DETERMINATION OF ROLE OF COMPLEMENT PATHWAY GENES, CFI & CFH, IN THE ONSET OF AGE RELATED MULTIFACTORIAL EYE DISORDERS

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FINAL APPROVAL

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DEDICATION

I dedicate this thesis to my parents, for without their support in more ways than can be mentioned, encouragement, advice and catharsis I wouldn't have been able to fare as I have. It would be remiss of me to not mention my incorrigible sisters and devoted friends, the mere presence of whom marks a great difference. You all have shared my burden in an unimaginable way and for that I'll be ever in your debt.

Zantasha Khalid

DECLARATION

It is hereby declared that the work present in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

Date 4 Aug 2015

Zantasha Khalid

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
DR	Diabetic Retinopathy
AMD	Age-related Macular Degeneration
RGCs	Retinal Ganglion Cells
ONH	Optic Nerve Head
IOP	Intraocular Pressure
AH	Aqueous Humor
POAG	Primary Open Angle Glaucoma
PACG	Primary Angle Closure Glaucoma
JOAG	Juvenile Onset Open Angle Glaucoma
NTG	Normal Tension Glaucoma
CG	Congenital Glaucoma
PEXG	Secondary Glaucoma-Pseudoexfoliative Glaucoma
CCT	Central Corneal Thickness
ET-1	Endothelin-1
NO	Nitric Oxide
H_2O_2	Hydrogen Peroxide
RPE	Retinal Pigment Epithelium
PUFA	Polyunsaturated Fatty Acids
BM	Bruch's Membrane
VEGF	Vascular Endothelial Growth Factor
CS	Complement System

MAC	Membrane Attack Complex
MBL	Mannose Binding Lectin
MASP	MBL Associated Serine Proteases
DAF	Decay Accelerating Factor
CFI	Complement Factor I
SCR	Short Consensus Repeat
FIMAC	FI and Membrane Attack Domain
LDLR	Low Density Lipoprotein Receptor Domains
MCP	Membrane Cofactor Protein
EDTA	Ethylene diamine tetra acetic acid
RBCs	Red Blood Cells
NH_4Cl	Ammonium Chloride
$NaHCO_3$	Sodium Bicarbonate
NaCl	Sodium Chloride
TE	Tris-EDTA
SDS	Sodium Dodecyl Sulphate

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INTRODUCTION

1.0 INTRODUCTION

Multifactorial diseases are complex diseases which do not follow the typical Mendelian patterns of inheritance. In such complex disorders, while the presence of disease associated genes increase the susceptibility of an individual to develop the disorder, however, it is not sufficient to develop the disease itself. The phenotypic development of the disease, therefore, depends on the interactions of multiple genes with lifestyle and environmental factors (Craig, 2008). To mention a few, these disorders include Diabetes, Obesity, Depression, Cancer, Cardiovascular diseases, Endocrine diseases, Multiple Sclerosis and Asthma (Stolk *et al.*, Lobo, 2008). Similarly, these complex factors (genetic and environmental) are also involved in causing eye disorders such as Dry Eye Syndromes (Javadi *et al.*, 2011), Diabetic Retinopathy (DR) (Kowluru, 2010), Age-related Macular Degeneration (AMD) (Swaroop *et al.*, 2007) and Glaucoma (Jindal, 2013). Among complex eye disorders Glaucoma and AMD are the most prevalent worldwide.

1.1 Glaucoma

Glaucoma comprises of a collection of multifactorial neurodegenerative eye disorders characterized by the irreversible degeneration of the retinal ganglion cells (RGCs) which results in cupping of the optic nerve head (ONH) due to an elevation in the intraocular pressure (IOP) (IOP > 21mm of Hg) (Menaa *et al.*, 2011).

1.1.1 Ocular Anatomy and Physiology Relevant to Glaucoma

1.1.1.1 Production and Outflow of Aqueous Humor

Aqueous humor (AH) is a transparent fluid circulating in the anterior segment of the eye. Production and outflow of aqueous humor needs to be strictly regulated in order to maintain

the intraocular pressure in the eye. Under normal conditions, IOP rises due to resistance offered by the anterior chamber angle to its outflow to maintain the AH value at 15 mm of Hg. However, in glaucomatous condition this resistance becomes unusually high resulting in an elevation of IOP to 21mm of Hg (Goel *et al.*, 2010).

The aqueous humor is actively secreted by the pigmented as well as non-pigmented cells of the ciliary body and exits the eye mainly via the trabecular meshwork in the anterior chamber angle and then via the Schlemm's canal into the episcleral veins. The inner wall of Schlemm's cell provides the greatest resistance to the aqueous outflow. This is known as trabecular or conventional outflow pathway, which is the main pathway for aqueous humor outflow and is pressure-dependant in nature i.e. as the IOP rises, the flow through this pathway increases (Toris *et al.*, 2011). The state of the actin cytoskeleton and adhesion of the trabecular meshwork cells are important determinants of fluid outflow through the trabecular meshwork (Tan *et al.* 2006). Similarly, a small quantity of the aqueous humor is drained via the ciliary muscle, the suprachoroidal space, and the sclera, via a process known as uveoscleral or unconventional outflow of aqueous humor (Goel *et al.*, 2010) (Fig 1).

1.1.1.2 Anatomy of the Optic Nerve Head

The optic nerve head with adjoining nerve fibres running down through the lamina cribrosa forms the optic nerve which acts as a bridge between the eye and the brain and carries communication between them. The retinal surface is irregularly covered with nerve fibers present largely above and below the disc and join at the edge of the disc called scleral ring and at the inner surface. This dense presence of nerve fibers inside the scleral ring forms the neurorctinal rim where the cup forms the central area. However a glaucomatous eye undergoes structural changes which aid in the disease progression. The cup becomes enlarged, disc haemorrhage is observed, neuroretinal rim thins down, an irregular cupping

between eyes of a patient is seen, the nerve fiber layer is lost and parapapillary atrophy is observed which is common in glaucomatous eyes (Bourne, 2006) (Fig 2).

1.1.2 Epidemiology of Glaucoma

Though the elevated IOP is a major highlight in glaucoma nevertheless IOP lowering therapies, whether medical or surgical, solely have not been successful for the treatment of this disease, hence, a continuing neural damage is observed as the disease progresses (Jindal, 2013). Due to this complex nature, glaucoma is reported to be the causative agent of affecting 70 million people, including both blind and at risk, worldwide (Goel *et al.*, 2010). It contributes to the 8% of total blindness, 39 million blind people worldwide, making it the second major cause of blindness where Africa has the highest frequency of the disease as compared to the other regions of the world. The number of people being affected by glaucoma tends to increase as the years advance. It is estimated that this number would reach up to 20 million by the year 2020 (Kyari *et al.*, 2013). The rapid increase in the number of blind people across the globe is a result of combined impact of several types of glaucoma contributing hand in hand to the total prevalence of the disease.

1.1.3 Classification of Glaucoma

The characterization of glaucoma into various types is based on the primary and secondary characteristics of the disease where, the age of onset and iridocorneal angle depict the primary characteristics, while IOP, pseudoexfoliations and developmental abnormalities depict the secondary characteristics. These characteristics form the foundation and confer to six main types of glaucoma:

1. Primary Open Angle Glaucoma (POAG)
2. Primary Angle Closure Glaucoma (PACG)

3. Juvenile Onset Open Angle Glaucoma (JOAG)
4. Normal Tension Glaucoma (NTG)
5. Congenital Glaucoma (CG)
6. Secondary Glaucoma-Pseudoexfoliative Glaucoma (PEXG)

Among these types, POAG is the most common form worldwide and is characterized by an open iridocorneal angle and elevated IOP. Its onset occurs at a late age and has been shown to follow a familial pattern of inheritance. Lack of the associated symptoms makes it undetectable until irreversible damage has occurred (Jannson, 2004; Bailey *et al.*, 2013).

The early onset of POAG (between 30 to 40 years) is termed as JOAG (Menaa *et al.*, 2011). Similarly, onset of Glaucoma prior to 3 years of age with open iridocorneal angle and elevated IOP is termed as Congenital Glaucoma. Another condition usually classed under the POAG category is NTG, which is characterized by open iridocorneal angle but normal IOP (Jannson, 2004; Rao *et al.*, 2013).

The second major type of Glaucoma is Primary Angle Closure Glaucoma (PACG) in which the iridocorneal angle is closed in addition to an elevated IOP. It differs from POAG in that the symptoms, such as headaches, nausea, pain and blurred vision, are more frequent hence making it easier to diagnose (Jannson, 2004; Rao *et al.*, 2013).

Secondary glaucoma, on the other hand, is caused by a collection of secondary characteristics such as pseudoexfoliation, developmental abnormalities or trauma. PEXG is the most common type of secondary glaucoma which is characterized with late onset, open iridocorneal angle and an elevated IOP along with deposition of exfoliative material on the lens (Ayub *et al.*, 2014).

Increased IOP is a common culprit in POAG, PCAG and PEXG yet the reason for the IOP elevation varies from type to type. In POAG and PCAG the intraocular pressure raises due to

an obstructed trabecular meshwork while blockage due to the deposition of white dandruff like exfoliation material in the trabecular meshwork causes the increased IOP in PEXG. The exfoliative material in PEXG is mainly composed of glycol-conjugates surrounding a protein core and are synthesized in response to oxidative stress in a variety of cells like non-pigmented ciliary epithelial cells (which are also responsible for the production of Aqueous Humor), trabecular meshwork cells and pre-equatorial lens epithelial cells. The raised IOP results in degeneration of retinal ganglion cells; though one type of Glaucoma, Normal Tension Glaucoma bears an exception to this. Absence of a major hallmark i.e. elevated IOP in NTG indicates that Glaucoma, as a disease, is caused by complex and heterogeneous mechanisms (Kurihara *et al.*, 2012).

1.1.4 Clinical Risk Factors

1.1.4.1 General Risk Factors

Among several risk factors of glaucoma, age is of prime importance. In POAG, the risk factor increases with age. This also supports the vascular theory for glaucoma since the ocular blood flow is reported to reduce with increasing age. While looking at the association of gender as a contributing factor for POAG the results are found to be very contradictory leading to no definite conclusion. In addition epidemiological studies have revealed that African natives are at a higher risk than their white counterparts as well as being subject to an earlier onset of glaucoma. Glaucoma is found to segregate in families thus the risk of having glaucoma is increased 10 times in the members of a family (Montgomery, 2007).

1.1.4.2 Ocular Risk Factors

Intraocular pressure remains a major risk factor for glaucoma as reduced levels of IOP tend to reduce the risk of glaucoma and vice versa. There is evidence that myopic patients are at a

higher risk of developing glaucoma than non-myopic individuals (Chen *et al.*, 2012). The variation in central corneal thickness (CCT) is reported to have a considerable impact on POAG specifically. The thinner the CCT is (<555μm), the more imminent becomes the risk of developing glaucoma as compared to a thicker CCT (>558μm) (Montgomery, 2007).

1.1.4.3 Systemic Risk Factors

It has also been observed in some studies that diabetics have two to three times greater risk of occurrence of POAG where as other studies show variable results. This has been postulated to be due to the fact that diabetic patients undergo regular examination, thus glaucoma is more likely to be diagnosed in such patients. While considering blood pressure as a contributing factor, varying results have been reported. This again can be explained with respect to the vascular theory of glaucoma. The cupping of the optic nerve head ultimately leads to microvascular damage and impaired blood flow, resulting in hypertension (Montgomery, 2007).

1.1.5 Pathophysiology of Glaucoma

Glaucoma is affected by multiple factors which directly or indirectly damage the optic nerve. Among these factors increased IOP and vascular damage is of main concern while secondary factors such as excitotoxic damage and oxidative damage from reactive oxygen species also play a negative role. The combined effect, however, of the primary and secondary elements lead to the irreparable blindness (Agarwal *et al.*, 2009).

1.1.5.1 Theories of Pathogenesis of Glaucoma

Pathogenesis of glaucoma has been related to two main theories. According to the "Mechanical Theory", glaucoma develops as the elevated IOP directly damages the axons of

the retinal ganglion cells by inducing cribriform plate compression of the axons at the level of the lamina cribrosa (Resch *et al.*, 2009).

On the other hand the "Vascular Theory" implies that the progression of glaucoma is marked by ischemia in the optic nerve; this includes glaucomatous ganglion cell damage due to insufficient blood supply in the optic nerve head. Several studies have highlighted the role of vascular factors such as ischemia and vascular dysregulation in the pathogenesis of glaucoma. Moreover, existence of various systemic vascular diseases such as Hypertension, Migraine, Vasospasm and transient nocturnal decrease of Systemic Blood Pressure also act as predisposing factors for glaucoma. Vascular dysregulation originates in the vascular endothelium lining the ocular blood vessels. Vascular endothelium acts as a single-celled barrier between the blood plasma and the blood vessels and performs vascular homeostasis via the production of several important vasoactive peptides such as Endothelin-1 (ET-1), Angiotensin-II, Thromboxane [Vasoconstrictors] and Nitric Oxide (NO), Prostacyclin, Hydrogen Peroxide (H_2O_2) [Vasodilators] (Resch *et al.*, 2009).

1.1.5.2 Neuronal loss in glaucoma by Apoptosis

In glaucoma the cupping of the optic nerve head arises due to the loss of the retinal ganglion cells. This loss has been associated to the programmed cell death universally known as apoptosis. The chains of events that ultimately lead to apoptosis include DNA fragmentation, chromosome clumping, cell shrinkage and blebbing of the cell membrane. Once the nuclear damage has occurred, the cell is broken down into numerous membrane bound vesicles which are engulfed by the adjacent cells consequently removed from the system. In other words, the abnormally elevated IOP due to increased aqueous humor resistance causes the death of the retinal ganglion cells, by apoptosis leading to the cupping of the optic nerve head (Agarwal *et al.*, 2009).

1.1.5.3 Damage by Secondary Factors

In hypoxic situations the ganglion cells release a neurotransmitter glutamate, found in the retina. When the glutamate levels increase from the normal physiological levels it becomes toxic to the surrounding neurons hence causing the apoptotic cell death of the retinal ganglion cells (Agarwal *et al.*, 2009).

Similarly nitric oxide (NO) in excess has also been reported to be associated with neurological conditions. NO functions as an important messenger and is involved in vasodilatation, neurotransmission, neurotoxicity and inflammation. The enzymatic action of nitric oxide synthetase (NOS) on L-arginine results in three isoforms namely NOS-1 neuronal, NOS-2 inducible and NOS-3 endothelial [vasodilation] (Stefan *et al.*, 2006).

It has been reported that glaucomatous patients have a significantly reduced antioxidant capability in the aqueous humor despite the embellishment of the ocular tissue with highly efficient antioxidant defence mechanisms. Under the oxidative stress the endothelial functions as well as the production of NO is disturbed. Therefore the excessive formation of free radicals and oxidative stress is considered pathophysiological in glaucoma leading to cell death (Agarwal *et al.*, 2009).

1.2 Age-related Macular Degeneration

AMD is a multifactorial blinding disease of the elderly caused due to the irreparable and continuous degeneration of the macula, an area in the retinal centre (Aslam *et al.*, 2014). The macular region is thickly populated with photoreceptors which allow detailed central vision. Thus damage to the macula leads to the loss of central vision (Yates *et al.*, 2007).

1.2.1 Ocular Anatomy and Physiology Relevant to AMD

1.2.1.1 The Macula

The posterior retina comprises of a yellow, capillary free segment comprising of xanthophyll pigments and ganglion cells layers. This region, of 5.5 mm diameter, located below the optic nerve head is known as macula. It is further divided into four zones fovea, foveola, parafovea and perifovea.

Fovea is the densely pigmented centre of the macula, forming about 1.5 mm of its total diameter. Foveola forms the centre of the fovea which has reduced thickness and contributes about 0.35 mm diameter of the macula. The reduced thickness occurs due to the lack of inner plexiform, ganglion cells and nerve fiber layers. The foveola only contains cones which are responsible for high acuity vision. The parafoveal zone forms a 0.5 mm wide ring surrounding the foveola and is the thickest part of the retina due to the presence of a thick mass of ganglion cells, inner nuclear layer neurons and both photoreceptor cells, the rods and cones. This arrangement is further surrounded by a 1.5 mm perifoveal zone where the macula ends. In other words, the thickest portion of the retina surrounds the thinnest portion, the foveolar zone (Milam *et al.*, 2006) (Fig 3).

1.2.2 Prevalence of AMD

AMD is a leading cause of progressive, irreparable visual impairment and ultimately blindness. It contributes to at least 8.7% of the total blindness (Wong *et al.*, 2014) and affects more than 50 million people across the globe. In America alone the number of individuals suffering from AMD is reported to be 1.75 million while reports for UK indicate 214000 individuals suffering from AMD (Hageman *et al.*, 2005). As AMD resides in elder population, above the age of 60, the ratio of AMD is expected to continuously grow

alongside the ageing population. On the basis of current knowledge about AMD, the number of people suffering from AMD by 2020 is estimated to be 196 million and will further increase up to 288 million by 2040 unless steps to improve the understanding of AMD are taken (Thornton *et al.*, 2005; Wong *et al.*, 2014). These figures indicating the total prevalence of AMD have been calculated on the basis of two types of AMD.

1.2.3 Classification of AMD

AMD is classified into two types, wet (exudative) and dry (non-exudative) AMD. Among the two forms, exudative AMD is less common and gives rise to approximately 10% whereas non exudative AMD, the commonly occurring form, contributes to the 90% of the total AMD cases. Wet AMD differs from dry AMD in that the wet form is characterized by the formation of abnormal blood vessels in the macula which cause hemorrhage, exudation, scarring or serous retinal detachment while in dry AMD the degradation of the retinal pigment epithelium (RPE) as well as the diminishing of the photoreceptor cells in the macula cause retinal and neighbouring tissue death (Kawa *et al.*, 2014; Aboobakar *et al.*, 2014). This atrophy is known to be caused by extracellular deposits known as drusen which deposit, on the Bruch's membrane (a layer in the retina) below the retinal pigment epithelium, with age. Although the exact composition and mechanism of drusen deposits is not known, depending upon the size and quantity of these deposits, they act as a major risk factor in AMD ultimately causing one of its two types henceforth loss of central vision (Jager *et al.*, 2008).

1.2.4 Clinical Risk Factors

1.2.4.1 General Risk Factors

AMD has a late age of onset (>60 years) therefore the risk of developing AMD increases with the advancing age. It is the most significant and non-modifiable risk factor of AMD.

However AMD does not occur in all older population. Evidence suggests that AMD is an autosomal dominant trait hence increasing the susceptibility of the disease in an affected family or population (Jager *et al.*, 2008). Similarly ethnicity has proven to be an effective risk factor as the Western countries have a high risk ratio of developing AMD as compared to Eastern countries. On the other hand gender has not shown any significant involvement in the development of the disease (Chakravarthy *et al.*, 2010). Smoking has shown significant association with this disorder. The risk of developing AMD increases two to three folds in smokers as compared to non-smokers however there is evidence indicating reversal of risk in ex-smokers which suggests the reversal of damage caused by smoking (Seddon, 2013).

1.2.4.2 Ocular Risk Factors

The presence of drusen deposits is the hallmark of AMD development hence known as one of the most significant risk factors of the disorder. Deposition, specifically of large and soft drunsen and hyperpigmentation in the RPE are considered as important indicators of early AMD stage (Jong *et al.*, 2006).

1.2.4.3 Systemic Risk Factors

Systemic hypertension has been found to be associated with AMD although the extent of these associations varies from population to population moreover relationship of AMD also depends on the type of AMD. Where neovascular AMD is shown to be associated with hypertension, the non-neovascular AMD is independent of it (Hyman *et al.*, 2000; Chakravarthy *et al.*, 2010).

1.2.5 Pathophysiology of AMD

Progression of AMD is associated with oxidative damage, lipofuscin accumulation and chronic inflammation. All these factors combined play a significant role in the pathogenesis of AMD hence causing vision loss (Zarbin, 2012).

1.2.5.1 Role of Oxidative Damage in AMD

It is widely accepted that oxygen, though necessary for aerobic respiration, also acts as toxic for many cells and tissues of the body. Various research data indicates a significant association of oxidative damage with the pathogenesis of AMD. The long term exposure of the photoreceptors to sunlight causes the polyunsaturated fatty acids (PUFA) (present in abundance in photoreceptor membranes) to undergo oxidation due to their high affinity with oxygen and oxygen derived radical species hence causing oxidative damage. Similarly the undegradable lipofuscin accumulation, which occurs throughout the life of an individual as a consequence of phagocytosis of the photoreceptors, in the RPE is also reported to be a response to oxidative stress and sunlight. This accumulation also plays a vital role in the pathogenesis of AMD. Choriocapillaris is another target site where light sensitive reactions may occur leading to AMD. Light exposure causes the activation of haemoglobin precursors in the red blood cells running through the choriocapillaris which in turn are involved in the generation of reactive oxygen species causing damage to the RPE and Bruch's membrane (Zarbin, 2012).

1.2.5.2 Pathogenesis of AMD

The progression of AMD is characterized by the deposition of cellular debris in the retina leading towards morphological changes. If left unchecked these changes affect the macula in

such a way as to take away the central vision. This visual impairment occurs in form of either wet or dry AMD.

Dry AMD occurs as a result of drusen deposits beneath the RPE located within Bruch's Membrane (BM) which acts as a barrier between the RPE and choroid. This barrier regulates the diffusion of nutrients, oxygen and metabolic waste between RPE and choriocappillaris. The morphological changes in BM due to age such as thickening, calcification, degeneration of collagen and elastin fibres etc impairs the exchange between RPE and choriocappillaris leading to the deposition of drusen material. The drusen deposits are of two types, hard and soft drusen. The presence of hard drusen is found normally in the ageing population however the amalgamation of several hard drusen gives rise to comparatively larger irregularly bordered soft drusen which is an important hallmark of AMD. The end stage in the pathogenesis of dry AMD involves geographical atrophy of the macula and RPE leading to cell death. Wet AMD on the other hand progresses as a result of abnormal angiogenesis of choroidal blood vessels into the retina and hence the macula. Angiogenesis is characterized by increased levels of vascular endothelial growth factor (VEGF) in the retina causing the proliferation of vascular endothelial cells. This choroidal neovascularization damages the BM barrier and invades the RPE and macula. Since this vasculature is abnormal it is characterized by continuous bleeding forcing the macula to bulge out and cause haemorrhaging and tissue scarring causing central vision loss. Where, dry AMD has a slow progression wet AMD can progress in mere months or weeks (Porte, 2012; Guilloty *et al.*, 2011).

1.3 Age as a Risk Factor for Glaucoma and AMD

Glaucoma and AMD both have several common risk factors such as age, familial inheritance, hypertension, oxidative stress and inflammation. However, age is the prime risk factor in both glaucoma and AMD. Aging is a normal and irreversible function of the body affecting all the

organs such as eye, heart, brain, bones etc and hence all the systems of the body. It is a process of slow yet continuous deterioration of the body. This gradual deterioration causes pathological affects hence the reason of growing number of disorders in the elderly population (Boss and Seegmiller, 1981). As the years add on, the risk of developing all three types of glaucoma (POAG, PCAG and PEXG) as well as AMD increases. There is no sufficient data related to both disorders which might give insight into the reasons as to why age plays a significant role in the onset of these disorders (Montgomery, 2007; Yates *et al.*, 2007) however age as a serious risk feature for glaucoma and AMD is an established fact but the common genetic components causing the diseases are not fully defined.

1.4 Complement System

The human body is equipped to respond to an infection by utilizing either the innate immune system, that does not rely on antigen specific lymphocytes or the adaptive immune system which is target specific and activated if the innate immune system is breached. Complement system (CS) is a multiple protein based first-line defence mechanism of the innate immune system effective immediately once the pathogen escapes and overpowers the initial barriers: the epithelial and antimicrobial barriers. The complement proteins are inactive soluble proteins, produced by the liver, which circulate the blood and other bodily fluids, activated in the event of an infection. The proteins involved in the complement system are mostly proteases which conduct a sequential cascade to cleave and activate one another. These sequentially activated proteins form different pathways of complement activation which are responsible to eliminate the pathogens either by a direct attack or facilitating its phagocytosis by other cells of the innate immune system (Sivaprasad and Chong, 2006; Chen *et al.*, 2010).

On the basis of their mode of activation there are three complement activation pathways:

1. The Classical Pathway
2. Lectin Pathway
3. Alternative Pathway

1.4.1 Nomenclature of Complement Proteins

The proteins of the classical pathway were first to be discovered and designated by the letter C and a number. The parent inactive proteins are named as C1, C2, C3, C4, C5, C6, C7, C8 and C9 while their cleaved products are given a lower case suffix such as C3 is cleaved into a smaller fragment C3a and a larger fragment C3b. The larger fragment is always designated with the suffix b, with one exception for C2 in which the larger fragment was named C2a. Similarly C1 protein is formed of three additional protein components C1q, C1r and C1s and it is worth mentioning here that these three proteins should not be mistaken as the cleavage products of C1. On the other hand the alternative pathway proteins are designated by letters B and D with 'a' and 'b' as suffix indicating large and small fragments of the cleavage product (Kemper *et al.*, 2014, Yates *et al.*, 2007).

1.4.2 Complement Pathways

Although all three pathways of the complement system have different triggers but they converge at a common point to initiate the terminal pathway where the C3 protein is cleaved followed by the formation of membrane attack complex (MAC) C5b-9 which is responsible for the pathogenic lysis (Nilsson *et al.*, 2009)

The activation of classical pathway takes place when the C1q binding component of the C1 protein encounters a pathogen directly or to antibodies already present on the pathogenic surface. This leads to a conformational change in the C1s:C1r complex which activates the C1r component in such a way that it cleaves the neighbouring C1s converting it into an active

serine protease followed by cleavage of C4 component into C4a and C4b and C2 component into C2a and C2b. The larger peptides gather to form the C3 convertase, C4b2a, which cleaves C3 component to release two peptides C3a, an inflammatory mediator and C3b, an opsonin to facilitate phagocytosis. The C3b fragment also has the potential to combine with the previously formed C3 convertase and generate the C5 convertase, C4b2a3b to form MAC (Kopp *et al.*, 2012).

The lectin pathway is triggered by the binding of carbohydrate binding proteins, the mannose binding lectin (MBL) and the ficolins which serve as pattern recognition receptors, with the carbohydrates on the pathogenic surface. The MBL complexes with the MBL associated serine proteases (MASP) 1, 2 and 3. When the MBL binds with the surface of a foreign intruder the MASP activity is activated due to conformational changes in the MBL complex leading to the cleavage of C4 and C2 components ending up with the generation of C3 convertase which is further responsible for the generation of C5 convertase (Rus *et al.*, 2005).

The alternative pathway can be activated in two different ways and has a C3 convertase different from the typical classical and lectin pathway C3 convertase. The first method involves utilizing the C3b fragment generated by either classical or lectin pathway attached to the microbial surface. This surface bound C3b has the ability to bind factor B further causing conformational changes in the factor B making it available for factor D protease which cleaves it into Ba and Bb fragments. The Bb fragment maintains its association with C3b to form C3bBb, the alternative pathway C3 convertase. The second method involves spontaneous hydrolysis of C3 to form iC3b which binds B factor and is later cleaved by factor D into Ba and Bb. The iC3b and Bb together form a complex, C3bBb, the C3 convertase of the alternative pathway which generates C5 convertase in the following steps (Sarma and Ward, 2011) (Fig 4).

1.4.3 Complement Pathway Regulation

There are several mechanisms involved which limit the actions of complement system to the pathogenic surface or on damaged host cells to protect the normal cells of the body. The regulation of these pathways involves C3 converatse stability by combined action of both soluble and membrane bound proteins which take part as either positive or negative regulators (Kopp *et al.,* 2012).

Properdin acts as a positive regulator for stabilizing the C3bBb complex. Similarly negative regulatory proteins include decay accelerating factor (DAF or CD55) which act by competing with the factor B hence dissociating the C3b component of already formed C3 convertase. The cell surface type I complement receptors (CR1 or CD35) has the same activity as DAF but has a limited tissue distribution.

The complement factor I (CFI) along with co-factors such as membrane cofactor of proteolysis (MCP or CD46) acts by cleaving C3b to inactive iC3b thus preventing C3 convertase formation. Likewise, complement factor H binds C3b and competes with factor B resulting in the displacement of Bb from alternative pathway C3 convertase. In addition to its direct role in the negative control of complement pathway CFH also acts as a cofactor for CFI and is abundantly present on vertebrate cells hence protecting the normal cells of the. Soluble proteins such as vitronectin and clusterin prevent cell lysis by binding with the C5b67 complement complexes (Sivaprasad and Chong, 2006).

1.4.4 Complement System in the Eye

Recent reports suggest the presence of local complement system pathways in the eye specifically in cornea, aqueous humor, tears and retina. The components such as C3, C5 and the MAC complex are found in the choriocappillaris and the vitreous of the eye. These tissues

also contain complement regulatory proteins such as DAF, MCP, CD59, CFI and CFH which are distributed randomly in the eye. The choriocappillaris, for example, are found to be rich in CFH and CD55 while CFB is present in choroid and found associated with the luminal surfaces of choroid endothelial cells. Another important component, the CFI is found excessively in the retina. Only CFD is found in both the retinal tissue and the choroid. Clusterin, vitronectin and CD59 show similar expression rate in the RPE and choroid while CD46 inhibitor of the alternative pathway is found in RPE, choroid and neural retina. Various findings have established that the choroid tissue is rich with activators and inhibitors of the complement system while retina and RPE express selective inhibitors of the complement system, CD46, CD55, CD59, CFH, CFI, clusterin and vitronectin (Jha *et al.*, 2007, Kawa *et al.*, 2014). If the complement system is not regulated efficiently it causes autoimmune responses and destruction of the tissues leading towards degenerative diseases of the eye (Fig 5).

1.4.5 Role of Complement System in Glaucoma and AMD

The presence of complement system in the eye plays a dual role. Although the initial purpose of CS is to provide first line of defence but it has also been implicated to play a role in the pathogenesis of many eye disorders including glaucoma and AMD as failure in proper regulation of the complement system due to increased risk factors or mutations in the CFI and CFH genes initiates an autoimmune response hence causing tissue degeneration.

In case of glaucoma the local complement system is activated during neurodegeneration of the RGCs. Under normally regulated conditions the CS recognizes the cellular debris and damaged tissue resulting from neural damage and aids in phagocytosis. However due to progressive neuronal damage in glaucomatous eye there is an increased activation of the

complement system or in some cases lack of regulation which leads to normal tissue damage (Tezel *et al.*, 2010).

The drusen deposits, major risk factor in AMD, are reported to be constituted of almost all the complement proteins including activators, regulators and terminal proteins. In case of AMD the drusen build-up offers mechanical damage to the eye and hence complement system is activated which causes the degeneration of the RPE cells and ultimately aids in the pathogenesis of AMD (Kawa *et al.*, 2014).

1.4.6 CFH and CFI: Structure and Function

CFH is located on chromosome 1q31 adjacent to the genes encoding factor H related proteins (CFHRs) and is responsible for encoding CFH protein. This blood soluble protein is present in a range of 116 to 711 mg/L in the plasma and is a 150 kDa single chain glycoprotein composed of 20 short consensus repeat (SCR) domains. Among which the N terminal (1-4 SCRs) and C terminal (19-20 SCRs) domains perform the important functions of complement regulation and target attachment respectively. This enables the CFH protein to inhibit the formation of C3 and C5 convertase, to compete with the factor Bb and hence degrade C3 convertase in the alternative pathway and to act as a co-factor for CFI protein, a product of CFI gene (Fig 6). The CFI protein has a serum concentration of 35 mg/L and plays an important regulatory role in all complement pathways (Malm *et al.*, 2012). Structurally it is a multidomain protein of about 85 kDa (Sim and Laich, 2000) which is proteolytically processed to give a heavy chain of 50 kDa and light chain of 38 kDa covalently linked by a disulphide bond. The heavy chain carries domains homologous to CD5, FI and membrane attack domain (FIMAC), the low density lipoprotein receptor domains 1 and 2 (LDLr1 and -2) while the catalytic serine protease (SP) domain is present on the light chain (Vyse *et al.*, 1994). CFI proteins act by cleaving the C4b and C3b components of the complement

activation pathways and can only function in the presence of its cofactors CFH, membrane cofactor protein (MCP) and complement receptor 1 (CR1) (Catterall *et al.,* 1987) (Fig 7).

1.4.7 CFH and CFI Association with Glaucoma and AMD

The onset of glaucoma and AMD both is dependent upon variable risk factors including both environmental and genetic which makes it hard for the researcher to pin down the exact cause of pathogenesis of the diseases. However, till date, there have been several gene association studies which have given us a better perspective towards these eye disorders. Some of these studies include positive association of estrogen SNP pathway with POAG among women (Pasquale *et al.,* 2013), association of POAG with genes that code for local factors involved in setting vascular tone (Kang *et al.*, 2014), association of BIRC6 gene polymorphisms with Pseudoexfoliative Glaucoma (Ayub *et al.,* 2014), association of AMD with allelic variants of genes such as ABCA4, APOE, FBLN5, ELOVL4, FBLN6 and TLR4 which are implicated as risk factors (Rivera *et al.,* Haines *et al.,* 2005). Similarly recent research has shown role of complement system (CS) in glaucoma and particularly CFH and CFI gene polymorphisms in pathogenesis of AMD (Scheetz *et al.*, 2013).

AMD, on the basis of current understanding, has been reported to be associated to the chromosome 1q31 region which is also the residing region of CFH. Secondly, the drusen deposits are composed of many components of the complement system. Thirdly, the two main important risk factors of AMD, smoking and age, increase the plasma levels of CFH protein (Klein *et al.,* 2005 and Bailey *et al.,* 2013). Similarly failure in regulation of CFI in AMD leads to eye inflammation. Amyloid beta is a component of drusen and has a high binding affinity to CFI, as a result, hampering the C3b cleaving potential of CFI (Alexander *et al.,* 2014). However the role of CFH and CFI in glaucoma is yet to be explored.

1.5 Aim of Study

The aim of this study was to investigate the potential role of complement pathway genes, CFH and CFI, in the onset of Glaucoma and AMD by means of conducting a case-control study based on AMD and two types of glaucoma namely POAG and PEXG.

Recent research implicates the involvement of complement pathway in both glaucoma and AMD however no significant studies confined to Pakistan have occurred therefore this study focuses on the Pakistani population in hopes to gain improved understanding of these diseases. It will allow us a better insight into their pathogenesis and ultimately take us a step closer towards finding means of treatment for both glaucoma and AMD.

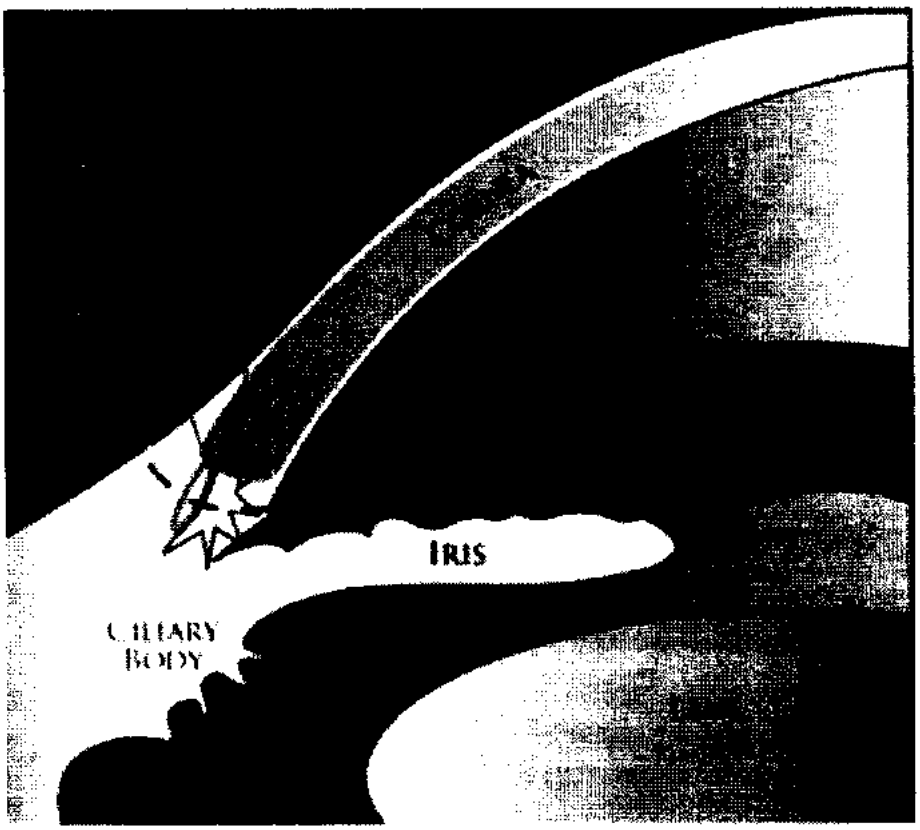

Figure 1 The trabecular meshwork conventional outflow pathway (Goel *et al.*, 2010)

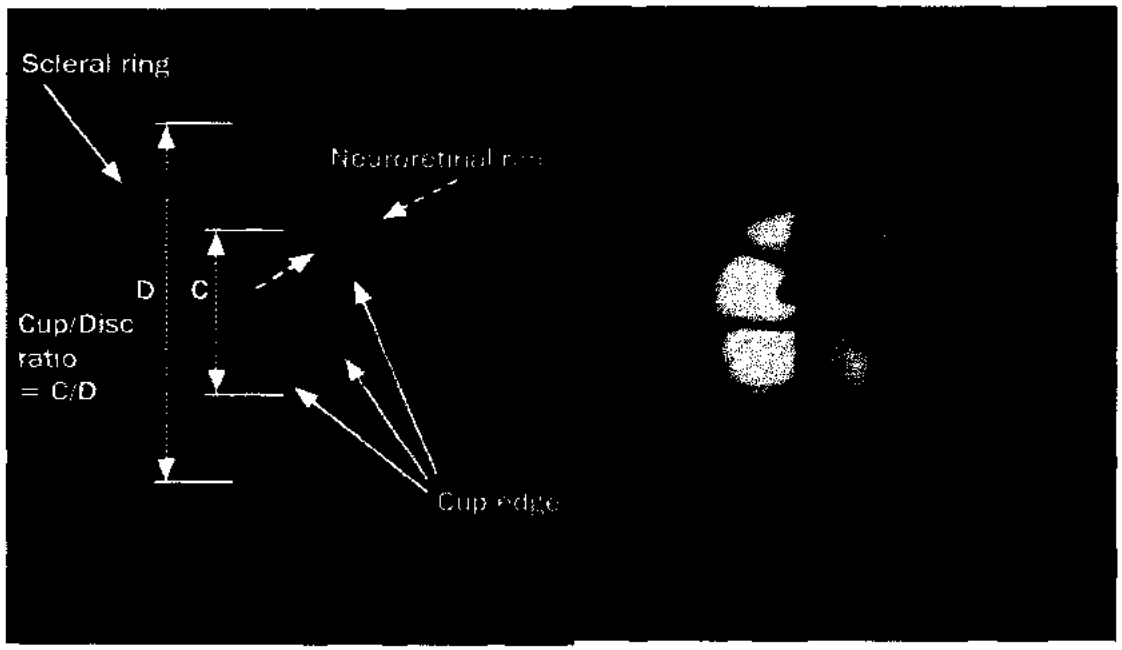

Figure 2 Representation of a Normal Optic Nerve Head [left] and Glaucomatous Neuropathy [right] (Bourne, 2006).

Figure 3 Macular Zones: Foveola (a), Fovea (b), Parafovea (c) and Perifovea (d).

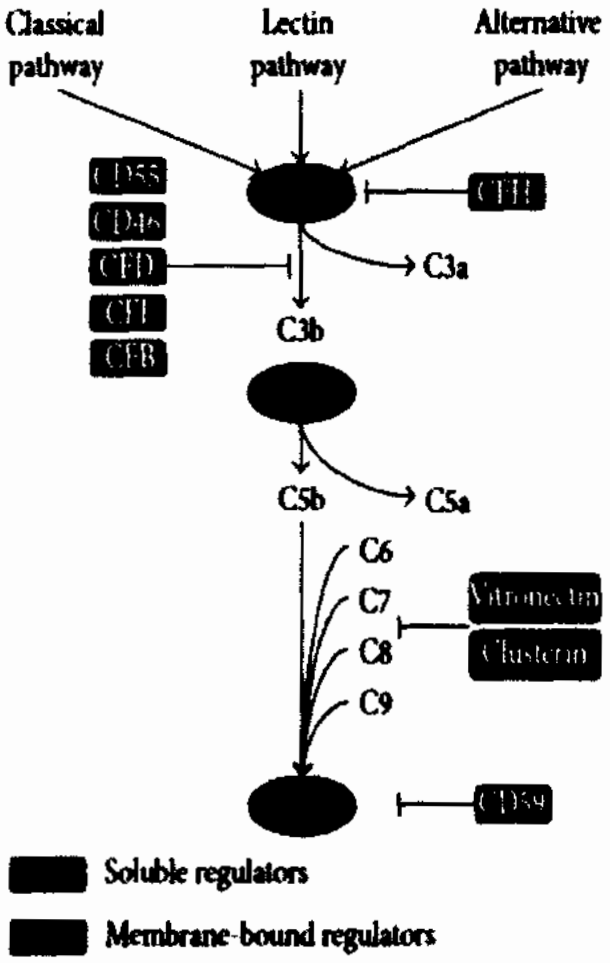

Figure 4 Complement Activation Pathways and their Regulators (Kawa *et al.*, 2014).

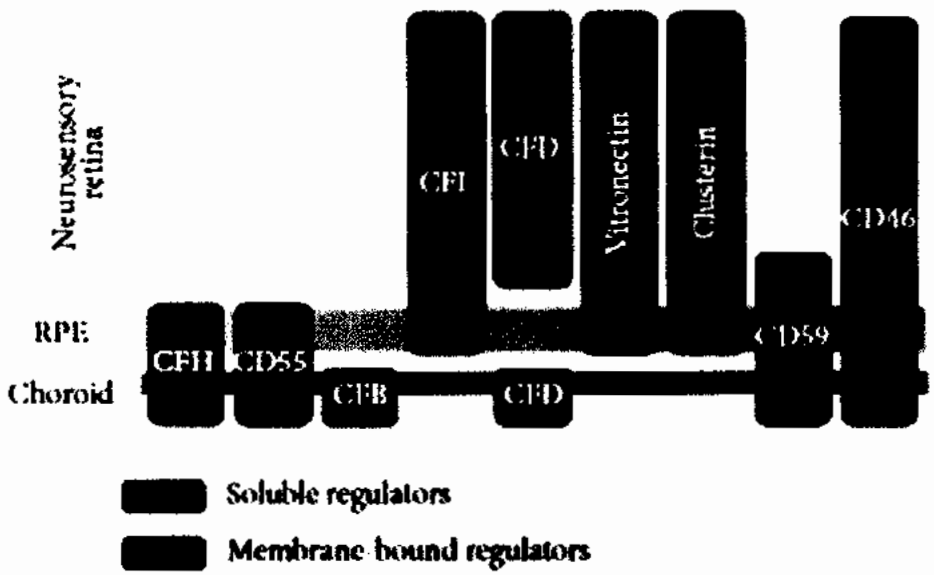

Figure 5 Complement system regulators in the Eye (Kawa *et al.*, 2014).

Figure 6 Structure of CFH protein (Kopp *et al.*, 2012)

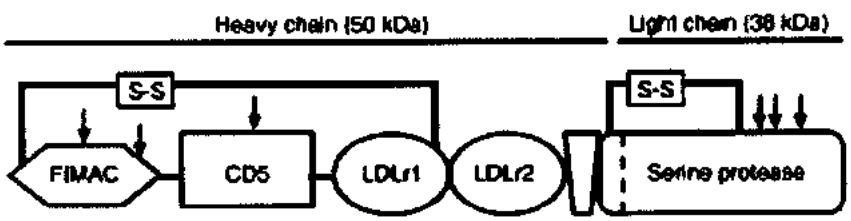

Figure 7 Structure of CFI protein (Nilsson *et al.*, 2010)

Chapter 2:

MATERIALS & METHODS

2.0 Materials and Methods

2.1 Glaucoma and AMD Patients

AMD, POAG and PXF samples were collected from Pakistan Institute of Ophthalmology, Al-Shifa Eye Trust Hospital, Rawalpindi. All the participants were informed about the purpose of the study and were selected after a detailed clinical assessment by the physician followed by obtaining written consent prior to collection of blood samples. Blood samples were also collected from healthy individuals to be used as controls.

2.2 Genomic DNA Extraction

For DNA extraction, 6ml of blood was collected in 8.5ml vacutainer™ tubes (BD, Franklin Lakes, NJ) containing ethylene diamine tetra acetic acid (EDTA) as the anti-coagulant agent.

2.2.1 DNA Extraction by Organic Method

The standard organic method of DNA extraction (Phenol-Chloroform method) was used to obtain high yield genomic DNA. The method comprised of four main steps; in the first step lysis of a nucleated red blood cells (RBCs) was done followed by removal of cellular debris, using RBC lysis buffer. In the next step lysis of white blood cells and their nuclei was carried out using nuclear lysis buffer. Following this step selective removal of cellular debris using a Phenol/ Chloroform wash was performed. The final step was the precipitation of DNA by isopropanol in the presence of sodium acetate, followed by washing of the genomic DNA with 70% ice-cold ethanol.

2.3 Material & Reagents

The stock solutions that were used for RBC lysis buffer, nuclear lysis buffer, phenol equilibration and Tris-EDTA buffer preparation are mentioned hereunder:

2.3.1 Ammonium Chloride (NH_4Cl)

1M stock solution was prepared by dissolving 53.5g of NH_4Cl in deionized water followed by adjusting the final volume to one liter.

2.3.2 Sodium Bicarbonate ($NaHCO_3$)

1M stock solution was prepared by dissolving 84g of sodium bicarbonate in deionized water and adjusting the final volume to one litre.

2.3.3 Ethylene Diamine Tetra Acetic Acid (EDTA)

1M stock solution was prepared by dissolving 2.923g of EDTA in deionized water, the pH of the solution was adjusted to 8.0 with sodium hydroxide and the final volume was adjusted to 500ml.

2.3.4 Tris-HCl (pH 8.2)

1M stock solution was prepared by dissolving 121.1g of Tris in deionized water, the pH of the solution was adjusted to 8.2 by adding concentrated hydrochloric acid (HCl) drop wise and the final volume was made up to one litre.

2.3.5 Sodium Chloride (NaCl)

1M stock solution was prepared by dissolving 58.44g of NaCl in deionized water followed by adjusting the final volume to one liter.

2.4 DNA Isolation Method

The following steps were performed for DNA isolation.

2.4.1 Step 1: RBC and Nuclear Lysis

2.4.1.1 Red Blood Cell (RBC) Lysis Buffer (pH7.4)

RBC lysis buffer was prepared by mixing 155ml 1M NH_4Cl, 10ml $NaHCO_3$ and 0.5ml 0.2M EDTA (pH 8.0), the volume was then made up to one liter with deionized water.

2.4.1.2 Nuclear Lysis Buffer (pH 8.2)

Nuclear lysis buffer was prepared by mixing 10ml 1M Tris-HCl (pH 8.2) and 5ml 0.2M EDTA (pH 8.0) solutions were mixed and the final volume was made up to one liter with deionized water.

2.4.1.3 Tris-EDTA (TE) Buffer (pH 8.0)

For the preparation of TE buffer 10ml 1M Tris-HCl (pH 8.2) and 5ml 0.2M EDTA solutions were mixed and the final volume was made up to one liter with deionised water.

During DNA extraction the proteins that were present in the cell were denatured and digested for which the following reagents were used.

2.4.1.4 Sodium Dodecyl Sulphate (SDS)

For the denaturation of proteins 20% SDS was prepared by dissolving 20g of SDS in deionized water and the final volume was made up to 100ml.

2.4.1.5 Proteinase K Solution

Proteinase K solution was prepared by mixing 20mg (lyophilized) Proteinase K, 50µl 1M Tris-HCl (pH 8.2), 1µl 1M $CaCl_2$, 500µl Glycerin and 450µl deionized water.

2.4.1.6 Procedure for RBC and Nuclear Lysis

3ml blood was taken in a 15ml falcon tube and to this 12ml of RBC lysis buffer was added. The tubes were incubated for 5min and during incubation they were inverted several times in order to completely lyse all the RBCs. The tubes were then centrifuged for 3min at 4100rpm, after this the supernatant was discarded. The cell pellet was washed by re-suspending it in 4ml RBC Lysis buffer followed by centrifugation at 4100rpm for 3min. The supernatant was discarded and the cell pellet was re-suspended in 400µl Nuclear lysis buffer (pH 8.2), to which 30µl SDS (20%) and 10µl Proteinase K were added, this reaction mix was then incubated overnight at 55°C in a water bath with constant shaking.

2.4.2 Step 2: Purification of DNA

The following reagents were prepared for DNA purification:

2.4.2.1 Phenol Preparation

Phenol was melted in a water bath at 55°C for 3hrs, followed by the addition of an equal volume of 1M Tris-HCl (pH 8.2). Few pellets of 8-Hydroxy Quinoline were added to this mixture. The solution was shaken repeatedly after which the upper aqueous phase was removed and an equal

volume of TE buffer was added. The solution was again mixed vigorously and the process was repeated until a pH of 8 to 8.4 was achieved.

2.4.2.2 Chloroform/Isoamyl alcohol (24:1) Preparation

The solution was prepared by adding chloroform and isoamyl alcohol in a ratio of 24:1. 1000ml of this solution was prepared by adding 960ml chloroform and 40ml isoamyl alcohol.

2.4.2.3 Procedure for DNA Isolation

After overnight incubation of the lysed RBSs in step 2.4.1.6, 3ml of phenol was added to the tubes followed by the addition of an equal volume of TE buffer. The tubes were then incubated at room temperature for 5min, during which they were inverted several times, followed by centrifugation for 10min at 4500rpm. The centrifugation resulted in the separation of two phases i.e. the lower organic phase containing the digested proteins and other cellular debris and the upper aqueous phase containing the dissolved DNA.

The aqueous phase was transferred to a new 15ml falcon tube followed by the addition of an equal volume of chloroform/isoamyl alcohol. The tubes were then mixed by inversion followed by centrifugation for 10min at 4500rpm; the upper aqueous phase was transferred to a new 15ml falcon tube.

2.4.3 Step 3: DNA Precipitation

The following reagents were prepared for DNA precipitation:

2.4.3.1 Sodium Acetate (3M)

Sodium acetate solution was prepared by dissolving 12.3g of sodium acetate in distilled water and final volume was made up to 50ml.

2.4.3.2 Isopropanol

Ice cold isopropanol at -20°C was used.

2.4.3.3 Method for DNA Precipitation

To the aqueous phase obtained in section 2.4.2.3, 1/10th volume 3M sodium acetate and two volumes of ice-cold isopropanol were added. The tubes were inverted several times to precipitate the DNA, followed by centrifugation for 10min at 4500rpm (4°C).

2.4.4 Step 4: DNA Washing

DNA washing was done with the help of the following reagents.

2.4.4.1 Ethanol 70%

70% ethanol was prepared by mixing 70ml absolute Ethanol and 30ml deionized water and stored at -20°C till used.

2.4.4.2 Procedure for DNA Washing

DNA pellet formed in step 2.4.3.3 was washed with ice cold 70% Ethanol followed by centrifugation for 10min at 4500rpm. The supernatant was discarded and the pellet was air dried or dried in an incubator at 37°C.

After the pellet was completely dried, it was re-suspended in 400µl TE buffer and the DNA was re-suspended by incubating the samples at 37°C for 30min, the samples were then labeled and stored at -20°C.

2.5 Stock DNA Dilution

The stock DNA qualification was done using horizontal gel electrophoresis and the concentration of DNA was determined by comparing the intensity of the band with a known concentration of Hind III DNA ladder. Then optimal concentration of template's genomic DNA for genotyping was obtained by diluting with GIBCO™ DNase, RNase free water.

2.6 Genotyping

The genotyping was done for the CFH and CFI gene polymorphisms as follows:

2.6.1 CFH and CFI Gene Polymorphisms

Both samples and controls were genotyped for the CFH and CFI gene polymorphisms based on the sequence information mentioned in Table 2.1. The genotypes were calculated to determine the association of CFH and CFI gene polymorphisms with AMD, POAG and PXF.

DNA dilutions were prepared in the ratio 1:10 for all the samples and Kompetitive Allele Specific PCR (KASP) genotyping was performed with 1.0 µl of DNA dilution, KASP assay mix based on two allele specific forward primers and a common reverse primer and a KASP master mix containing ROX™ reference dye, KASPTaq™ DNA polymerase, free nucleotides and $MgCl_2$ in a buffer solution.

2.7 Kompetitive Allele Specific PCR (KASP)

KASP for CFH and CFI gene polymorphisms was performed in the Applied Biosystems StepOne ™ and StepOnePlus ™ Real-Time PCR Systems (ABI, Foster City, CA).

The thermal cycling of KASP utilizes two temperature conditions. The denaturation of the DNA occurs at a high temperature while the lower temperature is for both, the annealing and extension of DNA strands.

For the amplification, a hot-start activation was given for 15 minutes at 94°C followed by a 10-cycles based denaturation step at 94° for 20 seconds and 55°C for 60 seconds and then a 26-cycles based annealing and extension step at 94°C for 20 seconds and 55°C for 60 seconds.

The raw data was analysed on the basis of fluorescence signals and interpreted using cluster plots to assign a genotype to the DNA samples.

2.8 Statistical Analysis

Percentages and means were calculated of the genotype and allele frequencies in AMD, POAG and PEXG cases as well as healthy controls and statistically analysed by chi square test and p-values were calculated using Epi Info Version 6.0 Stats Calculator (http://statpages.org/ctab2x2.html).

The Z test was performed using the online IFA services statistics Binomial proportions (www.fon.hum.uva.nl/Service/Statistics/Binomialproportions.html). Dominant and recessive models for the risk allele were also calculated by using Java Stat 2-way contingency table analysis (http://statpages.org/ctab2x2.html).

Table 2.1: Sequence information submitted for KASP™ Assay Design

Gene	Primer Sequence
CFI	RTCTTGGTCCACAAGTTTYACTTCAACTATTYCCTCTGAATCTGTATTTC[C/T]RTGN sTCAAGGAAACAMTAAASTTTCCTAAAATAAAAAAACRAAATAATG
CFH	CARGTGAATCAGYTKAATTTGTGTGTAAMYGKGGATATCGTCTTTCAWCA[C/T]GT TCTCACACATTRSGAACAACATGTTGGGATGGGAAACTGGAGTATCCA

Chapter 3:

RESULTS

3.0 CFI and CFH Genotype Distribution

A total of 434 patients were genotyped against a total of 196 healthy controls. Individually for CFI allele (rs141853578) 37 AMD, 46 POAG and 78 PXF samples were typed against 114 healthy controls. Similarly for CFH allele (rs121913059), 86 AMD, 94 POAG and 93 PXF samples were typed against 82 healthy controls. All controls were found to be in Hardy-Weinberg Equilibrium with $p>0.05$.

3.1 Overall Analysis of CFI Polymorphism

In controls, allele frequency (Table 3.1a) of risk allele 'T' was 86.40% where as that of the ancestral allele 'C' was 13.59%. Similarly the genotype frequency for homozygous ancestral allele CC was 6.14%, while that of heterozygous genotype 'CT' was 14.91% whereas 78.94% of individuals were homozygous variant with genotype 'TT'. In AMD patients (n=37), 16.2% were 'CC', 18.9% were heterozygous 'CT' and 64.8% were homozygous 'TT', while the frequencies of 'C' and 'T' alleles were 25.6% and 74.3%, respectively. In POAG patients (n=46), the genotype frequencies of CC, CT and TT were 0%, 0% and 100% respectively and the allele frequency of the risk allele 'T' was 100% and that of the ancestral allele 'C' was 0%. In PXF patients (n=78), the genotypic frequencies of CC, CT and TT were 7.69%, 33.33% and 58.97% respectively and the allele frequency of the risk allele 'T' was 75.64% and that of the ancestral allele 'C' was 24.36%. While table 3.1b indicates the values obtained from Z test.

3.1.1 Comparison between AMD and Controls

The data of the AMD patients and healthy controls were compared statistically (Table 3.1a). No significant difference was observed between the genotype frequencies of AMD samples

and healthy Controls (χ^2=4.30 [p=0.11]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=0.34, [95% CI=0.09-1.24], p=0.08) nor under the RM (OR=0.5, [95% CI=0.2-1.19], p=0.12).

A significant difference was observed between the allele frequencies of AMD and healthy controls (χ^2=5.9 [p=0.01]). Univariate logistic regression analysis also revealed a significant association between these two groups (OR=0.46, [95% CI=0.22-0.91], p=0.02).

3.1.2 Comparison between POAG and Controls

When statistically compared, a significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=11.4 [p=0.00]). Univariate logistic regression analysis did not reveal any significant association between these two groups neither under the DM (OR= Inf, [95% CI=0.52-Inf], p=0.20) but a marginally significant association was observed under the RM (OR=Inf, [95% CI=2.35-Inf], p=0.00). Significant difference was also observed between the allele frequencies of POAG samples and healthy controls (χ^2=13.8 [p=0.00]). Univariate logistic regression analysis also revealed a significant association between these two groups (OR=Inf, [95% CI=2.87-Inf], p=0.00).

3.1.3 Comparison between PXF and Controls

A very significant difference was observed between the genotype frequencies of PXF samples and healthy Controls (χ^2=9.79 [p=0.00]). Univariate logistic regression analysis did not reveal significant association between these two groups under the DM (OR=0.78, [95%CI=0.22-2.76], p=0.77) but significant association was observed under the RM (OR=0.38, [95%CI=0.19-0.76], p=0.00). Significant difference was also observed between allele frequencies of healthy controls and patients of PXF (χ^2=7.27 [p=0.00]). Univariate

logistic regression analysis also revealed marginally significant association between these two groups (OR=0.49, [95% CI=0.27-0.85], p=0.01).

3.2 Gender-based Comparison

Association between CFI gene polymorphisms and AMD, POAG and PXF was also studied on the basis of gender.

3.2.1 Comparison between Males (AMD, POAG, PXF and Controls)

In male controls (n=50), allele frequency (Table 3.2) of the risk allele 'T' was 86.0% whereas that of the ancestral allele 'C' was 14.0% and the genotype frequency of homozygous ancestral 'CC' was 6.0%, heterozygous 'CT' was 16.0% and homozygous 'TT' was 78.0%.

In male AMD samples (n=27), the genotype frequencies of CC, CT and TT were 14.81%, 25.92% and 59.3% respectively. The allele frequency of the risk allele 'T' was 72.22% and that of the ancestral allele 'C' was 27.77%.

For male POAG samples (n=38), the genotype frequencies of CC, CT and TT were 0%, 100% and 0%, respectively and the allele frequency of the risk allele 'T' and ancestral allele 'C' was 100% and 0% respectively.

For male PXF samples (n=58), the genotype frequencies of CC, CT and TT were 10.34%, 25.86% and 63.79% respectively. The allele frequency of the risk allele 'T' was 76.72% and that of the ancestral allele 'C' was 23.28%.

3.2.1.1 Comparison between Male AMD and Controls

No significant difference was observed between the genotype frequencies of AMD samples and healthy Controls (χ^2=3.24 [p=0.19]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=0.37,

[95% CI=0.06-2.17], p=0.23) nor under the RM (OR=0.41, [95% CI=0.13-1.28], p=0.11). A significant difference was observed between the allele frequencies of AMD samples and healthy controls (χ^2=4.35 [p=0.03]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=0.42, [95% CI=0.17-1.03], p=0.05).

3.2.1.2 Comparison between Male POAG and Controls

A significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=9.6 [p=0.00]). Univariate logistic regression analysis did not reveal any significant association between these two groups under the DM (OR=Inf, [95% CI=0.33-Inf], p=0.25) but it showed significance under the RM (OR=Inf, [95% CI=1.89-Inf], p=0.00). In addition a significant difference was observed between the allele frequencies of POAG samples and healthy controls (χ^2=11.6 [p=0.00]). Univariate logistic regression analysis revealed a considerable significant association between these two groups (OR=Inf, [95% CI=1.89-inf], p=0.00).

3.2.1.3 Comparison between Male PXF and Controls

No significant difference was observed between the genotype frequencies of male PXF samples and healthy male Controls (χ^2=2.6 [p=0.27]). Univariate logistic regression analysis did not reveal a significant association neither between these two groups under the DM (OR=0.55, [95%CI=0.10-2.69], p=0.50) nor under the RM (OR=0.49, 95%CI=0.19-1.26], p=0.14). No significant difference was observed between allele frequencies of healthy controls and patients of PXF (χ^2=3.0 [p=0.08]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=0.53, [95% CI=0.24-1.15], p=0.11).

3.2.2 Comparison between Females (AMD, POAG, PXF and Controls)

Table 3.3 shows the comparison between female controls, AMD, POAG and PXF. In female Controls (n=64), allele frequency of the risk allele 'T' was 86.72%, whereas that of the ancestral allele 'C' was 13.28% and the genotype frequency of homozygous ancestral 'CC' was 6.25%, heterozygous 'CT' was 14.06% and homozygous 'TT' was 79.68%.

In female AMD samples (n=10), the genotypic frequencies of CC, CT and TT were 20%, 0% and 80%, respectively. The allele frequency of the risk allele 'T' was 80% and that of the ancestral allele 'C' was 20%.

For POAG samples (n=8), the genotype frequencies of CC, CT and TT were 0%, 0% and 100%, respectively. The allele frequency of the risk allele 'T' was 100% and that of the ancestral allele 'C' was also 0%.

For PXF samples (n=20), the genotype frequencies of CC, CT and TT were 0%, 55.0% and 45.0%, respectively. The allele frequency of the risk allele 'T' was 72.5% and that of the ancestral allele 'C' was 27.5%.

3.2.2.1 Comparison between Female AMD and Controls

No significant difference was observed between the genotype frequencies of AMD female samples and healthy female Controls (χ^2=3.42 [p=0.18]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=0.27, [95% CI=0.03-2.51], p=0.18) nor under the RM (OR=1.02, [95% CI=0.17-7.91], p=1.00). No significant difference was observed between the allele frequencies of AMD samples and healthy controls (χ^2=0.64 [p=0.42]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=0.61, [95% CI=0.16-2.46], p=0.5).

3.2.2.2 Comparison between Female POAG and Controls

No significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=1.9 [p=0.37]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=Inf, [95% CI=0.06-Inf], p=1.00) nor under the RM (OR=Inf, [95% CI=0.32-Inf], p=0.33). No significant difference was observed between the allele frequencies of POAG samples and healthy controls (χ^2=2.4 [p=0.12]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=Inf, [95% CI=0.44-Inf], p=0.21).

3.2.2.3 Comparison between Female PXF and Controls

Significant difference was observed between the genotype frequencies of PXF samples and healthy Controls (χ^2=14.5 [p=0.00]). Univariate logistic regression analysis did not reveal a significant association between these two groups under the DM (OR=Inf, [95%CI=0.19-Inf], p=0.56) but significant association was found under the RM (OR=0.20, [95%CI= 0.06-0.68], p=0.00). There was a significant association in the allele frequencies of healthy female controls and female patients of PXF (χ^2=4.43 [p=0.03]). Univariate logistic regression analysis also did not reveal any substantial association between these two groups (OR=0.40, [95% CI=0.15-1.04], p=0.05).

3.3 Overall Analysis of CFH polymorphism

In controls, allele frequency (Table 3.4) of risk allele 'T' was 91.5% where as that of the ancestral allele 'C' was 8.53%. Similarly the genotype frequency for homozygous ancestral allele CC was 1.22%, while that of heterozygous genotype 'CT' was 14.63% whereas 84.15% of individuals were homozygous variant with genotype 'TT'. In AMD patients (n=86), 8.14% were 'CC', 34.88% were heterozygous 'CT' and 56.98% were homozygous

'TT', while the frequencies of "C" and "T" alleles were 25.60% and 74.40%, respectively. In POAG patients (n=94), the genotype frequencies of CC, CT and TT were 3.19%, 10.64% and 86.17% respectively and the allele frequency of the risk allele 'T' was 91.5% and that of the ancestral allele 'C' was 8.51%. In PXF patients (n=93), the genotypic frequencies of CC, CT and TT were 3.23%, 56.99% and 39.78% respectively and the allele frequency of the risk allele 'T' was 68.3% and that of the ancestral allele 'C' was 31.7%. While table 3.1b indicates the values obtained from Z test.

3.3.1 Comparison between AMD and Controls

The data of the AMD patients and healthy controls were compared statistically (Table 3.4). A significant difference was observed between the genotype frequencies of AMD samples and healthy Controls (χ^2=15.5 [p=0.00]). Univariate logistic regression analysis did not reveal any significant association between these two groups neither under the DM (OR=0.14, [95% CI=0.00-1.16], p=0.06) however under the RM (OR=0.25, [95% CI=0.11-0.54], p=0.00) a significant association was observed.

A significant difference was also observed between the allele frequencies of AMD and healthy controls (χ^2=17.1 [p=3.6 E-05]). Univariate logistic regression analysis also revealed a significant association between these two groups (OR=0.27, [95% CI=0.14-0.53], p=0.00).

3.3.2 Comparison between POAG and Controls

When statistically compared, no significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=1.32 [p=0.51]). Univariate logistic regression analysis did not reveal any significant association between these two groups neither under the DM (OR=0.37, [95% CI=0.02-4.2], p=0.62) nor did the data analysed under the RM (OR=1.17, [95% CI=0.47-2.91], p=0.83). No significant difference was observed

between the allele frequencies of POAG samples and healthy controls (χ^2=0.00 [p=0.99]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=1.00, [95% CI=0.45-2.25], p=1.00).

3.3.3 Comparison between PXF and Controls

A very significant difference was observed between the genotype frequencies of PXF samples and healthy Controls (χ^2=35.9 [p=0.00]). Univariate logistic regression analysis did not reveal significant association between these two groups under the DM (OR=0.37, [95%CI=0.01-4.10], p=0.62) but significant association was observed under the RM (OR=0.12, p=0.00, [95%CI=0.05-0.27]). Significant difference was also observed between allele frequencies of healthy controls and patients of PXF (χ^2=28.4 [p=0.00]). Univariate logistic regression analysis also revealed significant association between these two groups (OR=0.20, [95% CI=0.10-0.39], p=0.00).

3.4 Gender-based Comparison

Association between CFH gene polymorphisms and AMD, POAG and PXF was also studied on the basis of gender.

3.4.1 Comparison between Males (AMD, POAG, PXF and Controls)

In male controls (n=53), allele frequency (Table 3.5) of the risk allele 'T' was 92.45% whereas that of the ancestral allele 'C' was 7.55% and the genotype frequency of homozygous ancestral 'CC' was 1.88%, heterozygous 'CT' was 11.32% and homozygous 'TT' was 86.79%.

In male AMD samples (n=47), the genotype frequencies of CC, CT and TT were 8.51%, 38.29% and 53.19% respectively. The allele frequency of the risk allele 'T' was 72.34% and that of the ancestral allele 'C' was 27.66%.

For male POAG samples (n=61), the genotype frequencies of CC, CT and TT were 1.64%, 6.56% and 91.80%, respectively and the allele frequency of the risk allele 'T' and ancestral allele 'C' was 95.08% and 4.91% respectively.

For male PXF samples (n=69), the genotype frequencies of CC, CT and TT were 1.45%, 56.52% and 42.03% respectively. The allele frequency of the risk allele 'T' was 70.29% and that of the ancestral allele 'C' was 29.71%.

3.4.1.1 Comparison between Male AMD and Controls

A significant difference was observed between the genotype frequencies of AMD samples and healthy Controls (χ^2=13.7 [p=0.00]). Univariate logistic regression analysis did not reveal any significant association between these two groups neither under the DM (OR=0.20, [95% CI=0.00-2.09], p=0.18) but under the RM (OR=0.17, [95% CI=0.05-0.50], p=0.00) a significant association was observed. A significant difference was also observed between the allele frequencies of AMD samples and healthy controls (χ^2=14.2 [p=0.00]). Univariate logistic regression analysis also revealed significant association between these two groups (OR=0.21, [95% CI=0.08-0.53], p=0.00).

3.4.1.2 Comparison between Male POAG and Controls

No significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=0.8 [p=0.66]). Univariate logistic regression analysis did not reveal any significant association between these two groups neither under the DM (OR=1.15, [95% CI=0.03-43.48], p=1.00) nor under the RM (OR=1.70, [95% CI=0.44-6.71], p=0.54). In

addition no significant difference was observed between the allele frequencies of POAG samples and healthy controls (χ^2=0.7 [p=0.40]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=1.57, [95% CI=0.47-5.33], p=0.42).

3.4.1.3 Comparison between Male PXF and Controls

Significant difference was observed between the genotype frequencies of male PXF samples and healthy male Controls (χ^2=26.4 [p=0.00]). Univariate logistic regression analysis did not reveal a significant association between these two groups under the DM (OR=1.31, [95%CI=0.03-49.19], p=1.00) but significant association was found under the RM (OR=0.11, 95%CI=0.03-0.30], p=0.00). Significant difference was also observed between allele frequencies of healthy controls and patients of PXF (χ^2=18.3 [p=1.8 E-05]). Univariate logistic regression analysis also revealed significant association between these two groups (OR=0.19, [95% CI=0.07-0.45], p=0.00).

3.4.2 Comparison between Females (AMD, POAG, PXF and Controls)

Table 3.6 shows the comparison between female controls, AMD, POAG and PXF. In female Controls (n=29), allele frequency of the risk allele 'T' was 89.66%, whereas that of the ancestral allele 'C' was 10.34% and the genotype frequency of homozygous ancestral 'CC' was 0%, heterozygous 'CT' was 20.68% and homozygous 'TT' was 79.31%.

In female AMD samples (n=39), the genotypic frequencies of CC, CT and TT were 7.69%, 30.77% and 61.54%, respectively. The allele frequency of the risk allele 'T' was 76.92% and that of the ancestral allele 'C' was 23.07%.

For POAG samples (n=33), the genotype frequencies of CC, CT and TT were 6.06%, 18.18% and 75.76%, respectively. The allele frequency of the risk allele 'T' was 62.5% and that of the ancestral allele 'C' was also 37.5%.

For PXF samples (n=24), the genotype frequencies of CC, CT and TT were 8.33%, 58.33% and 33.33%, respectively. The allele frequency of the risk allele 'T' was 62.5% and that of the ancestral allele 'C' was 37.5%.

3.4.1.1 Comparison between Female AMD and Controls

No significant difference was observed between the genotype frequencies of AMD female samples and healthy female Controls (χ^2=3.6 [p=0.16]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=0.00, [95% CI=0.00-3.03], p=0.25) nor under the RM (OR=0.41, [95% CI=0.11-1.42], p=0.18). No significant difference was observed between the allele frequencies of AMD samples and healthy controls (χ^2=3.7 [p=0.05]). Univariate logistic regression analysis also did not reveal any significant association between these two groups (OR=0.38, [95% CI=0.12-1.13], p=0.06).

3.4.1.2 Comparison between Female POAG and Controls

No significant difference was observed between the genotype frequencies of POAG samples and healthy Controls (χ^2=1.8 [p=0.39]). Univariate logistic regression analysis also did not reveal any significant association between these two groups neither under the DM (OR=0.00, [95% CI=0.00-4.73], p=0.49) nor under the RM (OR=0.82, [95% CI=0.20-3.13], p=0.77). No significant difference was observed between the allele frequencies of POAG samples and healthy controls (χ^2=0.6 [p=0.42]). Univariate logistic regression analysis also did not reveal

any significant association between these two groups (OR=0.64, [95% CI=0.19-2.11], p=0.59).

3.4.1.3 Comparison between Female PXF and Controls

Significant difference was observed between the genotype frequencies of PXF samples and healthy Controls (χ^2=12.1 [p=0.00]). Univariate logistic regression analysis did not reveal a significant association between these two groups under the DM (OR=0.00, [95%CI=0.00-3.41], p=0.20) but significant association was found under the RM (OR=0.13, [95%CI= 0.03-0.52], p=0.00). There was a significant association in the allele frequencies of healthy female controls and female patients of PXF (χ^2=11.1 [p=0.00]). Univariate logistic regression analysis also revealed significant association between these two groups (OR=0.19, [95% CI=0.06-0.58], p=0.00).

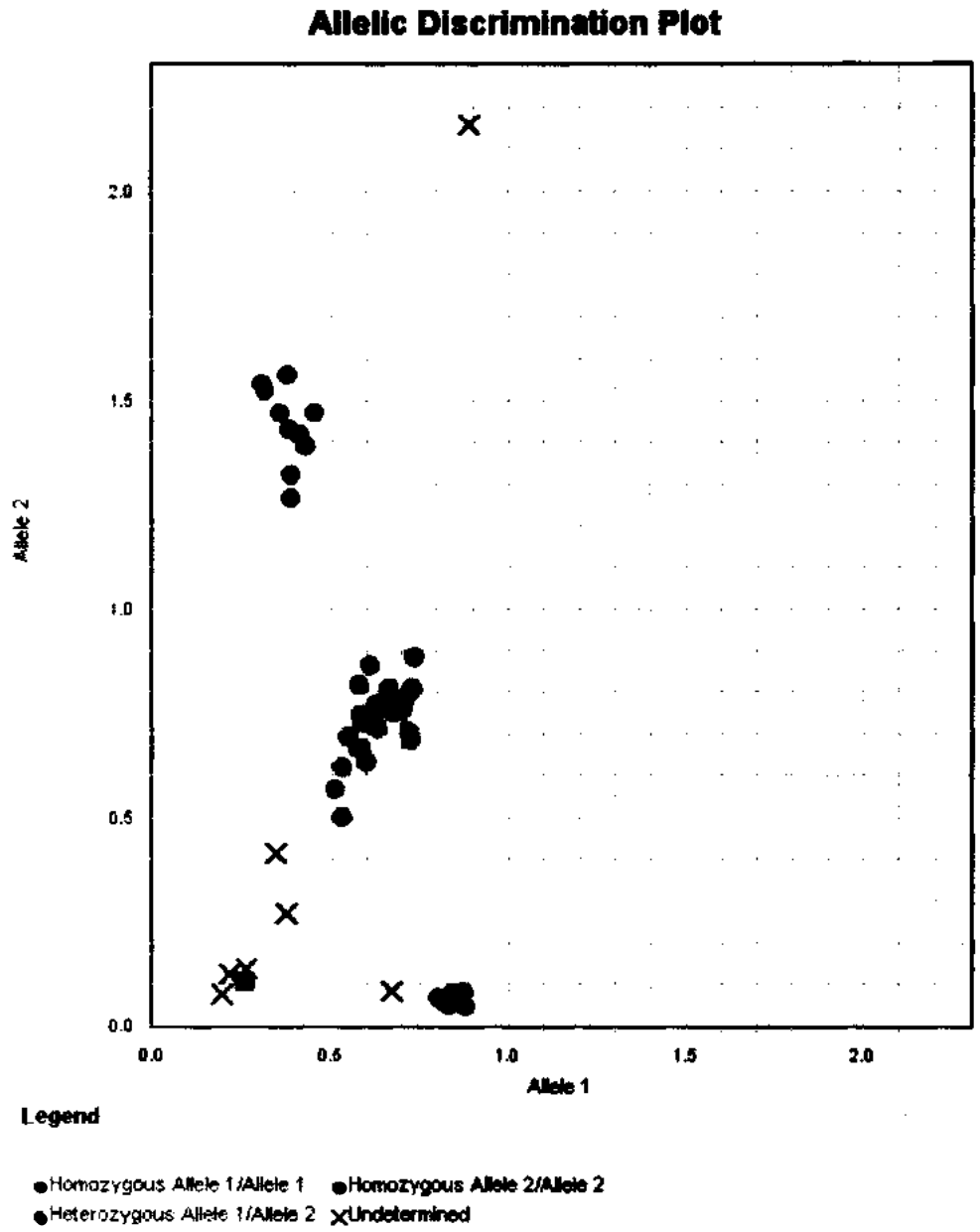

Figure 8 Cartesian or Cluster plot for PEX samples

Table 3.1a Genotype and allele frequency distribution of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls.

rs141853578 (CFI)

Genotype	Controls (N= 114)	AMD (N= 37)	POAG (N= 46)	PXF (N= 78)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
CC	7 (6.14%)	6 (16.2%)	0 (0%)	6 (7.69%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
TT	90 (78.94%)	24 (64.8%)	46 (100%)	46 (58.97%)	4.30 (0.11)	DM: 0.34 (0.09-1.24) (0.08)	11.4 (0.00)	DM: Inf (0.52-Inf) (0.2)	9.79 (0.00)	DM: 0.78 (0.22-2.76) (0.77)
CT	17 (14.91%)	7 (18.9%)	0 (0%)	26 (33.33%)		RM: 0.5 (0.2- 1.19) (0.12)		RM: Inf (2.35-Inf) (0.00)		RM: 0.38 (0.19-0.76) (0.00)
Allele Frequency	**Controls (N= 228)**	**AMD (N= 74)**	**POAG (N= 92)**	**PXF (N= 156)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
C	31 (13.59%)	19 (25.6%)	0 (0%)	38 (24.36%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
T	197 (86.40%)	55 (74.3%)	92 (100%)	118 (75.64%)	5.9 (0.01)	0.46 (0.22- 0.91) (0.02)	13.8 (0.00)	Inf (2.87-Inf) (0.00)	7.27 (0.00)	0.49 (0.27-0.85) (0.01)

Table 3.1b Z-Test values of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls.

Genotype	Controls (N= 114)	AMD (N= 37)	Z-Test (p-value)	POAG (N= 46)	Z-Test (p-value)	PXF (N= 78)	Z-Test (p-value)
CC	7 (6.14%)	6 (16.2%)	1.89 (0.05)	0 (0%)	-1.72 (0.08)	6 (7.69%)	0.42 (0.67)
TT	90 (78.94%)	24 (64.8%)	1.73 (0.08)	46 (100%)	3.4 (0.00)	46 (58.97%)	-2.99 (0.00)
CT	17 (14.91%)	7 (18.9%)	0.57 (0.56)	0 (0%)	-2.8 (0.00)	26 (33.33%)	3.00 (0.00)
Allele Frequency	**Controls (N= 228)**	**AMD (N= 74)**	**Z-Test (p-value)**	**POAG (N= 92)**	**Z-Test (p-value)**	**PXF (N= 156)**	**Z-Test (p-value)**
C	31 (13.59%)	19 (25.6%)	2.42 (0.01)	0 (0%)	-3.7 (0.00)	38 (24.36%)	2.69 (0.00)
T	197 (86.40%)	55 (74.3%)	-2.42 (0.01)	92 (100%)	3.7 (0.00)	118 (75.64%)	-2.69 (0.00)

Table 3.2a Genotype and allele frequency distribution of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Males.

rs141853578 (males)

Genotype	Controls (N= 50)	AMD (N=27)	POAG (N= 38)	PXF (N= 58)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
CC	3 (6.0%)	4 (14.81%)	0 (0%)	6 (10.34%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
TT	39 (78.0%)	16 (59.3%)	38 (100%)	37 (63.79%)	3.24 (0.19)	DM: 0.37 (0.06- 2.17) (0.23)	9.6 (0.00)	DM: Inf (0.33-Inf) (0.25)	2.6 (0.27)	DM: 0.55 (0.10-2.69) (0.50)
CT	8 (16.0%)	7 (25.92%)	0 (0%)	15 (25.86%)		RM: 0.41 (0.13- 1.28) (0.11)		RM: Inf (1.89-Inf) (0.00)		RM: 0.49 (0.19-1.26) (0.14)
Allele Frequency	**Controls (N= 100)**	**AMD (N= 54)**	**POAG (N= 76)**	**PXF (N= 116)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
C	14 (14.0%)	15 (27.77%)	0 (0%)	27 (23.28%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
T	86 (86.0%)	39 (72.22%)	76 (100%)	89 (76.72%)	4.35 (0.03)	0.42 (0.17- 1.03) (0.05)	11.6 (0.00)	Inf (2.33-Inf) (0.00)	3.0 (0.08)	0.53 (0.24-1.15) (0.11)

Table 3.2b Z-Test values of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Males.

Genotype	Controls (N= 50)	AMD (N= 27)	Z-Test (p-value)	POAG (N= 38)	Z-Test (p-value)	PXF (N= 58)	Z-Test (p-value)
CC	3 (6.0%)	4 (14.81%)	1.28 (0.2)	0 (0%)	-1.53 (0.12)	6 (10.34%)	0.81 (0.41)
TT	39 (78.0%)	16 (59.3%)	-173 (0.08)	38 (100%)	3.1 (0.00)	37 (63.79%)	-1.61 (0.10)
CT	8 (16.0%)	7 (25.92%)	1.04 (0.29)	0 (0%)	-2.5 (0.00)	15 (25.86%)	1.24 (0.21)
Allele Frequency	**Controls (N= 100)**	**AMD (N= 54)**	**Z-Test (p-value)**	**POAG (N= 76)**	**Z-Test (p-value)**	**PXF (N= 116)**	**Z-Test (p-value)**
C	14 (14.0%)	15 (27.77%)	2.08 (0.03)	0 (0%)	-3.4 (0.00)	27 (23.28%)	1.73 (0.08)
T	86 (86.0%)	39 (72.22%)	-2.08 (0.03)	76 (100%)	3.4 (0.00)	89 (76.72%)	-1.73 (0.08)

Table 3.3a Genotype and allele frequency distribution of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Females.

rs141853578 (females)	Genotype	Controls (N= 64)	AMD (N= 10)	POAG (N= 8)	PXF (N= 20)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
	CC	4 (6.25%)	2 (20%)	0 (0%)	0 (0%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
	TT	51 (79.68%)	8 (80%)	8 (100%)	9 (45.0%)	3.42 (0.18)	DM: 0.27 (0.03- 2.51) (0.18)	1.9 (0.37)	DM: Inf (0.06-Inf) (1.00)	14.5 (0.00)	DM: Inf (0.19-Inf) (0.56)
	CT	9 (14.06%)	0 (0%)	0 (0%)	11 (55.0%)		RM: 1.02 (0.17- 7.91) (1.00)		RM: Inf (0.32-Inf) (0.33)		RM: 0.20 (0.06-0.68) (0.00)
	Allele Frequency	**Controls (N= 128)**	**AMD (N= 20)**	**POAG (N= 16)**	**PXF (N= 40)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
	C	17 (13.28%)	4 (20%)	0 (0%)	11 (27.5%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
	T	111 (86.72%)	16 (80%)	16 (100%)	29 (72.5%)	0.64 (0.42)	0.61 (0.16- 2.46) (0.5)	2.4 (0.12)	Inf (0.44- Inf) (0.21)	4.4 (0.03)	0.40 (0.15-1.04) (0.05)

Table 3.3b Z-Test values of CFI gene SNP (rs141853578) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Females.

Genotype	Controls (N= 64)	AMD (N= 10)	Z-Test (p-value)	POAG (N= 8)	Z-Test (p-value)	PXF (N= 20)	Z-Test (p-value)
CC	4 (6.25%)	2 (20%)	1.48 (0.14)	0 (0%)	-0.72 (0.46)	0 (0%)	-1.14 (0.25)
TT	51 (79.68%)	8 (80%)	0.02 (0.98)	8 (100%)	1.40 (0.15)	9 (45.0%)	-2.99 (0.00)
CT	9 (14.06%)	0 (0%)	-1.26 (0.20)	0 (0%)	-1.13 (0.25)	11 (55.0%)	3.75 (0.00)
Allele Frequency	**Controls (N= 128)**	**AMD (N= 20)**	**Z-Test (p-value)**	**POAG (N= 16)**	**Z-Test (p-value)**	**PXF (N= 40)**	**Z-Test (p-value)**
C	17 (13.28%)	4 (20%)	0.80 (0.42)	0 (0%)	-1.55 (0.12)	11 (27.5%)	2.10 (0.03)
T	111 (86.72%)	16 (80%)	-0.80 (0.42)	16 (100%)	1.55 (0.12)	29 (72.5%)	-2.10 (0.03)

Table 3.4a Genotype and allele frequency distribution of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls.

rs121913059 (CFH)

Genotype	Controls (N= 82)	AMD (N= 86)	POAG (N= 94)	PXF (N= 93)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
CC	1 (1.22%)	7 (8.14%)	3 (3.19%)	3 (3.23%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
TT	69 (84.15%)	49 (56.98%)	81 (86.17%)	37 (39.78%)	15.5 (0.00)	DM: 0.14 (0.00-1.16) (0.06)	1.32 (0.51)	DM: 0.37 (0.02-4.2) (0.62)	35.9 (<0.00)	DM: 0.37 (0.01-4.10) (0.62)
CT	12 (14.63%)	30 (34.88%)	10 (10.64%)	53 (56.99%)		RM: 0.25 (0.11-0.54) (0.00)		RM: 1.17 (0.47-2.91) (0.83)		RM: 0.12 (0.05-0.27) (0.00)
Allele Frequency	**Controls (N= 164)**	**AMD (N= 172)**	**POAG (N= 188)**	**PXF (N= 186)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
C	14 (8.53%)	44 (25.6%)	16 (8.51%)	59 (31.7%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
T	150 (91.5%)	128 (74.4%)	172 (91.5%)	127 (68.3%)	17.1 (3.6 E-05)	0.27 (0.14-0.53) (0.00)	0.00 (0.99)	1.00 (0.45-2.25) (1.00)	28.4 (0.00)	0.20 (0.10-0.39) (0.00)

Table 3.4b Z-Test values of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls.

Genotype	Controls (N= 82)	AMD (N= 86)	Z-Test (p-value)	POAG (N= 94)	Z-Test (p-value)	PXF (N= 93)	Z-Test (p-value)
CC	1 (1.22%)	7 (8.14%)	2.10 (0.03)	3 (3.19%)	0.87 (0.38)	3 (3.23%)	0.88 (0.37)
TT	69 (84.15%)	49 (56.98%)	-3.85 (0.00)	81 (86.17%)	0.37 (0.70)	37 (39.78%)	-5.99 (2.08e-09)
CT	12 (14.63%)	30 (34.88%)	3.02 (0.00)	10 (10.64%)	-0.79 (0.42)	53 (56.99%)	5.78 (7.21e-09)
Allele Frequency	**Controls (N= 164)**	**AMD (N= 172)**	**Z-Test (p-value)**	**POAG (N= 188)**	**Z-Test (p-value)**	**PXF (N= 186)**	**Z-Test (p-value)**
C	14 (8.53%)	44 (25.6%)	4.13 (3.59e-05)	16 (8.51%)	-0.00 (0.99)	59 (31.7%)	5.32 (1e-07)
T	150 (91.5%)	128 (74.4%)	-4.13 (3.59e-05)	172 (91.5%)	0.00 (0.99)	127 (68.3%)	-5.32 (1e-07)

Table 3.5a Genotype and allele frequency distribution of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Males.

rs121913059 (males)

Genotype	Controls (N= 53)	AMD (N= 47)	POAG (N= 61)	PXF (N= 69)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
CC	1 (1.88%)	4 (8.51%)	1 (1.64%)	1 (1.45%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
TT	46 (86.79%)	25 (53.19%)	56 (91.80%)	29 (42.03%)	13.7 (0.00)	DM: 0.20 (0.00-2.09) (0.18)	0.8 (0.66)	DM: 1.15 (0.03-43.48) (1.00)	26.4 (0.00)	DM: 1.31 (0.03-49.19) (1.00)
CT	6 (11.32%)	18 (38.29%)	4 (6.56%)	39 (56.52%)		RM: 0.17 (0.05-0.50) (0.00)		RM: 1.70 (0.44-6.71) (0.54)		RM: 0.11 (0.03-0.30) (0.00)
Allele Frequency	**Controls (N= 106)**	**AMD (N= 94)**	**POAG (N= 122)**	**PXF (N= 138)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
C	8 (7.55%)	26 (27.66%)	6 (4.91%)	41 (29.71%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
T	98 (92.45%)	68 (72.34%)	116 (95.08%)	97 (70.29%)	14.2 (0.00)	0.21 (0.08-0.53) (0.00)	0.7 (0.40)	1.57 (0.47-5.33) (0.42)	18.3 (1.8 E-05)	0.19 (0.07-0.45) (0.00)

Table 3.5b Z-Test values of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Males.

Genotype	Controls (N= 53)	AMD (N= 47)	Z-Test (p-value)	POAG (N= 61)	Z-Test (p-value)	PXF (N= 69)	Z-Test (p-value)
CC	1 (1.88%)	4 (8.51%)	1.51 (0.12)	1 (1.64%)	-0.10 (0.92)	1 (1.45%)	-0.18 (0.85)
TT	46 (86.79%)	25 (53.19%)	-3.69 (0.00)	56 (91.80%)	0.86 (0.38)	29 (42.03%)	-5.03 (4.76e-07)
CT	6 (11.32%)	18 (38.29%)	3.15 (0.00)	4 (6.56%)	-0.89 (0.37)	39 (56.52%)	5.12 (2.92e-07)
Allele Frequency	**Controls (N= 106)**	**AMD (N= 94)**	**Z-Test (p-value)**	**POAG (N= 122)**	**Z-Test (p-value)**	**PXF (N= 138)**	**Z-Test (p-value)**
C	8 (7.55%)	26 (27.66%)	3.77 (0.00)	6 (4.91%)	-0.82 (0.40)	41 (29.71%)	4.28 (1.84e-05)
T	98 (92.45%)	68 (72.34%)	-3.77 (0.00)	116 (95.08%)	0.82 (0.40)	97 (70.29%)	-4.28 (1.84e-05)

Table 3.6a Genotype and allele frequency distribution of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Females.

rs121913059 (females)	Genotype	Controls (N= 29)	AMD (N= 39)	POAG (N= 33)	PXF (N= 24)	Controls vs AMD		Controls vs POAG		Controls vs PXF	
	CC	0 (0%)	3 (7.69%)	2 (6.06%)	2 (8.33%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
	TT	23 (79.31%)	24 (61.54%)	25 (75.76%)	8 (33.33%)	3.6 (0.16)	DM: 0.00 (0.00-3.03) (0.25)	1.8 (0.39)	DM: 0.00 (0.00-4.73) (0.49)	12.1 (0.00)	DM: 0.00 (0.00-3.41) (0.20)
	CT	6 (20.68%)	12 (30.77%)	6 (18.18%)	14 (58.33%)		RM: 0.41 (0.11-1.42) (0.18)		RM: 0.82 (0.20-3.13) (0.77)		RM: 0.13 (0.03-0.52) (0.00)
	Allele Frequency	**Controls (N= 58)**	**AMD (N= 78)**	**POAG (N= 66)**	**PXF (N= 48)**	**Controls vs AMD**		**Controls vs POAG**		**Controls vs PXF**	
	C	6 (10.34%)	18 (23.07%)	10 (15.15%)	18 (37.5%)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)	χ^2 (p-value)	OR (95%CI) (p-value)
	T	52 (89.66%)	60 (76.92%)	56 (84.84%)	30 (62.5%)	3.7 (0.05)	0.38 (0.12-1.13) (0.06)	0.6 (0.42)	0.64 (0.19-2.11) (0.59)	11.1 (0.00)	0.19 (0.06-0.58) (0.00)

Table 3.6b Z-Test values of of CFH gene SNP (rs121913059) polymorphism among AMD cases, POAG cases, PEXG cases and Controls in Females.

Genotype	Controls (N= 29)	AMD (N= 39)	Z-Test (p-value)	POAG (N= 33)	Z-Test (p-value)	PXF (N= 24)	Z-Test (p-value)
CC	0 (0%)	3 (7.69%)	1.52 (0.12)	2 (6.06%)	1.34 (0.17)	2 (8.33%)	1.58 (0.11)
TT	23 (79.31%)	24 (61.54%)	-1.56 (0.11)	25 (75.76%)	-0.33 (0.73)	8 (33.33%)	-3.38 (0.00)
CT	6 (20.68%)	12 (30.77%)	0.93 (0.35)	6 (18.18%)	-0.24 (0.80)	14 (58.33%)	2.81 (0.00)
Allele Frequency	**Controls (N= 58)**	**AMD (N= 78)**	**Z-Test (p-value)**	**POAG (N= 66)**	**Z-Test (p-value)**	**PXF (N= 48)**	**Z-Test (p-value)**
C	6 (10.34%)	18 (23.07%)	1.92 (0.05)	10 (15.15%)	0.79 (0.42)	18 (37.5%)	3.32 (0.00)
T	52 (89.66%)	60 (76.92%)	-1.92 (0.05)	56 (84.84%)	-0.79 (0.42)	30 (62.5%)	-3.32 (0.00)

Chapter 4:

DISCUSSION

4.0 DISCUSSION:

For the complement pathway gene CFI (rs141853578), the results indicate a significant association of CFI risk genotype 'TT' with POAG and PXF but not with AMD. There was a marked difference between the genotype frequencies of CFI 'TT' genotype with the highest in POAG (100%), AMD (64.8%) and PXF (58.97%).

Whereas for the CFH gene (rs121913059), a significant association was observed between the CFH risk genotype 'TT' and AMD and PXF. While the results indicate no association with POAG. However the genotype frequencies of CFH 'TT' genotype was highest with POAG (86.17%) followed by AMD (56.98%) and finally PXF (39.78%).

Complement system is a sensitive system comprising of a group of proteins working together to defend the body against foreign invaders, trigger inflammation, generate adaptive immune response and remove cellular and tissue debris. However the complement system function is not limited to the beneficial aspects only. The dysregulation of the complement system may influence detrimental effects known as autoimmune response on the body's normal cells therefore a strictly controlled system is required to avoid damaging effects by the complement system (Wu *et al.*, 2013). Since C3 protein is the fundamental component of the all three complement pathways as it is involved in generating C3a and C3b components which act as inflammatory mediators and a major opsonin respectively upon proteolytic cleavage to ultimately form the membrane attacking complex (MAC) the stability of the complement system, therefore, is achieved by regulation of C3 protein activity by both soluble and membrane bound proteins (Helgason *et al.*, 2013 and Nilsson *et al.*, 2009). The soluble regulatory components include CFH, CFI, CFD, CFB, Vitronectin and

Clusterin while CD46, CD55 and CD59 comprise the membrane bound regulatory agents of the complement system (Figure 5).

Similarly an actively functional local complement system in the eye specifically in cornea, aqueous humor, tears and retina is also present, completed by the existence of regulatory proteins such as DAF, MCP, CD59, CFI and CFH. However there is a varying distribution of these inhibitory proteins in different cells and tissues of the eye. CFH, for example, is abundantly present in the choriocapillaris however an elevated level of CFI is present in the retina. It is reported that unlike choroid tissue which is rich with all the regulatory components of the complement system, the retina and RPE cells only express selective inhibitors among which are included CFI and CFH proteins (Jha *et al.*, 2007, Kawa *et al.*, 2014). The involvement of both CFI and CFH as a risk factor for age related disorders of the eye have been studied in different populations and consequently reported in several studies (Seddon *et al.*, 2013; Raychaudhuri *et al.*, 2011; Scheetz *et al.*, 2013 and Aboobakar *et al.*, 2014). However the resulting data varies due to genetic variations among different populations around the globe. Therefore this study was directed towards evaluating the possibility of an association between the genetic variants of both CFI and CFH with age related eye disorders, glaucoma and AMD in Pakistani population.

AMD showed a positive association with the CFH variant indicating the 'C/T' missense mutation as an important genetic risk factor for the disease pathogenesis. The wild type CFH plays a vital role in controlling activation of the alternative pathway through the degradation of active C3 convertase (C3bBb) as well as acts as a cofactor of CFI for the degradation of C3b component. However the Arg1210Cys mutation is a rare variant known to affect the efficient interaction between the active components of C3 protein as well as cell surfaces. This renders the regulatory properties of CFH protein incompetent and thus the CFH involvement in the eye becomes

inflammatory due to excessive activation of the complement system, the outcome of which is the detrimental affects on the Bruch's membrane and retinal pigment epithelial cells consequently elevating the drusen deposits in the eye (Duvvari *et al.*, 2015; Ristau *et al.*, 2014; Sarma and Ward, 2011).

On the other hand the CFI gene variation did not reveal any significant association with the pathogenesis of AMD. The Gly119Arg mutation previously has been reported to be positively associated with AMD in other populations resulting in the low level secretion of the CFI variant protein due to its instability, hence an overcharged complement system activity is observed (van de Ven *et al.*, 2013; Den Hollander *et al.*, 2014). This variation causes the binding of amyloid beta component of the drusen which in turn hinders the C3b cleaving activity of CFI protein (Alexander *et al.*, 2014). However the absence of an association with CFI SNP suggests that this variant may be restricted to a particular set of population rather than being effective at a broad range.

On the basis of obtained results, POAG was shown to be associated with the CFI gene SNP. As mentioned earlier the Gly119Arg substitution causes a low level secretion of the CFI protein as a consequence of its instability thus rendering the activation of the complement system unchecked (van de Ven *et al.*, 2013; Den Hollander *et al.*, 2014). The resulting dysregulation of complement activation has been assigned as a contributing factor to the continuous degradation of the RGCs, their synapses and axons alike (Tezel *et al.*, 2010).

On the other hand POAG did not disclose an association with the CFH gene SNP. This could be linked to the fact that glaucoma is an autoimmune disorder which can form immune complexes and trigger the activation of the complement system. Reports based on the proteomic analysis data point out the presence of increased levels of various complement components in glaucoma patients in contrast to healthy controls. Furthermore the complement system is recognized to play a

significant role in the optimized activity of the CNS and visual system. This is achieved by the cropping of the inappropriate synaptic regions of the nerve cells. Although this process becomes dormant when the desired CNS and visual system development is formed however this process is revitalized in glaucomatous patients. Along with these observations it has also been indicated that the CFH levels in glaucoma suffering eyes have considerably reduced levels of CFH inhibitor. Data also suggests the association of glial cells of CNS with the elevation of IOP in retina. These cells play protective role in the CNS and initiate immune response via inflammatory agents such as protease, cytokines as well as complement system (Rieck, 2013; Ahmed *et al.,* 2004; Tezel *et al.,* 2010). Therefore in event of the damage to the RGCs occurring due to the elevated IOP the complement system is activated either by direct or indirect signals to initiate the removal of damaged cells and the CFH activity is suppressed in order to achieve a prolonged complement system activity. Along with this the absence of association of CFH variant indicates that the risk allele 'T' plays no significant role in the pathogenesis of POAG in Pakistani population.

Pseudoexfoliative glaucoma (PEX), on the other hand, was significantly associated with the genetic variants of both CFI and CFH implicating the missense mutations as an important genetic risk factor for PEX. This implies a significant role of oxidative stress in glaucoma particularly in the PEX form as the foliative material that deposits various ocular tissues, thus elevating the IOP, results in response to the oxidative stress in the eye. Studies reveal that the oxidative stability and related repair mechanisms are lost in the glaucomatous patients and induce inflammatory responses (Schlotzer-Schrehardt, 2010).

4.1 Conclusion:

Age related disorders such as glaucoma and AMD have become a common blindness causing agent throughout the world and due to the lack of understanding of the pathogenesis of these diseases the diagnosis and treatment is somewhat crippled in the area of medicine. However the increasing number of GWAS studies worldwide has improved the quality of knowledge available regarding these disorders (Fuse, 2010).

From the above discussion it can be concluded the CFI and CFH gene variations are substantially involved in the pathogenesis of PEX form of glaucoma. This association reflects the role of oxidative molecules in causing stress induced damage to the ocular tissues by triggering the autoimmune response via the complement systems as a potential risk factor for the pathogenesis of this disease and marks it as a significant target for the management of PEX glaucoma.

On the other hand, association of POAG with CFI and its lack thereof with CFH gene highlights not only the complexity of this disease but also its varying risk factors among different populations worldwide and hence proves yet again the significance of pin pointing the exact mechanism influencing the progression of this disease.

The role of both CFI and CFH gene in AMD has been reported in several studies. Yet in this study the Pakistani cohort was shown not to be associated with CFI gene while CFH gene was positively associated with the disease. These differences in the outcome of association studies of same genes among different ethnic groups specify the differences in the genetic makeup of these ethnic groups. Studies of this nature could help figure out the genetic alterations and hence aware us of their different therapeutic response towards the same treatment.

4.2 Future Prospect:

AMD and glaucoma both are irreparable progressive disorders affecting a large population. Detecting the genes playing a role as a risk factor in these disorders not only has the potential to facilitate the physicians in evaluating the risk for developing these particular diseases but it could also help in an efficient and timely diagnosis. However as the genetic makeup varies among different ethnic groups, which then tend to respond differently towards the risk alleles it becomes an important task for a researcher to conduct population specific research for gaining accurate understanding of these complex disorders. Therefore a detailed study should be conducted on a large Pakistani cohort to ascertain the role of complement pathway induced inflammatory response in the disease progression of glaucoma and AMD. Once that is established, a better therapeutic approach can be directed towards these disease such as suppressing the activation of complement pathway or gene therapy for altering the risk genes.

Chapter 5:

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5.0 References

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