plan of World Health Organization (WHO) to address avoidable blindness in VISION 2020 program (Kulkarni et al., 2013). The pathology of AMD occurs over an extended time frame whereby incidence of disease increases after the age of 70 years. It is a multifactorial disease that affects retinal pigment epithelium (RPE), choriocapillaries, Bruch's membrane and photoreceptors (Ding et al., 2009).

Several biological pathways are studied and associated with AMD pathology. The involvement of inflammation and immune mediated processes has also been suggested in AMD pathogenesis. In both exudative and geographic type of AMD, the degree of inflammation is of low grade during early stage that reaches a high level in later stage when maculopathy is complicated by CNV development (Campa et al., 2010). The abnormal levels of complement components C3a, C5, C5a, C5b-9, complement factor H (CFH) and membrane cofactor protein have been observed that show involvement of complement system in the AMD pathogenesis (Kumar and Fu, 2014).

In addition, it is suggested that C reactive protein (CRP) which is another inflammatory marker, is directly responsible for AMD pathogenesis, for the reason that it induces complement activation and ultimately leads to macular damage via complement mediated mechanisms (Parmeggiani et al., 2012). Under normal conditions, immunosuppressive signals like FAS ligand (FASL) are expressed by RPE that prevent leukocyte accumulation in subretinal space. AMD causes breakdown of this immunosuppressive environment and chronic accumulation of mononuclear phagocytes (MPs). They also express high levels of apoE in subretinal space (Levy et al., 2015). Apolipoprotein E (ApoE) is a multifunctional protein and is widely distributed in body tissues. It is known to be involved in the transport of lipids and lipoprotein metabolism. It is expressed in liver and is a major component of high density lipoproteins (HDL) in the blood and main lipoprotein in brain and retina (Anderson et al., 2001). It has been shown that retina synthesizes cholesterol and expresses proteins that mediate cholesterol transport and its removal. Cholesterol homeostasis is however considered relatively independent of the rest of the human

body due to presence of internal and external blood retina barriers. Endothelial cells and neural retina along with RPE cells synthesize and acquire cholesterol from LDL and HDL derived from blood circulation. Importantly, ratio of blood-borne and endogenously synthesized cholesterol is yet unknown (Parmeggiani et al., 2013).

ApoE along with ApoA1 is involved in reverse cholesterol transport by allowing cholesterol ester rich core expansion in HDL (Mahley et al., 2006) that in turn obstructs efficient lipid evacuation from RPE to the choroid and ultimately leads to drusen formation (Riddell et al., 2008). ApoE modulates the response of body to inflammation and is involved in oxidative disorders via its linkage with pro-inflammatory and anti-inflammatory cytokines (Zhang et al., 2011).

In addition, ApoE and APOA1 are able to extract cholesterol from lipid rafts, activate innate immunity receptor cluster in the absence of toll like receptor ligand and induce production of inflammatory cytokines including IL-6 (Smoak et al., 2010). Irregularity in the production of cytokines like tumor necrosis factor (TNF)-α, IL-1 β, IL-6 and IL-8 has been reported in autoimmune and inflammatory disorders. These cytokines are important components of pro-inflammatory response and intraocular inflammation (Evereklioglu et al., 2002).

One of the cytokines, IL-6 can initiate immune response by inducing acute phase of inflammation (Chung and Chang, 2003). IL-6 is a proinflammatory cytokine that is related to cellular and humoral immunity. A variety of tissues like adipocytes, leukocytes and endothelial cells produce this cytokine in response to IL-1, interferon-γ and TNF (Ridker et al., 2000). The serum IL-6 secreted from the adipose tissues under basal state is at least partly involved in the regulation of CRP production (Bastard et al., 2000). IL-6 dependent hepatic biosynthesis of CRP is one of the principal downstream mediators of acute phase inflammation (Pradhan et al., 2001).

Under normal physiological conditions, retinal pigment epithelium (RPE) utilizes several growth factors (GFs) to provide anti-angiogenic environment in addition to trophic support to neuroretina and choriocapillaries. An imbalance of these physiological functions resultsinto initiation of CNV in AMD. The evidence is provided by the expression of growth factors like VEGF, angiopoietin and platelet derived growth factor (PDGF) by new vessels in CNV. VEGF-A secretion by retinal pigment epithelium increases during hypoxic conditions and

maintainschroiocapillaries. Age related changes damage this balance leading to atrophy of choriocapillary that causes reduced oxygen diffusion towards neuroretina. This results into hypoxia in outer retina which is responsible for CNV formation and VEGF over-expression by RPE. It may also be related to decreased expression of inhibitors of angiogenesis like pigment epithelium derived factor (PEDF) that leads to pro-angiogenic state in aging eye (Schlingemann, 2004). Studies have shown that VEGF is a key regulator for angiogenesis that occurs as a result of hypoxia due to oxidative damage in RPE leading to CNV (Lim et al., 2012).

Cellular stress caused by AMD acts as a stimulator for the production of VEGF and another inflammatory marker HTRA1. Both VEGF and HTRA1 are regulated by NFκB dependent pathway. NFκB in association with HTRA1 and VEGF induces the expression of genes involved in chronic and acute inflammatory responses (Bressler, 2009). HTRA1 is a member of HTRA family of chymotrypsin like serine proteases which show temperature dependent proteolytic activities. It has N-terminal secretory signal peptide and a C-terminal HTRA domain (Ng et al., 2011). The size of HTRA1 protein is about 30kDa which is a secreted enzyme that cleaves substrates like vitronectin, fibromodulin and clusterin. These proteins are involved in complement pathway (Yang et al., 2006).

HTRA1 is although expressed ubiquitously but it is found in abundance in smooth muscle cells. HTRA1 gene promotor carries a single nucleotide polymorphism that results to elevated HTRA1 transcript levels associated with AMD progression. It is therefore suggested that HTRA1 disrupts pericellular matrix network (Polur et al., 2010). This serine protease also regulates TGF β signalling pathway. It mediates proteolysis of the prodomain of proTGF β with rough endoplasmic reticulum associated degradation (Muratoglu et al., 2013). Data from proteomics show that apolipoproteins like APOE are a substrate of HTRA1. Therefore HTRA1 is a target and risk marker for neurodegenerative diseases like AMD (Ehrmann et al., 2008).

Being a multifactorial disease, AMD has complex aetiology. In addition to genetic predisposition, environmental factors like smoking, obesity and hypercholesterolemia have also been suggested to play role in the pathogenesis of

AMD. In addition, evidence of the involvement of oxidative stress and inflammation in AMD also exists but yet not confirmed.

Materials and Methods

3.1 Blood Sampling

About 10 ml blood samples were drawn from the cubital vein of controls and AMD patients. For DNA extraction 5 ml of blood was collected into EDTA.K_3 vacuatiners (BD, USA), while remaining blood was collected in the gel tubes for serum separation (BD, USA).

3.2 Serum Separation

The blood was allowed to settle for 1 hr at room temperature without shaking in the yellow capped serum separation gel tubes and then centrifuged at 960 x g for 10 minutes (Eppendorff 5810R, Germany). Sera were collected and saved in aliquots at – 20 ºC until further analyses.

3.3 Lipid Profile

Serum lipid profile included the estimation of total cholesterol concentration, triglycerides (TG), high density lipoprotein cholesterol (HDLc) and low density lipoprotein cholesterol (LDLc). The analyses were carried out using commercially available diagnostic kits and following the instructions by the manufacturer on an automated Chemistry Analyser (Selectra, XL, The Netherlands). Incubation at 37°C was carried out in the laboratory incubator (Model INC108 med, Memmert, Germany).

3.3.1 Estimation of Triglycerides

Commercial kit (Diasys Diagnosis Systems GmbH, Germany) was used for the estimation of triglycerides

Reagent composition

The reagents contained 4-chlorophenol (4 mmol/L), Good's buffer (50 mmol/L) pH 7.2, Magnesium ions (15 mmol/L), Glycero kinase (≥0.4KU/L), peroxidase (2KU/L), ATP (2 mmol/L), 4-aminaoantipyrine (0.5 mmol/L), Lipoprotein lipase (≥4KU/L), Glycerol-3-phosphate-oxidase (≥ 1.5 KU/L).

Method

Enzymatic-colorimetric test using glycerol-3-phospahte-oxidae (GPO)

Principle

Quinoneimine is an indicator to determine TG after enzymatic splitting with lipoprotein lipase. It is produced from 4-aminoantipyrine 4-chlorophenol and under the catalytic action of peroxidase by hydrogen peroxide.

$$\text{Tryiglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerols + fatty acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerols-3- phosphate + ADP}$$

$$\text{Glycerols-3-phosphate} + O_2 \xrightarrow{\textit{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-Chlorophenol} + \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + HCl + 4\,H_2O$$

Procedure

Blank (10 µl) of dH_2O, standard and sample were mixed with 1 ml of reagent (R). These were mixed and the absorbance of the standard (AbsStd) and the sample (AbsS) were read after 10 min incubation at 37 ºC on a spectrophotometer at 500 nm wavelength with 1cm light path cuvette.

Calculation

Final concentration of triglycerides was calculated by the formula given as:

$$\text{Triglycerides}\left(\text{mg}/\text{dl}\right) = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{Conc. Std [mg/dl]}$$

Range

The test was developed to measure triglycerides concentration within a measuring range of 1-1000 ml/dl. Desirable range as mentioned in the kit protocol was < 200 mg/dl; borderline high concentration was 200-400 mg/dl whereas > 400 mg/dl was considered as elevated value.

World over, the triglycerides levels are considered normal when > 150 mg/dl, borderline high at 150-199 mg/dl, high at 200 - 499 mg/dl and very high when 500 mg/dl or above (NIH, 2012).

In the laboratory where the tests were carried out currently, triglycerides were considered normal, upto 200 mg/dl and high when above this range.

3.3.2 Estimation of Cholesterol

A commercial kit (Merck, France) was used for estimation of serum total cholesterol levels.

Reagent composition

The reagent contained phenol (24 mmol/L), pipes buffer (50 mmol/L) pH 6.7, 4-Aminoantipyrine (0.5 mmol/L), cholesterol oxidase (≥200 U/L), Sodium cholate (5 mmol/L), cholesterol esterase (≥180 U/L), peroxidase (≥1000 U/L)

Method

Enzymatic-colorimetric test

Principle

The estimation of cholesterol concentration was carried out through hydrolysis through enzyme and the oxidation. The quinoneimine served as indicator that was formed from hydrogen peroxide and 4-aminophenazone in presence of peroxidise and phenol.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\textit{Cholesterol}\text{esterase}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4- Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Qunineimine} + 4H_2O$$

Procedure

Blank (10 µl) of dH_2O, standard and sample were mixed with 1 ml of reagent (R). These were mixed and the absorbance of the standard (AbsStd) and the sample (AbsS) were read after 10 min incubation at 37 ºC in a spectrophotometer at 500 nm wavelength with 1cm light path cuvette.

Calculation

Final concentration was calculated by the following formula:

$$\text{Cholesterol}\left(\text{mg}/\text{dl}\right) = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{Conc. Std.}[\text{mg/dl}]$$

Range

The range of the kit used for estimation of cholesterol was 600 mg/dl. The manufacturers of the kit mentioned the normal range was < 200 mg/dl, cholesterol levels were suspicious at 200-240 mg/dl whereas it needed to be treated when levels were > 240 mg/dl.

US National Institutes of Health (NIH) and the European Atherosclerosis Society have defined an optimal cholesterol level in serum at ≤ 200 mg/dl. A level at 201-239 mg/dl is considered to be borderline high, whereas cholesterol levels >240 mg/dl, indicate high (NIH, 2001).

The range for normal cholesterol levels in the laboratory where the tests were run for current study was 120-200 mg/dl.

3.3.3 Estimation of High Density Lipoprotein Cholesterol (HDLc)

A commercial kit (Spinreact, Spain) was used for the estimation of HDLc in serum.

Reagent composition

The reagent 1 contained GOOD buffer pH 7.0, Peroxidase (<1300 U/L), cholesterol oxidase (< 1000 U/L), DSBmT (<1mM). Reagent 2 contained GOOD buffer, cholesterol esterase (<1500 U/L), detergent (<2%), ascorbic oxidase (< 3000U/L), 4-Aminoantopyrine (< 1mM),

Method

Enzymatic-colorimetric test

Principle

HDLc levels in serum were determined directly with no centrifugation or pre-treatment of the sample needed. The method depends upon the detergent properties that solubilise only HDL so that it is released and reacts with the cholesterol esterase, cholesterol oxidase and the chromogens to produce colour. The non HDL lipoproteins including LDL, VLDL and chylomicrons were not allowed to react with the enzymes

due to absorption of the detergents on their surface. The intensity of the colour produced was directly proportional to the sample HDLc concentration.

Procedure

Each of 3 µl of standard and serum sample was added to 300 µl of the reagent 1(R1). The contents were then mixed thoroughly and incubated at 37 ºC for 5 min. Then absorbance (A_1) of the sample as well as the standard was recorded at 600 nm wavelength with 1cm light path cuvette. Then 100 µl of reagent 2 (R2) was added to the solution, then mixed and incubated for 5 min. Again absorbance (A_2) of the sample and the standard were noted at 600nm wavelength with 1cm light path cuvette. R1 and R2 without the addition of any sample or standard were taken as blank solution. Increase in the absorbance was calculated through formula: $\Delta A = A_2 - A_1$

Calculation

Final concentration was calculated by the formula as follows:

$$\text{HDLc}\left(\text{mg}/\text{dl}\right) = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{Standard conc. [mg/dl]}$$

Range

The measuring range of kit was 2.5 - 200 mg/dl. The low risk HDL value as provided by the manufacturer of kit was > 50 ml/dl in men whereas in women it was > 60 mg/dl. Nominal risk related value was 35-50 mg/dl in men and 45-60 mg/dl in women, the high risk value was < 35 mg/dl in men and < 45 mg/dl in women.

According to the American Heart Association, the average males have HDL levels < 40 mg/dl. Whereas, the average levels in females is from < 50 mg/dl (Grundy et al., 2004).

In the laboratory where the analyses were carried out, the range of HDL for males was 30-65 mg/dl and for females it was 35-80 mg/dl.

3.3.4 Estimation of Low Density Lipoprotein Cholesterol (LDLc)

A commercial kit (Spinreact, Spain) was used for the estimation of LDLc in serum.

Reagent composition

The reagent 1 contained cholesterol oxidase (380 U/L), GOOD buffer (50 mmol/L pH 7.0, cholesterol oxidase (380 U/L), n-(2-hydroxy-3-sulfopropyl)-3, catalase (400 U/mL), 5-dimethoxyaniline (0.45 mmol/L). Reagent 2 contained 4-Aminoantopyrine (< 1 mmol/L), GOOD buffer (50 mmol/L) and peroxidase (1000 U/L)

Method

Enzymatic-colorimetric test

Principle

Direct determination of serum LDLc levels with no pre-treatment or centrifugation of the sample needed. The assay was carried out in two steps:

Estimation of lipoprotein no-LDL

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + fatty acid}$$

$$\text{Cholesterol + } O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{4-Cholestone + } H_2O_2$$

$$2\ H_2O_2 + \text{TODS + 4-AA} \xrightarrow{\text{Peroxidase}} \text{Qunineimine + 4 } H_2O$$

The intensity of colour produced was directly proportional to the concentration of LDLc in the sample.

Procedure

Four microliter standard and sample were mixed with 300 µl of reagent 1(R1). Theses constituents were mixed thoroughly and incubated at 37 ˚C for 5 minutes. Then 100 µl of the reagent 2 (R2) were added to the solution, mixed and incubated for 5 minutes and again absorbance (A_2) of the sample and the calibrator were noted at 600 nm wavelength with 1cm light path. The mixture of R1 and R2 without sample or calibrator was taken as blank. ΔA=A2-A1

Calculation

Final concentration was calculated by the formula as follows:

$$\text{LDLc}\left(\frac{\text{mg}}{\text{dl}}\right) = \frac{\Delta A \text{ of Sample}}{\Delta A \text{ of Standard}} \times \text{Standard conc.[mg/dl]}$$

Range

The detection limit of the kit was 3.7 mg/dl to 1000 mg/dl. The desirable LDL level as mentioned by the kit manufacturer was < 100 mg/dl, medium risk at 130-160 mg/dl and high risk at the LDL level > 160 mg/dl.

The American heart association defines optimal levels < 100 mg/dl, near or optimal at 100 to 129 mg/dl, border line high levels at 130 to 159 mg/dl, high risk related level at 160 to 189 mg/dl and very high risk levels at 190 mg/dl and above (Krauss et al., 2000).

In the laboratory where the analyses were carried out the normal range of LDL level was 90-160 mg/dl.

3.4 Estimation of Angiogenic Factors and Inflammatory Markers

The angiogenic factors and inflammatory markers including IL-6, IL-8, ApoE, leptin, HTRA1, VEGF, hs-CRP and CFH were estimated through enzyme linked immunosorbant assay (ELISA) in serum of all the studied subjects.

3.4.1 ELISA

The protein analyses of angiogenic factors and inflammatory markers wascarried out through enzyme linked immunosorbant assay (ELISA) which is a test that uses antibodies and color change to identify a substance. It is a preferred method than enzyme immunoassay (EIA) as the time for ELISA is practically much shorter (Ohkaru et al., 1995). EIA and ELISA were developed simultaneously but independently by the research groups of Anton Schuurs and Bauke van Weemen in The Netherlands and Peter Perlmann and Eva Engvall at Stockholm University in Sweden (Lequin, 2005).

There are several types of ELISA. In the indirect ELISA, antibody is detected through binding to an antigen. It is a method used mostly for HIV infection test. In the test, viral core proteins acting as antigen, are absorbed to the bottom of the well. The antibodies from the serum of patient are added to the coated well and allowed to bind to antigen. Finally, enzyme-linked antibodies to human antibodies are allowed to react in the well and unbound antibodies are removed by washing. Then, the substrate is applied. A reaction of the enzyme-linked antibodies bound to human antibodies suggestedthat the patient had antibodies to the viral antigen (Fig. 3.1) (Berg et al., 2002).

In the sandwich ELISA, is applied for detection and quantitation of antigen. Firstly, antibody of a particular antigen in absorbed to the bottom of well, secondly, serum or urine sample containing antigen is added to the wellthat binds to the antibody. Thirdly, a second different antibody to the antigen is added. This antibody is enzyme linked and is processed as described for indirect ELISA. The extent of reaction is directly proportional to the amount of antigen present. This methodpermits the quantification of small amount of antigen (Fig. 3.1) (Berg et al., 2002).

In the current study, several inflammatory factors including ApoE, IL-6, IL-8, CRP, CFH, HTRA1, leptin and VEGF were quantified in the serum samples of the AMD patients and normal subjects through ELISA.

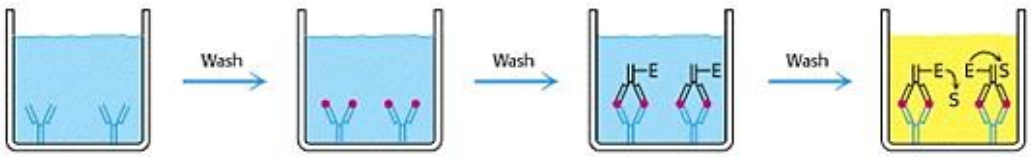
Anitgen-coated well

Specific antibody binds to antigen

Enzyme-linked antibody to specific antibody

Substrate is added and converted by enzyme into colored product; the rate of color formation is proportional to the amount of specific antibody

(A)



Monoclonal antibody-coated well

Antigen binds to antibody

A second monoclonal antibody, linked to enzyme, binds to immobilized antigen

Substrate is added and converted by enzyme into colored product; the rate of color formation is proportional to the amount of antigen

(B)

Fig. 3.1 (A)Indirect ELISA, the production of color indicates the amount of an antibody to a specific antigen. (B) Sandwich ELISA, the production of color indicates the quantity of antigen (Adopted from Berg et al., 2002)

3.5 Estimation of Interleukin 6 (IL-6)

The serum IL-6 was estimated using commercially available kit (Immunotech, France).

Method

IL-6 was estimated through enzyme linked immuniosorbent assay (ELISA)

Reagents Composition

Calibrator was one vial containing bovine serum albumin. It was reconstituted with the volume of distilled water to make 10ng/ml. The calibrator was calibrated by reference to the WHO IL-6 (89/548) standard. IL-6 ACE conjugate was lyophilized powder and was reconstituted using distilled water. Diluent contained material of human origin. It was reconstituted with the volume of distilled water stated on the vial label. Wash solution (20x) was a 50 ml vial of concentrated solution that was diluted in 950ml distilled water before use. Substrate was in the form of lyophilized powder and was reconstituted with distilled water. Stop solution was ready to use tacrine solution.

Principle

This ELISA is a one immunological step sandwich type assay. Samples and calibrators were incubated in the microtiter plate coated with the first monoclonal antibody anti-IL-6, in presence of the second anti-IL-6 monoclonal antibody linked to acetylcholine esterase (ACE). After incubation, the wells were washed and the bound enzymatic activity was detected by addition of a chromogenic substrate. The intensity of the coloration was proportional to IL-6 concentration in the sample or calibrator.

Procedure

The calibrator solution was diluted for different concentration using diluent solution.

In the first step, 100 µl of calibrator or sample was added per well and then 100 µl conjugate was added. Then it was incubated at 18-25°C for 2 hr while shaking (4625 Titer Plate Shaker, Labline, USA). Then wells were washed thoroughly three times with washing solution using the microtiter plate washer (KHB-ST-36W, China). Then, at step 2, 200 µl of substrate was added and incubation was carried out for at 18-25°C for 30 min in the dark with shaking. At step 3, 50 µl of stop solution was

added and absorbance was read at 405 nm through ELISA plate reader (KHB-ST 360, China).

Standard Point	Dilution	IL-6 (pg/ml)
P1	50 µl 10ng/ml Standard + 450 µl diluent	1000
P2	150 µl P1+ 300 µl Diluent	333
P3	150 µl P2+ 300 µl Diluent	111
P4	150 µl P3 + 300 µl Diluent	37.0
P5	150 µl P4 + 300 µl Diluent	12.3
P6	300 µl Diluent	0.0

Calculations

The sample results were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn, plotting on the horizontal axis the IL-6 concentration of the calibrators and on the vertical axis the corresponding absorbance. The absorbance for each sample was located on the vertical axis and corresponding IL-6 concentration was read on the horizontal axis. Using a computer, a curve fitting in the equation $Y = Ax^2 + Bx + C$ (quadratic mode) was drawn to calculated IL-6 concentration.

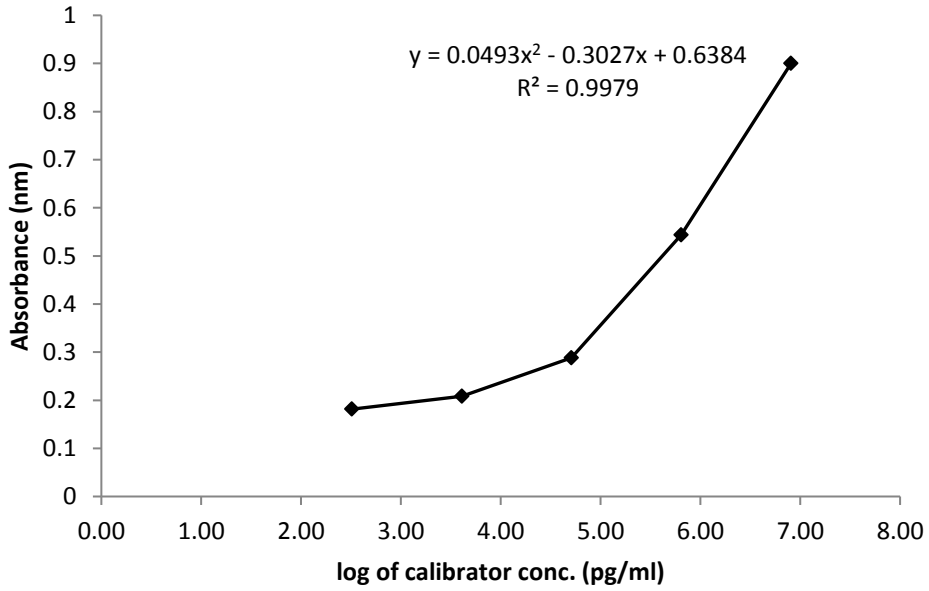


Fig 3.2 Standard Curve of IL-6

Analytical Range

The analytical range of the kit was 3 pg/ml – 150 ng/ml. Intra-assay precision was determined by assaying sera 9 times. Variation coefficient ranged between 1.6 and 6.8%. Inter-assay precision was determined by assaying sera 5 times in independent assays. Variation coefficient ranged between 7.9 and 14.6%.

3.6 Estimation of Interleukin 8 (IL-8)

The serum IL-8 was estimated using commercially available kit (Immunotech, France).

Method

IL-8 was estimated through enzyme linked immunosorbent assay (ELISA)

Reagents Composition

Calibrator was one vial containing bovine serum albumin. It was reconstituted with the volume of distilled water to prepare 20 ng/ml solution. The calibrator was calibrated by reference to the WHO IL-8 (89/520) standard. Biotinylated monoclonal antibody was ready to use and contained bovine serum albumin. Diluent was ready to use solution and contained bovine serum albumin. Streptavidin-HRP conjugate was ready to use. The vial contained streptavidine-HRP conjuguate and bovine serum albumin. Wash solution (20x) was a 50 ml vial of concentrated solution that was diluted in 950ml distilled water before use. Substrate was ready to use solution. Stop solution was also ready to use and contained a 2.0 N sulfuric acid solution.

Principle

This ELISA is a two immunological step sandwich type assay. In the first step the IL-8 was captured by a monoclonal antibody bound to the wells of a microtiter plate. In the second step, a biotinylated monoclonal antibody was added together with streptavidin-peroxidase conjugate. The biotinylated antibody bound to the solid phase antibody-antigen complex and, in turn, bound the conjugate. After incubation, the wells were washed and the antigen complex bound to the well detected by addition of a chromogenic substrate. The intensity of the coloration was proportional to the IL-8 concentration in the sample or calibrator.

Procedure

The calibrator solution was diluted for different concentration using diluent solution.

Standard Point	Dilution	IL-8 (pg/ml)
P1	50 µl 20ng/ml Standard + 450 µl diluent	2000
P2	150 µl P1+ 300 µl Diluent	500
P3	150 µl P2+ 300 µl Diluent	125
P4	150 µl P3 + 300 µl Diluent	31.2
P5	300 µl Diluent	0.0

In step 1, 150 µl of calibrator or sample was added per well and then was incubated for 2 hr at 18-25°C while shaking. Then wells were washed thrice thoroughly with washing solution (KHB-ST-36W, China). Then, in step 2, 50 µl of biotinylated antibody and 100 µl of streptavidin-HRP conjugate was added and incubated for 30 min at 18-25°C. In step 3, 100 µl of substrate was added and incubated for 20 min at 18-25°C while shaking (4625 Titer Plate Shaker, Labline, USA). Then 50 µl of stop solution was added and absorbance was read at 450 nm through an ELISA plate reader (KHB-ST 360, China).

Calculations

The sample results were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn, plotting on the horizontal axis the IL-8 concentration of the calibrators and on the vertical axis the corresponding absorbance. The absorbance for each sample was located on the vertical axis and corresponding IL-8 concentration was read on the horizontal axis.

Using a computer, a curve fitting in the equation $Y = Ax^2 + Bx + C$ (quadratic mode) was drawn to calculated IL-8 concentration.

Analytical range

The kit was sensitive to measure 8 pg/mL. Intra-assay precision was determined by assaying sera or plasma 10 times. Coefficient of variation ranged between 2.3 and 5.5%, whereas inter-assay precision was determined by assaying sera

or plasma 10 times in independent assays. Coefficient of variation ranged between 7.6 and 10.1%.

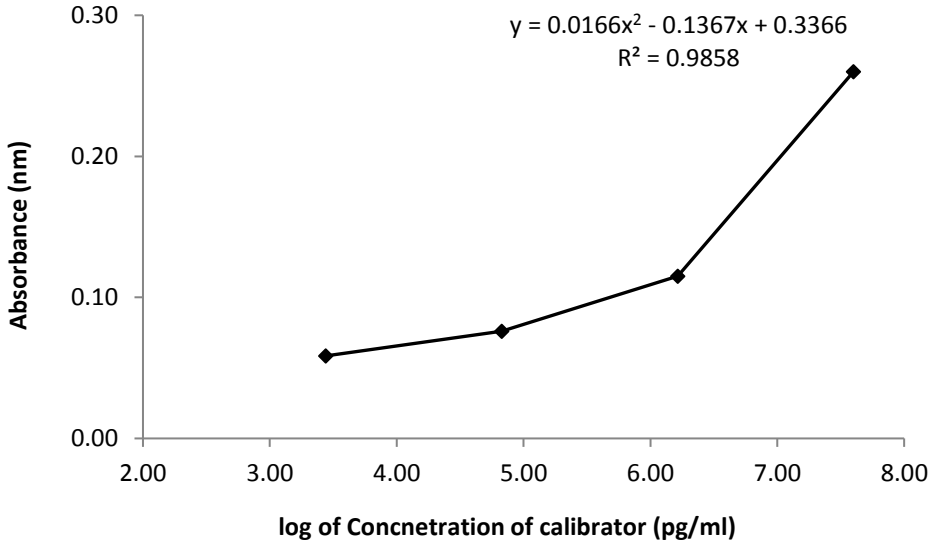


Fig 3.3 Standard curve of IL-8

3.7 Estimation of Apolipoprotein E (ApoE)

The serum apoE was estimated using commercially available kit AssayMax Human ELISA kit (Asaaypro, USA).

Method

ApoE was estimated through enzyme linked immuniosorbent assay (ELISA)

Reagents Composition

Calibrator was one vial containing 1µg of ApoE standard and was reconstituted with 0.5 ml of 2 µg/ml EIA diluent solution. Prior to making further dilutions, this stock solution was allowed to stand for 10 min with gentle agitation. Then standard points were prepared in duplication by serial dilution of the standard stock solution in 1:2 with EIA diluent to produce 1.0, 0.5, 0.25, 0.125, 0.625 and 0.0313 µg/ml concentration. Pure solution of EIA diluent served as zero standards (0 µg/ml).

Biotin Apo E Antibody was diluted by 1:100 with EIA diluent. Streptavidin-peroxidase conjugate (SP-conjugate) was diluted by 1:100 with EIA diluent. Wash solution (20x) contained 50 ml of concentrated solution that was diluted in 950 ml

distilled water before use. Stop solution was ready to use and contained a 0.5 N hydrochloric acid solution.

Standard Point	Dilution	ApoE (μg/ml)
P1	Standard (2 μg/ml)	2.00
P2	1 part P1 + 1 part EIA Diluent	1.00
P3	1 part P2 + 1 part EIA Diluent	0.50
P4	1 part P3 + 1 part EIA Diluent	0.25
P5	1 part P4 + 1 part EIA Diluent	0.125
P6	1 part P5 + 1 part EIA Diluent	0.063
P7	1 part P6 + 1 part EIA Diluent	0.031
P8	EIA Diluent	0.00

Principle

This ELISA employed a sandwich enzyme immunoassay quantitative technique that measured ApoE in about 4 hrs. A 96-good microplate with removable strips was pre coated with a polyclonal antibody specific for human ApoE. ApoE in standards solution and serum samples was sandwiched by the immobilized antibody and biotinylated polyclonal antibody that was specific for ApoE. It was recognized by a streptavidin-peroxidase conjugate. Then the unbound material was washed away thoroughly and a peroxidise enzyme substrate was added. The development of color was stopped and its intensity was estimated.

Procedure

The calibrator solution was diluted for different concentration using diluent solution. The serum samples were diluted to 1:400 for estimation. The entire assay was performed at room temperature (20-30 ºC).

To each well of the plate, 50 μl of calibrator or sample was added and incubated for two hours at room temperature after sealing with the tape. Wells were then washed 4-5 times with washing solution using microtiter plate washer (KHB-ST-36W, China). Then 50 μl of biotinylated ApoE was added and the contents were

incubated again for one hour. The plate was washed again and 50 µl of streptavidin-peroxidase conjugate was added and incubated again for about 30 min. The microtiter plate was washed again and 50 µl of chromogen substrate was added and incubated for 12 min or till the optimal color density developed. To stop the reaction, 50 µl stop solution was added to each well that changed the color from blue to yellow. Absorbance was recorded through a microplate reader (KHB-ST 360, China) at 450 nm and 570 nm. Reading at 570 nm was subtracted from reading at 450 nm.

Calculations

The mean value of duplicate readings of standards was calculated. The results of samples were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn by plotting concentrations of standard on x-axis and corresponding difference of absorbance at 450 nm and 570 nm, on y axis. The best fit line was determined through regression analyses using log-log curve. The sample concentration was calculated from standard curve and the value was multiplied by the dilution factor.

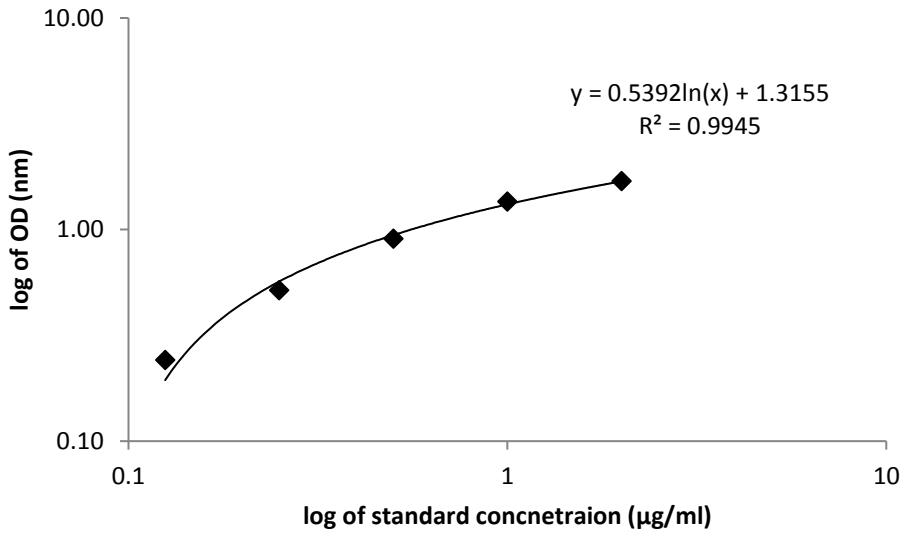


Fig. 3.4 Standard Curve of ApoE

Performance characteristics

The minimum level of ApoE detectable through the kit was ~0.03 µg/ml. It recognized ApoE-2, ApoE-3, and ApoE-4 isoforms. The inter-assay and intra-assay coefficients of variation were 7.4% and 4.6% respectively.

3.8 Estimation of Leptin

The serum Leptin was estimated using commercially available kit AssayMax Human ELISA kit (Asaaypro, USA).

Method

Leptin was estimated through enzyme linked immuniosorbent assay (ELISA)

Reagents Composition

The calibrator was contained in a vial containing 64 ng of human leptin standard that was reconstituted by adding 2 ml mix diluent to prepare stock solution having 32 ng/ml leptin. Prior to making further dilutions this stock solution was allowed to stand for 10 min with gentle agitation. Duplicate standard points were prepared by serial dilution of leptin standard solution (32 ng/ml) with diluent as 1:4 to make 8.0, 2.0, 0.5 and 0.125 ng/ml solutions. Pure mix diluent served as standard with zero concentration of leptin (0 ng/ml).

Standard Point	Dilution	Leptin (ng/ml)
P1	Standard (32 ng/ml)	32.000
P2	1 part P1 + 3 parts EIA Diluent	8.000
P3	1 part P2 + 3 part EIA Diluent	2.000
P4	1 part P3 + 3 part EIA Diluent	0.500
P5	1 part P4 + 3 part EIA Diluent	0.125
P6	Mix Diluent	0.000

Biotin leptin antibody was diluted by 1:50 with mix diluent. Streptavidin-peroxidase conjugate (SP-conjugate) was diluted by 1:100 with mix diluent. Wash solution (20x) consisted of a vial having 50 ml of concentrated solution that was diluted in 950 ml distilled water before use. Stop solution was ready to use and contained a 0.5 N hydrochloric acid solution.

Principle

This ELISA employed a technique for quantitative sandwich enzyme immunoassay that measured leptin in about 5 hrs. A 96-well microplate pre coated with polyclonal antibody specific for human leptin with removable strips was used.

Leptin in samples as well as standards was sandwiched by the immobilized antibody and leptin specific biotinylated polyclonal antibody that was recognized by the SP-conjugate. All the unbound material was then washed away thoroughly and then a peroxidase enzyme substrate was added. The development of color was stopped and the color intensity was estimated.

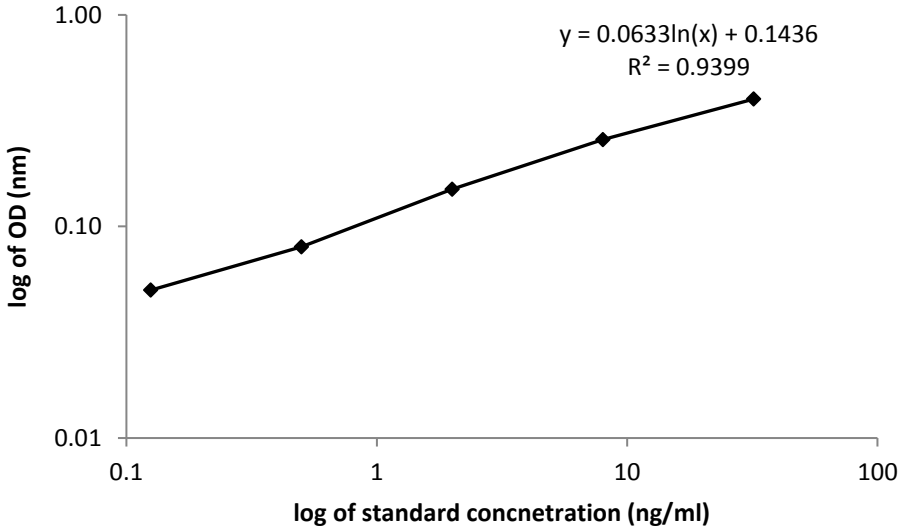


Fig 3.5 Standard curve of Leptin

Procedure

The calibrator solution was diluted for different concentration using mix diluent solution. The serum samples were diluted to 1:8 for estimation. The entire assay was performed at room temperature (20-30 ºC).

Each well of the plate was added with 50 µl of calibrator or sample, sealed with the tape and then incubated for 2 hr at room temperature. The wells were then washed 4-5 times with washing solution using microtiter plate washer (KHB-ST-36W, China). Then 50 µl biotinylated leptin antibody was added and it was incubated again for about 2 hrs. The plate was then washed again. Then 50 µl of SP- conjugate was added and incubated for about 30 min. The plate was washed again. Then, 50 µl of chromogen substrate was added and incubated for 30 min or till the optimal color density developed. Then stop solution (50 µl) was added to each well that changed the color from blue to yellow. Then the absorbance on micro plate reader (KHB-ST 360,

China) was read at 450 nm and 570 nm immediately. Reading at 570 nm was subtracted from reading at 450 nm.

Calculations

The mean value of duplicate readings of standards was calculated. The sample results were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn using concentration of standard on x-axis and corresponding mean absorbance difference at 450 nm and 570 nm, on y axis. The best fit line could be calculated through regression analyses applying log-log curve. The sample concentration was calculated from standard curve and multiplied by the dilution factor.

Performance Characteristics

The minimum measurable leptin level was ~ 0.12 ng/ml. The inter-assay and intra-assay coefficients of variation were 7.3% and 4.1% respectively.

3.9 Estimation of Serine Protease HTRA1

The serum HTRA1 was estimated using commercially available kit for human serine protease HTRA1 ELISA kit EIAab (Eiaab, China).

Method

HTRA1 was estimated through enzyme linked immuniosorbent assay (ELISA)

Reagents Composition

The standard solution was reconstituted with 1.0 ml sample diluents to prepare a stock solution of 5000 pg/ml. Prior to making further dilutions it was allowed to stand for 15 min with gentle agitation. Standard points were prepared in duplicate by serially diluting HTRA1 standard solution (5000 pg/ml) 1:2 with sample diluent to produce 2500, 1250, 625, 312, 156 and 78 pg/ml solutions. Sample diluent served as zero standard solution (0 pg/ml).

Detection Reagent A and B were diluted to 1:100 using Assay Diluent A and Brespectively. Wash solution (25x) was a 30 ml vial of concentrated solution that was diluted to 750ml with distilled water before use. Stop solution was ready to use and contained a 0.5 N Sulfuric acid solution.

Detection Reagent A and B were diluted to 1:100 using Assay Diluent A and Brespectively. Wash solution (25x) was a 30 ml vial of concentrated solution that was diluted to 750ml with distilled water before use. Stop solution was ready to use and contained a 0.5 N Sulfuric acid solution.

Standard Point	Dilution	HTRA1 (pg/ml)
P1	Standard (5000 pg/ml)	5000
P2	1 part P1 + 1 part Sample Diluent	2500
P3	1 part P2 + 1 part Sample Diluent	1250
P4	1 part P3 + 1 part Sample Diluent	625.0
P5	1 part P4 + 1 part Sample Diluent	312.0
P6	1 part P5 + 1 part Sample Diluent	156.0
P7	1 part P6 + 1 part Sample Diluent	78.00
P8	Sample Diluent	0.000

Principle

The antibody specific to HTRA1 was pre coated on microtiter plate provided with the kit. Then standards and samples were added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for serine protease HTRA1 and avidin conjugated to horseradish peroxidase (HRP) was added to each well andincubated. Then a TMB substrate solution was added to each well. Only those wells that contained for serine protease HTRA1, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of HTRA1 in the samples was then determined by comparing the optical density of the samples to the standard curve.

Procedure

The calibrator solution was diluted for different concentration using mix diluent solution. The serum samples were diluted to 1:2 for estimation. The entire assay was performed at 37 ºC using laboratory incubator (model INC108 med, Memmert, Germany).

Each well of the plate was added with 100 µl of calibrator or sample and then was incubated for 2 hr at 37 ºC after sealing with the tape. Then the liquid was removed from the plate and 100 µl of detection reagent A working solution was added to each well. It was then incubated for one hour at 37 ºC. Then the plate was washed three times with washing solution using microtiter plate washer (KHB-ST-36W, China). Then 100 µl of detection reagent B working solution was added and incubated again for 1 hr at 37 ºC. Then again the plate was washed and 90 µl of substrate solution was added. It was incubated for about 15-30 minutes at 37 ºC. Then 50 µl of stop solution was added to each well that changed the color from blue to yellow. Then the absorbance on micro plate reader (KHB-ST 360, China) was read at 450 nm.

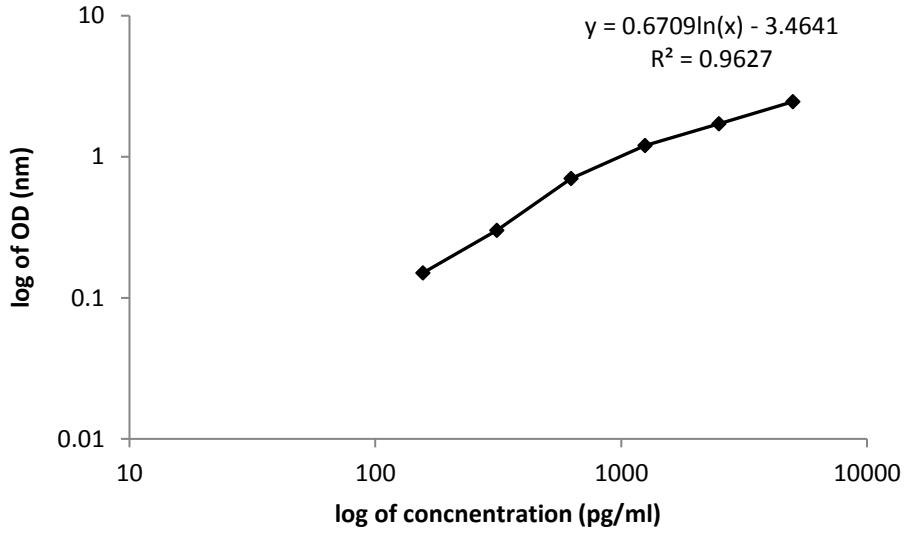


Fig 3.6 Standard curve of serine protease HTRA1

Calculations

The mean value of duplicate readings of standards was calculated. The concentration in the unknown samples was determined by interpolation from a calibrator curve. The curve was plotted using value of standard concentrations on x-axis and corresponding absorbance at 450 nm on y axis. The best fit line could be determined through regression analyses using log-log curve. The unknown concentration was calculated from standard curve and multiplied by the dilution factor.

Detection Range

The detection range of the kit was from78.0 to 5000 pg/ml.

3.10 Estimation of Vascular Endothelial Growth Factor (VEGF)

The serum VEGF was estimated using commercially available kit from Cusabio, China.

Method

VEGF was estimated through an enzyme linked immunosorbent assay (ELISA).

Reagents Composition

Standard was reconstituted with 1.0 ml sample diluents to prepare a stock solution of 2000 pg/ml. It was allowed to stand for 15 min with moderate shakeup prior to make further dilutions. Standard points were prepared in duplication by diluting the VEGF standard solution (2000 pg/ml) serially in 1:2 with sample diluent to produce solutions with 1000, 500, 250, 125, 62.5 and 31.25 pg/ml concetration. Sample diluent served as zero standard (0 pg/ml).

Standard Point	Dilution	VEGF (pg/ml)
P1	Standard (2000 pg/ml)	2000
P2	1 part P1 + 1 parts Sample Diluent	1000
P3	1 part P2 + 1 part Sample Diluent	500.0
P4	1 part P3 + 1 part Sample Diluent	250.0
P5	1 part P4 + 1 part Sample Diluent	125.0
P6	1 part P5 + 1 part Sample Diluent	62.5
P7	1 part P6 + 1 part Sample Diluent	31.25
P8	Sample Diluent	0.000

Biotin antibody was diluted to 100 folds with biotin diluents. HRP-avidin was diluted to 100-folds with HRP-avidin diluents**.** Wash buffer (25x) was a 20 ml vial of concentrated solution that was diluted in 500ml distilled water before use. Stop solution was ready to use and contained a 0.5 N hydrochloric acid solution.

Principle

This assay employed the quantitative sandwich enzyme immunoassay technique. A microplate was pre-coated with antibody specific for VEGF. Standards

and samples were pipetted into wells and any VEGF present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for VEGF was added to the wells. After washing, avidin conjugated Horeseradish peroxidase (HRP) was added to the wells. Following a wash to remove any avidin-enzyme reagent, a substrate solution was added to the wells and the color developed in proportion to the amount of VEGF bound in the initial step. The development of color was stopped and the intensity of color was estimated.

Procedure

The calibrator solution was diluted for different concentration using mix diluent solution. The serum samples were diluted to 1:2 for estimation. The entire assay was performed at 37 ºC using the laboratory incubator (Model INC108 med, Memmert, Germany).

Each well of the plate was added with 100 µl of standard or sample and then was incubated for 2 hr at 37 ºC after sealing with the tape. The contents were removed from the plate and 100 µl of biotin antibody solution was added to each well without washing. It was then incubated for one hour at 37 ºC. The plate was then washed three times with washing solution using microtiter plate washer (KHB-ST-36W, China). Then 100 µl of HRP-avindin solution was added and incubated again for one hour at 37 ºC. Then the plate was washed again and 90 µl of TMB solution was added and incubated for about 15-30 min at 37 ºC. Each well was then added with 50 µl stop solution that changed the color from blue to yellow. Then absorbance on micro plate reader (KHB-ST 360, China) was read at 450 nm and 570 nm immediately. Reading at 570 nm was subtracted from 450 nm reading.

Calculations

The mean value of duplicate readings of standards was calculated. The reading at 450 nm was subtracted from that at 570 nm. The sample results were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn using standard solution concentrations along x-axis and corresponding absorbance at 450 nm along the y-axis. The best fit line was determined through regression analyses using log-log curve. The unknown concentration in the serum samples was calculated from standard curve and multiplied by the dilution factor.

Detection Range

Detection range of the kit was 31.25 pg/ml – 2000 pg/ml. Intra-assay precision was noted to be < 8% coefficient of variation (CV) while inter-assay precision was< 10% CV.

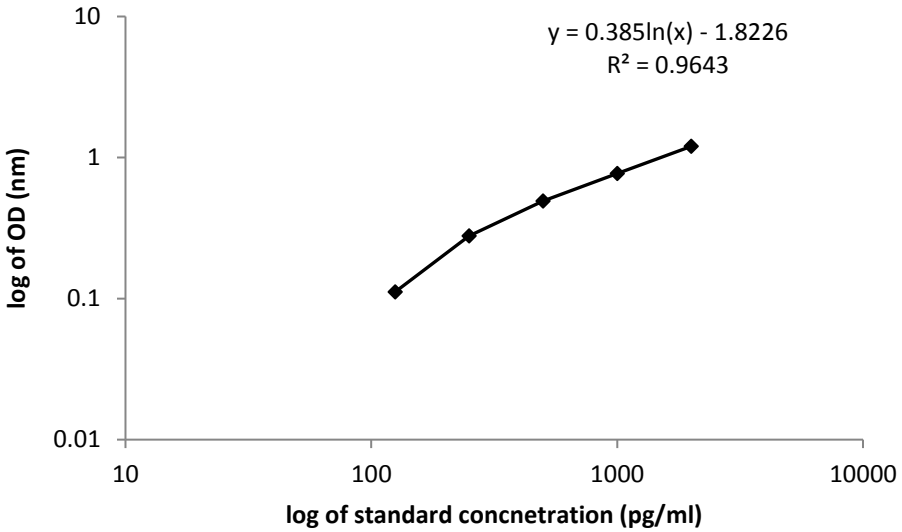


Fig 3.7 Standard curve of VEGF

3.11 Estimation of High Sensitivity C-Reactive Protein (hsCRP)

The hsCRP was estimated using commercially available kit for human CRP (Amgenix, USA).

Method

The hsCRP was estimated through enzyme linked immunosorbent assay (ELISA).

Reagents Composition

Standards with concentration 100, 50, 25, 10, 5 and 0 ng/ml were provided with the kit. CRP enzyme reagent and TMB solution were ready to use. Wash solution (50x) was a 15 ml vial of concentrated solution that was diluted in 750ml distilled water before use. Stop solution was ready to use and contained a 0.5 N Sulfuric acid solution.

Principle

The assay was based on the principle of a solid base ELISA that utilized a unique monoclonal antibody directed against a specific antigen determinant on the CRP molecule. This mouse monoclonal anti-CRP antibody was used for solid phase immobilization coated on the microtiter wells. The goat anti-CRP antibody was added in the antibody enzyme (Horseradish peroxidase) conjugate solution. The sample to be tested was allowed to react with the two antibodies simultaneously, resulting in the CRP molecules being sandwiched between the enzyme linked antibodies and solid phase. It was incubated at room temperature for 45 min and later the wells were washed to remove unbound labeled antibodies. A tetramethybenzidine reagent (TMB) was added and then incubated for 20 minutes that resulted to the development of blue color. Stop solution was added to stop the color development and the color was changed to yellow. The CRP concentration was directly proportional to the intensity of color in the test sample. Absorbance was measured spectrophotometrically at 450 nm.

Procedure

The serum samples were diluted to 1:100 with hsCRP sample diluents for estimation. The entire assay was performed at room temperature (18-22 ºC). Each well of the plate was added with 50 µl of calibrator or sample and then 50 µl of hsCRP sample diluents was added to each well. The contents were mixed thoroughly for 30 seconds and incubated at room temperature for 30 min. The plate was then washed five times with washing buffer using microtiter plate washer (KHB-ST-36W, China) and all the water residues were removed thoroughly. Then 100 µl of CRP enzyme conjugate reagent was added to each well, mixed thoroughly for 30 sec and incubated for 30 min. Plate was washed again as before and 100 µl of TMB reagent was added to each well. It was then mixed for about 5 seconds. Incubation was carried out for 30 min. Then stop solution was added that changed the blue color to yellow. Then reading was noted at 450 nm through ELISA reader (KHB-ST 360, China).

Calculations

The mean value of duplicate readings of standards was calculated. To draw the standard curve absorbance value was plotted on y-axis and CRP standard concentration on x- axis. Using the absorbance of the sample the corresponding

concentration was calculated. The obtained values were multiplied by the dilution factor.

Range

The kit manufacturer had mentioned that healthy adult individuals have CRP values from 68 to 8200 ng/ml (Clinical guide to laboratory tests, 1995).

Normal concentration in healthy human serum is usually lower than 10 ng/ml, it slightly increases with age. Higher levels are found in late pregnant women, mild inflammation and viral infections (10–40 ng/ml), active inflammation, bacterial infection (40–200 mg/l), severe bacterial infections and burns (> 200 ng/ml) (Clyne and Olshaker, 1999).

In the laboratory where the analysis was carried out, the CRP levels more than 200 ng/ml was considered positive while less than 200 ng/ml was considered negative for inflammation.

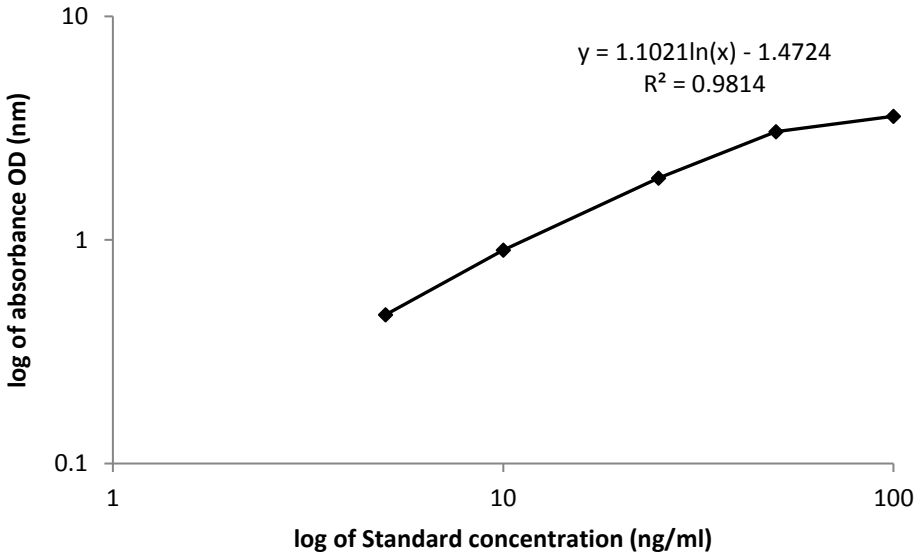


Fig 3.8 Standard curve of hs-CRP

3.12 Estimation of Complement Factor H (CFH)

The human CFH was estimated using commercially available kit from Cusabio, China.

Method

Human CFH was estimated through enzyme linked immunosorbent assay (ELISA).

Reagents Composition

Standard was reconstituted with 1.0 ml sample diluents to prepare a stock solution of 2000 pg/ml. It was allowed to stand for 15 min with gentle shaking prior to make dilutions further. Standard points in duplicate were prepared by serial dilution of the CFH standard solution (2000 pg/ml) in 1:2 with sample diluent to produce 1000, 500, 250, 125, 62.5 and 31.25 pg/ml solutions. Sample diluent served as zero standards (0 pg/ml). Biotin antibody was diluted to 100 folds with biotin diluents. HRP-avidin was diluted to 100-fold with HRP-avidin diluents**.** Wash buffer (25x) was a 20 ml vial of concentrated solution that was diluted in 500 ml distilled water before use. Stop solution was ready to use and contained a 0.5 N hydrochloric acid solution.

Standard Point	Dilution	CFH (ng/ml)
P1	Standard (2000 pg/ml)	2000
P2	1 part P1 + 1 parts Sample Diluent	1000
P3	1 part P2 + 1 part Sample Diluent	500.0
P4	1 part P3 + 1 part Sample Diluent	250.0
P5	1 part P4 + 1 part Sample Diluent	125.0
P6	1 part P5 + 1 part Sample Diluent	62.5.0
P7	1 part P6 + 1 part Sample Diluent	31.25
P8	Sample Diluent	0.00

Principle

This assay employed the quantitative sandwich enzyme immunoassay technique. CFH specific antibody was pre-coated onto a microplate. Standards or samples were pipetted into wells and any CFH present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for CFH was added to the wells. After washing, avidin, conjugated Horeseradish peroxidase (HRP) was added to the wells. Following a wash to remove any avidin-enzyme reagent, a substrate solution was added to the wells and the color developed in proportion to the amount of CFH bound in the initial step. The development of color was stopped and the intensity of color was measured.

Procedure

The calibrator solution was diluted for different concentration using mix diluent solution. The serum samples were diluted to 1:2000 for estimation. This dilution was achieved by adding 2 µl sample to 98 µl of sample diluent. The dilution was completed by adding 6 µl of the first solution to 234 µl of sample diluents. The entire assay was performed at 37 ºC using laboratory incubator (model INC108 med, Memmert, Germany).

Each well of the plate was added with 100 µl of standard or sample and then was incubated for 2 hr at 37 ºC after sealing with the tape. The contents were then removed from the plate and 100 µl of biotin antibody solution was added to each well without washing. It was then incubated for one hour at 37 ºC and washed three times with washing solution using microtiter plate washer (KHB-ST-36W, China). HRP-avindin solution (100 µl) was then added and incubated again for one hour at 37 ºC and washed again. Then 90 µl of TMB solution was added and incubated for about 15-30 min at 37 ºC. Then 50 µl of stop solution was added to each well that changed the color from blue to yellow. Then absorbance on micro plate reader (KHB-ST 360, China) was read at 450 nm and 570 nm immediately. Reading at 570 nm was subtracted from 450 nm reading.

Calculations

The mean value of duplicate readings of standards was calculated. The reading at 450 nm was subtracted from that at 570 nm. The sample results were calculated by interpolation from a calibrator curve that was performed in the same assay as that of the sample. The curve was drawn using value of standards on x-axis and corresponding absorbance value on y-axis. The best fit line was determined through regression analyses applying log-log curve. The unknown values were calculated from standard curve and multiplied by the dilution factor.

Detection Range

The detection range of the kit was 31.25 ng/ml-2000 ng/ml.

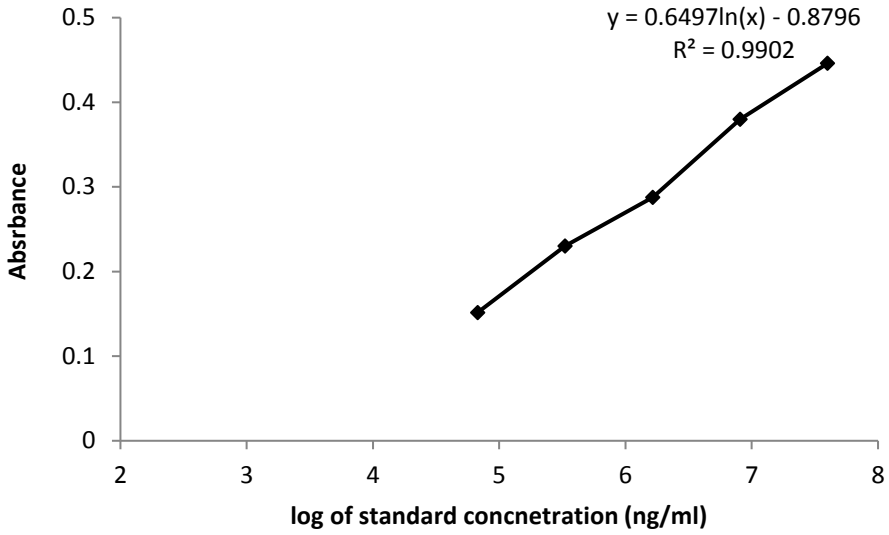


Fig 3.9 Standard curve of CFH

3.13 Statistical Analyses

All statistical analyses were performed on "IBM Statistical Product and Service Solutions (SPSS) Statistics" (version 18.0, Chicago, Illinois, USA) and Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). All data were presented as mean ± standard error of mean (SEM). The significance level was set at $P < 0.05$. Student's t-test was applied to analyse the difference of values between the diseased and control groups. When significance in Levene's test for equality of variance was ≤ 0.05, equal variance was assumed otherwise not. Pearson's correlation was applied to study the correlation of different parameters. Logistic regression analyses were applied to predict if the change in serum protein levelswere associated with the risk of disease.

The data were transformed using the method as described by Box and Cox (1964) for reducing heterogeneity of error and to permit the assumption of equal variance to be met. Data were transformed using XLSTAT (Version,2015USA). Transformed data were subjected to Multivariate analysis of variance (M-ANOVA) for statistical evaluation of the association of serum IL-6, IL-8, ApoE, leptin, VEGF, HTRA1, CRP and CFH levels with AMD.

Results

3.14 Lipid Profile

3.14.1 Cholesterol

The serum total cholesterol concentration in AMD patients was higher but it was non-significantly ($p< 0.092$) different from the control subjects (167.5 ± 3.84 vs 154.50 ± 4.0). In AMD patients76 patients had optimal level (< 200 mg/dl), 11 had borderline high (200-239 mg/dl) and 3 had high serum cholesterol level (240 mg/dl and above). In the control group 86 subjects had optimal level, 13 had borderline high and a single subject had high level (Table 3.1).

Male AMD patients had non-significantly increased levels as compared to healthy males (168.5 ± 4.78 vs 154.50 ± 5.17; $p< 0.523$). In AMD males, 51 patients had optimal cholesterol level, 6 had borderline high and 2 had high cholesterol level. In the control subjects 48 had optimal level, 6 had borderline high and a single subject had high serum cholesterol level (Table 3.2).

Infemale AMD patients, cholesterol concentrationwas significantly elevated than the normal females (168.0 ± 7.19 vs 156.0 ± 6.42; $p< 0.040$). In AMD patients, 25 patients had optimal level, 5 had borderline high and a single patient had high cholesterol level. In the control group, 39 had optimal level, 6 had borderline high cholesterol level (Table 3.3).

The cholesterol concentration in patients with dry AMD was non-significantly elevated as compared to patients with wet AMD (171.0 ± 6.68 vs 167.5 ± 4.64; $p< 0.21$). In dry AMD group,24 patients had optimal cholesterol level and 3 had borderline high level. In the wet AMD group, 52 patients had optimal level, 8 had borderline high and 3 had very high cholesterol level (Table 3.4).

3.14.2 Triglycerides (TG)

Triglyceride concentration in AMD patients (160.0 ± 8.07) was non-significantly elevated ($p< 0.409$) than the control subjects (132.0 ± 9.81). In AMD group, 40 patients had normal level (less than 150 mg/dl), 27 had borderline high (150-199 mg/dl) and 23 had high TG level (200-499 mg/dl). In the control group, 59 subjects had normal, 23 had borderline high, 17 had high levels and a single case showed very high level of serum triglycerides (500 mg/dl and above) (Table 3.1).

The serum triglyceride concentration in male AMD patients was non-significantly raised as compared to healthy males (155.0 ± 9.55 vs 129.0 ± 13.99; p< 0.987). In AMD group, 27 patients had normal level, 16 had borderline high, 16 had high and none of the patients had very high triglyceride level. In the control group, 32 subjects had normal triglyceride level, 13 had borderline high, 9 had high and a single case showed very high level of triglycerides (Table 3.2).

The concentration of triglycerides in females with AMD was non-significantly (p< 0.382) elevated as compared to the females of control group (166.0 ± 15.01vs132.0 ± 13.27). In AMD female patients, 13 had normal level,11 had borderline highand 7 had high TG level. In the control group, 26 subjects had normal level, 11 had borderline high and 8 subjects had high TG level (Table 3.3).

The triglyceride concentration in patients with dry AMD was non-significantly (p< 0.05) elevated when compared with the patients with wet AMD (168.0 ±14.89 vs 153.50 ±9.62). In dry AMD group, 15 patients had normal level, 9 had borderline high and 3 had high TG level. In the wet AMD group, 25 patients had normal, 18 had borderline high and 20 had high triglyceride 4level (Table 3.4).

3.14.3 HDL

HDL concentration in AMD patients (57.0 ± 1.49) was significantly elevated (p< 0.044) as compared to control subjects (51.00± 1.47). In AMD group, 79 patients had optimally high value (> 40 mg/dl) while 11 patients had risk related values (< 40 mg/dl). In the control group, 85 subjects had optimally high values and 15 had values related to high cardiac risk i.e., < 40 mg/dl (Table 3.1).

The HDL concentration in AMD male patients was slightly elevated as compared to healthy male subjects (56.5 ± 2.03vs 50.5 ± 1.98; p< 0.163). In male AMD patients, 49 had optimally high values and 10 had lower values. In the control male subjects48 had optimally high values and 7 had lower value (Table 3.2).

The concentration of HDL was non-significantly (p< 0.126) elevated in female patients with AMD (57.0 ± 2.19) as compared to normal females of the control group (53.5 ± 2.28). In AMD female patients, 25 had optimally high values and 6 had lower values. In the control group, 28 subjects had optimally high values and 17 had lower level of HDL (Table 3.3).

The concentration of HDL in dry AMD patients was non-significantly elevated when compared to the concentration in wet AMD patients (58.0 ± 2.77 vs 56.0 ±1.76; p< 0.08). In dry AMD patient group, 5 had optimally high values and 22 had risk related values. In the wet AMD patients, 6 had optimally high values related to low risk and 57 had lower values (Table 3.4).

3.14.4 LDL

The concentration of LDL in AMD patients (76.5 ± 2.85) was non-significantly elevated (p < 0.393) than the control subjects (72.50 ± 3.39). In AMD group, 73 patients had optimal level (less than 100 mg/dl), 16 had above optimal (100-129 mg/dl) and only a single patient had high LDL level (130-159 mg/dl). In controls, 80 subjects had optimal level, 15 had above optimal and 5 subjects had border line high levels of LDL (Table 3.1).

Serum LDL concentration in AMD male patients was non-significantly elevated as compared to control male subjects (73.5± 3.62 vs 73.0 ± 4.78; p < 0.966). In AMD males, 48 had optimal levels, 10 had above optimal and only a single patient had high LDL level. In control males, 43 subjects had optimal LDL level, 10 had above optimal and 2 subjects had border line high level while none of them had high LDL level (Table 3.2).

The LDL concentration in female AMD patients was non-significantly raised (p < 0.108) as compared to normal control females (87.0 ± 3.43vs 69.0 ± 4.96).In AMD group, 25 patientshad optimal LDL concentration and 6 had above optimal level. In the control group, 37 subjects had optimal level, 5 had above optimal and 3 subjects had border line high levels while none of them had high serum LDL level (Table 3.3).

The concentration of LDL in dry AMD was non-significantly reduced (p < 0.73) in patients with dry AMD when compared to concentration in patients with wet AMD (66.0 ± 5.56 vs 82.0 ± 3.28).In dry AMD patient group, 21 had optimal level and 6 had above optimal level. In wet AMD group, 52 subjects had optimal level,10 had above optimal and a single subjects had border line high LDL level (Table 3.4).

3.14.5 Cholesterol/HDL ratio

In AMD patients, the value of cholesterol/HDL ratio (2.86 ± 0.07) was non-significantly reduced (p< 0.832) than the control subjects (2.94 ± 0.06). In AMD

group, 78 patients the cholesterol/HDL ratio wasin optimal range (1-3.5) and in 11 patientsit was in high range (upto 5) while the value of ratio was > 5 in a single patient that is related to high risk of cardiovascular diseases. In controls, the ratio was in optimal range in 79 subjects and in 21 subjects it was in high range (Table 3.1).

In AMD patients cholesterol/HDL ratio was non-significantly reduced ($p< 0.520$) as compared to control subjects (2.84 ± 0.10 vs 3.0 ± 0.08). In 52 AMD patients the ratio was in optimal range while in 6 patients it was in high range and a single patient had > 5. The ratio was in optimal range in 41 control subjects whereas it was relatively high in 14 control subjects (Table 3.2).

The cholesterol/HDL ratio in AMD females was non-significantly ($p< 0.599$) elevated as compared to healthy females (2.89 ± 0.10 vs 2.78 ± 0.09). In AMD females, 26 patients had this ratio in the optimal range whereas in 5 patients the value was in high range. In control females, the ratio was in the optimal range in 38 subjects and in relatively high range in7 cases in control females (Table 3.3).

Thecholesterol/HDL ratio in patients with dry AMD was non-significantly ($p< 0.77$) lower as compared to patients with wet AMD (2.77 ± 0.09 vs 2.90 ± 0.09). In dry AMDgroup,25 patients had ratio in the optimal range while 2 had this ratio in the high range. In wet AMD patients, the ratio was in optimal range in 52 cases and in relatively high range in10 andstill higher in a single patient (Table 3.4).

3.14.6 Cholesterol/TG ratio

As compared to control subjects, the value of cholesterol/TG ratio in AMD patients was non-significantly reduced ($p< 0.250$; 1.11 ± 0.04 vs 1.17 ± 0.06). All the subjects in the two groups had the value of cholesterol/TG ratio <3.5 that was not related to the risk of cardiac diseases (Table 3.1).

The median value of cholesterol/TG ratio in AMD male patients was non-significantly reduced ($p< 0.396$) as compared to healthy control subjects (1.15 ± 0.06 vs 1.16 ± 0.07). All the subjects in the two groups had ratio <3.5(Table 3.2).

In female AMD patients, the cholesterol/TG ratio was non-significantly reduced ($p< 0.476$) as compared to normal healthy females of the control group (1.05 ± 0.08 vs 1.24 ± 0.09). All the subjects in the two groups had ratio less than 3.5(Table 3.3).

The cholesterol/TG ratio was non-significantly lower in patients with dry AMD as compared to wet AMD patients (1.05 ± 0.09 vs 1.14 ± 0.06; p< 0.12). All the subjects in the two groups had ratio < 3.5(Table 3.4).

3.14.7 Cholesterol/LDL ratio

The value of cholesterol/LDL ratio was found to be non-significantly elevated (p< 0.053) in AMD patients (2.14 ± 0.12) than the control subjects (2.02 ± 1.69). In AMD patients, cholesterol/LDL ratio was < 3.5 in 80 patients while in 87 subjects in the control group had the value of ratio > 3.5 (Table 3.1).

The cholesterol/LDL ratio in males was non-significantly (p < 0.068) elevated in AMD patients as compared to control males (2.14 ± 0.18 vs2.0 ± 0.56). In AMD group, 51 patients had <3.5 value of the ratiowhile in the control group 47 subjects had > 3.5 value (Table 3.2).

The cholesterol/LDL ratio in females with AMD (2.01 ± 0.14) was non-significantly different (p< 0.286) from the healthy females of the control group (2.06 ± 0.42). In 29 female AMD patients the value was< 3.5, while in the control group, 40 subjects had greater than 3.5 value of ratio (Table 3.3).

The level of cholesterol/LDL ratio in patients with dry AMD was non-significantly lower (p< 0.13) as compared to patients with wet AMD (2.19 ± 0.12 vs 2.61 ± 0.17). In dry AMD group, 25 patients had < 3.5 value of the ratio while in patients with wet AMD 55 subjects had cholesterol/LDL ratio< 3.5(Table 3.4).

3.14.8 LDL/HDL ratio

The LDL/HDL ratio in AMD patients (1.40 ± 0.07) was non-significantly (p< 0.769) reduced as compared to the control subjects (1.49 ± 0.07). In AMD patients 88 subjects had the value in safe range (≤ 2.5) and only two patients had higher values i.e. > 2.5. In control group, 92 subjects had value in safe range and 8 subjects had greater than 2.5value (Table 3.1).

In male AMD patients, LDL/HDL ratio was non-significantly (p< 0.578) reduced as compared to normal control subjects (1.39 ± 0.10 vs 1.5 ± 0.09). In AMD patient group, 58 subjects had the value in optimal range and only a single patient had higher value. In control group, the value was in the optimal range in 51 subjects and 4 subjects had higher values (Table 3.2).

The LDL/HDL ratio in AMD femaleswas non- significantly (p< 0.759) different from control females (1.42 ± 0.09 vs 1.42 ± 0.10). In female AMD patients, 30 subjects had the value in optimalrange and only a single patient had higher value. In control group, 41 subjects had the value in optimal range (Table 3.3).

In patients with dry AMD the value of LDL/HDL ratio was non-significantly (p< 0.42) lower as compared to patients with wet AMD (1.21 ± 0.11vs1.41 ± 0.09). In patients with dry AMD, 26 subjects hadLDL/HDL ratio in optimal range and only a single patient had value > 2.5. In patients with wet AMD, 61 subjects had the value in the optimal range and 2 patients had greater than 2.5 value of the ratio (Table 3.4).

3.14.9 HDL/LDL ratio

The HDL/LDL ratio was significantly raised (p> 0.046) in AMD patients (0.71 ± 0.06) as compared to the control subjects (0.67 ± 0.76). In the AMD group, 8 patients had ideal value (= 0.3), 2 had normal value (upto 0.4), 60 had slightly above normal (upto 1) and 20 patients had HDL/LDL ratio > 1. In the control group, 7 subjects had ideal value of ratio, 12 had normal value, 58 had slightly above normal and 23 had > 1(Table 3.1).

The HDL/LDL ratio in AMD patients was non-significantly (p> 0.065) elevated as compared to healthy control subjects (0.71± 0.09 vs0.66 ± 1.38). In the AMD patients, 5 had normal value, 1 had ideal value, 34 had slightly above normal and 19 patients had elevated values. In the control group, 8 subjects had optimal ratio, 13 had ideal, 29 had slightly above normal and 15 had elevated values (Table 3.2).

HDL/LDL ratio in AMD females was not significantly (p< 0.276) different from control healthy female subjects (0.70 ± 0.08 vs 0.70 ± 0.24). In the AMD group, a single patient had normal value, 3 had ideal value, 22 had slightly above normal and 5 patients had > 1 ratio. In the control group, 4 subjects had normal ratio, 4 hadideal ratio, 25 had slightly above normal and 12 had value > 1(Table 3.3).

The value of HDL/LDL ratio in patients with dry AMD was non-significantlyelevated as compared to patients with wet AMD (0.82 ± 0.17 vs 0.70 ± 0.06; p< 0.14). In the dry AMD patients, 1 had normal value, 1 had ideal value, 21had slightly above normal and 4 patients > 1 value of ratio. In the wet AMD patients, 1 had normal value, 7 had ideal value, 35 had slightly above normal and 20 had value > 1 (Table 3.4).

3.14.10 TG/HDL ratio

In AMD subjects, the value of TG/HDL ratio (2.66 ± 0.11) was non-significantly elevated (p> 0.885) than the control subjects (2.56 ± 0.01). In AMD group, 72 patients had value ≤ 3.5 which is safe, 13 had upto 5 which is in optimal limit and 5 had greater than 5 indicating increased risk of cardiac diseases (Bowden and Sinatra, 2012). In control group, 78 subjects had the value ≤ 3.5; 14 had upto 5 and 8 had > 5 (Table 3.1).

In male patients with AMD, median value of TG/HDL ratio was no different from healthy male subjects (2.64 ± 0.14 vs 2.64 ± 0.18; p> 0.763). In AMD group, 49 patients had value ≤ 3.5, 7 had ≤ 5.0 and 3 had > 5.0. In the control group, 43 subjects had TG/HDL ratio≤ 3.5, 9 had ≤ 5.0 and 3 had > 5.0 (Table 3.2).

The TG/HDL ratio in female patients with AMD was non-significantly (p< 0.599) elevated as compared to healthy females of control group (2.73 ± 0.21 vs 2.54 ± 0.16). In AMD group, 23 patientshad value upto 3.5, 6 had upto 5 and 2 had > 5. In control group, 36 subjects had the value ≤ 3.5,6 had ≤ 5 and 3 had > 5(Table 3.3).

In patients with dry AMD, the level of TG/HDL ratio was non-significantly elevated as compared to patients with wet AMD (2.84 ± 0.19 vs 2.66 ± 0.14; p< 0.12). In patients with dry AMD, 24 had value ≤ 3.5, 3 had ≤ 5 whereas in patients with wet AMD, 47 subjects had the value ≤ 3.5, 10 had ≤ 5and 6 had >5 value of the ratio (Table 3.4).

3.14.11 TG/LDL ratio

The value of TG/LDL ratio in AMD patients was non-significantly elevated (p > 0.062) (1.93 ± 0.31) than the control subjects (1.69 ± 2.60). In AMD patients, 77 had < 5 value and 13 had > 5.0while in control group, 89 cases had the ratio < 5.0 and 11 had > 5 value of TG/HDL ratio (Table 3.1).

The median value of TG/LDL ratio in AMD males was non-significantly (p> 0.102) reduced as compared to healthy males of the control group (1.86 ± 0.45 vs 1.91 ± 4.57). In AMD group, 50 patients had < 5.0 value and in control group 48 subjects had the ratio > 5.0 (Table 3.2).

TG/LDL ratio in females AMD patients was non-significantly elevated than the females of control group (2.02 ± 0.31vs 1.52 ± 1.08; p< 0.305). In AMD group,27

patients had value < 5.0 while in control group 40 cases had the ratio >5.0 (Table 3.3).

The level of TG/LDL ratio in patients with dry AMD was non-significantly ($p<0.13$) elevated as compared to patients with wet AMD (2.02 ± 0.89 vs 1.75 ± 0.22). In patients with dry AMD, 26 had the value < 5.0 while in wet AMD patient group, 51 cases had value of TG/LDL ratio >5.0 (Table 3.4).

Table 3.1 Concentrations of serum total cholesterol, HDL, LDL and Triglycerides (TG) in AMD patient vs healthy controls.

Parameters	Control (n=100)		AMD (n=90)		T-value	95% Confidence Interval of the Difference		
	Mean ± SEM	Range	Mean ± SEM	Range		Lower	Upper	p value
Cholesterol (mg/dl)	155.44 ± 4.0	66.0-252.0	166.17 ± 3.84	89.0-262.0	-1.729	-21.23	0.83	0.092
Triglycerides (mg/dl)	154.02 ± 9.81	33.0-486.0	164.67 ± 8.07	51.0-429.0	-0.691	-34.26	18.40	0.409
HDL (mg/dl)	53.17± 1.47	27.0-107.0	57.47 ± 1.49	27.0-98.0	-1.965	-8.50	-0.18	0.044*↑
LDL (mg/dl)	71.86 ± 3.39	1.20-154.0	75.75 ± 2.85	17.0-141.0	-1.010	-12.91	4.84	0.393
Cholesterol/HDL	2.99 ± 0.06	1.68- 4.38	2.97 ± 0.07	1.98-7.52	0.205	-0.17	0.21	0.832
Cholesterol/TG	1.26 ± 0.06	0.32 - 3.37	1.17 ± 0.04	0.44-2.43	1.143	-0.06	0.24	0.250
Cholesterol/LDL	5.80 ± 1.69	1.53-128.3	2.47 ± 0.12	1.44-11.06	1.894	-0.05	6.70	0.053
LDL/HDL	1.42 ± 0.07	0.02-2.97	1.40 ± 0.07	0.20-5.22	0.165	-0.16	0.22	0.769
HDL/LDL	2.45 ± 0.76	0.36-49.17	1.0 ± 0.06	0.20-5.0	2.031	0.02	3.05	0.046*↓
TG/HDL	2.82 ± 0.01	0.96-8.15	2.48 ± 0.11	1.30-6.44	-0.204	-0.36	0.31	0.885
TG/LDL	12.06 ± 4.89	0.46-164.26	2.79 ± 0.31	0.68-25.24	1.050	-0.47	19.00	0.062

*Shows significantly different from control at $p < 0.05$; ↑ shows significantly raised level; ↓ shows significantly reduced level

Table 3.2 Concentration of serum total cholesterol, HDL, LDL and Triglycerides (TG) in AMD males vs normal control males

Parameters	Control males (n=55)		AMD males (n=59)		T values	95% Confidence Interval of the Difference		
	Mean ± SEM	Range	Mean ± SEM	Range		Lower	Upper	p value
Cholesterol (mg/dl)	157.6 ± 5.17	68.0-252.0	162.10 ± 4.78	89.0 – 262.0	-0.639	-18.44	9.43	0.523
Triglycerides (mg/dl)	159.58 ± 13.99	47.0-486.0	163.51 ± 9.55	51.0- 429.0	-0.016	-36.06	35.48	0.987
HDL (mg/dl)	53.03 ± 1.98	30.0-107.0	57.03 ± 2.03	27.0-98.0	-1.408	-9.64	1.64	0.163
LDL (mg/dl)	72.06 ± 4.78	1.20.0-139.0	72.32 ± 3.62	17.0 – 141.0	-0.043	-12.02	11.51	0.966
Cholesterol/HDL	3.04 ± 0.08	1.68-4.38	2.95 ± 0.10	2.07- 7.52	0.646	-0.18	0.36	0.520
Cholesterol/TG	1.24 ± 0.07	0.32-3.02	1.16 ± 0.06	0.44 - 2.43	0.852	-0.11	0.28	0.396
Cholesterol/LDL	8.35 ± 3.09	1.53-28.33	2.58 ± 0.18	1.44 - 11.06	1.862	-0.44	12.00	0.068
LDL/HDL	1.46 ± 0.09	0.20-2.79	1.38 ± 0.10	0.20 - 5.22	0.559	-0.20	0.35	0.578
HDL/LDL	3.59 ± 1.38	0.36-49.17	0.97 ± 0.09	0.19 - 5.00	1.887	-0.16	5.40	0.065
TG/HDL	2.92 ± 0.18	1.02-8.15	2.86 ± 0.14	1.30 - 6.44	0.299	-0.38	0.52	0.763
TG/LDL	10.71 ± 4.57	0.55-164.23	3.02 ± 0.45	0.68 - 25.24	1.803	-1.81	34.18	0.102

Table 3.3 Serum concentration of total cholesterol, HDL, LDL and Triglycerides (TG) in AMD females vs normal control females

Parameters	Control females (n=45)		AMD females (n=31)		T value	95% Confidence Interval of the Difference		
	Mean ± SEM	Range	Median ± SEM	Range		Lower	Upper	p value
Cholesterol (mg/dl)	154.00± 6.42	66.0-235.0	173.93± 6.37	89.0-242.0	-2.093	-38.91	-0.96	0.040*↑
Triglycerides (mg/dl)	148.84 ± 13.27	33.0-463.0	166.90± 15.01	69.0-417.0	-0.879	-59.03	22.90	0.382
HDL (mg/dl)	53.27± 2.28	27.0-91.0	58.32 ± 2.01	36.0-83.0	-1.546	-11.57	1.46	0.126
LDL (mg/dl)	71.30 ± 4.96	8.0-154.0	82.29 ± 4.47	25.0-122.0	-1.536	-24.46	2.49	0.108
Cholesterol/HDL	2.94 ± 0.09	1.78-4.30	3.01 ± 0.10	1.98-4.27	0.528	-0.35	0.20	0.599
Cholesterol/TG	1.30 ± 0.09	0.40-3.37	1.21 ± 0.08	0.58-2.03	0.717	-0.17	0.35	0.476
Cholesterol/LDL	2.86 ± 0.42	1.53-18	2.29 ± 0.14	1.64-4.96	1.076	-0.48	1.62	0.286
LDL/HDL	1.40± 0.09	0.10-2.70	1.45 ± 0.10	0.40-2.42	-0.308	-0.32	0.23	0.759
HDL/LDL	1.15 ± 0.24	0.37-10.13	0.82 ± 0.08	0.41-2.50	1.097	-0.27	0.93	0.276
TG/HDL	2.69 ± 0.16	0.96-5.26	2.82 ± 0.21	1.39-5.75	-0.528	-0.66	0.38	0.599
TG/LDL	3.78 ± 1.08	0.46-44.63	2.38 ± 0.31	0.86-7.35	1.033	-1.30	4.11	0.305

*significantly different from control at $p < 0.05$;↑ significantly reduced from control group

Table 3.4 Comparison of serum total cholesterol, HDL, LDL and Triglycerides (TG) in wet and dry AMD patients

Parameters	Wet AMD(n=63)		Dry AMD (n=27)		T value	95% Confidence Interval of the Difference		
	Mean ± SEM	Range	Mean ± SEM	Range		Lower	Upper	p value
Cholesterol (mg/dl)	169.42 ± 4.64	89.0-262.0	159.00 ±6.82	100.0-229.0	1.257	-0.605	26.89	0.21
Triglycerides (mg/dl)	175.50 ±10.28	51.0-417.0	140.71 ±11.45	64.0-429.0	2.030	0.73	68.84	0.05
HDL (mg/dl)	59.24 ±1.84	27.0-98.0	53.57 ±2.45	36.0-85.0	1.775	-0.68	12.02	0.08
LDL (mg/dl)	75.08 ±3.65	20.0-141.0	77.25 ±4.46	17.0-129.0	-0.350	-14.50	10.16	0.73
Cholesterol/HDL	2.96 ±0.10	1.98-7.52	3.01 ±0.09	2.12-4.47	-0.297	-0.38	0.28	0.77
Cholesterol/TG	1.13 ±0.06	0.58-2.43	1.29 ±0.08	0.44-2.04	-1.567	-0.37	0.04	0.12
Cholesterol/LDL	2.61 ±0.17	1.44-11.06	2.19 ±0.12	1.64-4.45	1.520	-0.13	0.96	0.13
LDL/HDL	1.37 ±0.09	0.4-5.22	1.49 ±0.09	0.20-2.05	-0.809	-0.43	0.18	0.42
HDL/LDL	0.99 ±0.09	0.19-2.5	0.77 ±0.07	0.49-5.0	1.479	-0.07	0.50	0.14
TG/HDL	2.96 ±0.15	1.3-6.44	2.58 ±0.16	1.51-5.05	1.557	-0.10	0.86	0.12
TG/LDL	3.11 ±0.43	0.68-7.53	2.09 ±0.27	0.83-25.24	1.523	-0.31	2.35	0.13

3.15 Angiogenic Factors and Inflammatory Markers

These included IL-6, IL-8, ApoE, leptin, HTRA1, VEGF, CRP and CFH in the serum of the control and AMD subjects.

3.15.1 Interleukin 6 (IL-6)

Serum IL-6 concentration in patients with AMD was significantly ($p < 0.0005$) increased as compared to control subjects (27.80 ±2.25 vs 23.24 ± 0.64) (Table 3.5).

In male patients with AMD, IL-6 level (32.62 ± 3.29) was significantly ($p < 0.0071$) increased as compared to healthy males (22.82 ± 0.64) (Table 3.6). Likewise, in female AMD patients, IL-6 concentration (28.37 ±2.40) was significantly raised ($p < 0.009$) as compared to normal healthy females (25.17 ±0.96) (Table 3.7).

The concentration of IL-6 in patients with dry AMD was non-significantly elevated than the patients with wet AMD (35.67 ± 6.94 vs 25.89 ± 1.87; $p < 0.1787$) (Table 3.8; Fig 3.10).

3.15.2 Interleukin 8 (IL-8)

The concentration of IL-8 in serum of patients with AMD was highly significantly elevated ($p< 0.0001$) as compared to that in control subjects (56.05 ±0.79 vs 52.17 ± 0.94) (Table 3.5).

The level of IL-8 in male patients with AMD (56.05 ± 1.14) was highly significantly elevated ($p< 0.0001$) than control male subjects (42.42 ± 1.36) (Table 3.6). IL-8 level in females with AMD was non-significantly raised than the normal healthy females (56.05 ±1.36 vs 55.60 ±1.33; $p< 0.152$) (Table 3.7).

The IL-8 concentration in patients with dry AMD was non-significantly different from the patients with wet AMD (56.16 ± 2.35 vs 56.02 ± 0.71; $p < 0.4725$) (Table 3.8; Fig. 3.11).

3.15.3 ApoE

Serum ApoE concentration was significantly elevated ($p < 0.0024$) in AMD patientsas compared to the control subjects (52.60 ± 0.65 vs 51.25 ± 0.50) (Table 3.5). In male patients with AMD, ApoE concentration was non-significantly different from the healthy males of control group (52.31 ± 0.79 vs 51.23 ± 0.81; $p < 0.2507$) (Table 3.6). In female AMD patients, ApoE level (54.28 ± 1.13) was highly significantly elevated ($p < 0.011$) than the control females (51.46 ± 0.37) (Table 3.7). In patients

with dry AMD, the ApoE concentration (52.58 ± 1.02) was non- significantly ($p < 0.6475$) different from the patients with wet AMD (52.62 ± 0.83) group (Table 3.8; Fig. 3.12).

3.15.4 Leptin

The concentration of serum leptin was significantly lowered ($p < 0.008$) in patients with AMD as compared to the healthy control subjects (7.92 ± 0.03 vs 8.0 ± 0.03) (Table 3.5).

In male AMD patients, leptin concentration (7.88 ± 0.03) was significantly ($p < 0.0009$) reduced as compared to healthy males of control group (7.95 ± 0.02) (Table 3.6). Likewise, in female AMD patients, the concentration of leptin was significantly reduced than the normal healthy females (8.03 ± 0.06 vs 8.20 ± 0.06; $p < 0.017$) (Table 3.7).

Leptin concentration in patients with dry AMD (8.0 ± 0.06) was non-significantly ($p < 0.8735$) altered as compared to the patients with wet AMD (8.06± 0.04) (Table 3.8; Fig.3.13).

3.15.5 HTRA1

In patients with AMD, the HTRA1 concentration was significantly elevated ($p < 0.0001$) as compared to normal control subjects (1.35 ± 0.11 vs 0.89 ± 0.08) (Table 3.5).

In AMD male patients (1.61 ± 0.14) the HTRA1 concentration increased significantly ($p < 0.0005$) as compared to normal healthy males (0.89 ± 0.10) (Table 3.6). In female patients with AMD, serum HTRA1 concentration was significantly elevated than the healthy females of control group (1.19 ± 0.21 vs 0.87 ± 0.14; $p < 0.0162$) (Table 3.7).

The serum HTRA1 level in patients with dry AMD (1.45± 0.20) was non-significantly elevated ($p < 0.7475$) than the patients with wet AMD (1.35 ± 0.14) (Table 3.8; Fig. 3.14).

3.15.6 Vascular Endothelial Growth Factor (VEGF)

Serum VEGF concentration in patients with AMD was significantly raised as compared to healthy control subjects (104.83 ±0.40 vs 102.34 ±0.31; $p < 0.0008$) (Table 3.5).

Male patients with AMD had significantly elevated ($p < 0.013$) serum VEGF concentration (105.04 ± 0.67) than healthy males (102.71 ±0.41) (Table 3.6). In female AMD patients, the VEGF concentration was significantly increased than that of control females (104.52 ±0.50 vs 102.35 ±0.45; $p < 0.015$) (Table 3.7).

The concentration of VEGF in patients with dry AMD (105.36 ± 0.63) was non-significantly ($p < 0.5858$) higher than the patients with wet AMD (104.52 ± 0.59) (Table 3.8; Fig. 3.15).

3.15.7 C-Reactive Protein (CRP)

The concentration of CRP in patients with AMD was highly significantly increased as comparedto healthy control subjects (4.46 ±0.48 vs 2.42 ± 0.29; $p < 0.0001$) (Table 3.5).

In the male AMD patients (4.44 ± 0.70), the CRP concentration was highly significantly elevated ($p< 0.0001$) than healthy control males (2.16 ± 0.37) (Table 3.6). Likewise, in femalepatients with AMD significantly high CRP level was observed than the healthy females (4.54±0.45 vs 2.71 ±0.46; $p < 0.038$) (Table 3.7).

The concentration of CRP in patients with wet AMD was significantly elevated than the levels in patients with dry AMD (5.08 ± 0.61 vs 3.99 ± 0.69; $p < 0.016$) (Table 3.8; Fig. 3.16).

3.15.8 Complementary Factor H (CFH)

The concentration of CFH in the patients with AMD was highly significantly reduced when compared with normal healthy subjects of the control group (421.53 ±12.13 vs 522.82 ±16.88; $p < 0.0001$) (Table 3.5).

In male patients with AMD, the serum CFH concentration (407.93 ±18.72) was highly significantly reduced ($p < 0.0001$) than the normal males of the control group (506.38 ±22.79) (Table 3.6). In the female AMD patients, CFH concentration (434.26 ±26.01) was non-significantly reduced ($p < 0.3008$) than the healthy females of the control group (530.74 ± 25.42) (Table 3.7).

The serum CFH level in patients with wet AMD (423.44 ± 15.26) were non-significantly ($p < 0.2757$) higher as compared to the patients with dry AMD (398.37 ± 22.93) (Table 3.8; Fig. 3.17).

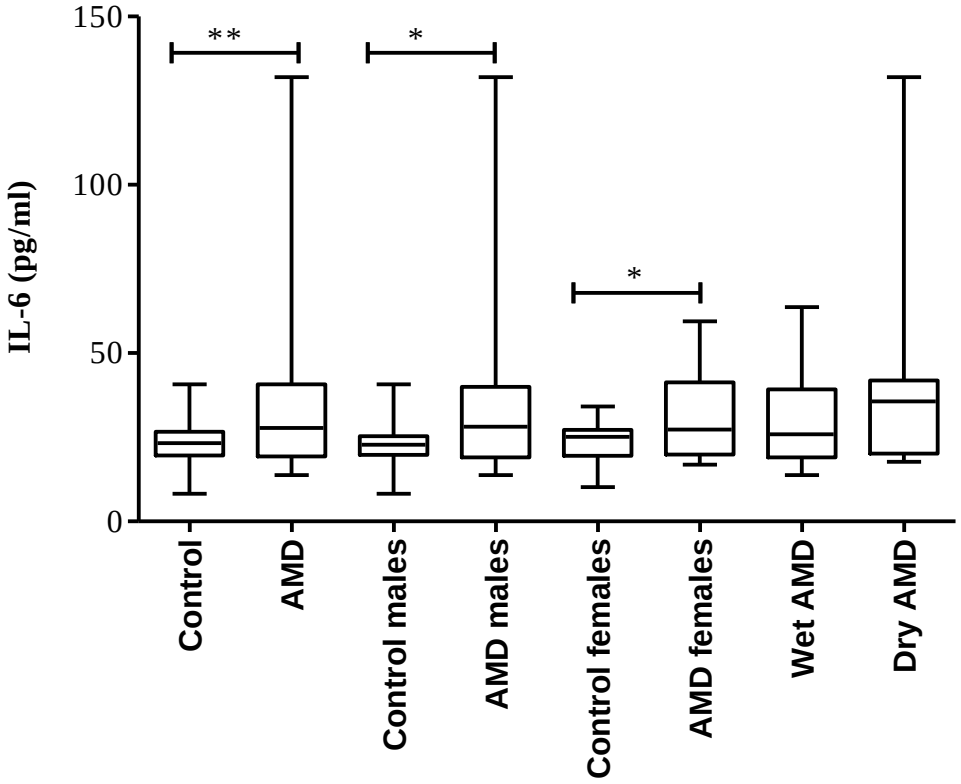


Fig. 3.10 Serum IL-6 concentration in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{**}p < 0.01$; $^{*}p < 0.05$

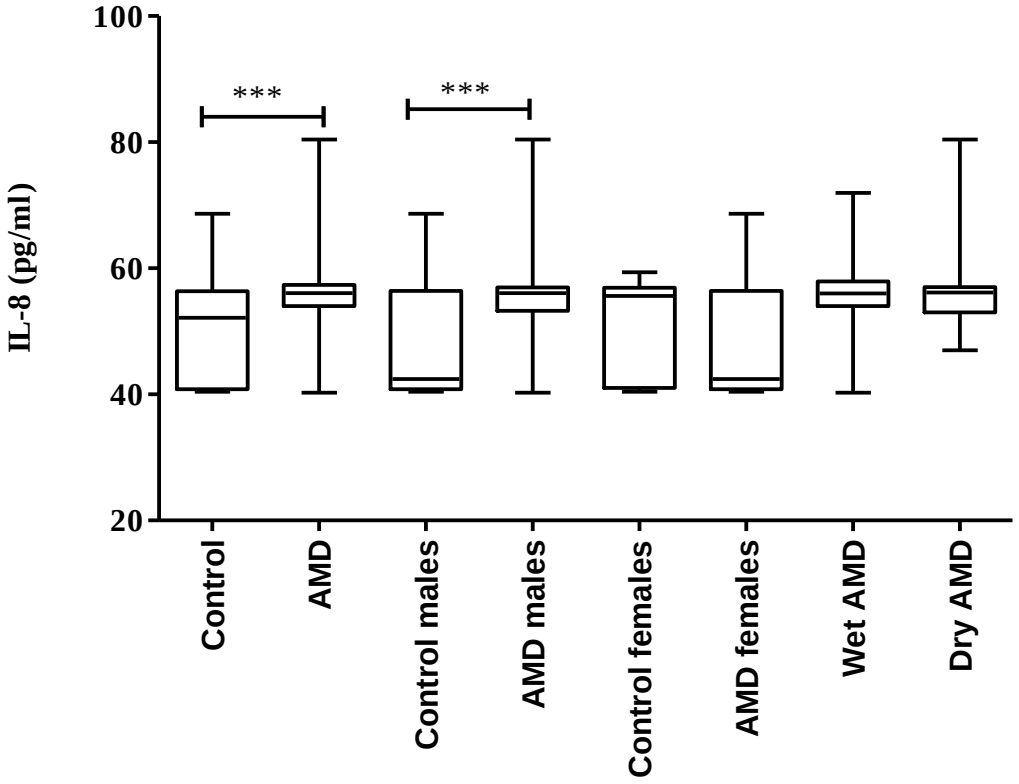


Fig. 3.11 Serum IL-8 concentration in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{***}p < 0.0001$

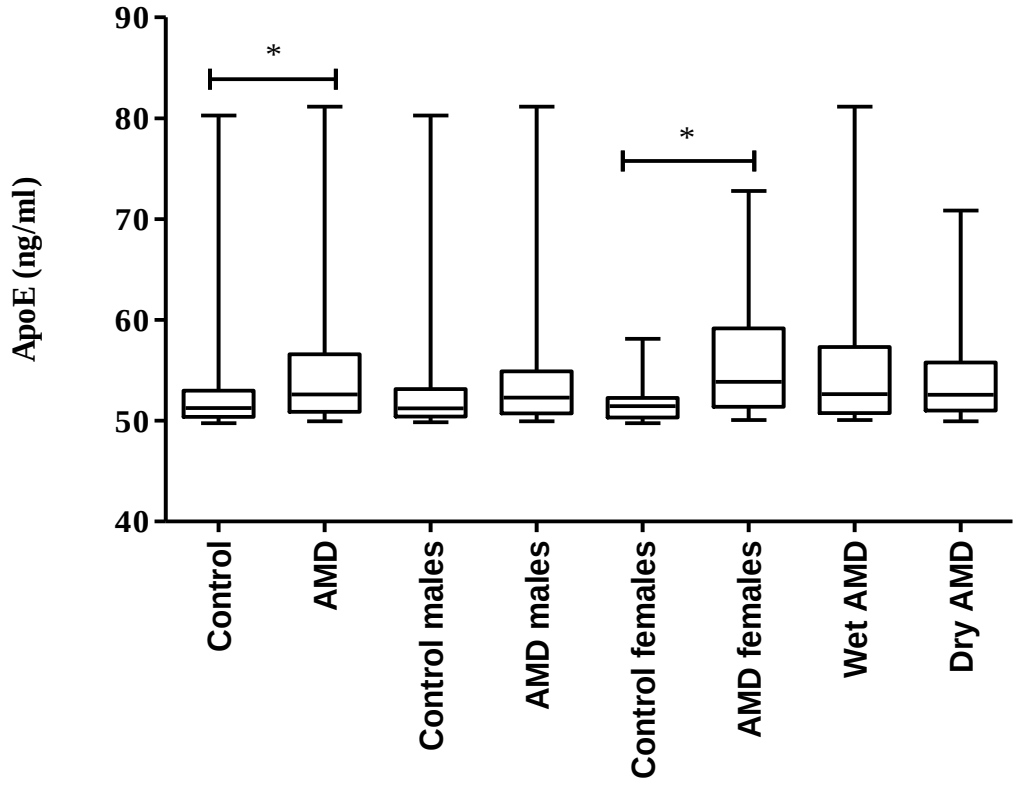


Fig. 3.12 Serum ApoE concentrations in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{*}p < 0.05$

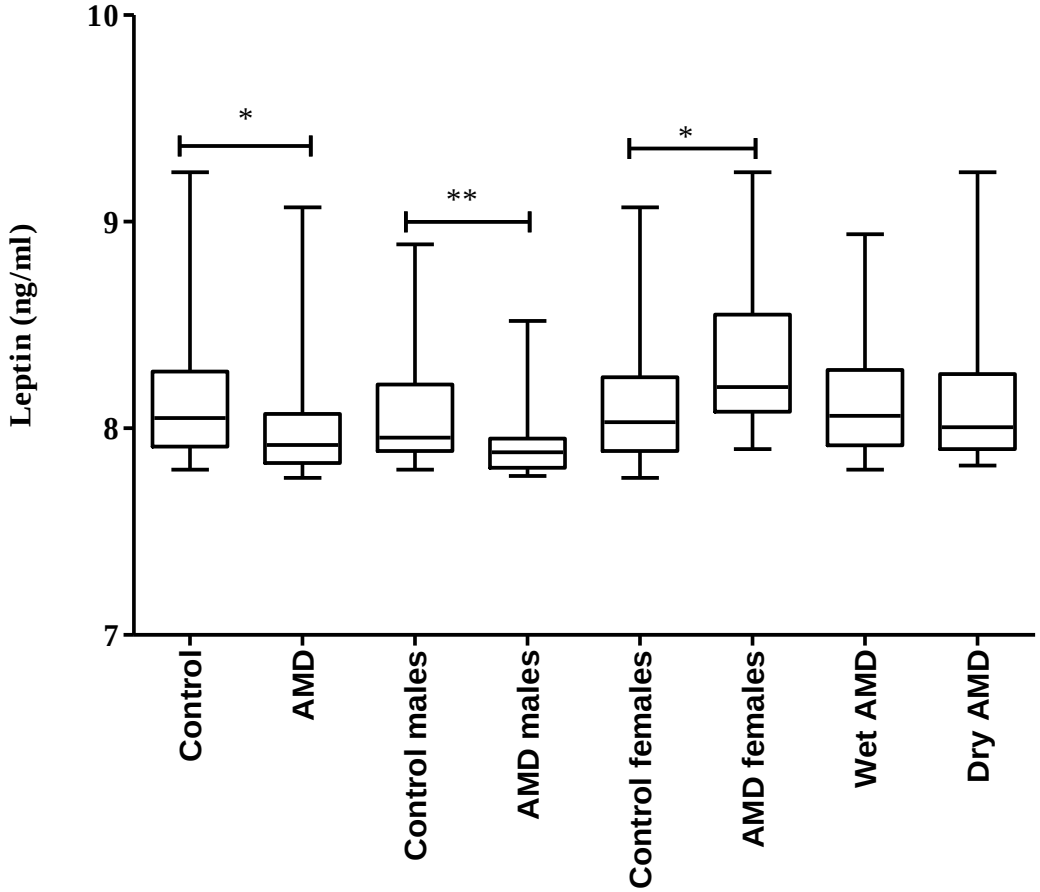


Fig. 3.13 Serum leptin concentrations in control subjects and AMD patients group. Bars represent median value and whiskers represent minimum and maximum values. $^{**}p < 0.01$; $^{*}p < 0.05$

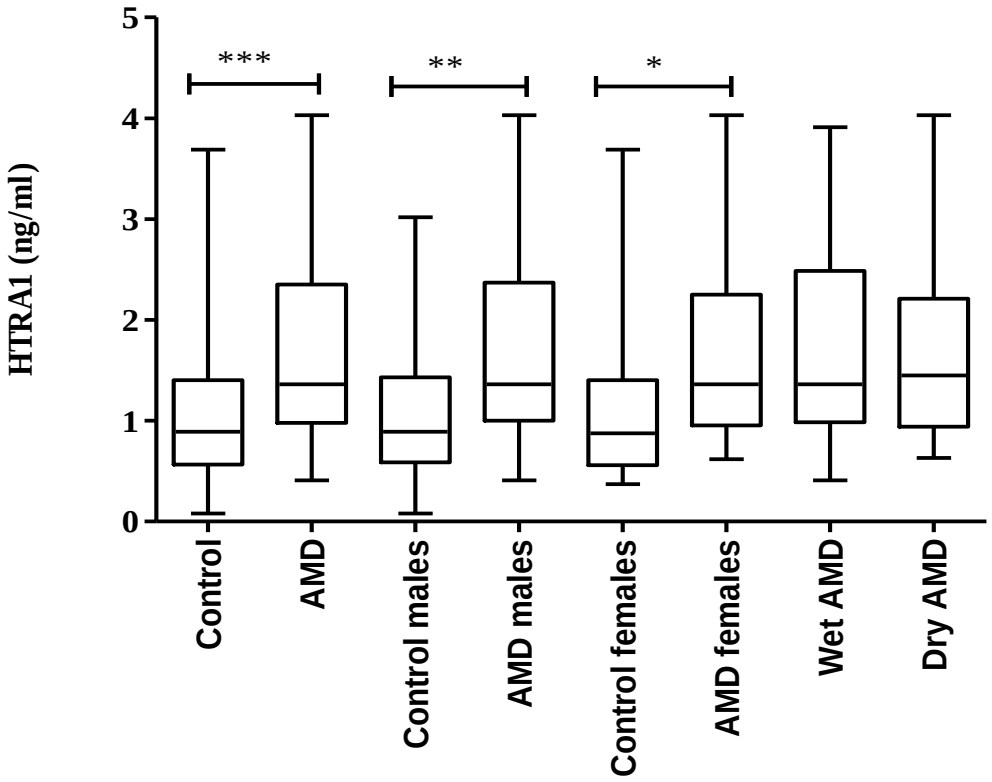


Fig. 3.14 Serum HTRA1 concentration in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{***}p < 0.0001$; $^{**}p < 0.01$; $^{*}p < 0.05$

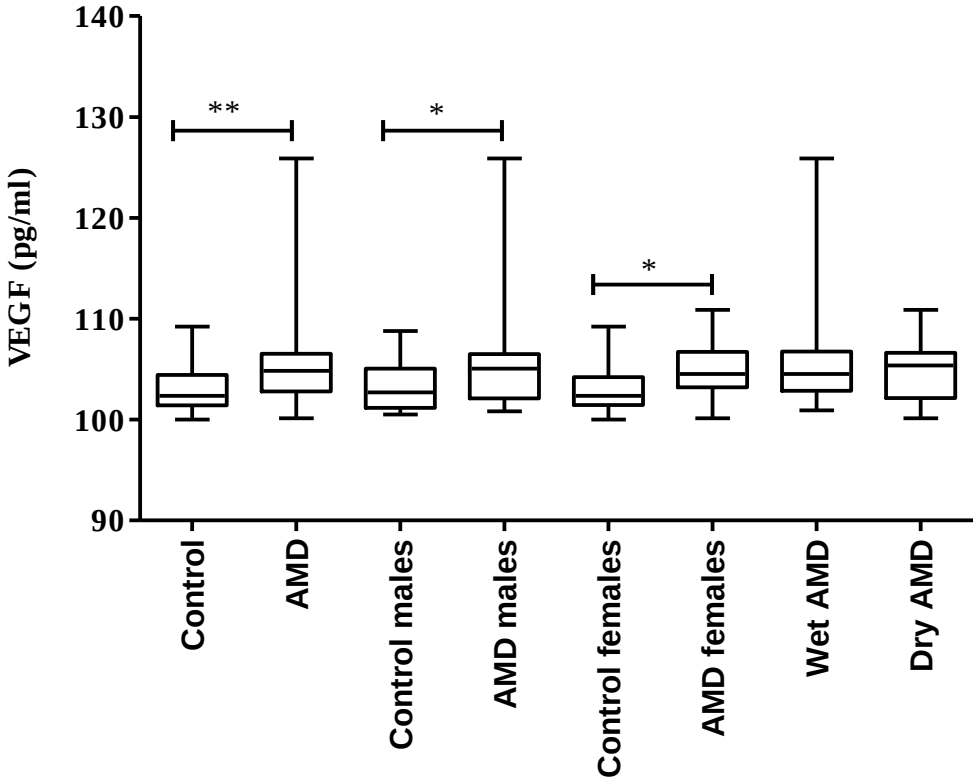


Fig. 3.15 Serum VEGF concentrations in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{***}p < 0.01$; $^{*}p < 0.05$

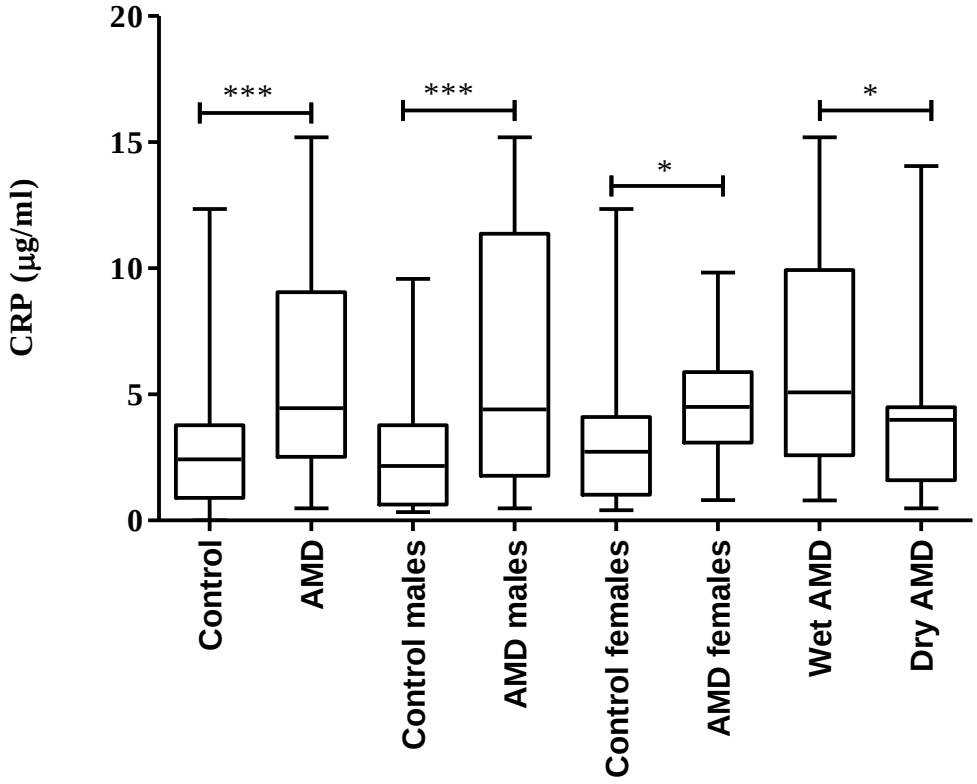


Fig. 3.16 SerumCRP concentrations in control subjects and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{***}p < 0.0001$; $^{*}p < 0.05$

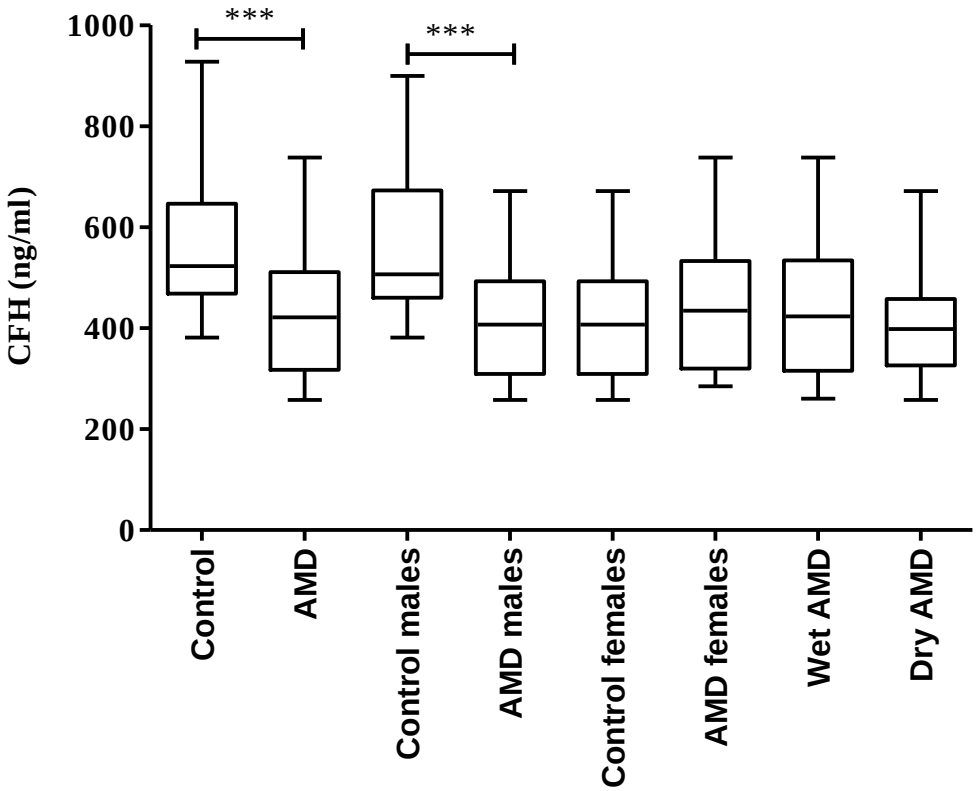


Fig.3.17 Serum CFH concentrations in control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{***}p < 0.0001$

Table 3.5 Serum parameters in control subjects and AMD patient group. (mean± Standard error of mean)

Parameters	Control (n=100) Mean ± SEM	Range	AMD(n=90) Mean ± SEM	Range	T value	95% Confidence Interval of the Difference		
						Lower	Upper	Sig.
IL6 (pg/ml)	23.14 ± 0.64	8.27-40.71	31.86 ± 2.25	13.80-131.98	-3.728	-13.37	-4.05	0.0005**↑
IL8 (pg/ml)	49.40 ± 0.94	40.43-68.67	56.37 ± 0.79	40.30-80.42	-5.724	-9.39	-4.54	0.0001***↑
ApoE (ng/ml)	52.25 ± 0.50	49.76-80.26	54.79 ±0.65	49.96-81.15	-3.094	-4.18	-0.92	0.0024*↑
Leptin (ng/ml)	8.15 ± 0.03	7.76-9.07	8.0 ± 0.03	7.80-9.24	-3.410	-0.06	-0.24	0.008*↓
HTRA1 (ng/ml)	1.01 ± 0.08	0.08-3.69	1.68 ± 0.11	0.41-4.03	-4.427	-0.937	-404	0.0001***↑
VEGF (pg/ml)	102.92 ± 0.31	100.02-109.22	105.17 ± 0.40	100.12-125.89	-3.445	-3.32	-1.16	0.0008**↑
CRP (µg/ml)	2.91 ± 0.29	0.33-12.35	5.77 ± 0.48	0.48-15.19	-5.069	-3.39	-1.74	0.0001***↑
CFH (ng/ml)	568.85 ± 16.88	380.98-927.73	441.14 ± 12.13	257.77-737.85	6.068	85.58	168.82	0.0001***↓

$^{***}p < 0.001$;$^{**}p < 0.01$;$^{*}p < 0.05$; ↑: increased from control; ↓: decreased from control

Table 3.6 Serum parameters incontrol males and AMD males. (mean± Standard error of mean)

Parameters	Control (n=55) Mean ± SEM	Range	AMD (n=59) Mean ± SEM	Range	T value	95% Confidence Interval of the Difference		
						Lower	Upper	Sig.
IL6 (pg/ml)	23.02 ± 0.64	8.27-40.71	32.62 ± 3.29	13.80-131.98	-2.820	-16.44	-2.74	0.0071*↑
IL8 (pg/ml)	48.47 ± 1.36	40.43-68.67	55.87 ± 1.14	40.30-80.42	-4.105	-10.94	-3.85	0.0001***↑
ApoE (ng/ml)	52.65 ± 0.81	49.86-80.26	53.96 ± 0.79	49.96-81.15	-1.157	-3.65	0.98	0.2507
Leptin (ng/ml)	8.06 ± 0.02	7.80-8.80	7.92 ± 0.03	7.77-8.52	-3.436	-0.23	-0.54	0.0009**↓
HTRA1 (ng/ml)	1.08 ± 0.10	0.08-3.02	1.72 ± 0.14	0.41-4.03	-3.650	-0.996	-0.292	0.0005**↑
VEGF (pg/ml)	103.39 ± 0.41	100.52-108.79	105.39 ± 0.67	100.83-125.89	-2.552	-3.59	-0.42	0.013*↑
CRP (ng/ml)	2.66 ±0.37	0.33-9.58	6.33 ± 0.70	0.48-15.19	-4.600	-5.32	-2.00	0.0001***↑
CFH (ng/ml)	568.09 ±22.79	380.98-899.41	417.93±18.72	257.77-727.82	5.091	91.71	208.60	0.0001***↓

***p < 0.0001; **p < 0.01; *p < 0.05;↑: increased from control; ↓: decreased from control

Table 3.7 Serum parameters in Control females and AMD females. (mean± Standard error of mean)

Parameters	Control (n=45) Mean ± SEM	Range	AMD (n=31) Mean ± SEM	Range	T value	95% Confidence Interval of the Difference		
						Lower	Upper	Sig.
IL6 (pg/ml)	23.28 ±0.96	10.18-34.12	30.56 ± 2.40	16.88-61.22	-2.813	-12.56	-1.99	0.009*↑
IL8 (pg/ml)	51.23 ±1.33	40.44-59.38	48.47 ±1.36	48.0-71.97	1.436	-1.04	6.56	0.152
ApoE (ng/ml)	51.67 ±0.37	49.76-58.11	56.71 ± 1.13	50.06-72.77	-3.983	-7.03	-2.17	0.011*↑
Leptin (ng/ml)	8.32 ± 0.06	7.76-9.07	8.11 ±0.06	7.90-9.24	-2.460	-0.377	-0.038	0.017*↓
HTRA1 (ng/ml)	1.07 ± 0.14	0.37-3.69	1.69 ±0.21	0.62-3.91	-2.498	-1.116	-0.131	0.0162*↑
VEGF (pg/ml)	103.17 ±0.45	100.02-109.22	104.86 ±0.50	100.12-110.87	-2.517	-3.03	-0.342	0.015*↑
CRP (ng/ml)	3.21 ± 0.46	0.41-12.35	4.59 ±0.45	0.81-9.83	-2.125	-2.668	-0.060	0.038*↑
CFH (ng/ml)	569.94 ±25.42	411.06-927.73	451.44 ±26.01	284.88-737.85	3.258	45.47	191.52	0.3008

*$p < 0.05$; ↑: increased from control; ↓: decreased from control

Table 3.8 Serum parameters in patients with Wet AMD and dry AMDs. (mean± Standard error of mean)

Parameters	Wet AMD (n=62) Mean ± SEM	Range	Dry AMD (n=28) Mean ± SEM	Range	T values	95% Confidence Interval of the Difference		
						Lower	Upper	Sig.
IL6 (pg/ml)	29.37 ± 1.87	13.80-63.64	39.45 ± 6.94	17.71-131.98	-2.002	-0.121	20.291	0.1787
IL8 (pg/ml)	55.90 ± 0.71	40.30-71.97	57.71 ± 2.35	47.00-80.42	-0.974	-1.842	5.459	0.4725
ApoE (ng/ml)	54.98 ± 0.83	50.06-81.15	54.38 ± 1.02	49.96-70.83	0.428	-3.417	2.208	0.6475
Leptin (ng/ml)	8.15 ± 0.04	7.80-8.94	8.14 ± 0.06	7.82-9.24	0.169	-0.155	0.130	0.8735
HTRA1 (ng/ml)	1.72 ± 0.14	0.41-3.91	1.64± 0.20	0.63-4.03	0.320	-0.596	0.431	0.7457
VEGF (pg/ml)	105.32 ± 0.59	100.93-125.89	104.85 ± 0.63	100.12-110.87	0.462	-2.515	1.569	0.5858
CRP (ng/ml)	6.49 ± 0.61	0.80-15.19	4.20 ± 0.69	0.48-14.05	2.261	-4.142	-0.444	0.016*↓
CFH (ng/ml)	430.74 ± 15.26	260.36-737.85	407.38 ± 22.93	257.77-671.19	0.988	-100.010	33.781	0.2757

*p< 0.05; ↓: decreased from wet AMD

3.16 Correlation Analyses

3.16.1 Correlation in control subjects and AMD patients

Correlation among different variables in control subjects and AMD patients is given in Table 3.9.

Table 3.9 Summary of correlation in control and AMD subjects collectively. (r=correlation coefficient)

Variables	Correlated to	r value	p value
Visual acuity left eye	Visual acuity corrected left eye	0.884	0.0001***
Visual acuity right eye	Visual acuity corrected right eye	0.871	0.0001***
Cholesterol	HDL	0.653	0.0001***
	LDL	0.646	0.0001***
TG	HDL	0.683	0.0001***
	TG/HDL	0.837	0.0001***
	Cholesterol/TG	-0.739	0.0001***
LDL	Cholesterol/HDL	0.763	0.0001***
	Cholesterol/TG	0.510	0.0001***
	LDL/HDL	0.839	0.0001***
Cholesterol/HDL	LDL/HDL	0.938	0.0001***
Cholesterol/TG	LDL/HDL	0.555	0.0001***
	TG/HDL	-0.768	0.0001***
Cholesterol/LDL	HDL/LDL	0.984	0.0001***
	TG/LDL	0.977	0.0001***
HDL/LDL	TG/LDL	0.937	0.0001***

***$p < 0.0001$

3.16.2 Correlation in AMD patient group

Correlation among different variables in AMD group is given in Table 3.10.

Table 3.10 Summary of correlation in AMD patients (r=correlation coefficient)

Variables	Correlated to	r value	p value
IL-6	IL-8	0.662	0.0001***
Visual acuity left eye	Visual acuity corrected left eye	0.921	0.0001***
Visual acuity corrected left eye	Visual acuity corrected right eye	0.514	0.0001***
Refractive equivalent left eye	Refractive equivalent right eye	0.543	0.0001***
Visual acuity right eye	Visual acuity corrected right eye	0.833	0.0001***
Cholesterol	TG	0.569	0.0001***
	HDL	0.644	0.0001***
	LDL	0.682	0.0001***
TG	HDL	0.640	0.0001***
	Cholesterol/LDL	0.584	0.0001***
	HDL/LDL	0.511	0.0001***
	TG/HDL	0.787	0.0001***
	TG/LDL	0.645	0.0001***
	Cholesterol/TG	-0.792	0.0001***
HDL	LDL/HDL	-0.540	0.0001***

***p< 0.0001 Continued --------

Variables	**Correlated to**	**r value**	**p value**
LDL	Cholesterol/HDL	0.711	0.0001***
	LDL/HDL	0.779	0.0001***
	Cholesterol/LDL	-0.669	0.0001***
	HDL/LDL	-0.722	0.0001***
	TG/LDL	-0.595	0.0001***
Cholesterol/HDL	LDL/HDL	0.954	0.0001***
	HDL/LDL	-0.576	0.0001***
Cholesterol/TG	LDL/HDL	0.509	0.0001***
	Cholesterol/LDL	-0.584	0.0001***
	HDL/LDL	-0.555	0.0001***
	TG/HDL	-0.759	0.0001***
	TG/LDL	-0.600	0.0001***
Cholesterol/LDL	HDL/LDL	0.988	0.0001***
	TG/LDL	0.986	0.0001***
	LDL/HDL	-0.636	0.0001***
LDL/HDL	HDL/LDL	-0.698	0.0001***
	TG/LDL	-0.553	0.0001***

***p< 0.0001 Continued --------

Variables	Correlated to	r value	pvalue
HDL/LDL	TG/LDL	0.949	0.0001***
Large drusen	Small drusen	0.773	0.0001***
Small drusen	CNV	0.582	0.0001***

***p< 0.0001

3.16.3 Correlation in different variables in males of AMD patient group

Detail of correlation among different parameters in AMD patients is given Table 3.11.

Table 3.11 Summary of correlation of parameters in male AMD Patient group

Variables	Correlated to	r value	pvalue
ApoE	VEGF	0.714	0.0001***
IL-6	IL-8	0.686	0.0001***
	VEGF	0.511	0.011*
Visual acuity left eye	Visual acuity corrected left eye	0.934	0.0001***
Visual acuity corrected left eye	Visual acuity right eye	0.524	0.0001***
Refractive equivalent left eye	Refractive equivalent right eye	0.575	0.0001***
Visual acuity right eye	Visual acuity right eye	0.848	0.0001***

***p< 0.0001 Continued --------

Variables	**Correlated to**	**r value**	**pvalue**
Cholesterol	TG	0.584	0.0001***
	HDL	0.615	0.0001***
	LDL	0.598	0.0001***
TG	HDL	0.671	0.0001***
	Cholesterol/LDL	0.685	0.0001***
	HDL/LDL	0.646	0.0001***
	TG/HDL	0.755	0.0001***
	TG/LDL	0.712	0.0001***
	Cholesterol/TG	-0.823	0.0001***
HDL	HDL/LDL	0.525	0.0001***
	Cholesterol/HDL	-0.562	0.011*
	LDL/HDL	-0.633	0.0001***
LDL	Cholesterol/HDL	0.719	0.0001***
	LDL/HDL	0.779	0.0001***
	Cholesterol/LDL	-0.630	0.0001***
	HDL/LDL	-0.687	0.0001***
	TG/LDL	-0.561	0.0001***
Cholesterol/HDL	LDL/HDL	0.964	0.0001***

***p< 0.0001 Continued --------

Variables	**Correlated to**	**r value**	**p value**
LDL/HDL	HDL/LDL	-0.644	0.0001***
AMD type	AMD pattern	-0.548	0.0001***
	CNV	-0.666	0.011*
Large drusen	Small drusen	0.791	0.0001***
Small drusen	CNV	0.600	0.0001***
Cholesterol/HDL	HDL/LDL	-0.514	0.0001***
Cholesterol/TG	Cholesterol/LDL	-0.552	0.0001***
	HDL/LDL	-0.543	0.0001***
	TG/HDL	-0.723	0.0001***
	TG/LDL	-0.552	0.0001***
Cholesterol/LDL	HDL/LDL	0.991	0.0001***
	TG/LDL	0.991	0.0001***
	LDL/HDL	-0.573	0.0001***

*** $p < 0.0001$; * $p < 0.05$

3.16.4 Correlation among different variables in females of AMD patient group

The detail of correlation among different parameters is given in Table 3.12.

Table 3.12 Summary of correlation of parameters in female AMD patients

Variables	Correlated to	r value	p value
HTRA1	Refractive equivalent right eye	0.550	0.015*
	GA	0.578	0.003*
IL-6	IL-8	0.601	0.003*
IL-8	CFH	0.544	0.029*
	IOP left eye	0.652	0.012*
CRP	Family history	0.506	0.014*
	Cholesterol	0.531	0.004*
	HDL	0.601	0.001*
IOP left eye	IOP right eye	0.778	0.0001***
Visual acuity left eye	Visual acuity corrected left eye	0.907	0.0001***
Visual acuity corrected left eye	Visual acuity corrected right eye	0.593	0.001**
Visual acuity right eye	Visual acuity corrected right eye	0.808	0.0001***
Cholesterol	TG	0.550	0.001*
	HDL	0.732	0.0001***

***p< 0.0001; ** p< 0.001; *p< 0.05 Continued --------

Variables	**Correlated to**	**r value**	**p value**
Cholesterol	LDL	0.796	0.0001***
TG	HDL	0.589	0.0001***
	TG/HDL	0.842	0.0001***
	TG/LDL	0.533	0.001*
	Cholesterol/TG	-0.735	0.0001***
LDL	Cholesterol/HDL	0.762	0.0001***
	Cholesterol/TG	0.503	0.003*
	LDL/HDL	0.831	0.0001***
	Cholesterol/LDL	-0.840	0.0001***
	HDL/LDL	-0.843	0.0001***
	TG/LDL	-0.789	0.0001***
Cholesterol/HDL	LDL/HDL	0.921	0.0001***
	Cholesterol/LDL	-0.738	0.0001***
	HDL/LDL	-0.807	0.0001***
	TG/LDL	-0.607	0.0001***
Cholesterol/TG	LDL/HDL	0.612	0.0001***
	Cholesterol/LDL	-0.722	0.0001***
	HDL/LDL	-0.609	0.0001***
	TG/HDL	-0.827	0.0001***

*** p< 0.0001; ** p< 0.001 Continued --------

Variables	**Correlated to**	**r value**	**p value**
Cholesterol/TG	TG/LDL	-0.829	0.0001***
Cholesterol/LDL	HDL/LDL	0.981	0.0001***
	TG/LDL	0.969	0.0001***
	LDL/HDL	-0.887	0.0001***
LDL/HDL	HDL/LDL	-0.886	0.0001***
	TG/LDL	-0.838	0.0001***
HDL/LDL	TG/LDL	0.903	0.0001***
TG/HDL	TG/LDL	0.608	0.0001***
AMD pattern	CNV	0.502	0.007*
RPE changes	Small drusen	0.596	0.001*
	Large drusen	0.680	0.0001***
Large drusen	Small drusen	0.876	0.0001***
Small drusen	CNV	0.543	0.002*

***p< 0.0001;**p< 0.001

3.16.5 Correlation in different variables in dry AMD patient group

The detail of correlation among different variables in dry AMD patient groups is given in Table 3.13.

Table 3.13 Summary of correlation in different parameters in dry AMD group

Variables	Correlated to	r value	p value
Leptin	Refractive equivalent right eye	0.566	0.022*
HTRA1	CRP	0.625	0.004*
	Age	0.586	0.008*
IL-6	IL-8	0.788	0.001*
	Age	0.535	0.033*
	Cholesterol	0.538	0.032*
IL-8	IOP	0.728	0.041*
	Cholesterol	0.605	0.017*
	LDL	0.564	0.029*
	Cholesterol/HDL	0.641	0.010*
CRP	TG	0.538	0.007*
	Cholesterol/LDL	0.596	0.002*
	HDL/LDL	0.580	0.003*
	TG/LDL	0.605	0.002*
	RPE changes	-0.590	0.003*
CFH	Refractive equivalent right eye	-0.603	0.029*

*p< 0.05 Continued -------

Variables	**Correlated to**	**r value**	**p value**
Family history	Refractive equivalent left eye	0.602	0.023*
	Refractive equivalent right eye	0.665	0.007*
IOP left eye	IOP right eye	0.762	0.001*
	AMD pattern	0.718	0.001*
	Visual acuity corrected right eye	-0.624	0.030*
IOP right eye	AMD pattern	0.571	0.021*
Visual acuity left eye	Visual acuity corrected left eye	0.833	0.0001***
	Visual acuity right eye	0.708	0.0001***
	Visual acuity corrected right eye	0.749	0.0001***
	Large drusen	0.503	0.014*
	Small drusen	0.503	0.014*
	CNV	0.610	0.002*
Visual acuity corrected left eye	Visual acuity right eye	0.676	0.002*
	Visual acuity corrected right eye	0.813	0.0001***
Refractive equivalent left eye	Refractive equivalent right eye	0.630	0.012*
Visual acuity right eye	Visual acuity corrected right eye	0.848	0.0001***

*** $p < 0.0001$; * $p < 0.05$ Continued ----

Variables	**Correlated to**	**r value**	**p value**
Cholesterol	HDL	0.685	0.0001***
	LDL	0.639	0.0001***
TG	HDL	0.700	0.0001***
	Cholesterol/LDL	0.808	0.0001***
	HDL/LDL	0.794	0.0001***
	TG/HDL	0.776	0.0001***
	TG/LDL	0.813	0.0001***
	Cholesterol/TG	-0.896	0.0001***
	LDL/HDL	-0.625	0.0001***
HDL	HDL/LDL	0.525	0.005**
	Cholesterol/HDL	-0.525	0.005**
	LDL/HDL	-0.527	0.005**
LDL	Cholesterol/HDL	0.765	0.0001***
	Cholesterol/TG	0.597	0.0001***
	LDL/HDL	0.852	0.0001***
	Cholesterol/LDL	-0.627	0.0001***
	HDL/LDL	-0.661	0.0001***
	TG/LDL	-0.588	0.001*
Cholesterol/HDL	LDL/HDL	0.932	0.0001***
	Cholesterol/LDL	-0.543	0.004*
Cholesterol/TG	LDL/HDL	0.744	0.0001***
	Cholesterol/LDL	-0.575	0.002*
	HDL/LDL	-0.576	0.002*

*** $p < 0.0001$; ** $p < 0.01$ Continued ----

Variables	**Correlated to**	**r value**	**p value**
Cholesterol/TG	TG/HDL	-0.839	0.0001***
	TG/LDL	-0.576	0.002*
Cholesterol/LDL	HDL/LDL	0.994	0.0001***
	TG/HDL	0.574	0.002*
	TG/LDL	0.995	0.0001***
	LDL/HDL	-0.695	0.0001***
LDL/HDL	HDL/LDL	-0.747	0.0001***
	TG/LDL	-0.637	0.0001***
HDL/LDL	TG/HDL	0.523	0.005*
	TG/LDL	0.978	0.0001***
TG/HDL	TG/LDL	0.617	0.001*
Large drusen	Small drusen	0.904	0.0001***

***$p < 0.0001$; *$p < 0.05$

3.16.6 Correlation in different variables in wet AMD patient group

The details of correlation among different parameters in patients with wet AMD are given in Table 3.14

Table 3.14 Summary of correlation of parameters in Wet AMD patients

Variables	Correlated to	r value	p value
ApoE	VEGF	0.584	0.0001***
IL-8	Small drusen	-0.505	0.001*
CFH	GA	-0.513	0.0001***
Visual acuity left eye	Visual acuity corrected left eye	0.954	0.0001***
Refractive equivalent left eye	Refractive equivalent right eye	0.595	0.0001***
Visual acuity right eye	Visual acuity corrected right eye	0.831	0.0001***
Cholesterol	TG	0.634	0.0001***
	HDL	0.636	0.0001***
	LDL	0.701	0.0001***
TG	HDL	0.613	0.0001***
	TG/HDL	0.793	0.0001***
	TG/LDL	0.619	0.0001***
	Cholesterol/TG	-0.787	0.0001***
HDL	LDL/HDL	-0.547	0.0001***
LDL	Cholesterol/HDL	0.714	0.0001***

***p< 0.0001; *p< 0.05 Continued ----

Variables	**Correlated to**	**r value**	**p value**
LDL	LDL/HDL	0.760	0.0001***
	Cholesterol/LDL	-0.775	0.0001***
	HDL/LDL	-0.806	0.0001***
	TG/LDL	-0.697	0.0001***
Cholesterol/HDL	LDL/HDL	0.960	0.0001***
	Cholesterol/LDL	-0.579	0.0001***
	HDL/LDL	-0.655	0.0001***
Cholesterol/TG	Cholesterol/LDL	-0.677	0.0001***
	HDL/LDL	-0.582	0.0001***
	TG/HDL	-0.734	0.0001***
	TG/LDL	-0.772	0.0001***
Cholesterol/LDL	HDL/LDL	0.985	0.0001***
	TG/LDL	0.974	0.0001***
	LDL/HDL	-0.725	0.0001***
LDL/HDL	HDL/LDL	-0.760	0.0001***
	TG/LDL	-0.646	0.0001***
HDL/LDL	TG/LDL	0.921	0.0001***
RPE changes	Large drusen	0.551	0.0001***
Large drusen	Small drusen	0.551	0.0001***
Small drusen	CNV	0.584	0.0001***

*** p< 0.0001 Continued ----

3.17 Logistic Regression

The level of significance for lipid profile in Omnibus tests was <0.0001 showing that the model best fitted for prediction of risk factors. The Cox & Snell R square value was 0.187 and Nagelkerke R Square was 0.250 showed that there was a strong effect of parameters on the disease. The classification table showed percentage correct equivalent to 89.4. The Hosmer and Lemeshow Test showed a significance level 0.999 which predicted the fitness of model (Table 3.15 A, B, C & D).

The regression coefficient Exp(B) for age was 1.103 ($p < 0.0001$), for IL-8 it was 1.483 ($p < 0.005$), for leptin 712.697 ($p < 0.038$) and for CRP 1.807 ($p < 0.007$). No other variable including gender and lipid profile showed any significant change related to the disease condition (Table 3.16). The risk of AMD increased with increasing age, elevated serum IL-8 and CRP levels and reduced leptin levels.

Table 3.15 A) Omnibus Tests of Model Coefficients

		Chi-square	Sig.
Step 1	Step	38.305	0.000
	Block	38.305	0.000
	Model	38.305	0.000

B) Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	217.245	0.187	0.250

C) Classification Table

Observed			Predicted		
			1		Percentage
			control	AMD	Correct
Step 1	1	control	30	4	88.2
		AMD	3	29	90.6
		Overall Percentage			89.4

D) Hosmer and Lemeshow Test

Step	**Chi-square**	**df**	**Sig.**
1	0.614	7	0.999

Table 3.16 Variables in Equation

Variables	B	S.E.	Wald	Sig.	Exp (B)
Gender	2.151	1.522	1.997	0.158	8.597
Age	0.098	0.020	23.106	0.0001***	1.103
IL-6	0.067	0.088	.585	0.444	1.070
IL-8	0.394	0.141	7.856	0.005**	1.483
ApoE	0.163	0.136	1.432	0.231	1.177
Leptin	6.569	3.165	4.307	0.038*	712.697
HTRA1	0.183	0.654	.079	0.779	1.201
VEGF	-0.352	0.230	2.339	0.126	.703
CRP	0.591	0.219	7.281	0.007**	1.807
CFH	-0.002	0.004	0.297	0.586	.998
Cholesterol	-0.025	0.037	0.477	0.490	.975
TG	0.005	0.008	0.509	0.476	1.005
HDL	0.053	0.042	1.657	0.198	1.055
LDL	0.028	0.037	0.561	0.454	1.028

* $p < 0.05$; ** $p < 0.01$; *** $P < 0.0001$

3.18 Transformation of Data

Box and Cox transformation provided an algorithm through which the optimal value of the transformation parameter λ was selected by the method of maximum likelihood. The analysis of variance for λ= 0.175 that yield the lowest error sums of squares was then used for hypothesis testing.

3.19 Multivariate Analyses of Variance

Multivariate analyses of variance (M-ANOVA) showed that serum levels of IL-6, IL-8, VEGF and CRP were significantly elevated in AMD patients ($p< 0.0001$; value of Wilk's lambda = 0.018; F= 466.55). The transformed data showed that serum concentration of IL-8 ($p< 0.015$) and VEGF ($p< 0.0108$) was significantly higher in AMD patients as compared to normal controls. However, serum ApoE concentration was significantly lowered in AMD patients as compared to that in control subjects ($p< 0.0032$) (Fig. 3.18-3.25; Table 3.17).

The levels of all these factors were significantly associated with type of AMD that is wet or dry ($p< 0.0001$; value of Wilk's lambda = 0.126; F= 53.455). The serum levels of VEGF were highly significantly raised in patients with wet AMD as compared to those with dry type of AMD ($p < 0.0001$) (Fig. 3.18-3.25; Table 3.18).

There was no significant difference in the serum levels of inflammatory factors with respect to age groups ($p > 0.075$; Wilk's lambda = 0.975; F=1.917) (Table 3.19-3.20). These levels were not found to be associated with the gender ($p < 0.843$; value of Wilk's lambda = 0.999; F= 0.170) (Table 3.21).

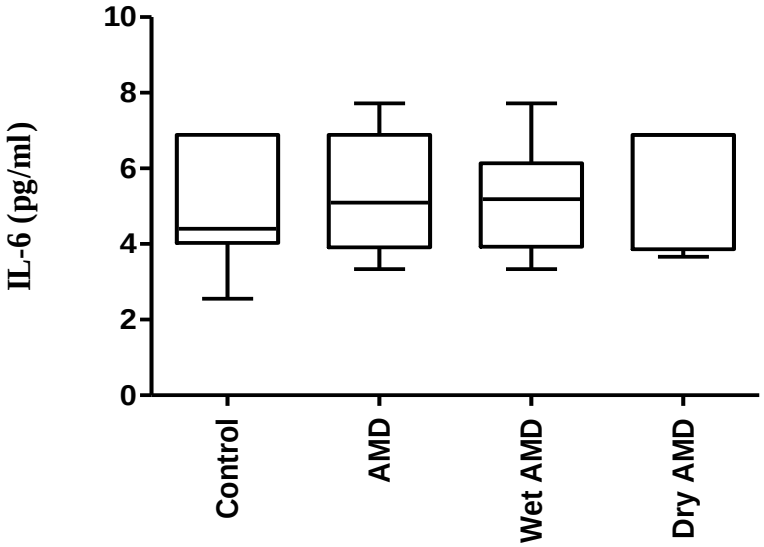


Fig. 3.18 Serum IL-6 concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values.

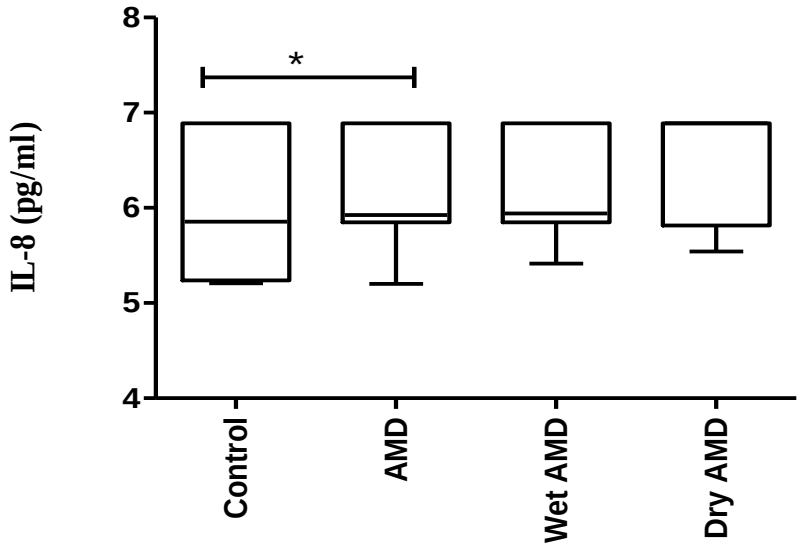


Fig. 3.19 Serum IL-8 concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{*}p < 0.05$

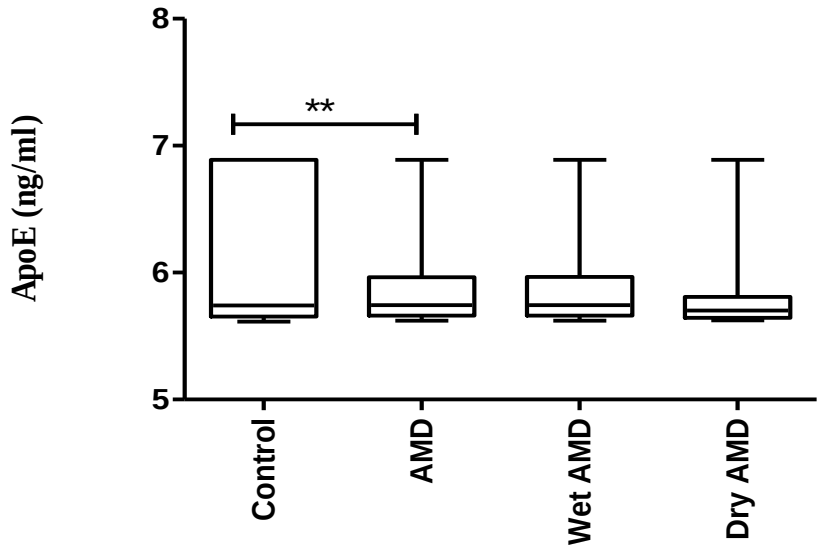


Fig. 3.20 Serum ApoE concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{**}p < 0.01$

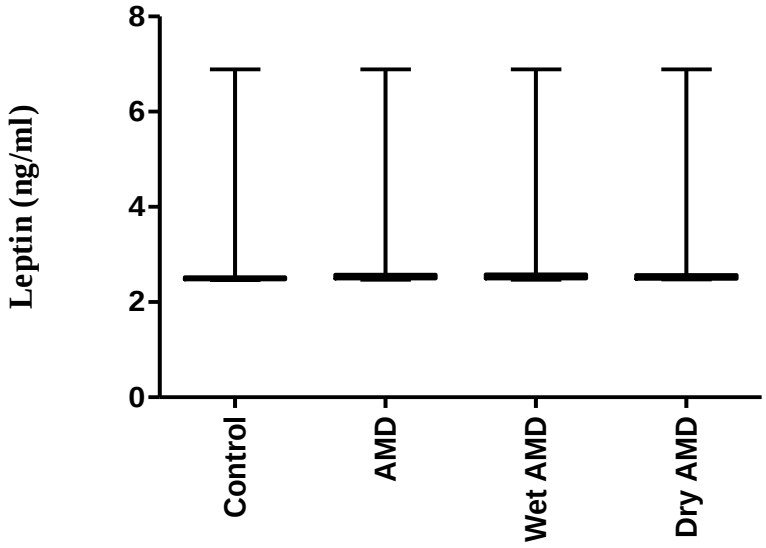


Fig. 3.21 Serum leptin concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values.

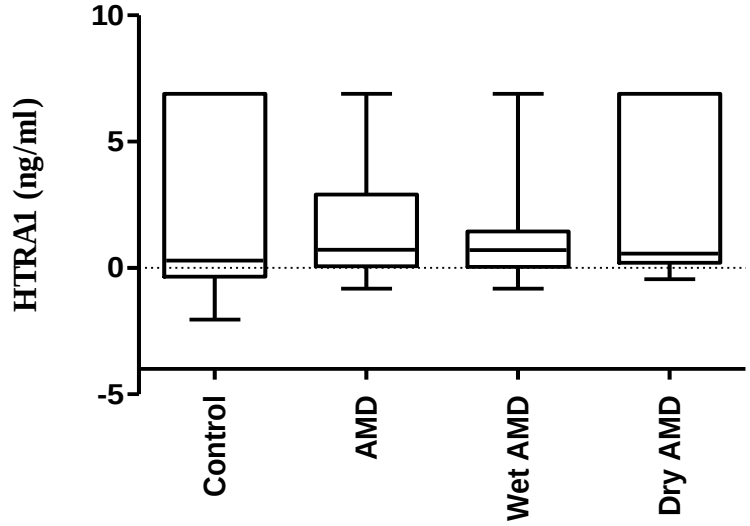


Fig. 3.22 Serum HTRA1 concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values.

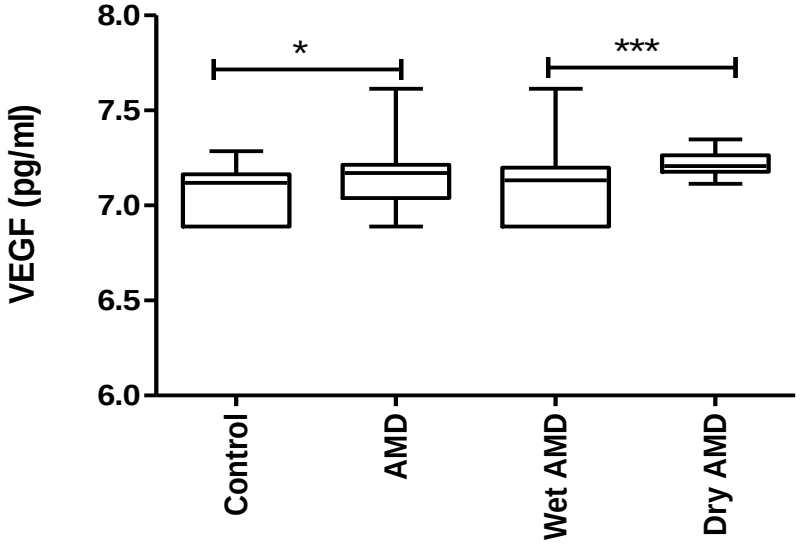


Fig. 3.23 Serum VEGF concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values. $^{*}p < 0.05$; $^{***}p < 0.0001$

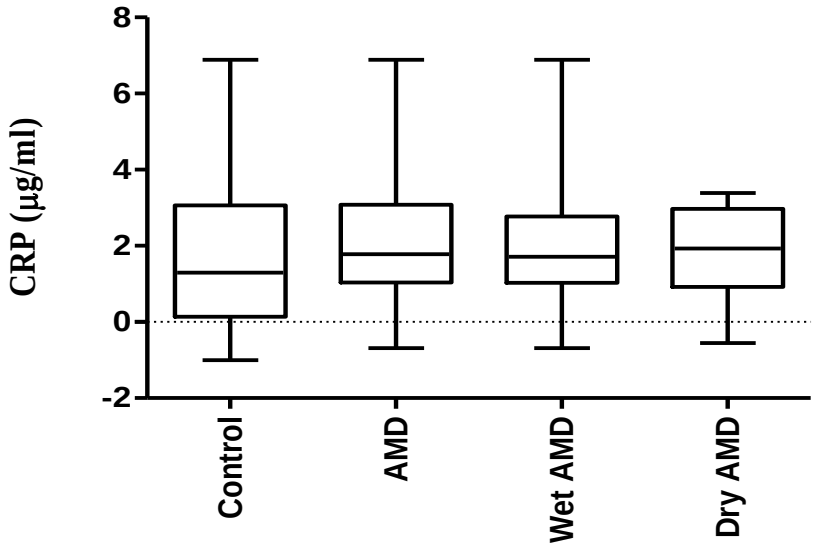


Fig. 3.24 Serum CRP concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values.

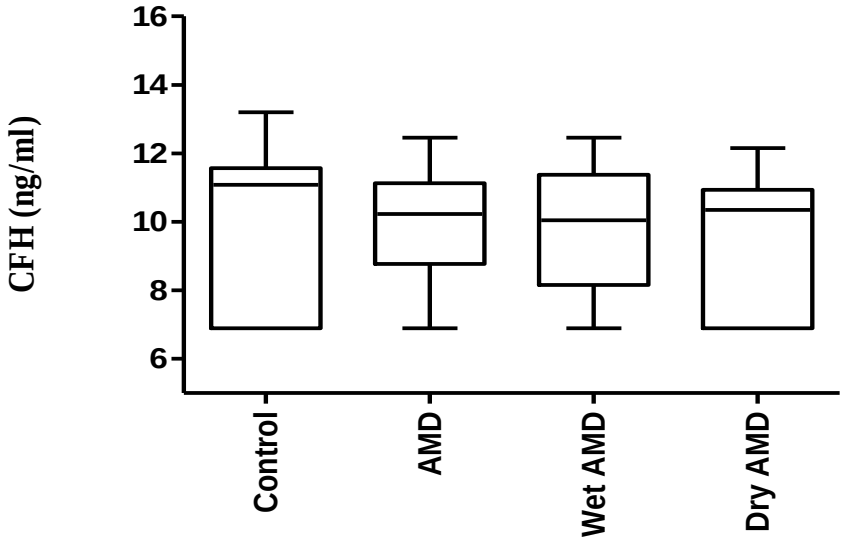


Fig. 3.25 Serum CFH concentrations in transformed data of control and AMD patients. Bars represent median value and whiskers represent minimum and maximum values.

Table 3.17 Comparison of serum levels of IL-6, IL-8, ApoE, leptin. HTRA1, VEGF, CRP and CFH in control sujects and AMD patients in transformed data (mean± Standard error of mean)

Parameters	Control (n=100)	AMD (n=90)	T value	95% Confidence Interval of the Difference		
				Lower	Upper	Sig.
IL6 (pg/ml)	4.97 ± 0.13	5.25 ± 0.13	1.481	-0.6439	0.08973	0.1404
IL8 (pg/ml)	5.95 ± 0.06	6.23 ± 0.05	3.227	-0.4476	-0.1093	0.0015*↑
ApoE (ng/ml)	6.13 ± 0.06	5.92 ± 0.04	2.989	0.07338	0.3528	0.0032*↓
Leptin (ng/ml)	2.57 ± 0.06	2.83 ± 0.11	1.927	-0.5118	0.004323	0.0561
HTRA1 (ng/ml)	2.15 ± 0.33	2.00 ± 0.30	0.3377	-0.7238	1.025	0.7360
VEGF (pg/ml)	7.07 ± 0.01	7.12 ± 0.01	2.576	-0.09457	-0.01284	0.0108*↑
CRP (μg/ml)	2.27 ± 0.28	2.32 ± 0.21	0.1287	-0.7258	0.6364	0.8978
CFH (ng/ml)	10.08 ± 0.22	9.79 ± 0.19	0.9883	-0.2882	0.8743	0.3243

*$p < 0.05$; ↑: increased from control; ↓: decreased from contro

Table 3.18 Comparison of serum levels of IL-6, IL-8, ApoE, leptin. HTRA1, VEGF, CRP and CFH in patients with wet and dry type of AMD in transformed data (mean± Standard error of mean)

Parameters	Wet AMD (n=63)	Dry AMD (n=27)	T value	95% Confidence Interval of the Difference		
				Lower	Upper	Sig.
IL6 (pg/ml)	5.21 ± 0.16	5.47 ± 0.29	0.7925	-0.9353	0.4080	0.4325
IL8 (pg/ml)	6.21 ± 0.07	6.39 ± 0.11	1.455	-0.4443	0.07183	0.1527
ApoE (ng/ml)	5.89 ± 0.04	5.78 ± 0.05	1.472	-0.03459	0.2274	0.1462
Leptin (ng/ml)	2.92 ± 0.16	2.69 ± 0.16	0.9629	-0.2385	0.6829	0.3389
HTRA1 (ng/ml)	1.87 ± 0.37	2.32 ± 0.59	0.6520	-1.853	0.9465	0.5176
VEGF (pg/ml)	7.10 ± 0.02	7.22 ± 0.01	5.322	-0.1689	-0.07685	0.0001***↑
CRP (µg/ml)	2.04 ± 0.23	1.83 ± 0.23	0.6406	-0.4440	0.8633	0.5239
CFH (ng/ml)	9.83 ± 0.24	9.63 ± 0.34	0.4733	-0.6438	1.041	0.6379

$p < 0.0001$; ↑: increased from control

Table 3.19 Comparison of transformed values serum IL-6 (pg/ml), IL-8 (pg/ml), ApoE(ng/ml) and Leptin (ng/ml) with respect to age groups in control subjects and AMD patientsin transformed data (mean± Standard error of mean)

Age group	Group	n	IL-6	IL-8	ApoE	Leptin
50-59	Control	45	4.78 ± 0.41	5.98 ± 0.41	5.79 ± 0.41	2.56 ± 0.41
	AMD	15	5.66 ± 0.40	6.58 ± 0.40	5.80 ± 0.40	2.57 ± 0.40
60-69	Control	30	5.56 ± 0.28	6.14 ± 0.28	5.81 ± 0.28	2.71 ± 0.28
	AMD	33	4.86 ± 0.27	6.01 ± 0.27	6.09 ± 0.27	3.31± 0.27
70-79	Control	21	4.89 ± 0.26	5.73 ± 0.26	5.89 ± 0.26	3.02 ± 0.26
	AMD	29	5.54 ± 0.25	6.35 ± 0.25	5.71 ± 0.26	2.53 ± 0.26
80-89	Control	4	5.08 ± 0.47	6.23 ± 0.65	5.97 ± 0.65	3.61 ± 0.65
	AMD	10	4.58 ± 0.63	6.02 ± 0.63	5.71 ± 0.63	2.51 ± 0.63
90-99	Control	0	None	None	None	None
	AMD	2	7.73 ± 0.41	6.70 ± 0.19	5.89 ± 0.08	2.55 ± 0.07
≥100	Control	0	None	None	None	None
	AMD	1	5.44	6.89	6.63	2.50

None: no subject in these age groups could be found in the control group

Table 3.20 Comparison of serum transformed values of HTRA1 (pg/ml), VEGF (pg/ml), CRP (ng/ml) and CFH (ng/ml) with respect to age groups in control subjects and AMD patientsin transformed data (mean± Standard error of mean)

Age group	Group	n	HTRA1	VEGF	CRP	CFH
50-59	Control	45	-0.27±0.41	7.12± 0.41	0.67±0.41	11.49± 0.41
	AMD	15	0.64±0.40	7.15± 0.40	1.25± 0.40	10.32± 0.40
60-69	Control	30	0.15± 0.28	7.14± 0.28	0.88± 0.30	11.07± 0.27
	AMD	33	2.74± 0.27	7.08± 0.27	2.48± 0.27	9.54± 0.26
70-79	Control	21	0.84± 0.26	7.14± 0.26	1.18± 0.26	11.06± 0.26
	AMD	29	0.54± 0.25	7.20± 0.25	1.87± 0.25	10.35± 0.25
80-89	Control	4	2.08± 0.65	7.08± 0.65	2.61± 0.65	11.06± 0.65
	AMD	10	0.56± 0.63	7.23± 0.63	2.05± 0.63	10.71± 0.63
90-99	Control	0	None	None	None	None
	AMD	2	3.88±3.0	7.03±0.14	-0.18±0.37	9.03±2.14
≥100	Control	0	NIL	NIL	NIL	NIL
	AMD	1	None	None	None	None

None control subject in these age groups could be found in the control group

Table 3.21 Association of gender with serum levels of IL-6 (pg/ml), IL-8 (pg/ml), ApoE (ng/ml) and Leptin (ng/ml), HTRA1 (pg/ml), VEGF (pg/ml), CRP (ng/ml) and CFH (ng/ml) in control subjects and AMD patients in transformed data. (mean± Standard error of mean)

Protein	Gender	Control	AMD
IL-6	Female	4.93±0.34	5.13±0.27
	Male	5.04±0.26	5.40±0.20
IL-8	Female	6.05±0.34	6.24±0.27
	Male	5.92±0.26	6.26±0.20
ApoE	Female	6.15±0.34	5.98±0.27
	Male	6.07±0.26	5.87±0.20
Leptin	Female	4.08±0.34	2.98±0.27
	Male	3.63±0.26	2.76±0.20
HTRA1	Female	2.35±0.34	1.58±0.27
	Male	1.73±0.26	2.14±0.20
VEGF	Female	7.05±0.34	7.13±0.27
	Male	7.09±0.26	7.12±0.20
CRP	Female	1.97±0.34	2.14±0.27
	Male	2.06±0.26	2.38±0.20
CFH	Female	9.89±0.34	10.06±0.27
	Male	10.36±0.26	9.67±0.20

Discussion

Average life expectancy has though increased upto 80 years in developed countries as a result of advancement in medical sciences yet the quality of life is highly compromised due to age related diseases. Age related macular degeneration (AMD) is one of such pathologies that are characterized by central visual loss that occurs as a result of degenerative and neovascular changes in the macula. AMD is emerging as a major public health burden because the physical impairment in the form of blindness badly affects the geriatric population and their families (Gehrs et al., 2006).

In the present study, angiogenic factors and inflammatory markers were determined in the sera collected from AMD patients and control subjects. Total lipid profile in AMD subjects was compared with the control subjects. Serum HDL levels as well as HDL/LDL ratio were significantly high in AMD patients as compared to control subjects, but overall, no significant change was observed in lipid profile in male patients when compared with normal male subjects. In contrast, serum cholesterol levels were significantly higher in females with AMD as compared to normal female subjects. Elevated serum cholesterol is positively correlated with the incidence of geographic atrophy (Tomany et al., 2004) and is a major component of drusen, one of the basic symptoms of AMD.It has been reported that total serum cholesterol is associated with elevated incidence of GA but decreased frequency of CNV in AMD patients. However, total cholesterol was not found to be associated with AMD prevalence (Smith et al., 2001).

A few studies have reported that HDL levels are increased in AMD patients but no explanation has been provided so far (Klein et al., 1993; Hyman et al., 2000). It has been reported that raised HDL levels were associated with increased risk of AMD, although total cholesterol levels were not related to AMD risk. It is suggested that ApoE genotype can modify this effect. Although, a previous study suggests that cholesterol transporter Apolipoprotein E (ApoE) gene is associated with AMD that affects cholesterol levels (vanLeeuwen et al., 2004).

Currently, serum ApoE levels elevated in AMD patients as compared to normal age matched control subjects. Increased ApoE levels induce IL-6 in mononuclear phagocytes (MPs) through the activation of TLR2-CD14 dependent

innate immunity receptor cluster. In turn RPE FasL expression is repressed by IL-6 that also prolongs subretinal MP survival. MP accumulation has been observed in retina of Cx3cr knock out mice. A study on these knock out mice suggest that ApoE plays a pro inflammatory role in subretinal space in AMD (Levy et al., 2015).

In the present study, significantly elevated serum concentration of VEGF was observed in the patients with AMD as compared to normal subjects. Clinical as well as experimental evidence suggests that VEGF is involved in the occurrence of CNV (Witmer et al., 2003). Surgically excised CNV membrane shows expression of VEGF. The patients with exudative type of AMD have significantly increased VEGF levels in vitreous humour as compared to healthy controls. In addition, VEGF blocking agents cure laser-induced CNV (Nakajima et al., 2014). The elevation of pro angiogenic factors like VEGF is the result of tissue hypoxia that is mediated by the family of transcription factors known as hypoxia inducible factors (HIFs). Therefore, it is suggested that hypoxia is a major stimulus for the submacular wound healing. In this scenario, CNV is another process that leads to HIF synthesis. The activation of HIFs is a key signal for hyperproduction of VEGF with subsequent anomalous neovascular growth (Campa et al., 2010).

Serum HTRA1 concentration showed significantincrease in the currently studied AMD patients as compared to age matched normal subjects. DeWan et al. (2006) has provided evidence that single nucleotide polymorphism (SNP) in HTRA1 promotor region is a genetic risk factor for wet AMD. The Chinese individuals with risk associated genotype had ten times more likelihood of developing wet AMD than those with wild type genotype. This risk related allele results into elevated expression of HTRA1 mRNA and protein in lymphocytes and RPE in Caucasian patients with wet AMD. Strong immunolabelling with HTRA1 antibodies has also been reported in drusen from AMD eyes. Therefore, it is suggested that HTRA1 plays a role in AMD pathogenesis through inflammatory pathway (Yang et al. 2006).

Presently, serum interleukins i.e., IL-6 and IL-8 levels were significantly elevated in AMD patients as compared to control subjects. Elevated IL-6 and CRP levels are related to AMD progression from early or intermediate stage to late stage. In addition, this shows that AMD is mediated through inflammatory and immune-related mechanisms (Seddon et al., 2005). The pro angiogeinc role of IL-6 though

VEGF-A upregulation in context of tumor angiogenesis is already well established. The signalling of IL-6 promotes RPE degeneration through lipopolysaccharide stimulation (Ambati et al., 2013). Thus, IL-6 performs dual functions in AMD pathology, one by promoting angiogenesis and other through RPE degeneration.

IL-8 is another mediator of angiogenesis that contributes to plaque formation. IL-8 production increases in several cell types under oxidative stress. The reactive oxygen species (ROS) contribute to IL-8 production through transduction pathways like mitogen activated protein kinases. Oxidative stress also triggers inflammatory processes as a result of ROS production. Therefore, it is suggested that in the current AMD patients, oxidative stress resulted into elevated IL-8 production due to oxidative inactivation of proteasome as studied in cultured RPE by Ricci et al(2013).

The concentration of CRP was significantly high in currently studied AMD patients as compared to that in normal control subjects. CRP is one of the physiological markers for systemic inflammation. Elevated CRP levels are related to the advanced stage of AMD as stated by a multicentre randomized study on a trail of antioxidant supplements (Age-Related Eye Disease Study Research Group, 2005). A positive relationship between the systemic inflammatory markers including IL-6 and CRP has been reported by Seddon et al, (2005).

Currently, serum leptin levels were significantly low in AMD patients as compared to control subjects. Leptin in involved in lipid deposition in macular lesions, especially in late AMD. Under normal conditions, leptin decreases the rate of triglyceride and fatty acid synthesis and inhibits intracellular accumulation of lipids. Moreover, it directly affects lipoprotein metabolism or lipase activities. Therefore, it is suggested that under conditions of reduced serum leptin levels, fatty acids synthesis would increase due to inhibition of mitochondrial uptake and oxidationas observed in current study (Spaide et al., 1999). The high level of polyunsaturated fatty acids in photoreceptor-RPE complex renders macula a highly susceptible site for oxidative damage and lipid peroxidation due to its near-arterial oxygen levels. With advancing age, accumulation of lipids and cholesterol esters within Bruch's membrane leads to accumulation of increased amount of peroxidized lipids and drusen formation (Haimovici et al., 2001).

In addition, reduced leptin levels in serum of patients with AMD results into the loss of lipidostatic function at cellular level and increased intracellular fatty acid accumulation leading to the lesions found in AMD patients (Evereklioglu et al., 2003). To confirm the plausible role of leptin in AMD further studies with estimation of vitreous leptin levels are required. It is suggested that increased lipidswith reduced leptin levels are one the risk factors in the aetiology of AMD.

In the present study, CFH levels in serum of AMD patients were significantly reduced as compared to normal subjects. Similar were the findings in a study on North Indian population by Sharma et al (2013). Another study by Silva et al. (2012) also observed reduced levels of complementary factor H in female subjects affected by AMD. CFH is expressed by the ocular tissues including RPE and choroid in human and mouse (Mandal and Ayyagari, 2006; Skerka et al., 2007). Any change in CFH expression may lead to activation of complement system that generates further events leading to cellular damage in RPE, drusen formation and ultimately the visual loss (Hageman et al., 2006). CFH, CRP and other complement proteins have been detected in the drusen isolated from the macular region, the RPE and around the choroid capillaries (Issa et al., 2011).

The activation of complement system in addition to hypoxia, together with inflammatory process leads to irregularities in pro/antiangiogeic balance. RPE cells overexpress proangiogenic growth factors leading to CNV (Nita et al., 2014). The current study provides evidence that AMD is a chronic low grade inflammatory disease induced by oxidative stress. Therefore, it is suggested that usage of therapies that focus on inhibition of chronic inflammation to treat late AMD will open new avenues.

CHAPTER # 4

Analyses of Gene Polymorphism

Introduction

Evidences have been provided by previous studies that strong genetic components can influence the occurrence of age related macular degeneration (AMD). The genetic studies on AMD are inherently difficult due to nature of the disease. The symptoms of the disease appear late in life due to which only one generation in the appropriate age is available while the parents of the patients are usually deceased and the children are too young to be affected. There is heterogeneity in the phenotypes of AMD symptoms that makes it an even challenging pathology. If all stages of AMD are studied together in an analysis along with different sets of genes responsible for different phenotypes, then it is difficult to pin point any one gene or region responsible for the disease. If the analysis is restricted to any one region or specific form of AMD, the sample size may not be sufficient to detect small or moderate effects. In addition, as AMD has complex inheritance patterns, it is very difficult to discover susceptibility gene in each individual due to multiple genetic and environmental effects and interactions. However, investigations through genetic epidemiological methods have contributed to progress in AMD genetic research (Haddad et al., 2006).

The rationale for determining genetic factors that play role in AMD is to improve knowledge regarding the AMD pathology and options for prevention and treatment so that the quality of life of the affected persons be improved. The understanding of susceptible genes in AMD pathology will also help to investigate the biological processes involved in the development and progression of AMD. The development in functional knowledge and improved diagnosis will ultimately lead to the development of effective therapies for each subtype of AMD. Moreover, development of new therapeutic measures is likely to develop preventive treatments using the current information about pathogenesis of the disease.

Role of certain gene loci in AMD has been investigated over the years. More than 30 risk loci have been discovered by Genome-Wide Association Studies (GWAS) that include 1q25-31, 9p13, 9p24, 10q26, 15q21 and 17q25. These loci are associated with several candidate genes i.e., CFH, C3, C2-CFB, CFI, HTRA1/ ARMS2, CETP, TIMP3, LIPC, VEGFA, COL10A1, TNFRSF10A and ApoE (Kumar and Fu, 2014).

Seddon and co-workers (2005) carried out population based twin study including both monozygotic/dizygotic and concordant/discordant sibling pairs. The study suggested that both hereditary as well as environmental factors contribute in AMD pathology. The heritability estimates for AMD ranged from 46% to 71%. There are certain epigenetic mechanisms like behavioural and nutritional factors that interact with susceptible genes leading to the pathology (Seddon et al., 2011). Genome wide association studies provide a rationale to study AMD-related gene variants, despite the unavoidable efforts required to plan genetic analyses of a complex disease with late onset (Parmeggiani et al., 2012).

It appears that genetic findings are different among different study populations and investigators. Single nucleotide polymorphism (SNP) is one of the most common expressions of genetic variation. A large number of studies have been performed to assess the association of SNPs with various diseases. It is explained partly by the fact that SNP can vary in minor allele frequency among different ethnic populations (Bai et al., 2008). SNPs serve as markers for a particular gene region and do not represent the actual disease contributing mutations. The associations often result from linkage disequilibrium or non-random cosegregation with the causal mutations (Miller, 2013).

4.1 Cytokine Genes

The variation in cytokine levels are influenced by environmental and genetic factors. Variations in genes of cytokines result into alteration in structure and expression of a cytokines leading to a number of chronic diseases and enhanced risk of infection (Smith et al., 2009). The genetic polymorphisms in different interleukin (IL) genes (such as IL-6 and IL-8) are known to modify the transcription and production of ILs (Tsai et al., 2008).

IL-8 is a member of CXC chemokine family produced by macrophages, monocytes, neutrophils, fibroblasts and keratinocytes (Rollins, 1997). It has been suggested that IL-8 is a sensitive serological marker to monitor the progress of several immune related diseases like recurrent aphthous ulcer (Sur et al., 2004) and Behcet's disease (Gür-Toy et al., 2005). It is an important angiogenesis mediator and contributes to plaque formation due to its angiogenic properties (Simonini et al., 2000).

During systemic inflammation endothelial cells, fibroblasts and monocytes/macrophages produce IL-6 (Heinrich et al., 1990) that effects on the sites other than its origin (Fernandez-Real et al., 2001). In humans, gene of IL-6 is located on shorter arm of chromosome number 7p15-p21. The gene consists of five exons and four introns (Fig 4.1) (Jeon et al., 2010). Currently three SNPs for IL-6 gene rs1800795; rs1800796 and rs1800797 were studied. These SNPs were in weak linkage disequilibrium as D´ = 1; r^2= 0.034 for rs1800797 and rs1800796; D´ = 1; r^2 = 0.035 for rs1800796 and rs1800795; D´ = 1; r^2 = 0.238 for rs1800797 and rs1800795 (https://caprica.genetics.kcl.ac.uk/~ilori/ld_ calculator_action.php).

The gene of interleukin 8 (IL-8) is located at chromosome 4q 13-21. It consists of four exons, three introns, and the proximal promoter region (Fig 4.2) (Tsai et al., 2008). The three SNPs studied for the currently for IL-8 gene were rs4073; rs2227306 and rs2227543. These were in weak linkage disequilibrium as D´ = -1; r^2= 0.88 for rs4073 and rs2227306; D´= -0.99; r^2= 0.89 for rs4073 and rs2227543; D´= 0.99; r^2= 0.97for rs2227306 and rs2227543 (https://caprica.genetics.kcl.ac.uk/~ilori/ld_ calculator_action.php). Due to its inflammatory properties, IL-8 represents a gene that can be linked to AMD development and progression. The variations in IL-8 gene need to be investigated to understand its biological role in the pathogenesis of disease (Ricci et al., 2013)

4.2 Vascular Endothelial Growth Factor (VEGF)

VEGF has angiogenic characteristics as it induces vascular leakage and inflammation by enhancing production and permeability of capillary endothelial cells (Ferrara et al., 2003). The VEGF-A gene is located on chromosome 6p21.3 and contains eight exons and seven introns (Fig 4.3). The two SNPs considered currently were rs699947 and rs3025039. There was strong linkage equilibrium in the two SNPs.For rs699947 and rs3025039 D´ = -0.02; r^2= $5.34x10^{-5}$(https://caprica.genetics.kcl.ac. uk/~ilori/ld_ calculator_action.php). Several single-nucleotide polymorphisms (SNPs) have been identified in the VEGF-A gene and are believed to have functional activity. Genetic variability of the VEGF-A gene may thus have an important role in determining and/or modifying the development and progression of AMD and the response to anti-VEGF therapy. Over the past decade, a considerable number of epidemiological studies have focused on the association

between VEGF-A polymorphisms and AMD susceptibility. However, these studies were limited by small or moderate sample sizes (Huang et al., 2013).

4.3 C-Reactive Protein (CRP) Gene

CRP was discovered back in 1930 by Tillet and Francis during the studies on patients with acute pneumonia. CRP is in fact an inflammatory marker as it is shown to be involved in pathogenesis and progression of AMD (Lelubreet al., 2013). CRP gene has been mapped to the proximal arm of chromosome 1 in 1q23.2 regions (Fig 4.4) (Whitehead et al., 1983). There was strong linkage equilibrium in the two SNPs considered for the current study. For rs1205 and rs1130864 D´ = -1; r^2= 0.21(https://caprica.genetics.kcl.ac.uk/~ilori/ld_calculator_action.php). The regulation of CRP expression occurs mostly at transcriptional level with IL-6 being the main inducer for its production (Kushner et al., 1995). Difference in serum CRP levels is associated with gene polymorphism (Hage et al., 2007).

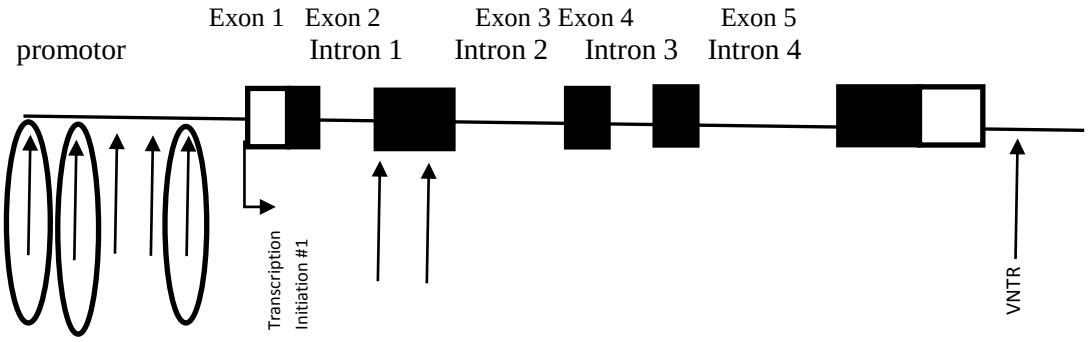


Fig 4.1 Schematic representation of IL-6 gene showing position of studied SNPs i.e., -174(rs1800795); -572(rs1800796); -597(rs1800797) (adapted and modified from Jeon et al., 2010)

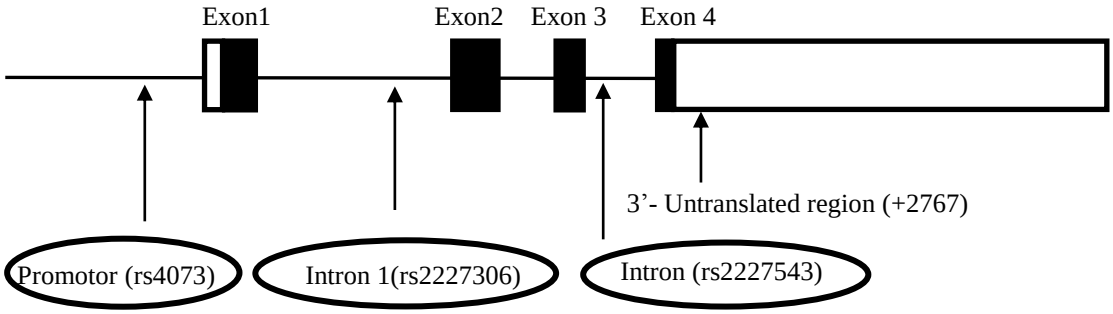


Fig 4.2 IL-8 gene showing position of studied SNPs (Adapted and modified from Tsai et al., 2008)

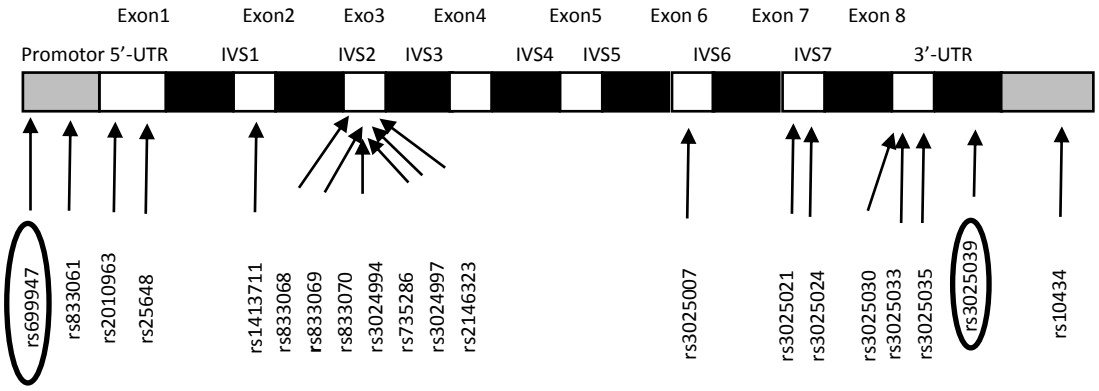


Fig. 4.3 VEGF-Agene polymorphisms studied currently. IVS: intervening sequence (Adapted and modified from Huang et al., 2013)

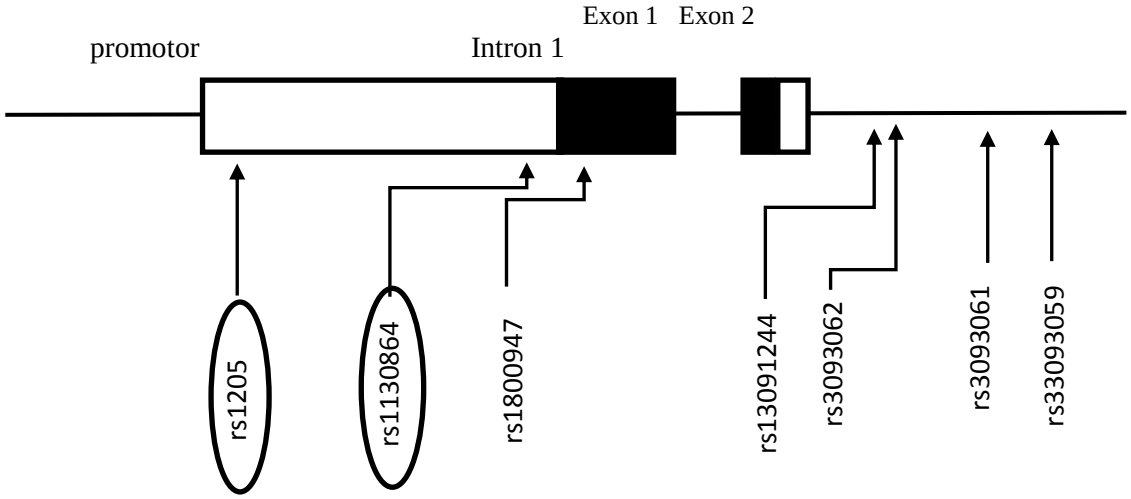


Fig. 4.4 Polymorphism in CRP gene showing studied SNPs (Adapted and modified from Rhodes et al., 2011)

Materials and Methods

4.4 Extraction of DNA

Genomic DNA was extracted from about 5 ml of blood of AMD patients and control subjects collected in ACD vacutainer (BD Vacutainer, USA) that contained 2.5 ml acid citrate dextrose solution and the final volume was 7.5 ml. The standard organic method was followed for the DNA extraction that using two organic chemicals that is phenol and chloroform (Sambrook and Russell, 2001). The method was slightly modified as per requirement. Kit method for DNA isolation (QIAamp DNA Mini, Qiagen Inc, USA) was used for the samples which were either less in quantity or somewhat clotted. Three days were given to each batch (20 samples) of blood samples to get reliable and large quantity of DNA.

4.4.1 DNA extraction through basic organic method

The detail of solution preparation is given in Appendix II. The procedure was as follows:

Day 1

The blood sample was transferred to a 50 ml centrifuge tube (Axygen Inc. USA) and three times cell lysis buffer ($KHCO_3$, NH_4Cl and 0.5 M EDTA) was added to it. The tubes were placed on ice for 30 minutes. The samples were centrifuged at 1200 rpm for 10 minutes at 4℃ in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130, Germany). The supernatant was then discarded and pellet at the base was re-suspended in the tube. If the pellet was reddish in color then further 10 ml of lysis buffer was added and the tubes were centrifuged again at 1200 rpm at 4℃ for 10 minutes. Then supernatant was discarded and the pellet was re-suspended, then 4.75 ml of STE and 250µl of 10% SDS was added to pallet. Ten micro litres (20 mg/ml) of proteinase K (Fermentas, UK) was then added to the same tube and the samples were incubated overnight at 55˚C in shaking water bath (Orbit Shaker Bath, Lab-Line, USA).

Day 2

The next day, equal quantity (5 ml) of equilibrated phenol (pH=8.0) was added to the samples, it was then agitated for 10 minutes and kept on ice for 10 minutes. The sample was spun at 3200 rpm for 30 minutes at 4℃. Then the supernatant was removed with 1 ml micropipette tip and shifted into a separately

labelled tube. To the samples, 5 ml of chloroform : isoamyl alcohol (24:1) solution was added, agitated for 10 minutes and kept on ice for 10 minutes. Then the samples were spun for 30 minutes at 3200 rpm at 4℃. The supernatant was then separated into 15 ml separately labelled tubes. The isolated supernatant was added with 500 µl of 10M ammonium acetate and 5 ml of chilled Isopropanol and agitated until DNA precipitate was visible as white thread. The tubes with samples were placed overnight at -20℃ (or for 15 minutes at -70℃).

Day 3

The next day, tubes were centrifuged at 3000 rpm for 60 minutes at 4℃. The supernatant was discarded and pellet was separated from the edge of tube by tapping the tube with fingers. The pellet was washed with 5 ml of chilled 70% ethanol by rotating at 3200 rpm for 40 minutes at 4℃ in refrigerated centrifuge. The supernatant was discarded and DNA was dried. The dried pellet was then dissolved in 10mM tris EDTA buffer (the volume buffer added according to the size of the pellet). The whole DNA was dissolved in TE buffer by incubation of the samples in shaking water bath at 55℃ (Orbit Shaker Bath, Lab-Line, USA). The samples were then transferred into 1.5 ml centrifuge tubes (Axygen, UK) and stored at 4℃.

4.4.2 DNA extraction through commercial kit

The manufacturer's instructions were followed for DNA extraction through kit method (Scibona, 2012). In a microcentrifuge tube 20 µl of Qiagen Proteinase was taken and 200 µl of blood was added to it. Buffer AL (200 µl) was then added to it, mixed and incubated for 10 minutes at 56 ºC. Then 200 µl of ethanol was added to the sample and the mixture was applied to the capped QIAamp Spin column (in a 2 ml collection tube). This column was centrifuged at 8000 rpm for 1 minute and then placed in another clean collection tube. To this column, Buffer AW1 (500 µl) was added and it was centrifuged at 8000 rpm for 1 minute. Then the column was again placed in a new collection tube and 500 µl Buffer AW2 was added to it. The column was then centrifuged at 14000 rpm for 3 minutes and placed in a 1.5 microcentrifuge tube. Then, 200 µl Buffer AE was added to the column; it was incubated at room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute. The DNA acquired in microcentrifuge tube was stored at 4 ºC.

4.4.3 Agarose gel electrophoresis

For the analyses of whole DNA after extraction from blood, 1% agarose gel electrophoresis was used.

For the preparation of agarose solution, powdered agarose (Promega, USA) was added to 1x tris-borate ethylene diamintetraacetic acid (TBE) buffer. It was boiled on hot plate or microwave oven until no granules of agarose were visible. It was then cooled upto 70 °C and 5 µl of ethidium bromide solution was added and mixed thoroughly. The temperature was allowed to be even low to pour the gel on a 20x20 cm gel plate of the gel tank (Horizon 58 Gel Elctrophoresis System, Life Technologies, USA) and comb was inserted at a suitable distance. After polymerization of the gel for about 30-45 minutes, the tank was filled with 1X TBE gel buffer and combs were removed.

For loading of the samples, 5 µl of sample DNA was mixed with 3 µl of bromophenol blue solution. The samples were loaded into specific wells onto the gel. Electrophoresis was run at 90V for 45 min to 1 hr (Power Pac Basic, Bio-Rad, Singapore). The gel was viewed on a gel documentation system and the images were prepared and visualized with the software using gel documentation system (GeneGenius, Syngene, UK).

4.4.4 Quality assessment and quantification of DNA

Each sample was diluted to 50 folds by addition of 6 µl of DNA to 294 µl distilled water and quantified on UV Spectrophotometer (UVD-2950, Labomed Inc. USA). The absorbance was recorded at 260 nm and 280 nm wavelength.

Optical density (OD) ratio for each sample was calculated as:

$$\text{OD} = \text{Absorbance at 260 nm}/\text{Absorbance at 280 nm}$$

If the ratio of absorbance at the two wavelengths was between 1.7-1.9, the DNA was of good quality. The concentration of DNA samples was calculated as:

$$\text{DNA concentration } (\mu g/ml) = \text{Absorbance at 260 nm} \times \text{Dilution factor} \times \text{Correction factor}$$

Value for dilution and correction factor is 50.

4.4.5 Working solution of DNA

The working solution for DNA was prepared from the stock as 40ng/µl. the dilution was prepared using the formula:

$$C_1V_1 = C_2V_2$$

The DNA was shifted to separate microcentrifuge tubes as aliquots and stored at -20˚C to be used as template for polymerase chain reaction (PCR).

4.5 Primer Designing

For genotyping, the primers were designed for rs1800795, rs1800796, rs1800797, rs4073, rs2227306, rs2227543, rs3025039, rs699947, rs1205, rs1130864, through previous reports in the literature andPrimer 3 (version 4.0) was used to assess the validity of primer sequence (Untergasser et al., 2012; Koressaar and Remm, 2007). The sequence was rechecked through genome Browser (Genome Bioinformatics Group, University of California Santa Cruz, USA) (Table 4.1).

4.6 Reconstitution of Primers

The primers were in the form of lyophilized powder and were reconstituted to prepare a stock solution of 100 nM for each primer by adding required amount of dH_2O. The working solution with 20 nM strength was prepared by adding 80 µl of dH_2O to 20 µl of primer stock that was used for PCR.

4.7 Polymerase Chain Reaction (PCR)

The optimization of PCR was carried out with reproducible results. All the samples were amplified for target DNA fragment spanning through SNP to be analysed. The PCR was carried out for each sample with a final volume of 20 µl. It contained 40 ng genomic DNA (2 µl), 2 µl of PCR buffer without Mg^{2+}(magnesium ions) (Invitrogen, Brazil), 1 µl of 25 mM $MgCl_2$ (magnesium chloride) (Invitrogen, Brazil), 0.2 µl of 5 U/µl Taq DNA polymerase (Invitrogen, Brazil), 1 µl of 2 mM dNTPs (deoxnucleotide triphosphate) (Invitrogen, Germany), 1 µl of each 20 nM forward and reverse primer (e-oligos, Gene Link, Inc, USA). The final volume was adjusted to 20 µl with PCR water.

The reaction was performed using thermal cycler (Thermo electron Corporation, Millford, USA). The programming for PCR conditions had 3 steps. In step 1, denaturation was carried out at 94 ºC for 5 minutes with 1 cycle. In step 2, denaturation at 94 ºC for 45 seconds, annealing was carried out for 45 seconds at the

temperature specific for each primer set (Table 4.2) and extension at 72 ºC for 1 minute. The number of cycles was 35. In step 3, final extension was carried out at 72˚C for 10 min.

For gel electrophoresis, 5 µl of PCR product was mixed with 5 µl of 6x gel loading buffer (Invitrogen, Brazil) and loaded on 2% agarose gel. The DNA ladder of 100 bp (Invitrogen, Brazil) was loaded as size reference for amplified DNA fragments in the first well. Gel electrophoresis (Maxicell Electrophoretic Gel System, USA) was done by running the gel in 1x TBE buffer at 200 volts using BioRad Power Pack 3000 (BioRad, USA). The gel was run for 40 minutes. Bands of PCR product were visualized under UV illumination and photographed with gel documentation system (GeneGenius, Syngene, UK).

4.8 Restriction Fragment Length Polymorphism (RFLP)

It is a molecular biological technique used to study variations in homologous DNA sequences detected by the presence of different lengths after digestion of DNA samples with specific restriction endonucleases. The RFLP markers are highly specific. In RFLP analysis the DNA is broken into pieces and digested by restriction enzymes that results into restriction fragments separated according to lengths by application of gel electrophoresis (Fig 4.5) (www.bio.davidson.edu/genomics /method/RFLP.html).

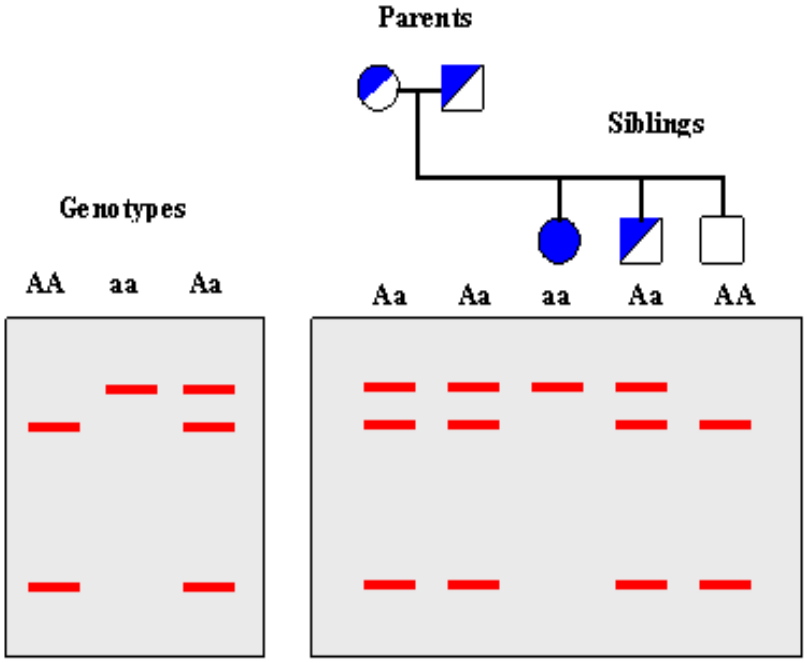


Fig. 4.5 Analysis and inheritance of allelic RFLP fragments**(**www.bio.davidson.edu/genomics/method/RFLP.html**)**

4.9 Restriction Fragment Length Polymorphism (RFLP) Assay

The amplified DNA fragment harbouring the site of SNP was digested with restriction enzyme (New England Biolabs Inc. UK) designed through NEB cutter V2.0 (Vincze et al., 2003). Each PCR product was mixed with 5 U of restriction enzyme. The restriction reaction was carried out at 37 ºC for 16 hours. The details of restriction enzymes for each primer set are given in Table 4.2.

The RFLP products were analyzed through 3 % agarose gel. Electrophoresis was carried out at 200 volts for 40 minutes using Bio-Rad Power PAC 3000. The size of RFLP bands was depicted with DNA reference size ladder (Invitrogen, Brazil).

4.10 Sanger DNA Sequencing

Sequencing of DNA can be accomplished by either chemical or enzymatic means. The enzymatic technique, Sanger sequencing, involves the use of dideoxynucleotides (2',3'-dideoxy) that terminate DNA synthesis therefore also called dideoxy chain termination sequencing.

The Sanger DNA sequencing protocol utilizes dideoxynucleotides (ddNTPs) to terminate chain elongation during the *in vitro* synthesis of DNA from a cloned template. Synthesis is initiated using a specific oligonucleotide primer. During the synthesis reaction a radioactive nucleotide (usually dATP) is incorporated into the elongating strands. Four separate reactions are carried out simultaneously, each of which contains all 4 dNTPs and a single ddNTP. The higher the concentration of ddNTP the more frequently chain elongation will terminate. Therefore, one can regulate the extent of sequence information obtainable by varying the dNTP/ddNTP ratio (Fig 4.6) (http://dwb.unl.edu/Teacher/NSF/C08/C08Links/www.piopio.school .nz/molmed.htm#sequencing).

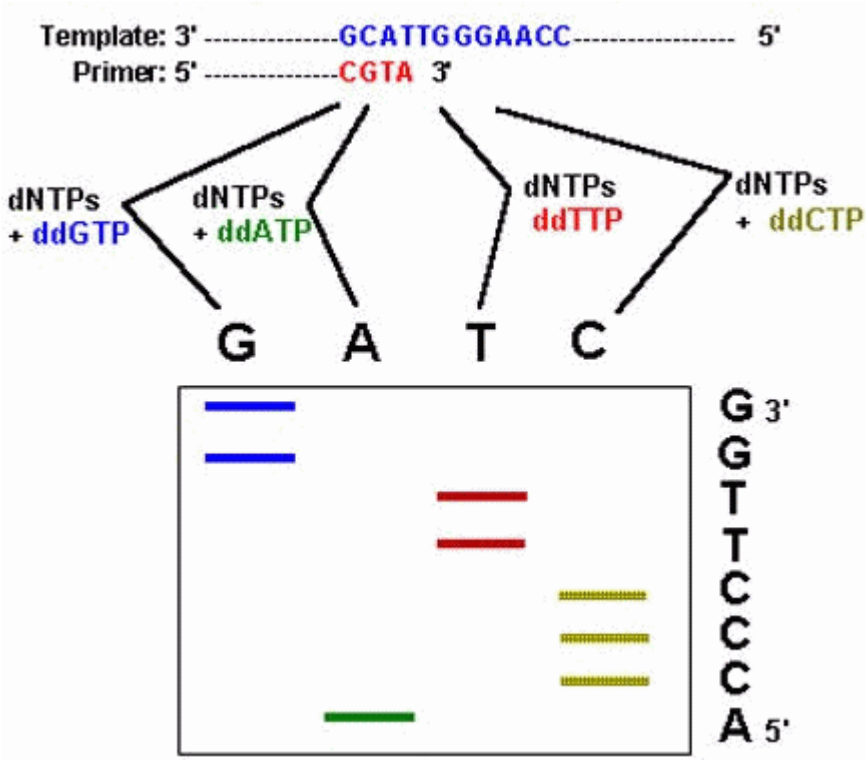


Fig 4.6 Sanger ddNTP chain termination sequencing (http://dwb.unl.edu/Teacher/NSF/C08/C08Links/www.piopio.school.nz/molmed.htm#sequencing)

Table 4.1 Primer sequence for PCR for identification of different SNPs

Accession number	Primers	Primer sequence	PCR product (bp)	Reference
rs1800795	Forward	5' TGACTTCAGCTTTACTCTTTGT3'	198	Fernandez-Real, et al. (2001)
	Reverse	5'CTGATTGGAAACCTTATTAAG3'		
rs1800796	Forward	5'GCAAAGTCCTCACTGGGAGGA3'	296	Tsai, et al. (2008)
	Reverse	5'TCTGACTCCATCGCAGCCC3'		
rs1800797	Forward	5'GGAGTCACACACTCCACCTG3'	419	Tsai, et al. (2008)
	Reverse	5'AGCAGAACCACTCTTCCTTTACTT3'		
rs4073	Forward	5′TCATCCATGATCTTGTTCTAA3'	542	Tsai, et al. (2008)
	Reverse	5′GGAAAACGCTGTAGGTCAGA3'		
rs2227306	Forward	5′CTCTAACTCTTTATATAGGAATT3'	203	Tsai, et al. (2008)
	Reverse	5′GATTGATTTTATCAACAGGCA3'		
rs2227543	Forward	5′CTGATGGAAGAGAGCTCTGT3'	397	Tsai, et al. (2008)
	Reverse	5′TGTTAGAAATGCTCTATATTCTC3'		

Continued.....

Accession number	Primers	Primer sequence	PCR product (bp)	Reference
rs3025039	Forward	5' AAGGAAGAGGAGACTCTGCGC3'	198	Primer 3.0
	Reverse	5' TATGTGGGTGGGTGTGTCTACAG3'		
rs699947	Forward	5'GGATGGGGCTGACTAGGTAAGC3'	324	Primer 3.0
	Reverse	5'AGCCCCCTTTTCCTCCAAC3'		
rs1205	Forward	5' GGAGTGAGACATCTTCTTG3'	227	Primer 3.0
	Reverse	5' CTTATAGACCTGGGCAGT3'		
rs1130864	Forward	5' AGCTCGTTAACTATGCTGGG3'	181	Primer 3.0
	Reverse	5' CTTCTCAGCTCTTGCCTTAT3'		

Table 4.2 Conditions for restriction reaction for different SNPs studied

Gene variant	Restriction Enzyme	Annealing temperature	Fragment size
rs1800795	*SfaN1*	56.0 ºC	198, 144, 54
rs1800796	*BsrBI*	64.0 ºC	296, 202, 94
rs1800797	*FokI*	64.0 ºC	419, 362, 57
rs4073	*MfeI*	60.0 ºC	542, 450, 92
rs2227306	*EcoR1*	56.0 ºC	203, 185
rs2227543	*NlaIII*	60.0 ºC	396, 233, 163
rs3025039	*HpyCH4 III*	58.0 ºC	198,112, 86
rs699947	*BglII*	55.0 ºC	324, 122, 202
rs1205	*HpyCH4 III*	50.0 ºC	227, 131, 96
rs1130864	*HpyCH4 III*	59.0 ºC	181, 156, 25

4.11 DNA Sequencing

For the validity of genotype acquired through PCR-RFLP, direct sequencing of at least 10 samples for each SNP from control and AMD patients each was carried out through automated capillary sequencing methods. The SNPs were amplified through the same primers as mentioned in Table 4.1.

4.11.1 PCR product purification

PCR product (25 µl) was mixed with 2.5 µl of 10M ammonium acetate and 50 µl chilled absolute ethanol and left on ice for 20 minutes. It was then spun at 14000 rpm (Eppendorf, 5810, Germany) for 10 minutes. Then supernatant was discarded and pellet was washed with 100 µl of 70 % chilled ethanol. This solution was again centrifuged at 14000 rpm. The supernatant was discarded and pellet was dried and re-suspended in 15 µl of distilled water.

4.11.2 Sequencing reaction

The purified PCR product that was harbouring the SNP was subjected to Sanger sequencing reaction. The forward primer for the target region was used in sequencing reaction. The reaction mixture contained 3µl of purified PCR product, 1 µl of 20 nM forward primer, 4 µl ABI big cycle dye terminator (Applied Biosystem, Life Technologies, USA) and the final volume was raised to 10 µl. The thermal cycle conditions were 25 cycles each at 95 ºC for 1 minute, 95 ºC for 15 seconds and 55 ºC for 4 minutes. After completion of sequencing reaction, the product was subjected to purification.

4.11.3 Purification of sequencing product

For purification of sequencing product, it was shifted to 0.5 ml eppendorf tube. To the product, 2.5 µl (125 mM) EDTA and 30 µl of absolute ethanol were added. The mixture was left on ice for 15 minutes and then spun at 14,000 rpm for 20 minutes. Then supernatant was discarded, pellet was washed with 100 µl of 70 % chilled ethanol. It was spun again at 14,000 rpm for 10 minutes. Then supernatant was discarded and pellet was dried at 55 ºC in the oven.

4.11.4 Denaturing the purified sequencing reaction product

The purified dry product after sequencing was added with 10 µl hiDi Formamide (Applied Biosystems, Life Technologies, USA). It was denatured at 95 ºC for 5 minutes and immediately cooled at 4 ºC on ice.

4.11.5 Sequencing

The purified sequencing reaction product was loaded in ABI 3130 genetic analyser (Applied Biosystems, Life Technologies, USA) for sequencing. The results of sequencing were read and the SNP genotypes were validated.

4.12 Statistical Analyses

All statistical analyses were performed on "IBM Statistical Product and Service Solutions (SPSS) Statistics" (version 18.0, Chicago, Illinois, USA) and Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). All data were presented as mean ± standard deviation (SD).

The allele and genotype frequency in AMD patients group was compared to that with control group through non-parametric Mann Whitney test. The association of genotype with serum protein levels was analysed by application of one way analyses of variance (ANOVA). The frequency of genotype and alleles were estimated though the observed number of each specific allele and genotype. The expected Hardy-Weinberg (H-W) frequencies for the alleles and genotypes were calculated by applying the H-W Equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

'p' and 'q' represent the frequencies of alleles and are calculated as:

$$\text{Allele frequency} = \frac{\text{No. of allele in study group}}{\text{Total no. of alleles}}$$

The deviation of data of genotypes from H-W equilibrium was tested through chi-square (χ^2). Confidence interval $p < 0.05$ was considered as significant.

The odds ratio and confidential interval for genotype and allele frequency was calculated through online software linkhttp://www.hutchon.net/ConfidOR.html (Bland and Altman, 2000).

Results

4.13 PCR Results of rs1800795

The amplicon generated from rs1800795gene after PCR was 198 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.13.1 RFLP analyses

When the PCR amplicon was digested with *SfaN1* restriction enzyme, it produced fragments of 144 bp and 54 bp in case of allele C while the product was not cut in case of allele G (Fig 4.7). The findings were validated through sequencing; homozygous individuals showed genotype GG while heterozygous individuals showed genotypes CG; however, none of the individual had genotype CC (Fig 4.8).

4.13.2 Genotype and allele frequency

In control subjects, frequency of genotype GG was 73, for GC it was 27 and none of the subjects in this group had genotype CC (χ^2= 2.43; $p < 0.11$) while in case of AMD patients the frequency of genotype GG was 50, for GC it was 14 and for CC it was 26 (χ^2= 39.80; $p < 0.0001$). The frequency of genotype CC was significantly high in AMD patients as compared to control subjects ($p < 0.002$; OR = 1.9596; CI: 0.9824-3.9086). In control group, frequency of allele G was 0.865 and for allele C it was 0.135. In patients with AMD, frequency of allele G was 0.633 and for allele C it was 0.367 (Reported allele frequency 87% and G 13% C in Lahore, Pakistan, http://asia.ensembl.org, 2015). The frequency of allele C was significantly high in AMD patients as compared to control subjects ($p < 0.0001$; OR= 3.4905; CI: 2.1864 – 5.5722) (Table 4.3).

In males of control subjects, frequency of genotype GG was observed in 42, GC in 13 and none of them had genotype CC (χ^2= 0.98; $p < 0.32$). In male patients with AMD, 34 had genotype GG, 9 had GC and 16 had genotype CC (χ^2= 25.98; $p < 0.0001$). The frequency of genotype CC was significantly high in AMD patients as compared to control subjects ($p < 0.004$; OR= 0.5815; CI= 0.2263-1.4942). In males of control group, frequency of allele G was 0.882 and that for allele C it was 0.118. In male patients with AMD frequency for allele G was 0.653 and for allele C it was 0.347. The frequency of allele C was significantly high in AMD patients as compared

to that of control male subjects ($p < 0.0001$; OR= 3.973; CI= 1.989-7.936) (Table 4.4).

In the females of control group 31 subjects had genotype GG and 14 had genotype GC while none of them had genotype CC (χ^2= 1.52; $p < 0.21$). In the female patients with AMD, 16 subjects showed genotype GG, 5 had GC and 10 patients had genotype CC (χ^2= 13.7; $p < 0.0002$). The frequency of genotype CC was significantly high in AMD female patients as compared to that in control female patients ($p < 0.0155$; OR= 0.4258; CI= 0.1353-1.34). In females of control group frequency of allele G was 0.844 and for allele C it was 0.156 while in female patients with AMD, frequency of allele G was 0.597 and for allele C it was 0.403. The frequency of allele C was significantly higher in female patients with AMD as compared to control females ($p < 0.006$; OR= 3.668; CI= 1.71 – 7.8682) (Table 4.5).

In the patients with wet AMD, the frequency of genotype GG was 27, for GC it was 13 and for genotype CC it was 23 (χ^2= 16.82; $p < 0.0001$). In patients with dry AMD frequency of genotype GG was 19, for GC it was 5 and for CC it was 3 (χ^2= 4.97; $p < 0.025$). The frequency of genotype CC was significantly high in wet AMD patients as compared to that with dry AMD patients ($p < 0.0089$; OR= 0.8774; CI= 0.286 – 2.6913). The frequency of allele G in patients with wet AMD was 0.532, and for allele C it was 0.468. In patients with dry AMD, the frequency of allele G was 0.796 and for allele C it was 0.204. The frequency of allele C was significantly higher in wet AMD as compared to dry AMD ($p < 0.0016$; OR= 0.3306; CI= 0.1722 – 0.6345) (Table 4.6).

4.14 PCR Results of rs1800796

The amplicon generated after PCR of rs1800796 was 296 bp in size. The size of the product was compared with the DNA marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.14.1 RFLP analyses

The PCR product was digested with *BsrB1* restriction enzyme. It was cut into fragments of 202 bp and 94 bp in case of allele G while the product was not digested with this enzyme in case of allele C (Fig. 4.9). The genotypes were validated through sequencing analyses; homozygous individuals showed genotypes GG and CC while GC was the genotype in heterozygous individuals (Fig. 4.10).

4.14.2 Genotype and allele frequency

In control group the frequency of genotype GG was 35, GC was 48 and CC was 17 (χ^2= 0.0061; p < 0.93). In patients with AMD, the frequency of genotype GG was 25, GC was 55 and CC was 10 (χ^2= 5.95; p < 0.01). The difference in genotype frequency between the two groups was non-significant (p < 0.7958; OR= 1.7024; CI= 0.9555 – 3.033). The frequency of allele G in case of control subjects was 0.59 and for allele C it was 0.41. In AMD patients the frequency of allele G was 0.583, and for allele C it was 0.417 (allele frequency 67% G and 33% C in Lahore, Pakistan, http://asia.ensembl.org, 2015). The allele frequency was non-significantly different between the two groups (p < 0.8957; OR = 1.0279; CI= 0.6829 – 1.5472) (Table 4.7).

In the males of control group, the frequency of genotype GG was 20, GC was 28 and for CC it was 7 (χ^2= 0.338; p < 0.56). In case of male patients with AMD, the frequency of genotype GG was 18, for GC it was 34 and for CC it was 7 (χ^2= 2.22; p < 0.13). No significant difference in the genotype frequency between the two groups was observed (p < 0.5789; OR= 1.3114; CI= 0.6263-2.7459). In control males the allele frequency for G was 0.618 and for allele C it was 0.382. In males of AMD group, frequency of allele G was 0.593 and for allele C it was 0.407. There was non-significant difference in the allele frequency between males of control and AMD patients group (p < 0.444; OR= 1.1102; CI= 0.6523-1.8897) (Table 4.8).

In case of females of control group, the frequency of genotype GG was 15, for GC it was 20 and for CC it was 10 (χ^2= 0.45; p < 0.50). In females of AMD group, frequency of genotype GG was 7, GC was 21 and frequency of genotype CC was 3 (χ^2= 4.24; p < 0.035). The difference in genotype frequency between the two groups was non-significant (p < 0.9953; OR=2.5229; CI= 1.0137- 6.2788). In control females, the frequency of allele G was 0.555 and for allele C it was 0.445. In females with AMD, frequency of allele G was 0.565 and for allele C it was 0.435. The difference between the two groups was non-significant (p < 0.9149; OR= 0.9645; CI= 0.5038-1.8465) (Table 4.9).

In dry AMD patients the frequency of genotype GG was 18, for GC it was 38 and for CC it was 7 (χ^2= 3.759; p < 0.05). In patients with wet AMD, the frequency of genotype GG was 7, for GC it was 17 and for CC it was 3 (χ^2= 2.23; p < 0.13). The difference between the two groups was non-significant (p < 0.9123; OR= 1.1184; CI= 0.4413-2.8346). The frequency of allele G in wet AMD patients was 0.587 and for

allele C it was 0.413. In dry AMD patients frequency of allele G was 0.574 and for allele C it was 0.426. The allele frequency was non-significantly different between the two groups ($p < 0.752$; OR= 1.0558; CI= 0.5536-2.0135) (Table 4.10).

4.15 PCR Results of rs1800797

The amplicon (PCR product) generated from the rs1800797gene was 419 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.15.1 RFLP analyses

When the PCR amplicon was digested with *Fok1* restriction enzyme, it produced fragments of 362 bp and 57 bp in case of allele A while the product was not cut in case of allele G (Fig. 4.11). The genotypes of selected samples were validated through sequencing (Fig. 4.12).

4.15.2 Genotype and allele frequency

The frequency of genotype GG in control subjects was 64, for GA it was 36 while none of the subjects was with genotype AA (χ^2= 4.818; $p < 0.028$). In case of AMD patients, 83 cases had genotype GG, 7 had genotype GA while none of the patients had genotype AA (χ^2= 0.147; $p < 0.701$). The frequency of genotype GA heterozygotes was significantly low in AMD patients as compared to that in control subjects ($p < 0.0001$; OR= 0.1499; CI= 0.0626-0.3588). The frequency of allele G was 0.82 in control subjects and for allele A it was 0.18. In AMD patients, frequency of allele G was 0.96 and for allele A it was 0.04 (allele frequency 86% G and 14% A in Pakistan, http://asia.ensembl.org, 2015). The frequency of allele A was significantly low in AMD patients as compared control subjects ($p < 0.0001$; OR= 0.1843; CI= 0.0798-0.4258) (Table 4.11).

In males of control group, the frequency of genotype GG was 38; GA was 17 while none of the subjects was with genotype AA (χ^2= 1.838; $p < 0.175$). In case of male AMD patients, the frequency of genotype GG was 54, for GA it was 5 while none of the patients was having the genotype AA (χ^2= 0.115; $p < 0.734$). The frequency of genotype GA was significantly low in AMD male patients as compared to that in control male subjects ($p < 0.0026$; OR= 0.2398; CI= 0.0949-0.6059). The frequency of allele G in control group was 0.846 and for allele A it was 0.154. In

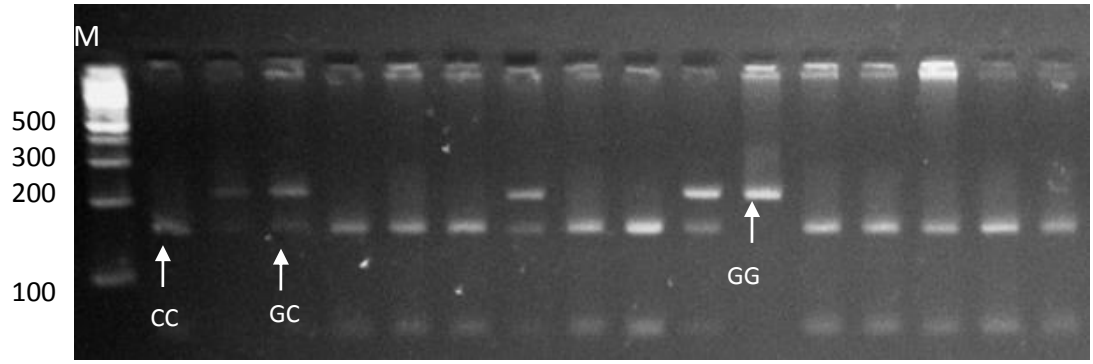
AMD patients, frequency of allele G was 0.97 and for allele A it was 0.043. The frequency of allele A was significantly low in male patients with AMD as compared to normal males ($p < 0.0001$; OR= 0.2778; CI= 0.1155-0.6682) (Table 4.12).

In females of control group, the frequency of genotype GG was 26, GA was 19 and none of the patients had genotype AA ($\chi^2= 3.22$; $p < 0.07$). In females with AMD, 29 patients had genotype GG and 2 had genotype GA ($\chi^2= 0.034$; $p < 0.853$). The frequency of genotype GA was significantly low in AMD female patients as compared to control females ($p < 0.002$; OR= 0.0944; CI= 0.02-0.4449). The frequency of allele G in control females was 0.789 and frequency of allele A was 0.211. In female patients with AMD, the frequency of allele G was 0.968 and that of allele A was 0.032. The frequency of allele A was significantly low in AMD female patients as compared to females in control group ($p < 0.0001$; OR= 0.1246; CI= 0.0279-0.5568) (Table 4.13).

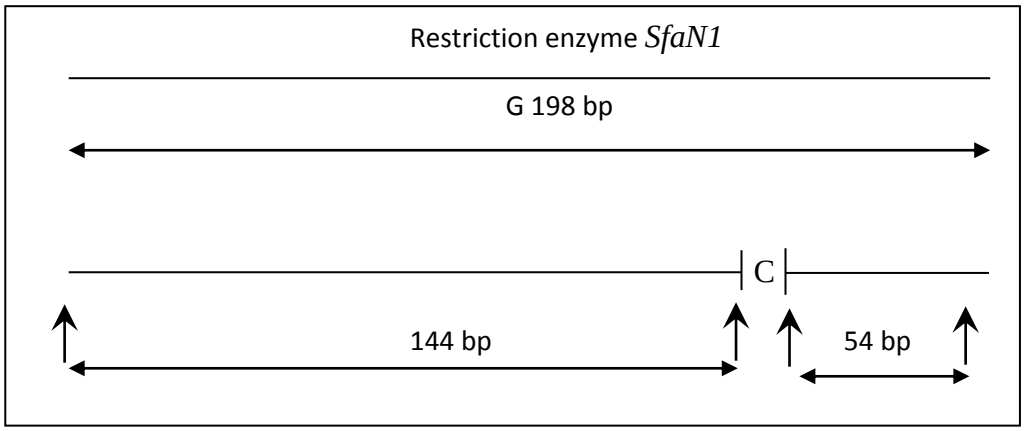
In patients with wet AMD, frequency of genotype GG allele was 60, for GA it was 3 ($\chi^2= 0.037$; $p < 0.846$) while in dry AMD patients, frequency of genotype GG was 23 and for GA it was 4 ($\chi^2= 0.173$; $p < 0.68$). The difference between the genotype frequencies of the two groups was non-significant ($p < 0.1597$; OR= 3.4783; CI= 0.722-16.7567). The frequency of allele G in wet AMD patients was 0.976 and for allele A it was 0.024. The frequency of allele G in dry AMD patients was 0.926 and for allele A it was 0.074. The frequency of allele A was significantly high in dry AMD patients as compared to patients with wet AMD ($p < 0.002$; OR= 3.28; CI= 0.7084-15.1877) (Table 4.14).

4.16 Relationship between Serum IL-6 Levels and Genotype

There was no significant difference in the mean serum IL-6 level in any of the genotypes of rs1800795in controls. However, mean serum IL-6 concentration in AMD patients with genotype GG for rs1800795 was significantly high as compared to both GC ($p < 0.006$) and CC ($p < 0.004$) genotypes. No significant change in serum IL-6 levels was observed in any of the genotypes for rs1800796 and rs1800797 in controls as well as AMD patients (Table 4.15).



(A)

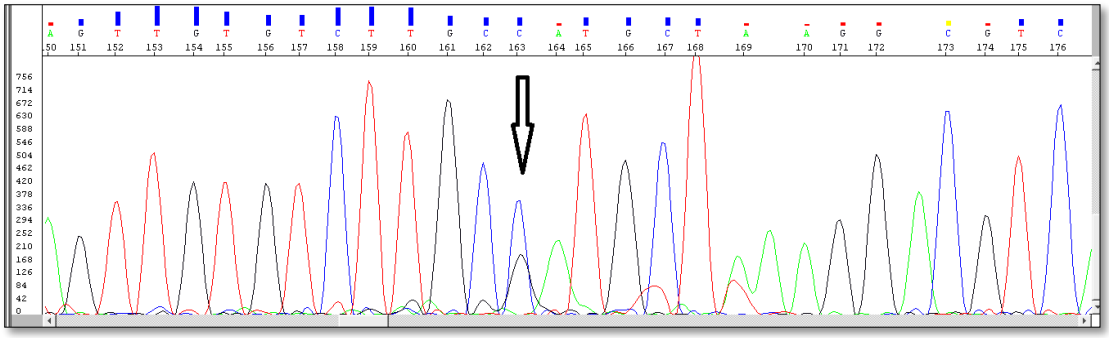


(B)

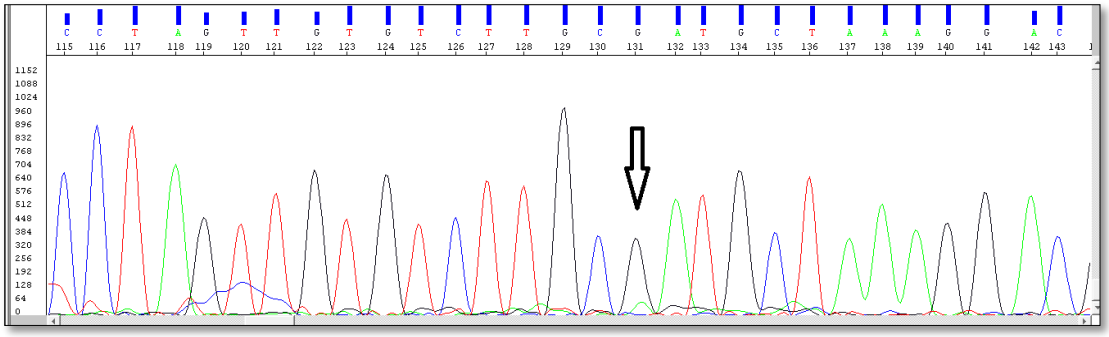
Fig. 4.7 (A) Agarose gel with fragments of PCR product for identification of genotypes after RFLP for rs1800795SNP.

(B)A schematic diagram illustrating rs1800795RFLP analysis

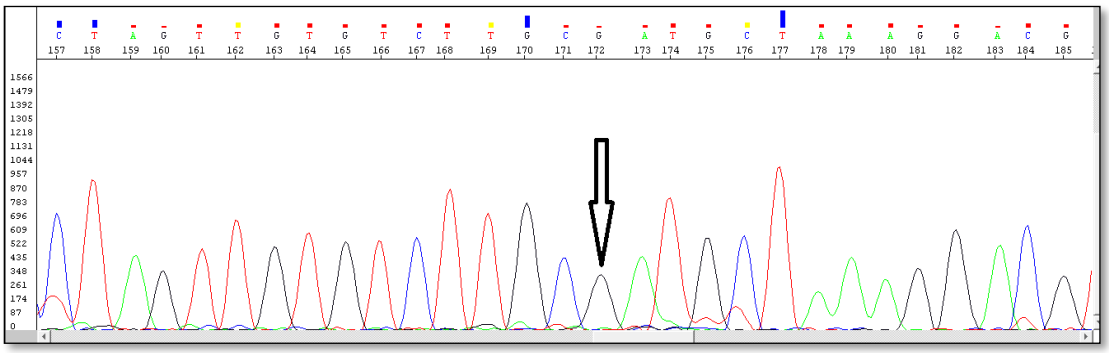
M	100 bp ladder DNA marker
GG	198 bp
CG	198+144+54 bp
CC	144+54 bp



(A)



(B)



(C)

Fig. 4.8 Sequence analyses of rs1800795polymorphism.

Sequence (A) revealed heterozygous genotype GC and (B) revealed homozygous individual GG(C) shows homozygous individual CC. Arrow indicates position of G> C inversion.

Table 4.3 (A) Frequency of genotypes and (B) alleles ofrs1800795SNP in control and AMD patients after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	73	74.8	50(55.5)	36.1	0.002
GC	27	23.4	14 (15.5)	41.8	
CC	0	1.8	26 (29)	12.1	
Var allele frequency	0.14		0.37		
χ^2	2.43		39.80		
p value	0.11		0.0001		

OR= 1.9596

CI= 0.9824 – 3.9086

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
G	173 (86.5)	114 (63.3)	0.0001
HWE allele frequency	0.865	0.633	
C	27(13.5)	66 (36.6)	
HWE allele frequency	0.135	0.367	

OR= 3.4905

CI =2.1864 – 5.5722

Table 4.4 (A) Genotype and (B) allele frequency of rs1800795SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	42 (76)	42.8	34 (57.6)	25.1	0.004
GC	13 (24)	11.5	9 (15.2)	26.8	
CC	0	0.8	16 (27.2)	7.1	
Var allele frequency	0.12		0.35		
χ^2	0.98		25.98		
p value	0.32		0.0001		

OR= 0.5815

CI= 0.2263 – 1.4942

(B)

Allele	**Control males (%)**	**AMD males (%)**	**pvalue**
G	97 (88.2)	77 (65.3)	0.0001
HWE allele frequency	0.882	0.653	
C	13 (11.8)	41 (34.7)	
HWE allele frequency	0.118	0.347	

OR= 3.973

CI= 1.989 – 7.936

Table 4.5 (A) Genotype and (B) allele frequency of rs1800795SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	31 (69)	32.1	16 (51.6)	11	0.0155
GC	14 (31)	11.8	5 (16.2)	14.9	
CC	0	1.1	10 (32.2)	5	
Var allele frequency	0.16		0.4		
χ^2	1.52		13.7		
p value	0.21		0.0002		
OR= 0.4258					
CI= 0.1353 – 1.34					

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
G	76 (84.4)	37 (59.7)	0.006
HWE allele frequency	0.844	0.597	
C	14 (15.6)	25 (40.3)	
HWE allele frequency	0.156	0.403	
OR=3.668			
CI= 1.71 – 7.8682			

Table 4.6 (A) Genotype and (B) allele frequency in patients with wet and dry type of AMD of rs1800795 SNP after RFLP

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	27 (43)	17.8	19 (70.4)	17.1	0.0089
GC	13 (20.6)	31.4	5 (18.5)	8.8	
CC	23 (36.4)	13.8	3(11.1)	1.1	
Var allele frequency	0.47		0.20		
χ^2	21.6		4.97		
p value	0.0001		0.025		

OR= 0.8774

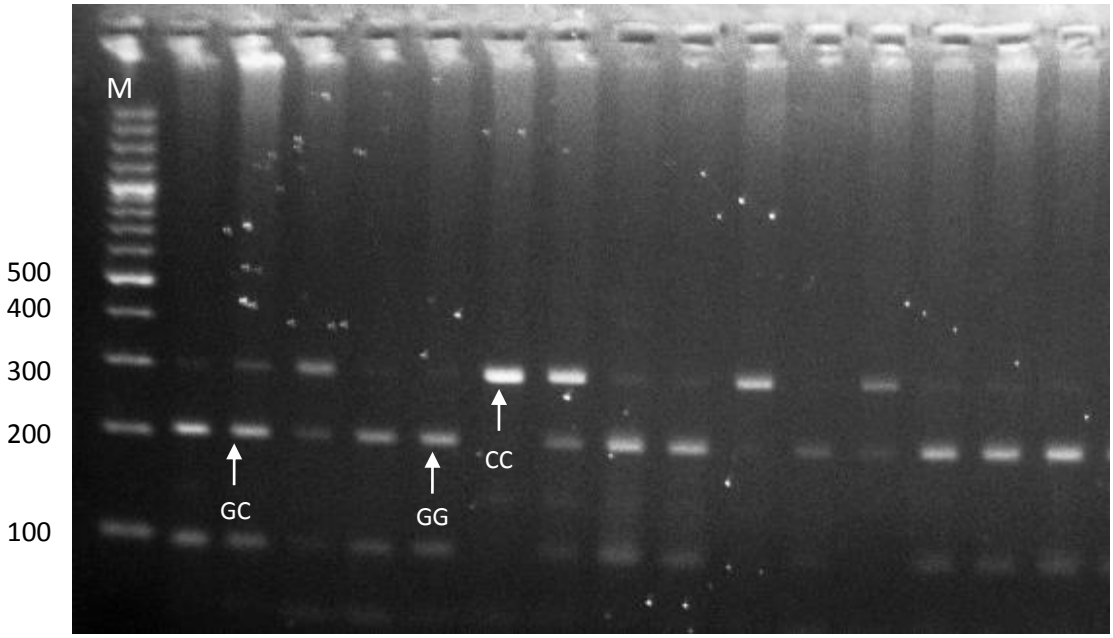
CI= 0.286 – 2.6913

(B)

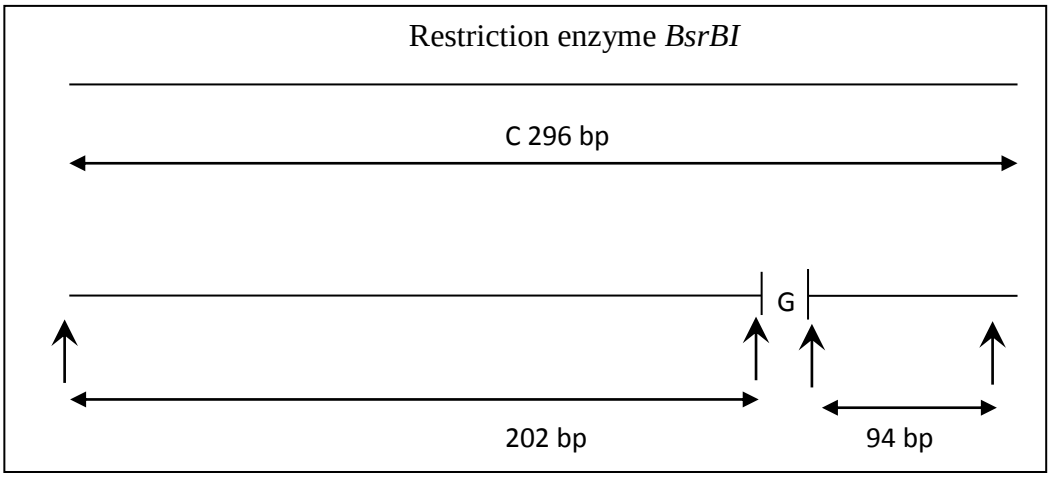
Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
G	67 (53.17)	43 (79.6)	0.0016
HWE allele frequency	0.532	0.796	
C	59 (46.8)	11 (20.4)	
HWE allele frequency	0.468	0.204	

OR= 0.3306

CI= 0.1722 – 0.6345



(A)

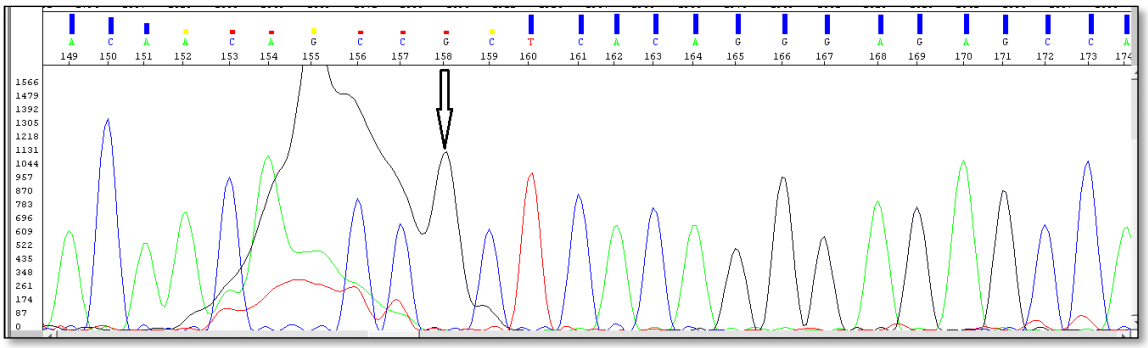


(B)

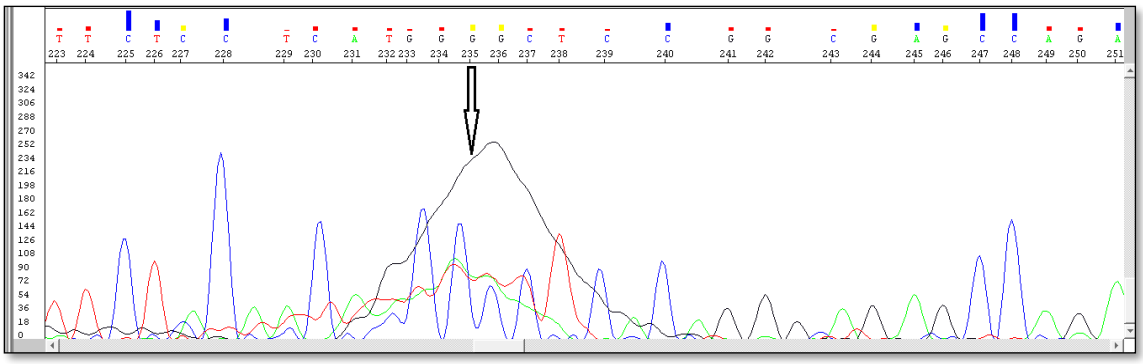
Fig. 4.9 (A)Agarose gel with fragments of PCR product for identification of genotypes after RFLP for rs1800796SNP

(B)Schematic diagram of RFLP analyses of rs1800796SNP

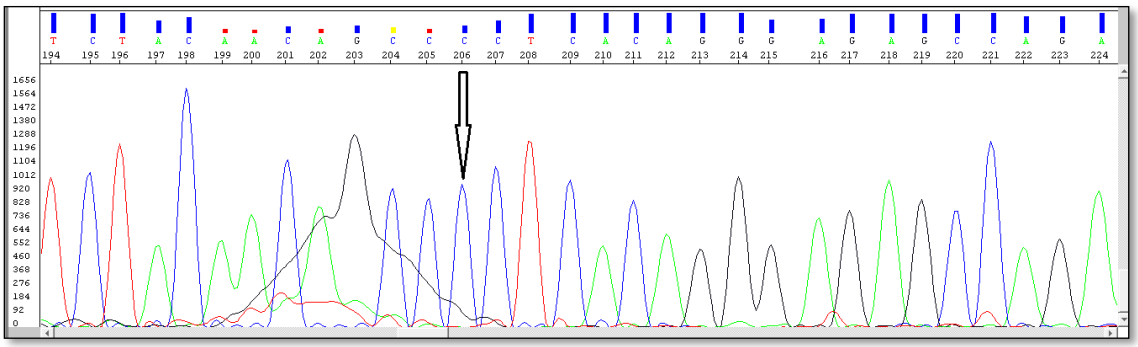
M	100 bp ladder DNA marker
GG	202 + 94 bp
CC	296 bp
GC	202 + 94 + 296 bp



(A)



(B)



(C)

Fig. 4.10 Sequence analyses of rs1800796polymorphism.

Sequence (A) revealed homozygous GG genotype and (B) revealed heterozygous GC individual(C) showed CC homozygous individual. Arrow indicates position of G> C inversion.

Table 4.7 (A) Genotype and (B) allele frequency of rs1800796SNP after RFLP in control vs AMD patients

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	35	34.8	25 (27.8)	30.6	0.7958
GC	48	48.4	55 (61.1)	43.8	
CC	17	16.8	10 (11.1)	15.6	
Var allele frequency	0.41		0.42		
χ^2	0.0061		5.95		
p value	0.93		0.01		
OR= 1.7024					
CI= 0.9555 – 3.033					

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
G	118 (59)	105 (58.3)	0.8957
HWE allele frequency	0.59	0.583	
C	82 (41)	75 (41.7)	
HWE allele frequency	0.41	0.417	
OR= 1.0279			
CI = 0.6829- 1.5472			

Table 4.8 (A) Genotype and (B) allele frequency ofrs1800796SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	20 (36.4)	21.0	18 (30.5)	20.8	0.5789
GC	28 (50.9)	26.0	34 (57.6)	28.5	
CC	7 (12.7)	8.0	7 (11.9)	9.8	
Var allele frequency	0.38		0.41		
χ^2	0.338		2.22		
p value	0.56		0.13		

OR= 1.3114

CI= 0.6263 – 2.7459

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
G	68 (61.8)	70 (59.3)	0.444
HWE allele frequency	0.618	0.593	
C	42 (38.2)	48 (40.7)	
HWE allele frequency	0.382	0.407	

OR= 1.1102

CI= 0.6523 – 1.8897

Table 4.9 (A) Genotype and (B) allele frequency of rs1800796SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	15 (33.3)	13.9	7 (22.5)	10.7	0.9953
GC	20 (44.5)	22.2	21 (67.8)	15.6	
CC	10 (22.2)	8.9	3 (9.7)	5.7	
Var allele frequency	0.44		0.44		
χ^2	0.45		4.24		
p value	0.50		0.035		

OR= 2.5229

CI= 1.0137 – 6.2788

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
G	50 (55.5)	35 (56.5)	0.9149
HWE allele frequency	0.555	0.565	
C	40 (44.5)	27 (43.5)	
HWE allele frequency	0.445	0.435	

OR= 0.9645

CI= 0.5038 – 1.8465

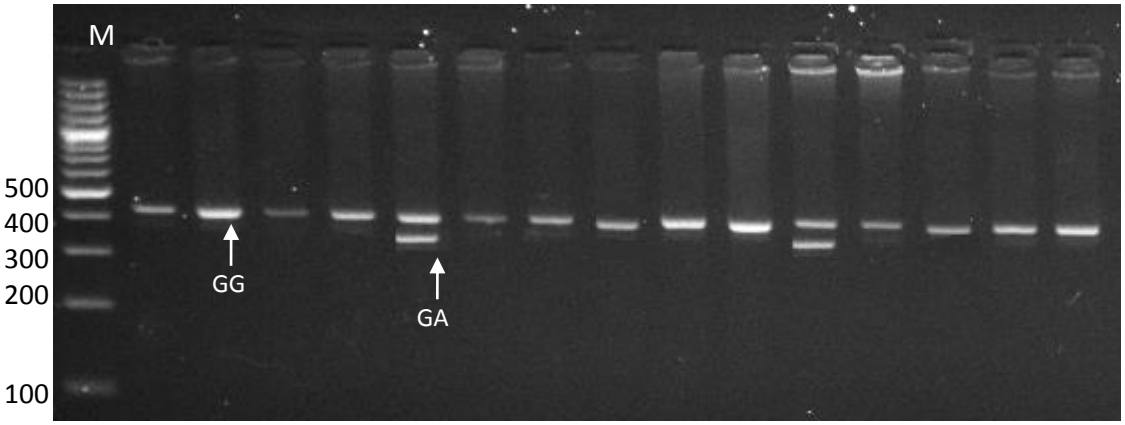
Table 4.10 (A) Genotype and (B) allele frequency in patients with wet and dry type of AMD of rs1800796SNP after RFLP

(A)

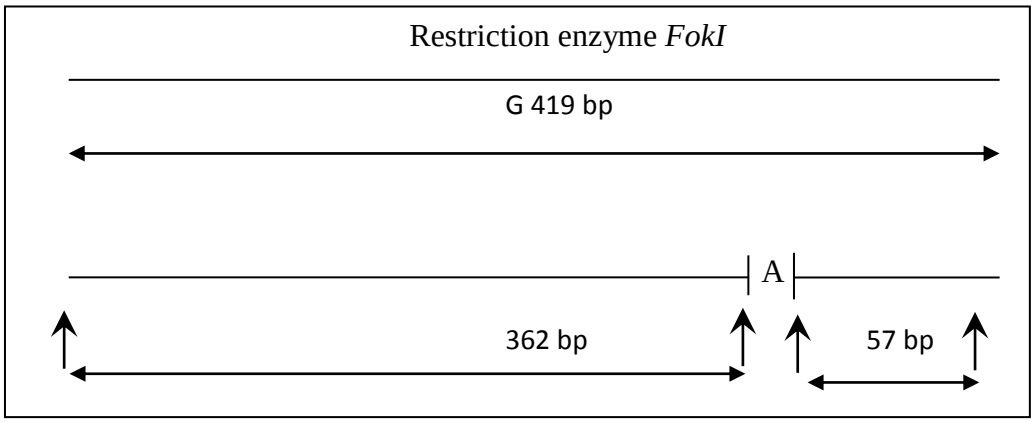
Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	18 (28.6)	21.7	7 (25.9)	8.9	0.9123
GC	38 (60.3)	30.5	17 (63)	13.2	
CC	7 (11.1)	10.7	3 (11.1)	4.9	
Var allele frequency	0.41		0.43		
χ^2	3.759		2.23		
p value	0.05		0.13		
OR= 1.1184					
CI= 0.4413 – 2.8346					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
G	74 (58.7)	31 (57.4)	0.752
HWE allele frequency	0.587	0.574	
C	52 (41.3)	23 (42.6)	
HWE allele frequency	0.413	0.426	
OR= 1.0558			
CI= 0.5536 – 2.0135			



(A)



(B)

Fig. 4.11 (A) Agarose gel with fragments of PCR product for identification of genotypes after RFLP for rs1800797SNP

(B) Schematic diagram of rs1800797RFLP

M	100 bp ladder DNA marker
AA	57 + 362 bp
AG	419 + 57 + 362 bp
GG	419 bp

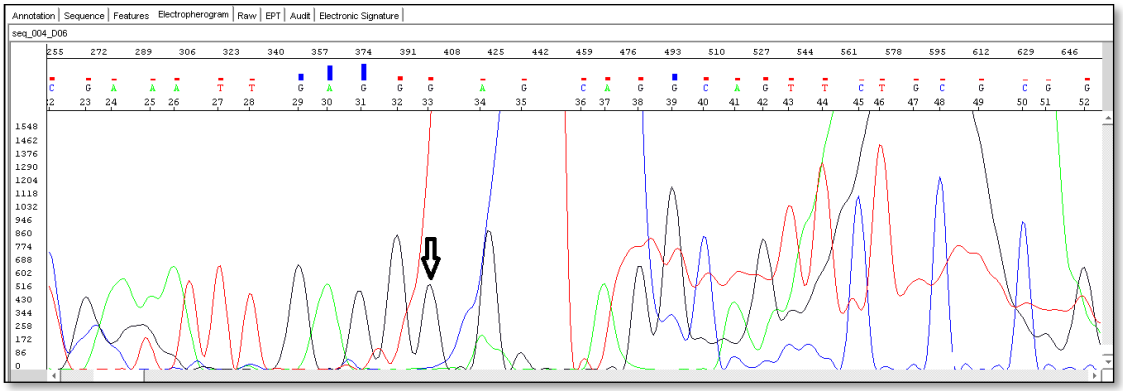


Fig 4.12 Sequence analyses of rs1800797containing G > A polymorphism.

Sequence revealed homozygous GG genotype. Arrow indicates position of G > A inversion.

Table 4.11 (A) Genotype and (B) allele frequency ofrs1800797 SNP in control vs AMD patients after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	64	67.2	83 (92.2)	83.1	0.0001
GA	36	29.5	7 (7.8)	6.7	
AA	0	3.2	0	0.1	
Var allele frequency	0.18		0.04		
χ^2	4.818		0.147		
p value	0.028		0.701		
OR= 0.1499					
CI= 0.0626 – 0.3588					

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
G	164 (82)	173 (96)	0.0001
HWE allele frequency	0.82	0.96	
A	36 (18)	7 (4)	
HWE allele frequency	0.18	0.04	
OR= 0.1843			
CI = 0.0798 – 0.4258			

Table 4.12 (A) Genotype and (B) allele frequency of rs1800797 SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	38 (69.1)	39.3	54 (91.5)	54.1	0.0026
GA	17 (30.9)	14.4	5 (8.5)	4.8	
AA	0	1.3	0	0.1	
Var allele frequency	0.15		0.04		
χ^2	1.838		0.115		
p value	0.175		0.734		

OR= 0.2398

CI= 0.0949 – 0.6059

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
G	93(84.6)	113 (95.7)	0.0001
HWE allele frequency	0.846	0.97	
A	17 (15.47)	5 (4.23)	
HWE allele frequency	0.154	0.043	

OR= 0.2778

CI= 0.1155 – 0.6682

Table 4.13 (A) Genotype and (B) allele frequency of rs1800797 SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	26 (57.8)	28.0	29 (93.5)	29.0	0.002
GA	19 (42.2)	15.0	2 (6.5)	1.9	
AA	0	2.0	0	0.0	
Var allele frequency	0.21		0.03		
χ^2	3.22		0.034		
p value	0.07		0.853		

OR= 0.0944

CI= 0.02 – 0.4449

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
G	71 (78.9)	60 (96.8)	0.0001
HWE allele frequency	0.789	0.968	
A	19 (21.1)	2 (3.22)	
HWE allele frequency	0.211	0.032	

OR= 0.1246

CI= 0.0279 – 0.5568

Table 4.14 (A) Genotype and (B) allele frequency in patients with wet and dry type AMD of rs1800797SNP after RFLP

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	60 (95.2)	60.0	23 (85.2)	23.1	0.1597
GA	3 (4.8)	2.9	4 (14.8)	3.7	
AA	0	0.0	0	0.1	
Var allele frequency	0.02		0.07		
χ^2	0.037		0.173		
p value	0.846		0.68		
OR= 3.4783					
CI= 0.722 – 16.7567					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
G	123 (97.6)	50 (92.6)	0.002
HWE allele frequency	0.976	0.926	
A	3 (2.4)	4 (7.41)	
HWE allele frequency	0.024	0.074	
OR= 3.28			
CI= 0.7084 – 15.1877			

Table 4.15 Comparison of serum IL-6 concentration in different genotypes. (Values are shown as mean ± SD)

	Control (n=100)			AMD (n=90)			
Genotype	**IL 6 concentration (n)**	**p value**	**F value**	**IL 6 concentration (n)**	**p value**	**F value**	**t test**
rs1800795							
GG	23.65 ± 0.66 (73)	0.105	2.707	38.74 ± 3.22 (50)	0.0001***	8.801	0.0001***
GC	21.05 ± 1.76 (27)			21.30 ± 1.96a** (14)			0.916
CC	NA (0)			20.57 ± 1.20** (26)			NA
rs1800796							
GG	28.87 ± 1.27 (35)	0.514	0.671	27.50 ± 2.39 (25)	0.293	1.254	0.010*
GC	23.12 ± 0.82 (48)			34.50 ± 3.40 (55)			0.0001***
CC	21.62 ± 1.43 (17)			26.18 ± 3.41 (10)			0.044*
rs1800797							
GG	24.10 ± 0.678 (64)	3.154	0.081	31.36 ± 2.50 (83)	0.254	0.616	0.0001***
GA	21.86 ± 1.15 (36)			35.33 ± 2.72 (7)			0.733
AA	NA (0)			NA (0)			NA

*P < 0.05; **p < 0.01; significantly different from serum levels in GG genotype

4.17 PCR Results of rs4073

The amplicon (PCR product) generated from the rs4073 gene was 542 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.17.1 RFLP analyses

When the PCR amplicon was digested with *Mfe1* restriction enzyme, it produced fragments of 448 bp and 94 bp in case of allele A while the product was not cut in case of allele T (Fig. 4.13). The genotype of the selected samples was validated through sequencing (Fig. 4.14).

4.17.2 Genotype and allele Frequency

In control subjects, frequency of genotype TT was 38, AT was 49 and AA was 13 (χ^2= 0.20; p < 0.65). In AMD patients, frequency of TT genotype was 28, for AT it was 50 and AA it was 12 (χ^2= 1.95; p < 0.16). The difference in genotype frequency between control and AMD patients was non-significant (p < 0.4239; OR=1.301; CI= 0.7347-2.3039). In control subjects, frequency of allele T was 0.625 and for A it was 0.375. In AMD patients, the frequency of allele T was 0.589 and for allele A it was 0.411 (frequency of allele T 64% and A 36% in Pakistan, http://asia.ensembl.org, 2015). The allele frequency was non-significantly different between two groups (p < 0.2366; OR= 1.1635; CI= 0.7703-1.7575) (Table 4.16).

In males of control group, the frequency of genotype TT was 18, AT was 27 and AA was 10 (χ^2= 0.0005; p < 0.98). In males with AMD, frequency of genotype TT was 21, AT was 31 and AA was 7 (χ^2= 0.761; p < 0.383). The genotype frequency was non significantly different between two groups (p < 0.4351; OR= 1.1481; CI= 0.5504-2.395). In the control males, frequency of allele T was 0.573 and for A, it was 0.427. In AMD patients, frequency of allele T was 0.619 and for A it was 0.381. The allele frequency between the two groups was non-significantly different (p < 0.4851; OR= 0.8263; CI= 0.4865-1.4036) (Table 4.17)

In female subjects of control group the frequency of genotype TT was 20, AT was 22 and AA was 3 (χ^2= 0.889; p < 0.345). In female patients with AMD, frequency of genotype TT was 7, AT was 19 and AA was 5 (χ^2= 1.653; p < 0.198). The frequency of genotype TT was significantly high in control subjects as compared to

that in AMD patients ($p < 0.0346$; OR= 1.6366; CI= 0.6576 - 4.073). The frequency of allele T in control females was 0.689 and for A it was 0.311. In AMD females, the frequency of allele T was 0.532 and for allele A, it was 0.468. The allele frequency was non-significantly different between the two groups ($p < 0.0510$; OR=1.9423; CI= 0.9979 -3.7806) (Table 4.18).

The frequency of genotype TT in wet AMD patients was 19, for AT it was 35 and for AA it was 9 ($\chi^2= 1.23$; $p < 0.27$). In case of patients with dry AMD, frequency of genotype TT was 9, AT was 15 and AA was 3 ($\chi^2= 0.76$; $p < 0.38$). There was non-significant difference between the genotype frequency of both groups ($p < 0.6822$; OR= 1; CI= 0.4057-2.4651). The frequency of allele T in wet AMD patients was 0.58 and for allele A it was 0.42. In dry AMD patients, frequency of allele T was 0.6 and of A it was 0.4. The difference in allele frequency of both groups was non-significant ($p < 0.6938$; OR= 0.8777; CI= 0.46 -1.6748) (Table 4.19).

4.18 PCR Results of rs2227306

The amplicon (PCR product) generated from the rs2227306gene was 203 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.18.1 RFLP analyses

When the PCR amplicon was digested with *EcoR1* restriction enzyme, it produced fragments of 185 bp and 18 bp in case of allele C while the product was not cut in case of allele T (Fig. 4.15). The validation of genotype of selected samples was done through sequencing (Fig. 4.16).

4.18.2 Genotype and allele frequency

The frequency of genotype CC in control subjects was 58, CT was 30 and TT was 12 ($\chi^2= 5.71$; $p < 0.01$). In AMD patients, frequency of genotype CC was 65, CT was 21 and TT was 4 ($\chi^2= 1.68$; $p < 0.19$). There was non-significant difference in genotype frequency of both the groups ($p < 0.0547$; OR= 0.7101; CI= 0.371-1.3592) (Table 3.41). The frequency of allele C in control subjects was 0.73 and that of allele T was 0.27. In AMD patient group frequency of allele C was 0.84 and that of allele T was 0.16 (frequency of allele T 26% and C 74%, Pakistan, http://asia.ensembl.org,

2015). The frequency of allele T was significantly low in patients with AMD ($p < 0.0001$; OR= 0.5193; CI= 0.3133-0.8608) (Table 4.20).

In males of control group, the frequency of genotype CC was 28, for CT it was 18 and for TT it was 9 ($\chi^2= 3.62$; $p < 0.06$). In males of AMD patient group, the frequency of genotype CC was 41, CT was 14 and TT was 4 ($\chi^2= 2.79$; $p < 0.09$). The frequency of genotype CC was significantly high in males with AMD as compared to normal males ($p < 0.0476$; OR= 0.5114; CI= 0.2306-1.1342). In control male subjects the frequency of allele C was 0.673 and for allele T it was 0.327. In AMD male patients the allele frequency of allele C was 0.813 and for allele T it was 0.187. The frequency of allele C was significantly high in male AMD patients as compared to normal males ($p < 0.0150$; OR= 0.5901; CI= 0.3194-1.0902) (Table 4.21).

In females of control group, the frequency of genotype CC was 30, for CT it was 12 and for TT it was 3 ($\chi^2= 1.25$; $p < 0.26$) and in female patients with AMD, the frequency of genotype CC was 24, CT was 7 while none of them had genotype TT ($\chi^2= 0.50$; $p < 0.478$). There was non-significant difference between the two groups ($p < 0.2576$; OR= 0.8021; CI= 0.2751-2.339). The frequency of allele C in control female subjects was 0.80 while that of allele T was 0.20. In case of female AMD patients allele frequency of C was 0.89 and that of T was 0.11. The frequency of allele C was significantly high in AMD female patients as compared to normal females ($p < 0.003$; OR= 0.5091; CI= 0.1987-1.3046) (Table 4.22).

The frequency of genotype CC in wet AMD patients was 43, for CT it was 17 and for TT it was 3 ($\chi^2= 0.578$; $p < 0.446$). In dry AMD patients, the frequency of genotype CC was 22, CT was 4 and TT was 1 ($\chi^2= 1.69$; $p < 0.19$). There was non-significant difference between the two groups ($p < 0.2173$; OR= 0.5103; CI= 0.1768-1.473). The frequency of allele C in wet AMD patients was 0.82 and that of T was 0.18. In dry AMD patients the frequency of allele C was 0.889 and T was 0.111. The allele frequency was non-significantly different between the two groups ($p < 0.2345$; OR= 0.5912; CI= 0.249 -1.4038) (Table 4.23).

4.19 PCR Results ofrs2227543

The amplicon (PCR product) generated from the rs2227543gene was 396 bp long double stranded DNA fragment. The size of amplicon was estimated by

comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.19.1 RFLP analyses

When the PCR amplicon was digested with *NlaIII* restriction enzyme, it produced fragments of 233 bp and 163 bp in case of allele C while the product was not cut in case of allele T (Fig. 4.17). The genotype results were validated through sequencing (Fig. 4.18).

4.19.2 Genotype and allele frequency

The frequency of genotype CC in control subjects was 39, for CT it was 54 and that of TT was 7 (χ^2= 4.12; p < 0.042). In AMD patients, frequency of genotype CC was 27, CT was 46 and for TT it was 17 (χ^2= 0.11; p < 0.73). The frequency of genotype TT was significantly higher in AMD patients as compared to that in control group (p < 0.0371; OR= 0.8906; CI= 0.5034-1.5756). The frequency of allele C in control subjects was 0.66 and that of T was 0.34. The frequency of allele C in AMD patients was 0.555 and that of T was 0.445 (allele frequency 68% C and 32% T in Pakistan, http://asia.ensembl.org, 2015). The frequency of allele T was significantly high in AMD patients as compared to the control group (p < 0.0372; OR= 1.5529; CI= 1.0257-2.3511) (Table 4.24).

In male subjects of controlgroup, the frequency of genotype CC was 20, CT was 32 and that of TT was 3 (χ^2= 4.52; p < 0.033). In male AMD patients, frequency of genotype CC was 19, CT was 29 and that of TT was 11 (χ^2= 0.0001; p < 0.991). There was non-significant difference in genotype frequency between the two groups (p < 0.1896; OR= 0.6948; CI= 0.3315-1.4563). The frequency of allele C in male control subjects was 0.655 and that of allele T was 0.345. The frequency of allele C in AMD male patients was 0.568 and that of T was 0.432. There was non-significant difference in the allele frequency between the two groups (p < 0.1812; OR= 1.4423; CI= 0.844-2.4647) (Table 4.25).

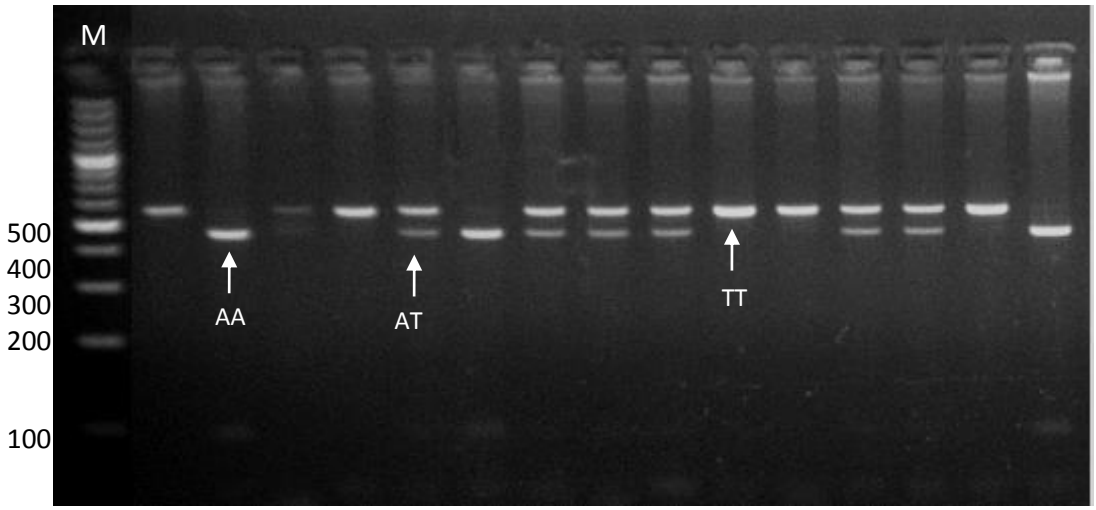
The frequency of genotype CC in control females was 19, CT was 22 and TT was 4 (χ^2= 0.45; p < 0.55). In females of AMD group, frequency of genotype CC was 8, CT was 17 and that of TT was 6 (χ^2= 0.318; p < 0.572). There was non-significant difference between the two groups (p < 0.1318; OR= 1.2649; CI= 0.5096 -3.1401). The frequency of allele C in control females was 0.667 and that of allele T was 0.333.

In females with AMD, frequency of allele C was 0.532 and that of allele T was 0.468. The allele frequency was non-significantly different between the two groups ($p < 0.0962$; OR= 1.7546; CI= 0.9054 – 3.4002) (Table 4.26).

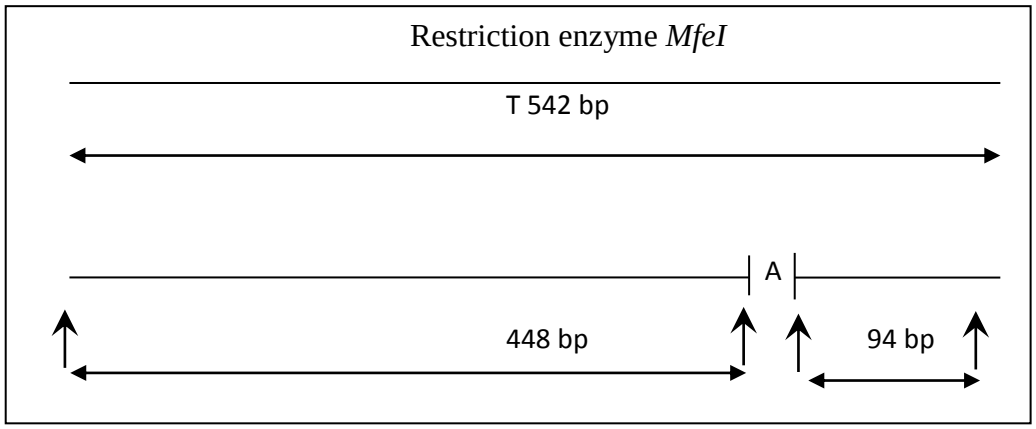
In patients with wet AMD type, the frequency of genotype CC was 18, CT was 32 and that of TT was 13 ($\chi^2= 0.031$; $p < 0.859$). In dry AMD patients, the frequency of genotype CC was 9, CT was 14 and that of TT was 4 ($\chi^2= 0.147$; $p < 0.701$). The difference between the two groups was non-significant ($p < 0.3788$; OR= 1.0433; CI= 0.4233-2.5716). The frequency of allele C in wet AMD patients was 0.54 and that of allele T was 0.46. In dry AMD patients, frequency of allele C was 0.59 and that of allele T was 0.41. There was non-significant difference between the two groups ($p < 0.5154$; OR= 0.806; CI= 0.4224-1.5379) (Table 4.27).

4.20 Relationship between Serum IL-8 Levels and Genotype

The concentration of IL-8 did not change significantly in both heterozygous and homozygous individuals for rs4073 SNP in control group ($p < 0.368$) as well as AMD patients ($p < 0.340$). In case of rs2227306 the serum IL-8 levels were non-significantly change in both heterozygotes and homozygotes in controls ($p < 0.125$) and AMD patients ($p < 0.951$). For rs2227543, the serum IL-8 levels were non significantly change in control subjects ($p < 0.710$) while the serum IL-8 concentration in patients with TT was significantly elevated as compared to patients with genotype CT ($P < 0.009$) and CC ($p < 0.003$) (Table 4.28).



(A)

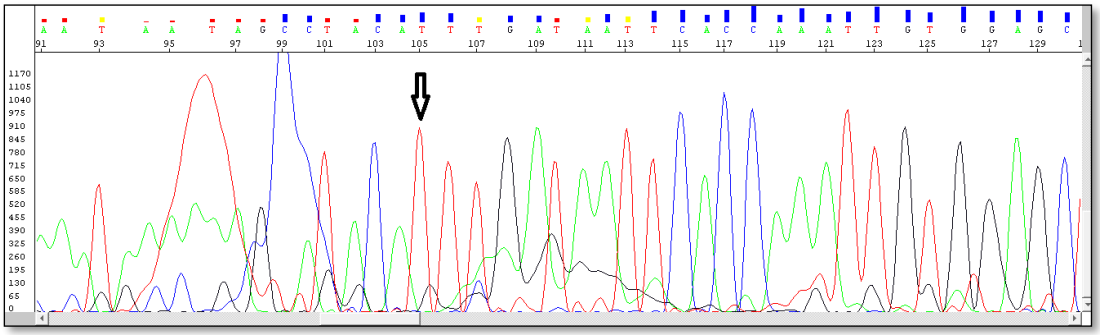


(B)

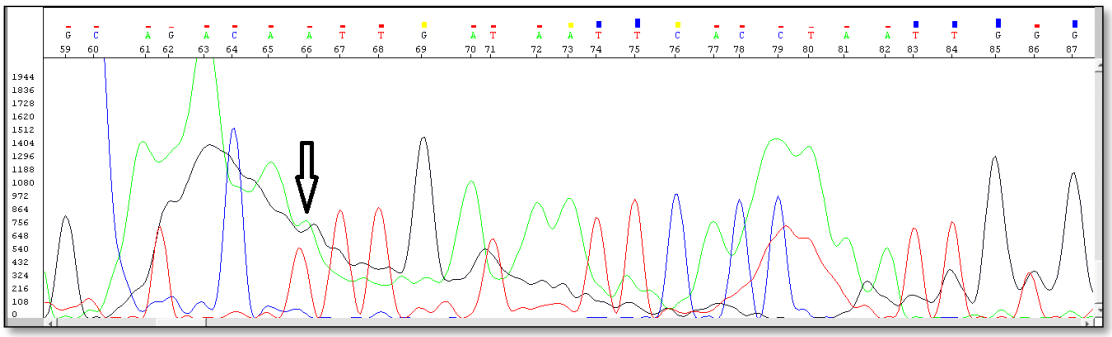
Fig. 4.13 (A) Agarose gel with fragments of PCR product for identification of genotypes after RFLP for rs4073SNP

(B) Schematic diagram of rs4073illustrating RFLP analyses

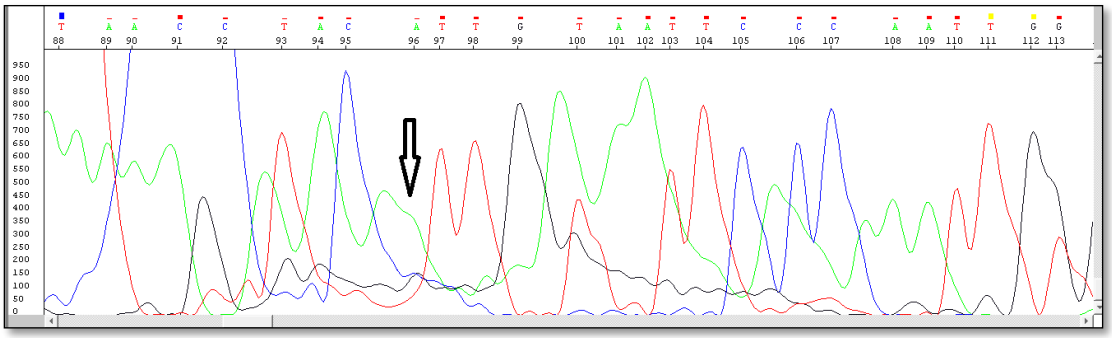
M	100 bp ladder DNA marker
AA	94 + 448
TT	542
AT	542 + 94 + 448



(A)



(B)



(C)

Fig. 4.14 Sequence analyses of rs4073 containing T> A polymorphism.

Sequence (A) revealed homozygous genotype TT and (B) revealed heterozygous genotype AT individual (C) showed genotype AA homozygous. Arrow indicates position of A<T inversion.

Table 4.16 (A) Genotype and (B) allele frequency ofrs4073SNP in control vs AMD patients after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	38	39.1	28 (31.1)	31.2	0.4239
AT	49	46.9	50 (55.5)	43.6	
AA	13	14.1	12 (13.4)	15.2	
Var allele frequency	0.38		0.41		
χ^2	0.20		1.95		
p value	0.65		0.16		
OR= 1.301 CI= 0.7347 – 2.3039					

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
T	125 (62.5)	106 (58.9)	0.2366
HWE allele frequency	0.625	0.589	
A	75 (37.5)	74 (41.1)	
HWE allele frequency	0.375	0.411	
OR= 1.1635 CI = 0.7703 – 1.7575			

Table 4.17 (A) Genotype and (B) allele frequency of rs4073 SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	18 (32.7)	18	21 (35.6)	22.6	0.4351
AT	27 (49.1)	26.9	31 (52.5)	27.8	
AA	10 (18.2)	10	7 (11.9)	8.6	
Var allele frequency	0.43		0.38		
χ^2	0.0005		0.761		
p value	0.98		0.383		
OR= 1.1481					
CI= 0.5504 – 2.395					

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
T	63 (57.3)	73 (61.9)	0.4851
HWE allele frequency	0.573	0.619	
A	47 (42.7)	45 (38.1)	
HWE allele frequency	0.427	0.381	
OR= 0.8263			
CI= 0.4865 – 1.4036			

Table 4.18 (A) Genotype and (B) allele frequency of rs4073SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	20 (44.3)	21.4	7 (22.6)	8.8	0.0346
AT	22 (49)	19.3	19 (61.3)	15.4	
AA	3 (6.7)	4.4	5 (16.1)	6.8	
Var allele frequency	0.31		0.47		
χ^2	0.889		1.653		
p value	0.345		0.198		
OR= 1.6366					
CI= 0.6576 – 4.073					

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
T	62 (68.9)	33 (53.2)	0.0510
HWE allele frequency	0.689	0.532	
A	28 (31.1)	29 (46.8)	
HWE allele frequency	0.311	0.468	
OR= 1.9423			
CI= 0.9979 – 3.7806			

Table 4.19 (A) Genotype and (B) allele frequency in patients with wet and dry type of AMD of rs4073 SNP after RFLP

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	19 (30.2)	21.1	9 (33.4)	10.1	0.6822
AT	35 (55.5)	30.7	15 (55.5)	12.8	
AA	9 (14.3)	11.1	3 (11.1)	4.1	
Var allele frequency	0.42		0.39		
χ^2	1.23		0.76		
p value	0.27		0.38		

OR= 1

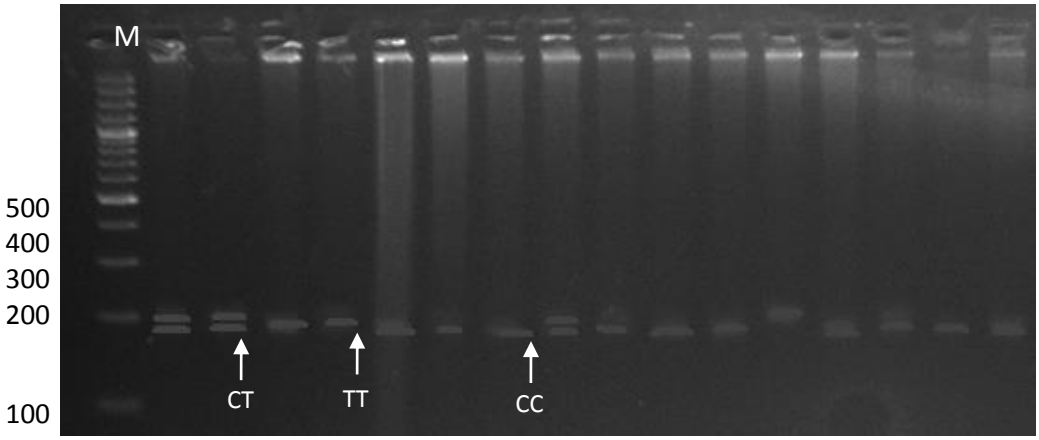
CI= 0.4057 – 2.4651

(B)

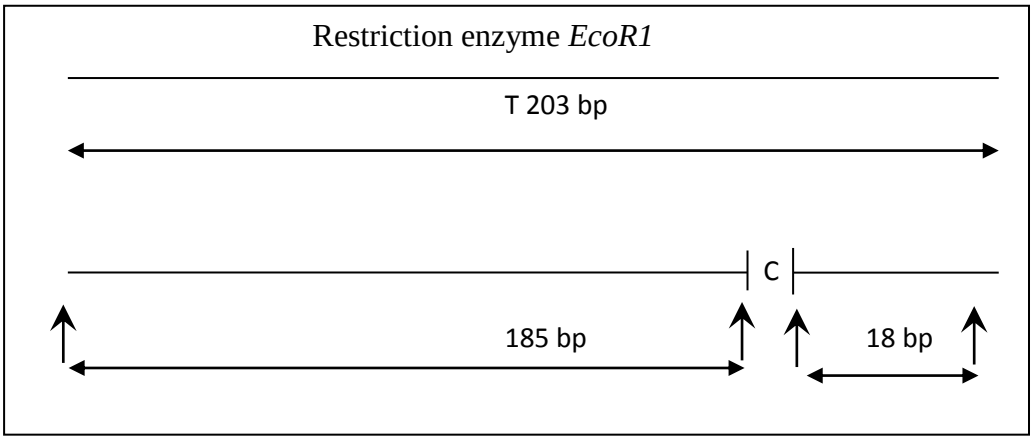
Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
T	73 (58.0)	33 (61.1)	0.6938
HWE allele frequency	0.58	0.6	
A	53 (42.0)	21 (39.9)	
HWE allele frequency	0.42	0.4	

OR= 0.8777

CI= 0.46 – 1.6748



(A)

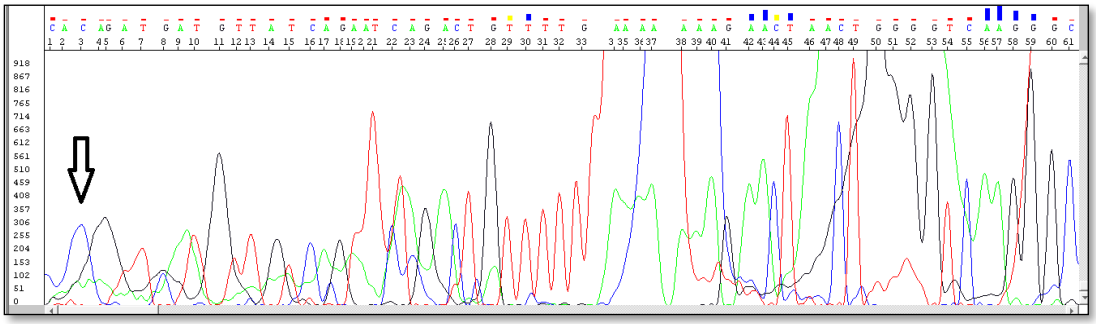


(B)

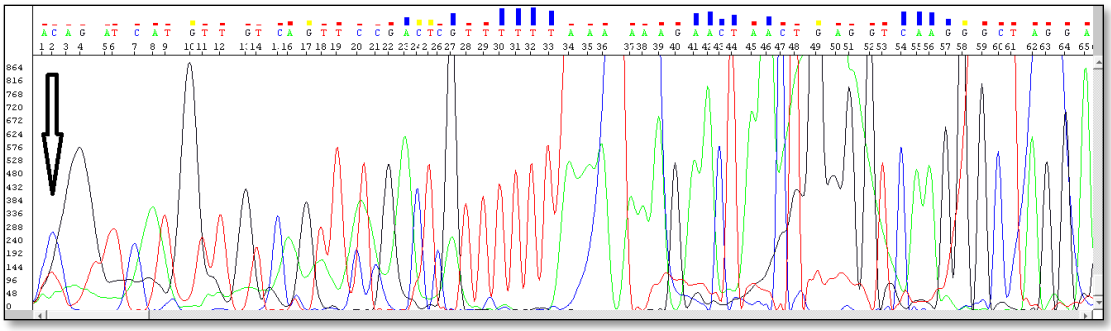
Fig. 4.15 (A) Agarose gel after RFLP showing fragmented product according to genotype for rs2227306SNP

(B) Schematic diagram of rs2227306illustrating RFLP analyses

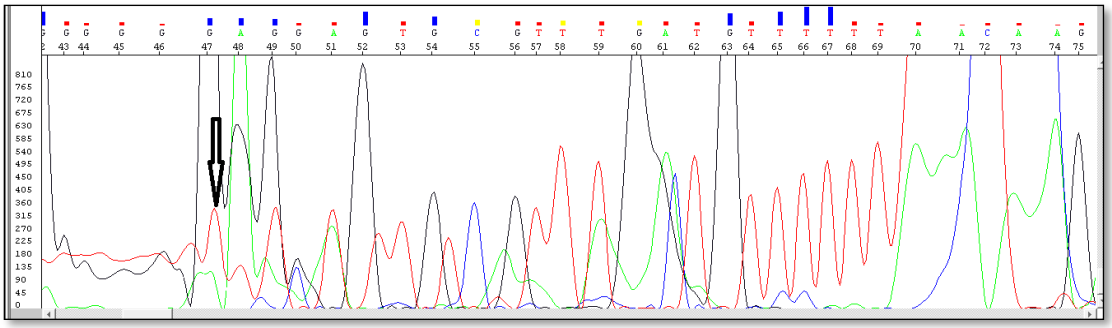
M	100 bp ladder DNA marker
CC	185bp
CT	185+203 bp
TT	203bp



(A)



(B)



(C)

Fig. 4.16 Sequence analyses of rs2227306 containing C > T polymorphism.

Sequence (A) revealed homozygous genotype CC and (B) revealed heterozygous CT individual (C) showed TT homozygous individual. Arrow indicates position of C> T inversion.

Table 4.20 (A) Genotype and (B) allele frequency of rs2227306 in control vs AMD patients group after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	58	53.3	65 (72.2)	63.3	0.0547
CT	30	39.4	21 (23.3)	24.3	
TT	12	7.3	4 (4.5)	2.3	
Var allele frequency	0.27		0.16		
χ^2	5.71		1.68		
p value	0.01		0.19		

OR= 0.7101

CI = 0.371 – 1.3592

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
C	146 (73)	151 (84)	0.0001
HWE allele frequency	0.73	0.84	
T	54 (27)	29 (16)	
HWE allele frequency	0.27	0.16	

OR = 0.5193

CI = 0.3133 – 0.8608

Table 4.21 (A) Genotype and (B) allele frequency of rs2227306 SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	28 (51)	24.9	41 (69.5)	39.1	0.0476
CT	18 (32.7)	24.2	14 (23.7)	17.9	
TT	9 (16.3)	5.9	4 (6.8)	2.1	
Var allele frequency	0.33		0.19		
χ^2	3.62		2.79		
p value	0.06		0.09		

OR= 0.5114

CI= 0.2306 – 1.1342

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	74 (67.3)	96 (81.3)	0.0150
HWE allele frequency	0.673	0.813	
T	36 (32.7)	22 (18.7)	
HWE allele frequency	0.327	0.187	

OR= 0.5901

CI= 0.3194 – 1.0902

Table 4.22 (A) Genotype and (B) allele frequency of rs2227306SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	30 (66.7)	28.8	24 (77.4)	24.4	0.2576
CT	12 (26.7)	14.4	7 (22.6)	6.2	
TT	3 (6.6)	1.8	0	0.4	
Var allele frequency	0.20		0.11		
χ^2	1.25		0.50		
p value	0.26		0.478		

OR= 0.8021

CI= 0.2751 – 2.339

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
C	72 (80)	55 (89)	0.003
HWE allele frequency	0.80	0.89	
T	18 (20)	7 (11)	
HWE allele frequency	0.20	0.11	

OR= 0.5091

CI= 0.1987 – 1.3046

Table 4.23 (A) Genotype and (B) allele frequency in patients with dry and wet type of AMD for rs2227306 after RFLP

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	43 (68.2)	42.1	22 (81.5)	21.3	0.2173
CT	17 (27)	18.8	4 (14.8)	5.3	
TT	3 (4.8)	2.1	1 (3.7)	0.3	
Var allele frequency	0.18		0.11		
χ^2	0.578		1.69		
p value	0.446		0.19		

OR = 0.5103

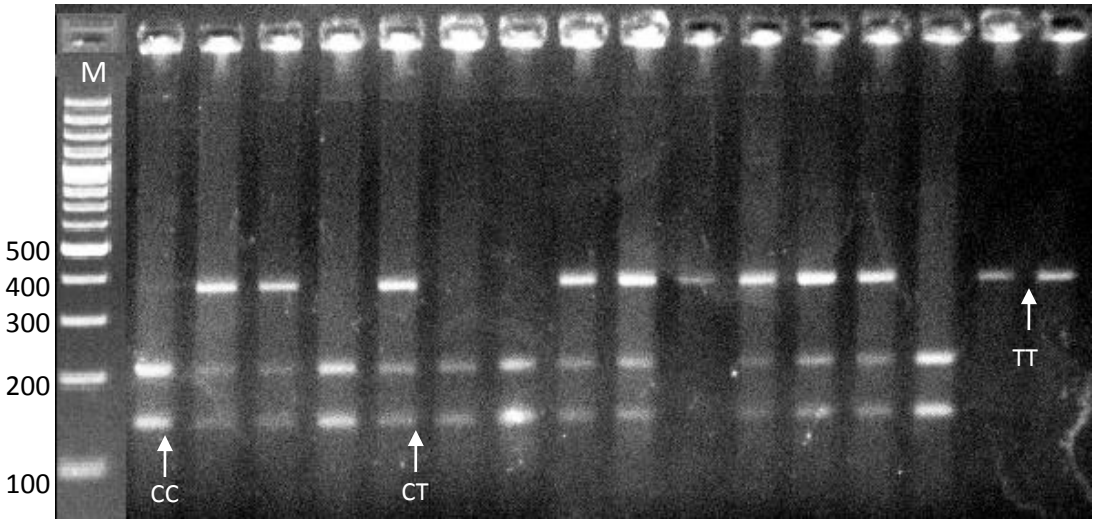
CI = 0.1768 -1.473

(B)

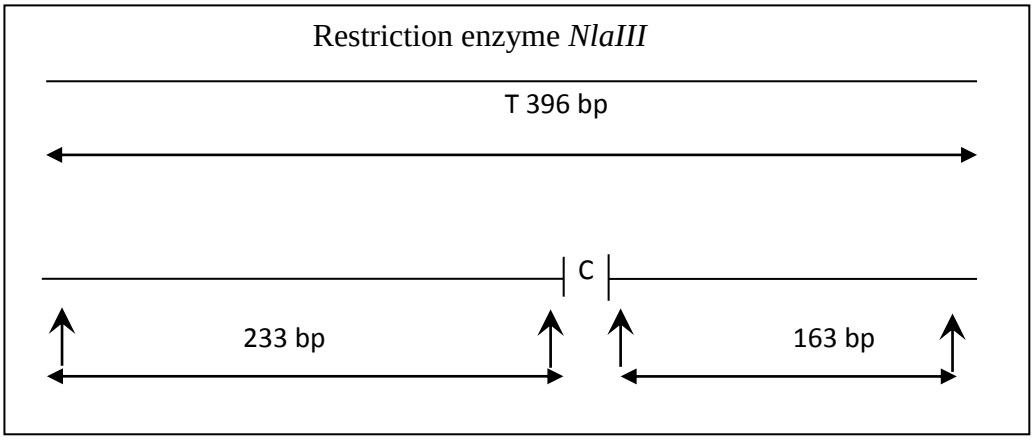
Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
C	103 (82)	48 (88.9)	0.2345
HWE allele frequency	0.82	0.889	
T	23 (18)	6 (11.1)	
HWE allele frequency	0.18	0.111	

OR = 0.5912

CI = 0.249 – 1.4038



(A)



(B)

Fig 4.17 (A) Agarose gel after RFLP showing fragmented product according to genotype for rs2227543SNP

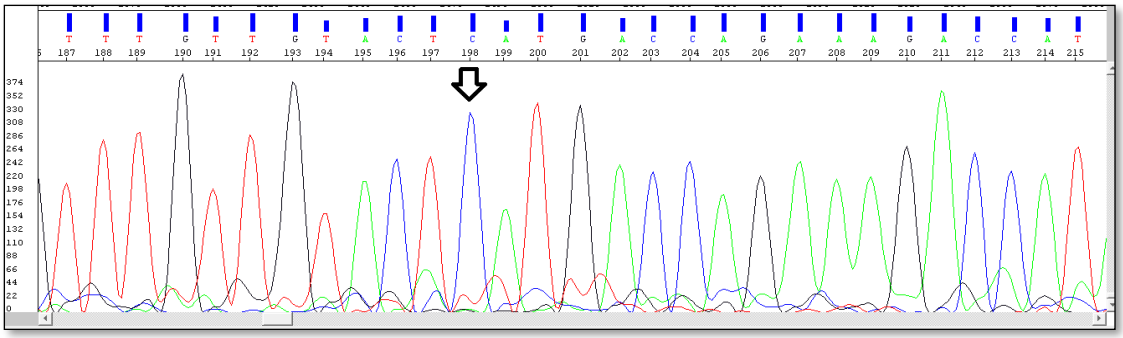
(B) Schematic diagram of rs2227543illustrating RFLP analyses

M 100 bp ladder DNA marker

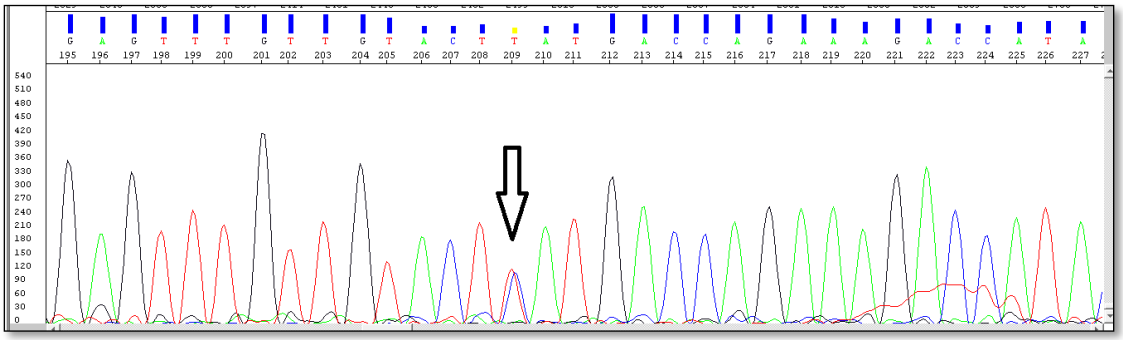
CC 233 + 163

CT 396 + 233 + 163

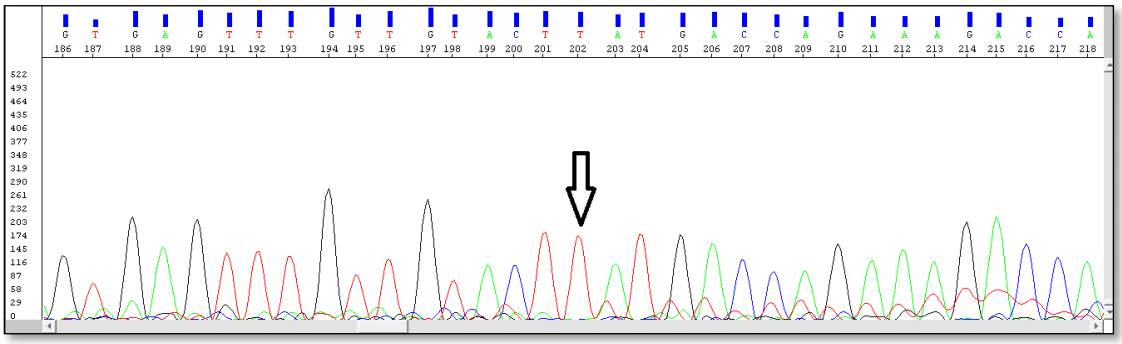
TT 396



(A)



(B)



(C)

Fig 4.18 Sequence analyses of rs2227543 containing C > T polymorphism. Sequence (A) revealed homozygous genotype CC and (B) revealed heterozygous CT individual (C) showed TT homozygous individual. Arrow indicates position of C> T inversion.

Table 4.24 (A) Genotypes frequency and (B) alleles frequencyrs2227543SNP in control and AMD patients after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	39	43.6	27(30.1)	27.8	0.0371
CT	54	44.9	46 (51.1)	44.4	
TT	7	11.5	17 (18.4)	17.8	
Var allele frequency	0.34		0.44		
χ^2	4.12		0.11		
P value	0.042		0.73		
OR= 0.8906 CI= 0.5034 – 1.5756					

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
C	132 (66)	100 (55.5)	0.0372
HWE allele frequency	0.66	0.555	
T	68 (34)	80 (44.5)	
HWE allele frequency	0.34	0.445	
OR= 1.5529 CI = 1.0257 – 2.3511			

Table 4.25 (A) Genotype and (B) allele frequency of rs2227543SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	20 (35.7)	23.6	19 (31.7)	19	0.1896
CT	32 (58.9)	24.9	29 (49.2)	29	
TT	3 (5.4)	6.6	11 (19.0)	11	
Var allele frequency	0.35		0.43		
χ^2	4.52		0.0001		
P value	0.033		0.991		

OR= 0.6948

CI= 0.3315 – 1.4563

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	72 (65.5)	67 (56.8)	0.1812
HWE allele frequency	0.655	0.568	
T	38 (34.5)	51 (43.2)	
HWE allele frequency	0.345	0.432	

OR= 1.4423

CI= 0.844 – 2.4647

Table 4.26 (A) Genotype and (B) allele frequency of rs2227543SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	19 (42.2)	20.0	8 (25.8)	8.8	0.1318
CT	22 (48.8)	20.0	17 (55)	15.4	
TT	4 (9.1)	5.0	6 (19.2)	6.8	
Var allele frequency	0.33		0.47		
χ^2	0.45		0.318		
p value	0.55		0.572		

OR= 1.2649

CI= 0.5096 – 3.1401

(B)

Allele	**Control females (%)**	**AMD females (%)**	**p value**
C	60 (66.7)	33 (53.2)	0.0962
HWE allele frequency	0.667	0.532	
T	30 (33.3)	29 (46.8)	
HWE allele frequency	0.333	0.468	

OR= 1.7546

CI= 0.9054 – 3.4002

Table 4.27 (A) Genotype and (B) allele frequency in patients with wet and dry type of AMD of rs2227543SNP after RFLP

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	18 (27.9)	18.3	9 (33.3)	9.5	0.3788
CT	32 (50.8)	31.3	14 (52.4)	13.0	
TT	13 (21.3)	13.3	4 (14.3)	4.5	
Var allele frequency	0.46		0.41		
χ^2	0.031		0.147		
p value	0.859		0.701		

OR= 1.0433

CI= 0.4233 – 2.5716

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
C	68 (54.0)	32 (59)	0.5154
HWE allele frequency	0.54	0.59	
T	58 (46.0)	22 (41)	
HWE allele frequency	0.46	0.41	

OR= 0.806

CI= 0.4224 – 1.5379

Table 4.28 Comparison of IL-8 concentration in different genotypes for IL-8 SNPs (mean ± SEM)

	Control (n=100)			AMD (n=90)			
Genotype	**IL-8 concentration (n)**	**p value**	**F value**	**IL-8 concentration (n)**	**p value**	**F value**	**T test**
rs4073							
TT	50.35 ± 1.44 (38)	0.368	1.015	55.85 ±1.0 (28)	0.340	1.099	0.0001***
AT	49.31 ± 1.37 (49)			55.81 ± 1.95 (50)			0.007**
AA	47.33± 2.65 (13)			60.30 ± 3.06 (12)			0.146
rs2227306							
CC	43.33 ± 1.22 (58)	0.125	2.148	55.93 ±1.04 (65)	0.951	0.050	0.0001***
CT	50.83± 1.70 (30)			57.71 ±1.45 (21)			0.0001***
TT	54.37 ± 2.27 (12)			56.16 ± 0.139 (04)			0.137
rs2227543							
CC	48.13 ± 1.53 (39)	0.710	0.495	54.54 ± 1.30 (27)	0.002*	6.80	0.0001***
CT	49.42 ± 1.34 (54)			54.82 ± 0.77 (46)			0.0001***
TT	52.37 ± 2.59 (07)			60.99 ±2.14 (17) a**b**			0.833

***p < 0.0001; **p < 0.001

4.21 PCR Results of rs3025039

The amplicon (PCR product) generated from the rs3025039gene was 198 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.21.1 RFLP analyses

When the PCR amplicon was digested with *HpyCH4III* restriction enzyme, it produced fragments of 112 bp and 86 bp in case of allele C while the product was not cut in case of allele T (Fig. 4.19).The validation of results was done through sequencing (Fig. 4.20).

4.21.2 Genotype and allele frequency

The frequency of genotype CC in control subjects was 85, for CT it was 11 and that of TT was 4 (χ^2= 12.98; $p < 0.0003$). In AMD patients, frequency of genotype CC was 74, CT was 9 and for TT it was 7 (χ^2= 27.36; $p < 0.0001$). The genotype frequency between the two groups was non-significantly different ($p < 0.5124$; OR= 0.899; CI= 0.3544 – 2.2807). The frequency of allele C in control subjects was 0.905 and that of allele T was 0.095. The frequency of allele C in AMD patients was 0.872 and that of allele T was 0.128 (allele frequency 93% C and 7% T in Pakistan, http://asia.ensembl.org, 2015). There was no significant difference in allele frequency between the two groups ($p < 0.310$; OR= 1.3956; CI= 0.7329-2.6577) (Table 4.29).

In male subjects of control group, the frequency of genotype CC was 47, CT was 7 and that of TT was 1 (χ^2= 1.28; $p < 0.26$). In male AMD patients, frequency of genotype CC was 46, CT was 8 and that of TT was 5 (χ^2= 13.34; $p < 0.0002$). There was non-significant difference in genotype frequency between the two groups ($p < 0.1503$; OR= 1.0748; CI= 0.3643-3.1714). The frequency of allele C in male control subjects was 0.918 and that of allele T was 0.082. The frequency of allele C in AMD male patients was 0.847 and that of allele T was 0.153. There was non-significant difference in the allele frequency between the two groups ($p < 0.1409$; OR= 1.963; CI= 0.8801 – 4.3784) (Table 4.30).

The frequency of genotype CC in control females was 38, CT was 4 and TT was 3 (χ^2= 13.6; $p < 0.0001$). In females of AMD group, frequency of genotype CC

was 28, CT was 1 and 2 had genotype TT (χ^2= 6.72; p < 0.009). There was non-significant difference between the two groups (p < 0.4938; OR= 0.4028; CI= 0.0644 - 2.519). The frequency of allele C in control females was 0.889 and that of allele T was 0.111. In AMD female patients, frequency of allele C was 0.92 and that of allele T was 0.08. There was non-significant difference in the allele frequency between the two groups (p < 0.5397; OR= 0.7116; CI= 0.2414 – 2.0977) (Table 4.31).

In patients with wet type AMD, the frequency of genotype CC was 52, CT was 6 and that of TT was 5 (χ^2= 20.50; p < 0.0001). In dry AMD patients, the frequency of genotype CC was 22, CT was 3 and that of TT was 2 (χ^2= 6.96; p < 0.008). The difference between the two groups was non-significant (p < 0.9264; OR= 1.1875; CI= 0.2742 – 5.1425). The frequency of allele C in wet AMD patients was 0.87 and that of allele T was 0.13. In dry AMD patients, frequency of allele C was 0.87 and that of allele T was 0.13. There was non-significant difference between the two groups (p < 0.6934; OR= 1.0239; CI= 0.3953 – 2.6518) (Table 4.32).

4.22 PCR Results of rs699947

The amplicon (PCR product) generated from the rs699947 gene was 324 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.22.1 RFLP analyses

When the PCR amplicon was digested with *BglII* restriction enzyme, it produced fragments of 202 bp and 122 bp in case of allele A while the product was not cut in case of allele C (Fig. 4.21). The genotype of selected samples was validated through sequencing (Fig. 4.22).

4.22.2 Genotype and allele frequency

The frequency of genotype CC in control subjects was 43, for CA it was 44 and that of AA was 13 (χ^2= 12.98; p < 0.0003). In AMD patients, frequency of genotype CC was 27, CA was 44 and for AA it was 19 (χ^2= 24.26; p < 0.0001). The genotype frequency between the two groups was non-significantly different (p < 0.052; OR= 1.0114; CI= 0.4079 – 2.5078). The frequency of allele C in control subjects was 0.65 and that of allele A was 0.35. The frequency of allele C in AMD patients was 0.54 and that of allele A was 0.46 (allele frequency 59% C and 41% A in

Pakistan, http://asia.ensembl.org,2015). The frequency of allele A in AMD patients was significantly high as compared to control subjects ($p < 0.0363$; OR=1.5539; CI= 1.0283-2.3482) (Table 4.33).

In male subjects of control group, the frequency of genotype CC was 22, CA was 26 and that of AA was 7 ($\chi^2= 0.025$; $p < 0.87$). In male AMD patients, frequency of genotype CC was 17, CA was 30 and that of AA was 12 ($\chi^2= 0.035$; $p < 0.852$). There was non-significant difference in genotype frequency between the two groups ($p < 0.2351$; OR= 1.1538; CI= 0.5531-2.4071). The frequency of allele C in male control subjects was 0.636 and that of allele A was 0.364. The frequency of allele C in AMD males was 0.542 and that of allele A was 0.458. There was non-significant difference in the allele frequency between the two groups ($p < 0.1509$; OR= 1.4766; CI= 0.8682 – 2.5114) (Table 4.34).

The frequency of genotype CC in control females was 21, CA was 18 and AA was 6 ($\chi^2= 0.45$; $p < 0.502$). In females of AMD group, frequency of genotype CC was 10, CA was 14 and that of AA was 7 ($\chi^2= 0.24$; $p < 0.623$). There was non-significant difference between the two groups ($p < 0.1301$; OR= 1.2353; CI= 0.4897–3.116). The frequency of allele C in control females was 0.667 and that of allele A was 0.333. In AMD female patients, frequency of allele C was 0.548 and that of allele A was 0.452. There was non-significant difference between the two groups ($p < 0.1421$; OR= 1.6471; CI= 0.847 – 3.2029) (Table 4.35).

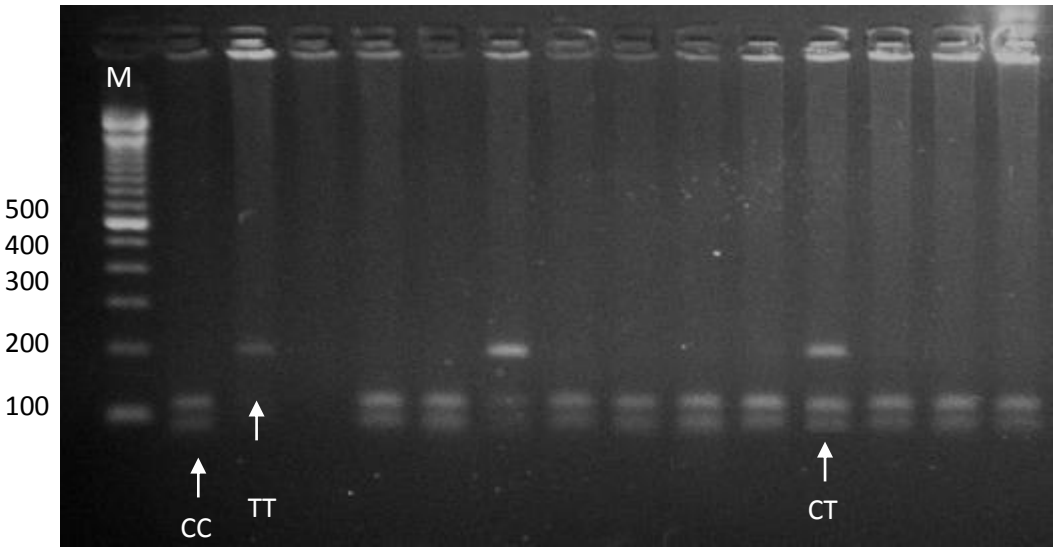
In patients with wet type AMD, the frequency of genotype CC was 17, CA was 35 and that of AA was 11 ($\chi^2= 0.927$; $p < 0.336$). In dry AMD patients, the frequency of genotype CC was 10, CA was 9 and that of AA was 8 ($\chi^2= 2.93$; $p < 0.086$). The difference between the two groups was non-significant ($p < 0.6957$; OR= 0.415; CI= 0.1693 – 1.0716). The frequency of allele C in wet AMD patients was 0.548 and that of allele A was 0.452. In dry AMD patients, frequency of allele C was 0.537 and that of allele A was 0.463. There was non-significant difference between the two groups ($p < 0.8978$; OR= 1.0433; CI= 0.5511 – 1.9754) (Table 4.36).

4.23 Relationship between Serum VEGF Levels and Genotype

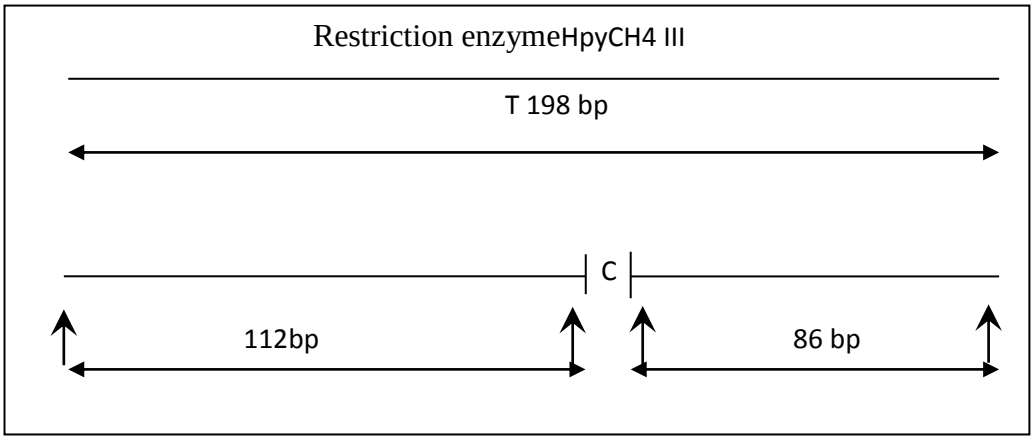
The concentration of serum VEGF was not significantly different in heterozygous and homozygous control subjects for rs3025039variant ($p < 0.62$; F= 0.481). However, significantly increased VEGF levels were found in AMD patients

with genotype TT for ($p < 0.038$; $F= 3.441$) as compared to patients with genotype CC and CT. No significant change in serum VEGF concentration was observed in any of the genotypes between control and AMD patient group (Table 4.37).

The results for rs699947showed that none of the genotype frequency was significantly different with respect to VEGF serum levels between control ($p < 0.755$; $F= 0.283$) and AMD patients ($p < 0.938$; $F= 0.396$). Likewise, serum VEGF concentration was not significantly different among the three genotypes of controls as well as AMD patients (Table 4.37).



(A)

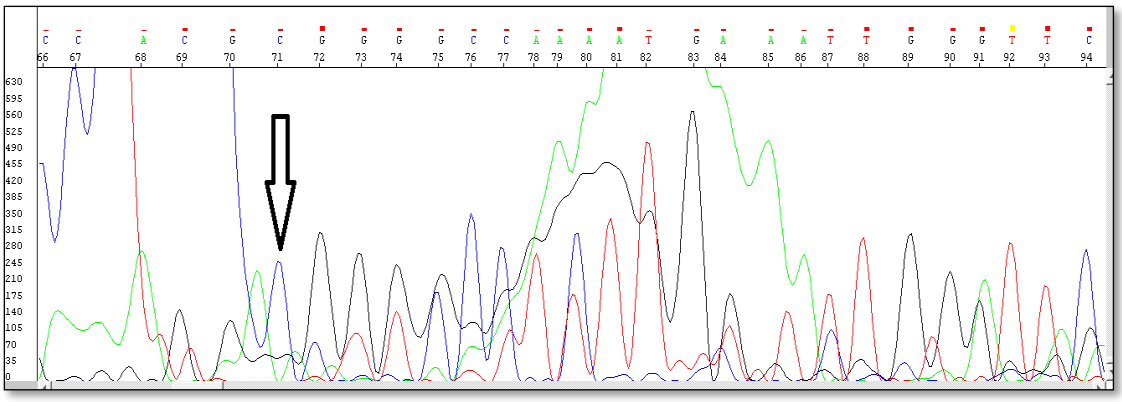


(B)

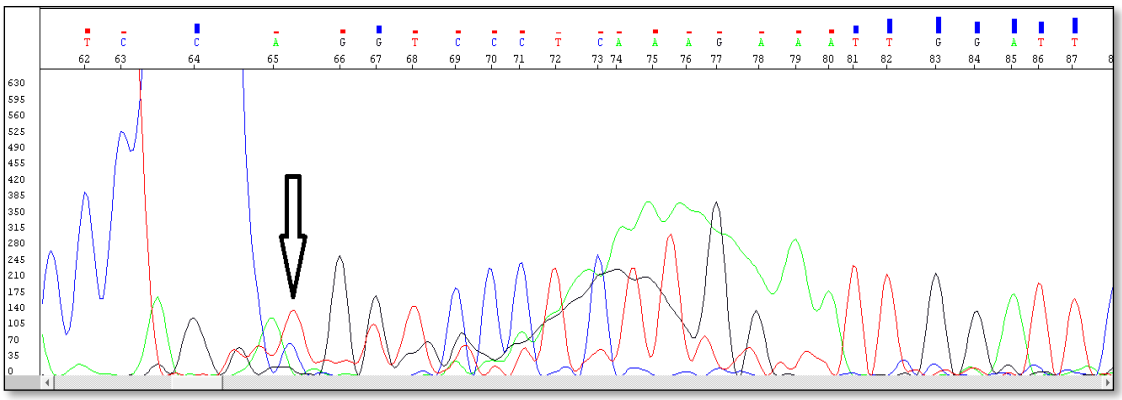
Fig 4.19 (A) Agarose gel after RFLP showing fragmented product according to genotype forrs3025039SNP

(B) Schematic diagram ofrs3025039 RFLP

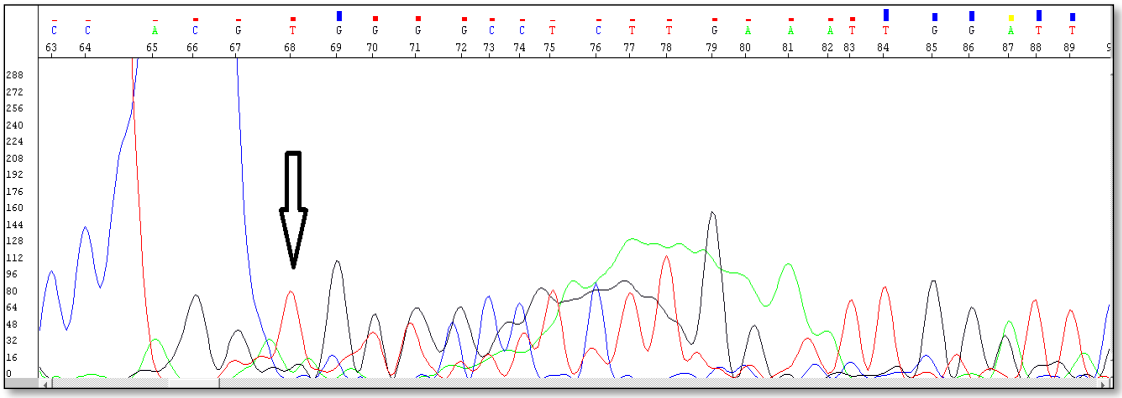
M	100 bp ladder DNA marker
CC	112 + 86 bp
CT	198+ 112 + 86 bp
TT	198 bp



(A)



(B)



(C)

Fig 4.20 Sequence analyses of rs3025039 polymorphism.

Sequence (A) revealed homozygous CC (B) revealed heterozygous CT individual (C) showed TT homozygous individual. Arrow indicates position of C> T inversion.

Table 4.29 (A) Genotype and (B) allele frequency ofrs3025039in control vs AMD patients group after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	85	81.9	74 (82.2)	68.5	0.5124
CT	11	17.2	9 (10)	20.1	
TT	4	0.9	7 (7.8)	1.5	
Var allele frequency	0.10		0.13		
χ^2	12.98		27.36		
p value	0.0003		0.0001		

OR= 0.899

CI = 0.3544 – 2.2807

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
C	181 (90.5)	157 (87.2)	0.310
HWE allele frequency	0.905	0.872	
T	19 (9.5)	23 (12.8)	
HWE allele frequency	0.095	0.128	

OR = 1.3956

CI = 0.7329 – 2.6577

Table 4.30 (A) Genotype and (B) allele frequency of rs3025039SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	47 (85.5)	46.4	46 (78)	42.4	0.1503
CT	7 (12.7)	8.3	8 (13.5)	15.3	
TT	1 (1.8)	0.4	5 (8.5)	1.4	
Var allele frequency	0.08		0.15		
χ^2	1.28		13.34		
p value	0.26		0.0002		

OR= 1.0748

CI= 0.3643 – 3.1714

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	101 (91.8)	100 (84.7)	0.1409
HWE allele frequency	0.918	0.847	
T	9 (8.2)	18 (15.3)	
HWE allele frequency	0.082	0.153	

OR= 1.963

CI= 0.8801– 4.3784

Table 4.31 (A) Genotype and (B) allele frequency ofrs3025039SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	38 (84.4)	34	28 (90.3)	26.2	0.4938
CT	4 (8.9)	11.9	1 (3.2)	4.6	
TT	3 (6.7)	1	2 (6.5)	0.2	
Var allele frequency	0.11		0.06		
χ^2	13.6		6.72		
p value	0.0001		0.009		

OR= 0.4028

CI= 0.0644 – 2.519

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	80 (88.9)	57 (92)	0.5397
HWE allele frequency	0.889	0.92	
T	10 (11.1)	5 (8)	
HWE allele frequency	0.111	0.08	

OR= 0.7116

CI= 0.2414 – 2.0977

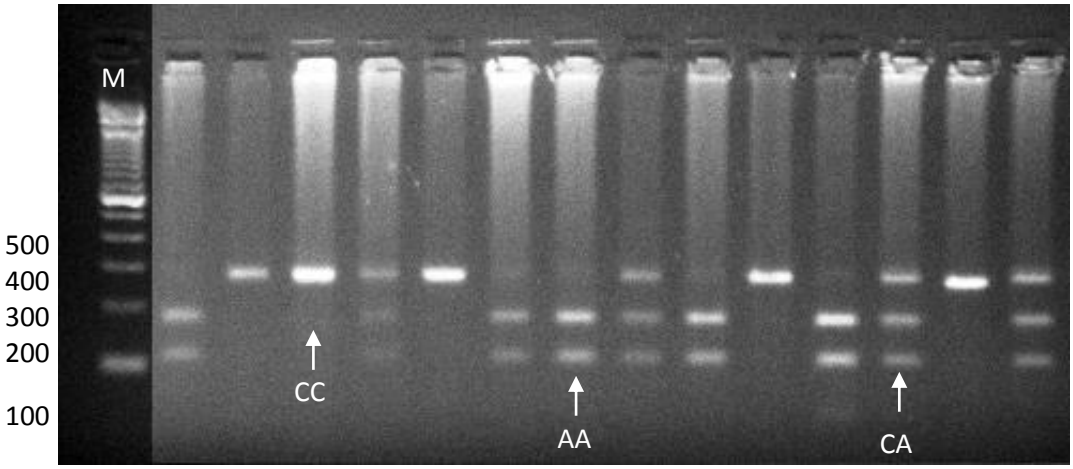
Table 4.32 (A) Genotype and (B) allele frequency of rs3025039 SNP after RFLP in wet AMD vs dry AMD patients with AMD

(A)

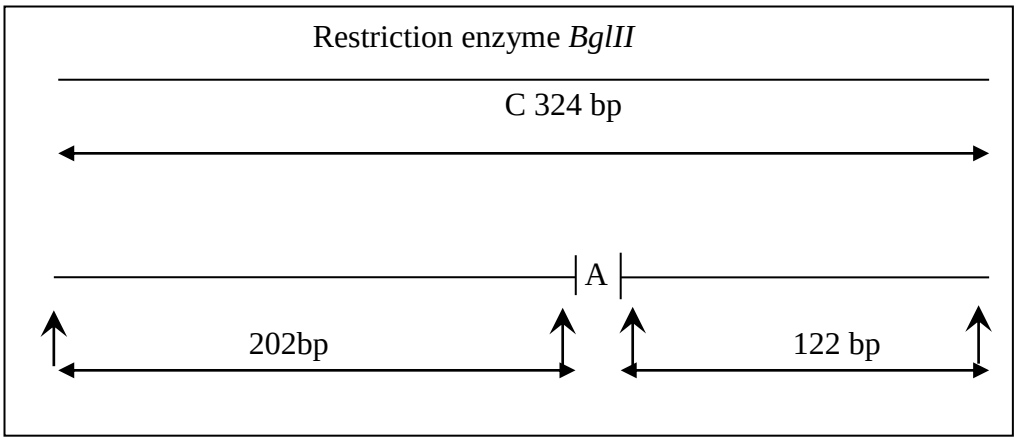
Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	52 (82.5)	0.48	22 (81.5)	20.5	0.9264
CT	6 (9.5)	14	3 (11.1)	6.1	
TT	5 (8)	1	2 (7.4)	0.5	
Var allele frequency	0.13		0.13		
χ^2	20.50		6.96		
p value	0.0001		0.008		
OR= 1.1875					
CI= 0.2742 – 5.1425					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
C	110 (87.3)	47 (87.0)	0.6934
HWE allele frequency	0.87	0.87	
T	16 (12.7)	7 (13)	
HWE allele frequency	0.13	0.13	
OR= 1.0239			
CI= 0.3953 – 2.6518			



(A)



(B)

Fig 4.21 (A) Agarose gel after RFLP showing fragmented product according to genotype for rs699947 SNP

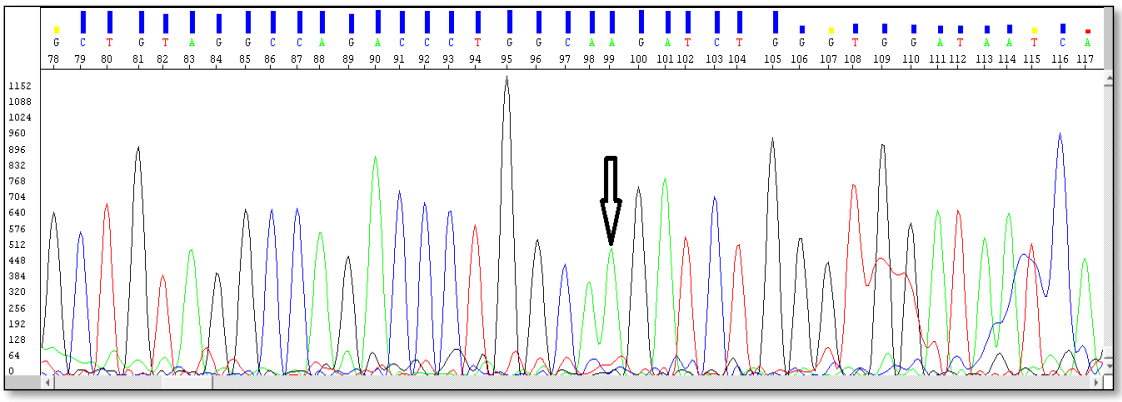
(B) Schematic diagram of rs699947RFLP

M 100 bp ladder DNA marker

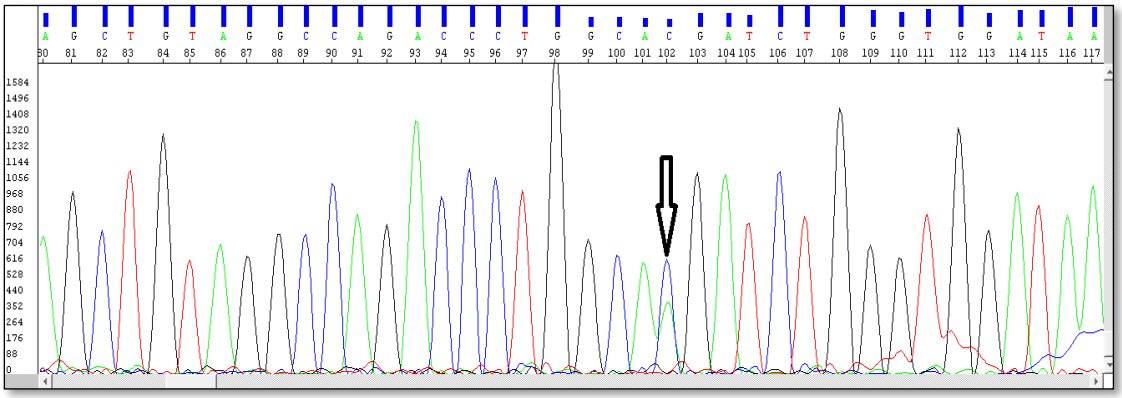
CC 324 bp

CA 324+ 202 + 122 bp

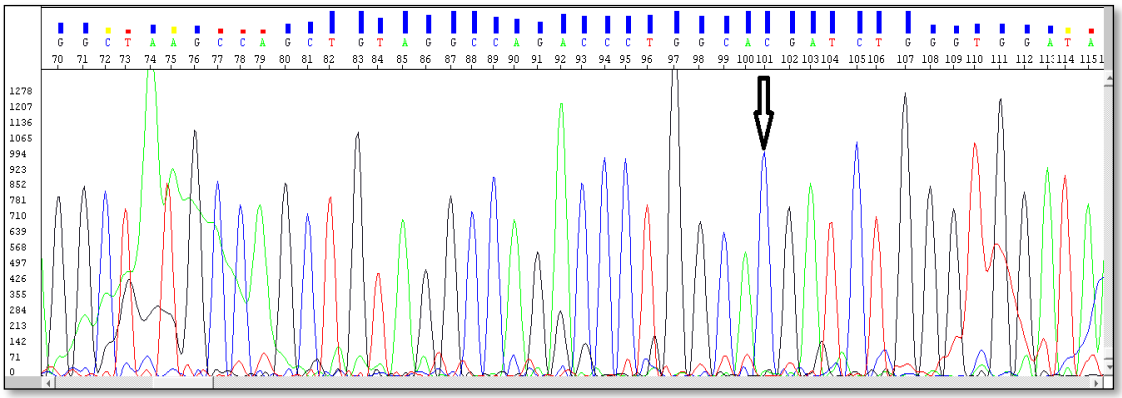
AA 202 + 122 bp



(A)



(B)



(C)

Fig 4.22 Sequence analyses of rs699947 polymorphism.

Sequence (A) revealed homozygous AA genotype (B) revealed heterozygous CA individual (C) showed CC homozygous individual. Arrow indicates position of A > C inversion.

Table 4.33 (A) Genotype and (B) allele frequency of rs699947 in control vs AMD patients group after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	43	39.8	27 (30.5)	67.6	0.052
CA	44	39.4	44 (48.4)	20.8	
AA	13	9.8	19 (21.1)	1.6	
Var allele frequency	0.10		0.13		
χ^2	12.98		24.26		
p value	0.0003		0.0001		

OR= 1.0114

CI = 0.4079 – 2.5078

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
C	130 (65)	98 (54.5)	0.0363
HWE allele frequency	0.65	0.54	
A	70 (35)	82 (45.5)	
HWE allele frequency	0.35	0.46	

OR = 1.5539

CI = 1.0283 – 2.3482

Table 4.34 (A) Genotype and (B) allele frequency ofrs699947SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	22 (40)	22.3	17 (28.8)	17.4	0.2351
CA	26 (47.3)	25.5	30 (50.8)	29.3	
AA	7 (12.7)	7.3	12 (20.4)	12.4	
Var allele frequency	0.36		0.46		
χ^2	0.025		0.035		
p value	0.87		0.852		

OR= 1.1538

CI= 0.5531 – 2.4071

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	70 (63.6)	64 (54.2)	0.1509
HWE allele frequency	0.636	0.542	
A	40 (36.4)	54 (45.8)	
HWE allele frequency	0.364	0.458	

OR= 1.4766

CI= 0.8682 – 2.5114

Table 4.35 (A) Genotype and (B) allele frequency of rs699947 SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	21 (46.7)	21	10 (32.2)	9.3	0.1301
CA	18 (40)	18	14 (45.2)	15.4	
AA	6 (13.3)	6	7 (22.6)	6.3	
Var allele frequency	0.33		0.45		
χ^2	0.45		0.24		
p value	0.502		0.623		

OR= 1.2353

CI= 0.4897 – 3.116

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
C	60 (66.7)	34 (54.8)	0.1421
HWE allele frequency	0.667	0.548	
A	30 (33.3)	28 (45.2)	
HWE allele frequency	0.333	0.452	

OR= 1.6471

CI= 0.847 – 3.2029

Table 4.36 (A) Genotype and (B) allele frequency ofrs699947SNP after RFLP in wet AMD vs dry AMD patients with AMD

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
CC	17 (27)	18.9	10 (37)	7.8	0.6957
CA	35 (55.5)	31.2	9 (33.3)	13.4	
AA	11 (17.5)	12.9	8 (29.7)	5.8	
Var allele frequency	0.45		0.46		
χ^2	0.927		2.93		
p value	0.336		0.086		
OR= 0.415 CI= 0.1693 – 1.0716					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
C	69 (54.8)	29 (53.7)	0.8978
HWE allele frequency	0.548	0.537	
A	57 (45.2)	25 (46.3)	
HWE allele frequency	0.452	0.463	
OR= 1.0433 CI= 0.5511 – 1.9754			

Table 4.37 Comparison of VEGF concentration in different genotypes for VEGF SNPs (mean ± SEM)

	Control (n=100)			AMD (n=90)			
Genotype	**VEGF concentration (n)**	**p value**	**F value**	**VEGF concentration (n)**	**p value**	**F value**	**T test**
rs3025039							
CC	103.33 ± 0.35 (85)	0.62	0.481	104.73 ± 0.39 (74)	0.038*	3.441	0.801
CT	102.59 ± 0.59 (11)			105.05 ± 1.02 (9)			0.258
TT	104.10 ± 1.29 (4)			108.34 ± 2.63 (7)			0.342
rs699947							
CC	103.63 ± 0.15 (43)	0.755	0.283	104.29 ± 0.47 (27)	0.938	0.396	0.349
AC	103.14 ± 0.40 (44)			105.45 ± 0.51 (44)			0.134
AA	103.11 ± 0.89 (13)			105.91 ± 1.73 (19)			0.295

*$p < 0.05$

4.24 PCR Results of rs1205

The amplicon (PCR product) generated from the rs1205 gene was 227 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.24.1 RFLP analyses

When the PCR amplicon was digested with *HpyCH4III* restriction enzyme, it produced fragments of 131 bp and 96 bp in case of allele C while the product was not cut in case of allele T (Fig. 4.23). Genotype was validated through sequencing of certain selected samples (Fig 4.24).

4.24.2 Genotype and allele frequency

The frequency of genotype TT in control subjects was 16, for TC it was 83 and that of CC was 1 (χ^2= 48.75; $p < 0.0001$). In AMD patients, frequency of genotype TT was 18, TC was 63 and for CC it was 9 (χ^2= 15.44; $p < 0.0001$). The genotype frequency between the two groups was non-significantly different ($p < 0.6849$; OR= 0.4779; CI= 0.2398 – 0.9524). The frequency of allele T in control subjects was 0.575 and that of allele C was 0.425. The frequency of allele T in AMD patients was 0.55 and that of allele C was 0.45 (allele frequency 25% T and 75% C in Pakistan, http://asia.ensembl.org, 2015). There was no significant difference in allele frequency between the two groups ($p < 0.342$; OR= 1.107; CI= 0.7376-1.6614) (Table 4.38).

In male subjects of control group, the frequency of genotype TT was 8, TC was 47 and none had genotype CC (χ^2= 30.61; $p < 0.0001$). In male AMD patients, genotype frequency of TT was 12, TC was 41 and that of CC was 6 (χ^2= 9.64; $p < 0.001$). There was non-significant difference in genotype frequency between the two groups ($p < 0.7184$ OR= 0.4071; CI= 0.1703 – 0.9733). The frequency of allele T in male control subjects was 0.573 and that of allele C was 0.427. The frequency of allele T in AMD male patients was 0.55 and that of allele C was 0.45. There was non-significant difference in the allele frequency between the two groups ($p < 0.7408$; OR= 1.0925; CI= 0.648 – 1.8419) (Table 4.39).

The frequency of genotype TT in control females was 8, TC was 36 and CC was 1 (χ^2= 18.41; $p < 0.0001$). In females of AMD group, frequency of genotype TT

was 6, TC was 22 and CC was 3 (χ^2= 5.81; p < 0.015). There was non-significant difference in the genotype frequency between the two groups (p < 0.6532; OR= 0.6111; CI= 0.2106 – 1.7734). The frequency of allele T in control females was 0.578 and that of allele C was 0.422. In AMD female patients, frequency of allele T was 0.548 and that of allele C was 0.452. There was non-significant difference in the allele frequency between the two groups (p < 0.7219; OR= 1.1269; CI= 0.5871 – 2.163) (Table 4.40).

In patients with wet type AMD, the frequency of genotype TT was 11, TC was 45 and that of CC was 7 (χ^2= 11.88; p < 0.0005). In dry AMD patients, the frequency of genotype TT was 7, TC was 18 and that of CC was 2 (χ^2= 3.91; p < 0.047). The difference between the two groups was non-significant (p < 0.4974; OR= 0.8; CI= 0.3036 – 2.1084). The frequency of allele T in wet AMD patients was 0.532 and that of allele C was 0.468. In dry AMD patients, frequency of allele T was 0.408 and that of allele C was 0.592. There was non-significant difference in the allele frequency between the two groups (p < 0.1278; OR= 0.6054; CI= 0.3174 – 1.1549) (Table 4.41).

4.25 PCR Results of rs1130864

The amplicon (PCR product) generated from the rs1130864gene was 181 bp long double stranded DNA fragment. The size of amplicon was estimated by comparing it with a DNA molecular size marker (100 bp ladder DNA, cat no. 11400, Norgen Biotek, Corporation, Canada) run on the same gel.

4.25.1 RFLP analyses

When the PCR amplicon was digested with *HpyCH4III* restriction enzyme, it produced fragments of 156 bp and 25 bp in case of allele A while the product was not cut in case of allele G (Fig. 4.25). The genotype of certain selected samples was validated through sequencing (Fig. 4.26).

4.25.2 Genotype and allele frequency

The frequency of genotype GG in control subjects was 60, for GA it was 37 and that of AA was 3 (χ^2= 0.924; p < 0.336). In AMD patients, frequency of genotype GG was 48, GA was 34 and for AA it was 8 (χ^2= 0.307; p < 0.58). There was no significant difference in genotype frequency between the two groups (p < 0.2411; OR= 1.0338; CI= 0.5739 – 1.8623). The frequency of allele G in control subjects was 0.785 and that of allele A was 0.215. The frequency of allele G in AMD patients was

0.722 and that of allele A was 0.278 (allele frequency 72% G and 28% A in Pakistan, http://asia.ensemble.org, 2015). No significant difference in allele frequency between the two groups was observed ($p < 0.1560$; OR= 1.4043; CI= 0.8784 -2.2452) (Table 4.42).

In male subjects of control group, the frequency of genotype GG was 32, GA was 22 and AA was 1 (χ^2= 1.636; $p < 0.201$). In male AMD patients, frequency of genotype GG was 31, GA was 22 and that of AA was 6 (χ^2= 0.489; $p < 0.484$). There was no significant difference in genotype frequency between the two groups ($p < 0.4070$; OR= 0.8919; CI= 0.4194-1.8969). The frequency of allele G in male control subjects was 0.782 and that of allele A was 0.218. The frequency of allele G in AMD male patients was 0.712 and that of allele A was 0.288. There was non-significant difference in the allele frequency between the two groups ($p < 0.2271$; OR= 1.4504; CI= 0.7937 – 2.6504) (Table 4.43).

The frequency of genotype GG in control females was 28, GA was 15 and AA was 2 (χ^2= $2.47x10^{-5}$; $p < 0.996$). In females of AMD group, frequency of genotype GG was 17, GA was 12 and AA was 2 (χ^2= 0.004; $p < 0.95$). The genotype frequency was non-significantly different between the two groups ($p < 0.5092$; OR= 1.2607; CI= 0.4878 – 3.2583). The frequency of allele G in control females was 0.789 and that of allele A was 0.211. In AMD female patients, frequency of allele G was 0.742 and that of allele A was 0.258. There was no significant difference in allele frequency between the two groups ($p < 0.5023$; OR= 1.301; CI= 0.6049 – 2.7983) (Table 4.44).

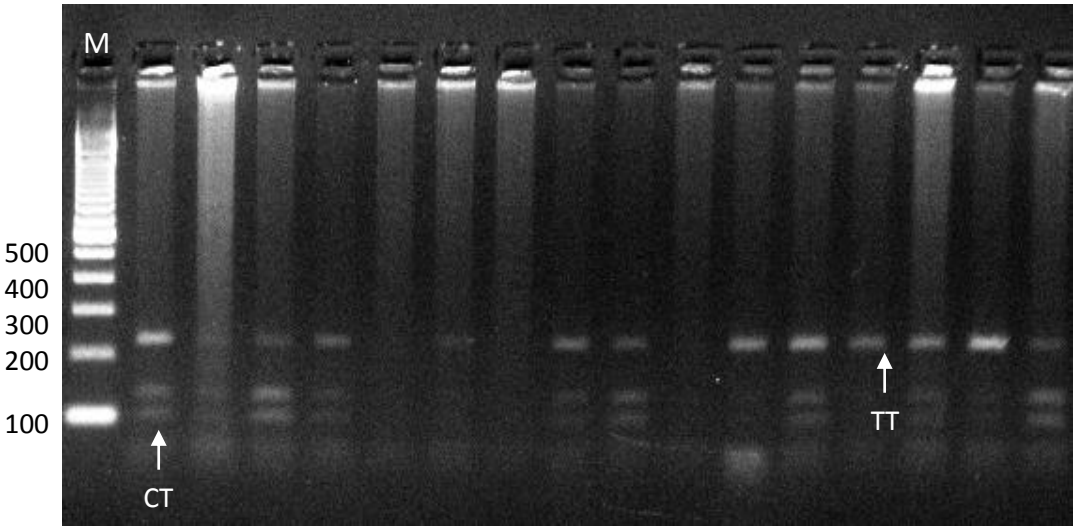
In patients with wet type AMD, the frequency of genotype GG was 36, GA was 22 and that of AA was 5 (χ^2= 0.387; $p < 0.533$). In dry AMD patients, the frequency of genotype GG was 12, GA was 12 and that of AA was 3 (χ^2= $2.63x10^{-31}$; $p < 1.0$). The difference between the two groups was non-significant ($p < 0.2748$; OR= 1.4928; CI= 0.5921 – 3.7636). The frequency of allele G in wet AMD patients was 0.746 and that of allele A was 0.254. In dry AMD patients, frequency of allele G was 0.667 and that of allele A was 0.333. There was non-significant difference between the two groups ($p < 0.4031$; OR= 1.482; CI= 0.7288 – 3.0137) (Table 4.45).

4.26 Relationship between Serum CRP Levels and Genotype

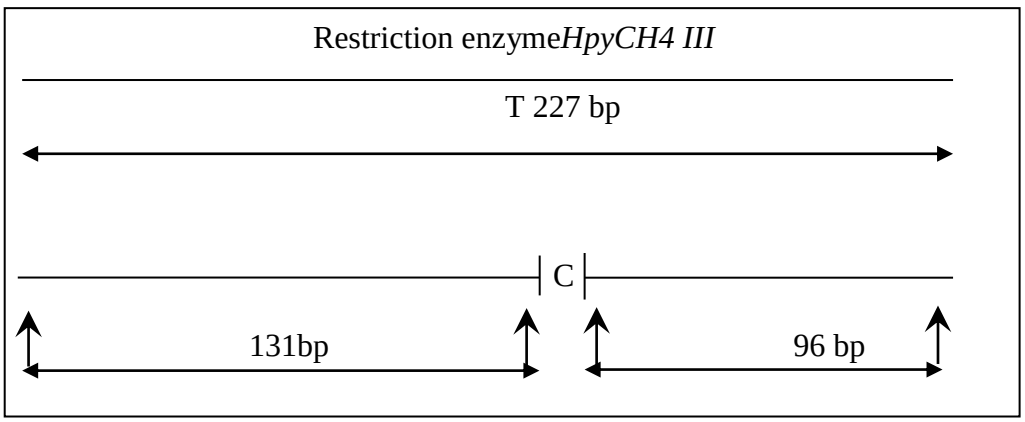
The serum concentration of CRP was not significantly different in heterozygous and homozygous control subjects for rs1205variant ($p < 0.233$; F=1.487) and AMD patients ($p < 0.618$; F= 0.484). The serum CRP level in AMD

patients with genotype TC was significantly increased as compared to control subjects with TC genotype ($p < 0.0001$) (Table 4.46).

The serum CRP levels were not significantly different in heterozygous and homozygous individuals for gene variant rs1130864in controls ($p < 0.171$; $F= 1.810$) as well as AMD patients ($p < 0.534$; $F= 0.633$). The CRP concentration in AMD patients with genotype GG was significantly elevated as compared to normal control with same genotype ($p < 0.001$). Similarly, AMD patients with genotype GA had significantly high serum CRP level as compared to control subjects with genotype GA ($p < 0.002$) (Table 4.46).



(A)

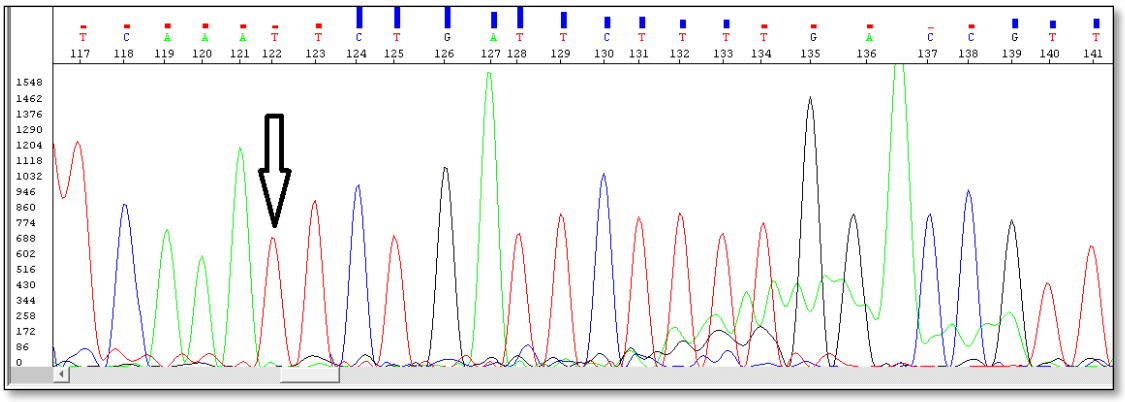


(B)

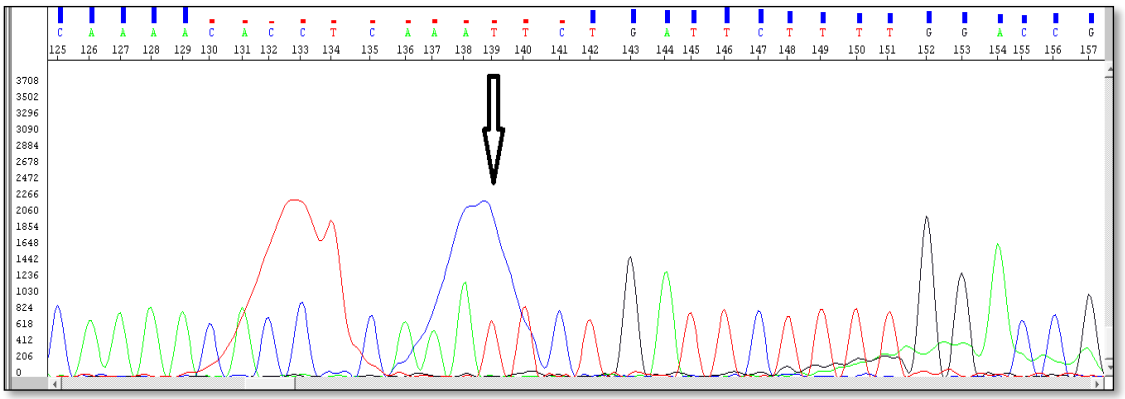
Fig 4.23 (A) Agarose gel after RFLP showing fragmented product according to genotype for rs1205SNP

(B) Schematic diagram of rs1205illustrating RFLP analyses

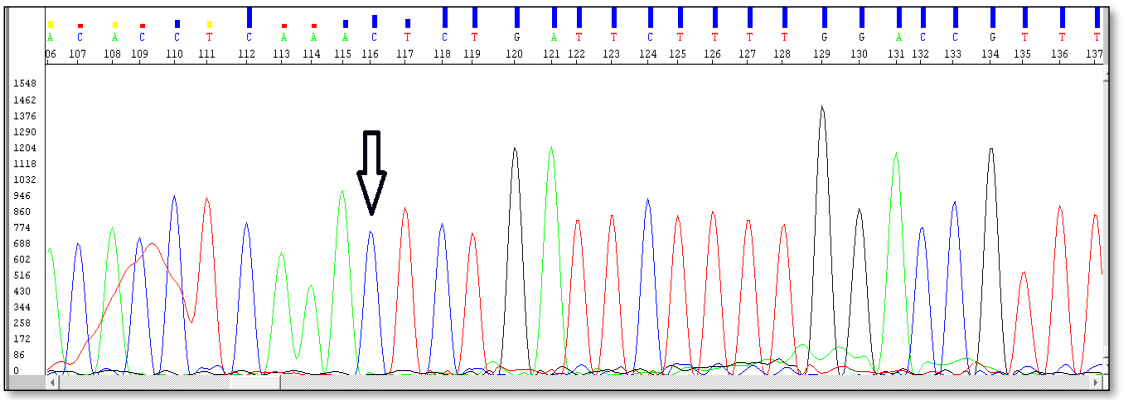
M	100 bp ladder DNA marker
TT	227 bp
CT	227 + 131 + 96 bp
CC	131 + 96 bp



(A)



(B)



(C)

Fig 4.24 Sequence analyses of rs1205polymorphism.

Sequence (A) revealed homozygous genotype TT and (B) revealed heterozygous TC individual (C) showed CC homozygous individual. Arrow indicates position of T>C inversion.

Table 4.38 (A) Genotype and (B) allele frequency of rs1205in control vs AMD patients group after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	16	33.1	18 (20)	27.2	0.6849
TC	83	48.9	63 (70)	44.6	
CC	1	18.1	9 (10)	18.2	
Var allele frequency	0.43		0.45		
χ^2	48.75		15.44		
p value	0.0001		0.0001		

OR= 0.4779

CI = 0.2398 – 0.9524

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
T	115 (57.5)	99 (55)	0.342
HWE allele frequency	0.575	0.55	
C	85 (42.5)	81 (45)	
HWE allele frequency	0.425	0.45	

OR = 1.107

CI = 0.7376 – 1.6614

Table 4.39 (A) Genotype and (B) allele frequency of rs1205SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	8 (14.5)	18	12 (20.3)	17.9	0.7184
TC	47 (85.5)	26.9	41 (69.5)	29.2	
CC	0	10	6 (10.2)	11.9	
Var allele frequency	0.43		0.45		
χ^2	30.61		9.65		
P value	0.0001		0.001		
OR= 0.4071					
CI= 0.1703 – 0.9733					

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
T	63 (57.3)	65 (55)	0.7408
HWE allele frequency	0.573	0.55	
C	47 (42.7)	53 (45)	
HWE allele frequency	0.427	0.45	
OR= 1.0925			
CI= 0.648 – 1.8419			

Table 4.40 (A) Genotype and (B) allele frequency of rs1205SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	8 (17.8)	15	6 (19.3)	9.3	0.6532
TC	36 (80)	22	22 (71)	15.4	
CC	1 (2.2)	8	3 (9.7)	6.3	
Var allele frequency	0.42		0.45		
χ^2	18.41		5.81		
p value	0.0001		0.015		

OR= 0.6111

CI= 0.2106 – 1.7734

(A)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
T	52 (57.8)	34 (54.8)	0.7219
HWE allele frequency	0.578	0.548	
C	38 (42.2)	28 (45.2)	
HWE allele frequency	0.422	0.452	

OR= 1.1269

CI= 0.5871 – 2.163

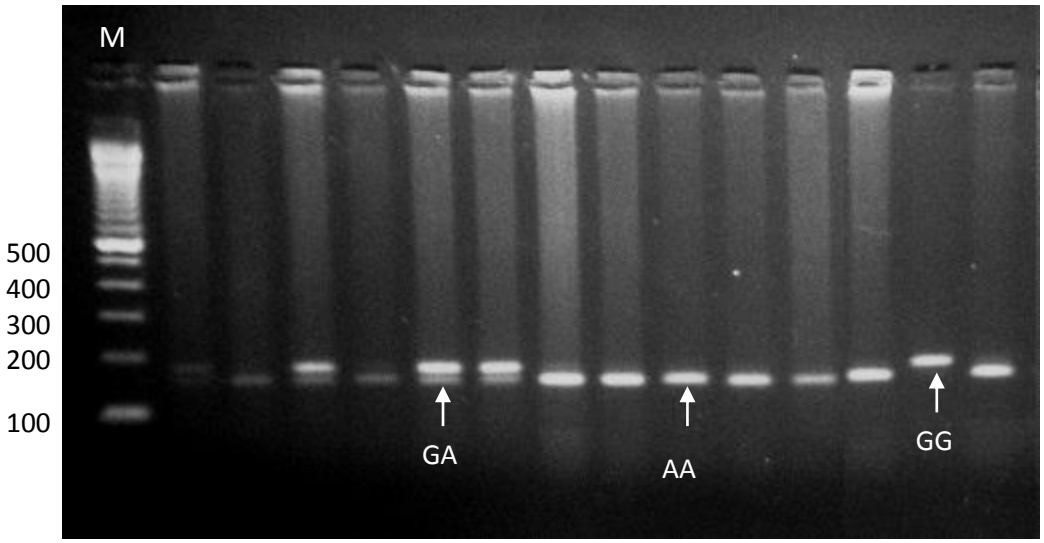
Table 4.41 (A) Genotype and (B) allele frequency of rs1205SNP after RFLP in wet AMD vs dry AMD patients with AMD

(A)

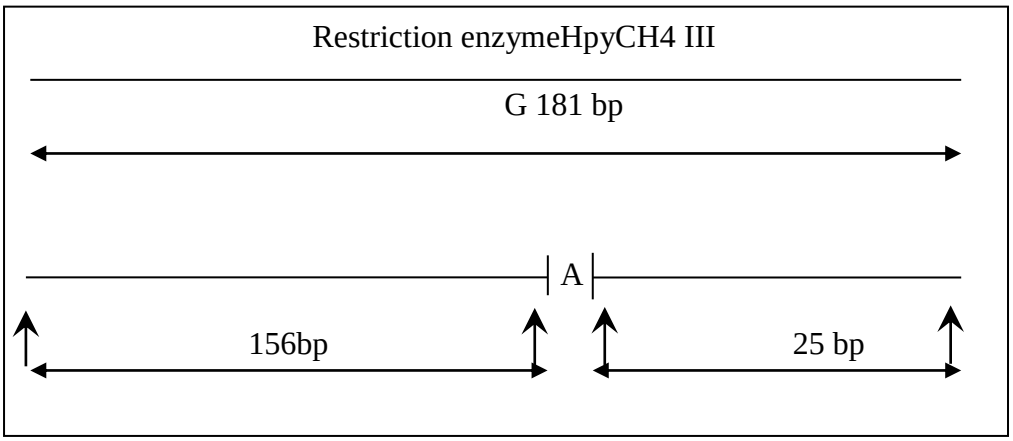
Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
TT	11 (17.4)	17.8	7 (25.9)	9.5	0.4974
TC	45 (71.4)	31.4	18 (66.7)	13	
CC	7 (11.2)	13.8	2 (7.4)	4.5	
Var allele frequency	0.47		0.41		
χ^2	11.88		3.91		
p value	0.0005		0.047		
OR=0.8					
CI= 0.3036 – 2.1084					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
T	67 (53.2)	22 (40.8)	0.1278
HWE allele frequency	0.532	0.408	
C	59 (46.8)	32 (59.2)	
HWE allele frequency	0.468	0.592	
OR= 0.6054			
CI= 0.3174 – 1.1549			



(A)



(B)

Fig 4.25 (A) Agarose gel after RFLP showing fragmented product according to genotype for rs1130864SNP

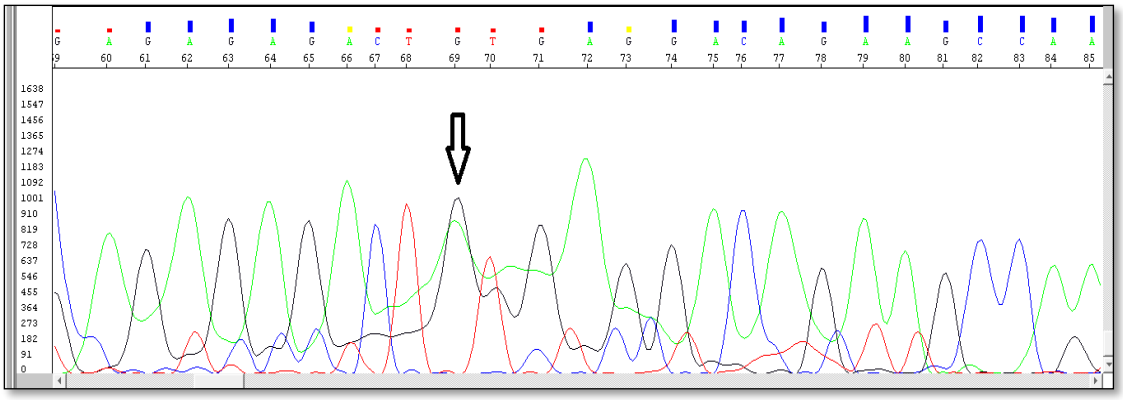
(B) Schematic diagram of rs1130864RFLP

M 100 bp ladder DNA marker

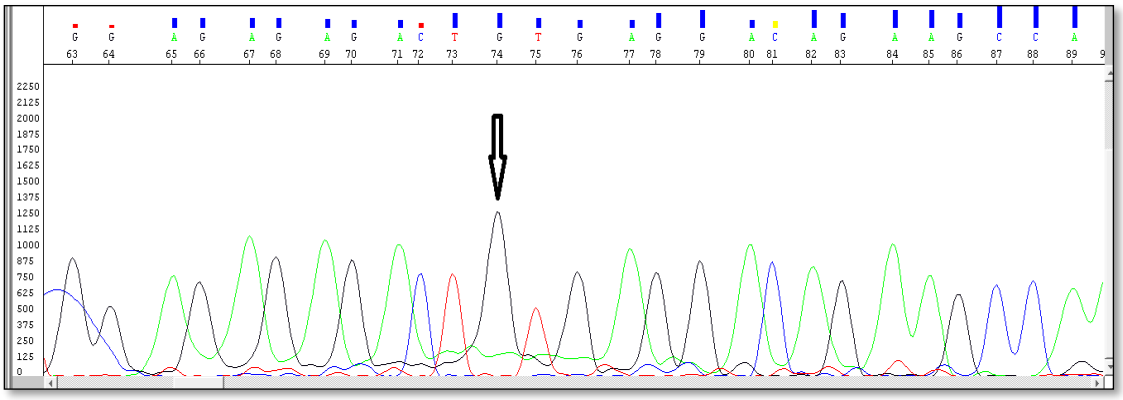
GG 181 bp

GA 181+ 156 + 25 bp

AA 156 + 25 bp



(A)



(B)

Fig. 4.26 Sequence analyses of rs1130864polymorphism.

Sequence (A) revealed heterozygous genotype GA and (B) revealed homozygous GG individual Arrow indicates position of G >A inversion.

Table 4.42 (A) Genotype and (B) allele frequency of rs1130864in control vs AMD patients group after RFLP

(A)

Genotype	**Control (%) n=100**		**AMD (%) n=90**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	60	61.6	48 (53.3)	46.9	0.2411
GA	37	33.8	34 (37.8)	36.1	
AA	3	4.6	8 (8.9)	6.9	
Var allele frequency	0.22		0.28		
χ^2	0.924		0.307		
p value	0.336		0.58		
OR= 1.0338					
CI = 0.5739 – 1.8623					

(B)

Allele	**Control (%)**	**AMD (%)**	**p value**
G	157 (78.5)	130 (72.2)	0.1560
HWE allele frequency	0.785	0.722	
A	43 (21.5)	50 (27.8)	
HWE allele frequency	0.215	0.278	
OR =1.4043			
CI = 0.8784 – 2.2452			

Table 4.43 (A) Genotype and (A) allele frequency ofrs1130864SNP after RFLP in control male vs male patients with AMD

(A)

Genotype	**Control males (%) n=55**		**AMD males (%) n=59**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	32 (58.2)	33.6	31 (52.5)	29.9	0.4070
GA	22 (40)	18.8	22 (37.3)	24.2	
AA	1 (1.8)	2.6	6 (10.2)	4.9	
Var allele frequency	0.22		0.29		
χ^2	1.636		0.489		
p value	0.201		0.484		
OR= 0.8919					
CI= 0.4194 – 1.8969					

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
G	86 (78.2)	84 (71.2)	0.2271
HWE allele frequency	0.782	0.712	
A	24 (21.8)	34 (28.8)	
HWE allele frequency	0.218	0.288	
OR= 1.4504			
CI= 0.7937 – 2.6504			

Table 4.44 (A) Genotype and (B) allele frequency rs1130864SNP after RFLP in control female vs female patients with AMD

(A)

Genotype	**Control females (%) n=45**		**AMD females (%) n=31**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	28 (62.2)	28	17(54.8)	17.1	0.5092
GA	15 (33.3)	15	12 (38.7)	11.9	
AA	2 (4.5)	2	2 (6.4)	2.1	
Var allele frequency	0.21		0.26		
χ^2	2.47×10^{-5}		0.004		
p value	0.996		0.95		
OR= 1.2607					
CI= 0.4878 – 3.2583					

(B)

Allele	**Control males (%)**	**AMD males (%)**	**p value**
G	71 (78.9)	46 (74.2)	0.5023
HWE allele frequency	0.789	0.742	
A	19 (21.1)	16 (25.8)	
HWE allele frequency	0.211	0.258	
OR= 1.301			
CI= 0.6049 – 2.7983			

Table 4.45 (A) Genotype and (B) allele frequency of rs1130864SNP after RFLP in wet AMD vs dry AMD patients with AMD

(A)

Genotype	**Wet AMD (%) n=63**		**Dry AMD (%) n=27**		**p value**
	Observed	**Expected**	**Observed**	**Expected**	
GG	36 (57.1)	35.1	12 (44.4)	12	0.2748
GA	22 (35)	23.9	12 (44.4)	12	
AA	5 (7.9)	4.1	3 (11.2)	3	
Var allele frequency	0.25		0.33		
χ^2	0.387		$2.63x10^{-31}$		
p value	0.533		1.0		
OR= 1.4928					
CI= 0.5921 – 3.7636					

(B)

Allele	**Wet AMD (%)**	**Dry AMD (%)**	**p value**
G	94 (74.6)	36 (66.7)	0.4031
HWE allele frequency	0.746	0.667	
A	32 (25.4)	18 (33.3)	
HWE allele frequency	0.254	0.333	
OR= 1.482			
CI= 0.7288– 3.0137			

Table 4.46 Comparison of CRP concentration in different genotypes for CRP SNPs (mean ± SEM)

	Control (n=100)			AMD (n=90)			
Genotype	**CRP concentration (n)**	**p value**	**F value**	**CRP concentration (n)**	**p value**	**F value**	**T test**
rs1205							
TT	4.05 ± 0.96 (16)	0.233	1.487	4.72 ± 0.94 (18)	0.618	0.484	0.856
TC	2.67± 0.29 (83)			5.97 ± 0.59 (63)			0.0001***
CC	2.84 (1)			6.13 ± 1.62 (9)			0.123
rs1130864							
GG	2.80 ± 0.35 (60)	0.171	1.810	5.50 ± 0.64 (48)	0.534	0.633	0.001**
GA	2.79 ± 0.43(37)			5.89 ± 0.76 (34)			0.002**
AA	5.97 ± 3.44 (3)			7.32 ± 2.13 (8)			0.905

***$p < 0.0001$; **$p < 0.01$

Discussion

The present study was carried out to investigate the association ofgenotypes of inflammatory markers and angiogenic factors with the risk of AMDoccurrence in patients from Pakistan. A significant association was observed between AMD and SNPs in IL-6, IL-8 and VEGF genes. However, no significant association was observed between AMD and genetic variants of CRP. A genetic analysis of AMD is a challenge due to several reasons. One is that the symptoms appear relatively late in life therefore the parents and older siblings of the index patient are usually no more living and the descendants are too young to show symptoms. In addition, the genetic study on AMD is further complicated by the fact that it is a heterogeneous disease with both neovascular and nonvascular forms. In addition, phenotypic characterization of AMD progression still needs refinement, complicating the efforts to correlate genotype and phenotype (Swaroop et al., 2007).

There was significant difference in genotype and allele frequency for rs1800795 between control subjects and AMD patients. Moreover, AMD patients with genotype GG had significantly higher serum IL-6 levels as compared to those with genotype GC and CC. Elevated serum IL-6 levels have been reported to be associated with genotype GG for -174 G > C by Cornateanu et al (2006). This polymorphism is functionally important, since it influences the transcription rate of the gene and the plasma concentration of the IL-6 protein (Terry et al, 2000). The transcriptional activity of IL-6 gene and plasma IL-6 are associated with a single G > C exchange polymorphism at -174 position (Pascual et al., 2000). The presence of IL-6 -174 G > C polymorphism has been suggested to be involved in some autoimmune and inflammatory diseases like diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis and juvenile chronic arthritis (Fernandez-Real et al., 2000; Schotte et al, 2000; Vasku et al. 2003).

No significant change was observed in genotype and allele frequency between AMD patients and control subjects for SNP rs1800796. However, in case of SNP IL -6 597 G>A frequency of genotype GG was significantly high in AMD patients as compared to control subjects. The results were same when comparison was made between male as well as femalessubjects. Serum concentration of IL-6 did not show any significant variation as regards the genotype of AMD patients and control subjects forrs1800796and rs1800797gene variants. Previous studies show complete

linkage disequilibrium in IL-6 -597 and -174 (Fedetz et al., 2001; Villuendas et al., 2002).

In the current study, there was no significant difference in genotype and allele frequency for SNP rs4073between AMD patients and the control subjects. Genotype frequency of genotype TT was significantly low in AMD female patients as compared to control females. However, non-significantly elevated IL-8 levels were observed in AMD patients with genotype AA. The -251 A > T polymorphism is present in the promotor zone of IL-8 gene that affects its expression. The mutant allele A is less common but its presence is related to increased production of IL-8 cytokine (Vairaktaris et al., 2007) as observed in current set of AMD patients.

No significant difference in genotype frequency was the outcome in case of SNP rs2227306but the frequency of allele C was significantly high in AMD patients (both males and females) as compared to control subjects. However, no significant difference in allele and genotype frequency was observed between wet and dry AMD patients. Forrs2227543, the genotype frequency of TT was significantly high in AMD patients as compared to control subjects. However, no such significance was observed in comparison of AMD males with control males and AMD females with control females. The serum IL-8 levels were significantly high in AMD patients with genotype TT as compared to AMD patients with genotype CC and CT for rs2227543. Therefore, presence of genotype TT forrs2227543increased the risk of occurrence of AMD in Pakistani patients.

Genetic polymorphisms in several cytokine genes have been demonstrated to influence gene transcription, leading to inter-individual variations in cytokine production. Therefore, it is reasonable to speculate that genetic polymorphisms may be important determinants of pathogenesis of AMD. IL-8 is a multifunctional cytokine and a member of the family of chemokines. It is involved in acute and chronic inflammatory processes (Tsai et al., 2008). IL-8 is an important regulator of the inflammatory response as it recruits and activates acute inflammatory cells. It mediates the activation and migration of neutrophils. This cytokine is produced early in inflammation and its presence persists for a longer period of time (Zhang et al., 2014).

Currently, no significant difference was observed in genotype frequency for rs3025039between control and AMD patients. Similarly, in case of rs699947, no

significant difference in genotype frequency was the outcome when compared between AMD patients and control subjects. In addition, the serum VEGF levels were significantly high in AMD patients with genotype TT for SNP rs3025039 as compared to AMD patients with genotype CC and CT therefore; presence of genotype TT increased the risk of disease. Similar were the findings in a study by Lin et al. (2008) who reported that allele T in SNP rs3025039 was significantly increased in Chinese wet AMD patients as compared to the controls. However, this association was not replicated in the other groups, i.e., in one Caucasian population (Watson et al., 2010) and one Chinese population (Stevens et al., 2003).

Contrarily, it has been reported in a meta-analysis that the polymorphisms in SNPs in VEGF-A gene including rs2010963 and rs3025039 were not a risk factor for AMD. When stratifying for the subtype of the disease, there was no significant difference in genotype distribution between wet AMD cases and controls (Huang et al., 2013). Several studies report that VEGF polymorphism is associated with the VEGF production, however the results are inconsistent. Renner et al., (2011) reported that -936T allele is associated with lower VEGF plasma levels. Similar were the findings by Kripppl et al., (2003) when lower plasma VEGF levels were related to -936T allele in breast cancer patients.

Presently, when genotype frequency for CRP gene SNP 1846 G > A was compared between control and AMD subjects, no significant difference was observed. Similarly, the genotype frequency was non-significantly different between AMD patients and normal control subjects when compared for SNP 1444 C > T. Serum CRP levels also did not change significantly with respect to the genotype. It has been reported that CRP is directly responsible for macular damage through complement mediated mechanisms, especially in cases with CFH-Y402H gene polymorphism (Vine et al., 2005). However, mechanism of CRP influence on AMD is yet not fully understood (Nita, 2014).

It has been suggested that SNP in a candidate gene that shows strongest association with the disease is likely to be most causally related. Moreover, subjects with two copies of the variant related to the risk are at more risk to develop the disease than the ones with a single copy. In addition, the association of studies carried out in past and future are important considerations. Consistency in different population is a strong evidence of causality. Likewise, absence of replication in other

studies on other populations does not necessarily indicate lack of causality (Tabor et al., 2002).

The current investigation provides a clue for the possibility that genetic variation in inflammatory markers is related to incidence of AMD. The findings of the study will be confirmed when the country wide surveys will be carried out and meta-analyses of small regional studies are done combining the data of thousands of AMD patients and controls. In addition, consideration of gene polymorphisms for CRP, HTRA1, ApoE and leptin are required to be studied for the same population. The study on interrelationship between inflammatory system and AMD is worthy for future investigations. The identification of SNPs in AMD patients will open the door for a variety of new and potentially fruitful avenues of investigation to be utilized in future for effective therapy.

CHAPTER # 5
GENERAL DISCUSSION

GENERAL DISCUSSION

Age related macular degeneration (AMD) is characterized by the occurrence of damage to inner layers of retina as a consequence of aging process. The factors and mechanisms that might lead to AMD are neither fully understood nor well-defined but deficiency of anti-oxidants, accumulation of toxic material in the retinal pigment epithelium (RPE), genetic and some angiogenic factors are proven to be involved in the emergence of AMD (Evans, 2001). In this regard, the role of immune processes has also been highlighted in the development, progression and treatment of AMD (Ambati et al., 2013).

In addition to the undefinedaetiology, limited number of therapeutic measures available for AMD has motivated the need of vigorous research, to identify the role of risk factors in the pathology of AMD.Estimation of these factors in the serum samples of affected patients along with the identification of single nucleotide polymorphisms (SNPs) in a sample from Pakistani population was the focus of present study.The present study was designed and carried out to study the role of different angiogenic growth factors and mediators of inflammation in AMD. Apart from scant studies on the prevalence of AMD, no comprehensive studies have been undertaken on AMD in Pakistan.

In addition to total lipid profile, serum levels of IL-6, IL-8, ApoE, CRP, leptin, VEGF, CFH and HTRA1 were analysed in AMD patients and age matched control subjects. The SNPs in the genes of IL-6, IL-8, VEGF and CRP were also investigated and genotype and allele frequencies between AMD patients and control subjects were compared.

5.1 Risk Factors for AMD

The risk factors for wet AMD where intervention might be possible include current smoking, hypertension, greater caloric intake, low serum carotenoids, low serum antioxidant index, elevated serum cholesterol levels and light exposure, whereas, positive family history and light color of iris are non-modifiable risk factors. However, for dry type of AMD, elevated vitamin A levels, high caloric intake and smoking are controllable risk factors while positive family history and lighter iris color cannot be modified like in case of wet AMD.

Extensive research in western countries has been devoted to define the risk factors for AMD (Berrow et al., 2011). In comparison, literature especially from eastern developing countries is scant, so much so that authentic reports on simple biochemical parameters known to be involved in AMD are lacking. In the current data collection, 64% AMD patients were in the age range of 60-79 years; above this age the survival is relatively low, as the average life expectancy in Pakistan is 67.05 (males 64.9; females 66.9) years (https://www.cia.gov 2014). Some other studies like the Beaver Dam Study have shown similar findings where the cumulative incidence of late AMD was upto 8% in people having age ≥ 75 years (Klein et al., 2007).

Currently, the gender ratio was not significantly different between the control and diseased groups however, number of males was significantly greater in the AMD patient group compared to females. No significant difference was found between the two genders as regards in dry or wet type of AMD. In contrast, studies undertaken in other parts of the world have shown females to be at greater risk of developing AMD (Ferris et al., 1984). This deviation is likely due to the fact that females in Pakistan, report less incidentally in the hospitals due to the closed social set up. However, on the whole, the number of patients having wet type AMD was greater as compared to those with dry AMD. This finding comes parallel to a previous another report by Klaver et al. (1998).

In the present population, addiction was found significantly related to the risk of geographic atrophy. It has been reported in a report from United Kingdom that cigarette pack per year is strongly associated with the risk of both GA and CNV therefore smoking increases the risk of AMD in addition to the genetic factors (Khan et al., 2006).

5.2 Lipid Profile

Although none of the presently investigated subjects was suffering from any systemic disease like hypertension or diabetes, a significant elevation in serum total cholesterol concentration in female AMD patients, predicted elevated serum cholesterol level to be a key risk factor for developing AMD especially in females. A study by Reynolds et al. (2010) also reported thathigher levels of total cholesterol in AMD patients irrespective of sex are associated with the risk of developing AMD. Colak et al. (2011) have also shown that elevated plasma lipid concentration could play an important role in the development of AMD in elderly people.

Presently, care was taken to include subjects from the same ethnic background to circumvent aspect of ethnic variability in the assessment of lipid profile, such that the difference between the two groups was based solely on the incidence of AMD in the diseased group. A little bias in the selection of subjects could have however occurred due to socioeconomic and demographic factors which could not be ruled out. Furthermore, cases and controls were screened from the same institute and with same ophthalmologic practices according to a similar protocol; in addition, all subjects were same as regards the distribution of country of residence.

Cholesterol level is known to raise due to some diet rich in saturated fats, smoking, sedentary life style and increased visceral fats (Despres, 2007). Previous studies haveassociated AMD with moderate and severe hypertension particularly in patients receiving antihypertensive treatment (Hyman et al., 2000). The present is the first study carried out in subjects with normal blood pressure which reports elevated cholesterol levels in female AMD patients as compared to normal females. A positive correlation of serum cholesterol and AMD has been shown by the Eye Disease Case-Control Study Group (Parekhet al., 2009) and Belds et al, (2001). In contrast, some other studies found either no association (Delcourt et al., 2001) or negative association as Klein et al, (1993) found lowered cholesterol levels in AMD patients.

In the present AMD patients, LDL, HDL and triglyceride concentrations were found correlated with total cholesterol. It has been shown that oxidized LDL that gets deposited in the BM, may lead to neovascularization possibly by two mechanisms; one is direct as it causes the release of angiogenic cytokines, while the other is indirect as it causes damage or death of RPE cells that are responsible to inhibit angiogenesis by producing inhibitory substances (Spaide et al., 1999).

Additionally, it is also worth mentioning that there is inconsistency as regards the cholesterol lowering medications in relation to AMD. Rotterdam and several other studies did not find any relationship between cholesterol lowering medication, including statins, and the risk of AMD (Pauleikhoffet al., 1990; Klein et al., 2003). In contrast, McGwin et al. (2003) studied the same in a relatively larger population and found significant relationship between AMD and the use of cholesterol lowering medications.

5.3 Angiogenic Growth Factors and Inflammatory Mediators

As regards the inflammatory cytokines and growth factors associated with AMD, the present study on AMD patients revealed elevation of serum ApoE, IL-6, IL-8, HTRA1, CRP and VEGF levels, while a reduction was noticeable in serum leptin and CFH levels. It is already known that inflammatory proteins and angiogenic factors play prominent role in the progression of disease in the form of increased number of drusen, geographic atrophy (GA) and neovascularization, all leading to a complete loss of central vision (Patel and Chen, 2008; Campa et al., 2010).

Elevated levels of proinflammatory cytokines including the IL-6 and IL-8 are in fact related to the production of reactive oxygen species (ROS) that in turn leads to the production of such cytokines. A positive feedback is thus created that not only promotes the inflammatory process but also enhances vascularization thus contributing to disease progression (Szmitko et al., 2003). I*n vitro* and *in vivo* studies have further shown that due to its proangiogenic properties, elevated concentration of serum IL-8 is associated with elevated vascularity that occur in CNV during AMD (Higgins et al., 2003). It promotes inflammatory and angiogenic processes in cornea, retina and orbit (Ghasemi et al., 2011). In this regard, the present is the first study that reports changes in the levels of IL-8 in the serum of AMD patients.

CFH is another regulator of inflammatory pathways that prevents the formation of C3 convertase enzyme and enhances its dissociation (Ricklin et al., 2010). Over recent years, several studies have indicated that mutations in complement gene are involved in AMD pathology, although underlying mechanism is still unclear (Kopp et al., 2012). CFH SNP was reported to be strongly associated with AMD (Edwards et al., 2005), but in another study no difference was observed in AMD patients with CFH SNP (Klein et al., 2005). In the present AMD patients, serum CFH levels were found significantly decreased. Reduced CFH concentration appears to be associated with other risk factors for AMD such as, hypertension and obesity (Silva et al., 2012).

CRP has been established as a marker for systemic inflammation (Seddon et al., 2010).Elevated CRP levels that were observed in the serum of present AMD patients indicated that AMD is in fact a chronic systemic inflammatory disease associated with age.Logistic regressionanalyses demonstrated that elevation of serum CRP increases the risk of developing AMD. CFH His402 variant has shown elevated

CRP levels. Currently, CRP was negatively correlated to CFH. It has been suggested that high CRP levels and insufficient CFH at retina or choroid may lead to uncontrolled complement activation associated with cell and tissue damage (Bhutto et al., 2011). Drusen in AMD have also been reported to express CRP (Johnson et al., 2006). In contrast, another study showed that plasma CRP levels were not changed in the AMD patients as compared to controls and were not influenced by the CFH SNP or the age of a patient (Silva et al., 2012).

Presently, leptin levels were found significantly reduced in AMD patients under study as compared to normal controls. Leptin has a direct metabolic effect on lipoprotein metabolism or lipase activity (Auwerx and Staels, 1998). In case of leptin deficiency, fatty acid synthesis would increase due to the inhibition of mitochondrial fatty acid uptake and oxidation resulting into elevated intracellular fatty acids and triglyceride levels. Thus intracellular lipid accumulation in RPE is an indicative of occurrence of AMD in the form of Bruch's membrane deposits and drusen. Reduced leptin levels are therefore expected to be a causative factor in the pathogenesis of AMD leading to enhanced inflammation acting through lipid peroxidation modulation (Evereklioglu et al., 2003).

Presently, ApoE levels were found elevated in AMD patients. Studies have shown that ApoE4 is protective SNP against AMD. The lipoproteins that pass through and accumulate in RPE and Bruch's membrane provide substrate for initial changes like drusen formation in the onset of AMD. These deposits also provide substrate for chronic inflammation. The evidence is provided by the presence of ApoE in drusen (Miller, 2013), the present study provides indication for the association of serum ApoE levels with AMD pathology. The underlying mechanism of ApoE might be the altered regulation of VEGF in RPE cells by the ApoE isoforms (Bojanowski et al., 2006).

In current AMD patients, serum VEGF was found significantly increased as compared to normal subjects. VEGF is produced as a result of hypoxia and is elevated in the adjacent necrotic areas. Retina expresses VEGF receptors but the exact role of VEGF in retina and eye is still not clear (Grisanti and Tatar, 2008). A previous study by Lip et al. (2001) has suggested involvement of angiogenic markers like VEGF in the homeostasis and endothelial dysfunction in the pathogenesis of AMD.

The present study relates inflammatory markers ApoE, leptin, IL-6, IL-8, CRP and CFH with the pathogenesis of AMD. It is the first report from Pakistan that has revealed changes in serum levels of the inflammatory markers and angiogenic factors. The study further suggests that AMD is in fact a disease that involves a number of processes leading to inflammation and angiogenesis.

5.4 Genetic Archaeology of Pakistan

The region of Pakistan has an ancient history with respect to population diversity. This area of the world has been under use as a highway by many immigrants and emigrants. It has been used as a corridor for the dispersal of modern humans as well (Cann, 2001). Therefore, this region has enormous linguistic, cultural and genetic diversity.

The Indo-Aryan migration is a theory based on the migration of Aryans from the central Asia who migrated to the northern part of Indian subcontinent more than two millennia ago. The primary basis of Aryan immigration theory is the linguistics of the population, although evidence through skeletal examination is lacking. Craniometric data have shown similarity of the people of this area with Iranian plateau and Western Asia (Ratnagar, 1995).

The geographical location of Pakistan including its southern coasts is proposed to be the route followed by modern *Homo sapiens* from Africa and is therefore the area that was perhaps inhabited by modern humans as early as 60,000–70,000 years ago. Fragmentary fossil evidence of cave dwellers has been found in Northwest of Pakistan (Hussain, 1997). At Mehargarh, in Southwest of Pakistan, Neolithic settlements of 7,000 BC have been discovered (Jarrige, 1991). It was followed by the Indus Valley Civilization that flourished in the 3rd and 2nd millennia BC (Dales, 1991). The Indo-European nomadic tribes called Aryans crossed Hindu Kush mountain range to enter Subcontinent. Later the area was invaded by Alexander the Great (327-325 BC) and Arab conquerors from 711 AD onwards (Wolpert, 2000).

The studies of near past show that the Indian subcontinent has been subjected to a series of Indo-European migrations around 1500 BC. The earliest inhabitants in India are supposed to be the Austro-Asiatic tribes; later Indo-European tribes drove the Dravidian speaking tribes to the south. The major influx was in the North Western

areas that also include the regions included in Pakistan after partition of the Sub-continent in 1947 (Basu et al., 2003).

The current population of Pakistan counts more than 180 million individuals belonging to at least 18 different ethnic groups speaking more than 60 languages (Grimes, 2006). Mostly these languages are Indo-European but may also include isolated groups like a Dravidian language called Burushaski and Brahui which is a Sino-Tibetan language. Punjabi is the language spoken by majority of Pakistanis although it represents multiple ethnic groups and castes (Ibbetson, 1883).

Accordingly, Pakistan is the area that contains combination of genes of different origins. Mutations of these genes along with the traditional consanguineous marriages have led to the accretion of gene pools and their transmission to next generations. This suggests complexity in the origin of local inhabitants with admixture of migrations, including those from Central Asia on one hand and farther East on the other hand. The diversity in the haplogroups in Y chromosome in Pakistani population having Baruhi origin supports novel gene pool of the area (Qamar et al., 2002).

Currently, SNPs of genes of inflammatory and angiogenic factors were studied in AMD patients, who mainly belonged to Punjab Province. Although AMD is suggested to be an inflammatory disease but its relationship with inflammation is still under debate. Since gene transcription is influenced by genetic polymorphism of several cytokines, inter-individual variations therefore lead to variation in cytokine production. Hence, assumption of genetic polymorphism is an important determinant of AMD pathogenesis (Tsai et al., 2008).

5.5.1 Interleukin gene polymorphism

In the present case control study, six SNPs of two interleukins were examined to determine whether these were related to the increased risk of AMD. Present data showed that presence of allele C in SNP rs1800795 increases the risk of AMD in Pakistani population. In addition, allele A in SNP rs1800797 is a risk related allele. These SNPs are present in the promoter region of IL-6 gene (Tsai et al., 2008). Variation in these regions is related to functional alteration of the protein product which ultimately leads to AMD pathology. The polymorphism in rs1800795 in IL-6

gene has already been found to be associated with altered plasma levels of IL-6 in healthy subjects (Fishman et al., 1998).

Frequency of allele C in SNP rs2227306 for IL-8 gene in AMD patients was significantly higher as compared to control subjects that suggestit to be a risk related allele for AMD. In addition, for SNP rs2227543, allele G appears to be a risk related allele as its frequency was higher in AMD patients as compared to normal controls. IL-8 gene is located on chromosome 4, area q13-q21 that consists of three introns and four exons (Mukaida et al., 1989). A significant elevation of serum IL-8 levels in AMD patients indicates that these SNPs might be functional variants of the gene. It has been shown previously that significantly greater IL-8 concentrations were found in the vitreous of AMD patients having genotype TT for rs4073 (Tsai et al., 2008).

5.5.2 VEGF gene polymorphism

Being an important angiogenic factor VEGF has been shown to enhance the progressive exudative AMD (Boltz et al., 2012). Many SNPs are shown by VEGF gene involved in quantitative and qualitative expression of VEGF protein (Watson et al., 2000; Renner et al., 2001; Ruggiero et al., 2011).

In the current study, elevated serum VEGF levels were observed in AMD patients with allele T for VEGF SNP 936 C > T. However, no other variation in VEGF gene was found related to serum levels of VEGF. A meta-analyses study suggests that VEGF polymorphisms are associated with enhanced risk of AMD, particularly the wet type of AMD (Liu et al., 2014).

Variation in serum VEGF levels is related to the VEGF gene polymorphism including 634 C > G variant increasing susceptibility of the carrier to diseases like diabetic retinopathy (Petrovic et al., 2008). Recently, a study on Tunisian population showed that VEGF genetic variants contribute to vulnerability of subjects to AMD and their response to anti-VEGF treatment (Habibi et al., 2014). These findings were related to the study carried out on Polish population by Janik-papis et al. (2009) and in Taiwan Chinese population by Lin et al. (2008). However, Boltz et al. (2012) did n**o**t find any relationship between VEGF SNPs and response of patients to anti-VEGF therapy in exudative AMD patients. Likewise, in the Rotterdam study and Anglo-Celtic subpopulation, VEGF gene polymorphism and chances of AMD occurrence could not be confirmed (Richardson et al., 2007; Boekhoorn et al., 2008; Haas et al.,

2011). These conflicting findings might be due to variation on sample size, heterogeneity of patients and choice of analyzed SNPs located in the promoter region along with methods used to study genotypes. It is now established that cumulative effect of several predictive alleles identified in the full haplotype information determines genetic susceptibility to complex diseases like AMD (Habibi et al., 2014).

The population aged 50 years or more is the largest growing part of society and so is the burden of senility related diseases like AMD in this millennium. Considering the financial and social burden, identification of risk factors with improved therapeutic measures to treat AMD pathology is required. Many different approaches are in focus to treat AMD including anti-angiogenic therapy and transplant surgery (Zarbin, 2004). The current study adds to the information for eye care professionals to counsel their patients regarding AMD risk factors.

Aetiological research has suggested that AMD is a complex disease which is caused by actions and interactions of multiple environmental and genetic factors. From a molecular perspective, changes in DNA sequence represent either a normal variation or a mutation but from a medical perspective, a change in DNA sequence represents the development of a pathological condition (Donoso et al., 2006). It may involve change of a single nucleotide or an entire chromosome. SNPs represent by far the most common DNA sequence variation (Li, 2001).

5.5.3 CRP gene polymorphism

Currently no influence of CRP genotype was observed on the AMD pathology as regards the studied SNPs including -1846 A >G and -1444 C > T. Kim et al. (2008) acquired similar results regarding CRP gene in wet AMD patients and suggested that genetic susceptibility of an individual in complex diseases like AMD is the result of cumulative effect of several predictive alleles studied in the full haplotype information rather than a single allelic variation. Brull et al., (2003) reported that CRP SNP -1444 C > T contributes to CRP phenotype but there is uncertainty in the findings as yet. CRP is considered as an inflammatory marker and is also involved in cardiovascular diseases and atherosclerosis. Its involvement in endothelial dysfunction has also been suggested. It has been reported previously that CRP levels show a heritable characteristic. By the identification of genetic variations in CRP gene some risk related genotypes can be worked out that will add to the information for overall risk estimation (Verma et al., 2004).

Conclusion

The present study concludes that elevated levels of cholesterol are directly related to AMD pathogenesis in Pakistani subjects. The present study shows that inflammatory markers viz., ApoE, HTRA1, leptin, IL-6, IL-8, VEGF, CRP and CFH are the factors involved in AMD pathogenesis. It is suggested that AMD is in fact an inflammatory pathology that involves a number of processes leading to inflammation and angiogenesis. A significant association of IL-6, IL-8 and VEGF gene polymorphism with AMD in Pakistani population was observed. However, the number of patients and controls was relatively less so it does not represent the whole population of the country. Nevertheless, it shows a trend towards higher expression of IL-6, IL-8 and VEGF in the sera of AMD patients having risk related alleles.

FUTURE PROSPECTS

The current study does not cover the patients from the whole country, although the tertiary care hospital from where samples of patientswere collected covers a large catchment area, but still many major districts of Pakistani could not be covered for the current study. Pakistan is a multi-ethnic country with a population of about 120 million. Several ethnic groups including Punjabis, Saraikis, Sindhis, Muhajirs, Pashtuns, Balochis, Hindkowans, Gujarati and Chitralis with other smaller groups live in Pakistan. In addition, approximately 1.7 million Afghan refugees live in Pakistan. The majority of Afghans in Pakistan are ethnic Pakhtuns from southeastern Afghanistan, who have settled in Pakistan due to the civil war in their home country. Further rigorous functional studies are required to confirm the investigations with larger and diverse samples from the same population so that the evidence of role of inflammatory and angiogenic factors is confirmed in the pathogenesis of AMD. The sampling of patients is required to be extended to primary, secondary and tertiary health care units in the far-off areas of the country in order to cover most of the population.

Social awareness regarding risk factors for AMD is also required. Family marriages need to be discouraged and, population counselling as regards signs and symptoms of AMD is required for timely diagnosis of the disease. Therapy at an early stage of the disease has better outcome rather than late stage when the therapy becomes least possible and the patient gets permanently blind.

In future, studies on the association of gene polymorphism for HTRA1, ApoE, CFH and leptin are required to be investigated in the same population. The estimation of inflammatory factors and angiogenic factors in the vitreous humor of AMD patients will provide a direct evidence of role of inflammation in AMD. The expression of these factors can be studied through immunohistochemistry in cadaveric eyes to confirm the presence of these factors in AMD eyes. It is predicted that the therapeutic agents focusing specific complement-modulation agents and nonspecific anti-inflammatory drugs might prove effective to treat AMD.

Publications from Current Study

Full Length Papers

1. **Fareeha Ambreen**, Irfan Zia Qureshi and Muhammad Ismail. (2015). Association of serum levels of inflammatory and angiogenic factors with gene polymorphism in Pakistani patients with age related macular degeneration. *Molecular Vision,* 21:985-999. (Impact factor: 1.986)
2. **Fareeha Ambreen**, Wajid Ali Khan, Nadeem Qureshi and Irfan Zia Qureshi. (2014). Assessment of serum lipids in patients with age related macular degeneration from Pakistan. *Journal of Pakistan Medical Association*, 64: 664-669. (Impact Factor: 0.409)
3. Irfan Zia Qureshi and **Fareeha Ambreen**. Serum APOE, leptin, CFH and HTRA1 levels in Pakistani age related macular degeneration patients. *Journal of Pakistan Medical Association*. In process.

Conference Presentations

1. **Fareeha Ambreen**, Irfan Z. Qureshi. Inflammatory Markers and Angiogenic Mediators in Patients with Age Related Macular Degeneration from Pakistan. AOPT (Association of Ocular Pharmacology and Therapeutics) 12th Scientific Meeting. Feb 26th- March 1st, 2015, Charleston, South Carolina, USA.
2. **Fareeha Ambreen**, Wajid Ali Khan, Nadeem Qureshi, Shahnaz Murtaza and Irfan Zia Qureshi. Effect of Age related Macular Degeneration on Lipid Profile: A study in Pakistani Patients. Pakistan Congress of Zoology. Pakistan Museum of Natural History, Islamabad. 2nd – 4th April, 2013.
3. **Fareeha Ambreen** and Irfan Zia Qureshi. (2012). Determination of Lipid Profile in Pakistani AMD Patients. National Science Conference. PMAS Arid Agriculture University, Rawalpindi, Pakistan. 10th – 12th January, 2012.

APPENDIX I

STUDY PERFORMA

Dear fellow,

A study is being conducted on the biochemical and genetic polymorphism of Age Related Macular Degeneration (AMD) in Pakistani subjects. For this we need some personnel information from the subjects suffering from this disease along with normal controls. It is assured that the information provided will be kept confidential.

Subject Identity

1. Hospital no. _____
2. Patient study no._____

Anthropometric Information:

3. Name: _____
4. Sex: Male ☐ Female ☐
5. Province:_____
6. Address & contact number: _____
7. Current Age: _____
8. Marital status: Single ☐ Married ☐
9. Parental relationship: Family ☐ Non-family ☐

Clinical History

10. Diabetes Mellitus Yes☐ No ☐ If yes for how long _____
11. Hypertension Yes☐ No ☐ If yes for how long _____
12. Smoking Yes☐ No ☐ If yes for how long _____
13. Any other systemic disease Yes☐ No ☐If yes for how long _____

Information Regarding AMD Diagnosis

14. Any other family member suffering from AMD Yes☐ No ☐
15. Age at onset of disease: _____
16. Unaided visual acuity Left eye _____ Right eye _____
17. Visual acuity corrected Left eye _____ Right eye _____
18. Slit lamp examination:
    a. Anterior segment: _____
    b. Fundus: _____
19. Optic nerve and macular examination: _____

20. Fundus fluorescence angiography findings: _____
21. Optical coherence tomography: _____
22. Presence of small drusen: Yes ☐ No ☐
23. Presence of large drusen: Yes ☐ No ☐
24. Presence of coarse RPE changes: Yes ☐ No ☐
25. Presence of Geographic atrophy: Yes ☐ No ☐
26. Presence of CNV: Yes ☐ No ☐
27. Type of AMD : Dry ☐ Wet ☐
28. Pattern of AMD: Maculopahty ☐ Atrophic☐ Exudative ☐
29. Associated anomaly: _____
30. Treatment given: _____
31. Comments: _____

_____

INFORMED CONSENT

I agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally AcceptableRepresentative:

_____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date: _____/_____/_____

Name of the Witness: _____

Thank you very much for your cooperation.

APPENDIX II

Solution Preparation for DNA Extraction

Phenol preparation

Solidified phenol was warmed at 65°C in a water bath (Julabo SW22, Germany) and equal quantity of distilled water (dH_2O) was added to phenol solution. It was kept in dark for overnight. Next day, water was separated from the top and fresh dH_2O was added again. The process was repeated three times and then aliquots were made and frozen.

Sucrose

Sucrose (MW=342.2 g) solution of 0.32M was prepared by dissolving 1.1 g sucrose in 100 ml dH_2O.

Ethidium Bromide

It was prepared by mixing 10 mg of ethidium bromide per ml of dH_2O. It was stored at 4 ºC.

Bromophenol blue

Bromophenol blue (25 mg) and 4 g of sucrose was added to 10 ml of dH_2O. It was stored at 4 ºC.

10X TBE buffer

Tris-base (Promega, USA) 107.8 gm/litre

Boric acid (Promega, USA) 55.02 gm/litre

EDTA (Ethylene diamine tetra acetic acid) (Bio-Rad, USA) 9.04 gm/litre

All the chemicals were added to 800 ml dH_2O and mixed with magnetic stirrer. The pH was raised to 8.4. the final volume of solution was raised to 1000 ml.

1% Agarose Solution

For agarose gel (1%), 1g of agarose was dissolved in 100 ml 1x TBE buffer.

For agarose gel (2%), 2g of agarose was dissolved in 100 ml 1X TBE buffer.

For agarose gel (3%), 3g of agarose was dissolved in 100 ml 1X TBE buffer.

Composition of the solutions used in DNA Extraction

Cell Lysis Buffer

This buffer is composed three chemicals as:

i. $KHCO_3$ (Potassium Carbonate) 1gm/L
ii. NH_4Cl (Ammonium Chloride) 8.29gm/L
iii. 0.5 M EDTA (Ethylene Diamine Tetra Acetate) 0.34gm

Cell lysis buffer cleaves the cell membranes and exposes the chromatin material of the nucleated cells.

STE (Saline Tris EDTA) buffer

This buffer provides a saline environment to the newly exposed chromatin material

i. 3M NaCl (Sodium Chloride) 33.3ml
ii. 1M Tris-HCl buffer (pH8.0) 4.0ml
iii. 0.5M EDTA (pH8.0) 2.0ml

The above reagents were mixed and volume was made up to 1 liter with dH_2O.

DNA Dissolving Buffer

This buffer is used for dissolving the DNA and is composed of

i. 10mM Tris-HCl (pH8.0)
ii. 0.1mM EDTA

SDS (Sodium Dodecyl Sulfate) solution

Solution of sodium dodecyl sulfate or sodium lauryl sulfate (10%) helps in protein degrading and also the activity of proteinase K in enhanced in its presence.

Chloroform-Isoamyl Alcohol

This solution is made in 24:1 v/v for 500 ml total volume as:

i. Chloroform 480ml (24)
ii. Isoamyl alcohol 20ml (1)

REFERENCES

REFERENCES

Abalain, J. H., Carre, J. L., Leglise, D., Robinet, A., Legall, F., Meskar, A., Floch, H. H. &Colin, J. (2002). Is age-related macular degeneration associated with serum lipoprotein and lipoparticle levels? *Clinica Chimica Acta*, **326**(1): 97-104.

Adamis, A. P., Shima, D. T., Yeo, K. T., Yeo, T. K., Brown, L. F., Berse, B., Damore, P. A., &Folkman, J. (1993). Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. *Biochemical and Biophysical Research Communications*, **193**(2): 631-638.

Age-Related Eye Disease Study Research Group. (2000). 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*, **107**(12): 2224-2232.

Age-Related Eye Disease Study Research Group. (2005). The Age-Related Eye Disease Study severity scale for age-related macular degeneration: AREDS report no. 17. *Archives of Ophthalmology*, **123**(11): 1484-1498.

Aiello, L. P., Northrup, J. M., Keyt, B. A., Takagi, H., & Iwamoto, M. A. (1995). Hypoxic regulation of vascular endothelial growth factor in retinal cells. *Archives of Ophthalmology*, **113**(12): 1538-1544.

Ambati, J., Ambati, B. K., Yoo, S. H., Ianchulev, S., & Adamis, A. P. (2003). Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. *Survey of Ophthalmology*, **48**(3): 257-293.

Ambati, J., Atkinson, J. P., &Gelfand, B. D. (2013). Immunology of age-related macular degeneration. *Nature Reviews Immunology*, **13**(6): 438-451.

Amin, H. I., McDonal, R. H., Johnson, R. N., AI, E., &Schatz, H. (2006). Acquired macular diseases. In: Duane's ophthalmology. Lippincott Williams and Wilkins, USA.

Amin, R., Puklin, J. E., & Frank, R. N. (1994). Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **35**(8): 3178-3188.

Anderson, D. H., Mullins, R. F., Hageman, G. S., &Johnson, L. V. (2002). A role for local inflammation in the formation of drusen in the aging eye. *American Journal of Ophthalmology*, **134**(3): 411-431.

Anderson, D. H., Ozaki, S., Nealon, M., Neitz, J., Mullins, R. F., Hageman, G. S., & Johnson, L. V. (2001). Local cellular sources of apolipoprotein E in the human retina and retinal pigmented epithelium: implications for the process of drusen formation. *American Journal of Ophthalmology*, **131**(6): 767-781.

Applegate, R. A. (2000). Limits to vision: can we do better than nature? *Journal of Refractive Surgery*, **16**(5): S547-S551.

Apte, R. S. (2010). Regulation of angiogenesis by macrophages. In: Retinal Degenerative Diseases. Anderson, R. E., Hollyfield, j. G., LaVail, M. M. (eds.) Springer, New York, USA. pp. 15-19.

AREDS (Age-Related Eye Disease Study Research Group). (2001). A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Archives of Ophthalmology*,**119**: 1417–1436.

Arstila, A. U., Smith, M. A., &Trump, B. F. (1972). Microsomal lipid peroxidation: morphological characterization. *Science*, **175**(4021): 530-533.

Asensio-Sanchez, V. M., Rodriguez-Martin, T., Gala-Molina, I., & Rodriguez-Fernandez, I. (2006). Age-related macular degeneration: its association with the epsilon4 allele of the apolipoprotein E gene. *Archivos de la Sociedad Española de Oftalmología*, **81**(1): 9–12.

Auwerx, J., & Staels, B. (1998). Leptin.The *Lancet*, **351**(9104): 737-742.

Bai, J., Lin, M., Zeng, X., Zhang, Y., Wang, Z., Shen, J., Jiang L., Gao, F., & Chen, Q. (2008). Association of polymorphisms in the human IFN-γ and IL-4 gene with oral lichen planus: a study in an ethnic Chinese cohort. *Journal of Interferon & Cytokine Research*, **28**(6): 351-358.

Baird, P. N., Guida, E., Chu, D. T., Vu, H. T., &Guymer, R. H. (2004). The ε2 and ε4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **45**(5): 1311-1315.

Bastard, J. P., Jardel, C., Bruckert, E., Blondy, P., Capeau, J., Laville, M., Vidal, H., & Hainque, B. (2000). Elevated Levels of Interleukin 6 Are Reduced in Serum and Subcutaneous Adipose Tissue of Obese Women after Weight Loss 1. *The Journal of Clinical Endocrinology & Metabolism*, **85**(9): 3338-3342.

Basu, A., Mukherjee, N., Roy, S., Sengupta, S., Banerjee, S., Chakraborty, M., Dey, B, Roy, M., Roy, B., Bhattacharyya, N. P., Roychoudhury, S., &Majumder, P. P. (2003). Ethnic India: a genomic view, with special reference to peopling and structure. *Genome Research*, **13**(10): 2277-2290.

Beatty, S., Koh, H. H., Phil, M., Henson, D., &Boulton, M. (2000). The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Survey of Ophthalmology*, **45**(2): 115-134.

Beatty, S., Murray, I. J., Henson, D. B., Carden, D., Koh, H. H., & Boulton, M. E. (2001). Macular pigment and risk for age-related macular degeneration in subjects from a Northern European population. *Investigative Ophthalmology & Visual Science*, **42**(2): 439-446.

Becerra, S. P. (1997). Structure-function studies on PEDF. Church, F. C., Cunningham, D. D., Ginsburg, D., Hoffman, M., Stone, S. T. andTollefsen, D. M. (editors), In: *Chemistry and Biology of serpins. Vol. 425:* Springer US. pp. 223-237.

Berendschot, T. T., Willemse-Assink, J. J., Bastiaanse, M., de Jong, P. T., & van Norren, D. (2002). Macular pigment and melanin in age-related maculopathy in a general population. *Investigative Ophthalmology & Visual Science*, **43**(6): 1928-1932.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002).Immunology provides important techniques with which to investigate protein. In: Biochemistry. 5th Edition.W. H Freeman. New York.

Berrow, E., Eperjesi, H. B., Eperjesi, F., &Gibson, J.M. (2011). Risk factors for age-related macular disease. *European Ophthalmic Review*, **5**(2): 143-153.

Betsholtz, C., Lindblom, P., & Gerhardt, H. (2005). Role of pericytes in vascular morphogenesis. In: *Mechanisms of Angiogenesis*. Clauss, M., Breier, G. (Eds.) Birkhäuser Basel, Switzerland. pp. 115-125.

Bhutto, I. A., Baba, T., Merges, C., Juriasinghani, V., McLeod, D. S., & Lutty, G. A. (2011). C-reactive protein and complement factor H in aged human eyes and eyes with age-related macular degeneration. *British Journal of Ophthalmology*, **95**(9): 1323-1330.

Bhutto, I. A., McLeod, D. S., Hasegawa, T., Kim, S. Y., Merges, C., Tong, P., & Lutty, G. A. (2006). 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*, **82**(1): 99-110.

Bhutto, I. A., McLeod, D. S., Merges, C., Hasegawa, T., & Lutty, G. A. (2006). Localisation of SDF-1 and its receptor CXCR4 in retina and choroid of aged human eyes and in eyes with age related macular degeneration. *British Journal of Ophthalmology*, **90**(7): 906-910.

Biarnés, M., Monés, J., Alonso, J., & Arias, L. (2011). Update on geographic atrophy in age-related macular degeneration. *Optometry & Vision Science*, **88**(7): 881-889.

Bird, A. C., Bressler, N. M., Bressler, S. B., Chisholm, I. H., Coscas, G., Davis, M. D. de Jong P.T.V.M., Klaver,C.C. W., Klein, B.E.K., Klein, R., Mitchell P., Sarks, J. P.,Sarks, S. H., Soubrane, G., Taylor, H. R.,&Vingerling, J. R.(1995). An international classification and grading system for age-related maculopathy and age-related macular degeneration. *Survey of Ophthalmology*, **39**(5): 367-374.

Bland, J. M., & Altman, D. G. (2000). Statistics Notes: The odds ratio. *British Medical Journal*,**320** (7247):1468.

Boekhoorn, S. S., Isaacs, A., Uitterlinden, A. G., van Duijn, C. M., Hofman, A., de Jong, P. T., & Vingerling, J. R. (2008). Polymorphisms in the vascular endothelial growth factor gene and risk of age-related macular degeneration: the Rotterdam Study. *Ophthalmology*, **115**(11): 1899-1903.

Bojanowski, C. M., Shen, D., Chew, E. Y., Ning, B., Csaky, K. G., Green, W. R., Chan, C.C., & Tuo, J. (2006). An apolipoprotein E variant may protect against age-related macular degeneration through cytokine regulation. *Environmental and Molecular Mutagenesis*, **47**(8): 594-602.

Boltz, A., Ruiß, M., Jonas, J. B., Tao, Y., Rensch, F., Weger, M., Garhöfer, G., Frantal, S., El-Shabrawi, Y., & Schmetterer, L. (2012). Role of vascular endothelial growth factor polymorphisms in the treatment success in patients with wet age-related macular degeneration. *Ophthalmology*, **119**(8): 1615-1620.

Bonilha, V. L. (2008). Age and disease-related structural changes in the retinal pigment epithelium. *Clinical Ophthalmology*, **2**(2): 413-424.

Botto, M., Fong, K. Y., So, A. K., Koch, C., & Walport, M. J. (1990). Molecular basis of polymorphisms of human complement component C3. *The Journal of Experimental Medicine*, **172**(4): 1011-1017.

Bowden, J., &Sinatra, S. (2012). The great cholesterol myth: Why lowering your cholesterol won't prevent heart disease-and the statin-free plan that will. In: Cholesterol is harmless. Fair Winds Press, Beverly, USA.pp- 29-43.

Box, G. E. P., Cox, D. R. (1964). An analysis of transformations. *Journal of Royal Statistical Scoiety Series B,* **26**:211-252.

Bressler, N. M. (1999). Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials—TAP report 1. *Archives of Ophthalmol*ogy, **117**(10): 1329-1345.

Bressler, N.M. (2005). Guidelines for using verteporfin (Visudyne) in photodynamic therapy for choroidal neovascularization due to age-related macular degeneration and other causes: update. *Retina,***25**(2): 119–134.

Bressler, N.M., **Silva, J.C**., **Bressler, S.B**., **Fine, S.L**., **& Green, W.R**. (1994). Clinicopathologic correlation of drusen and retinal pigment epithelial abnormalities in age-related macular degeneration.*Retina,* **14**(2):130-142.

Bressler, S. B. (2009). Introduction: Understanding the role of angiogenesis and antiangiogenic agents in age-related macular degeneration. *Ophthalmology*, **116**(10): S1-S7.

Brull, D. J., Serrano, N., Zito, F., Jones, L., Montgomery, H. E., Rumley, A., Sharma, P., Lowe, G. D., World, M.J., Humphries, S. E., & Hingorani, A. D. (2003). Human CRP gene polymorphism influences CRP levels implications for

the prediction and pathogenesis of coronary heart disease. *ArteriosclerosisThrombosis and Vascular Biology*, **23**(11): 2063-2069.

Cai, W., Rook, S. L., Jiang, Z. Y., Takahara, N., & Aiello, L. P. (2000). Mechanisms of hepatocyte growth factor–induced retinal endothelial cell migration and growth. *Investigative Ophthalmology & Visual Science*, **41**(7):1885-1893.

Campa, C., Costagliola, C., Incorvaia, C., Sheridan, C., Semeraro, F., De Nadai, K.,Sebastiani, A., & Parmeggiani, F. (2010). Inflammatory mediators and angiogenic factors in choroidal neovascularization: pathogenetic interactions and therapeutic implications. *Mediators of Inflammation*.doi: 10.1155/2010/546826.

Cann, R. L. (2001). Genetic clues to dispersal of human populations: Retracing the past from the present. *Science*, **291**(5509): 1742-1748.

Cao, W., Tombran-Tink, J., Elias, R., Sezate, S., Mrazek, D., & McGinnis, J. F. (2001). In vivo protection of photoreceptors from light damage by pigment epithelium–derived factor. *Investigative Ophthalmology & Visual Science*, **42**(7): 1646-1652.

Carlson, C. S., Aldred, S. F., Lee, P. K., Tracy, R. P., Schwartz, S. M., Rieder, M., Liu, K., & Reiner, A. P. (2008). Polymorphisms within the C- reactive protein (CRP) promoter region are associated with plasma CRP levels. *American Journal of Human Genetics*, **82**(1): 251.

Carlson, N. B., & Kurtz, D. (2003). Visual acuity (VA) minimum legible using a LogMAR chart. In: Clinical Procedures of Ocular Examination. 3rd edition. McGraw Hill. USA. pp. 19-23.

Cayouette, M., Smith, S. B., Becerra, S. P., & Gravel, C. (1999). Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. *Neurobiology of Disease*, **6**(6): 523-532.

Chader, G. J. (2001). PEDF: Raising both hopes and questions in controlling angiogenesis. *Proceedings of the National Academy of Sciences*, **98**(5): 2122-2124.

Chakravarthy, U., Soubrane, G., Bandello, F., Chong, V., Creuzot-Garcher, C., Dimitrakos, S. A., Korobelnik, J. F., Larsen, M., Monés, J., Pauleikhoff,

D., Pournaras, C. J., Staurenghi, G., Virgili, G., &Wolf, S. (2006). Evolving European guidance on the medical management of neovascular age related macular degeneration. *British Journal of Ophthalmology*, **90**(9): 1188-1196.

Chase, H. P., Cooper, S., Osberg, I., Stene, L. C., Barriga, K., Norris, J., Eisenbarth, J., & S. Rewers, M. (2004). Elevated C-reactive protein levels in the development of type 1 diabetes. *Diabetes*, **53**(10): 2569-2573.

Chen, L. J., Liu, D. T., Tam, P. O., Chan, W. M., Liu, K., Chong, K. K., Lam, D. S., &Pang, C. P. (2006). Association of complement factor H polymorphisms with exudative age-related macular degeneration. *Molecular Vision*, **12**(5): 1536.

Cheung, C. M. G., Tai, E. S., Kawasaki, R., Tay, W. T., Lee, J. L., Hamzah, H., & Wong, T. Y. (2012). Prevalence of and risk factors for age-related macular degeneration in a multiethnic Asian cohort. *Archives of Ophthalmology*, **130**(4): 480-486.

Cho, E., Hung, S., Willett, W. C., Spiegelman, D., Rimm, E. B., Seddon, J. M., Colditz, G. A., &Hankinson, S. E. (2001). Prospective study of dietary fat and the risk of age-related macular degeneration. *American Journal of Clinical Nutrition,***73**: 209–218.

Chopdar A. (1996). Fundus fluorescein angiography. Oxford: Butterworth-Heinmann. Oxford, UK. pp 4-5.

Chopdar, A., Chakravarthy, U., & Verma, D. (2003). Age related macular degeneration. *British Medical Journal*, **326**(7387): 485-488.

Chung, Y. C., & Chang, Y. F. (2003). Serum interleukin-6 levels reflect the disease status of colorectal cancer. *Journal of Surgical Oncology*, **83**(4): 222-226.

Clyne, B., &Olshaker, J. S. (1999). The C-reactive protein. *The Journal of Emergency Medicine*, **17**(6): 1019-1025.

Colak, E., Jaković, N. K., Žorić, L., Radosavljević, A., Stanković, S., & Singh, N. M. (2011). The association of lipoprotein parameters and C-reactive protein in patients with age-related macular degeneration. *Ophthalmic Research*, **46**: 125-132.

Colak, E., Majkic-Singh, N., Zoric, L., Radosavljevic, A., & Kosanovic-Jakovic, N. (2012). The role of CRP and inflammation in the pathogenesis of age-related macular degeneration. *Biochemia Medica*, **22**(1): 39-48.

Coleman, H. R., Chan, C. C., Ferris, F. L., & Chew, E. Y. (2008). Age-related macular degeneration. *The Lancet*, **372**(9652): 1835-1845.

Crabb, J. W., Miyagi, M., Gu, X., Shadrach, K., West, K. A., Sakaguchi, H., Kamei, M., Hasan, A., Yan, L., Rayborn, M. E., Salomon, R. G., &Hollyfield, J. G. (2002). Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proceedings of the National Academy of Sciences*, **99**(23): 14682-14687.

Cruickshanks, K. J., Klein, R., & Klein, B. E. (1993). Sunlight and age-related macular degeneration: The Beaver Dam Eye Study. *Archives of Ophthalmology*, **111**(4): 514-518.

Curcio, C. A., &Millican, C. L. (1999). Basal linear deposit and large drusen are specific for early age-related maculopathy. *Archives of Ophthalmology*, **117**(3): 329-339.

Dales, G. F. (1991). The phenomenon of the Indus civilization. Jansen, M., Mulloy, M., Urban G. (Eds.). Forgotten cities on the Indus: Early civilization in Pakistan from the 8th to the 2nd millennia BC. Verlag Philipp von Zabern, Mainz, Germany. pp. 129–144.

Danis, R. P., & Bingaman, D. P. (1997). Insulin-like growth factor-1 retinal microangiopathy in the pig eye. *Ophthalmology*, **104**(10): 1661-1669.

Dargel, R. (1992). Lipid peroxidation—a common pathogenetic mechanism? *Experimental and Toxicologic Pathology*, **44**(4): 169-181.

Darzins, P., Mitchell, P., & Heller, R. F. (1997). Sun Exposure and Age-related Macular Degeneration: An Australian Case-Control Study. *Ophthalmology*, **104**(5): 770-776.

Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H. J., Benedict, W., & Bouck, N. P. (1999). Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science*, **285**(5425): 245-248.

de Jong, P. T. (2006). Age-related macular degeneration. *New England Journal of Medicine*, **355**(14): 1474-1485.

De La Paz, M. A., & Anderson, R. E. (1992). Lipid peroxidation in rod outer segments. Role of hydroxyl radical and lipid hydroperoxides. *Investigative Ophthalmology & Visual Science*, **33**(7): 2091-2096.

Delcourt, C., Michel, F., Colvez, A., Lacroux, A., Delage, M., &Vernet, M. H. (2001). Associations of cardiovascular disease and its risk factors with age-related macular degeneration: the POLA study. *Neuro-Ophthalmology*, **8**(4): 237-249.

Despres, J. P. (2007). Cardiovascular disease under the influence of excess visceral fat. *Critical Pathways in Cardiology*,**6**(2): 51-59.

DeWan, A., Liu, M., Hartman, S., Zhang, S. S. M., Liu, D. T., Zhao, C., Tam, P. O., Chan, W. M., Lam, D. S., Snyder, M., Barnstable, C., Pang, C. P., &Hoh, J. (2006). HTRA1 promoter polymorphism in wet age-related macular degeneration. *Science*, **314**(5801): 989-992.

Dineen, B., Bourne, R. R. A., Jadoon, Z., Shah, S. P., Khan, M. A., Foster, A., Gilbert, C. E., &Khan, M. D. (2007). Causes of blindness and visual impairment in Pakistan.The Pakistan national blindness and visual impairment survey. *British Journal of Ophthalmology*, **91**(8): 1005-1010.

Ding, X., Patel, M., & Chan, C. C. (2009). Molecular pathology of age-related macular degeneration. *Progress in Retinal and Eye Research*, **28**(1): 1-18.

Dithmar, S., Curcio, C. A., Le, N. A., Brown, S., & Grossniklaus, H. E. (2000). Ultrastructural changes in Bruch's membrane of apolipoprotein E–deficient mice. *Investigative Ophthalmology & Visual Science*, **41**(8): 2035-2042.

Donoso, L. A., Kim, D., Frost, A., Callahan, A., & Hageman, G. (2006). The role of inflammation in the pathogenesis of age-related macular degeneration. *Survey of Ophthalmology*, **51**(2): 137-152

Edwards, A. O., Ritter, R., Abel, K. J., Manning, A., Panhuysen, C., & Farrer, L. A. (2005). Complement factor H polymorphism and age-related macular degeneration. *Science*, **308**(5720): 421-424.

Ehrmann, M., Egensperger, R., & Tennstadt, A. (2008). U.S. Patent Application *12/666,578*.

Enge, M., Bjarnegård, M., Gerhardt, H., Gustafsson, E., Kalén, M., Asker, N. Hammes, H. P., Shani, M., Fässler, R., & Betsholtz, C. (2002). Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *The EMBO Journal*, **21**(16): 4307-4316.

Eong, K. (2006). Age-related macular degeneration: an emerging challenge for eye care and public health professionals in the Asia Pacific region. *Annals-Academy of Medicine Singapore*, **35**(3): 133-135.

Erke, M. G., Bertelsen, G., Peto, T., Sjølie, A. K., Lindekleiv, H., & Njølstad, I. (2012). Prevalence of Age-related Macular Degeneration in Elderly Caucasians: The Tromsø Eye Study. *Ophthalmology*, **119**(9): 1737-1743.

Evans, J. R. (2001). Risk factors for age-related macular degeneration. *Progress in Retinal and Eye Research*, **20**(2): 227-253.

Evereklioglu, C., Doganay, S., Er, H., Cekmen, M., Özerol, E. & Otlu, B. (2003). Serum leptin concentrations are decreased and correlated with disease severity in age-related macular degeneration: a preliminary study. *Eye*, **17**(3): 350-355.

Evereklioglu, C., Er, H., Türköz, Y., & Çekmen, M. (2002). Serum levels of TNF-α, sIL-2R, IL-6, and IL-8 are increased and associated with elevated lipid peroxidation in patients with Behçet's disease. *Mediators of Inflammation*, **11**(2): 87-93.

Fedetz, M., Matesanz, F., Pascual, M. (2001). The -174/-597 promoter polymorphisms in the interleukin-6 gene are not associated with susceptibility to multiple sclerosis. *Journal of Neurological Sciences,* **190**: 69–72.

Fernández-Real, J. M., Broch, M., Vendrell, J., Gutiérrez, C., Casamitjana, R., Pugeat, M., Richart, C., & Ricart, W. (2000). Interleukin-6 gene polymorphism and insulin sensitivity. *Diabetes*, **49**(3): 517-520.

Fernandez-Real, J. M., Vendrell, J., Richart, C., Gutierrez, C., &Ricart, W. (2001). Platelet count and interleukin 6 gene polymorphism in healthy subjects.*BMC Medical Genetics*, **2**(1): 6.

Ferrara, N., Gerber, H. P., & LeCouter, J. (2003). The biology of VEGF and its receptors.*Nature Medicine*, **9**(6): 669-676.

Ferris, F. L., Fine, S. L., & Hyman, L. (1984). Age-related macular degeneration and blindness due to neovascular maculopathy. *Archives of Ophthalmology*, **102**(11): 1640-1642.

Fine, S. L., Berger, J. W., Maguire, M. G., & Ho, A. C. (2000). Age-related macular degeneration. *New England Journal of Medicine*,**342**(7): 483-492**.**

Fisher, S. A., Abecasis, G. R., Yashar, B. M., Zareparsi, S., Swaroop, A., Iyengar, S. K.,Klein, B. E., Klein, R., Lee, K. E., Majewski, J., Schultz, D. W., Klein, M. L., Seddon, J. M., Santangelo. S. L., Weeks, D. E., Conley, Y. P., Mah, T. S., Schmidt, S., Haines, J. L., Pericak-Vance, M. A., Gorin, M. B., Schulz, H. L., Pardi, F., Lewis, C. M., & Weber, B. H. (2005). Meta-analysis of genome scans of age-related macular degeneration. *Human Molecular Genetics*, **14**(15): 2257-2264.

Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudkin, J. S., Humphries, S., & Woo, P. (1998). The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *Journal of Clinical Investigation*, **102**(7): 1369-1376.

Forrester, J., Dick, A., McMenamin, P., &Lee, W. (1996). The Eye: Basic sciences in practice. W.B. Saunders Company Ltd, London. UK. pp. 28.

Frank, R. N., Amin, R. H., Eliott, D., Puklin, J. E., & Abrams, G. W. (1996). Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. *American Journal of Ophthalmology*, **122**(3): 393-403.

Freyberger, H., Bröcker, M., Yakut, H., Hammer, J., Effert, R., Schifferdecker, E. Schatz, H., & Derwahl, M. (2000). Increased levels of platelet-derived growth factor in vitreous fluid of patients with proliferative diabetic retinopathy. *Experimental and Clinical Endocrinology & Diabetes*, **108**(02): 106-109.

Friedman, D. S., Katz, J., Bressler, N. M., Rahmani, B., & Tielsch, J. M. (1999). Racial differences in the prevalence of age-related macular degeneration: The Baltimore eye survey. *Ophthalmology*, **106**(6): 1049-1055.

Friedman, E. (2000). The role of the atherosclerotic process in the pathogenesis of age-related macular degeneration. *American Journal of Ophthalmology*, **130**(5): 658-663.

Fritsche, L. G., Loenhardt, T., Janssen, A., Fisher, S. A., Rivera, A., Keilhauer, C. N., & Weber, B. H. (2008). Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nature Genetics*, **40**(7): 892-896.

Fritsche, L. G., Chen, W., Schu, M., Yaspan, B.L., Yu, Y., Thorleifsson, G., Zack, D.J., Arakawa, S., Cipriani, V., Ripke, S. & Igo Jr, R.P. (2013). Seven new loci associated with age-related macular degeneration. *Nature Genetics*, **45(**4): 433-9.

Futter, C. E. (2006). The molecular regulation of organelle transport in mammalian retinal pigment epithelial cells. *Pigment Cell Research*, **19**(2): 104-111.

Gass, J. D. M. (1997). Stereoscopic Atlas of Macular Diseases: Diagrams and Treatment, vol. 1, Mosby, St. *Louis, Mo, USA.*

Gehrs, K. M., Anderson, D. H., Johnson, L. V., & Hageman, G. S. (2006). Age-related macular degeneration-emerging pathogenetic and therapeutic concepts. *Annals of Medicine*, **38**(7), 450-471.

Ghasemi, H., Ghazanfari, T., Yaraee, R., Faghihzadeh, S., & Hassan, Z. M. (2011). Roles of IL-8 in ocular inflammations: a review. *Ocular Immunology and Inflammation*, **19**(6): 401-412.

Giannakis, E., Jokiranta, T. S., Male, D. A., Ranganathan, S., Ormsby, R. J., Fischetti, V. A., Mold, C., &Gordon, D. L. (2003). A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. *European Journal of Immunology*, **33**(4): 962-969.

Gold, B., Merriam, J. E., Zernant, J., Hancox, L. S., Taiber, A. J., Gehrs, K., Cramer, K., Neel, J., Bergeron, J., Barile, G. R., Smith, R. T., Hageman, G. S., Dean, M., &Allikmets, R. (2006). Variation in factor B (BF) and

complement component 2 (C2) genes is associated with age-related macular degeneration. *Nature Genetics*, **38**(4): 458-462.

Gottsch, J. D., Bynoe, L. A., Harlan, J. B., Rencs, E. V., &Green, W. R. (1993). Light-induced deposits in Bruch's membrane of protoporphyric mice. *Archives of Ophthalmology*, **111**(1): 126-129.

Grau, S., Richards, P. J., Kerr, B., Hughes, C., Caterson, B., Williams, A. S., Junker, U., Jones, S. A., Clausen, T., & Ehrmann, M. (2006). The role of human HtrA1 in arthritic disease. *Journal of Biological Chemistry*, **281**(10): 6124-6129.

Green W. R. (1999). Histopathology of age-related macular degeneration. *Molecular Vision*,**5**: 27.

Green W. R., & Enger, C. (1993). Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture.*Ophthalmology*, **100**:1519-1535.

Green, W. R., & Key, S. N. (1977). Senile macular degeneration: a histopathologic study. *Transactions of the American Ophthalmological Society*,**75**:180- 254.

Grimes, B. F. (2006). Ethnologue: Languages of the World.Summer Institute of Linguistics, Dallas, TX.

Grisanti, S., & Tatar, O. (2008). The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. *Progress in Retinal and Eye Research*, **27**(4): 372-390.

Grossniklaus, H. E., & Green, W. R. (1998). Histopathologic and ultrastructural findings of surgically excised choroidal neovascularization. *Archives of Ophthalmology*, **116**(6): 745-749.

Grossniklaus, H., & Gass, J. D. (1998). Clinicopathologic correlations of surgically excised type 1 and type 2 submacular choroidal neovascular membranes. *American Journal of Ophthalmology*, **126**(1): 59-69.

Grundy, S. M, Brewer, H. B.J., Cleeman, J. I., Smith, S. C., &Lenfant, S. (2004). Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*,**109**(3): 433-438.

Gupta, N., Brown, K. E., &Milam, A. H. (2003). Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. *Experimental Eye Research*, **76**(4): 463-471.

Gür-Toy, G., Lenk, N., Yalcin, B., Aksaray, S., & Alli, N. (2005). Serum interleukin-8 as a serologic marker of activity in Behçet's disease. *International Journal of Dermatology*, **44**(8): 657-660.

Guyer, D. R., Yannuzzi, L. A., Slakter, J. S., Sorenson, J. A., Hope-Ross, M., &Orlock, D. R. (1994). Digital indocyanine-green video angiography of occult choroidal neovascularization. *Ophthalmology*, **101**(10): 1727-1735.

Haas, P., Steindl, K., Aggermann, T., Schmid-Kubista, K., Krugluger, W., Hageman, G. S., & Binder, S. (2011). Serum VEGF and CFH in exudative age-related macular degeneration. *Current Eye Research*, **36**(2): 143-148.

Habibi, I., Sfar, I., Chebil, A., Kort, F., Bouraoui, R., Jendoubi-Ayed, S., Makhlouf, M., Abdallah, T., B., Matri, L., & Gorgi, Y. (2014). Vascular endothelial growth factor genetic polymorphisms and susceptibility to age-related macular degeneration in Tunisian population. *Biomarker Research*, **2**(1): 15.

Haddad, S., Chen, C. A., Santangelo, S. L., & Seddon, J. M. (2006). The genetics of age-related macular degeneration: a review of progress to date. *Survey of Ophthalmology*, **51**(4): 316-363.

Hageman, G. S., Chong, N. H. V., Keonin, J., Amin, S., Klaver, C. W., Frennesson, C. I., Russell, S. R., Luthert, P. J., Mullins, R. F., &Kuehn, M. H. (2002). Topographical differences in the structure and composition of bruch's membrane may explain the predilection of the macula for degeneration in individuals with age-related macular degeneration. *Investigative Ophthalmology and Visual Sciences*, Suppl. **43**: S1988.

Hageman, G. S., &Mullins, R. F. (1999). Molecular composition of drusen as related to substructural phenotype. *Molecular Vision*, ***5***: 28.

Hageman, G. S., Hancox, L. S., Taiber, A. J., Gehrs, K. M., Anderson, D. H., Johnson, L. V., Radeke, M. J., Kavanagh, D., Richards, A., Atkinson, J., Meri, S., Bergeron, J., Zernant, J., Merriam, J., Gold, B., Allikmets, R., Dean, M. AMD Clinical Study Group., & Dean, M. (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. *Annals of Medicine*, **38**(8): 592-604.

Hageman, G. S., Luthert, P. J., Victor Chong, N. H., Johnson, L. V., Anderson, D. H., &Mullins, R. F. (2001). 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. *Progress in Retinal and Eye Research*, **20**(6): 705-732.

Hageman, G. S., Mullins, R. F., Russell, S. R., Johnson, L. V., &Anderson, D. H. (1999). Vitronectin is a constituent of ocular drusen and the vitronectin gene is expressed in human retinal pigmented epithelial cells. *The FASEB Journal*, **13**(3): 477-484.

Haines, J. L., Hauser, M. A., Schmidt, S., Scott, W. K., Olson, L. M., Gallins, P., Spencer, K. L., Kwan, S. Y., Noureddine, M., Gilbert, J. R., Schnetz-Boutaud, N., Agarwal, A., Postel, E. A., & Pericak-Vance, M. A. (2005). Complement factor H variant increases the risk of age-related macular degeneration. *Science*, **308**(5720): 419-421.

Hall, N. F., Gale, C. R., Syddall, H., Phillips, D. I., & Martyn, C. N. (2001). Risk of macular degeneration in users of statins: cross sectional study. *British Medical Journal*, **323**(7309): 375-376.

Halliwell, B., & Gutteridge, J. (1989). Free Radicals in Biology and Medicine (3rd ed), Oxford University Press Inc., New York (USA), pp. 1–35.

Hammond, B. R., Wooten, B. R., & Snodderly, D. M. (1996). Cigarette smoking and retinal carotenoids: implications for age-related macular degeneration. *Vision Research*, **36**(18): 3003-3009.

Hammond, C. J., Webster, A. R., Snieder, H., Bird, A. C., Gilbert, C. E., & Spector, T. D. (2002). Genetic influence on early age-related maculopathy: a twin study. *Ophthalmology*, **109**(4): 730-736.

Hangai, M., Moon, Y. S., Kitaya, N., Chan, C. K., Wu, D. Y., Peters, K. G., Ryan, S. J., & Hinton, D. R. (2001). Systemically expressed soluble Tie2 inhibits intraocular neovascularization. *Human Gene Therapy*, **12**(10): 1311-1321.

Heeschen, C., Jang, J. J., Weis, M., Pathak, A., Kaji, S., Hu, R. S., Tsao, P. S., Johnson, F. L., & Cooke, J. P. (2001). Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nature Medicine*, **7**(7): 833-839.

Heinen, S., Hartmann, A., Lauer, N., Wiehl, U., Dahse, H. M., Schirmer, S., Gropp, K., Enghardt, T., Wallich, R., Halbich, S., Mihlan, M., Schlotzer-Schrehardt, U., Zipfel, P. F., & Skerka, C. (2009). Factor H–related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood*, **114**(12): 2439-2447.

Heinrich, P. C., Castell, J. V., & Andus, T. (1990). Interleukin-6 and the acute phase response. *Biochemical Journal*, **265**(3): 621-636.

Heinzmann, A., Ahlert, I., Kurz, T., Berner, R., & Deichmann, K. A. (2004). Association study suggests opposite effects of polymorphisms within< i> IL8</i> on bronchial asthma and respiratory syncytial virus bronchiolitis. *Journal of Allergy and Clinical Immunology*, **114**(3): 671-676.

Hellstrom, A., Perruzzi, C., Ju, M., Engstrom, E., Hard, A. L., Liu, J. L., Albertsson-Wikland, K., Carlsson, B., Niklasson, A., Sjodell, L., LeRoith, D., Senger, D. R., & Smith, L. E. (2001). Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. *Proceedings of National Academy of Sciences USA*, ***98***(10): 5804–5808.

Hernández, C., Carrasco, E., García-Arumí, J., Maria Segura, R., & Simó, R. (2004). Intravitreous levels of hepatocyte growth factor/scatter factor and vascular cell adhesion molecule-1 in the vitreous fluid of diabetic patients with proliferative retinopathy. *Diabetes & Metabolism*, **30**(4): 341-346.

Heuberger, R. A., Mares-Perlman, J. A., Klein, R., Klein, B. E., Millen, A. E., &Palta, M. (2001). Relationship of dietary fat to age-related maculopathy in the Third National Health and Nutrition Examination Survey. *Archives of Ophthalmology*, **119**(12): 1833-1888.

Higgins, G. T., Wang, J. H., Dockery, P., Cleary, P. E., & Redmond, H. P. (2003). Induction of angiogenic cytokine expression in cultured RPE by ingestion of oxidized photoreceptor outer segments. *Investigative Ophthalmology & Visual Science*, **44**(4): 1775-1782.

Holekamp, N. M., Bouck, N., & Volpert, O. (2002). Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. *American Journal of Ophthalmology*, **134**(2): 220-227.

Hollyfield, J. G., Salomon, R. G., Crabb, J. W., &Gu, X. (2007). *U.S. Patent No. 7,172,874*. Washington, DC: U.S. Patent and Trademark Office.

Holz, F. G., Bellman, C., Staudt, S., Schütt, F., & Völcker, H. E. (2001). Fundus autofluorescence and development of geographic atrophy in age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **42**(5): 1051-1056.

http://dwb.unl.edu/Teacher/NSF/C08/C08Links/www.piopio.school.nz/molmed.htm#sequencing. Retrieved on 10.05.2015

http://www.hutchon.net/ConfidOR.html

https://www.cia.gov/library/publications/the-world-factbook/rankorder/2102 rank. html. Retrieved on 17/09/2014

http://asia.ensembl.org/Homo_sapiens/Variation/Population. Retrieved on23/06/2015

https://caprica.genetics.kcl.ac.uk/~ilori/ld_calculator_action.php. Based on the 1000 genomes phase1 version 3, March 2012 release

Huang, C., Xu, Y., Li, X., & Wang, W. (2013). Vascular endothelial growth factor A polymorphisms and age-related macular degeneration: a systematic review and meta-analysis. *Molecular Vision*, ***19***: 1211-1221.

Hughes, A. E., Orr, N., Esfandiary, H., Diaz-Torres, M., Goodship, T., &Chakravarthy, U. (2006). A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nature Genetics*, **38**(10): 1173-1177.

Hussain, J. (1997). A history of the peoples of Pakistan towards independence. Oxford University Press, Karachi, Pakistan.

Hutchinson, J., & Tay, W. (1874). Symmetrical central choroido-retinal disease occurring in senile persons. *Ophthalmic Hospital Reports and Journal of the Royal London Ophthalmic Hospital*, **8**: 231-244.

Hyman, L. G., Lilienfeld, A. M., Ferris, F. L., & Fine, S. L. (1983). Senile macular degeneration: a case-control study. *American Journal of Epidemiology*, **118**(2): 213-227.

Hyman, L., Schachat, A. P., He, Q., & Leske, M. C. (2000). Hypertension, cardiovascular disease, and age-related macular degeneration. *Archives of Ophthalmology*, **118**(3): 351-358.

Ibbetson, D. (1883). Panjab castes. Sang-e-Meel, Lahore, Pakistan.p. 161. 2001 (Reprint) pp. 338.

Ishibashi, T., Hata, Y., Yoshikawa, H., Nakagawa, K., Sueishi, K., & Inomata, H. (1997). Expression of vascular endothelial growth factor in experimental choroidal neovascularization. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **235**(3): 159-167.

Issa, P. C., Chong, N. V., & Scholl, H. P. (2011). The significance of the complement system for the pathogenesis of age-related macular degeneration—current evidence and translation into clinical application. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **249**(2): 163-174.

Ito, T., Nakano, M., Yamamoto, Y., Hiramitsu, T., & Mizuno, Y. (1995). Hemoglobin-induced lipid peroxidation in the retina: a possible mechanism for macular degeneration. *Archives of Biochemistry and Biophysics*, **316**(2): 864-872.

Jablonski, M. M., Tombran-Tink, J., Mrazek, D. A., &Iannaccone, A. (2000). Pigment epithelium-derived factor supports normal development of photoreceptor neurons and opsin expression after retinal pigment epithelium removal. *The Journal of Neuroscience*, **20**(19): 7149-7157.

Jadoon, M. Z., Dineen, B., Bourne, R. R., Shah, S. P., Khan, M. A., Johnson, G. J., Gilbert, C. E., & Khan, M. D. (2006). Prevalence of blindness and visual impairment in Pakistan: The Pakistan national blindness and visual impairment survey. *Investigative Ophthalmology & Visual Science*, **47**(11): 4749-4755.

Jager, R. D., Mieler, W. F., & Miller, J. W. (2008). Age-related macular degeneration. *New England Journal of Medicine*, **358**(24): 2606-2617.

Jakobsdottir, J., Conley, Y. P., Weeks, D. E., Mah, T. S., Ferrell, R. E., & Gorin, M. B. (2005). Susceptibility genes for age-related maculopathy on chromosome 10q26. *The American Journal of Human Genetics*, **77**(3): 389-407.

Janik-Papis, K., Zaras, M., Krzyzanowska, A., Wozniak, K., Blasiak, J., Szaflik, J., & Szaflik, J. P. (2009). Association between vascular endothelial growth factor gene polymorphisms and age-related macular degeneration in a Polish population. *Experimental and Molecular Pathology*, **87**(3): 234-238.

Jarrige, J. F. (1991). Mehrgarh: Its place in the development of ancient cultures in Pakistan. Jansen, M., Mulloy, M., Urban, G. (Eds.). Forgotten cities on the Indus: early civilization in Pakistan from the 8th to the 2nd millennia BC. Verlag Philipp von Zabern, Mainz, Germany, pp. 34–50.

Jarva, H., Jokiranta, T. S., Hellwage, J., Zipfel, P. F., & Meri, S. (1999). Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8–11. *The Journal of Immunology*, **163**(7): 3957-3962.

Jeon, J. Y., Kim, H. A., Kim, S. H., Park, H. S., & Suh, C. H. (2010). Interleukin 6 gene polymorphisms are associated with systemic lupus erythematosus in Koreans. *The Journal of Rheumatology*, **37**(11): 2251-2258.

Johnson, L. V., Leitner, W. P., Staples, M. K., & Anderson, D. H. (2001). Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Experimental Eye Research*, **73**(6): 887-896.

Johnson, L. V., Ozaki, S., Staples, M. K., Erickson, P. A., & Anderson, D. H. (2000). A potential role for immune complex pathogenesis in drusen formation. *Experimental Eye Research*, **70**(4): 441-449.

Johnson, P. T., Betts, K. E., Radeke, M. J., Hageman, G. S., Anderson, D. H., & Johnson, L. V. (2006). Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. *Proceedings of the National Academy of Sciences*,**103**(46): 17456-17461.

Johnson, P. T., Lewis, G. P., Talaga, K. C., Brown, M. N., Kappel, P. J., Fisher, S. K., Anderson, D. H., & Johnson, L. V. (2003). Drusen-associated degeneration in the retina. *Investigative Ophthalmology & Visual Science*, **44**(10): 4481-4488.

Jonisch, J., & Shah, G. (2011). Diagnosis of age-related macular degeneration. In: Age-related Macular Degeneration Diagnosis and Treatment. Springer New York. pp. 23-38.

Józsi, M., & Zipfel, P. F. (2008). Factor H family proteins and human diseases. *Trends in Immunology*, **29**(8): 380-387.

Jumper, J. M., Fu, A. D., McDonald, H. R., Johnson, R. N., &Ai, E. (2006). In: Alfaro III DV, Liggett, P. E., Mieler, W. F., Quiroz-Mercado, H., Jager, R. D., Tano, Y. (eds.) Age-related Macular Degeneration: A Comprehensive Textbook. Lippincott Williams and Wilkins. Philadelphia. pp 86-101.

Kalayoglu, M. V., Bula, D., Arroyo, J., Gragoudas, E. S., D'Amico, D., & Miller, J. W. (2005). Identification of Chlamydia pneumoniae within human choroidal neovascular membranes secondary to age-related macular degeneration. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **243**(11): 1080-1090.

Kanda, A., Chen, W., Othman, M., Branham, K. E., Brooks, M., Khanna, R., He, S., Lyons, R., Abecasis, G. R., &Swaroop, A. (2007). A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. *Proceedings of the National Academy of Sciences*,**104**(41): 16227-16232.

Kayatz, P., Thumann, G., Luther, T. T., Jordan, J. F., Bartz–Schmidt, K. U., Esser, P. J., & Schraermeyer, U. (2001). Oxidation causes melanin fluorescence. *Investigative Ophthalmology & Visual Science*, **42**(1): 241-246.

Kevany, B. M., & Palczewski, K. (2010). Phagocytosis of retinal rod and cone photoreceptors. *Physiology*,**25**(1): 8-15.

Khan, J. C., Thurlby, D. A., Shahid, H., Clayton, D. G., Yates, J. R. W., Bradley, M., Moore A. T., & Bird, A. C. (2006). Smoking and age related macular degeneration: the number of pack years of cigarette smoking is a major

determinant of risk for both geographic atrophy and choroidal neovascularisation. *British Journal of Ophthalmology*, **90**(1): 75-80.

Killingsworth, M. C., Sarks, J. P., & Sarks, S. H. (1990). Macrophages related to Bruch's membrane in age-related macular degeneration. *Eye*, **4**(4): 613-621.

Kim, I. K., Ji, F., Morrison, M. A., Adams, S., Zhang, Q., Lane, A. M., Capone, A., Dryja, T. P., Ott, J., Miller, J. W., & DeAngelis, M. M. (2008). Comprehensive analysis of CRP, CFH Y402H and environmental risk factors on risk of neovascular age-related macular degeneration. *Molecular Vision*, **14**: 1487-1495.

Kinose, F., Roscilli, G., Lamartina, S., Anderson, K. D., Bonelli, F., Spence, S. G., Ciliberto, G., Vogt, T. F., Holder, D. J., Toniatti, C., &Thut, C. J. (2005). Inhibition of retinal and choroidal neovascularization by a novel KDR kinase inhibitor. *Molecular Vision*, **11***:* 366-373.

Klaver, C. C., Wolfs, R. C., Assink, J. J., Van Duijn, C. M., Hofman, A., &de jong, P. T. (1998). Genetic risk of age-related maculopahty. Population -based familial aggregation study. *Archives of Ophthalmology*,**116**(12): 1646-51.

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C., & Hoh, J. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science*, **308**(5720): 385-389.

Klein, R. (2007). Overview of progress in the epidemiology of age-related macular degeneration. *Ophthalmic Epidemiology*,**14**(4): 184–187.

Klein, R., Cruickshanks, K. J., Nash, S. D., Krantz, E. M., Nieto, F. J., Huang, G. H., Pankow, J. S., & Klein, B. E. (2010). The prevalence of age-related macular degeneration and associated risk factors. *Archives of Ophthalmology*, **128**(6): 750.

Klein, R., Klein, B. E. K., Jensen, S. C., & Meuer, S. M. (1997). The five-year incidence and progression of age-related maculopathy: The Beaver Dam Eye Study. *Ophthalmology*,**104**(1):7-21.

Klein, R., Klein, B. E., & Franke, T. (1993). The relationship of cardiovascular disease and its risk factors to age-related maculopathy: The Beaver Dam Eye Study. *Ophthalmology*, **100**(3): 406-414.

Klein, R., Klein, B. E., & Moss, S. E. (1998). Relation of smoking to the incidence of age-related maculopathy. The Beaver Dam Eye Study. *American Journal of Epidemiology*, **147**(2): 103-110.

Klein, R., Klein, B. E., Knudtson, M. D., Meuer, S. M., Swift, M., &Gangnon, R. E. (2007). Fifteen-year cumulative incidence of age-related macular degeneration: The Beaver Dam Eye Study. *Ophthalmology*, **114**(2): 253-262.

Klein, R., Klein, B. E., Marino, E. K., Kuller, L. H., Furberg, C., Burke, G. L., & Hubbard, L. D. (2003). Early age-related maculopathy in the cardiovascular health study. *Ophthalmology*, **110**(1): 25-33.

Klein, R., Klein, B. E., Tomany, S. C., Danforth, L. G., & Cruickshanks, K. J. (2003). Relation of statin use to the 5-year incidence and progression of age-related maculopathy. *Archives of Ophthalmology*, **121**(8): 1151.

Klein, R., Klein, B. E., Tomany, S. C., Meuer, S. M., &Huang, G. H. (2002). Ten-year incidence and progression of age-related maculopathy: The Beaver Dam Eye Study. *Ophthalmology*, **109**(10): 1767-1779.

Klein, R., Rowland, M. L., &Harris, M. I. (1995). Racial/ethnic differences in age-related maculopathy: Third National Health and Nutrition Examination Survey. *Ophthalmology*, **102**(3): 371-381.

Klein, R., Klein, B. E., &Linton, K. L.(1992). Prevalence of age-related maculopathy. The Beaver Dam Eye Study.*Ophthalmology.* **99**(6):933-43.

Kliffen, M., Sharma, H. S., Mooy, C. M., Kerkvliet, S., &de Jong, P. T. (1997). Increased expression of angiogenic growth factors in age-related maculopathy. *British Journal of Ophthalmology*, **81**(2): 154-162.

Kopp, A., Hebecker, M., Svobodová, E., & Józsi, M. (2012). Factor H: a complement regulator in health and disease, and a mediator of cellular interactions. *Biomolecules*, **2**(1): 46-75.

Koressaar T., & Remm, M. (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics*, **23**(10):1289-91

Krauss, R. M., Eckel, R. H., Howard, B., Appel, L. J, Daniels, S. R., Deckelbaum, R. J., Erdman, J. W., Etherton, P. K., Goldberg, I. J., Kotchen, T. A., Lichtenstein, A. H., Mitch, W. E., Mullis, R., Robinson, K., Rosett, J. W., Jeor, S., Suttie, J., Tribble, D. L., & Bazzarre, T. L. (2000). AHA Scientific Statement. AHA Dietary Guidelines: Revision 2000: A Statement for Healthcare Professionals from the Nutrition Committee of the American Heart Association. *Circulation-Hagertown*, **102**(18): 2284-2299.

Krippl, P., Langsenlehner, U., Renner, W., Yazdani-Biuki, B., Wolf, G., Wascher, T. C., Paulweber, B., Haas, J., & Samonigg, H. (2003). A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk. *International Journal of Cancer*, **106**(4): 468-47.

Krzystolik, M. G., Afshari, M. A., Adamis, A. P., Gaudreault, J., Gragoudas, E. S., Michaud, N. A., Li, W., Connolly, E., O'Neill, C. A., &Miller, J. W. (2002). Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Archives of Ophthalmology*, **120**(3): 338-346.

Kukreja, R. C., &Hess, M. L. (1992). The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardiovascular Research*, **26**(7): 641-655.

Kulkarni, S. R., Aghashe, S. R., Khandekar, R. B., & Deshpande, M. D. (2013). Prevalence and determinants of age-related macular degeneration in the 50 years and older population: a hospital based study in Maharashtra, India. *Indian Journal of Ophthalmology*, **61**(5): 196-201.

Kumar, S., & Fu, Y. (2014). Age Related Macular Degeneration: a Complex Pathology. *Austin Journal of Genetics and Genomic Research*, **1**(1): 5.

Kushner, I., Jiang, S. L., Zhang, D., Lozanski, G., & Samols, D. (1995). Do Post-Transcriptional Mechanisms Participate in Induction of C-reactive Protein and Serum Amyloid A by IL-6 and IL-I? *Annals of the New York Academy of Sciences*, **762**(1): 102-107.

Kvanta, A. (1995). Expression and regulation of vascular endothelial growth factor in choroidal fibroblasts. *Current Eye Research*, **14**(11): 1015-1020.

Kvanta, A. (2006). Ocular angiogenesis: the role of growth factors. *Acta Ophthalmologica Scandinavica*, **84**(3): 282-288.

Kvanta, A., Algvere, P. V., Berglin, L., &Seregard, S. (1996). Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Investigative Ophthalmology & Visual Science*, **37**(9): 1929-1934.

Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Lehtinen, M. J., Lindeman, N., Anderson, D. H., Johnson, P. T., Jarvela, I., Jokiranta, T. S., Hageman, G. S., Immonen, I., &Meri, S. (2007). Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *The Journal of Immunology*, **178**(6): 3831-3836.

Langmann, T. (2007). Microglia activation in retinal degeneration. *Journal of Leukocyte Biology*, **81**(6): 1345-1351.

Lashkari, K., Hirose, T., Yazdany, J., McMeel, J. W., Kazlauskas, A., & Rahimi, N. (2000). Vascular endothelial growth factor and hepatocyte growth factor levels are differentially elevated in patients with advanced retinopathy of prematurity. *The American Journal of Pathology*, **156**(4): 1337-1344.

Lee, B. C., Lim, J. I., &Grossniklaus, H. E. (1996). Clinicopathologic features of indocyanine green angiographically-imaged, surgically excised choroidal neovascular membranes. *Retina*, **16**: 64–69.

Lequin, R. M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical Chemistry*, **51**(12): 2415-2418.

Leuschen, J. N., Schuman, S. G., Winter, K. P., McCall, M. N., Wong, W. T., Chew, E. Y., Hwang, T., Srivastava, S., Sarin, N., Clemons, T., Harrington M., & Toth, C. A. (2013). Spectral-domain optical coherence tomography characteristics of intermediate age-related macular degeneration. *Ophthalmology*, **120**(1): 140-150.

Levy, O., Calippe, B., Lavalette, S., Hu, S. J., Raoul, W., Dominguez, E., Housset, M., Paques, M., Sahel, J.A, Bemelmans, A. P., Combadiere, C., Guillonneau,

X., & Sennlaub, F. (2015). Apolipoprotein E promotes subretinal mononuclear phagocyte survival and chronic inflammation in age-related macular degeneration. *EMBO Molecular Medicine*, e201404524.

Li, C. M., Clark, M. E., Chimento, M. F., & Curcio, C. A. (2006). Apolipoprotein localization in isolated drusen and retinal apolipoprotein gene expression. *Investigative Ophthalmology & Visual Science*, **47**(7): 3119-3128.

Li, M., Atmaca-Sonmez, P., Othman, M., Branham, K. E., Khanna, R., Wade, M. S., Li, Y., Liang, L., Zareparsi, S., Swaroop, A., &Abecasis, G. R. (2006). CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nature Genetics*, **38**(9): 1049-1054.

Lim, L. S., Mitchell, P., Seddon, J. M., Holz, F. G., & Wong, T. Y. (2012). Age-related macular degeneration. *The Lancet*, ***379***(9827): 1728-1738.

Lin, J. M., Wan, L., Tsai, Y. Y., Lin, H. J., Tsai, Y., Lee, C. C., Tsai, C. H., Tseng, S. H., & Tsai, F. J. (2008). Vascular endothelial growth factor gene polymorphisms in age-related macular degeneration. *American Journal of Ophthalmology*, **145**(6): 1045-1051.

Lip, P. L., Blann, A. D., Hope-Ross, M., Gibson, J. M., & Lip, G. Y. (2001). Age-related macular degeneration is associated with increased vascular endothelial growth factor, hemorheology and endothelial dysfunction. *Ophthalmology*, **108**(4): 705-710.

Liu, Y., Hou, S., Lang, W., Dai, D., Wang, Z., Ji, X., Zou, Y., & Wang, J. (2014). Roles of three common VEGF polymorphisms in the risk of age-related macular degeneration. *Genetic Testing and Molecular Biomarkers*, **18**(4): 245-252.

Lokki, M. L., & Koskimies, S. A. (1991). Allelic differences in hemolytic activity and protein concentration of BF molecules are found in association with particular HLA haplotypes. *Immunogenetics*, **34**(4): 242-246.

Lopez, P. F., Sippy, B. D., Lambert, H. M., Thach, A. B., & Hinton, D. R. (1996). Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular

degeneration-related choroidal neovascular membranes. *Investigative Ophthalmology & Visual Science*, **37**(5): 855-868.

Losonczy, G., Fekete, Á., Vokó, Z., Takács, L., Káldi, I., Ajzner, É., Kasza, M., Vajas, A., Berta, A., & Balogh, I. (2011). Analysis of complement factor H Y402H, LOC387715, HTRA1 polymorphisms and ApoE alleles with susceptibility to age-related macular degeneration in Hungarian patients. *Acta Ophthalmologica*, **89**(3): 255-262.

Mahley, R. W., Huang, Y., & Weisgraber, K. H. (2006). Putting cholesterol in its place: apoE and reverse cholesterol transport. *Journal of Clinical Investigation*, **116**(5): 1226.

Majewski, J., Schultz, D. W., Weleber, R. G., Schain, M. B., Edwards, A. O., Matise, T. C.,Acott, T. S., Ott, J.,& Klein, M. L. (2003). Age-related macular degeneration—a genome scan in extended families. *The American Journal of Human Genetics*, **73**(3): 540-550.

Malek, G., Li, C. M., Guidry, C., Medeiros, N. E., & Curcio, C. A. (2003). Apolipoprotein B in cholesterol-containing drusen and basal deposits of human eyes with age-related maculopathy. *The American Journal of Pathology*, **162**(2): 413-425.

Mandal, N. A., & Ayyagari, R. (2006). Complement factor H: spatial and temporal expression and localization in the eye. *Investigative Ophthalmology & Visual Science*, **47**(9), 4091-4097.

Mares-Perlman, J. A., Brady, W. E., Klein, R., Klein, B. E., Bowen, P., Stacewicz-Sapuntzakis, M., & Palta, M. (1995). Serum antioxidants and age-related macular degeneration in a population-based case-control study. *Archives of Ophthalmology*, **113**(12): 1518-1523.

Mares-Perlman, J. A., Brady, W. E., Klein, R., VandenLangenberg, G. M., Klein, B. E., &Palta, M. (1995). Dietary fat and age-related maculopathy. *Archives of Ophthalmology*, **113**(6): 743-748.

Marmorstein, A. D., Finnemann, S. C., Bonilha, V. L., & Rodriguez-Boulan, E. N. R. I. Q. U. E. (1998). Morphogenesis of the Retinal Pigment Epithelium:

Toward Understanding Retinal Degenerative Diseasesa. *Annals of the New York Academy of Sciences*, **857**(1): 1-12.

Mauschitz, M. M., Fonseca, S., Chang, P., Göbel, A. P., Fleckenstein, M., Jaffe, G. J.,Holz, F. G., &Schmitz-Valckenberg, S. (2012). Topography of geographic atrophy in age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **53**(8): 4932-4939.

McCarty, C. A., Mukesh, B. N., Fu, C. L., Mitchell, P., Wang, J. J., &Taylor, H. R. (2001). Risk factors for age-related maculopathy: The Visual Impairment Project. *Archives of Ophthalmology*, **119**(10): 1455-1462.

McGwin, G., Owsley, C., Curcio, C. A.&Crain, R. J. (2003).The association between statin use and age related maculopathy. *British Journal of Ophthalmology*,**87**: 1121-5.

Memon, M. S. (1992). Prevalence and causes of blindness in Pakistan. *JPMA. The Journal of the Pakistan Medical Association*, **42**(8):196.

Miller, J. W. (2013). Age-related macular degeneration revisited–piecing the puzzle: the LXIX Edward Jackson memorial lecture. *American Journal of Ophthalmology*, **155**(1): 1-35.

Misasi, R., Huemer, H. P., Schwaeble, W., Sölder, E., Larcher, C. &Dierich, M. P. (1989). Human complement factor H: An additional gene product of 43kDa isolated from human plasma shows cofactor activity for the cleavage of the third component of complement. *European Journal of Immunology*, **19**(9): 1765-1768.

Mitchell, P., Wang, J. J., Foran, S., & Smith, W. (2002). Five-year incidence of age-related maculopathy lesions: The Blue Mountains Eye Study. *Ophthalmology*, **109**(6): 1092-1097.

Mori, K., Gehlbach, P., Yamamoto, S., Duh, E., Zack, D. J., Li, Q. Berns, K. I., Raisler, B. J., Hauswirth, W. W., &Campochiaro, P. A. (2002). AAV-mediated gene transfer of pigment epithelium-derived factor inhibits choroidal neovascularization. *Investigative Ophthalmology & Visual Science*, **43**(6): 1994-2000.

Mueck, A. O., Seeger, H., & Wallwiener, D. (2001). Further evidence for direct vascular actions of statins: effect on endothelial nitric oxide synthase and

adhesion molecules. *Experimental and Clinical Endocrinology & Diabetes*, **109**(03): 181-183.

Mukaida, N., Shiroo, M., &Matsushima, K.(1989). Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *The Journal of Immunology*, **143**(4): 1366-1371.

Mullins, R. F., Russell, S. R., Anderson, D. H., & Hageman, G. S. (2000). Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *The FASEB Journal*, **14**(7): 835-846.

Muratoglu, S. C., Belgrave, S., Hampton, B., Migliorini, M., Coksaygan, T., Chen, L.Mikhailenko, I,& Strickland, D. K. (2013). LRP1 protects the vasculature by regulating levels of connective tissue growth factor and HtrA1. *Arteriosclerosis Thrombosis and Vascular Biology*, **33**(9): 2137-2146.

Nagaoka, T., Kuo, L., Ren, Y., Yoshida, A., & Hein, T. W. (2008). C-reactive protein inhibits endothelium-dependent nitric oxide-mediated dilation of retinal arterioles via enhanced superoxide production. *Investigative Ophthalmology &Visual Science*, **49**(5): 2053-2060.

Naghza, D., Karam, N., Amin, F., Zaka, M., Khan, D. H., Khan, S. D., & Khan, D. R. (2013). Epidemiology of age related macular degeneration (AMD) and its associated ocular conditions and concomitant systemic diseases. *Pakistan Journal of Ophthalmology*, **29**(4), 221-224.

Nakajima, T., Hirata, M., Shearer, T. R., & Azuma, M. (2014). Mechanism for laser-induced neovascularization in rat choroid: Accumulation of integrin α chain-positive cells and their ligands. *Molecular Vision*, **20**: 864-871.

National Institutes of Health. (2001). Third Report of the national cholesterol education program expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *Bethesda*, Md: National Institutes of Health. (*NIH Publication no.* 01-3670).

Nazimul, H., Rohit, K., & Anjli, H. (2008). Trend of retinal diseases in developing countries.*Expert Review of Ophthalmology*,**3**(1): 43-50.

Newsome, D. A., Swartz, M., Leone, N. C., Elston, R. C., & Miller, E. (1988). Oral zinc in macular degeneration. *Archives of Ophthalmology*, **106**(2): 192-198.

Ng, T. K., Yam, G. H., Chen, W. Q., Lee, V. Y., Chen, H., Chen, L. J., Choy, K.W., Yang, Z., & Pang, C. P. (2011). Interactive expressions of HtrA1 and VEGF in human vitreous humors and fetal RPE cells. *Investigative Ophthalmology & Visual Science*, **52**(6): 3706-3712.

Nita, M., Grzybowski, A., Ascaso, F. J., & Huerva, V. (2014). Age-related macular degeneration in the aspect of chronic low-grade inflammation (pathophysiological parainflammation). *Mediators of Inflammation*, Article ID 930671.

Nowak, M., Swietochowska, E., Marek, B., Szapska, B., Wielkoszynski, T., Kos-Kudla, B.,Karpe, J., Kajdaniuk, D., Sieminska, L.,Glogowska-Szelag, J., &Nowak, K. (2005). Changes in lipid metabolism in women with age-related macular degeneration. *Clinical and Experimental Medicine*, **4**(4): 183-187.

Nozaki, M., Raisler, B. J., Sakurai, E., Sarma, J. V., Barnum, S. R., Lambris, J. D., Chen, Y., Zhang, K., Ambati, B. K., Baffi, J. Z., &Ambati, J. (2006). Drusen complement components C3a and C5a promote choroidal neovascularization. *Proceedings of the National Academy of Sciences of the United States of America*, **103**(7): 2328-2333.

Ogata, N., Wada, M., Otsuji, T., Jo, N., Tombran-Tink, J., & Matsumura, M. (2002). Expression of pigment epithelium-derived factor in normal adult rat eye and experimental choroidal neovascularization. *Investigative Ophthalmology & Visual Science*, **43**(4): 1168-1175.

Ohia, S. E., Opere, C. A., & LeDay, A. M. (2005). Pharmacological consequences of oxidative stress in ocular tissues. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **579**(1): 22-36.

Ohkaru, Y., Asayama, K., Ishii, H., Nishimura, S., Sunahara, N., Tanaka, T., & Kawamura, K. (1995). Development of a sandwich enzyme-linked immunosorbent assay for the determination of human heart type fatty acid-binding protein in plasma and urine by using two different monoclonal antibodies specific for human heart fatty acid-binding protein. *Journal of Immunological Methods*, **178**(1): 99-111.

Oka, C., Tsujimoto, R., Kajikawa, M., Koshiba-Takeuchi, K., Ina, J., Yano, M., Tsuchiya, A., Ueta, Y., Soma, A., Kanda, H., Matsumoto, M., &Kawaichi, M. (2004). HtrA1 serine protease inhibits signalling mediated by Tgfβ family proteins. *Development,* **131**(5): 1041-1053.

Okamoto, H., Umeda, S., Obazawa, M., Minami, M., Noda, T., Mizota, A., Honda, M., Tanaka, M., Koyama, R., Takagi, I., Sakamoto, Y., Saito, Y., Miyake, Y., &Iwata, T. (2006). Complement factor H polymorphisms in Japanese population with age-related macular degeneration. *Molecular Vision*, **12**: 156-158.

Organisciak, D. T., Darrow, R. M., Barsalou, L., Darrow, R. A., Kutty, R. K., Kutty, G., & Wiggert, B. (1998). Light history and age-related changes in retinal light damage. *Investigative Ophthalmology & Visual Science*, **39**(7): 1107-1116.

Ormsby, R. J., Ranganathan, S., Tong, J. C., Griggs, K. M., Dimasi, D. P., Hewitt, A. W., Burdon. K. P., Craig, J. E., Hoh, J., &Gordon, D. L. (2008). Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **49**(5): 1763-1770.

Oshima, Y., Oshima, S., Nambu, H., Kachi, S., Takahashi, K., Umeda, N. Shen, J., Dong, A., Apte, R. S., Duh, E., Hackett, S. F., Okoye, G., Ishibashi, K., handa, J., melia, M., Wiegand, S., Yancopoulos, G., Zack, D. J., & Campochiaro, P. A. (2005). Different effects of angiopoietin-2 in different vascular beds: new vessels are most sensitive. *The FASEB Journal*, **19**(8): 963-965.

Owen, C. G., Fletcher, A. E., Donoghue, M., & Rudnicka, A. R. (2003). How big the burden of visual loss caused by age related macular degeneration in the United Kingdom? *British Journal of Ophthalmology*, **87**(3): 312-317.

Owsley, C., Jackson, G. R., Cideciyan, A. V., Huang, Y., Fine, S. L., Ho, A. C., Maguire, M. G., Lolley, V., &Jacobson, S. G. (2000). Psychophysical evidence for rod vulnerability in age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **41**(1): 267-273.

Panda-Jonas, S., Jonas, J. B., & Jakobczyk-Zmija, M. (1996). Retinal pigment epithelial cell count, distribution, and correlations in normal human eyes. *American Journal of Ophthalmology*, **121**(2): 181-189.

Pankow, J. S., Folsom, A. R., Cushman, M., Borecki, I. B., Hopkins, P. N., Eckfeldt, J. H., & Tracy, R. P. (2001). Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. *Atherosclerosis*, **154**(3): 681-689.

Parekh, N., Voland, R. P., Moeller, S. M., Blodi, B. A., Ritenbaugh, C., Chappell, R. J., Wallace, R. B., &Mares, J. A. (2009). Association between dietary fat intake and age-related macular degeneration in the Carotenoids in Age-Related Eye Disease Study (CAREDS): an ancillary study of the Women's Health Initiative. *Archives of Ophthalmology*, **127**(11): 1483.

Parmeggiani, F., Romano, M. R., Costagliola, C., Semeraro, F., Incorvaia, C., D'Angelo, S. Perri, D. P., Nadai, K. D., & Sebastiani, A. (2012). Mechanism of inflammation in age-related macular degeneration. *Mediators of Inflammation*, Article ID 546786.

Pascual, M., Nieto, A., Mataran, L., Balsa, A., Pascual-Salcedo, D., & Martin, J. (2000).IL-6 promoter polymorphisms in rheumatoid arthritis. *Genes and Immunity*, **1**(5): 338-340.

Patel, M. & Chan, C. C. (2008). Immunopathological aspects of age-related macular degeneration. *In: Seminars in Iimmunopathology*, **30**(2): 97-110.

Pauleikhoff, D., Chen, J. C., Chisholm, I. H., &Bird, A. C. (1990). Choroidal perfusion abnormality with age-related Bruch's membrane change. *American Journal of Ophthalmology*, **109**(2): 211-217.

Penfold, P. L., Wong, J. G., Gyory, J., & Billson, F. A. (2001). Effects of triamcinolone acetonide on microglial morphology and quantitative expression of MHC-II in exudative age-related macular degeneration. *Clinical &Experimental Ophthalmology*, **29**(3): 188-192.

Petrovič, M. G., Korošec, P., Košnik, M., Osredkar, J., Hawlina, M., Peterlin, B., & Petrovič, D. (2008). Local and genetic determinants of vascular endothelial

growth factor expression in advanced proliferative diabetic retinopathy. *Molecular Vision*, **14**: 1382-1387.

Pierce, E. A., Avery, R. L., Foley, E. D., Aiello, L. P., & Smith, L. E. (1995). Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proceedings of the National Academy of Sciences*, **92**(3): 905-909.

Polur, I., Lee, P. L., Servais, J. M., Xu, L., & Li, Y. (2010). Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. *Histology and Histopathology*,**25**(5): 599- 608.

Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E., & Ridker, P. M. (2001). C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *Journal of American Medical Association*,**286**(3): 327-334.

Prosser, B. E., Johnson, S., Roversi, P., Herbert, A. P., Blaum, B. S., Tyrrell, J., Jowitt, T. A., Clark, S. J., Tarelli, E., Uhrin, D., Barlow, P. N., Sim, R. B., Day, A. J., &Lea, S. M. (2007). Structural basis for complement factor H–linked age-related macular degeneration. *The Journal of Experimental Medicine*, **204**(10): 2277-2283.

Qamar, R., Ayub, Q., Mohyuddin, A., Helgason, A., Mazhar, K., Mansoor, A., Zertaj, T., Smith, C. T., &Mehdi, S. Q. (2002). Y-chromosomal DNA variation in Pakistan. *The American Journal of Human Genetics*, **70**(5), 1107-1124.

Rao R. (2012). Understanding age related macular degeneration. Monthly health focus (July)

Ratnagar, S. (1995). Archaeological perspectives of early Indian societies. In: *Recent perspectives of early Indian history.* Thapar R. (ed.) Popular Prakashan, Bombay, India. pp. 1-52.

Reich, S. J., Fosnot, J., Kuroki, A., Tang, W., Yang, X., Maguire, A. M., Bennett, J., & Tolentino, M. J. (2003). Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Molecular Vision*, **9**(5): 210-216.

Reinisalo, M. (2012) Studies on Retina-specific Gene Expression.Kosma, V., Turunen, H., & Gröhn, O. (Edoitors), Honkakoski, P. and Urtti, A. (Supervisors).

Publications of the University of Eastern Finland.*Dissertations in Health Sciences,***119**: 3.

Renner, W., Gerger, A., Hofmann, G., Thurner, E. M., Kapp, K. S., & Langsenlehner, U. (2011). Impact of VEGF gene polymorphisms and haplotypes on radiation-induced late toxicity in prostate cancer patients. *Strahlentherapie and Onkologie*, **187**(12): 784-791.

Renner, W., Kotschan, S., Hoffmann, C., Obermayer-Pietsch, B., & Pilger, E. (2001). A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. *Journal of Vascular Research*, **37**(6): 443-448.

Reynolds, R., Rosner, B., & Seddon, J. M. (2010). Serum lipid biomarkers and hepatic lipase gene associations with age-related macular degeneration. *Ophthalmology*, **117**(10): 1989-1995.

Rhodes, B., Fürnrohr, B. G., & Vyse, T. J. (2011). C-reactive protein in rheumatology: biology and genetics. *Nature Reviews Rheumatology*, **7**(5): 282-289.

Ricci, F., Staurenghi, G., Lepre, T., Missiroli, F., Zampatti, S., Cascella, R., Borgiani, P., Marsella, L. T., Maria, C. E., Cusumano, A., Novelli, G., & Giardina, E. (2013). Haplotypes in IL-8 gene are associated to age-related macular degeneration: a case-control study. *PloS One*, **8**(6): e66978.

Richardson, A. J., Islam, F. M., Guymer, R. H., Cain, M., & Baird, P. N. (2007).A tag-single nucleotide polymorphisms approach to the vascular endothelial growth factor-A gene in age-related macular degeneration. *Molecular Vision*, **13**: 2148-2152.

Ricklin, D., Hajishengallis, G., Yang, K., & Lambris, J. D. (2010). Complement: a key system for immune surveillance and homeostasis. *Nature Immunology*, **11**(9): 785-797.

Rickman, C. B., Farsiu, S., Toth, C. A., & Klingeborn, M. (2013). Dry age-related macular degeneration: mechanisms, therapeutic targets, and imaging. *Investigative Ophthalmology & Visual Science*, **54**(14): ORSF68-ORSF80.

Riddell, D. R., Zhou, H., Atchison, K., Warwick, H. K., Atkinson, P. J., Jefferson, J., Xu, L., Aschmies, S., Kirksey, Y., Hu, Y., Wagner, E., Parratt, A., Xu, J., Li, Z., Zaleska, M.M., Jacobsen, J.S., Pangalos, M.N., & Reinhart, P. H. (2008). Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. *The Journal of Neuroscience*, **28**(45): 11445-11453.

Ridker, P. M., Rifai, N., Stampfer, M. J., & Hennekens, C. H. (2000). Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation*, **101**(15): 1767-1772.

Rivera, A., Fisher, S. A., Fritsche, L. G., Keilhauer, C. N., Lichtner, P., Meitinger, T., & Weber, B. H. (2005). Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Human Molecular Genetics*, **14**(21): 3227-3236.

Robinson, G. S., Pierce, E. A., Rook, S. L., Foley, E., Webb, R., & Smith, L. E. (1996). Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. *Proceedings of the National Academy of Sciences*, **93**(10): 4851-4856.

Rodríguez de Córdoba, S., Esparza-Gordillo, J., Goicoechea de Jorge, E., Lopez-Trascasa, M., & Sánchez-Corral, P. (2004). The human complement factor H: functional roles, genetic variations and disease associations. *Molecular Immunology*, **41**(4): 355-367.

Rollins, B.J. (1997). Chemokines.*Blood*,**90**(3):909–928.

Roth, F., Bindewald, A., & Holz, F. G. (2004). Keypathophysiologic pathways in age-related macular disease. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **242**(8): 710-716.

Ruggiero, D., Dalmasso, C., Nutile, T., Sorice, R., Dionisi, L., Aversano, M., Bröet, P., Leutenegger, A.L., Bourgain, C. & Ciullo, M. (2011). Genetics of VEGF serum variation in human isolated populations of cilento: importance of VEGF polymorphisms. *PloS One*, **6**(2), e16982.

Sambrook, J., &Russell, D. W. (2001). Molecular Cloning.A laboratory manual.3rd Edition.Volume 1. Cold Spring Harbour Laboratory Press, New York, USA.

Schaumberg, D. A., Christen, W. G., Hankinson, S. E., & Glynn, R. J. (2001). Body mass index and the incidence of visually significant age-related maculopathy in men. *Archives of Ophthalmology*, **119**(9): 1259-1265.

Schlingemann, R. O. (2004). Role of growth factors and the wound healing response in age-related macular degeneration. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **242**(1): 91-101.

Schmid-Kubista, K. E., Tosakulwong, N., Wu, Y., Ryu, E., Hecker, L. A., Baratz, K. H., Brown, W. L., & Edwards, A. O. (2009). Contribution of copy number variation in the regulation of complement activation locus to development of age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **50**(11): 5070-5079.

Schmidt-Erfurth, U., Kriechbaum, K., & Oldag, A. (2007). Three-dimensional angiography of classic and occult lesion types in choroidal neovascularization. *Investigative Ophthalmology & Visual Science*, **48**(4): 1751-1760.

Schneider, S., Greven, C. M., & Green, R. W. (1998). Photocoagulation of well-defined choroidal neovascularization in age-related macular degeneration: clinicopathologic correlation. *Retina*, **18**(3): 242-250.

Schotte, H., Schlüter, B., Rust, S., Assmann, G., Domschke, W., & Gaubitz, M. (2001). Interleukin-6 promoter polymorphism (− 174 G/C) in Caucasian German patients with systemic lupus erythematosus. *Rheumatology*, **40**(4): 393-400.

Scibona, P., Redal, M.A., Garfi, L.G., Arbelbide, J., Argibay, P.F., & Belloso, W.H. (2012). Prevalence of CYP2C9 and VKORC1 alleles in the Argentine population and implications for prescribing dosages of anticoagulants. *Genetics and Molecular Research*, **11**(1): 70-76.

Seddon, J. M., Ajani, U. A., & Mitchell, B. D. (1997). Familial aggregation of age-related maculopathy. *American Journal of Ophthalmology*, **123**(2): 199-206.

Seddon, J. M., Ajani, U. A., Sperduto, R. D., Hiller, R., Blair, N., Burton, T. C., Marilyn, D. F., Gragoudas, E. S., Haller, J., Miller, D. T., yannuzzi, L. A., & Willett, W. (1994). Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. *The Journal of the American Medical Association*, **272**(18): 1413-1420.

Seddon, J. M., Cote, J., &Rosner, B. (2003). Progression of age-related macular degeneration: association with dietary fat, transunsaturated fat, nuts, and fish intake. *Archives of Ophthalmology,***121**: 1728–1737.

Seddon, J. M., Cote, J., Page, W. F., Aggen, S. H., & Neale, M. C. (2005). The US twin study of age-related macular degeneration: relative roles of genetic and environmental influences. *Archives of Ophthalmology*, **123**(3): 321-327.

Seddon, J. M., Gensler, G., & Rosner, B. (2010). C-reactive protein and CFH, ARMS2/HTRA1 gene variants are independently associated with risk of macular degeneration. *Ophthalmology*, **117**(8): 1560-1566.

Seddon, J. M., George, S., Rosner, B., & Rifai, N. (2005). Progression of age-related macular degeneration: prospective assessment of C-reactive protein, interleukin 6, and other cardiovascular biomarkers. *Archives of Ophthalmology*, **123**(6): 774-782.

Seddon, J. M., Reynolds, R., Shah, H. R., & Rosner, B. (2011). Smoking, dietary betaine, methionine, and vitamin D in monozygotic twins with discordant macular degeneration: epigenetic implications. *Ophthalmology*, **118**(7): 1386-1394.

Seddon, J. M., Santangelo, S. L., Book, K., Chong, S., & Cote, J. (2003). A genomewide scan for age-related macular degeneration provides evidence for linkage to several chromosomal regions. *The American Journal of Human Genetics*, **73**(4): 780-790.

Seddon, J. M., Willett, W. C., Speizer, F. E., & Hankinson, S. E. (1996). A prospective study of cigarette smoking and age-related macular degeneration in women. *The Journal of the American Medical Association*, **276**(14):1141-1146.

Seitsonen, S., Lemmela, S., Holopainen, J., Tommila, P., Ranta, P., Kotamies, A., Moilanen, J., Palosaari, T., Kaarniranta, K., Meri, S., Immonen I., & Jarvela, I. (2006). Analysis of variants in the complement factor H, the elongation of very long chain fatty acids-like 4 and the hemicentin 1 genes of age-related macular degeneration in the Finnish population. *Molecular Vision*, **12**: 796-801.

Sesso, H. D., Buring, J. E., Rifai, N., Blake, G. J., Gaziano, J. M., & Ridker, P. M. (2003). C-reactive protein and the risk of developing hypertension. *The Journal of American Medical Association*, **290**(22): 2945-2951.

Sharma, N. K., Gupta, A., Prabhakar, S., Singh, R., Sharma, S. K., Chen, W., & Anand, A. (2013). Association between CFH Y402H polymorphism and age related macular degeneration in North Indian cohort. *PloS One*, **8**(7), e70193.

Shinoda, K., Ishida, S., Kawashima, S., Wakabayashi, T., Matsuzaki, T., Takayama, M., Shinmura, K., & Yamada, M. (1999). Comparison of the levels of hepatocyte growth factor and vascular endothelial growth factor in aqueous fluid and serum with grades of retinopathy in patients with diabetes mellitus. *British Journal of Ophthalmology*, **83**(7): 834-837.

Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*, **359**(6398): 843-845.

Silva, A. S., Teixeira, A. G., Bavia, L., Lin, F., Velletri, R., Belfort Jr, R., & Isaac, L. (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**: 2288-99.

Simonelli, F., Frisso, G., Testa, F., di Fiore, R., Vitale, D. F., Manitto, M. P., Brancato, R., Rinaldi, E. & Sacchetti, L. (2006). Polymorphism p. 402Y> H in the complement factor H protein is a risk factor for age related macular degeneration in an Italian population. *British Journal of Ophthalmology*, **90**(9): 1142-1145.

Simonini, A., Moscucci, M., Muller, D. W., Bates, E. R., Pagani, F. D., Burdick, M. D., & Strieter, R. M. (2000). IL-8 is an angiogenic factor in human coronary atherectomy tissue. *Circulation*, **101**(13): 1519-1526.

Simundic, A. M. (2011). New insights in the pathophysiology of inflammation. *Biochemia Medica*, **21**(3): 243-244.

Simundic, A. M., Bilic-Zulle, L., Nikolac, N., Supak-Smolcic, V., Honovic, L., Avram, S., Beregovaja E., Dobreanu, M., Guimaraes, J., Kovacs, G. L., Singh, N. M., Sierra-Amor, R. I., Sypniewska, G. & Zima, T. (2011). The

quality of the extra-analytical phase of laboratory practice in some developing European countries and Mexico–a multicentric study. *Clinical Chemistry and Laboratory Medicine*, **49**(2): 215-228.

Sivaprasad, S., Adewoyin, T., Bailey, T. A., Dandekar, S. S., Jenkins, S., Webster, A. R. & Chong, N. V. (2007). Estimation of systemic complement C3 activity in age-related macular degeneration. *Archives of Ophthalmology*, **125**(4): 515.

Sjöberg, A. P., Trouw, L. A., Clark, S. J., Sjölander, J., Heinegård, D., Sim, R. B., Day, A. J., & Blom, A. M. (2007). The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. *Journal of Biological Chemistry*, **282**(15): 10894-10900.

Skerka, C., Lauer, N., Weinberger, A. A., Keilhauer, C. N., Sühnel, J., Smith, R.,Weinberger, A. A., Keilhauer, C. N., Sühnel, J., Smith, R., Schlötzer-Schrehardt, U., Fritsche, L., Heinen, S., Hartmann, A., Weber, B. H,& Zipfel, P. F. (2007). Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Molecular Immunology*, **44**(13): 3398-3406.

Smith, A. J., & Humphries, S. E. (2009). Cytokine and cytokine receptor gene polymorphisms and their functionality. *Cytokine & Growth Factor Reviews*, **20**(1): 43-59.

Smith, L. E., Shen, W., Perruzzi, C., Soker, S., Kinose, F., Xu, X., Robinson, G., Driver, S., Bischoff, J., Zhang, B., Schaeffer, J. M. & Senger, D. R. (1999). Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-1 receptor. *Nature Medicine*, **5**(12): 1390-1395.

Smith, L. E., Kopchick, J. J., Chen, W., Knapp, J., Kinose, F., Daley, D., Foley, E., Smith, R. G.&Schaeffer, J. M.(1997). Essential role of growth hormone in ischaemia-induced retinal neovascularization. *Science*, **276**(5319): 1706–1709.

Smith, W., Assink, J., Klein, R., Mitchell, P., Klaver, C. C., Klein, B. E., Hofman, A., Jensen, S., Wang, J. J., de Jong, P. M. T. V., & de Jong, P. T. (2001). Risk

factors for age-related macular degeneration: pooled findings from three continents. *Ophthalmology*, **108**(4): 697-704.

Smith, W., Mitchell, P. & Leeder, S. R. (2000). Dietary fat and fish intake and age-related maculopathy. *Archives of Ophthalmology*, **118**(3): 401-404

Smith, W., Mitchell, P., & Wang, J. J. (1997). Gender, oestrogen, hormone replacement and age-related macular degeneration: Results from the Blue Mountains Eye Study. *Australian and New Zealand Journal of Ophthalmology*, **25**(4): 13-15.

Smoak, K. A., Aloor, J. J., Madenspacher, J., Merrick, B. A., Collins, J. B., Zhu, X., Cavigiolio, G., Oda, M.N., Parks, J.S., & Fessler, M. B. (2010). Myeloid differentiation primary response protein 88 couples reverse cholesterol transport to inflammation. *Cell Metabolism*, **11**(6): 493-502.

Sommer, A., Tielsch, J. M., Katz, J., Quigley, H. A., Gottsch, J. D., Javitt, J. C. Martone J. F., Royall, R. M., Witt, K. A. & Ezrine, S. (1991). Racial differences in the cause-specific prevalence of blindness in east Baltimore. *New England Journal of Medicine*, **325**(20): 1412-1417.

Souied, E. H., Leveziel, N., Richard, F., Dragon-Durey, M. A., Coscas, G., Soubrane, G., Benlian, P. & Fremeaux-Bacchi, V. (2005). Y402H complement factor H polymorphism associated with exudative age-related macular degeneration in the French population. *Molecular Vision*, **11**: 1135-1140.

Spaide, R. F. (2006). Rationale for combination therapies for choroidal neovascularization. *American Journal of Ophthalmology*, **141**(1): 149-156.

Spaide, R. F., Ho-Spaide, W. C., Browne, R. W., & Armstrong, D. (1999). Characterization of peroxidized lipids in Bruch's membrane. *Retina*, **19**(2): 141-147.

Sparrow, C. P., Burton, C. A., Hernandez, M., Mundt, S., Hassing, H., Patel, S., Rosa, R., Hermanowski-Vosatka, A., Wang, P. R., Zhang, D., Peterson, L., Detmers, P. A., Chao, Y. S., Wright, S. D.& Wright, S. D. (2001). Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arteriosclerosis Thrombosis, and Vascular Biology*, **21**(1): 115-121.

Spencer, K. L., Olson, L. M., Anderson, B. M., Schnetz-Boutaud, N., Scott, W. K., Gallins, P., Agarwal, A., Postel, E. A., Pericak-Vance, M. A., Haines, J. L.& Haines, J. L. (2008). C3 R102G polymorphism increases risk of age-related macular degeneration. *Human Molecular Genetics*, **17**(12): 1821-1824.

Sperduto, R. D. (1993). Antioxidant status and neovascular age-related macular degeneration. *Archives of Ophthalmology*, **111**(1): 104-109.

Spraul, C. W., Lang, G. E., & Lang, G. K. (1998). Value of optical coherence tomography in diagnosis of age-related macular degeneration. Correlation of fluorescein angiography and OCT findings. *Klinische Monatsblatter fur Augenheilkunde*, **212**(3): 141-148.

Steinmetz, R. L., Haimovici, R., Jubb, C., Fitzke, F. W., &Bird, A. C. (1993). Symptomatic abnormalities of dark adaptation in patients with age-related Bruch's membrane change. *British Journal of Ophthalmology*,**77**: 549-54.

Stellmach, V., Crawford, S. E., Zhou, W. & Bouck, N. (2001). Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proceedings of the National Academy of Sciences*, **98**(5): 2593-2597.

Stone, W. L., Farnsworth, C. C. & Dratz, E. A. (1979). A reinvestigation of the fatty acid content of bovine, rat and frog retinal rod outer segments. *Experimental Eye Research*, **28**(4): 387-397.

Sundelin, S. P., Nilsson, S. E. G. & Brunk, U. T. (2001). Lipofuscin-formation in cultured retinal pigment epithelial cells is related to their melanin content. *Free Radical Biology and Medicine*, **30**(1): 74-81.

Sung, C.H. &Chuang, J.Z. (2010.) The cell Biology of vision. *The Journal of Cell Biology*,**190**(6): 953-963.

Sunness, J. S., Bressler, N. M., Tian, Y., Alexander, J., & Applegate, C. A. (1999). Measuring geographic atrophy in advanced age-related macular degeneration. *Investigative Ophthalmology & Visual Science*, **40**(8): 1761-1769.

Sunness, J. S., Rubin, G. S., Applegate, C. A., Bressler, N. M., Marsh, M. J., Hawkins, B. S., & Haselwood, D. (1997). Visual function abnormalities and

prognosis in eyes with age-related geographic atrophy of the macula and good visual acuity. *Ophthalmology*, **104**(10): 1677-1691.

Swaroop, A., Branham, K. E., Chen, W., & Abecasis, G. (2007). Genetic susceptibility to age-related macular degeneration: a paradigm for dissecting complex disease traits. *Human Molecular Genetics*, **16**(R2): R174-R182.

Szalai, A. J., Wu, J., Lange, E. M., McCrory, M. A., Langefeld, C. D., Williams, A., Zakharkin, S. O., & Kimberly, R. P. (2005). Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. *Journal of Molecular Medicine*, **83**(6): 440-447.

Szmitko, P. E., Wang, C. H., Weisel, R. D., de Almeida, J. R., Anderson, T. J., & Verma, S. (2003). New markers of inflammation and endothelial cell activation part I. *Circulation*, **108**(16): 1917-1923.

Tabor, H. K., Risch, N. J., & Myers, R. M. (2002). Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nature Reviews Genetics*, **3**(5), 391-397.

Takagi, H., Koyama, S., Seike, H., Oh, H., Otani, A., Matsumura, M. &Honda, Y. (2003). Potential role of the angiopoietin⁄Tie-2 system in ischaemia-induced retinal neovascularization. *Investigative Ophthalmology &Visual Science*,**44**: 393–402.

Takeda, A., Hata, Y., Shiose, S., Sassa, Y., Honda, M., Fujisawa, K., Sakamoto, T. & Ishibashi, T. (2003). Suppression of experimental choroidal neovascularization utilizing KDR selective receptor tyrosine kinase inhibitor. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **241**(9): 765-772.

Tanemura, M., Miyamoto, N., Mandai, M. & Honda, Y. (2001). Estrogen may enhance VEGFR-2 expression in the process of laser-induced CNV formation. *Investigative Ophthalmology & Visual Science*, **42**(4): S96-S96.

Taylor, H. R., Tikellis, G., Robman, L. D., McCarty, C. A. & McNeil, J. J. (2002). Vitamin E supplementation and macular degeneration: randomised controlled trial. *British Medical Journal*, **325**(7354): 11.

Terry, C. F., Loukaci, V., & Green, F. R. (2000). Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *Journal of Biological Chemistry*, **275**(24): 18138-18144.

The eye diseases prevalence research group. (2004). Causes and prevalence of visual impairment among adults in the United States.*Archives of Ophthalmology*,**122**: 477-485.

Thiruvagounder, M., Khan, S., & Sheriff, D. S. (2010). The prevalence of metabolic syndrome in a local population in India. *Biochemia Medica*, **20**(2): 249-252.

Thornton, J., Edwards, R., Mitchell, P., Harrison, R. A., Buchan, I., & Kelly, S. P. (2005). Smoking and age-related macular degeneration: a review of association. *Eye*, **19**(9): 935-944.

Tomany, S. C., Klein, R., & Klein, B. E. (2001). The relation of coffee and caffeine to the 5-year incidence of early age-related maculopathy: The Beaver Dam Eye Study. *American Journal of Ophthalmology*, **132**(2): 271-273.

Tomany, S. C., Wang, J. J., van Leeuwen, R., Klein, R., Mitchell, P., Vingerling, J. R., Klein, B. E., Smith, W, & de Jong, P. T. (2004). Risk factors for incident age-related macular degeneration: pooled findings from 3 continents. *Ophthalmology*, **111**(7): 1280-1287.

Tombran-Tink, J., Chader, G. G. & Johnson, L. V. (1991). PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Experimental Eye Research*, **53**(3): 411-414.

Tong, Y., Liao, J., Zhang, Y., Zhou, J., Zhang, H., & Mao, M. (2010). LOC387715/HTRA1 gene polymorphisms and susceptibility to age-related macular degeneration: A HuGE review and meta-analysis. *Molecular Vision*, **16**: 1958-1981.

Tsai, Y. Y., Lin, J. M., Wan, L., Lin, H. J., Tsai, Y., Lee, C. C., Tsai, C. H., Tsai, F. J., & Tseng, S. H. (2008). Interleukin gene polymorphisms in age-related

macular degeneration. *Investigative Ophthalmology & Visual Science*, **49**(2): 693-698.

Tsoumakidou, M., Demedts, I. K., Brusselle, G. G., &Jeffery, P. K. (2008). Dendritic cells in chronic obstructive pulmonary disease: new players in an old game. *American Journal of Respiratory and Critical Care Medicine*,**177**(11): 1180–1186.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research*, **40**(15): e115-e115.

Utheim, O. A., Ritland, J. S., Utheim, T. P., Espeseth, T., Lydersen, S., Rootwelt, H., Semb, S. O., & Elsås, T. (2008). Apolipoprotein E genotype and risk for development of cataract and age-related macular degeneration. *Acta Ophthalmologica*, **86**(4): 401-403.

Vairaktaris, E., Yapijakis, C., Serefoglou, Z., Derka, S., Vassiliou, S., Nkenke, E., Vylliotis, A., Wiltfang, J, Avgoustidis, D., Critselis, E., Neukam, F. W., & Patsouris, E. (2007). The interleukin-8 (− 251A/T) polymorphism is associated with increased risk for oral squamous cell carcinoma. *European Journal of Surgical Oncology (EJSO)*, **33**(4): 504-507.

van der Schaft, T. L., Mooy, C. M., De Bruijn, W. C., & De Jong, P. T. (1993). Early stages of age-related macular degeneration: an immunofluorescence and electron microscopy study. *British Journal of Ophthalmology*, **77**(10): 657-661.

Van Kuijk, F. J., &Buck, P. (1992). Fatty acid composition of the human macula and peripheral retina. *Investigative Ophthalmology & Visual Science*, **33**(13): 3493-3496.

van Leeuwen, R., Klaver, C. C., Vingerling, J. R., Hofman, A., & de Jong, P. T. (2003). The risk and natural course of age-related maculopathy: follow-up at $6^{1/2}$ years in the Rotterdam Study. *Archives of Ophthalmology*, **121**(4): 519.-526

van Leeuwen, R., Klaver, C. C., Vingerling, J. R., Hofman, A., van Duijn, C. M., Stricker, B. H. C., & de Jong, P. T. (2004). Cholesterol and age-related macular degeneration: is there a link? *American Journal of Ophthalmology*, **137**(4): 750-752.

VandenLangenberg, G. M., Mares-Perlman, J. A., Klein, R., Klein, B. E., Brady, W. E. & Palta, M. (1998). Associations between antioxidant and zinc intake and the 5-year incidence of early age-related maculopathy in the Beaver Dam Eye Study. *American Journal of Epidemiology*, **148**(2): 204-214.

Vašků, A., Souček, M., Goldbergova, M., & Vacha, J. (2003). Office blood pressure, heart rate and A (-596) G interleukin-6 gene polymorphism in apparently healthy Czech middle-aged population. *Physiological Research*, **52**(2): 291-297.

Verma, S., Szmitko, P. E., & Yeh, E. T. (2004). C-reactive protein structure affects function. *Circulation*, **109**(16), 1914-1917.

Vickers, M. A., Green, F. R., Terry, C., Mayosi, B. M., Julier, C., Lathrop, M., Ratcliffe. P. T., Watkins, H. C., & Keavney, B. (2002). Genotype at a promoter polymorphism of the interleukin-6 gene is associated with baseline levels of plasma C-reactive protein. *Cardiovascular Research*, **53**(4): 1029-1034.

Villuendas, G., San, Millan, J.L., Sancho, J., & Escobar-Morreale, H.F. (2002). The -597 G—>A and -174 G—>C polymorphisms in the promoter of the IL-6 gene are associated with hyperandrogenism. *Journal of Clinical Endocrinology and Metabolism*,**87**(3): 1134–41.

Vincze, T., Posfai, J., & Roberts, R.J. (2003). NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research*,**31**: 3688-3691

Vine, A. K., Stader, J., Branham, K., Musch, D. C., & Swaroop, A. (2005). Biomarkers of cardiovascular disease as risk factors for age-related macular degeneration. *Ophthalmology*, **112**(12): 2076-2080.

Vingerling, J. R., Dielemans, I., Bots, M. L., Hofman, A., Grobbee, D. E., & de Jong, P. T. (1995). Age-related Macular Degeneration Is Associated with Atherosclerosis: The Rotterdam Study. *American Journal of Epidemiology*,**142**(4): 404-409.

Vinores, S. A., Xiao, W. H., Aslam, S., Shen, J., Oshima, Y., Nambu, H., Liu, H., Carmeliet, P., & Campochiaro, P. A. (2006). Implication of the hypoxia response element of the Vegf promoter in mouse models of retinal and choroidal

neovascularization, but not retinal vascular development. *Journal of Cellular Physiology*, **206**(3): 749-758.

Wang, L., Clark, M. E., Crossman, D. K., Kojima, K., Messinger, J. D., Mobley, J. A., & Curcio, C. A. (2010). Abundant lipid and protein components of drusen. *PLoS One*, **5**(4): e10329.

Wang, Y., Wang, V. M., & Chan, C. C. (2011). The role of anti-inflammatory agents in age-related macular degeneration (AMD) treatment. *Eye*, **25**(2): 127-139.

Watson, C. J., Webb, N. J., Bottomley, M. J., & Brenchley, P. E. (2000). Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine*, **12**(8): 1232-1235.

Watzke, R. C., Klein, M. L., Hiner, C. J., Chan, B. K., & Kraemer, D. F. (2000). A comparison of stereoscopic fluorescein angiography with indocyanine green videoangiography in age-related macular degeneration. *Ophthalmology*, **107**(8): 1601-1606.

West, S., Vitale, S., Hallfrisch, J., Munoz, B., Muller, D., Bressler, S., & Bressler, N. M. (1994). Are antioxidants or supplements protective for age-related macular degeneration? *Archives of Ophthalmology*, **112**(2): 222-227.

Whitehead, A. S., Bruns, G. A., Markham, A. F., Colten, H. R., & Woods, D. E. (1983). Isolation of human C-reactive protein complementary DNA and localization of the gene to chromosome 1. *Science*, **221**(4605): 69-71.

Wilkinson-Berka, J. L., Babic, S., de Gooyer, T., Stitt, A. W., Jaworski, K., Ong, L. G., Kelly, D. R., & Gilbert, R. E. (2004). Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy. *The American Journal of Pathology*, **164**(4): 1263-1273.

Witmer, A. N., Vrensen, G. F. J. M., Van Noorden, C. J. F., & Schlingemann, R. O. (2003). Vascular endothelial growth factors and angiogenesis in eye disease. *Progress in Retinal and Eye Research*, **22**(1): 1-29.

Wolpert, S. (2000). A new history of India. Oxford University Press, New York.

Wong, W. L., Su, X., Li, X., Cheung, C. M. G., Klein, R., Cheng, C. Y., & Wong, T. Y. (2014). Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *The Lancet Global Health*, **2**(2): e106-e116.

Woo, J. H., Sanjay, S., & Au Eong, K. G. (2009). The epidemiology of age-related macular degeneration in the Indian subcontinent. *Acta ophthalmologica*, **87**(3): 262-269.

Wu, M. S., Huang, S. P., Chang, Y. T., Shun, C. T., Chang, M. C., Lin, M. T., Wang, H. P.,& Lin, J. T. (2002). Tumor necrosis factor-alpha and interleukin-10 promoter polymorphisms in Epstein-Barr virus associated gastric carcinoma. *The Journal of Infectious Diseases*,**185**: 106–109.

Xu, H., Chen, M., & Forrester, J. V. (2009). Para-inflammation in the aging retina. *Progress in Retinal and Eye Research*, **28**(5): 348-368.

Xu, W., Grunwald, J. E., Metelitsina, T. I., DuPont, J. C., Ying, G. S., Martin, E. R., Dunaief, J. L.,& Brucker, A. J. (2010). Association of risk factors for choroidal neovascularization in age-related macular degeneration with decreased foveolar choroidal circulation. *American Journal of Ophthalmology*, **150**(1): 40-47.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., & Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature*, **407**(6801): 242-248.

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., & Zhang, K. (2006). A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. *Science*, **314**(5801): 992-993.

Yang, Z., Tong, Z., Chen, Y., Zeng, J., Lu, F., Sun, X. Zhao, C., Wang, K., Davey, L., Chen, H., London, N., Muramatsu, D., Salasar, F.,Carmona, R., Kasuga, D., Wang, X., Bedell, M., Dixie, M., Zhao, P., Yang, R., Gibbs, D., Liu, X., Li, Y., Li, C., Li, Y., Campochiaro, B., Constantine, R., Zack, D. J., Campochiaro, P., Fu, Y., Li, D. Y., Katsanis, N., & Zhang, K. (2010).

Genetic and functional dissection of HTRA1 and LOC387715 in age-related macular degeneration. *PLoS Genetics*, **6**(2): e1000836.

Yannuzzi, L. A., Sorenson, J. A., Sobel, R. S., Daly, J. R., Derosa, J. T., Seddon, J. M., & Phillips, D. A. (1992). Risk-factors for neovascular age-related macular degeneration. *Archives of Ophthalmology*, **110**(12): 1701-1708.

Yates, J. R., Sepp, T., Matharu, B. K., Khan, J. C., Thurlby, D. A., Shahid, H., Clayton, D. G., Hayward, C., Morgan, J., Wright. A. F., Armbrecht, A. M., Dhillon, B., Deary, I. J., Redmond, E., Bird, A. C., &Moore, A. T. (2007). Complement C3 variant and the risk of age-related macular degeneration. *New England Journal of Medicine*, **357**(6): 553-561.

Yildirim, Z., Ucgun, N. I.., & Yildirim, F. (2011). The role of oxidative stress and antioxidants in the pathogenesis of age-related macular degeneration. *Clinics (Sao Paulo)*, **66**(5): 743-746.

Yilmaz, A., Davis, M. E., & Simmen, R. C. (1999). Reproductive performance of bulls divergently selected on the basis of blood serum insulin-like growth factor I concentration. *Journal of Animal Science*, **77**(4): 835-839.

Zafar, S. N., & Afghani, T. (2009). Prevalence Study of age related macular degeneration in a target population of Rawalpindi division**.** *Al-Shifa Journal of Ophthalmology*,**5**(1): 24-27.

Zarbin, M. A. (2004). Current Concepts in the Pathogenesis of Age-Related Macular Degeneration.*Archives of Ophthalmology*, **122**:598-614.

Zeiss, C. J. (2010). Animals as models of age-related macular degeneration. An imperfect measure of the truth. *Veterinary Pathology Online*, **47**(3): 396-413.

Zhang, H., Wu, L. M., & Wu, J. (2011). Cross-talk between apolipoprotein E and cytokines. *Mediators of Inflammation*, ID 949072.

Zhang, L., Lim, S. L., Du, H., Zhang, M., Kozak, I., Hannum, G., Wang, X., Ouyang, H., Hughes, G., Zhao, L., Zhu, X., Lee, C., Su, Z., Zhou, X., Shaw, R., Geum, D., Wei, X., Zhu, J., Ideker, T., Oka, C., Wang, N., Yang, Z., Shaw, P. X., & Zhang, K. (2012). High Temperature Requirement Factor A1 (HTRA1) Gene Regulates Angiogenesis through Transforming Growth Factor-β

Family Member Growth Differentiation Factor 6. *Journal of Biological Chemistry*, **287**(2): 1520-1526.

Zhang, N., Xu, Y., Zhang, B., Zhang, T., Yang, H., Zhang, B., Zufei, F., & Zhong, D. (2014). Analysis of interleukin-8 gene variants reveals their relative importance as genetic susceptibility factors for chronic periodontitis in the Han population. *PloS One*, **9**(8): e104436.

Zhou, J., Pham, L., Zhang, N., He, S., Gamulescu, M. A., Spee, C., Ryan, S. J., & Hinton, D. R. (2005). Neutrophils promote experimental choroidal neovascularization. *Molecular Vision*, **11**: 414-424.

Zipfel, P. F., & Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nature Reviews Immunology*, **9**(10): 729-740.

Zipfel, P. F., Edey, M., Heinen, S., Józsi, M., Richter, H., Misselwitz, J., Hoppe, B., Routledge, D., Strain, L., Hughes, A. E., Goodship, J. A., Licht, C., Goodship, T. H., &Skerka, C. (2007). Deletion of Complement Factor H–Related Genes CFHR1 and CFHR3 Is Associated with Atypical Hemolytic Uremic Syndrome. *PLoS Genetics*, **3**(3): e41.

Zipfel, P. F., Lauer, N., & Skerka, C. (2010). The role of complement in AMD. In: *Inflammation and Retinal Disease: Complement Biology and Pathology*. Lambris, J. D. and Adamis, A. P. (Editors.). Springer New York. USA. pp. 9-24.