UNIVERSITY OF SOUTHAMPTON

PhD Thesis

Immunogenetic pathways in Age related macular degeneration

By,

Srinivas Goverdhan

Clinical Neurosciences Division

Mailpoint 806, Southampton General Hospital

Southampton SO16 6YD

UK

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AMD	Age-related macular degeneration
ARM	Age-related maculopathy
RPE	Retinal pigment epithelium
CNV	Choroidal neovascularisation
GA	Geographic atrophy
ETDRS	Early treatment of diabetic retinopathy study
PDT	Photodynamic therapy
VEGF	Vascular endothelial growth factor
PEDF	Pigment epithelium derived factor
SNP	Single nucleotide polymorphism
SSCP	Single strand conformational polymorphism
DHPLC	Denaturing High Performance Liquid Chromatography
TEAA	Triethylammonium acetate
ELB	Erythrocyte Lysing Buffer
NLB	Nuclear lysis buffer
PCR	Polymerase chain reaction
TAE	Tris acetate buffer
HLA	Human Leukocyte Antigen
LD	Linkage disequilibrium
LOXL1	Lysyl oxidase like-1
CRP	C-reactive protein
CRYAA	Crystallin - alpha A
AREDS	Age-Related Eye Disease Study
EMC	Enzyme mismatch cleavage
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
TCS	Treacher Collins Syndrome
KIR	Killer-cell Immunoglobulin-like Receptors
CAD	Coronary Artery Disease

Abstract

Recent evidence supports inflammation and immunological modification as being important for age related macular degeneration (AMD). Immune response gene polymorphisms could explain both the role of inflammation and genetic predisposition reported in AMD. We therefore explored immunogenetic pathways in AMD by testing several interlinked hypotheses. These included, analysing HLA genotypes which may genetically predispose AMD patients towards immune mediated AMD responses, measuring serum biomarkers like C-reactive protein and soluble intercellular adhesion molecule-1 along with studying functional polymorphisms in these and other related genes (Complement factor H, cytokines like Interleukins and VEGF). Also, mutation screening of candidate genes coding Bruch's membrane proteins which may have functional implications in AMD was undertaken (LOXL1 gene, PEDF and CRYAA gene Polymorphisms). Results from the HLA study showed that individuals harbouring the HLA Cw*0701 allele had an increased risk of developing AMD with localization of HLA class I and DR antigens in the choriocapillaris and drusen, respectively. These novel HLA-AMD associations were reproducible in a cohort of AMD cases and controls from Michigan. Further analysis of KIR genotypes (which are ligands for HLA) showed that, HLA C*0701 allele in combination with the inhibitory KIR AA haplotype was strongly associated with AMD status. Our CRP results confirmed previous associations with AMD. The interleukin IL8-251A/T variant showed a pro-inflammatory association in AMD while the ICAM1 12959G/A variant appears to raise ICAM1 levels especially in cigarette smokers and could predict AMD development. The strong association of the CFH Y402H coding polymorphism with AMD was confirmed in our UK cohort, while no association could be found between CRP levels and the CFH Y402H polymorphism in AMD. CFH Y402H association was significantly strongest in the predominantly classic CNV group both for CC and CT genotypes. Visual outcomes following PDT was significantly poorer in the CFH genotype groups; 50% of CC cases (n=13) and 45% of the CT cases (n=12) lost 15 or more ETDRS letters. Mutation screening of the LOXL1 gene responsible for basement membrane integrity and elastinogenesis, and testing for promoter region SNP's in PEDF and CRYAA genes did not yield significant results. These null findings need to be confirmed by studying other population cohorts and also perhaps by LD based studies. In summary, this research has provided important evidence for our hypothesis of genetic predisposition to inflammation as being important in a significant proportion of AMD cases.

CHAPTER ONE: INTRODUCTION AND STUDY AIMS

An introduction to age-related macular degeneration (AMD), the current known pathways for disease aetiology, available treatment options, and the main aims of this research study are briefly summarised in this chapter.

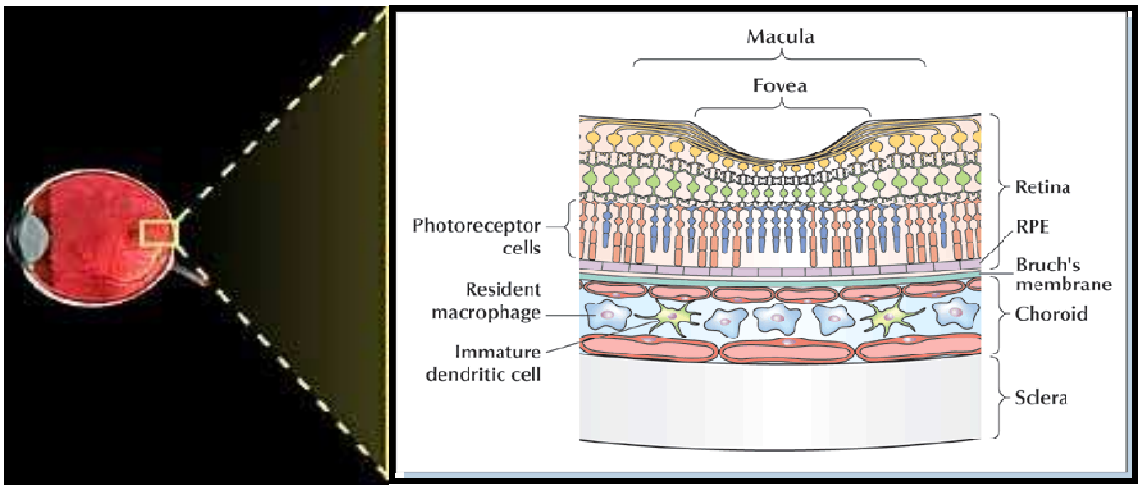


Figure 1. Gross and microscopic anatomy of the macula in the retina (modified from Forrester JV, Macrophages eyed in macular degeneration, Nat Med. 2003 Nov; 9(11):1390-7).

1. 1 ANATOMY OF THE MACULA

The macula is the central portion of the retina at the back of the eye responsible for the fine and detailed vision of the human eye. Beneath the retina are retinal pigment epithelial cells (RPE), and below that is the choroid containing a bed of tiny blood vessels that help to bring oxygen and nutrients to the eye and remove waste products that are generated through normal cellular metabolism (fig.1).

1. 2 AGE RELATED MACULAR DEGENERATION (AMD)

Macular degeneration is a degenerative retinal disease that causes progressive loss of function of the portion of the eye responsible for central vision. While some forms of macular degeneration result from monogenetic hereditary diseases, most cases occurring in patients aged 50 or older are known as age-related macular degeneration (AMD).

1.2.1 AMD DISEASE EPIDEMIOLOGY

AMD is a major health problem. It accounts for approximately 50% of all cases of registered blindness in the western world. It affects up to 30% of the population over the age of 75 and as the population ages its overall prevalence will continue to rise [2]. Although not life threatening, AMD has been judged on the basis of a spectrum of measures of patient disability to be the third most disabling disease in the US population after diabetes and cancer[1]. Because central vision makes detailed vision possible, macular degeneration may significantly diminish quality of life and the ability to function independently in individuals who suffer from this disease. As the disease progresses patients lose their ability to read and write, watch television or even recognise people's faces. As expected, this interferes with work, hobbies, mobility, communication, and social interaction. It also leads to an increased risk of falls with hip fractures and social isolation[2]. Choroidal neovascularisation (CNV) is responsible for the majority of cases of severe central visual loss, often sudden in onset with devastating psychological and practical consequences. Unfortunately current treatments are rarely sight saving. Therefore new insights into the disease with emphasis on defining an 'at risk' population are necessary so that specific preventive and disease modification measures may be applied. In this context research to identify the basic pathological processes which lead to this disease is paramount to develop more effective treatment strategies.

1.2.2 AMD CLASSIFICATION AND DISEASE PHENOTYPES

The characteristic ophthalmoscopic lesions of AMD are drusen with areas of increased or irregular pigmentation at the level of the retinal pigment epithelium (RPE). If these are the only lesions present, the condition is best referred to as early age related maculopathy (ARM), reserving the terms late ARM or AMD to describe more advanced changes which are likely to be associated with visual impairment. The classification and grading of ARM

and AMD has been complicated by the large variation in location, size, number, and types of lesion that may occur. Over the years, many definitions have been used in epidemiological studies making comparisons difficult. This problem was simplified to some extent by the International Age-related Maculopathy Epidemiological Study Group which proposed a classification and grading system for ARM and AMD based on detailed assessment of colour fundus photographs[3].

AMD can take two forms, dry AMD; also referred to as geographic atrophy (GA) or non-exudative AMD (seen in 90% cases) and wet AMD; also known as neovascular, exudative or disciform AMD (seen in 10% cases). The dry form is characterised by thinning of the macular retina with/without drusen, while the wet form is characterised by the growth of abnormal new blood vessels from the choroid beneath the retina. These new vessels (choroidal neovascularisation – CNV) can leak fluid and blood causing scarring, which can threaten vision. Using fluorescein angiography these vessels have been classified into 'classic' if they can be seen clearly and 'occult' if they cannot (figs. 2 and 3). If the picture is mixed they would be then classed as either 'minimally classic' or 'predominantly classic'. Wet macular degeneration usually occurs in people who already have dry macular degeneration and this variety of macular degeneration progresses more quickly with severe visual loss.

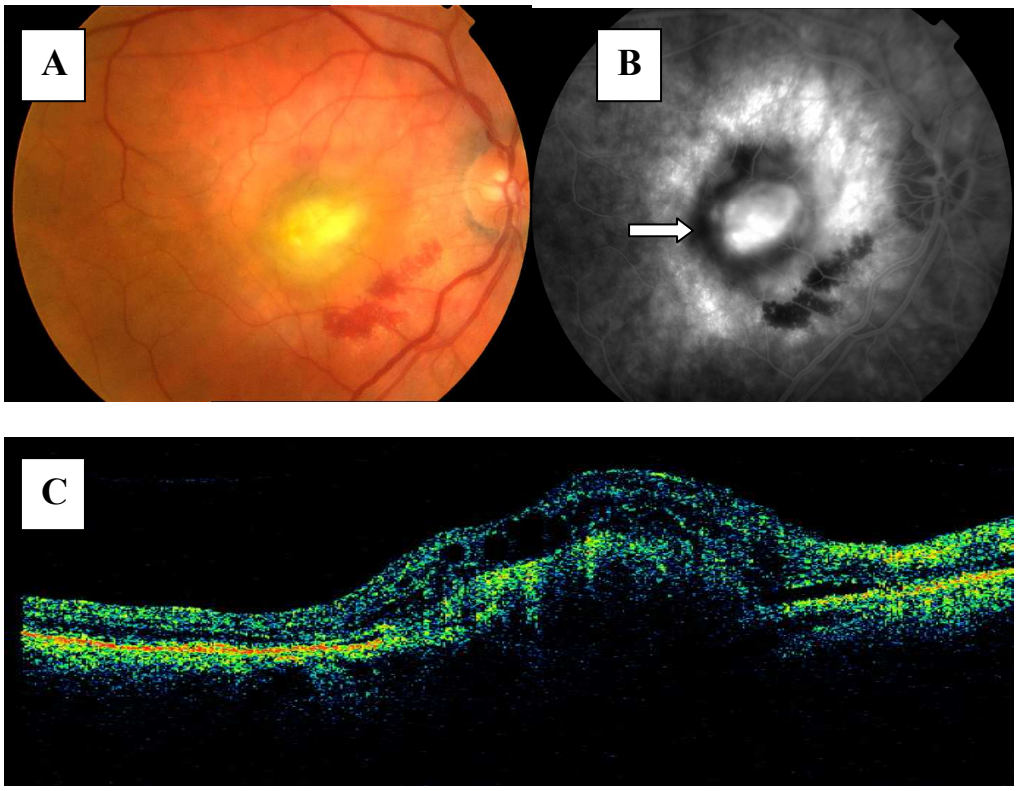


Figure 2. Patient with classic CNV.
A - Fundus appearance. B - Fluorescein angiography appearance showing central leakage from CNV (arrow). C - Corresponding Optical Coherence Tomography (OCT) scan showing sub-retinal and intra-retinal fluid from CNV.

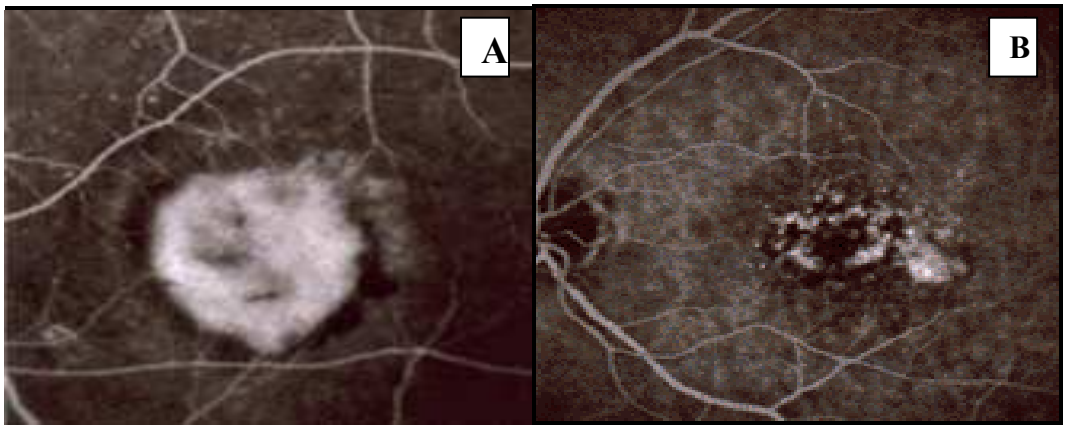


Figure 3. Fluorescein angiographic appearances of Classic (A) and Occult (B) types of CNV.

1.2.3 DRUSEN IN AMD

Drusen are extracellular deposits that accumulate between the basal lamina of the retinal pigmented epithelium (RPE) and the inner collagenous layer of Bruch's membrane in the human eye. They are typically associated with advancing age and are commonly observed in AMD. 'Drusen' has been derived from the German word for node or geode, and were first described by F.C. Donders in 1854. He described "reflective spheres" surrounded by rings of hyperpigmentation after studying the eyes of a 70 year old woman. Müller confirmed Donders' description of the frequent presence of hyperpigmentation around the rims of drusen, and suggested that they may be due to the displacement of RPE cells away from the apices of drusen, this is now commonly accepted.

The presence of large, soft, and/or numerous macular drusen is a strong risk factor for the development of both Dry and Wet AMD[4]. In addition to macular drusen, extramacular drusen also appear to be a significant risk factor for the development of AMD. Pauleikhoff et al demonstrated that the size, number, and extent of confluency of drusen are important determinants for the risk of developing AMD[5]. The presence of soft, large and/or confluent drusen is correlated to the occurrence of choroidal neovascularization, a clinically devastating manifestation of AMD. The risk of developing neovascular complications in patients with bilateral drusen has been estimated at 3-4% per year. A previous report from the Macular Photocoagulation Study Group shows a relative risk of 2.1 for developing choroidal neovascularization in eyes possessing five or more drusen, and a risk of 1.5 in eyes with one or more large drusen [6].

1.2.4 ORIGIN AND STRUCTURE OF DRUSEN

Ultrastructural studies suggest that drusen constituents are derived from the RPE [7]. Many components of drusen have been identified which provide crucial information towards

understanding the aetiology of AMD. Wolter and Falls made the observation in two human donor eyes that drusen stain with oil red O, indicating the presence of neutral lipids in at least some drusen[8]. Farkas et al performed a many experiments in which drusen composition was analyzed by enzymatic digestion, organic extraction, and histochemical staining methods in order to provide information on drusen-associated molecules[9]. They concluded that drusen are comprised of sialomucins (glycoproteins with O-glycosidically linked oligosaccharides), cerebrosides and/or gangliosides.

In an immunohistochemical study, Newsome et al[10] described labeling of soft drusen with antibodies directed against fibronectin, and both hard and soft drusen with antibodies directed against IgG and IgM. In more recent studies, a weak reaction of drusen with antibodies directed against beta amyloid[11] and complement factors (C1q, C3c, C3d, and C4) was observed[12]. More intense labelling with antibodies directed against ubiquitin, TIMP-3, and advanced glycation end products have been reported. Hageman et al have identified a number of drusen-associated carbohydrate moieties, specifically binding sites for Concanavalin A, LFA, WGA, and RCA-I [13]. They also reported vitronectin to be a major constituent of hard and soft drusen.

1.2.5 AMD RISK FACTORS

AMD is a "complex disease." This means an interplay between genetic and environmental risk factors result in it's development[14]. The relative significance of environmental and genetic risk will vary between individual patients with some having a mainly genetic aetiology and others mostly environmental. In this context, smoking is the most consistently identified and easily modifiable environmental risk factor. In others, genetic predisposition is the main reason they develop AMD. An early age of onset and family history, in the

absence of smoking, suggest a strongly genetic cause for AMD. In some patients it is likely they have a weak genetic risk of AMD but this tips the balance to developing disease because they also smoke. In others who have never smoked it is more likely they have a major genetic risk independent of environment.

1.2.6 GENETIC FACTORS IN AMD

The aetiology for this blinding disease still remains elusive. However, a number of studies have implicated both genetic and environmental factors in AMD aetiology. In addition to age, the evidence for heredity in AMD is compelling and nearly 20% of AMD patients have been shown to have a positive family history[15;16].

Until recently the search for AMD genes has been frustratingly slow. This is because AMD is difficult to study genetically because of its late age of onset and the consequent lack of large families available for study. This limits application of the most successful method of gene discovery called "positional cloning." This method requires three generation families to be available for study. Genes are then identified by association of affected family members with genetic markers which segregate with disease and can be traced through the family. Finding such segregation of marker and phenotype then positions a disease to a specific chromosomal location. Candidate genes can then be screened within the region and the disease gene identified or "cloned."

Despite these limitations multiple studies have confirmed that AMD is inherited. These studies include twin studies where a higher prevalence of AMD is seen among monozygotic twins as compared to their spouses and first-degree relative[17], population based segregation analyses[18], familial aggregation studies, gene variation association studies, studies

identifying AMD chromosomal loci by linkage[19] and more recently by actual AMD genes identified through studies of related dominant macular dystrophies[20-27].

Twin studies have demonstrated a high concordance of AMD in monozygotic twins [3; 4]. The Rotterdam Eye Study suggested that a genetic (as opposed to environmental) cause was responsible in 76 % of subjects with a family history of AMD [6]. In one large family with AMD, a genetic locus has actually been identified by conventional genetic mapping [7]. Thus it is clear that either a single gene or several genes may cause age related macular degeneration. Klein et al reported 8 of 9 monozygotic (MZ) twins concordant for AMD[28]. Meyers et al reported 100% concordance in 25 MZ twins compared to 42% concordance in 12 dizygotic (DZ) twins[29]. Gottfredsdottir et al found 90% concordance in 50 MZ twins which was significantly higher than for twin/spouse pairs[30]. Further evidence of a genetic predisposition to ARM was provided by Piguet et al who compared the characteristics of drusen in spouses and siblings of AMD patients and found a marked concordance between sibs but not with spouses[31]. Smith et al confirmed in a questionnaire study that family history is a risk factor, obtaining an odds ratio of 4.2[32]. Two studies from the United States have investigated the frequency of AMD in siblings of index cases and controls. Hyman et al found 29/146 (20%) of the siblings of index cases were affected, compared to 12/152 (8%) of the siblings of controls, giving an odds ratio of 2.9 [33]. In the study by Seddon et al the corresponding figures were 35/98 (36%) for cases and 15/112 (13%) for controls, giving an odds ratio of 2.7 [34]. In a similar case-control study from Belfast, fundus photographs were obtained in half of the siblings but diagnosis in the rest relied on reports from ophthalmologists or opticians: 20/81 (25%) siblings of index cases were affected but only 1/78 (1%) siblings of controls, giving a relative risk of 19 [35]. Klaver et al studied fundus photographs of first degree relatives of 87 index cases with late AMD and 135 controls and

obtained a relative risk of 4.2 [17]. There was evidence that relatives of cases expressed features of ARM at a younger age suggesting that genetic susceptibility may play an important role in determining the onset of disease. All studies cited here had moderate sample size with some having methodological shortcomings. Despite this, when taken together there is considerable proof for a genetic component in AMD although uncertainty exists about the magnitude and nature of this genetic effect and whether it varies with the type of AMD or with the age of disease onset.

Studies of inherited retinal disorders such as retinitis pigmentosa have revealed how genetically heterogeneous seemingly specific retinal phenotypes are. The number of cloned retinal genes has increased exponentially over the last twenty years and updated reports of this can be viewed at the Retnet website (fig.4). We now know that monogenetic retinal diseases involve functional sequence variations in numerous genes, sometimes singly and sometimes in combination e.g. digenic retinitis pigmentosa.[36]

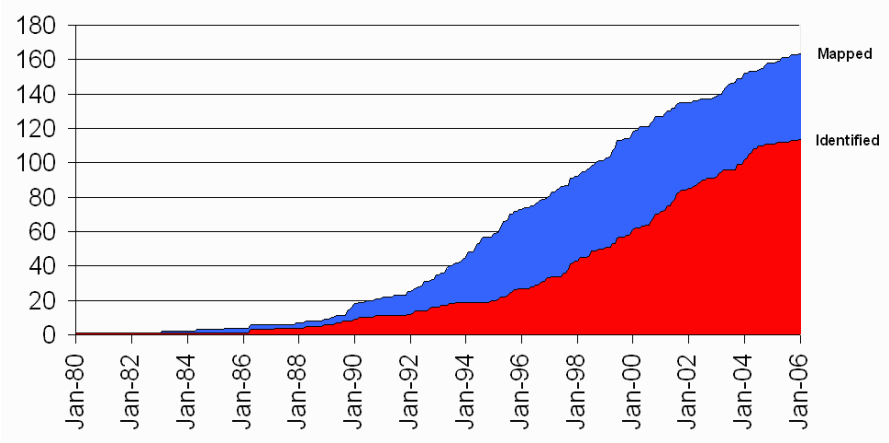


Figure 4. Mapped and Cloned Retinal disease genes (1980 to 2006) (http://www.sph.uth.tmc.edu/Retnet/home.htm)

An initial strategy to identify AMD genes was to use positional cloning to identify genes that cause early onset inherited macular diseases. The hope being that these genes would also be mutated in AMD. Positional cloning is easier in such early onset macular disease because more family generations are available. Many dominant macular dystrophy genes

have been identified (table 1. and website - http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). However none has convincingly been demonstrated to be involved in a clinically significant fraction of AMD[21;23;24;27]. The hereditary macular disease that is clinically most similar to "typical" AMD is Malattia Leventinese (Doyne's honeycomb retinal dystrophy). This is caused by a single mutation (Arg345Trp) in the fibulin-3 gene (also known as EFEMP1)[37]. The discovery that fibulin 3 was mutated in a juvenile form of macular degeneration, made the fibulin gene family potential candidate genes for AMD (table 2). In a recent analysis of the fibulin group of genes in 402 patients with AMD and 429 age matched controls 7 different missense mutations were identified in the fibulin-5 gene[38]. These sequence changes were seen in 1.7% of patients, but none were found in the control individuals (P = 0.006). Fibulin 5 which is essential for elastinogenesis therefore appears important in the development of AMD.

Genes other than those associated with hereditary retinal dystrophies can also be candidate genes for AMD (table 2). Klaver et al[39] have reported that the ApoE ε4 allele is associated with a reduced risk of AMD (odds ratio 0.43) and the ε2 allele with a slightly increased risk (odds ratio 1.5). ApoE is an important receptor for cholesterol and lipid clearance and transport. In a large AMD family with 10 affected members, Schultz et al[40] have identified that fibulin 6 (hemicentin-1) is mutated in a single family with AMD but not in simplex cases. Other genes which have been evaluated recently with some positive findings include cystatin C (CST3)[41], superoxide dismutase (SOD)[42] toll-like receptor 4 (TLR4)[43] and angiotensin-converting enzyme (ACE) where the ACE Alu+/+ genotype was protective[44].

Finally, as in other complex diseases, the additional effect exerted by multiple gene variants by their interaction with environmental factors cannot be discounted. Environmental components such as smoking therefore could trigger the development of AMD in genetically predisposed individuals. There is also increasing evidence that low grade inflammation is important in AMD, for example; raised serum C-Reactive Protein levels have been associated with AMD in one retrospective study[45].

Progress is being made in the molecular mechanisms of AMD. In the last year there has been an exciting discovery in the molecular genetics of AMD. Four groups have recently reported the association of a common coding polymorphism (Y402H) present in the CFH (complement factor H) complement regulation gene in nearly 50% cases of AMD. If a patient carries one or two copies of this mutant gene the risk of developing AMD is increased by over 2.4 to 7.4 fold[46-48]. These studies now suggest that in up to 50 % of cases genetic variation in a regulator of the alternative complement pathway, when combined with a triggering event, such as infection, may result in a major proportion of AMD cases.

This intriguing result has renewed interest in the complement pathway. Complement activation can arise through the classical, lectin, or alternative pathways[49]. All three pathways lead to the generation of a C3 convertase enzyme and subsequent activation of the immune response, terminal pathway pore-like membrane attack complex (C5b-9) formation then leads to cell lysis. CFH is an essential inhibitor of the alternative complement pathway preventing uncontrolled complement activation. The Y402H polymorphism is particularly interesting since it is located within the cluster of positively charged amino acids implicated in the binding of heparin, CRP and M proteins and various microbes[49] (figure 2). CHF through its direct binding to CRP is known to reduce the deposition of C5b-9 complexes.

Several components of the complement cascade and other terminal components of the complement pathway along with other markers of inflammation have been shown to be deposited in drusen and the choroid of eyes with AMD[50]. Abnormal regulation of the alternative pathway of complement activation by CFH seems to be consistent with these observations. Thus alteration of the binding properties of CHF may have functional implications for AMD. CFH represents the first major susceptibility gene for this complex disorder which is now directing research to the role of inflammation and innate immunity in the aetiology of AMD. We are now closer to our goal of being able to genetically screen individuals even at an early age to assess their genetic predisposition for developing AMD; so that effective interventions can be planned optimally even before the disease develops.

Table 1. Hereditary retinal dystrophy candidate genes evaluated in AMD.

Gene associated (Symbols)	location	Hereditary retinal dystrophy and Protein evaluated	Summary of findings in AMD	Authors (references)
VMD2	11q13	Best Disease, Protein: Bestrophin	1.5% of AMD cases had VMD2 mutations, protein localizes to the basolateral plasma membrane of the RPE.	Lotery 2000 Marmorstein 2000 Stone 1992
EFEMP1 FBLN3	2p16.1	Dominant radial macular drusen (Doyne's dystrophy, Malattia Leventinese), Protein: EGF containing fibrillin-like extracellular matrix protein 1 (fibulin 3).	Arg345Trp mutation found in affected individuals, normal protein is secreted from RPE but mutant protein is misfolded and retained in RPE; both proteins accumulate between the RPE and drusen, but not within drusen.	Marmorstein 2002 Stone 1999 Edwards 1998 Gregory 1996
ABCA4 ABCR	1p22.1	AR Stargardt's disease, Protein: ATP-binding cassette transporter - retinal	Conflicting results from studies with Stone et al finding no significant association in AMD.	Allikmets 2000 Allikmets 1997 Stone 1998
TIMP-3 SFD	22q12.3	Sorsby Fundus Dystrophy, Protein: Tissue inhibitor of metalloproteinases-3	Mutations found in TIMP3 in affected members of two SFD pedigrees.	Felbor 1997 Jacobson 1995 Weber 1994
ELOVL4	6q14.1	AD Stargardt disease, Protein: elongation of very long fatty acids protein	Large North American family with 5 bp deletion within the protein-coding region of ELOVL4, a retinal photoreceptor-specific gene.	Zhang 2001 Kniazeva 2000 Zhang 1999
Peripherin-RDS	6p21.2	Pattern Dystrophy, Protein: Peripherin 2	No association found with AMD, protein present in both cone and rod outer segment disks.	Ali 2000 Arikawa 1992
CLN3	16p11.2	Batten's Disease, Protein: Batten disease protein	23 mutations found in CLN3, No association found with AMD; protein in Golgi membranes.	Munroe 1997 Gardiner 1990

Table 2. Genes significantly associated with AMD.

Gene Associated (Symbols)	Chromosomal location	AMD disease form and protein evaluated	Summary and AMD disease risk	Authors (references)
CFH HF1	1q31.3	AMD, Protein: Complement Factor H	Linkage mapping and association studies; a common, polymorphic Tyr402His amino acid substitution in control domain 7 of CFH gene increases the life-time risk of AMD 2 to 7 fold in Americans of European origin.	Edwards 2005 Hageman 2005 Haines 2005 Klein 2005 Zareparsi 2005
HTRA1	10q26.13	AMD, Protein: HTRA serine peptidase 1	Candidate gene association study, a SNP (rs11200638), which is 512 bp 5' of HTRA1 in a region on10q linked to AMD has the highest association with AMD. The population attributable risk is 49.3%.The protein is present in AMD drusen and may regulate degradation of extracellular matrix; the HTRA1-LOC387715 associated SNPs are 6 kb apart.	Yang 2006 Weger 2007
LOC387715	10q26.13	AMD, Protein: hypothetical protein	Candidate gene association study, a SNP (rs10490924), within LOC387715 in a region on 10q linked to AMD has the second highest association with AMD. The SNP encodes a possible serine risk allele (Ala69Ser). The predicted gene product is a hypothetical protein of unknown function.	Jakobsdottir 2005; Rivera 2005
ARMD1 Hemicentin1 FBLN6	1q31.1	Dominant AMD	Gln5345Arg mutation found to segregate with disease in large families with AMD.	Klein 1998 Schultz 2003 Weeks 2001
LR4	9q33.1	AMD, Protein: Toll-like receptor 4	Association study; a polymorphic Asp299Gly amino acid substitution in TLR4 increases the life-time risk of AMD 2 fold (OR=2.65, p=0.025) in Caucasian carriers; TLR4 produces a widely-expressed transmembrane protein involved in innate immunity and cholesterol transport.	Zareparsi 2005
FBLN5	14q32.12	Familial AMD, Protein: fibulin 5	Missense changes found in 1.7% of AMD patients, presumed to be dominant acting; fibulins are extracellular matrix proteins with multiple EGF domains.	Stone 2004.

1.2.7 INFLAMMATORY FACTORS AND AMD

Inflammation is associated with AMD. However until recently it has not been clear whether this was a secondary phenomenon or part of the primary disease process. Circumstantial evidence has included the presence of inflammatory proteins in drusen, these include complement regulatory proteins such as vitronectin[51]. In addition, drusen contain several acute-phase reactants, immunoglobulins, activated complement, and proteins that are involved in the induction or activation of the immune response[52].

Histological studies dating back to the 1970's have implicated inflammatory cells including macrophages[53], multinucleate giant cells[54], fibroblasts and mast cells in the breakdown of Bruch's membrane associated with AMD[55]. Macrophages are observed in the choroid adjacent to drusen. These cells have been implicated in the development of choroidal neovascularisation [55-57], but might also contribute to drusen accretion, directly or indirectly, by synthesizing and secreting drusen-associated molecules such as complement C5, apolipoprotein E, Factor X, and prothrombin[51]. Both soft and hard drusen also exhibit intense HLA-DR immunoreactivity. Chronic inflammatory cellular components have also been demonstrated in early, intermediate and late AMD [58]. This suggests that a low grade chronic inflammatory response focused at the level of Bruch's membrane is involved in the pathogenesis of AMD. Recently, an animal model with the chemokine, cc motif, receptor 2 knockout mouse (CCR2) -/- has been shown to have an AMD phenotype with drusen and choroidal neovascularisation [59]. All these suggest that these inflammatory changes are causal rather than an epi-phenomenon. Auto-antibodies, indicative of autoimmune disease, have also been detected in the sera of some AMD patients [60,61]. In addition, a serological association has recently been reported between Chlamydia pneumoniae infection and AMD

[62]. A unifying hypothesis to explain both the genetic inheritance of AMD and the role of inflammation in AMD would be that AMD is, at least in part, an immune mediated disease.

1.2.8 BIOMARKERS IN AMD

Following the renewed interest in inflammation and immunological modification in the pathogenesis of AMD, many research groups are currently involved in identifying potential inflammatory biomarkers like CRP, ICAM1, interleukins and VEGF which could be important for AMD progression. The importance of identifying these in AMD lies in providing new insights into its basic pathogenesis and to help define an 'at risk' population for preventive measures to become applicable. This is particularly true, as there has been a recent paradigm shift in the understanding of atherosclerosis and heart disease which may be relevant to AMD. Atherosclerosis, like AMD, is a complex prevalent disease of the elderly sharing many environmental risk factors. Raised CRP levels have been demonstrated to be significantly associated with atherosclerosis, even in the presence of low cholesterol levels[63] and have been shown to be an independent predictor of the risk of first time heart disease or stroke. Treatment of atherosclerosis and coronary heart disease has benefited from this insight as it has been shown that aspirin and statins are most effective in reducing the risk of heart attacks in patients with elevated CRP levels. Indeed patients taking statins have been shown to have lower rates of AMD[64], possibly due to an anti-inflammatory effect.

Auto-antibodies indicative of autoimmune disease, have been detected in the sera of some AMD patients [60]. A retrospective study which was ancillary to the Age-Related Eye Disease Study (AREDS) has reported that elevated CRP could be a possible independent risk factor in AMD development[45], while a recent report by the same author showed that higher levels of systemic inflammatory markers (CRP and Interleukin-6) were independently associated with progression of AMD in a prospective study[65]. During the course of my research, we

found a similar association of elevated serum CRP levels with AMD in a UK case-control cohort[66]. CRP may thus be a useful biomarker to predict those patients who will develop severe AMD.

1.2.9 AMD PATHOGENESIS

While the exact cause of AMD is not known, the disease process begins when the transport of nutrients and waste products via the RPE begins to slow down, leading to the accumulation of waste products. Small deposits of these waste products, known as drusen, can be observed even before macular degeneration begins. The presence of drusen does not always indicate that visual loss will occur, but a portion of individuals with drusen will progress to either the dry or wet form of AMD. Some of the new vessels may also grow up into the retina, creating blind spots. A scar forms once the bleeding has stopped, and this scar may create a large blind spot in central vision.

Despite much research, the primary pathological processes remain poorly understood and this delays the development of rational therapies. In the normal eye throughout life there is a regular turnover of the photoreceptor outer segments which contain the photosensitive visual pigments. Material shed from the outer segments is engulfed and digested by the RPE and ultimately cleared by the capillaries of the choroid. The RPE is separated from the choriocapillaris by a multilayered structure called Bruch membrane. It is known that this membrane thickens with age and that in the early stages of ARM/AMD there is focal accumulation of extracellular eosinophilic material between the basement membrane of the RPE and inner collagenous zone of Bruch membrane, which contributes to the development of drusen, one of the characteristic clinical features of early ARM [67]. It is likely that this material is derived from the debris being continually discharged from the RPE. In addition there are degenerative changes evident in the RPE and choriocapillaris [67]. It is not known

why material accumulates in Bruch membrane, why some patients are more susceptible than others and what determines the subsequent progression to GA, detachment of the RPE or CNV. It has been suggested that genetic and other factors predisposing to the development of ARM/AMD might exert their effect by increasing outer segment turnover, reducing activity of RPE degradative enzymes causing free radical damage to the substrate of degradation, modifying the processes of ageing in Bruch membrane such as the cross linkage of collagen, or reducing the clearance of material from Bruch membrane.

1.2.10 PREVENTION STRATEGIES

The Age-Related Eye Disease Study (AREDS) reported significant reduction (25%) in the progression of disease from an intermediate stage to advanced neovascular AMD by the use of high-dose antioxidant, vitamin, and zinc supplementation given over 6.3 years. There are numerous alternative food supplements ranging from lutein and zeaxanthin to selenium which claim to reduce the risk of progression of AMD. At present their efficacy is not well established.

There is conflicting evidence concerning the association between AMD and hypertension[68;69], raised cholesterol[70] and raised C-reactive protein (CRP)[45]. Among all identified potential environmental factors, only age, diet and smoking are agreed upon by all to increase the risk of AMD. People with AMD would therefore benefit from lifestyle counselling in relation to diet, smoking and other known modifiable risk factors of CNV development.

1.2.11 TREATMENT OF AMD

Argon laser therapy has been traditionally used to coagulate new vessels in wet macular degeneration. However the procedure itself may permanently impair vision, especially if the vessels are very close to the fovea and recurrence is common. Laser photocoagulation is only effective in cases of classic CNV and is still the treatment of choice for extrafoveal CNV as described by the Macular Photocoagulation Study (MPS)[6]. Classic subfoveal CNV is best treated with photodynamic therapy (PDT).

1.2.12 PHOTODYNAMIC THERAPY (PDT)

Photodynamic therapy (Visudyne®) is an approved laser therapy for the treatment of wet AMD (subfoveal CNV). PDT for AMD is a two stage process requiring administration of both verteporfin and nonthermal laser. This is achieved by a 10 minute intravenous infusion of 6mg/kg verteporfin followed by activation 5 minutes later by a 689nm diode laser for 83 seconds at 50J/cm2. Lesions up to 5400 mm in greatest linear diameter can be treated. The photosensitive verteporfin is selectively taken up by the rapidly proliferating endothelial cells within the target CNV reaching its peak concentration at 15 minutes. Microvascular thrombosis of the CNV is then caused by endothelial damage from reactive oxygen radicals generated by the light activated verteporfin.

Evidence of clinical effectiveness of PDT with verteporfin comes from two major multicentre, randomised controlled clinical trials; the Treatment of AMD with Photodynamic therapy (TAP)[71;72] and Verteporfin In Photodynamic therapy (VIP)[73] trials which showed that vision outcomes were significantly better in treated than in untreated eyes throughout a two year study period. The TAP trial included 609 patients with subfoveal predominantly classic CNV having lesions of 5400 mm size, and best corrected visual acuity between 20/40 and 20/200. Of those patients treated with verteporfin, 59% of those on verteporfin had lower

vision loss compared to 31% of those on placebo (p<0,001) at 24 months. The VIP study analysed a subgroup of patients with subfoveal lesions composed of occult with no classic CNV. Some benefits were shown in reducing the risk of moderate visual loss in the verteporfin-treated group; especially in those with recent disease progression (29% in treated versus 47% in placebo, *P*=0 .004).

1.2.13 TREATMENT WITH ANTIANGIOGENIC DRUGS

There is now new evidence[74] that CNV is caused by a low grade inflammation, which can be reduced with drug treatment. VEGF (vascular endothelial growth factor), a key cytokine controlling angiogenesis and has been implicated in neovascularization during AMD[75]. The biological actions of VEGF are mediated via two receptors, VEGFR-1 and VEGFR-2. Increased expression of VEGF and pigment epithelium derived factor (PEDF) has been found in the maculae of human eyes with AMD and also in surgically removed human CNV membranes[75].

Over the last year, there has been a rapid uptake in the use of intravitreal anti-VEGF therapies for treating choroidal neovascularisation (CNV) in AMD. Of these, Pegaptanib - a non-immunogenic compound which binds with high affinity and high specificity to VEGF[76], Bevacizumab or Avastin - a 149-kDa full-length humanized antibody against VEGF-A isoforms and Ranibizumab or Lucentis - a recombinant humanized monoclonal antibody fragment directed towards VEGF-A isoforms have all been shown to be clinically effective both for vision and for reducing macular thickening in many studies.[77-79] Ranibizumab[80;81] is currently the approved first line of therapy for CNV in AMD.

1.2.14 GENE THERAPY AND GENE DIRECTED THERAPY

Gene discoveries will lead to more effective therapies for AMD by identifying specific underlying disease mechanisms that may be corrected by gene therapy or gene directed drug therapy. Once the disease gene has been characterized, gene functions can be dissected and biological processes involved in the normal and pathogenic states confirmed. The resulting information could be used to design novel therapies utilising gene products, vaccine, or genetic material. Gene therapy encompasses many strategies where genes, gene segments or oligonucleotides are transferred directly into diseased RPE or retinal cells within a patient (*in vivo* gene therapy) using viral vectors[82], or cells may be removed from the patient and the genetic material inserted into them *in vitro*. Once inside the desired cells, the expressed genes help produce a product that the patient lacks. Currently there is an ongoing human AMD gene therapy trial using pigment epithelium-derived factor (PEDF)[83]. The gene for this potent inhibitor of angiogenesis has been incorporated into an adenoviral vector and delivered into the eye by intravitreal injections to inhibit growth of CNV[84].

1. 3 STUDY AIMS

Literature review points to multiple lines of evidence which suggest an important role for inflammatory events in the pathogenesis of AMD. In fact, among the various factors involved in the pathogenesis of AMD, there has been a renewed interest in inflammation and immunological modification as being important for AMD progression. Recent Complement gene studies have provided more evidence for AMD to be an immune mediated disease[85-88]. Immune response gene polymorphisms may therefore modulate susceptibility to AMD which could explain both the role of inflammation and genetic predisposition reported in AMD. The overall objective of this research was to explore the immunogenetic pathways in AMD. This will be achieved by testing novel interlinked immune hypothesis and by

mutation screening of suitable AMD candidate genes. The study will therefore attempt to answer the following questions,

1. Are there specific HLA genotypes which may genetically predispose AMD patients to become susceptible to immune mediated responses which cause AMD?
2. Are biomarkers like CRP and soluble intercellular adhesion molecule-1(sICAM-1) associated with AMD? And do functional polymorphisms in CRP, Complement factor-H and ICAM1 genes influence serum expression levels in AMD?
3. Do expression-related cytokine polymorphisms (Interleukin/VEGF) result in genetic susceptibility to AMD?
4. Could genes encoding Bruch's membrane components be important for AMD? Candidate gene mutation screening will be undertaken by a case-control approach for;

    A. LOXL1 gene
    B. PEDF gene Polymorphisms
    C. CRYAA gene Polymorphisms
    D. TCOF1 2055 del AG mutation

5. Do mutations/polymorphisms in these genes affect AMD phenotype or outcome to treatments such as with photodynamic therapy?
6. Are HLA Cw and it's cognate KIR (killer-cell immunoglobulin-like receptor) ligand genotypes/haplotypes associated with AMD?

CHAPTER TWO: MUTATION DETECTION METHODS

This chapter summarises the molecular mutation detection techniques used in this research.

2.1 SINGLE NUCLEOTIDE POLYMORPHISMS

In any two unrelated individuals, 99.9% of the DNA sequence is similar and only 0.1% contains genetic variants that influence how people differ in their risk of disease. Single nucleotide polymorphisms (SNPs) which are pronounced as 'snips' are sites in the genome where DNA sequences differ by a single base. For example, some people may have a chromosome with an A at a particular site where others have a chromosome with a G. As we have two copies of all chromosomes except the sex chromosomes, for the above SNP a person could have the genotype AA, AG, or GG. Each form is further called an allele and the set of alleles that a person has is called a genotype. A "Haplotype" refers to a set of associated SNP alleles in a region of the chromosome. Most chromosome regions have only a few common haplotypes (each with a frequency of at least 5%), which account for most of the variation from person to person in a population.

2.2 MUTATIONS

Both "Mutation" and "polymorphism" refer to a heritable change in the DNA, they differ mainly in allele frequency (Mutation < 1% and Polymorphism > 1% frequency). Mutations could be a genome mutation such as a change in chromosome complement e.g. Down's syndrome (trisomy 21), a chromosomal mutation such as a translocation, or a gene mutation involving a number of bases. Gene mutations include base pair substitutions, deletions or insertions. They may originate through the normal process of DNA replication or because of mutagens. If they occur in the germline they could be passed on to offspring.

A point mutation in a gene may not confirm it as the disease causing mutation. The mean heterozygosity of human genomic DNA has been estimated to be 0.0037. This means that 1 in 250 to 1 in 300 bases are different between allelic sequences[89]. This sequence variation is lower in conserved gene sequences, but in an average gene several neutral (non-pathogenic) variants are still likely to be found; without functional testing it may be impossible to differentiate a neutral from pathological variant. Mutation detection usually accomplishes one of two goals. Firstly, to detect or exclude known mutations (*specific mutation testing*) or secondly, to scan known genes or exons for *any* mutation (*mutation scanning*). There are several methods available for detecting mutations in candidate genes. The chosen method varies with available resources and the researcher's experience and preference.

2.3 MUTATION DETECTION METHODS

Apart from DNA sequencing mutation detection methods fall into two groups. The first is based on the detecting aberrant migration of mutant molecules during electrophoresis. This includes heteroduplex analysis, single-strand conformation polymorphism analysis and denaturing gradient gel electrophoresis. These methods while simple do not reveal the position of the mutation and do not have 100% sensitivity[90]. The second group of methods relies on cleavage of RNA or DNA molecules prior to analysis and includes chemical and enzymatic mismatch cleavage. Their advantages include localisation of the mutation and the ability to scan DNA up to 1.6 kb in length[90].

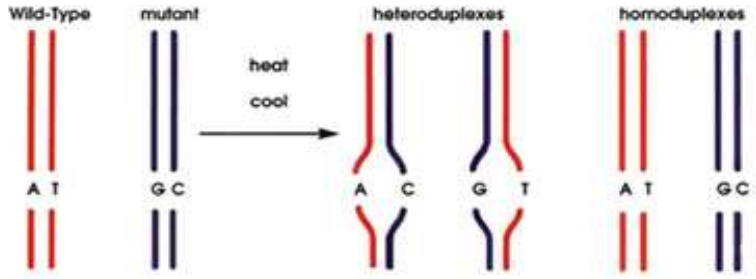
2.3.1 SINGLE-STRAND CONFORMATION POLYMORPHISM ANALYSIS

The most widely used mutation screening method is probably SSCP (single strand conformational polymorphism) a technically simple method with moderate sensitivity. Single-strand Conformation Polymorphism Analysis (SSCP) is based on the principle that

single stranded DNA folds up and develops a sequence dependent secondary structure (conformers) under non-denaturing conditions. The electrophoretic mobility of such DNA on non-denaturing gels depends not only on its chain length but also on its secondary structure which is dictated by the DNA sequence. DNA differing by as little as a single base substitution can form different conformers and migrate differently on a denaturing polyacrylamide gel[91]. The precise pattern seen on a gel is very dependent on conditions used and conditions need to be optimised for each exon being assessed. Wild type controls need to be run on the gel as well. SSCP is most sensitive for detecting base substitutions for DNA fragments of ≤150 bp. It becomes inefficient for fragments greater than 200 bp and does not reveal the position or nature of the mutation[92]. 100 % sensitivity is claimed if SSCP is performed on short DNA segments using two different electrophoretic conditions[90]. If dideoxy fingerprinting, a modified version of SSCP, is used, close to 100 % sensitivity is claimed with one electrophoretic condition[90].

2.3.2 DHPLC ANALYSIS

DHPLC (Denaturing High Performance Liquid Chromatography) identifies mutations without sequencing. This method can screen large numbers of crude PCR products for mutations and polymorphisms and direct sequencing can be used to confirm DHPLC results. The main principle of DHPLC is "Heteroduplex formation" through hybridization after heating and cooling the PCR products. Thus, mutations and polymorphisms are detected by heteroduplex formation between mismatched nucleotides in PCR amplified DNA. The sequence variation creates a mixed population of heteroduplexes and homoduplexes during reannealing of wild type and mutant DNA.



The DNA is mixed with an ion-pairing agent Triethylammonium acetate (TEAA), and the mix passes through a column (polystyrene-divinylbenzene copolymer) which binds the DNA. A linear gradient of acetonitrile allows separation of fragments based on size and/or presence of heteroduplexes. When this mixed population is analyzed under partially denaturing temperatures, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature. As the fragments elute, they are UV detected (260 nm).

2.3.3 ENZYMATIC CLEAVAGE OF MISMATCHES

In these techniques, a labelled wild type sequence is mixed with a test sequence. If the sequences differ, heteroduplexes form which can be cleaved by chemical or enzymatic methods[93]. Cleaved products are detected as short labelled fragments on a gel. The chemical method although very sensitive involves the use of toxic chemicals. Two enzymatic methods are used commonly; ribonuclease cleavage and enzyme mismatch cleavage (EMC). Enzymatic techniques offer (in principle) a greater simplicity of use while retaining the advantages of high sensitivity and specificity. Most enzymatic cleavage methods work on heteroduplex molecules, cleaving the helix at the distorted region caused by the mismatch. This means that homozygous or hemizygous mutations would not be detected unless a control sample is included to form the mismatched heteroduplex.

For mutation scanning, the enzymatic methods currently available are less widely used than physical and chemical alternatives such as SSCP and DGGE. This is because, despite some very encouraging reports no single enzymatic method has yet been able to match these methods for simplicity or sensitivity. An additional problem lies with high cost, or availability of some of these mismatch recognition proteins.

2.4 LABORATORY TECHNIQUES

2.4.4 DNA EXTRACTION[94]

1. Blood samples (10 ml) were defrosted and mixed for 1 hr on a rotary mixer in the cold room.

2. ELB (Erythrocyte Lysing Buffer) washes x 3:

In a 50ml conical tube: Add at least 30ml cold ELB to 10ml blood sample, mix and place on ice for 15 mins, mixing gently on the shaker (set to speed 3). Then spin at 1500rpm for 10 mins at 4◦C (in large centrifuge). Pour supernatant into Virkon. Add another 30ml cold ELB, mix and place on ice for 15 mins, mixing gently on the shaker. Again spin at 1500rpm for 10 mins at 4◦C. Pour off supernatant. Add final 30ml cold ELB, mix and place on ice for 15 mins, mixing gently on the shaker. Spin blood tubes at 1500rpm for 10 mins at 4◦C. Pour off all supernatant, keeping the tube upright drain the excess ELB and blot end of tube on tissue. If pellet falls onto the tissue it may be cut out and put back into the tube.

3. Overnight Protein Digestion:

Add 1ml NLB (Nuclear lysis buffer) to all tubes then pour each pellet into a fresh 15ml conical tube, rinse the 50ml tube with a further 2ml and discard it. Add 250µl 10% SDS (no.5 on pipette) and 150µl 40mg/ml Protease (no.3 on pipette) to each of the tubes. Mix and incubate at 37◦C overnight in the shaker incubator.

4. Ethanolic Precipitation of DNA:

As the DNA will be in a high concentration of salt solution, it is best to complete this stage in batches of 10-12 samples at a time. Allow the tubes to cool to room temperature (approximately 30 minutes).

Add 1ml saturated NaCL solution, shake tubes vigorously for 15 seconds, and then spin at 4000rpm for 20mins at room temperature. Mark up a new set of 15ml conical tubes. Using a P1000 pipette, take as much as possible (3-4ml) of the supernatant into the fresh tubes. If the last ml of supernatant is not clear, transfer it to a 1.5ml eppendorf tube, label it clearly and spin in the microfuge at 14,000rpm for 5mins. Then pour this into the corresponding 15ml tube. Using the 5ml Gilson pipette, add exactly twice the volume of cold Absolute Ethanol (Example: If tube contains 4ml supernatant, add 2x 4 ml absolute ethanol), invert the tube gently until the DNA precipitates out and forms a fluffy pellet.

Add 1ml of 70% Ethanol to labelled 1.5ml eppendorfs. Remove the sticky pellet using a yellow tip on a 200µl pipette and place it in the corresponding eppendorf. This may be left in the 70% ethanol for several hours. Repeat the wash if the DNA looks very brown. Pulse spin the eppendorfs in microfuge (hold pulse button for 10-15seconds), pour off the ethanol, blot the end of the tube and allow the DNA to nearly dry (~15mins). Add 500µl TE Buffer and allow DNA to dissolve overnight at room temperature and then transfer to the freezer.

2.4.5 PICOGREEN DNA QUANTIFICATION

Defrost DNA samples and mix gently. Pulse spin samples to remove DNA from lid, if necessary make a 1 in 20 dilution with ddH2O in a 96-well PCR plate e.g. 38µl water + 2µl sample. Mix well and pulse spin plate. In a flat bottom welled reaction plate, add 50µl TE Buffer to each well + 2µl dilute sample or standards (Use 5µl of undiluted Picogreen reagent for every 1ml of water in the Picogreen mix). Add 50µl Picogreen mix to each well and

incubate for 15 mins in dark to allow the Picogreen to bind to DNA in the samples. Then scan the plate on flourimager as follows,

1. Carefully insert plate into 3 plate holder and insert into Flourimager, Use 'tricia pg530' template, highlight the area you would like to scan (ex, B-H, 1-5), then press 'scan', choose a file name and location to be saved (D drive). Then, select 'Analysis', 'Volume report Set-up', 'Object name' and 'Median' to be ticked, finally click OK.

2. Auto tracer set-up: Threshold 0.1 Region 40x40 Set, Use the autotracer tool to select each region of the plate and adjust their position using the pointer. Then select 'Analysis', 'Volume report' and highlight all regions. When closing subsequent results window, click 'Yes' to activate Excel and save or print the results.

2.4.6 THE POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a technique which is used to amplify the number of copies of a defined target segment of DNA from a heterogeneous DNA source (genomic or cDNA population), in order to produce enough DNA to be adequately tested. Initially described in the 1980's[95] it has now become an indispensable technique in molecular genetics. The reaction requires the presence of a buffered solution containing DNA polymerase with the cofactor MgCl2, the four deoxynucleotide building blocks of DNA precursors (the deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP), selected oligonucleotide primers and the DNA source. The PCR mixture proceeds through replication cycles consisting of three temperature states:

1. One to several minutes at 94-96 degrees C, during which the DNA is denatured into single strands;

2. One to several minutes at 50-65 degrees C, during which the primers hybridize or "anneal" (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence;

3. One to several minutes at 72 degrees C, during which the polymerase binds and extends a complementary DNA strand from each primer.

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty such cycles, a theoretical amplification factor of one billion is attained. A typical 35 cycle experiment theoretically produces a 2^{34} amplification of the original desired sequence (plus an insignificant 35 copies of products with variable 3' ends). In practice this means that one can start with nanogram amounts of DNA and following PCR have a sufficient quantity to visualise the desired product by a convenient means such as agarose gel electrophoresis. Two important innovations were responsible for automating PCR. First, a heat-stable DNA polymerase was isolated from the bacterium Thermus aquaticus, which inhabits hot springs. This enzyme, called the Taq polymerase, remains active despite repeated heating during many cycles of amplification. Second, DNA thermal cyclers were invented that use a computer to control the repetitive temperature changes required for PCR.

General PCR amplification conditions used in the study were as follows. These conditions were varied according to the primers used, but in general the following was used:

Denaturation at94°C for 3 min
Denaturing 94°C for 1 min
Annealing 55°C for 1 min } 30-35 cycles
Extension 72°C for 1 min
Final extension72°C for 10 min

2.4.7 DESIGNING PRIMER OLIGONUCLEOTIDES

Primers were custom made using "Primer 3" software[96] using the following general principles,

1. If using genomic DNA, oligonucleotides should be greater than 20 bases (up to 24 bases) in length to ensure sequence specificity. It is extremely unlikely with this base length that a second sequence would exist which would be a perfect match to the target region. Primer length is also proportional to annealing efficiency: in general, the longer the primer, the more inefficient the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product.
2. Primers should not contain runs of a single base or a tandem repeat (Poly G or Poly C stretches). This avoids matching to known repetitive DNA sequences (which could produce non-specific products). The base composition of primers should be between 45% and 55% GC. Poly A and Poly T stretches are also to be avoided as these will open up stretches of the primer-template complex lowering the efficiency of amplification. The ideal primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56°C – 62°C.
3. To reduce primer dimer formation there should be no base homology internally or at the 3' end of primers. The inclusion of a G or C residue at the 3' end (GC Clamp) helps to ensure correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues.
4. The %GC content plus length of the primers should allow for the melting temperature (T_m) of the two primers to be equal. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will mis-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures. The T_m is the temperature corresponding to the midpoint in the observed transition of double stranded to single stranded DNA. The PCR annealing stage is usually most efficient if set at a

temperature corresponding to the T_m -5 ^{0}C of the chosen primers. The T_m (^{0}C) may be calculated approximately by the equation: T_m (^{0}C) = 2 × no. of (A + T) bases + 4 × no. of (G + C) bases.

2.4.8 AGAROSE GEL PREPARATION AND ELECTROPHORESIS

1. Mix 10 g of agarose with 500 µl of Tris acetate buffer (TAE) 1x.
2. Bring to boil until agarose is dissolved.
3. Cool gel to 65 ^{0}C in a water bath.
4. Add 25 µl of ethidium bromide and mix well.
5. Insert combs into the slots on the gel tank and pour the gel slowly, remove any bubbles on the gel.
6. Ensure adequate buffer level in the gel tank. Allow the gel to set for 30 mins before loading the samples (samples can be diluted in bromophenol blue -BPB loading buffer).
7. A 100kb DNA ladder is loaded into the end lane as a marker.
8. Run at 150 Volts (approx 20mins for 250bp sample separation) or until products have run 1/2 of the way down the gel.
9. View and document the gel on a UV transilluminator.
10. The image can be saved to disk as a windows “TIFF” (tagged image file format file).
11. The gel may be clear plastic wrapped and stored at 4^{0}C for several days.

2.4.9 DIRECT DNA SEQUENCING

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The most popular method for doing this is called the "dideoxy method". Automated sequencing is used more commonly now, this uses fluorescent labelling where each of the four different dNTPs is labelled with a different fluorophore and run in a single lane. For this project, Big Dye® Terminator v1.1.1 Cycle Sequencing Kit and the ABI PRISM® 3100 Genetic Analyser were used. Samples to be sequenced were selected and a 50µl PCR

reaction carried out for each. Samples were then electrophoresed on a 2% Agarose Gel to ensure adequate product formation. These appeared as a distinct band and corresponding to the right size when compared to a 100bp PCR ladder.

The products were then purified using the Qiagen purification Kit (Qiagen Ltd, UK) following the manufacturer's protocol. After purification, a 20µl sequencing reaction (using half strength Big Dye) was carried out on the Thermal Cycler according to protocol. This reaction contains 4µl Big Dye kit, 2µl 5X Buffer (Abgene), 3.2µl of 1µM primer, 3 – 10ng of the purified PCR product and 10.8µl sterile water. Both forward and reverse sequencing reactions were performed per sample fragment. A Qiagen DyeEx 2.0 Spin Kit (Qiagen Ltd, UK) was used to remove excess big dye and precipitate the DNA from the sequencing reaction. The products from this were then re-suspended in HiDi Formamide and transferred to a 96-well optical plate approved for use in the ABI PRISM® 3100 Genetic Analyser. This was then set to denature at 94ºC for ten minutes and 4ºC after that until it is ready to be loaded into the sequencer. The sequencer was loaded according to the settings recommended and left to run as long as necessary (~ 45mins for 16 samples). Results were exported and sequences analysed by visual inspection of the sequence printout on sequence analysing software (BioEdit Version 7.0) and by sequence comparison using the BLAST algorithm from NCBI. Each sample was checked by two observers (SVG and AJL). Any deviation between normal and affected or between the sample and the published sequence was noted.

2.4.10 RECRUITMENT OF AMD CASES AND CONTROLS

The study was approved by the Southampton and Southwest Hants Local Research Ethics Committee and was performed in accordance with the revised Helsinki declaration of 1983.

After informed and written consent, Caucasian subjects over the age of 55 with a diagnosis of AMD were recruited by qualified ophthalmologists from the ophthalmology clinics of the Southampton Eye Unit and research clinics (SG and AJL) on the Island of Guernsey (UK). Likewise age-matched normal Caucasian controls over age 55 were also recruited from the same clinics. Patients for the study underwent a detailed, dilated Ophthalmic examination to evaluate for the presence or absence of AMD. Fundus photographs were recorded using a Topcon digital retinal camera (model TRC50IX) at Southampton, while a portable camera was used in Guernsey. These photographs were graded by a masked observer into 4 groups of increasing disease severity as described in the AREDS study[97]. AREDS 1 changes were considered as normal and these cases were included in the control group during analysis. Each patient provided a 10 ml peripheral blood sample from which DNA was extracted in the laboratory using a rapid salting method as described in section 2.4.4. [98]

CHAPTER THREE : HLA GENE POLYMORPHISMS ARE ASSOCIATED WITH AMD

3.1 INTRODUCTION

3.1.1 HLA POLYMORPHISMS

The Human Leukocyte Antigen (HLA) gene complex is located in the MHC region at chromosome 6p21.3. It encodes HLA Class I (A, B, and C) and Class II (DR, DP and DQ) antigens. HLA genes are the most polymorphic within the human genome, with at least 396 HLA-A, 699 HLA-B, 198 HLA-C, 494 DRB, 28 DQA1, 66 DQB1, 23 DPA1 and 119 DPB1 alleles currently recognised. A current listing of all alleles can be electronically accessed from the European immunogenetic database [99]. More than 2000 HLA alleles are now known to be encoded by the Class I and II loci. The encoded polypeptides of these alleles differ from each other by one or more amino acid substitutions. These polymorphisms are known to influence the repertoire of peptides presented by both the HLA Class I and Class II antigens. HLA allele frequencies exhibit ethnic variations, with some alleles like DRB1*0401 distributed widely among populations while others are exclusive to a particular ethnic group like the DRB1*0302 allele seen only in afrocarribeans. The majority of polymorphism occurs in the exons that encode the α-1 and α -2 Class I (exons 2 and 3) and the α -1 and β-1 Class II (exon 2) domains which bind processed peptides and present them to T cells [100]. T cells then recognise the antigen as a peptide bound by a particular allelic variant of an HLA molecule, but will not recognise the same peptide bound to other HLA molecules. Most HLA alleles differ from one another by multiple amino acid substitutions, and these differences are focused on the peptide-binding site and adjacent regions that make direct contact with the T-cell receptor. At least three properties of HLA molecules are affected by HLA polymorphism: the range of peptides bound; the conformation of the bound peptide; and the direct interaction of the HLA molecule with the

T-cell receptor. Thus the highly polymorphic nature of the HLA system has functional consequences, and the evolutionary selection for these polymorphisms suggest that it is critical in the immune response.

3.1.2 HLA AND EYE DISEASES

The phenomenon of "immune privilege" has been described in the human eye for tissues like the cornea, anterior chamber (AC), lens, vitreous cavity, and the subretinal space. In comparison to peripheral blood the eye permits prolonged survival of foreign tumour and tissue allografts in the AC while inducing systemic tolerance to eye derived antigens which suppress the TH1 immune response. Ocular immune privilege is attributed to multiple anatomical, physiological and immunoregulatory factors; these include ocular blood barriers, the absence of lymphatic pathways, presence of soluble immunomodulatory factors in aqueous humour and the presence of tolerance promoting antigen presenting cells [101]. Also ocular tissue cells and fluids express various immunomodulatory ligands like CD95L, TGF-β, complement regulatory proteins, macrophage migration inhibitory factor, α-melanocyte stimulating hormone, etc[102]. Another important mechanism attributed to ocular immune privilege is an active process of differential cellular HLA expression (or absence thereof) of Class I and Class II molecules in the eye. In the cornea, Class I antigens are highly expressed on the epithelium and keratocytes but less so on the endothelium[103]. Similarly, Class I antigen expression is low in parenchymal cells of the uvea, the retinal pigment epithelium and cells in the neural retina. Normal retinal expression includes Class I and II antigens on microglia [53];[104] and Class I antigens on uveal pigment epithelium and vascular endothelium [105] Under normal conditions none of these cells express Class II antigens. In contrast, increased HLA Class II immunoreactivity has been observed in the human retina affected with age related macular degeneration (AMD) and related to drusen formation [106]. Interestingly, and possibly of clinical importance, this differential HLA Class

II antigen expression is reduced with intravitreal triamcinolone acetonide [107]. This may partly explain the benefit seen with intravitreal triamcinolone acetonide in AMD treatment [108;108-110].

3.1.3 HLA AND AGE RELATED MACULAR DEGENERATION (AMD)

Human leukocyte antigen (HLA) gene products have been implicated in the pathogenesis of an increasing number of eye diseases, mainly inflammatory in nature[111]. Accumulating evidence implicates an immune component to the aetiology of AMD. Research has shown that the retinal pigment epithelium (RPE) is replete with the ability to synthesize molecules involved in the immune response [112]. Also dendritic cells, which are potent antigen-presenting cells, have been intimately associated with drusen development along with key complement pathways both within drusen and along the RPE-choroid interface. Drusen are a constant feature and an important risk factor in all AMD phenotypes [12]. HLA-DR and certain immune-related cluster differentiation (CD) antigens including CD1a, CD83, and CD86 have been associated with core domains within drusen[12]. Previous immunostaining and ultrastructural studies have confirmed that these cores are derived from cell processes of choroidal dendritic cells that breach the Bruch's membrane[12].

A unifying hypothesis is that immune response gene polymorphisms modulate susceptibility to AMD. This could explain both the role of inflammation and genetic predisposition reported in AMD. In this context, human leukocyte antigen (HLA) polymorphisms, encoded within the major histocompatibility complex (MHC) are of particular interest. In other eye diseases, such as Birdshot chorioretinopathy specific HLA genotypes are a significant risk factor for the development of the disease. In this part of my study therefore, I evaluated whether specific HLA genotypes are also a risk factor for the development of

AMD and in addition whether HLA class I antigens are differentially expressed in the elderly human eye.

3.2 METHODS

This part of the study was approved by the Southampton and Southwest Hants Local Research Ethics Committee (approval no. 347/02/t) and followed the tenets of the Declaration of Helsinki.

3.2.1 PATIENTS AND CONTROLS

After informed and written consent, Caucasian subjects over the age of 55 with a diagnosis of AMD and normal Caucasian controls over age 55 were recruited from ophthalmology clinics at the Southampton General Hospital. Patients for the study underwent a detailed ophthalmic examination and fundus photographs were recorded using a Topcon digital retinal camera (model TRC50IX). These photographs were graded by a masked observer into 4 groups of increasing disease severity as described in the AREDS study[113]. Each patient provided a peripheral blood sample from which DNA was extracted using methods described in section 2.4.4. [114]

3.2.2 HLA GENOTYPING BY PCR-SSP

Genotyping was performed in two stages, initially for principal allele groups in a cohort of 100 AMD cases and 92 controls, and then alleles/allele groups which showed a trend towards significance ($P < 0.1$) on initial typing were genotyped in the next 98 cases and 102 controls from the same cohort. HLA class I A, B and Cw and class II DRB1 and DQB1 genotyping for principal allele groups was performed by PCR-SSP (section 2.3.1) [115,116]. PCR amplification was performed using panels of primers specific to 92 HLA Class I A,B,Cw and 30 class II DRB1 and DQB1 allele groups, plus primers specific for the HLA-

DRB3, 4 and 5 genes. Primer sequences were derived both from the literature[117-120] and also in house using published HLA-DRB allele sequences. Primers were combined into 24 separate PCR reaction mixes to detect 21 HLA-A alleles or allele groups, 48 reactions for 46 HLA-B alleles or allele groups and 23 reactions for 23 HLA-Cw alleles. Similarly, primers were combined into 23 reaction mixes for DRB typing, detecting 22 HLA-DRB1 alleles or allele groups plus the presence of the HLA-DRB3, DRB4 or DRB5 genes (encoding the DR52, 53 and 51 sub-types, respectively). Eight primer mixes for DQB typing were employed, detecting 8 HLA-DQB1 alleles or allele groups. Each PCR reaction mix contained separate allele or allele-group specific PCR primers, combined with an 'internal control' PCR primer pair to test for successful PCR amplification. Pre-tested PCR reaction mixes were aliquoted into 96 well plates (for single sample HLA-A, B, Cw typing or 3 arrays/plate for HLA-DRB1/DQB1 genotyping). The total volume of each PCR reaction was 10 µl. The reaction mix for each primer pair was: 1 µl 10x buffer (200 mM NH_4SO_4, 750 mM Tris-HCL pH 9.0, 0.1% Tween 20 (Advanced Biotechnologies Ltd, London), 1 µl dNTP mix (2mM), 0.6 µl $MgCl_2$ (25 mM), 0.05 µl Taq polymerase (5 u/µl), 0.5 µl DNA template. Each PCR mix contained 5′- and 3′- allele or allele group specific PCR primers at a final concentration of 0.5 µM. Each reaction mix also contained a second primer pair (at a final concentration of 0.05 µM) which amplified a 429 base pair fragment of the human growth hormone gene and functioned as an internal positive control for each PCR reaction. PCR products were visualized by running the entire reaction mix on a 2% agarose gel (Sigma, Dorset), prestained with ethidium bromide (Sigma, 0.5mg/ml gel), at 100 V for 20 minutes, in 1 x TAE buffer. Gels were photographed under ultraviolet transillumination and images interpreted by two independent observers (SVG and WMH). Genotyping was validated by random repeat typing in 10% of cases and controls, for each of the HLA

genotypes which showed a significant association with AMD in the study. PCR conditions used were:

1. 96° for 1:00
2. 96° for 0:15
3. 65° for 0:50 } steps 2 to 4 x 10 cycles
4. 72° for 0:40
5. 96° for 0:10
6. 60° for 0:50 } steps 5 to 7 x 20 cycles
7. 72° for 0:40
8. End / 4° forever

3.2.3 HLA CLASS I IMMUNOHISTOCHEMISTRY

Immunohistochemistry was done in collaboration with Prof. Rob Mullins who obtained four donor human eyes, ranging in age from 76 to 100 years of age, from the Iowa Lions Eye bank (Iowa City, IA, USA). Cryostat sections containing the retinal pigment epithelium (RPE), choroid, and sclera, were obtained from a sagittal wedge spanning from the ora serrata to the fovea embedded in optimal cutting temperature compound (Ted Pella, Inc; Redding, CA) without fixation. The neural retina was included in one specimen. Frozen sections were incubated with a monoclonal antibody directed against a shared epitope on expressed HLA class I A, B, and C molecules (clone W6/32HL; Chemicon, Temecula, CA, USA) at a concentration of 2µg/mL. In some experiments, the same sections were also incubated with antibodies directed against von Willebrand factor (Chemicon) at a concentration of 5µg/mL, in order to visualize the choroidal vasculature. Immunohistochemical labeling was performed as described previously[121] using Alexa-488 and Alexa-546 conjugated secondary antibodies (Molecular Probes, Eugene, OR). Negative controls included omission of primary antibody and comparison with antibodies directed against irrelevant antigens. In order to compare the labeling of MHC class I and class II antigens in drusen, adjacent unfixed cryostat sections were labeled with monoclonal antibody W6/32HL and with a monoclonal antibody directed against MHC class II proteins

HLA-DP/DQ/DR (Dako, clone CR3/43). Digital photomicrographs were collected using an Olympus BX41 microscope with fluorescence attachment and a SPOT-RT digital camera.

3.2.4 STATISTICAL METHODS AND ANALYSIS

Power calculations were performed utilizing the Epi Info statistical package (Revision 2, 2003) provided by the Center for Disease Control and Prevention (CDC). These calculations were based upon the number of cases and controls to be genotyped and the known frequency of specific HLA alleles. Allelic distributions for each locus were tested for conformity to Hardy-Weinberg equilibrium. This was achieved using PyPop statistical software[122] from the International Histocompatibility Working Group (IHWG) based on a methodology described by Guo and Thompson[123]. Alleles at each loci were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($p>0.05$) from expected frequencies. PyPop was also utilized for linkage disequilibrium (LD) analysis and haplotype estimation where complete data were available.

3.2.5 TWO-LOCUS STRATIFICATION ANALYSIS

Genotype frequencies after first and second stage typing were compared in cases and controls by 2x2 contingency tables and two-tailed *P* values calculated using Fisher's exact test. Adjustment for multiple comparisons was made after second stage typing using the Bonferroni method i.e. *P*-values for each comparison less than or equal to 0.05 were multiplied by the total number of study comparisons (nine in the full study group). Alleles showing significant positive or negative associations were then compared for all two-locus combinations using methodology for HLA association studies as described by Svejgaard et al[124], involving stratification of each allele against the other in order to determine whether the HLA associations were independent of each other or reflective of linkage disequilibrium

(LD) between the HLA loci concerned. The basic data for the analyses were 2x4 tables, giving the frequencies of the four phenotypic combinations from patients and controls for the two alleles being compared. These data were analyzed as 2x2 tables involving stratification of one of the two factors against the other. Two-tailed *P* values were calculated using Fisher's exact test for all of these analyses. When looking at two locus associations with AMD, *P* values were corrected by a multiple of the number of alleles tested for at the two loci involved i.e. 1 for HLA-B, 3 for Cw, 2 for DRB1 and 3 for DQB1. The level of statistical significance was set at $P < 0.05$ and $P_c < 0.05$ after application of the Bonferroni correction. Odds Ratios were estimated using the approximation of Woolf to avoid problems when critical entries were zero. Logistic regression analysis was carried out for each of the significant HLA alleles using data on age, sex, body mass index (BMI) and smoking status as covariables in the regression model. This was done between all the AREDS groups and with the control group. All statistical analyses were performed using the SPSS statistical software package version 12.0.

3.3 RESULTS

3.3.1 HLA GENOTYPING

Baseline demographics of the unrelated caucasian cohort recruited from a single clinic population (198 cases with AMD and 194 normal controls) are described in table 3. AREDS grading data from fundus photographs were available for 174 (87%) of the 200 AMD cases (table 4). Alleles were distributed in accordance with Hardy-Weinberg equilibrium for all loci tested. The results from genotyping repeats performed in 10% of cases and controls were found to be 100% concordant with those obtained during initial genotyping. In total, genotyping data for 92 HLA class I A, B and Cw and 30 class II DRB1 and DQB1 principal allele groups were available for analysis after the first stage of genotyping. Nine alleles revealed a trend towards significance on initial typing, based on uncorrected *P* values < 0.1

i.e., B*4001 (P=0.001), Cw*0302 (P=0.099), Cw*0701 (P=0.011), Cw*0702 (P=0.035), DRB1*1301 (P=0.0006), DRB1*11 (P=0.035), DQB1*0301 (P=0.056), DQB1*0302 (P=0.019) and DQB1*0303 (P=0.034). These alleles were then genotyped in the next 98 cases and 102 controls. The allele frequencies for the above antigens in the control group were similar to those previously reported for Caucasian UK populations[125-127]. HLA genotype frequencies for both class I and class II alleles in the AMD group (n=200) were compared with those in the normal controls (n=192). Data analysis demonstrated a positive association with class I Cw*0701 (P=0.003) and class II DQB1*0303 (P=0.023) alleles, while a negative association was found with HLA class I alleles B*4001 (P=0.004) and HLA class II alleles DRB1*1301 (P=0.0005) and DQB1*0302 (P=0.013) (table 3). After correcting for multiple comparisons, the positive association of Cw*0701 (P_c=0.027) and the negative associations of B*4001 (P_c=0.036) and DRB1*1301 (P_c=0.004) remained significant.

Two- locus stratification of alleles showed that B*4001 was negatively associated with AMD in DRB1*1301 negative (test 4) but not in the DRB1*1301 positive subjects (test 3) (tables 6-8). Likewise DRB1*1301 was associated with AMD in B*4001 negative but not positive individuals (test 6). Tests 9 and 10 did not demonstrate any association (*i.e.,* LD) between B*4001 and DRB1*1301 in either patients or controls. The results for comparisons between DRB1*1301 vs. DQB1*0302, B*4001 vs. DQB1*0302 and Cw*0701 vs. DQB1*0303 were similar (table 8). DRB1*1301 and DQB1*0302 demonstrated strong apparent negative LD in the control population (test 10). An elevated odds ratio (OR) of 5.82 was observed only in individuals carrying both Cw*0701 and DQB1*0303 positively associated alleles (test 8) but was not significant after a Bonferroni correction (P=0.025, P_c=0.23). Similarly individuals expressing both B*4001 and DQB1*0302 alleles had a reduced odds ratio of 0.1 (P=0.023, P_c=0.07, OR=0.10, CI-0.01 to 0.84), while a smaller

effect was seen when both B*4001 and DRB1*1301 were analyzed in combination (P=0.045, P_c=0.09, OR=0.21, CI-0.04 to 1.04). However in each case these effects were not significant after correction for multiple comparisons.

These significant HLA associations were further confirmed by logistic regression analysis. Correcting for sex, age, BMI, current smoker or previous smoker status again produced significant HLA associations for alleles B*4001 (P=0.001), Cw*0701 (P=0.02) and DRB1*1301 (P=0.0001) (tables 9 -11). This regression model was also applied for two-locus HLA analysis using frequencies from all possible pair wise combinations of the 5 significant alleles found in the study. In total, ten possible pair wise combinations were tested. None of these alleles were found to influence or predict the second allele in the combination, thus excluding confounding LD between the alleles.

Further categorization was possible in 174 subjects in the AMD group, who were divided into 4 groups of increasing AMD severity using AREDS criteria. No significant difference was found in comparison of the 4 AREDS groups and alleles B*4001, Cw*0701, DRB1*1301, DQB1*0302 or DQB1*0303 alleles. A multivariate logistic regression analysis for these 5 alleles in relation to age, sex, BMI and smoking status as covariables was performed between AREDS group IV compared to AREDS group I. The only positive association was with current smokers. Smoking increased the risk of developing advanced AMD 4 fold. This was seen irrespective of the allele tested (P=0.019 to 0.017, OR=4.55 to 4.79). No significant associations were found between the same covariables in the other AREDS groups or compared with the control group.

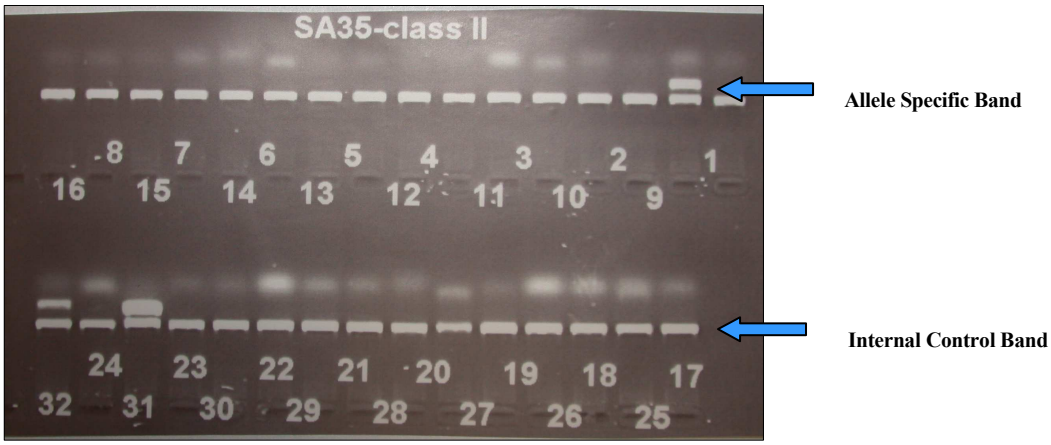


Figure 5. Gel run photograph for HLA Class II Genotyping in an AMD patient.

Table 3. Basic demographic characteristics of the HLA study population.

	AMD CASES	CONTROLS
Age	n = 198 (%)	n = 194 (%)
Mean	76.26	71.57
Range	55 - 95	55 - 91
SD	7.36	9.43
Sex	n= 198 (%)	n=194 (%)
Female	119 (60.1)	116 (59.8)
Male	81 (39.9)	76 (41.2)
BMI	n=137	n=173
Mean	26.73	27.19
SD	4.75	5.13
Smoking status	n=198(%)	n=194(%)
Current smokers	28(14.1)	24 (12.3)
Past smokers	97(50.0)	93(47.9)
Non-smokers	75(37.9)	77(39.8)

Table 4. AREDS sub-grouping of cases according to the severity of AMD in affected eye/s.

AREDS Grading	**Phenotypic Characteristics of AMD (changes seen in worst affected eye)**	**AMD Cases n = 174 (%)**
Grade I	non-extensive small drusen or no drusen in fellow eye (normal)	02 (1.2)
Grade II	extensive small or ≥ 1 intermediate size drusen, pigment changes	71 (40.8)
Grade III	≥ 1 large drusen-63μ diameter or Geographic Atrophy < 1/8 DD	39 (22.4)
Grade IV	GA ≥ 1/8 DD or Neovascular AMD (RPE detachments and CNV)	62 (35.6)

Table 5. HLA - A, B, Cw, DRB1 and DQB1 genotype frequency (%) tables for AMD cases and normal controls, first table after first stage genotyping and second table after final stage genotyping.

HLA Allele	AMD Group *n*=100(%)	Control Group *n*=94(%)	*P* value† Significant*	Odds Ratio‡	95% CI
B*4001	4(4)	18(19.1)	0.0011*	0.176	0.058 to 0.542
Cw*0302	14(14)	22(23.4)	0.0999	0.533	0.254 to 1.116
Cw*0701	46(46)	26(27.6)	0.0113*	2.228	1.224 to 4.056
Cw*0702	20(22)	32(34.0)	0.0348*	0.484	0.253 to 0.928
DRB1*1301	02(2)	15(15.9)	0.0006*	0.107	0.024 to 0.484
DRB1*11	22(22)	10(10.6)	0.0353*	2.369	1.055 to 5.319
DQB1*0301	46(46)	30(31.9)	0.0557	1.817	1.012 to 3.264
DQB1*0302	12(12)	25(26.6)	0.0108*	0.376	0.177 to 0.802
DQB1*0303	10(10)	02(2.1)	0.0339*	5.111	1.089 to 23.988

Table 6 (cont)

HLA allele	AMD cases *n*=198 (%)	Controls *n*=194(%)	*P* value†	P_c value§	Odds Ratio‡	95% CI
B*4001	16 (8.0)	35 (18.0)	0.004*	0.036*	0.39	0.21 to 0.75
Cw*0302	30 (15.0)	27 (13.9)	0.776		1.09	0.62 to 1.91
Cw*0701	84 (42.0)	54 (28.1)	0.003*	0.027*	1.91	1.25 to 2.91
Cw*0702	43 (21.5)	55 (27.8)	0.161		0.71	0.45 to 1.13
DRB1*1301	14 (7.0)	37 (19.7)	0.0005*	0.004*	0.32	0.16 to 0.61
DRB1*11	32 (16.5)	25 (12.9)	0.323		1.34	0.76 to 2.34
DQB1*0301	73 (36.5)	69 (35.05)	0.834		1.06	0.70 to 1.69
DQB1*0302	27 (13.5)	46 (23.7)	0.013*	0.117	0.50	0.30 to 0.86
DQB1*0303	22 (11.5)	9 (4.6)	0.023*	0.207	2.57	1.15 to 5.73

†Two-sided *P* values using Fisher's exact test (*Significant values); P_c indicates *P* values after application of Bonferroni correction factor (n=9). ‡Odds Ratio using the approximation of Woolf.

Tables 7a/6b. Showing construct of basic data which was utilised for 2x2 stratification analysis

Table 6a.

HLA 'A' allele	HLA 'B' allele	AMD cases(n)	Controls(n)
+	+	X1	Y1
+	-	X2	Y2
-	+	X3	Y3
-	-	X4	Y4

Table 6b.

Test	Values a	for b	2x2tables c	d	Allele frequencies tested with interpretation for each test
1	X1+X2	X3+X4	Y1+Y2	Y3+Y4	A+ vs. A-, individual 'A' association tested
2	X1+X3	X2+X4	Y1+Y3	Y2+Y4	B+ vs. B-, individual 'B' association tested
3	X1	X3	Y1	Y3	A+ vs. B+, association of 'A' independent of 'B'
4	X2	X4	Y2	Y4	A+ vs. B+, association of 'A' independent of 'B'
5	X1	X2	Y1	Y2	B+ vs. A+, association of 'B' independent of 'A'
6	X3	X4	Y3	Y4	B+ vs. A+, association of 'B' independent of 'A'
7	X2	X3	Y2	Y3	Difference between 'A' and 'B' associations tested
8	X1	X4	Y1	Y4	Combined association of 'A' and 'B' tested
9	X1	X2	X3	X4	LD tested between 'A' and 'B' in AMD cases
10	Y1	Y2	Y3	Y4	LD tested between 'A' and 'B' in controls

Table 8. Frequencies for individual significant alleles (basic data) used in the 2x2 stratification analysis.

B*4001	DRB1*1301	DQB1*0302	Cw*0701	DQB1*0303	AMD patients (n=198)	Controls (n=194)
+ve	+ve				2	7
+ve	-ve				14	28
-ve	+ve				12	30
-ve	-ve				172	129
	+ve	+ve			0	0
	+ve	-ve			14	37
	-ve	+ve			27	46
	-ve	-ve			159	111
+ve		+ve			1	7
+ve		-ve			15	28
-ve		+ve			26	39
-ve		-ve			158	120
			+ve	+ve	9	2
			+ve	-ve	75	52
			-ve	+ve	14	7
			-ve	-ve	102	132

Table 9. Results for two-locus stratification of alleles analyzed by 2x2 tables.

Allele Comparison		**Individual associations**		**Independent A association**		**Independent B association**		**Difference between A & B associations**	**Combined association**	**Association between A and B (testing LD)**	
Allele A	Allele B	(1) A	(2) B	(3) + + vs.– +	(4) + – vs. – –	(5) + + vs. + –	(6) + – vs. – –	(7) + – vs. – +	(8) + + vs.– –	(9) Patients	(10) Controls
B*4001	DRB1*1301	0.004 (0.39)	0.0005 (0.31)	1.000 (0.71)	0.0047 (0.37)	0.701 (0.57)	0.0008 (0.30)	0.814 (1.25)	0.045 (0.21)	0.310 (2.04)	0.817 (1.07)
DRB1*1301	DQB1*0302	0.0005 (0.31)	0.0097 (0.50)	NP	<0.0001 (0.26)	NP	0.0009 (0.40)	0.334 (0.64)	NP	0.224 (0.20)	<0.0001 (0.03)
B*4001	DQB1*0302	0.004 (0.39)	0.0097 (0.50)	0.245 (0.21)	0.0084 (0.40)	0.409 (0.26)	0.018 (0.50)	0.687 (0.80)	0.023 (0.10)	0.701 (0.40)	0.664 (0.76)
Cw*0701	DQB1*0303	0.006 (1.83)	0.0158 (2.67)	0.441 (2.25)	0.0082 (1.83)	0.200 (3.18)	0.065 (2.58)	0.632 (0.70)	0.025 (5.82)	0.825 (0.87)	1.000 (0.71)

Data from tests (1 to 10) indicate *Pc* values for individual two-allele comparisons while those in parentheses are Odds Ratios. (NP = Fisher's exact test not possible as one column of 2x2 table had zeros, LD= linkage disequilibrium).

Table 10. Multivariate logistic regression analysis in AMD cases and controls.

Logistic regression analysis	**HLA allele (total n=310)**		**Age (n=310)**	**Sex (M/F) (n=310)**	**BMI (n=310)**	**Current smokers (n=50)**	**Past smokers (n=198)**
Multivariate-OR	B*4001	0.20	1.09	1.06 (0.82)	1.03	1.10	0.72
		(0.001)†	(0.0001)†		(0.34)	(0.82)	(0.27)
AMD cases (n=198)	Cw*0701	1.82	1.09	1.00	1.03	1.26	0.77
vs.		(0.02)†	(0.0001)†	(0.99)	(0.24)	(0.60)	(0.35)
Controls (n=194)	DR*1301	0.12	1.10	0.97	1.03	1.25	0.91
		(0.0001)†	(0.0001)†	(0.91)	(0.27)	(0.62)	(0.76)
	DQ*0302	0.73	1.09	1.05	1.03	1.27	0.80
		(0.33)	(0.0001)†	(0.84)	(0.26)	(0.57)	(0.44)
	DQ*0303	3.19	1.09	1.06	1.02	1.18	0.74
		(0.01)†	(0.0001)†	(0.83)	(0.36)	(0.71)	(0.29)

† Significant *P* values shown in parenthesis below each Odds Ratio.

Table 11. Logistic regression analysis adjusting for Age**.**

Regression variable	**B*4001**	**Cw*0701**	**DRB1*1301**	**DQB1*0302**	**DQB1*0303**
OR without Age	0.39 (0.003)	1.85 (0.004)	0.31 (0.001)	0.50 (0.009)	2.67 (0.016)
Age adjusted OR	0.39 (0.005)	1.71 (0.018)	0.24 (0.0001)	0.44 (0.004)	3.22 (0.006)

P values shown in parenthesis beside each Odds Ratio (OR).

3.3.2 HLA CLASS I IMMUNOHISTOCHEMISTRY

Immunohistochemical detection of HLA class I antigens was performed on four human donor eyes without remarkable pathology, using a pan-HLA class I monoclonal antibody. The most notable labeling was observed in endothelial cells, particularly in the choriocapillaris but also in large vessels of the choroid (Figure 6A). This pattern was different from that of von Willebrand factor which was detected in capillaries but showed much stronger labeling in large veins and arteries of the outer choroid (Figure 6B). Labeling of large retinal vessels was also apparent with relatively little localization to retinal capillaries. Occasional RPE cells showed minor labeling along the basal aspect (data not shown). Vessels in the sclera as well as a population of scleral cells with an elongated morphology were immunoreactive. In contrast to MHC class II antigens[128], drusen present in these specimen did not exhibit strong immunoreactivity for HLA class I (Figure 6. C, D).

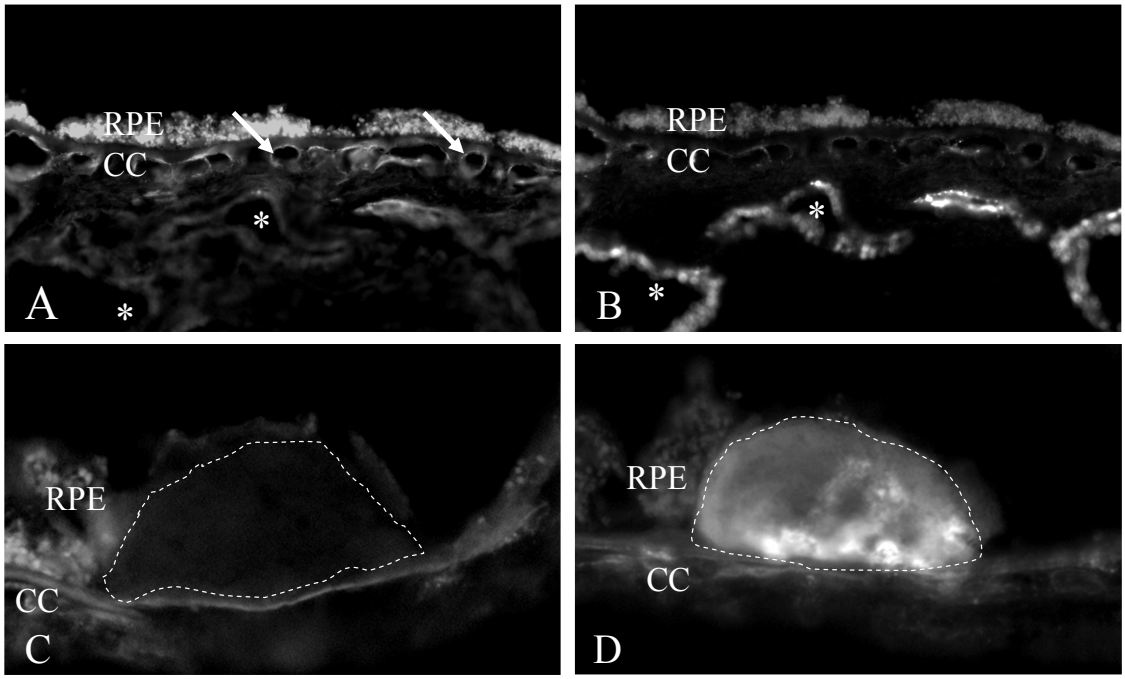


Figure 6. Immunolabeling of the RPE and choroid.
Monoclonal antibody directed against HLA class I antigens (A) and polyclonal antibodies directed against von Willebrand factor (B) on the same section. The fluorescence of the RPE is due to lipofuscin autofluorescence. The endothelial cells with the most notable

HLA labeling are cells of the choriocapillaris (CC, arrows), although other vessels are also weakly positive (asterisks). In contrast, robust labeling of large vessels is noted with antibodies directed against von Willebrand factor (B, asterisks). Immunolocalization of HLA class I (C) and class II (D) molecules in eyes with drusen reveals that, unlike MHC class II antigens, MHC class I antigens are not detectable as major components of drusen or other subRPE deposits. Drusen borders are indicated by the dashed line.

3.4 DISCUSSION

The importance of defining associations between HLA antigens and AMD lies in providing new insights into its basic pathogenesis and to help define an 'at risk' population for preventive measures to become applicable. This is particularly true, since the overwhelming majority of HLA gene polymorphisms result in functional amino acid substitutions in the expressed HLA molecules, thereby modulating the immune response to a vast range of antigens, with inter-individual differences in immune responses to complex or simple antigens[129]. Due to the critical role of HLA in regulating the immune response, combined with its extensive polymorphism, it is perhaps unsurprising that many HLA gene polymorphisms have been linked to susceptibility in a large number of immunologically mediated diseases including the retina and uvea of the eye, the skin, gut, endocrine and joint systems[130].

Association studies for a disease like AMD which has multiple etiological factors are difficult and require a robust study design and execution. In our study, several steps have been taken to avoid confounding variables. These include ethnically matched cases and controls of similar ages, with both groups having been screened for AMD; appropriate study sample sizes and the application of stringent statistical methods to detect the strength of our associations.

Due to the extreme polymorphic nature of the HLA region, sampling variation between cases and controls can easily lead to both false-positive and false-negative associations. Accordingly, application of a Bonferroni correction factor for the number of allelic subtypes tested is recommended and was applied in this study. A two-step genotyping procedure was followed. This permitted a realistic Bonferroni correction for a small group of candidate alleles, rather than an over-conservative correction for all possible HLA alleles, which may cause even true positive associations to be discarded. The two-stage method of genotyping used in our study has been modified from a previously reported similar study [131] where candidate alleles were identified initially by comparing frequencies in patient versus population controls. These short listed candidate alleles were then genotyped and compared between the two groups. This approach reduces the number of multiple comparisons made after the second stage and helps minimize the type I error rate. In addition, DNA based typing was utilized, which has a better allelic resolution and accuracy as compared with serological HLA typing.

Finally, LD occurs frequently between alleles of different HLA loci, whereby an allele from one locus often occurs more frequently in combination with an allele of a second locus than is expected from their individual allele frequencies. This can complicate studies of HLA and disease predisposition. In cases where alleles of more than one locus show positive or negative associations with disease, it is therefore important to determine whether such associations are independent or reflect LD between the alleles concerned. Methods such as the two-locus stratification analysis described[132] and the use of relevant genomic software such as PyPoP therefore assume importance in excluding LD and were applied in the present investigation. PyPoP also enables haplotype estimation. Such haplotype estimation was attempted in our study, but this was not possible for the whole study group since full

genotyping data were available only in the first half of cases and controls. However, we did obtain haplotype estimates for 2 and 3 locus combinations for the first 100 cases and 92 controls where complete genotype data was available. Each 2 locus haplotype estimation revealed between 85 to 161 haplotypes, and their corresponding frequencies were too small for any meaningful analysis between the groups (data not shown).

After controlling for known AMD risk factors (smoking, age and BMI) by logistic regression analysis alleles B*4001, DRB1*1301 and Cw*0701 remained significantly associated with AMD. Allele Cw*0701 increased susceptibility to AMD while HLA-B*4001 and DRB1*1301 decreased susceptibility to AMD. Two-locus stratification and logistic regression analysis indicated that these associations were independent and not reflective of LD between alleles. For example, two-locus stratification and logistic regression analysis demonstrated that the negative association of HLA-B*4001 with AMD was observed in DRB1*1301 negative (but not positive) individuals and the negative association of DRB1*1301 with AMD was seen in B*4001 negative (but not positive) individuals (table 6). The apparent negative LD found between DRB1*1301 and DQB1*0302 in our control but not AMD subjects is most probably a reflection of well-described strong LD between DRB1*1301 and DQB1*06 alleles in Caucasian UK populations[133;134]. After Bonferroni correction alleles DQB1*0302 and DQB1*0303 were not significantly associated with AMD. However as their uncorrected p values were < 0.05 these alleles would still merit further investigation in an independent/larger study group.

The mean age of our control population was slightly lower compared to our cases (mean age 71.57 vs. 76.26). Therefore some of our controls may subsequently develop AMD. However if so, the HLA associations described would be an underestimate. HLA associations with

AMD may therefore be stronger than we describe. In addition, controlling for age during regression analysis did not alter the described HLA associations. This study had 80% power to detect significant differences in allele frequencies between cases and controls at an alpha level of 0.05. For example, for an allele occurring at a frequency of 10% in the control population, detectable odds ratios are 2.3 for a predisposing allele and 0.5 for a protective allele with 95% confidence. We may therefore have not detected smaller HLA associations with this sample size.

This study broadens the role of HLA associations in ophthalmic disease. Previous eye associations have all been in uveitic diseases i.e. HLA-B27 is strongly associated with anterior uveitis[135;136]. HLA-A29 confers an increased risk of birdshot chorioretinopathy up to 224 times higher than for non-carriers[137;138]. Sympathetic ophthalmia is associated with HLA-B1*04 and intermediate uveitis with HLA-DRB1*03[139;140]. Our statistical correction for multiple comparisons is at least as conservative, and in some cases more conservative, than that used in these accepted HLA associations with uveitic eye disease.

More than one HLA locus may be involved with disease predisposition. This is not uncommon and has been described for other diseases, including celiac disease[141] and insulin dependent diabetes mellitus. HLA- DQA1 and DQB1 alleles have been associated with the former, while in the latter case, a number of predisposing and protecting alleles and allele combinations have been identified[142]. Based on our findings, multi-allelic HLA associations may also be important in AMD.

Logistic regression analysis was performed to evaluate relationships between the five HLA alleles initially associated with AMD (table 9) and their effect on disease severity. Smoking

in association with the HLA alleles conferred a four fold increased risk of developing advanced AMD (AREDS group IV) (table 10). Further investigation of these five HLA alleles in a larger study would be useful in determining the amount of risk contributed by these alleles towards advanced AMD and possibly CNV. Previously, significant associations have been reported between HLA-DRB1*15 (P_c=0.000001) and DQB1*06 (P_c=0.00001) alleles and the development of CNV in presumed ocular histoplasmosis syndrome[143]. These findings, along with the data from the current study, support the notion that certain HLA alleles are associated with CNV in various retinal diseases.

The localization of HLA class I antigens to choroidal endothelial cells is consistent with previous studies using tissues with longer post-mortem times[144]. It is interesting that choriocapillary endothelial cells exhibit more robust expression than the endothelium of larger vessels, as visualized by the von Willebrand factor dual labeling experiments. Endothelial cells in some tissues express HLA antigens constitutively and cultured choroidal endothelial cells increase their expression of HLA class I antigens following exposure to certain cytokines[145]. Whether these antigens are up regulated in endothelial cells or other ocular cell types in eyes with AMD remains to be determined, but this is an intriguing area of future study. It is also interesting that the expression patterns differ between HLA-I and HLA-II antigens in the choroid and in sub-RPE deposits, suggesting possible differences in how these molecules may confer risk in macular disease. Notably, drusen contain MHC class II antigens but do not appear to contain MHC class I proteins. The high level of expression of HLA class I antigens by choriocapillary endothelial cells could potentially place the choriocapillaris at risk for T-cell mediated attack in AMD and other inflammatory diseases.

The underlying mechanisms by which HLA genes determine susceptibility to various ocular diseases has not been fully elucidated yet and it should be stressed that an HLA association determined by a case-control study does not necessarily implicate HLA polymorphism in causality, but may reflect LD with causal polymorphisms in other genes, for example, LD between HLA-A*03 and causal polymorphisms in the HFE gene which determine susceptibility to hereditary hemochromatosis[146]. While we cannot exclude LD with non-HLA polymorphisms as causal in AMD, a number of lines of evidence, both indirect and direct, suggest a direct role for HLA in the disease process.

Firstly, HLA-A29 transgenic mice have been shown to develop a histologically similar disease to birdshot chorioretinopathy in humans, confirming the functional role of the HLA-A29 molecule in the pathogenesis of this condition[147]. Thus, certain HLA alleles are known to promote the development of other inflammatory ocular diseases. Secondly, and more importantly, accumulating evidence implicates an immune as well as genetic and environmental components in the aetiology of AMD. Research shows that the RPE is replete with the ability to synthesize molecules involved in the immune response[148]. Also dendritic cells, which are potent antigen-presenting cells, have been intimately associated with drusen development along with key complement pathways both within drusen and along the RPE-choroid interface. Drusen are a constant feature and an important risk factor in all AMD phenotypes, seen even in sub-clinical AMD phenotypes as typical small drusen[12]. HLA-DR and certain immune-related cluster differentiation (CD) antigens have been associated with core domains within drusen[12]. Immunohistochemical and ultrastructural studies have suggested that these cores are derived from cell processes of choroidal antigen presenting cells that breach the Bruch's membrane[12]. Accordingly, the HLA associations found here suggest a potential direct role for HLA molecules in the cellular immune mediated processes

directed against specific local antigens or newly created antigens particularly during drusen and AMD evolution.

Thirdly, another mechanism which might indicate a direct role for HLA polymorphism in determining susceptibility to AMD is "molecular mimicry," where antigenic cross reactions occur between HLA antigens and extraneous trigger antigens possibly from an invading organism. The latter itself may not induce a strong immune response, but instead induce a localized autoimmune reaction responsible for the associated pathology. Interestingly, anti-Chlamydia pneumoniae antibodies have been found to be elevated in patients with AMD, suggesting an association of this pathogen with AMD[62]. The pathogenesis for this could be similar to that reported in acute anterior uveitis (AAU) where potential Gram-negative bacterial antigens have been shown to alter the immune response of resident antigen-presenting HLA-DR(+) dendritic cells in the uvea[149]. Our findings reinforce the possible autoimmune nature of AMD as being a disordered response to inflammation or superantigens.

It is well established in genetic epidemiology research that it is important to evaluate any initial associations in at least one separate cohort. For this purpose DNA samples were obtained from a cohort of 576 AMD cases and 288 controls from Michigan, USA (courtesy of Prof. Anand Swaroop), these have now been genotyped and results summarised below. Following on from this hypothesis-generating study, further larger HLA-AMD association studies are needed in other populations to confirm these novel HLA associations. In addition, these studies where possible should be complemented by functional studies to determine the contribution of these genes to the development of AMD. Dissecting the role of HLA and immune pathways in AMD may ultimately lead to opportunities to modulate

these pathways by precise pharmacological means and thus improve the visual outcome in this devastating disease.

3.5 HLA REPLICATION STUDY IN MICHIGAN COHORT

HLA genotyping for the 3 significant alleles found in the UK cohort was carried out in the Michigan cohort using the same methodology as previously described above. Frequencies for the three tested HLA alleles in the Michigan AMD group (*n*=576) were compared with those in their normal controls (*n*=288). Data analysis demonstrated a similar trend as reported previously[150] in the UK cohort (table 12). The positive AMD association with class I HLA Cw*0701 allele (*P*=0.016, OR=1.81), the negative AMD associations found with HLA class I allele B*4001 (*P*=0.044, OR=0.67) and class II allele DRB1*1301 (*P*=0.006, OR=0.60) were all found to be significant. After correcting for multiple comparisons, the positive association of Cw*0701 (P_c=0.048) and the negative association of DRB1*1301 (P_c=0.018) remained significant while the B*4001 (P_c=0.132) did not reach significance. The lower HLA-Cw*0701 frequencies seen in the Michigan normal controls as compared to the normal UK controls seem to mirror reported population differences in allelic distributions (US populations vs. Western Europe, 0.13 and 0.17 respectively)[151].

Table 12. HLA - B*4001, Cw*0701 and DRB1*1301 genotype frequencies in AMD cases and normal controls of the Michigan cohort.

HLA allele	AMD Group *n*=576	Control Group *n*=288	*P* value†	P_c value§	Odds Ratio‡	95% CI
Cw*0701	78 (0.14)	23 (0.08)	0.016*	0.048*	1.81	1.11-2.94
B*4001	71 (0.13)	50 (0.18)	0.044*	0.132	0.67	0.45-0.99
DRB1*1301	85 (0.15)	64 (0.22)	0.006*	0.018*	0.60	0.42-0.87

†Two-sided *P* values using chi-squared test (*Significant values); P_c indicates *P* values after application of Bonferroni correction factor (n=3). ‡Odds Ratio using the approximation of Woolf.

In this study we have been able to replicate to a major extent the previously reported HLA - AMD associations in a different population cohort. Although the AMD risk (nearly 2 fold) conferred by the presence of the C*0701 allele seems to be similar in both Southampton and Michigan AMD cohorts (odds ratios of 1.92 and 1.81 respectively), the strength of the HLA-Cw*0701 association with AMD in the US cohort seems to be lower than that seen in the UK cohort. One possible explanation for this could be the lower Cw*0701 allele frequencies seen in the Michigan cohort due to reported population differences in allelic distributions (US vs. Western Europe, 0.13 and 0.17 respectively)[152] . It is also well recognized that the first report describing an association between a gene and a disease often overestimates the fraction of disease caused by mutation in the gene in question.[153] This emphasizes the need for replication studies such as ours to assess the HLA effect in further AMD populations.

In summary, the novel HLA-AMD associations found in this study were reproducible to a major extent in a different population cohort. It is therefore logical that these HLA associations could predictably play an important role in AMD among most western populations. Larger HLA-AMD studies in different ethnic populations are needed to further confirm these novel HLA-AMD associations.

CHAPTER FOUR: C-REACTIVE PROTEIN, ICAM-1, INTERLEUKIN, VEGF AND COMPLEMENT FACTOR H GENE VARIANTS IN AMD.

4.1 INTRODUCTION

Currently, both genetic and environmental risk factors are known to be associated with AMD [154;155]. Genetic associations include sequence changes in the ApoE, fibulin 5, complement factor H, toll like receptor 4 and HLA genes [150;156-162]. In addition elevated serum C-reactive protein and smoking have been associated with AMD [45;163].

There are now multiple lines of evidence suggesting an important role for inflammatory events in the pathogenesis of AMD. Histologically, drusen, which are extra-cellular deposits seen in the retina in patients with AMD, contain proteins which modulate the body's response to inflammation. These proteins include vitronectin, complement and immunoglobulins[164]. Auto-retinal antibodies have been detected in the sera of some AMD patients[165] and a serological association has also been reported between Chlamydia pneumoniae infection and AMD [62]. Recent research suggests that serum CRP could be an important biomarker for AMD [45;65]. However it is unclear why CRP levels associate with AMD.

The factors influencing basal CRP levels and levels reached during inflammation are not fully understood. Interleukin-6, the main inflammatory cytokine stimulus for CRP[166] has also been recently associated with AMD [65]. In the absence of acute inflammation, CRP levels appear stable[167], with absolute levels being influenced by age[168], sex[169], body mass index (BMI)[170], and smoking[171]. Studies in families have suggested that CRP level is a heritable trait [172;173], but the genes involved in this regulation are unknown[169]. However

several single nucleotide polymorphisms (SNP's) have been demonstrated recently in the human CRP gene, many of which appear functional. Of these, 1059G/C (exon 2, rs1800947) and 1444C/T (3' UTR region, rs113012959) seem to influence CRP levels both at baseline and during inflammation[174]. A recent study has also identified two functional promoter SNP's (-409G/A, rs3093062 and -390C/T/A, rs3091244) in the CRP gene which seem to affect protein transcription and baseline CRP levels[175].

The recent finding of a complement factor H coding polymorphism (1277T/C, rs1061170) in association with nearly 50% of AMD cases[46-48;176] may also be of particular interest in the context of CRP. Firstly, because it is located near a suspected AMD susceptibility locus on chromosome 1q, a region showing substantial linkage disequilibrium and secondly, this Y402H variant located on SCR7 domain of the CFH protein has been implicated as a binding site for CRP at the protein level. It has been proposed that CFH through its direct binding to CRP normally reduces complement mediated deposition in AMD and that this process may therefore be defective due to the variant CFH protein. We therefore analysed the CFH variant 1277T/C (Y402H) in our study cohort to evaluate any possible correlation with CRP levels.

Adhesion molecules such as Intercellular Adhesion Molecule-1(ICAM1) play a key role in autoimmune disorders and soluble forms (sICAM1) of these adhesion molecules can be measured in serum. CRP is known to have a direct pro-inflammatory effect on endothelial cells by stimulating ICAM1 expression [177], which may be relevant in AMD. In the eye, ICAM1 is expressed on retinal pigment epithelium (RPE) and choroidal vascular endothelial cell surfaces where it mediates leucocyte adhesion and migration[178]. In an animal model of choroidal neovascularization, ICAM-1 deficiency is associated with less severe choroidal

neovascular membranes than wild type, indicating that ICAM-1 is necessary in the pathogenesis of advanced AMD[179].

Elevated ICAM1 expression has been demonstrated on vascular elements and RPE cells in sub-foveal choroidal neovascular membranes (CNV) from AMD patients[180]. Indeed, recent clinical trials using intravitreal triamcinolone acetonide in the treatment of neovascular AMD seem to support this role of ICAM1 in the pathogenesis of AMD[108;109;181]. This is because one modality by which triamcinolone seems to exerts its effect is by reducing expression of ICAM1 in RPE and vascular endothelial cells[182].

Raised CRP has previously also been associated with increased expression of ICAM1 in atherosclerosis, especially in the coronary artery endothelial cells[183]. We therefore investigated CRP and ICAM1 in AMD as both inflammatory markers seem interrelated and important for the development of atherosclerosis[184;185], a disease which shares many epidemiological risk factors with AMD. Two coding SNPs reported in the ICAM-1 gene (a single copy gene) are known to be common in all populations and have been associated with several inflammatory diseases. These are located in exons 4 (12959G/A, R241G, rs1799969) and 6 (13848G/A, E469K, rs5498). While, R241G has been significantly associated with rheumatoid arthritis[186], inflammatory bowel disease[187] and giant cell arteritis[188], E469K has been predominantly associated with Bechet's disease (both ocular and systemic)[189-191] and Alzheimer's disease [192].

Increasingly evidence is emerging that variations in cytokine expression (and in the underlying genes which control their expression) may modulate susceptibility to age related macular degeneration (AMD). Cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8)

and vascular endothelial growth factor (VEGF) are released by degenerating retinal pigment epithelial cells and associated with drusen in AMD[12;193]. VEGF induces the expression of ICAM1 on vascular endothelium[194]. Studies have shown increased VEGF expression in the RPE of donor eyes with neovascular AMD[195] and in cultured RPE from surgically removed CNV[196]. C-reactive protein (CRP) has been The main inflammatory cytokine stimulus for CRP (shown to be a biomarker for AMD 45;65) is interleukin-6 and this has also been recently associated with AMD.[65;166]

Pro-inflammatory cytokines have also been shown to up regulate intercellular adhesion molecule-1 (ICAM1) at microglial sites[194] which along with locally released cytokines may lead to the recruitment of inflammatory cells seen in AMD. IL-8 is a primary mediator of angiogenesis and this has been demonstrated in various carcinomas[197;198]. IL-10 is a potent anti-inflammatory cytokine produced by T cells, macrophages, and retinal pigment epithelial cells.[199;200] It strongly inhibits antigen-specific T cell proliferation, cytokine production, and down regulates both HLA class 1 and class 2 antigen expression[201;202]. IL-10 is also important in the induction of antigen-specific anergy or tolerance.[203] Genotypes associated with high IL-10 expression have a protective role (possibly via inhibition of angiogenesis)[204;205] while genotypes associated with high IL-8 expression could be risk factors for stimulation of angiogenesis in AMD.

This part of the study was therefore done to determine if biomarkers like CRP and sICAM1 were independent risk factors for AMD development in a well defined UK cohort. Functional polymorphisms for biomarkers (CRP -409G/A, -390C/T/A, 1059G/C, 1444C/T and ICAM1 12959G/A, 13848G/A) were evaluated for any effect on serum levels. Based on the evidence for a possible role of cytokines in AMD we chose four known expression-

related cytokine polymorphisms in interleukin genes (pro-inflammatory IL 1β -511C/T (rs16944), IL 6 -174C/G (rs1800795), IL 8 -251A/T (rs4073) and anti-inflammatory IL 10 -1082G/A (rs1800896) to test the hypothesis that they may be associated with AMD.

Additionally, other expression-related cytokine polymorphisms in VEGF (-2578C/A or rs699947, -1154G/A or rs1570360, -634G/C or rs2010963, +936C/T or rs3025039) genes were evaluated for any association with AMD. Finally, the study also explores the plausible link between serum CRP and the CFH Y402H polymorphism as this CFH variant alters the binding properties of CFH to CRP which may lead to complement mediated AMD damage.

4.2 METHODS

4.2.1 SUBJECTS AND METHODS

AMD patients and normal controls over age 55 were recruited as per methods described in chapter 2 (section 2.4.10). AMD photographs were graded by a masked observer into 4 groups of increasing disease severity as described in the AREDS study [206]. The CNV group was further categorised into occult, minimally classic and predominantly classic sub-groups. General health was assessed and care was taken to exclude patients who reported any infective illness in the preceding month. Patients with a history of chronic joint or organ related inflammation were also excluded from the study. Information was also obtained about relevant past medical history, smoking history, ocular history, use of medications, vitamin or dietary supplementation; and height, weight, body mass index (BMI) measurements recorded. Serum separated during DNA extraction and stored at –20º C was utilised for CRP analysis.

4.2.2 MEASUREMENT OF CRP AND ICAM1

Serum CRP levels were measured using an automated Advia 1650 clinical analyser (Bayer). In this method, CRP in the serum sample combines specifically with anti-human CRP antibodies in the buffer-antibody reagent mix to form an aggregate. This insoluble aggregate causes an increased turbidity which is then measured optically; this is proportional to the amount of CRP in the sample. The sensitivity or linear range of detection for CRP levels with this analyser was 1 to 180 mg/l. Quality control for the analyser was done using the recommended graphical calibration software using recommended controls and diluents from the manufacturer.

Immunoassay for sICAM-1 was done using a solid phase ELISA kit (R & D systems Inc, UK). This assay employs a quantitative sandwich enzyme immunoassay technique using recombinant human ICAM-1 and antibodies raised against the recombinant factor. The intensity of colour development was measured using a microplate reader (Anthos 210) at 450 nm and with correction set at 620 nm. All samples were analysed using the accompanying software (stingray), and results obtained showed recommended linear curves that were parallel to the standard obtained using the recombinant parameter kit standards.

4.2.3 SNP GENOTYPING USING 5' NUCLEASE ASSAY

All SNP's were genotyped using the 5' nuclease assay for allelic discrimination. Primers and individual fluorogenic TaqMan probes (consisting of an oligonucleotide labeled with both a fluorescent reporter dye - FAM™ and a quencher dye -VIC®) were designed using Primer Express software (version 2.0; sequences shown in table 13). For the CRP -390 triallelic SNP, an additional TET probe was selected. These were then obtained from Applied Biosystems (UK). All assays were performed using the supplied Q-PCR buffer (2.5 µl),

PCR probe mix (0.125 µl) and 20 ng of genomic DNA in 2.375 µl dH2O to make a 5 µl reaction volume (384-well plate). The PCR thermocycling protocol consisted of 10 min @ 95°C, followed by 40 cycles of 15 sec @ 92°C and 1 min @ 60°C. Each genotyping plate contained 8 wells without any DNA template (water controls) and randomly selected duplicate samples (10% of plate samples). Allelic level genotyping from fluorescence measurements were then obtained using the ABI PRISM® 7900HT Sequence Detection System. SDS version 2.1 software was used to analyse real time and endpoint fluorescence data (figure 7).

Table 13 . Primer and probe sequences used for 5' nuclease (TaqMan) genotyping.

Primer / Probe	**Sequence**
CRP 1444 forward	5' GGTCTGGGAGCTCGTTAACTATG 3'
CRP 1444 reverse	5' CCAACTTGAAATAAAAATGAAAACAAAACACCT 3'
CRP 1444 T probe	VIC -TTTTGGACC**A**TTTCCCA – MGB
CRP 1444 C probe	FAM - TTGGACC**G**TTTCCCA – MGB
CRP 1059 forward	5' TGGAAATGTGAACATGTGGGACTT 3'
CRP 1059 reverse	5' CCCGCCAAGATAGATGGTGTTAATC 3'
CRP 1059 G probe	VIC - CATCTGGTGA**C**AGCACA – MGB
CRP 1059 C probe	FAM - TCTGGTGA**G**AGCACA – MGB
CRP-409 forward	5' GGGCTGAAGTAGGTGTTGGA 3'
CRP-409 reverse	5' GGTAACATATTAAACGAGTGGCCATCT 3'
CRP-409 G probe	VIC - CTACCAC**C**TGCACCC - MGB
CRP-409 A probe	FAM - CTACCAC**T**TGCACCC - MGB
CRP-390 forward	5' CTACCACGTGCACCCAGATG 3'
CRP-390 reverse	5' TATCCTGCGAAAATAATGGGAAA 3'
CRP-390 T probe	VIC - CCACT**A**GTTTAATATGTTACC - MGB
CRP-390 C probe	FAM - CCACT**G**GTTTAATATGTT - MGB
CRP-390 A probe	TET - CCACT**T**GTTTAATATGTTACC - MGB
CFH 1277 forward	5' CCATGCCTCAGAAAATGTTATTTTCCTT 3'
CFH 1277 reverse	5' GGCAGGCAACGTCTATAGATTTACC 3'
CFH 1277 C probe	VIC - TTTCTTCCAT**G**ATTTTG - MGB
CFH 1277 T probe	FAM - TTCTTCCAT**A**ATTTTG - MGB
ICAM1 12959 forward	5' CAGGGGACCGTGGTCTGTTCC 3'
ICAM112959 reverse	5' AACTTGGGGTGTCAGTGGATAC 3'
ICAM1 12959 A probe	VIC - TGGGAACAGCC**T**GTC - MGB
ICAM1 12959 G probe	FAM - TGGGAACAGCC**C**GTC - MGB
ICAM1 13848 forward	5' TCGAGATCTTGAGGGCACCTA 3'
ICAM1 13848 reverse	5' ATGTGCTCTGTGAGTGAGCCG 3'
ICAM1 13848 A probe	VIC - CACGGTCACCT**T**GCGGGT - MGB

ICAM1 13848 G probe	FAM - ACGGTCACCT**C**GCGGGTG - MGB
IL 1B-511 forward	5' GGTCTCTACCTTGGGTGCTGTT 3'
IL 1B-511 reverse	5' TCCTCAGAGGCTCCTGCAAT 3'
IL 1B-511 C probe	VIC - TGCCTC**G**GGAGCT - MGB
IL 1B-511 T probe	FAM - TCTGCCTC**A**GGAGC - MGB
IL 6-174 forward	5' GCTGCACTTTTCCCCCTAGTT 3'
IL 6-174 reverse	5' GCTGATTGGAAACCTTATTAAGATTGT 3'
IL 6-174 G probe	VIC - CTTTAGCAT**C**GCAAGAC - MGB
IL 6-174 C probe	FAM - CTTTAGCAT**G**GCAAGAC - MGB
IL 8-251 forward	5' GTCACATGGTCTATGATAAAGTTATCTAGAAATA 3'
IL 8-251 reverse	5' TACATTTAAAATACTGAAGCTCCACAATTT 3'
IL 8-251 A probe	VIC - AAGCATACA**T**TTGAT AATT - MGB
IL 8-251 T probe	FAM - AAGCATACA**A**TTGATAATT - MGB
IL 10-1082 forward	5' ACACACAAATCCAAGACAAACACTACTAA 3'
IL 10-1082 reverse	5' GGAGGTCCCTTACTTTCCTCTTACC 3'
IL 10-1082 G probe	ATCCCTACTTCCCC**C**TCCCAAAGAA - MGB
IL 10-1082 A probe	CCCTACTTCCCC**T**TCCCAAAGAAGC - MGB
VEGF 2578 forward	5' TCAGTCCATGCCTCCACAGA 3'
VEGF 2578 reverse	5' GGAACAAAGTTGGGGCTCTGA 3'
VEGF 2578 A probe	VIC - TATCCACCCAGATC**T**TGCCAGGGTC - MGB
VEGF 2578 C probe	FAM - CCACCCAGATC**G**TGCCAGGGT - MGB
VEGF 1154 forward	5' CGGGCCAGGCTTCACTG 3'
VEGF 1154 reverse	5' GGCGGGGACAGGCGA 3'
VEGF 1154 A probe	VIC - CTCAGCCC**T**TCCACA - MGB
VEGF 1154 G probe	FAM - CTCAGCCC**C**TCCAC - MGB
VEGF 634 forward	5' TCCAGAGAGAAGTCGAGGAAGAGA 3'
VEGF 634 reverse	5' CCCCAAAAGCAGGTCACTCA 3'
VEGF 634 C probe	VIC - TGCCCCTGTC**G**CTTTCGCTG - MGB
VEGF 634 G probe	FAM - TTGCCCCTGTC**C**CTTTCGCTG - MGB
VEGF 936 forward	5' ACTCCGGCGGAAGCATTC 3'
VEGF 936 reverse	5' AGCAAGAAAAATAAAATGGCGAATCCA 3'
VEGF 936 C probe	VIC - CAAGAGGGACC**G**TGCTG - MGB
VEGF 936 T probe	FAM - AAGAGGGACC**A**TGCTG - MGB

Forward and reverse sequences are primers. VIC, FAM and TET probe sequences have positions of single nucleotide polymorphisms marked in bold case. MGB, minor groove binding probe.

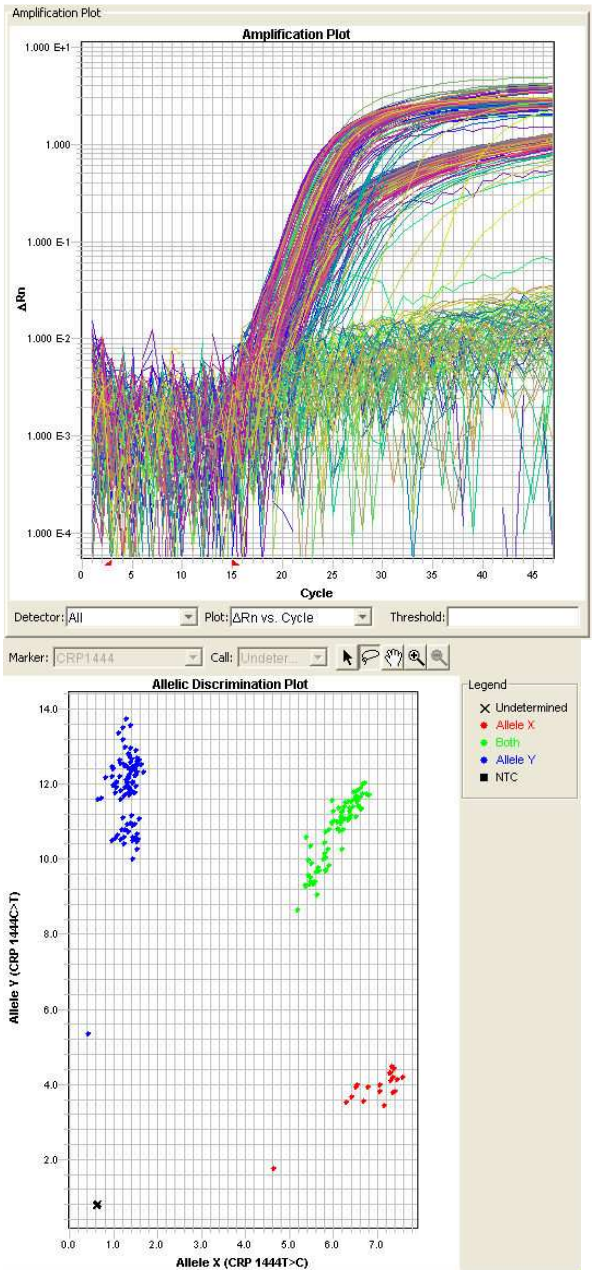


Figure 7. Real time amplification and allelic discrimination plots from Taqman genotyping assay.

4.2.4 STATISTICAL METHODS

Power calculations were performed utilizing the Epi Info statistical package (Revision 2, 2003) provided by the Center for Disease Control and Prevention (CDC). Calculations revealed that the study would have a power of 89% based upon 500 cases and 500 controls to detect a 15% difference in CRP and sICAM levels between the groups. Power calculations for gene polymorphisms were done using reported UK/European allele

frequencies. *P* values were evaluated by two sided Chi-square tests with Yate's correction and 1^{df}. The level of statistical significance in the study was set at $P_C < 0.05$.

Allelic and genotype distributions for each SNP were tested for conformity to Hardy-Weinberg equilibrium using control subjects. Alleles at each locus were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($P>0.05$) from expected frequencies. *P* values were evaluated using Pearson's chi-squared statistic. The level of statistical significance in the study was set at $P < 0.05$. All statistical analyses were performed using the SPSS statistical software package version 12.0. Haploscore software was utilized for CRP, ICAM1, IL and VEGF haplotype frequency estimation to test haplotypic effects on CRP and ICAM1 levels.

Non-parametric data (CRP and sICAM) underwent log transformation to achieve a normal distribution of variables before any statistical parametric testing. For the CRP analysis, the total study sample was analysed in groups based on CRP levels > 2 mg/L, > 4 mg/L and > 5 mg/L similar to previous CRP studies[45;207;208]. The effect of each CRP/ICAM1 genotype on CRP/sICAM1 levels between cases and controls was assessed by ANOVA and Chi-square two-tailed tests. Logistic regression analysis was carried out for CRP and sICAM1 levels using data on age, sex, body mass index (BMI), smoking status and the CRP or ICAM1 genotype as covariates in the regression model. This was done between all the AREDS groups and with the control group. All statistical analyses were performed using the SPSS statistical software package version 12.0.

4.3 RESULTS

Baseline demographics of the unrelated Caucasian cohort recruited from a clinic population (581 cases with AMD and 593 normal controls) are described in table 14. AREDS grading data from fundus photographs were available for 573 (98.6%) of the 581 AMD cases (table 16), based on this criteria the group was divided into 4 groups of increasing AMD severity. For the interleukin analysis, data from 478 cases with AMD and 555 normal controls was available. CNV grading data from fundus photographs and angiograms were available for 283 (59.2%) AMD cases in this cohort.

Table 14. Basic demographic characteristics of the caucasian study population.

	AMD CASES total n = 581 (%)	**CONTROLS total n = 593 (%)**
Age	n=581	n=593
Mean	78.4	69.1
Range	55 - 101	55 - 91
SD	8.65	9.76
Sex	n=581	n=593
Female	330 (64.2)	308 (55.0)
Male	184 (35.8)	252 (45.0)
BMI	n=237	n=407
Mean	26.48	27.27
SD	4.76	4.70
Smoking status	n=460	n=432
Current smokers	140(30.4)	81(18.7)
Past smokers	145(31.5)	213(49.3)
Non-smokers	175(38.0)	138(31.9)

4.3.1 CRP LEVELS AND CRP GENE POLYMORPHISMS

CRP levels were available for 305 cases and 384 controls (table 15). Mean levels were significantly higher in AMD cases as compared to controls (5.75 mg/l vs. 3.66 mg/l, *P*=0.0001, CI=0.83-3.27). CRP levels were also higher in AREDS groups II, III and IV as compared to AREDS group I (table 15). In the multivariate regression analysis based on different CRP values, all the three CRP groups had significantly higher odds ratio (OR) for AMD when compared to the control group (table 17). The disease association was nearly 2 fold in the CRP > 2 mg/l (OR=1.68, P_c=0.002) group, and over 2 fold in the CRP > 4 mg/l (OR=2.11, P_c=0.0004) and CRP > 5 mg/l (OR=2.28, P_c=0.0001) groups. The most significant CRP association was seen in AMD patients who were current smokers (OR=1.92, P_c=1.28e-007), the strongest correlation being in the CRP >4mg/l and CRP>5mg/l groups (table 17).

Table 15. CRP and sICAM1 levels in AMD cases and controls.

	CRP, mg/l, n=305	**sICAM1, ng/ml, n=384**
AMD cases, n=305	4.79 ± 4.78 (1.35 ± 0.74)	283.50 ± 76.98 (5.62 ± 0.24)
Controls, n=384	3.51 ± 3.14 (1.06 ± 0.59)	264.87 ± 62.65 (5.55 ± 0.21)
OR, *P* value	1.90, 3.20e-005	3.34, 0.014
OR, P_c value*	1.91, 1.28e-003	2.90, 0.037

Quantitative data are presented as mean with standard deviation. Corresponding log values are presented in parenthesis. *P_c value corrected for current smoking status.

Table 16. AREDS sub-grouping of AMD cases and mean CRP levels.

AREDS Grading n = 573 (%)	**AMD Cases with CRP levels, n=305 (%)**	**Mean CRP levels ± SD***
Grade I – 29 (5.1)	20 (6.5)	4.40 ± 2.71
Grade II - 84 (14.7)	53 (17.4)	4.09 ± 3.29
Grade III - 159 (27.7)	85 (27.9)	4.74 ± 4.68
Grade IV - 301 (52.5)	147 (48.2)	5.11 ± 5.03

*SD = Standard Deviation.

Table 17. Multivariate logistic regression analysis for CRP and sICAM1 levels in AMD cases and controls.

	CRP > 2 mg/l	**CRP > 4 mg/l**	**CRP > 5 mg/l**	**sICAM1**
Cases (n),Controls (n)	305, 384	154, 104	131, 86	194, 278
CRP/sICAM1	1.68 (0.002)*	2.11 (0.0004)*	2.28 (0.0001)*	1.50 (0.37)
Age (years)	1.14 (0.01)*	0.13 (0.01)*	1.13 (0.01)*	1.12 (0.01)*
Sex (F/M)	1.45 (0.08)	1.18 (0.40)	1.16 (0.45)	1.54 (0.06)
BMI (kg/m^2)	1.02 (0.32)	1.00 (0.82)	1.00 (0.80)	1.02 (0.32)
Current smokers	1.36 (0.06)	2.77 (4.91e-010)*	2.26 (5.50e-005)*	4.86 (1.53e-006)*
Past smokers	1.0 (0.99)	0.96 (1.01)	0.99 (0.98)	1.37 (0.33)

*Significant corrected P_c values shown in parenthesis next to each Odds Ratio.

The observed genotype and allele frequencies for the CRP polymorphisms tested in the control population was found to be in Hardy-Weinberg equilibrium. CRP genotype frequencies in AMD cases and normal controls were as shown in table 18. The genotype distributions were similar for the 1444C/T 3' UTR, 1059G/C exonic, -409G/A promoter and the triallelic promoter -390T/C/A CRP polymorphisms in both groups. No significant genotype associations were found with CRP levels or AMD status on analysis after correcting for multiple variables (data not shown).

Following the AREDS categorization in the AMD group, a multivariate logistic regression analysis was performed between CRP levels within these AREDS groups and the different CRP genotypes (1444C/T, 1059G/C, -409G/A and -390 C/T/A) in relation to age, sex, BMI and smoking status. No significant associations were found with these comparisons.

Table 18. CRP genotypes and CRP associations for individual 1444C/T, 1059G/C, -409G/A & -390 T/C/A CRP polymorphisms.

Genotypes	AMD cases	CRP mg/L (n)	Controls	CRP mg/L (n)
1444C/T	n=506	n=217	n=552	n=295
rs113012959				
CC	233	4.43± 4.16 (114)	277	3.22± 2.57 (169)
CT	229	3.95± 3.80 (98)	210	3.51± 3.06 (142)
TT	39	4.48± 3.99 (21)	63	3.15± 2.29 (37)
1059G/C	n=480	n=205	n=559	n=295
rs1800947				
GG	404	4.26 ± 4.16 (194)	486	4.27± 4.06 (194)
GC	61	3.65± 3.02 (30)	72	3.38± 2.88 (45)
CC	15	5.47± 4.75 (9)	01	1.90±0.00 (1)
-409G/A	n=505	n=232	n=559	n=304
rs3093062				
GG	500	4.27± 4.08 (228)	557	3.38± 3.24 (302)
GA	05	2.97± 2.15 (4)	02	1.90± 0.00 (2)
AA	-	-	-	-
-390T/C/A	n=477	n=204	n=476	n=253
rs3091244				
CC	195	4.52 ± 4.24 (91)	185	3.30± 2.36 (113)
CT	167	4.17± 4.05 (72)	171	3.53± 3.22 (114)
AC	40	3.74± 3.32 (22)	53	3.48± 3.02 (34)
AT	31	4.47± 2.57 (14)	23	2.65± 1.23 (16)
TT	37	4.41± 4.00 (20)	40	4.38± 3.80 (24)
AA	01	-	03	2.30± 0.69 (3)

*P_C values for each genotype and CRP levels or AMD status after adjusting for age, sex, BMI, Current smokers and Past smokers were not Significant (data not shown).

4.3.2 ICAM1 LEVELS AND ICAM1 GENE POLYMORPHISMS

sICAM1 levels were found to be higher in AMD cases as compared to controls (Mean levels of 301.5 ng/ml in cases vs. 271.8 ng/ml in controls, P=0.014), this remained significant (P_c=0.037) when corrected for AMD co-variables (table. 15). However, when the smoking

status was introduced into the regression model (table. 17), a strong association was noted only with current smokers (OR=4.86, P_c=1.53e-006) in the AMD group. A similar association with smoking was not seen in the control group.

Table 19. ICAM1 genotypes and sICAM1 associations for individual 12959G/A (rs1799969) & 13848G/A (rs5498) polymorphisms in the study groups.

Genotypes	**AMD cases**	**sICAM1 (ng/ml)**	**Controls**	**sICAM1 (ng/ml)**	**Genotype-sICAM1 association P_C value (OR,CI)**
12959G/A rs1799969	n=504	n=125	n=549	n=231	
GG	400	288.31± 68.84	444	281.30± 172.96	0.004 (2.88, 1.05-4.07)*
GA	100	275.89± 114.46	104	244.16± 46.57	NS
AA	04	-	01	-	
13848G/A rs5498	n=503	n=127	n=552	n=230	
GG	147	285.31± 66.74	151	312.74± 316.23	NS
GA	240	277.84± 74.85	278	263.98± 64.12	NS
AA	116	300.60± 95.35	123	264.96± 65.28	NS

* P_C values for sICAM1 levels in the total group irrespective of case or control status. P_C values for each genotype and AMD status after adjusting for age, sex, BMI, Current smokers and Past smokers were not Significant (data not shown). NS = Not Significant.

Observed genotype and allele frequencies for the ICAM1 polymorphisms were in Hardy-Weinberg equilibrium. Frequency distributions in AMD cases and normal controls were as shown in table 19. Genotype distributions for the two ICAM1 polymorphisms were similar among AMD cases and controls. On performing logistic regression analysis, sICAM1 levels were found to be significantly associated with the 12959 GG genotype irrespective of the case or control status (OR=2.88, *P*=0.004). None of the 13848 genotypes showed any association with sICAM1 levels. Also, no significant associations were found on performing logistic regression analysis for sICAM1 levels and the different ICAM1 12959G/A and

13848G/A genotypes in relation to age, sex, BMI and smoking status between the four AREDS groups (data not shown).

4.3.3 CFH Y402H POLYMORPHISM AND CRP LEVELS

CFH 1277 C/T (Y402H) genotyping data was available for 581 AMD cases and 593 controls. Of these, 227 AMD cases and 272 controls had both genotyping and CRP data. Genotype frequency distributions were as shown in table 20. The heterozygous CT genotype had the commonest representation with similar frequencies in cases and controls. The homozygous CC genotype was more often present in AMD cases (0.31 vs. 0.14, P_c= 6.92e-008) giving an OR for disease of 4.43. Correspondingly, the homozygous TT genotype was over represented in controls. Individual allelic distributions also differed in the two groups, with the C allele being more significantly represented in AMD cases (P_c=1.54e-007, OR=2.05). Mean CRP levels were similar for the individual CFH genotypes in cases and controls, both ANOVA and logistic regression analysis did not reveal any significant association for any of these CFH genotypes with CRP levels in the study groups. Finally, adjusting for age during the logistic regression analysis for all the SNP's analysed in the study did not alter the odds ratio for disease significantly. On the other hand, regression with BMI as the dependant variable for all the CRP, CFH and ICAM1 SNP's tested revealed large significant odds ratios of nearly 5 fold magnitude (table 21).

Six common and unique haplotypes were inferred from the Haplotypic analysis of the CRP SNP's, these were selected based on their minimum frequency being greater than 0.05 in either cases or controls. Each haplotype was then tested for association with CRP levels. The overall haplotypic distribution was largely similar between cases and controls with the most frequent haplotype being GCGC (0.38 in cases vs. 0.42 in controls). None of the CRP

haplotypes with moderate frequencies showed any association with any category of CRP levels. When looking at higher CRP levels of >3mg/l (n=32 AMD cases, n=21 controls), the association of Y402H with AMD in these individuals was no longer significant.

Table 20. CFH 1277 T/C genotype (rs1061170) and allele distribution in AMD cases and controls.

1277 T/C Genotypes rs1061170	**AMD cases n=581**	**CRP mg/l n=227**	**Controls n=593**	**CRP mg/l n=272**	**Genotype-AMD association P_C value (OR,CI)***	**Genotype-CRP association P_C value (OR,CI)***
CC	0.31 (167)	3.96± 2.90 (n=92)	0.14 (75)	3.21± 2.76 (n=42)	6.92e-008 (4.43, 3.54-4.90)	NS
CT	0.44 (258)	5.41± 5.34 (n=130)	0.47 (261)	3.54± 3.23 (n=161)	-	NS
TT	0.25 (132)	4.82± 4.41 (n=68)	0.39 (215)	3.42± 3.14 (n=145)	-	NS
C Allele	0.54	-	0.37	-	1.54e-007 (2.05, 1.74-2.67)	-
T Allele	0.46	-	0.63	-	-	-

* $P_C = P$ values for genotypes after adjusting for age, sex, BMI, Current smokers and Past smokers. NS = Not Significant.

4.3.4 INTERLEUKIN AND VEGF GENE POLYMORPHISMS

Genotype distributions were largely similar for most of the four IL and VEGF variants tested in the study and in Hardy-Weinberg equilibrium (tables 22 and 23). Genotype distributions were in Hardy-Weinberg equilibrium in the control groups. The IL 8-251AA genotype was found in a larger proportion of AMD cases (35% vs.27%). This genotype was significantly associated with AMD both before (P=0.037, OR=1.21, 95% CI=1.01-1.44) and after correcting for co-variables like age, sex, BMI, current smoking and past smoking status (P_C= 0.043, OR=1.20, 95% CI=1.0 to 1.50). Fluorescein angiographic data was available for 283 AMD patients. This subset of patients were then further categorised into CNV or dry AMD phenotypes (table 25). The CNV group included occult, minimally classic and predominantly classic CNV forms. However, no significant difference in allele frequencies was found on testing IL 8-251A risk allele frequencies in total CNV vs. dry AMD and total

CNV vs. controls. Further analysis stratifying by CNV subtype showed a marginally significant difference in allele distribution between the minimally classic CNV group and normal controls, without correcting for multiple testing (*P*=0.04, OR=1.59, 95% CI =0.92 - 2.71). Consistent with established data, we found evidence for a strong association between AMD status and smoking behaviour dichotomised as never smoked / ever smoked ($P = 2.0 \times 10^{-14}$). However, we found no significant association between IL-8 genotype status and smoking behaviour (data not shown).

Table 21. Available demographic characteristics of the Interleukin study population.

	AMD CASES total n = 478 (%)	**CONTROLS total n = 555 (%)**
Age	n=478	n=555
Mean	78.8	69.0
Range	55 - 101	55 - 91
SD	7.9	9.7
Sex	n=478	n=555
Female	312 (65.4)	306 (55.1)
Male	166 (34.2)	249 (44.9)
BMI	n=469	n=551
Mean	26.3	26.5
Range	13.9 – 43.0	15.7 – 55.4
SD	4.7	4.9
Smoking status	n=478	n=555
Ever smoked	301 (63.0)	217 (39.1)
Never smoked	177 (37.0)	338 (60.9)

Table 22. Logistic regression analysis for the gene variants adjusting for age and BMI.

Regression variable	**CRP 1444C/T**	**CRP 1059G/C**	**CRP -409G/A**	**CRP -390T/C/A**	**ICAM 12959G/A**	**ICAM 13848G/A**	**CFH 1277C/T**
OR without Age	1.33 (0.03)*	0.89 (0.29)	2.75(0.23)	1.01(0.96)	0.89 (0.39)	1.00 (0.89)	3.57 (3.61e-011)
Age adjusted OR	1.34 (0.04)*	0.96 (0.75)	2.32(0.39)	1.06(0.63)	0.94 (0.70)	0.91 (0.38)	3.72 (7.15e-011)
OR for BMI	4.86 (1.54e-006)	5.01 (7.46e-007)	5.02 (6.89e-007)	5.24 (2.33e-007)	4.94 (1.03e-006)	4.95 (9.66e-007)	5.05 (6.01e-007)

P values shown in parenthesis after each Odds Ratio, * significant values.

Table 23. IL genotype frequencies and their association with AMD cases and normal controls.

	IL 1β -511C/T (rs16944)		IL 6 -174G/C (rs1800795)		IL 8 -251A/T* (rs4073)		IL 10 -1082G/A (rs1800896)	
	Case n=475 (%)	Control n=548 (%)	Case n=462 (%)	Control n=553 (%)	Case n=474 (%)	Control n=540 (%)	Case n=473 (%)	Control n=551 (%)
IL Genotype	CC 218 (46)	251 (46)	GG 156 (34)	178 (32)	AA 166 (35)	147 (27)	GG 126 (26)	140 (25)
	CT 225 (47)	240 (43)	GC 223 (48)	270 (49)	AT 206 (43)	269 (50)	GA 230 (49)	270 (49)
	TT 32 (7)	57 (11)	CC 83 (18)	105 (19)	TT 102 (22)	124 (23)	AA 117 (25)	141 (26)
IL Allele	C 661 (70)	742 (68)	G 535 (58)	626 (57)	A 538 (57)*	563 (52)	G 482 (51)	550 (49)
	T 289 (30)	354 (32)	C 389 (42)	480 (43)	T 410 (43)	517 (48)	A 464 (49)	552 (51)
P value	0.36		0.56		0.037*		0.64	
OR (95% CI)	1.09 (0.91-1.32)		1.05 (0.88-1.26)		1.21 (1.01-1.44)		1.04 (0.88-1.24)	

* *P values by* χ^2 test with 1^{df}. P_C = 0.043, OR=1.20, 95% CI=1.0 to 1.50 (*P* value adjusted for age, sex, BMI, Current smoking and Past smoking status). PS: sample size (n) data is less for some IL genotypes due to few failed genotyping reactions.

Table 24. VEGF genotype frequencies in AMD cases and normal controls.

VEGF-634G/C		VEGF+936C/T		VEGF-1154G/A		VEGF-2578A/C	
Case n=490	**Control n=558**	**Case n=459**	**Control n=549**	**Case n=487**	**Control n=547**	**Case n=490**	**Control n=560**
CC 49 (0.10)	57 (0.10)	CC 345 (0.75)	397 (0.72)	AA 50 (0.10)	55 (0.10)	AA 135 (0.27)	148 (0.26)
CG 193 (0.39)	244 (0.44)	CT 104 (0.23)	141 (0.26)	AG 203 (0.42)	226 (0.41)	AC 235 (0.48)	269 (0.48)
GG 248 (0.51)	257 (0.46)	TT 10 (0.02)	11 (0.02)	GG 234 (0.48)	266 (0.49)	CC 120 (0.25)	143 (0.26)

Table 25. Risk for CNV associated with IL 8 -251A/T genotypes and alleles in comparison to dry AMD and control groups.

IL 8 -251A/T Genotype	**All CNV n= 150 (%)**	**Dry AMD n=133 (%)**	**Controls n=540 (%)**
AA	48 (32.0)	39 (29.3)	147 (27.0)
AT	68 (45.3)	67 (50.4)	269 (50.0)
TT	34 (22.7)	27 (20.3)	124 (23.0)
IL 8 -251A/T Allele			
A	164 (54.7)	145 (54.5)	563 (52.0)
T	136 (45.3)	121 (45.5)	517 (48.0)
†Versus Dry AMD (*P* value, OR, 95% CI)	0.48, 1.0* (0.72-1.40)		0.24, 1.10**
‡Versus Controls (*P* value, OR, 95% CI)	0.21, 1.10 (0.85 - 1.43)		(0.84 - 1.44)

P values by χ^2 test with 1^{df}. Analysis was restricted to 283 patients in the AMD group where fluorescein angiographic data was available. †Allele association for CNV as compared to dry AMD and as compared to normal controls‡. *Odds ratio for minimally classic CNV vs. dry AMD was 1.59 (*P*= 0.04, 95% CI= 0.92 - 2.71). **Allele association for dry AMD as compared to normal controls.

4.4 DISCUSSION

Identifying genetic determinants associated with AMD[47;176;209;210] provides new insights into disease pathogenesis and also assists in defining an 'at risk' AMD population. This may allow preventive measures to be instituted. In this study cytokine polymorphisms that functionally influence transcription levels and are associated with autoimmune diseases[192;211] were tested for association with AMD. The postulated common pathway of these inflammatory molecules prompted us to examine the contribution of gene polymorphisms in the encoding genes to AMD susceptibility. To the best of our knowledge, none of the polymorphisms selected have been studied so far for association with AMD.

The recent association of CFH with up to 50 % of cases of AMD has created a paradigm shift in our understanding of the pathogenesis of this disease. This association implies that in many cases AMD results from uncontrolled complement activation. Evidence for another mechanism which may contribute to this susceptibility for immune mediated attacks on RPE or endothelial cells comes from studies of the HLA (Human Leukocyte Antigen) system, which is essential for the immune regulation of self and foreign peptides. Localization of HLA class I and DR antigens have been reported in the choriocapillaris and drusen, respectively[106;212], and individuals harbouring the HLA Cw*0701 allele have been found to have an increased risk of developing AMD[212]. We therefore hypothesised that genetic predisposition to inflammation may be important in a significant proportion of AMD cases who could be prone to developing higher CRP or ICAM1 levels.

AMD is similar to atherosclerosis which are both complex prevalent diseases of the elderly sharing many environmental risk factors. Raised CRP levels have been demonstrated to be significantly associated with atherosclerosis (almost two fold increased risk), even in the

presence of low cholesterol levels[63;213] and have been shown to be an independent predictor of the risk of first time heart disease or stroke. Treatment of atherosclerosis and coronary heart disease has benefited from this insight as aspirin and statins have been shown to be most effective in patients with elevated CRP levels[214]. The present study, to our knowledge, is the first to investigate functional gene polymorphisms for CRP and ICAM1 in AMD and to explore the CFH Y402H polymorphism in relation to CRP.

ICAM-1 is a ligand for lymphocyte function associated-1 (LFA-1) and complement receptor-3 (MAC-1) proteins making it an important participant in many immune or inflammation mediated processes[215]. While IL-1beta, IL-6 and IL-8 all have regulatory functions in immune responses, inflammation and wound healing, contributing synergistically to disease changes. IL-1beta stimulates the production of IL-6 which is pro-stimulatory for CRP secretion. These pro-inflammatory effects have been demonstrated to include permeability changes involving microglia and Muller cells in the retina (Claudio et al., 1994). IL-8 is an important activator and chemoattractant for microglia and other inflammatory cells, it is induced by pro-inflammatory cytokines, bacterial or viral products and cellular stress [192].

Our results from the CRP analysis suggest a significant disease association at levels as low as 2 mg/l. This association seems to be linear with the disease risk rising by a factor of 3 for higher CRP levels greater than 5 mg/l. Although, a trend for association of increasing CRP levels with disease severity was identified, this was not significant in our study possibly due to reduced patient numbers (and insufficient power) in the AREDS sub groups. The CRP association in our cohort seems to be stronger compared to other similar studies. The levels reported by Seddon et al were in the range of 3.4 mg/l in cases and 2.7 mg/l among controls

(P=0.02)[45] while Swaroop et al recently reported a similar level of association with levels of 3.42 mg/l in AMD cases and 2.30 mg/l in controls (P=0.03)[216]. Contrastingly, a retrospective study by McGwin et al reported no difference in CRP levels in their AMD cases and controls (1.76 vs. 1.77). However, this negative result should be interpreted with caution as identification of AMD cases in this study was done by photographs taken 10 years after the CRP samples were collected for analysis[217].

After correcting for co-variables which affect both CRP and AMD, none of the CRP genotypes were found to associate with CRP levels. The presence of the 1444 T allele has been previously associated with both baseline and stimulated CRP levels in coronary heart disease studies[218;219], however this association was not seen in our study.

The reason/s underlying a CRP association with AMD remain unclear. One possibility is that the soluble levels of CRP are simply reflecting local or systemic inflammation associated with AMD. Alternatively CRP may be directly involved in the pathogenesis of AMD. Also, the exact mechanism by which CRP polymorphisms influence CRP gene expression and CRP levels is not fully known. CRP activates the classic complement pathway leading to the formation of membrane attack complexes (C5b-9) which results in cell lysis. Studies which recently reported a strong 43% attributable risk for AMD in patients with the Y402H CFH gene polymorphism (increased risk of AMD by 2.4 to 7.4 fold)[46-48] pointed to an interesting CRP binding site on the SCR7 domain of the CFH protein[48]. CHF through its direct binding to CRP functions to reduce the deposition of C5b-9 complexes in normal individuals. The Y402H CFH variant was therefore implicated in altering the binding properties of CFH to CRP in turn leading to complex mediated cell damage in AMD. However, in our study the presence of the CFH Y402H variant did not

correlate with CRP levels. This suggests the association of serum CRP with AMD is independent of CFH genotype.

Our finding of a trend for higher serum ICAM1 levels in AMD suggests a possible role for this molecule in the AMD disease process although this needs further evaluation. Continued smoking seems to be strongly additive to ICAM1 as demonstrated in our results giving a large five fold risk for development of AMD. ICAM1 is a mediator of VEGF-induced angiogenesis[220] and has been reported to mediate oxidative damage in AMD [221]. Experimental studies on ICAM1 and leukocyte function-associated antigen (LFA-1) deficient mice have shown significantly less CNV development in response to laser photocoagulation[179], while increased expression on RPE cells has been demonstrated in sub-foveal CNV from AMD patients[180]. Abnormal expression of ICAM1 may therefore lead to increased susceptibility to immune mediated attacks.

Of the ICAM1 SNP's tested in the study, the 12959 GG variant correlated with increased ICAM1 levels showing a 3 fold AMD risk even after correcting for co-variables like smoking. The 12959G/A (R241G) polymorphism is located in a macrophage adhesion molecule-1 (MAC-1) binding domain and sICAM1 levels have been previously associated with this polymorphism [222]. ICAM1 polymorphisms also appear to show different racial distributions[223], which might be important for AMD. In contrast to European populations, the korean and Japanese populations do not appear to exhibit the ICAM1 12959G/A (R241G) polymorphism[224] and both early and late stage ARM has been reported to be less common as compared to western populations[225].

The interleukin 8 -251A/T polymorphism has been previously associated with many inflammatory diseases and cancers[226;227]. Our results show a similar association for AMD and the homozygous IL 8-251AA genotype (P=0.037). This significance was preserved even after correcting for factors such as smoking (P_C= 0.043). This is of note as IL 8 production has previously been associated with smoking and the IL8 -251A/T genotype has been found to deter the initiation of smoking behaviour in a Japanese population.[228] This pro-angiogenic genotype has also been shown to induce angiogenesis in rat cornea.[229] Human recombinant IL-8 implanted into rat cornea was found to induce vascular proliferation and a similar proliferation and chemotaxis was also seen in human umbilical vein endothelial cells in this study. The IL 8-251 AA genotype seems to act by influencing IL 8 production. In a study of patients with respiratory bronchiolitis the highest serum levels of IL-8 were associated with the AA genotype.[226] Alternatively, this polymorphism may also be in linkage disequilibrium with functional variants elsewhere in the IL 8 loci or in neighbouring genes.

During the above analysis, the mean age of our control population was found to be lower compared to our cases (mean age 69.1 vs. 78.4), however, controlling for age during regression analysis did not alter the described associations. BMI was a significant covariable in the study which seemed to correlate with all the CRP, CFH and ICAM1 SNP's tested by a 5 fold magnitude. All cases and controls in the study were aged 55 or older and therefore in the at risk age group/range in which AMD is defined as occuring[230-232]. Although the mean age of our controls was slightly lower, this would mean that any genotype association found would represent an underestimate rather than an overestimate. True genotype associations with AMD may therefore be stronger than that indicated by this study.

In summary, our results demonstrate that the homozygous IL 8-251 AA genotype (A allele) is a risk factor for AMD. Furthermore, our findings indicate that although the ICAM1 R241G polymorphism does not have an independent effect on the risk of AMD, it may interact synergistically with the IL-8 polymorphism especially in AMD patients with a smoking history. While ICAM1 R241G polymorphism seems to directly induce ICAM1 production in AMD as suggested by our data, the exact mechanism by which the IL-8-251 AA genotype affects the risk of AMD is unclear. Individuals carrying one or both of these high-risk IL and ICAM1 genotypes could be predisposed to chronic inflammatory responses in the retina, which in turn could lead to AMD progression. The association of raised CRP levels with AMD in the study confirms recent findings. Although none of the tested CRP gene variants could be linked to CRP levels or AMD status, it is possible that an unidentified but causal gene variant exists in the CRP locus. Although a recent study has shown significant linkage to the VEGF region [233], none of the VEGF variants tested in this study were found to associate with AMD.

Following on from these hypothesis-generating study, replication studies are needed to confirm these associations. In addition, where possible these should be complemented by functional studies to determine the contribution of this gene to the development of AMD and the angiogenic processes in AMD. This would help develop genetic screening tests to identify individuals at-risk of developing AMD or those who may benefit from focused anti-inflammatory treatments.

CHAPTER FIVE: MUTATION SCREENING OF CANDIDATE GENES IN AMD

5.1 INTRODUCTION

AMD is characterized by many retinal changes including structural changes in the Bruch membrane[234;235]. The changes that develop within the Bruch membrane include diffuse thickening, accumulation of drusen, basal laminar, and basal linear deposits[234], collagen cross-linking in the inner and outer collagen layer and calcification or fragmentation of the elastin layer[235]. These age-related changes within the bruch's membrane has also been recently reported to affect the gene expression profile of the RPE[236].

Many candidate genes have been associated with AMD status, of which the fibulin group of genes are significant. Among these, fibulin 5 and fibulin 6 (hemicentin-1) have been strongly associated with AMD, while fibulin 3 is mutated in Doynes disease. Other genes like cystatin C (CST3)[41], superoxide dismutase (SOD)[42], toll-like receptor 4 (TLR4)[156]and ApoE have all shown some positive findings in recent evaluations.

In a recent analysis of the fibulin group of genes, Stone et al screened fibulin 1, 2, 4 and 5 genes in a cohort of 402 patients with AMD and 429 age matched controls. They reported 7 different missense mutations in the fibulin-5 gene[38]. These sequence changes were seen in 1.7% of AMD patients, but none were found in the control individuals ($P = 0.006$). A similar mutation screening of the fibulin 5 gene in our cohort at Southampton had recently found AMD associations with 2 novel missense mutations. Fibulin 5 which is essential for elastinogenesis therefore appears to be important in the development of AMD whereby mutations in this region could disrupt elastin formation in the bruch's membrane.

Elastin is a major part of the bruch's membrane with barrier functions in the retina, this has been shown to be affected during the AMD disease process (as above). Tropoelastin is the building block of elastin which requires a key polymerisation process catalysed by lysyl oxidase. Further, the process of repair and elastinogenesis requires the interaction of fibulin 5 gene with lysyl oxidase like-1 (LOXL1; shown to be expressed in the human eye), and this interaction has been found to be essential for elastic fibre homeostasis (figure 8). We therefore hypothesised and screened for mutations in this gene which could affect elastin formation in bruch's membrane, thereby contributing to AMD.

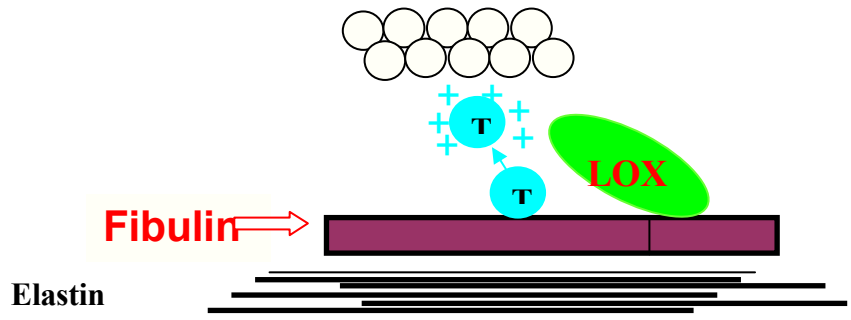


Figure 8. Fibulin5 and LOXL1 interactions at the protein level.

5.2 METHODS

376 AMD patients and 376 normal controls were recruited for this part of my study. The inclusion criteria and AMD phenotypying methodology used was as described previously.

5.2.1 PRIMER DESIGN

The LOXL1 gene sequence was obtained from the Ensembl genome browser (www.ensembl.org) and primers were designed using the Primer 3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer lengths were kept between 20 and 25 base pairs long, Tm was ≤ 61°C and product length to < 300 base pairs long. Although LOXL1 has only 8 exons, a total of 20 primers sets were needed to amplify this gene in smaller dHPLC recommended amplicons (Table 2.1). Exon 1 in particular had

a very large amplicon and in order to maintain a product size of < 300 base pairs, 8 overlapping primer sets were needed to amplify all the regions in this exon. All primers were obtained from Operon Biotechnologies (www.operon.com) in lyophilised form. These were then diluted to a concentration of 100µM/µl (using deionised water) and stored at -20ºC. Sub-aliquots of 20µM were made and stored at -4ºC for easy use and to prevent any degradation by repeated defrosting cycles.

5.2.2 OPTIMISING PCR

Optimisation was done to provide the best conditions for PCR and to ensure adequate single band product amplification. All primer sets were optimised by variying two parameters, 1) Mg++ concentration and 2) Annealing temperature. PCR was carried out in a total volume of 10µl (each 10µl reaction containing 1X strength PCR buffer, 0.4µM of each primer, 0.2mM dNTPs, 1.0-2.0mM MgCl (varied), 0.2U/µl *Taq* polymerase (Invitrogen Corporation, USA), 50ng of genomic DNA and PCR according to the gradient program/protocol on a MJ research PTC225 Peltier Thermal Cycler). The quality of the PCR product was then checked on 1% agarose gel.

In some cases were optimisation failed, 1.3mM Betaine (a PCR enhancing agent) was added to the reaction and a touch down PCR method was used to achieve better results. The touch-down method involves starting at an annealing temperature ≈ 5°C above the predetermined T_m and followed by a 20 cycle touch down phase in which the annealing temperature dropped by 0.5°C to 5°C below the primer T_m. After the 20 cycle touch down phase, the PCR then follows a normal cycle of denature (at 94°C for 30secs), then Annealing (at 55°C for 30secs), and finally synthesis (at 72°C for 30secs) which is repeated for an additional 14 cycles and then ends at 10°C forever.

5.2.3 DHPLC MUTATION SCREENING

Following successful PCR amplication, DHPLC analysis was employed to screen the LOXL-1 amplicons in the above cohort. DHPLC identifies mutations and polymorphisms based on detection of heteroduplex formation between mismatched nucleotides in PCR amplified DNA by liquid chromatography. DNA is mixed with an ion-pairing agent like triethylammonium acetate (TEAA) and is passed through a column comprised of a polystyrene-divinylbenzene copolymer which binds the DNA. A linear gradient of acetonitrile allows separation of fragments based on size and/or presence of heteroduplexes. When this mixed population is analyzed by HPLC under partially denaturing temperatures, the heteroduplexes elute from the column earlier than the homoduplexes because of their reduced melting temperature. As the fragments elute, they are UV detected (260 nm) and the resulting wave forms produced on a computer.

5.2.4 WAVEMAKER METHODS AND DHPLC CONDITIONS

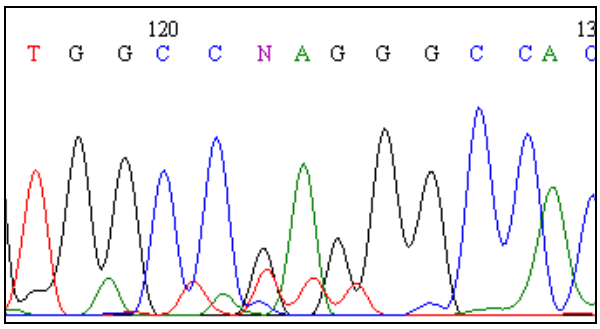
First, the target region's sequence was evaluated for uniformity of T_m with an amplicon size < 300 base pairs. If this condition was not satisfied, alternative primers were chosen. Mutation screening assay methods for each amplicon was then designed using established protocols using the WAVEMAKER software. This software allowed both the calculation of melting profiles and generation of elution profiles. Melting curves of the PCR product were calculated and the highest temperature (Oven temperature or DHPLC melt $T_{m)}$ at which the target sequence was predicted to be >90% duplex was chosen for assay performance. Sometimes, WAVEMAKER suggested an assay temperature that was too high for optimal resolution of sequence variation, while for the GC rich regions the predictions made tended to be generally too low. Therefore empirical melt evaluation and optimisation for screening

these amplicons was also done. In total, each amplicon was screened using 2 to 3 WAVEMAKER methods with varying DHPLC melt temperatures (table 25).

All buffers were freshly made up every week. Loading, elution and washing of the DHPLC column was carried out with varying combinations of three buffers injected at a flow rate of 0.9 ml/min: Buffer A contains 100 mM triethylamine acetate (TEAA), pH 7.0 and 0.025% acetonitrile, Buffer B contains 25% acetonitrile, 100 mM TEAA, pH 7.0, and 0.1 mM EDTA, and Buffer D contains 75% acetonitrile. Loading and elution buffers were combinations of buffers A and B, whose relative proportions formed a gradient over a specified time interval. Buffer D was used to wash the column. DHPLC elution buffer gradients were generated by WAVEMAKER software. The column was cleaned after each run with 100% buffer C at a flow-rate of 0.9 ml/min and a temperature of 80 ºC for 15 minutes. The temperature was then reduced to 50 °C and the column equilibrated with 50:50 A and B for at least 30 mins before each run. Prior to running samples through the system needle and injection port were washed 15 times. Following the gradient elution, all remaining bound material was washed from the column for 36 seconds with buffer D and the column was re-equilibrated with the loading buffer for 156 seconds. Sample elution was monitored by absorbance at 260 nm. All DNA samples which showed suspected mutation wave patterns were further analysed by automated sequencing to confirm the location and nature of the mutation as described below. Some suspected mutations were also screened by restriction digests and Taqman genotyping in a larger cohort of AMD cases and normal controls.

5.2.5 RESTRICTION DIGESTS AND GENOTYPING

A suspected LOXL1 exon 4 mutation pattern was seen in 21 AMD cases (n=192) and 15 normal controls (n=192) following dHPLC. BsaJ-1 enzyme derived from Bacillus stearothermophilus (New England Biolabs inc, UK) was used to analyse the bigger case-control cohort for the suspected change seen on sequencing ie, 5' CC**G/T**AGG 3' (see adjoining figure). The enzyme recognition site was between bases in bold letters, 5' CC**NN**GG 3'. All DNA samples were incubated with the provided NE buffer at 60° C.



Taqman genotyping was utilised (described previously) using appropriate designed probes/primers to genotype the LOXL1 3' UTR T/C Change (rs3522) in the larger study cohort (seen in 53/188 AMD cases and 20/188 controls with dHPLC).

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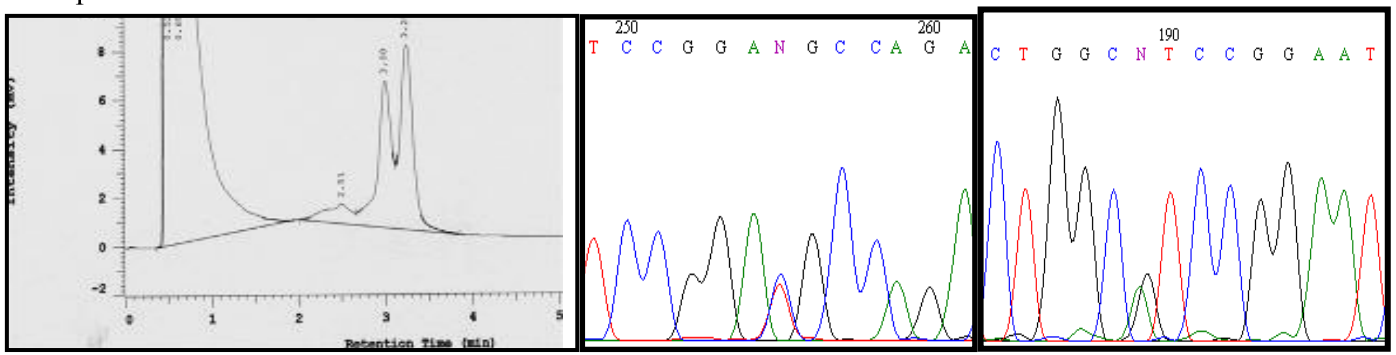
Table 26. LOXL1 primer design and DHPLC conditions.

LOXL1 exons	Exon size (bp)	Forward primer (5'-3' sequence)	Reverse primer (5'-3' sequence)	Amplicon size (bp)	Annealing Tm	DHPLC Melt Tm
1A	1428	TCCCAGCCTGTTGCTTATTC	TCCGATGACAGCATTTCAAG	186	60	66.8, 69.0
1B		AAATGCTGTCATCGGAGGAG	AGGCCTGGTGGACAGAGAG	154	60	67.0, 68.0
1C		CTGCAGAGGGGTCACCAT	GTACACCTGCCCGTTGTTCT	166	58	62.0, 63.0
1D		CTCAGCGCTCCGAGAGTAG	ACCTGGCCAAAGCCGAAT	165	58	64.0, 63.0
1E		ATTCGGCTTTGGCCAGGT	TTCTCGTACTGGCTGACGAA	198	60	64.0, 65.0
1F		TTCGTCAGCCAGTACGAGAA	GGTACTCGGGCAGCTCTTC	176	58	62.0, 63.0
1G		GAAGAGCTGCCCGAGTACC	AGGGTAGGCCTGCTCGAAG	153	58	60.0, 61.0
1H		AGCAGGCCTACCCTGACC	CTGGTTGGGCCGGTACAC	233	62	68.0, 69.0
2	109	CAGTGGCCAACCTGATGCTC	ACAGCTAGGCTGGGTTCTGG	253	60	62.3, 63.0
3	138	CTGGGTTCTGGTGTCACTGT	TGGCCCAGAGGAGAAGTGGA	397	55	64.8, 63.0
4	157	GATGAGGACAGGATGAGAGG	CCAGAAGCCTTGGCACTTGC	326	55	61.0, 62.8
5	96	GGTGGGCCAGAAACTCCTGA	ACAGGCCCAGAGGCTGAAGC	274	56	62.7, 63.2
6	116	CCACCTTCTCTGGTGAGCAG	TTCGGCCATCAAGGTGACCT	306	55	60.3, 61.9
7	305	TGCCCTGCCACGAGGGATCT	GATGATGCCTAAGGACATCC	494	55	62.5, 64.6

Figure 9. DHPLC chromatogram showing a double peaked heterozygous change in LOXL1 3' UTR region (T/C Change) in AMD sample.



5.2.6 AUTOMATED DNA SEQUENCING

Automated sequencing was done to characterise the mutations detected by dHPLC. The Big Dye® Terminator v1.1.1 Cycle Sequencing Kit and the ABI PRISM® 3100 Genetic Analyser were used for this sequencing method. Samples to be sequenced were selected and batch analysed (50µl PCR reactions were carried out for each sample and then electrophoresed on a 2% Agarose Gel to ensure adequate PCR and a single product). The clear band product corresponding to the right sized amplicon when compared to a 100bp PCR ladder was then purified using the Qiagen purification Kit (Qiagen Ltd, UK) following the manufacturer's protocol. After purification, a 20µl sequencing reaction (using half strength Big Dye) was carried out on the Thermal Cycler. The reaction contains 4µl Big Dye kit, 2µl 5X Buffer, 3.2µl of 1µM primer, 3 – 10ng of the purified PCR product and 10.8µl sterile water. Two sequencing reactions were performed per sample fragment (One in the forward orientation using the forward primer and one in the reverse orientation using the reverse primer). A Qiagen DyeEx 2.0 Spin Kit (Qiagen Ltd, UK) was used to remove excess big dye and precipitate the DNA from the sequencing reaction. The products from this were then re-suspended in HiDi-formamide and transferred to a 96-well optical plate approved for use in the ABI PRISM® 3100 Genetic Analyser. This is then set to denature at 94ºC for ten minutes and 4ºC after that until it is ready to be loaded into the sequencer. The sequencer is loaded according to the settings recommended in the handbook and left to run as long as necessary (~ 45mins for 16samples). Sequencing results were analysed by visual inspection of the sequence printout on sequence analysing software (BioEdit Version 7.0) and by sequence comparison using the BLAST algorithm from NCBI.

5.3 LOXL1 RESULTS

Following the initial dHPLC screening, possible mutation changes were detected in Exons-4,5 and in the 3' UTR region. The LOXL1 exon 4 G/T change which seemed plausible on sequencing was not confirmed on restriction digests, while the exon 5 change was a synonymous one (ATC/ATT change, Ile 520 Ile) which was therefore not investigated further. The 3'UTR (rs3522) genotype distribution was found to be similar in cases and controls without any significance (table 27).

Table 27. LOXL1 3'UTR (rs3522) genotype distribution in the cohort.

LOXL1 genotype	**AMD cases n=354**	**Normal controls n=352**	***P* values**
CC (%)	107 (30.2)	99 (28.1)	0.59
CT (%)	188 (53.1)	178 (50.5)	0.54
TT (%)	59 (16.6)	75 (21.3)	0.13

Chi-square *P* values using Yate's correction and 1^{df}. Chi-square *P* value with 2^{df} = 0.28.

5.4 PEDF AND CRYAA FUNCTIONAL MUTATIONS

Pigment Epithelium-Derived Factor (PEDF), a serine protease inhibitor (serpin) expressed in RPE cells is an important candidate gene for inherited retinal diseases and other photoreceptor retinal degenerations. It is a potent inhibitor of angiogenesis and has been shown to be highly expressed in the young adult RPE with progressive loss during cell senescence[237]. A significant reduction of PEDF has been reported in the choroid of AMD subjects when compared to the choroid of aged control subjects[238], suggesting that a loss of PEDF in the eye could be functionally important in the pathogenesis of AMD (CNV development). In mice models of retinal neovascularization, intravitreous injections of a PEDF vector has been shown to significantly inhibit neovascularization[239]. This

antiangiogenic activity has also now been shown in a human phase I clinical trial of AMD patients after an intravitreal injection of PEDF expressing adenoviral vector[240].

The Crystallin - alpha A (CRYAA) gene encodes a member of the small-heat-shock protein (sHSP) family of molecular chaperones and is primarily expressed in the ocular lens. Missense mutations in *CRYAA* have been associated with an autosomal dominant form of 'nuclear' cataract segregating in a four-generation Caucasian family. This may be important AMD as both senile cataract and AMD share common risk factors like age, smoking, UV exposure, etc.

To explore the possible role of functional SNP's in this single-copy PEDF gene in AMD, we identified and genotyped two SNP's which may affect transcription in the 5'UTR (rs12948385) and 5'flanking promoter regions (rs12948385). Aditionally, the functional CRYAA promoter SNP (rs13053109) was also tested for any association with AMD.

5.5 METHODS

233 AMD patients and 233 normal controls were recruited for this study. The inclusion criteria and AMD phenotyping methodology used was as described previously. Primers and individual fluorogenic TaqMan probes were designed using Primer Express software. Genotyping was done using the 5' nuclease assay for allelic discrimination.

5.6 PEDF AND CRYAA RESULTS

Genotype distributions for the PEDF and CRYAA promoter SNP's tested were largely similar in the study groups and in Hardy-Weinberg equilibrium (tables 28 and 29). Among the PEDF promoter SNP's tested, a common GG genotype for rs12948385 was found more

commonly in the control group with a *P* value nearing significance. No significance was found on chi-square testing for the CRYAA promoter SNP genotype distribution in the study group.

Table 28. Genotype distributions for PEDF 5' promoter SNP's in the study population.

rs9913583 genotypes	**AMD cases n=232**	**Normal controls n=233**	***P* values**	**rs12948385 genotypes**	**AMD cases n=222**	**Normal controls n=226**	***P* values**
CC (%)	218 (93.3)	227 (97.6)	0.07	GG (%)	84 (37.8)	106 (46.9)	0.05
CA (%)	14 (6.7)	06 (2.4)	0.07	GA (%)	100 (45.1)	92 (40.7)	0.39
AA (%)	0 (0)	0 (0)		AA (%)	38 (17.1)	28 (12.4)	0.18

Chi-square *P* values using Yate's correction and 1^{df}. Chi-square *P* value with 2^{df} for rs12948385 = 0.11.

Table 29. CRYAA promoter SNP (rs13053109) genotype distribution in the study cohort.

rs13053109 genotypes	**AMD cases n=233**	**Normal controls n=230**	***P* values**
GG (%)	104 (44.6)	104 (45.2)	0.92
GC (%)	107 (45.9)	97 (42.2)	0.45
CC (%)	22 (9.5)	29 (12.6)	0.30

Chi-square *P* values using Yate's correction and 1^{df}. Chi-square *P* value with $2^{df} = 0.48$.

5.7 DISCUSSION

Understanding the mechanism of AMD development is critical to developing new treatments for this disease. In addition to testing various immune pathways, we evaluated critical genes encoding structural membrane proteins which may have functional implications in AMD. This is important as previous studies have shown that the structural changes within Bruch's membrane precede and influence RPE changes by at least one or two decades[234;241].

Although the fibulin group of genes have been strong associated of with AMD and novel fibulin5 missense mutations have been found in our cohort, mutation screening of LOXl1 gene (responsible for basement membrane integrity and elastinogenesis), and testing for promoter region SNP's in PEDF and CRYAA genes did not yield significant results in this study. This was despite an adequately powered study (80% power to detect significant differences in genotype frequencies between cases and controls at an alpha level of 0.05, for genotypes occurring at 10% frequency in controls the detectable odds ratios were 2.3 for a predisposing genotype and 0.5 for a protective genotype with 95% confidence.). These findings need to be further confirmed by studying other population cohorts and also perhaps by LD based studies.

5.8 EVALUATING A NOVEL TCOF1 MUTATION ASSOCIATED WITH A TREACHER COLLINS- MACULAR DEGENERATION PHENOTYPE IN AMD

Treacher Collins Syndrome (TCS) results from defects in a nucleolar trafficking protein (Treacle) coded for by the TCOF1 gene[242]. The purpose of this report is firstly, to describe an isolated male with TCS associated with macular degeneration who also had a novel TCOF1 gene mutation, and secondly to evaluate this mutation in a well characterised cohort of 95 patients with age-related macular degeneration (AMD).

5.8.1 CASE REPORT

A 44 year old male presented with a 1 month history of metamorphopsia to Southampton Eye Unit. He had minimal dysmorphic features but was noted to have an antimongoloid slant of the palpebral fissures with mild flattening of the midface. Sensorineural deafness

had been diagnosed from childhood but the external ears were normal in appearance. Best corrected visual acuity was 6/9 (-2.25 DS) right eye and 6/24 (-1.50 DS) left eye. Ocular examination revealed bilateral posterior embryotoxon with adhesions between iris and Schwalbe's line, and iris hypoplasia. No eyelid colobomata were present. Posterior segment examination (fig.10) revealed atrophic macular degeneration in both eyes and a choroidal neovascular membrane (CNV) in the left eye confirmed on fluorescein angiography. No drusen were seen in either eye. Mutation screening of *TCOF1* gene was instigated because his facial appearance and deafness suggested possible TCS. A mutation was identified in exon 13 (2055 del AG), which is predicted to create a premature stop codon. In view of this unique genotype and phenotype we wished to evaluate whether this mutation in the *TCOF1* gene was commonly associated with macular degeneration. 95 Caucasian patients with AMD were therefore screened for the 2055 del AG mutation by denaturing high performance liquid chromatography (dHPLC)[243], using the DNA from our patient with TCS as a positive control (fig. 11). The spectrum of AMD in this cohort was AREDS grade I (21 patients); AREDS grade II (20 patients); AREDS grade III (19 patients) and AREDS grade IV (35 patients)[244]. No abnormal chromatograms were detected in any of these patients.

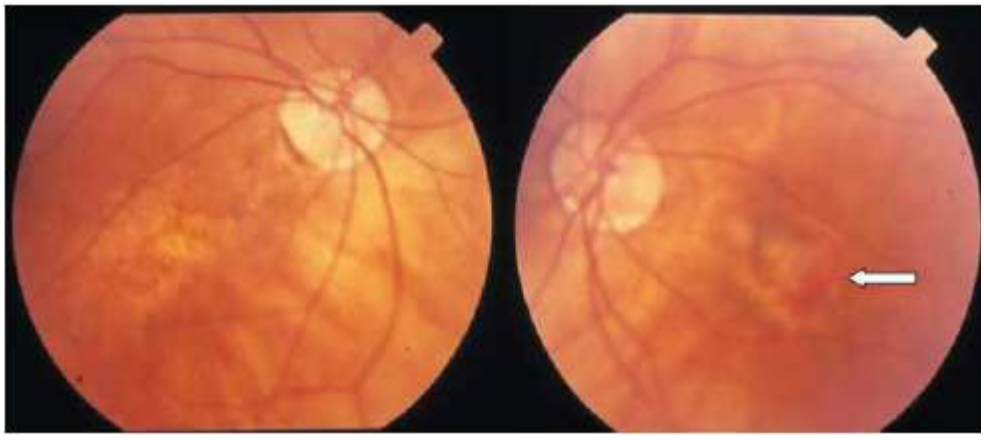


Figure 10. Both eyes showing atrophic macular degeneration. The left eye in addition has subretinal hemorrhage (white arrow) with CNV.

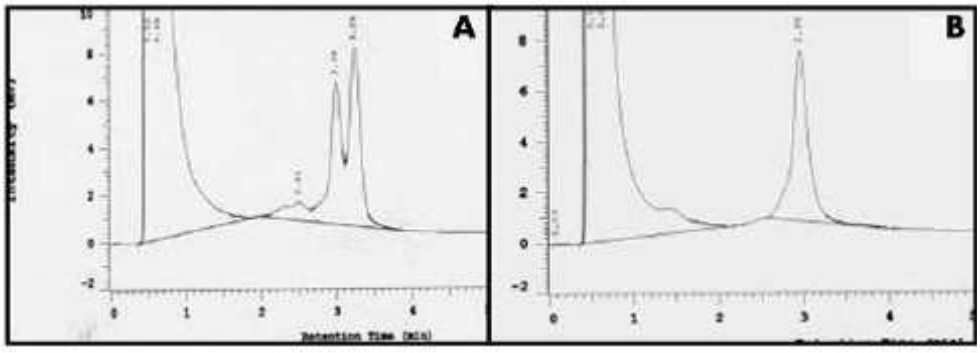


Figure 11. Mutation screening chromatograms. A. a double peak is seen indicating the base pair mismatch in the proband. B. a normal wave tracing from a screened AMD sample.

5.8.2 DISCUSSION

Ophthalmological features in TCS may be extensive, but rarely involve intraocular structures. Common features include astigmatism, defective inferior lateral angle of the orbit, caudal displacement of the superolateral orbit, coloboma of the lateral part of the lower lid, pseudocoloboma of the eyelids, lateral canthal dystopia, nasolacrimal obstruction, orbital and limbal dermoids and microphthalmos[245]. Hansen et al[246] have observed bilateral iris, choroid and optic nerve colobomata. Cataracts, lacrimal duct atresia, pupillary ectopia, distichiasis, and uveal colobomas have been reported less frequently. A solitary case with aniridia, sclerocornea and retinal maldevelopment has also been reported [247]. We believe this is the first report of atrophic macular degeneration and CNV demonstrated in a patient with a proven molecular diagnosis of TCS.

Disease expressivity is highly variable in TCS, ranging from the clinically undetectable to death in the perinatal period [248]. The late clinical presentation in our case may be explained by the mild phenotype in the spectrum of TCS. The TCS Collaborative Group[249] first identified different mutations in the *TCOF1* gene in each of 5 unrelated families with TCS. All of the mutations were predicted to result in a premature stop codon leading to premature

termination of the protein product. Since then over 100 disease causing mutations have been reported[250] throughout the *TCOF1* gene in patients with TCS, which represented a detection rate of 60%. Our patient has a 2055 del AG mutation in exon 13 of the *TCOF1* gene which has not been previously reported. We speculated that there may be a causal relationship between the mutation and the macular degeneration seen in our patient with TCS.

The specific role of *TCOF1* in the molecular pathogenesis of TCS remains elusive, but mechanisms such as abnormal neural crest cell migration and abnormal cell death seem important[251]. *TCOF1* is expressed in the human eye and apoptotic regression has been described in organs such as ears and kidneys in animal models of TCS[252]. Therefore, it seemed possible that this *TCOF1* mutation may also trigger cellular apoptosis resulting in atrophic macular degeneration. However, we tested for the presence of this mutation in 95 patients with AMD but did not identify any mutation carriers in this cohort. It is possible that this macular finding is incidental to TCS and the *TCOF1* mutation. The early age of onset of macular degeneration in this patient is also atypical for AMD. We also note that the macular degeneration seen in this patient is more severe than would be expected with his low degree of myopia. It is therefore possible that patients with TCS may have an increased risk of developing macular degeneration.

In summary, we describe a new clinical phenotype in a patient with molecularly proven TCS and a novel *TCOF1* mutation. Although most cases of TCS present early in life, ophthalmologists need to review adults with TCS to see if macular degeneration is more widespread than reported. This mutation, however, does not appear to be implicated in AMD.

CHAPTER SIX: EVALUATING COMPLEMENT FACTOR H IN AMD AND CARDIOVASCULAR DISEASES.

Among the various factors involved in the pathogenesis of epidemiologically similar diseases like AMD, atherosclerosis and heart disease, inflammation has been foremost with recent evidence pointing towards various immune related pathways. Inflammation often leads to tissue damage caused by lymphocyte mediated attack against self proteins. The activation of these and other tissue based macrophage cells is further influenced by various inflammatory mediators like, pentraxins (C-reactive protein or CRP), complement components, prostaglandins, chemokines, cytokines, cellular adhesion molecules, proteases, protease inhibitors, and free radicals.

6.1 COMPLEMENT FACTOR H IN UK COHORT AND RESPONSE TO PDT TREATMENT

Complement factor H (CFH), a key inhibitor of the alternative complement pathway has been implicated in patients with age-related macular degeneration (AMD). Many key publications have reported a highly significant CFH gene coding polymorphism (rs1061170, Y402H) which has been seen in nearly 50% cases of AMD[46-48;176] . A defective factor H protein has been hypothesised to influence AMD related inflammatory processes including angiogenic processes leading to choroidal neovascularisation (CNV) formation[253].

This CFH variant may also partly answer the question as to why increased serum CRP levels are associated with AMD. CRP is known to activate the classic complement pathway leading to formation of the membrane attack complex (C5b-9). Further, drusen with terminal complement deposition indistinguishable from AMD have been observed in eyes

from patients with a kidney disease (membranoproliferative glomerulonephritis type II) that can be caused by mutations in CFH[50]. It is likely that allele-specific changes in the activities of the binding sites for heparin and CRP may alter CFH's ability to suppress complement-related damage to arterial walls, and might ultimately lead to vessel injury and subsequent neovascular AMD.

Photodynamic therapy (PDT) is an effective treatment for AMD related CNV. PDT produces CNV regression by various cellular mechanisms which include a variety of immunological processes. Some of these may influence the complement pathway[254]. PDT has been shown to produce a transient inflammatory response by increasing cytokine levels. Of these, interleukin-1β, interleukin-2 and TNF-7α all seem to be important[254]. Also, antigen presenting cells (dendritic or Langerhans) have been reported to become less active while HLA class II antigens are significantly down regulated after PDT which may also affect immune responses[254]. These inflammatory responses might stimulate wound healing or paradoxically lead to recurrent CNV.

CFH is present in the retina of AMD patients. Immunolocalisation studies demonstrate CFH co-localizing within drusen, the choroid bordering drusen and the retinal pigment epithelium (RPE), and in both the basement membrane and wall of choriocapillaries[176]. Functionally, a normal CFH has been shown to limit immune complex deposition and prevent inflammatory scarring in mice models of kidney disease[255]. Damage to the neurosensory retina is reported as minimal and reversible after PDT[254]. However, PDT may also have undesirable consequences including retinal pigment epithelium damage and choroidal hypoperfusion[254;256]. Further, the potential for collateral damage to other retinal cell types

including glial cells from multiple PDT applications cannot be discounted. Inflammatory scarring may therefore be important in PDT.

We therefore analysed the CFH Y402H (1277T/C) variant in our study cohort to confirm the AMD associations reported. Aditionally we hypothesised that mutant CFH could affect PDT outcomes by altering immune or scarring related processes. To answer this, we first evaluated the magnitude of AMD/CNV risk conferred by the CFH Y402H polymorphism in our UK cohort and then tested whether any of the high-risk CFH genotypes associated with visual outcomes following PDT for CNV in AMD patients.

6.2 SUBJECTS AND METHODS

AMD and normal controls over age 55 were recruited as per previous methodology. ETDRS visual acuities were recorded. Stereoscopic fundus photographs and fluorescein angiograms were graded independently by two masked observers (SG and SH) into 4 groups of increasing disease severity as described in the AREDS study[257]. 27 patients who underwent PDT treatments were identified from the AMD group and followed up during a period of 15 months.

SNP GENOTYPING ASSAY

Genotyping was done using the 5' nuclease assay for allelic discrimination as described previously (see chapter 4, section 4.2.3 and table 13).

STATISTICAL METHODS

Genotype distributions for the CFH polymorphism were tested for conformity to Hardy-Weinberg equilibrium in both cases and controls. Alleles at each locus were said to be in

Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly (P>0.05) from expected frequencies. Individual CFH genotypes were analysed for AMD/CNV association using Fisher's exact two-tailed tests. The association with degree of visual loss following PDT therapy was analysed by non-parametric Mann-Whitney tests. Logistic regression analysis was performed using data on age, sex, body mass index (BMI), and smoking status as covariates in regression models. The level of statistical significance in the study was set at $P_C < 0.05$. All statistical analyses were performed using the SPSS statistical software package version 14.0.

Table 30. Primer and probe sequences used for 5' nuclease (TaqMan) genotyping (see chapter 4, table 13).

Table 31. Basic demographic characteristics of the Caucasian study population.

	AMD cases Total n = 557 (%)	**Controls Total n = 551 (%)**	**PDT cases Total n=27**
Age	n=557	n=551	n=27
Mean	78.4	69.1	78.6
Range	55 - 101	55 - 91	65-93
SD	8.65	9.76	6.13
Sex	n=557	n=551	n=27
Female	330 (59.2)	308 (55.9)	13 (48.1)
Male	227 (40.8)	243 (44.1)	14 (51.8)
BMI	n=237	n=407	n=24
Mean	26.5	27.3	25.4
SD	4.76	4.70	4.98
Smoking status	n=460	n=432	N=27
Current smokers	140(30.4)	81(18.7)	8 (29.9)
Past smokers	145(31.5)	213(49.3)	12 (44.4)
Non-smokers	175(38.0)	138(31.9)	7 (25.7)

6.3 RESULTS

Baseline demographics of the unrelated Caucasian cohort recruited from a clinic population (557 cases with AMD, 551 normal controls and 27 cases from the AMD group who underwent PDT) are described in table 31. CFH 1277 C/T (Y402H) genotyping data was available for all individuals. Genotype frequency distributions were as shown in table 32. The homozygous CC genotype was more often present in AMD cases (0.28 vs. 0.13, $P <$ 0.0001) giving an OR for disease of 3.62. The association with AMD was also significant in the larger heterozygous CT group but with a lesser risk of AMD than in the CC group (OR=1.61, P= 0.0008). Overall, the odds ratio for AMD was 1.90 for the high risk C allele (< 0.0001). Adjusting for age during the logistic regression analysis did not alter the odds ratio for disease significantly.

Table 32. CFH Y402H genotype association in AMD cases and controls.

CFH Genotypes	**AMD cases n=557 (%)**	**Controls n=551 (%)**	**Genotype association OR, 95%CI (P_C value)***
CC	167 (0.28)	75 (0.13)	3.62, 2.56 - 5.14 (< 0.0001)
CT	258 (0.44)	261 (0.44)	1.61, 1.22 - 2.12 (0.0008)
TT	132 (0.23)	215 (0.36)	-
CFH Allele			
C	592	411 (0.37)	1.90, 1.60 - 2.25 (< 0.0001)
T	522	691 (0.63)	-

*P_C = P values for genotypes (compared to TT) after adjusting for age, sex, BMI, Current smokers and Past smokers.

Further categorization was possible in 416 subjects in the AMD group, who were divided into 4 groups of increasing AMD severity using AREDS criteria (table 33). The

homozygous CC genotype was found to associate with all the four AREDS groups with the strongest association seen in AREDS-4 group (OR=5.17, $P < 0.0001$). Although the odds ratio in AREDS-1 was similar to that in AREDS-3 (OR of 2.64 vs. 2.69), the strength of this association in AREDS-1 was much weaker ($P = 0.03$ vs. $P = 0.0008$). The heterozygous CT genotype was found to associate with AREDS-4 group only (OR=1.78, $P = 0.008$). The C allele showed a similar risk trend for association in all the four AREDS groups with odds ratios ranging from (1.61 to 2.33).

The risk of various subtypes of CNV was then analysed in comparison to controls (table 34). CFH genotypes were found to associate with the predominantly classic CNV group only (OR for CC genotype=17.87, $P<0.0001$, OR for CT genotype=9.06, $P=0.0002$, combined OR=10.87, $P<0.0001$). Odds ratio for the presence of the high-risk C allele was 2.97 ($P<0.0001$) in this CNV subtype. Allele associations were also seen in the occult CNV (OR=3.09, $P<0.0001$) and minimally classic CNV (OR=2.52, $P=0.001$) subtypes, this was however not significant when genotypes were analysed in these groups.

The risk of developing classic or predominantly CNV within the wet AMD group was then compared to the minimally classic and occult CNV (including PED) group. Analysis was done for association with CFH genotypes (CC/CT) and alleles, no significant difference in the frequency of CFH genotypes or alleles was found between the two groups (data not shown).

Overall 76% (425/557) of AMD patients had the CC or CT genotype. Of these, 4.8% patients (n=27/557) had undergone PDT treatments. In the PDT group, 93% (25/27) patients carried the high-risk C allele (CC or CT). This difference in the genotype distributions in the

PDT group and the AMD group was significant for the CC genotype (93% vs. 76%, OR=5.48, $P = 0.015$).

Table 33. CFH Y402H genotype association in the AREDS subtypes compared to controls.

CFH Genotype	**AREDS-1, n=51(%) (OR, *P* value, CI)***	**AREDS-2, n=73(%) (OR, *P* value, CI)***	**AREDS-3, n=113(%) (OR, *P* value, CI)***	**AREDS-4, n=179(%) (OR, *P* value, CI)***	**Controls n=551 (%)**
CC	12 (23.5)	25 (34.2)	31 (27.4)	65 (36.3)	75 (0.13)
	(2.64, *P*=0.03, 1.16-6.05)*	(4.48, *P*<0.0001, 2.27-8.84)*	(2.69, *P*=0.0008, 1.54-4.69)*	(5.17, *P*<0.0001, 3.19-8.40)*	
CT	26 (51.0)	32 (43.8)	49 (43.4)	78 (43.6)	261 (0.44)
				(1.78, *P*=0.008, 2.59-16.54)*	
TT	13 (25.5)	16 (22.0)	33 (29.2)	36 (20.1)	215 (0.36)
CFH Allele					
C	50 (0.49)	82 (0.56)	111 (0.49)	208 (0.58)	411 (0.37)
	(1.61, *P*=0.02, 1.07-2.42)*	(2.15, *P*<0.0001, 1.51-3.05)*	(1.62, *P*=0.001, 1.21-2.16)*	(2.33, *P*<0.0001, 1.82-2.97)*	
T	52 (0.51)	64 (0.44)	115 (0.51)	150 (0.42)	691 (0.63)

**P* values by Fisher's exact test (two sided values), only significant values shown.

Table 34. Risk for CNV subtypes associated with CFH Y402H genotypes and alleles in comparison to controls.

CFH Genotype	**Occult CNV n= 49 (%)**	**Serous PED n= 12 (%)**	**Minimally Classic n= 25 (%)**	**Predominantly Classic n=36 (%) (OR, *P* value, 95%CI)***	**Classic n= 17 (%)**	**Controls n=551 (%)**
CC	21 (42.9)	4 (33.3)	10 (40.0)	12 (33.3) (17.87, *P*<0.0001, 3.76-78.66)*	2 (11.8)	75 (0.13)
CT	15 (30.6)	5 (41.7)	10 (40.0)	22 (61.2) (9.06, *P*=0.0002, 2.10-38.98)*	9 (52.9)	261 (0.44)
TT	13 (26.5)	3 (25.0)	5 (20.0)	2 (5.5)	6 (35.3)	215 (0.36)
CFH Allele						
C	57 (64.7) (3.09, *P*<0.0001, 1.96-4.86)*	13 (54.2)	30 (60.0) (2.52, *P*=0.001, 1.41-4.50)*	46 (63.9)_ (2.97, *P*<0.0001, 1.81-4.88)*	13 (38.2)	411 (37.0)
*T	31 (35.3)	11 (45.8)	20 (40.0)	26 (36.1)	21 (62.8)	691 (63.0)

P values by Fisher's exact test (two sided values comparing CC/CT vs. TT genotypes and C vs. T alleles), **Combined OR for CC+CT in Predominantly Classic= 10.87, *P*<0.0001, 95%CI=2.58-45.76. Only significant values shown.

In the PDT group, 27 patients who had predominantly classic or 100% classic CNV (Snellen VA ≥ 6/60) were followed after PDT for a period ranging from 3 to 22 months (median = 22 months). Each patient on average had received at least two PDT treatments (median = 2, range = 1 to 5 treatments). Other demographics of this PDT cohort are described in table 31. In this group, the CC and CT were the predominant genotypes observed in 48% and 44% cases respectively.

Table 35. CFH Y402H risk genotypes (CC and CT) and visual acuity following PDT.

CFH Genotype	**PDT cases n=27**	**ETDRS acuity loss (median, range)**	**Genotype vs. ETDRS acuity loss (*P* value)**
CC	13 (0.48)	-12.0 (+23.0 to -70.0)	*P* = 0.038*
CT	12 (0.44)	-3.5 (+20.0 to -80.0)	*P* = 0.087
TT	2 (0.08)	-70.0 (-65.0 to -75.0)	-

* Significant *P* values (CC and CT genotypes compared to TT genotype using nonparametric Mann-Whitney test).

Visual acuity data after PDT for each genotype are presented in table 35. Overall, 48% of CC cases (n=13) and 44% of the CT cases (n=12) had lost 15 or more ETDRS letters. The numbers were too small in the TT group for analysis (n=2). The degree of ETDRS acuity loss was statistically significant in the CC group (median loss of 12 letters, *P*=0.038). The loss of ETDRS letters in the CT group was not significant (median loss of 3.5 letters, *P*=0.087). Although acuity loss was greatest in the TT group (median loss of 70 letters), the sample size was small and *P* values were not obtainable.

6.4 DISCUSSION

CFH is an essential inhibitor of the alternative complement pathway preventing uncontrolled complement activation[49]. Recent data also suggest that the CFH Y402H polymorphism is a risk factor for several AMD phenotypes including choroidal neovascularisation (CNV), geographic atrophy and soft drusen [258;259]. This study has sought to identify the risk of AMD and particularly CNV in a UK cohort conferred by the presence of this CFH polymorphism additionally exploring any effect of these CFH genotypes on PDT outcomes.

In this UK study, the strength of the AMD association with the high risk CC and the heterozygous CT genotypes was similar to those reported in other Caucasian cohorts. The CC genotype showed a 5-fold risk in the AREDS-4 group compared to controls. With in the AMD group, the association of predominantly classic CNV with the CFH C allele was highly significant. The magnitude of this risk was 5 times greater in patients with CC or CT genotypes. When compared to the control group, the risk of predominantly classic CNV further increased by 17 fold in the CC group and 9 fold in the CT group. Recently, Haines et al reported similar raised odds ratios of 3.4 and 5.6 for the CT and CC genotypes when their analysis was restricted to CNV[46]. Zareparsi et al noted a similar raised frequency of CC genotypes in CNV[260]. In a recent report by Sepp et al, 81.8% of AMD patients with CNV were found to exhibit the high risk CFH genotypes, the odds ratios for CNV being 5.1 for CC and 2.7 for CT genotypes[258]. The reported odds ratio for CNV risk was 4.34 in a recent report from an Australian AMD cohort[209]. Our risk figures for CNV seem to be similar to the above reported rates. However, the correlation of CFH genotypes with predominantly classic CNV was higher than any of these reported associations. We are not aware of any previous analysis of predominantly classic or other CNV subtypes with CFH genotype. The

higher correlation for this CNV type may therefore reflect a tendency for the CFH mutant protein to result in this subtype of CNV.

A significantly increased frequency of CC and CT genotypes (93%) was seen in the PDT group.The high risk CC genotype was found to associate with the degree of visual loss following PDT in this study, while visual acuity was unrelated to the presence of heterozygous CT and homozygous TT genotypes. Calculations revealed that the PDT sample size (n=27) had 80% power to detect a minimum difference of 20 ETDRS letters (alpha error level = 5%) and 90% power to detect a 24 letter difference. The power to detect any smaller differences in acuity would have been limited.

Although sample sizes are adequate, the power to detect the association of CFH Y402H genotype with predominantly classic choroidal neovascularization may be limited given the close prevalence of CFH in the various membrane types, classic, minimally classic or occult. In addition, the type of neovascularisation may change over time in an individual and it is possible that some individuals in the AMD group would have developed some form of occult CNV making them ineligible for PDT therapy. However if so, the associations described for the predominantly classic form of CNV would be an underestimate. CFH risk genotype associations with predominantly classic CNV may therefore be stronger than we describe.

In conclusion, there was a highly significant trend for patients with the risk Y402H genotypes to develop predominantly classic CNV in AMD. The CFH Y402H gene variant was also found to associate with visual outcomes following PDT treatments for CNV. It may be that once CNV begins, downstream events such as VEGF expression and scarring

predict final visual outcome in addition to CFH genotype. Visual acuity responses to PDT therefore seem to be related to CFH Y402H genotype. This may be because of CFH related damage to the retinal pigment epithelium (RPE) allowing CNV to develop more easily above the RPE. This is however an exploratory hypothesis testing study. Further studies are needed to confirm the association reported here of a predominantly classic CNV subtype in patients with Y402H CFH genotypes. Such genotype – phenotype correlations may ultimately assist in clinical treatment algorithms.

6.5 COMPLEMENT FACTOR H IN CORONARY ARTERY DISEASE AND ATHEROSCLEROSIS

Inflammation is one of the most important factors underlying the pathogenesis of atherosclerosis and coronary heart disease, with recent evidence indicating the involvement of various immune related pathways. Several components of the complement cascade along with other inflammation mediators have been associated with the development of atherosclerosis and myocardial infarction (MI). Elevated serum levels of complement C3 and C5a, and circulating immune complex (CICs) have been reported to be independent risk factors for MI [261] [262] [263]. In addition, complement factor H (CFH) is present in the early atherosclerotic lesions and has been implicated in atherogenesis[264].

There are many similarities in the pathological changes between AMD and atherosclerosis. In particular, the pathogenesis of both diseases involves inflammation and lipid accumulation in affected tissues. In addition, genome wide linkage studies have shown evidence of linkage of a region on chromosome 2 both with AMD and with coronary artery disease[265;266,267]. Therefore, we hypothesised and examined a possible association of the CFH Y402H polymorphism with coronary heart disease and atherosclerosis.

6.5.1 SUBJECTS AND METHODS

We studied a group of 1170 Caucasian patients with coronary artery disease and a group of 560 healthy control subjects. The patients all had angiographically confirmed coronary artery disease and were recruited at the Wessex Cardiothoracic Unit, Southampton General Hospital as previously described[268] . Characteristics of the patients have been described in the same publication [268]. The study was approved by the South and West Local Research Ethics Committee (number 298/99), and all subjects gave written consent. The controls were healthy Caucasian individuals without CAD, recruited from ophthalmic clinics at Southampton General Hospital. Information obtained for the controls included relevant past medical and cardiovascular history, smoking history, ocular history, use of medications, vitamin or dietary supplementation; height, weight, body mass index (BMI) measurements were also recorded. From the above CAD patients and control subjects, we identified a sub-set of 233 pairs of age and gender matched patients and controls.

CFH GENOTYPING ASSAY

Genotyping was done using the 5' nuclease assay for allelic discrimination as described in chapter 4.

6.5.2 STATISTICAL METHODS AND ANALYSIS

Allelic and genotype distributions were tested for conformity to Hardy-Weinberg equilibrium by χ^2 analysis. One-way ANOVA and Chi-square tests (*P* values with Yate's correction and 1^{df}) were carried out to examine differences in quantitative and categorical variables between groups, and between CFH genotypes. In these analyses, triglyceride values were log transformed to normalize the distribution. Logistic regression analysis was used to evaluate the effects of genotypes on severity of CAD and occurrence of MI. In these analyses, the number of diseased vessels or occurrence of MI was entered as

dependent variable and the genotypes as independent variable, while age, gender, body mass index (BMI), smoking status, diabetes, hypertension, cholesterol and triglyceride levels, and family history of CAD as covariates in the regression model. The level of statistical significance in the study was set at $P < 0.05$. All statistical analyses were performed using the SPSS statistical software package version 12.0.

6.5.3 RESULTS

Baseline demographics of the CAD patients (n=1170) and normal controls (n=560) are as described in table 36. The observed CFH genotype distributions in both groups were in Hardy-Weinberg equilibrium.

CFH POLYMORPHISM AND CAD

The CFH genotype distributions were similar between CAD cases and normal controls, both in the sample as a whole and in a subset of 233 pairs of age and sex matched cases and controls (table 36). Analysis in the entire CAD patient group (n=1170) showed that there was no difference in CFH genotype distribution between patients with either one, two or three diseased coronary arteries (table 37), or between patients with an MI and those without (table 38).

CFH GENOTYPES AND CARDIOVASCULAR RISK FACTORS

In the CAD patient group, genotype frequencies were similar in males and females. There was no significant difference in mean age between the genotype groups. There was no significant difference in plasma levels of cholesterol and triglyceride, between the CFH genotypes, neither was there any association of the CFH polymorphism with BMI, smoking status, diabetes, hypertension or a family history of CAD (data not shown).

Table 36. Basic demographic and clinical characteristics of the subjects.

		CHD cases total n = 1170	**Controls total n = 560**
Age		63.29 (9.96)	69.10 (9.76)
Sex	Female	274 (23.4%)	308 (55.0%)
	Male	896 (76.6%)	252 (45.0%)
Smoking status		n=872	n=294
Current and ex-smokers		872 (74.5%)	294(53.0%)
Body mass index (kg/m2)		27.49 (4.25)	27.27 (4.70)

Quantitative data are presented as mean (standard deviation).

Table 37. CFH genotype distribution in the age and sex matched cohort.

CFH genotype	**CHD cases n=220**	**Normal controls n=228**	***P* values**
CC (%)	43 (19.5)	48 (21.1)	0.72
CT (%)	99 (45.0)	94 (41.2)	0.44
TT (%)	78 (35.5)	86 (37.7)	0.62

Chi-square *P* value using Yate's correction and 1^{df}

Table 38. CFH genotype distribution in patients with 1, 2 or 3 diseased coronary vessels and in patients with and without MI.

CFH genotype total n=1122	**1 vessel n=454**	**2 vessels n=380**	**3 vessels n=288**	**With MI n=615**	**Without MI n=507**
CC (%)	78 (17.2)	66 (17.4)	48 (16.7)	101 (16.4)	91 (17.9)
CT (%)	214 (47.1)	168 (44.2)	147 (51.0)	291 (47.3)	238 (46.9)
TT (%)	162 (35.7)	146 (38.4)	93 (32.3)	223 (36.3)	178 (35.1)

$P = 0.49$ and $P = 0.78$ (Chi-square values with 1^{df}).

6.5.4 DISCUSSION

Complement activation can arise through the classical, lectin, or alternative pathways[49]. All three pathways lead to the generation of a C3 convertase enzyme and subsequent activation

of the immune response, terminal pathway pore-like membrane attack complex (C5b-9) formation then leads to cell lysis. CFH is an essential inhibitor of the alternative complement pathway preventing uncontrolled complement activation[49]. The CFH Y402H polymorphism is particularly interesting since it locates to the SCR7 domain of the CFH protein, implicated as a binding site for heparin, c-reactive protein (CRP), streptococcal M proteins and various microbes[49]. CHF through its direct binding to CRP is known to reduce the deposition of C5b-9 complexes. It has been proposed that CFH through its direct binding to CRP normally reduces complement mediated deposition and this process may therefore be defective due to the variant CFH protein in atherosclerosis.

In this study, we did not find evidence of an association of the CFH Y402H variant with CAD susceptibility, CAD severity, occurrence of MI, or cardiovascular risk factors such as plasma levels of cholesterol and triglycerides, diabetes, and hypertension. This was despite an adequate power in the study (80% power to detect significant differences in genotype frequencies between cases and controls at an alpha level of 0.05). For example, for a genotype occurring at a frequency of 10% in the control population, detectable odds ratios were 1.84 for a predisposing genotype and 0.45 for a protective genotype with 95% confidence. Our study is hypothesis driven with the results suggesting no association between CAD and CFH Y402H polymorphism. By virtue of the study design, direct evidence for non-functionality of this polymorphism cannot be provided, and it is important to note that a different unidentified CFH gene variant might exist which is not in linkage disequilibrium with the analysed CFH polymorphism. To conclude, in this study based on a UK Caucasian population, no evidence was found to associate the complement factor H Y402H gene variant with coronary heart disease, severity of CAD, and cardiovascular risk factors.

7. CHAPTER SEVEN: HLA CW*0701 GENOTYPE AND NATURAL KILLER CELL RECEPTOR AA HAPLOTYPES ARE ASSOCIATED WITH AMD.

7.1 INTRODUCTION

There are now multiple lines of evidence suggesting an important role for inflammatory events in the pathogenesis of AMD. Histologically, extra-cellular drusen deposits seen in the retina of AMD patients have been shown to contain proteins which modulate the body's response to inflammation. These proteins include vitronectin, complement and immunoglobulins[269]. Inflammatory cells including macrophages[53;270;271], multinucleate giant cells[271;272,273], fibroblasts and mast cells have been observed in association with Bruch's membrane in AMD donor eyes[55]. Some characteristics of AMD have also been described in mice with macrophage defects[59]. Natural Killer (NK) cells which are lymphocytes of the innate immune system, have been reported to be present in sub-retinal neovascular lesions seen in AMD patients [274]. NK cells may therefore also have a role in AMD.

The Human Leukocyte Antigen (HLA) system is essential for the immune regulation of self and foreign peptides via presentation of processed antigenic peptides to both CD4 helper and CD8 cytotoxic T lymphocytes. We previously reported the association of HLA with age-related macular degeneration (AMD) in a UK cohort[150]. Evidence from that study points to an important mechanism which may contribute to susceptibility for immune mediated attacks on RPE or endothelial cells in AMD. Individuals harbouring the HLA Cw*0701 allele were found to have an increased risk of developing AMD [150].

HLA class I molecules are ligands for killer-cell immunoglobulin-like receptors (KIRs), a group of regulatory molecules which are expressed predominantly by natural killer (NK) cells and also T cells. Natural killer cells are involved in the early responses against infected

or transformed cells by production of cytokines and direct cytotoxicity. They also cross-talk with macrophages and dendritic cells. Thus, KIR and HLA in combination are involved in both innate and adaptive immune responses.

Healthy cells are protected from spontaneous killing when they express an appropriate HLA class I ligand for an inhibitory KIR receptor expressed on NK cells. This observation corresponds with the reported phenotypic dominance of KIR-mediated inhibition over activation[275]. However, aberrant or reduced levels of HLA class I expression can result in spontaneous destruction by NK cells. In this context, the expression patterns of HLA class I and class II antigens in the choroid and sub-RPE deposits seen in our previous AMD study seem important. Notably, the presence of class II antigens in drusen and RPE cells[276] and the apparent lack of class I antigens.

NK cells interact with HLA class I (A,B,C) ligands through KIR receptors. Particularly relevant to NK recognition by KIRs are polymorphic HLA-C molecules.Through interaction with inhibitory KIRs, HLA-C molecules are able to modulate NK cell function.Thus, KIR genes are likely to play a significant role in the control of the immune response . It follows that certain combinations of HLA C and KIR gene variants may influence susceptibility to AMD. To test this hypothesis we have analysed HLA C and KIR genotypes both individually and in combination for association with AMD.

7.2 METHODS

AMD subjects and normal controls over age 55 were recruited as per previous methodology. Retinal photographs and angiograms were graded into geographic atrophy or choroidal

neovascularization (CNV) sub-groups. The CNV AMD group was further categorised into occult, minimally classic and predominantly classic CNV sub-groups.

HLA AND KIR GENOTYPING BY PCR-SSP

Data from HLA genotyping for principal HLA class I allele groups including the Cw allele was available for the Southampton cohort of 104 AMD cases and 93 age-matched healthy controls. This group of patients and controls were then also genotyped for KIR genes.

The KIR locus contains polymorphic and homologous genes mapping to chromosome 19q13.4. KIR genotyping can be locus or allele specific. Locus-specific genotyping which detects the presence or absence of each gene in a given individual was employed in this study. The PEL-FREEZ KIR genotyping kit (Dynal Biotech®) was employed to detect the presence or absence of 16 KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1, 3DP1). This kit utilises PCR sequence-specific primer amplification methods (PCR-SSP) published previously[277;278]. The primer sets amplify 16 KIR alleles described by the international nomenclature committee of WHO (http://www.ebi.ac.uk/ipd/kir/).

Gel images of PCR products were photographed under ultraviolet transillumination and interpreted by two independent observers (SVG and AJL). Genotyping was validated by random repeat typing in 10% of cases and controls, for each of the HLA and KIR genotypes which showed a trend for significance (*P* value <1.0) for association with AMD in the study.

7.3 STATISTICAL METHODS AND ANALYSIS

Power calculations were performed utilizing the Epi Info statistical package (version 3.4.1, 2007) provided by the Center for Disease Control and Prevention (CDC). These calculations were based upon the number of cases and controls to be genotyped and the known frequency of specific HLA/KIR alleles. Allelic distributions for each locus were tested for conformity to Hardy-Weinberg equilibrium. Alleles at each locus were said to be in Hardy-Weinberg equilibrium if the observed homozygote and heterozygote frequencies did not differ significantly ($p>0.05$) from expected frequencies.

KIR and HLA genotype frequencies were compared in cases and controls by two-tailed *P* values calculated using the chi-squared test. Adjustment for multiple comparisons was made using the Bonferroni method i.e. *P*-values for each comparison less than or equal to 0.05 were multiplied by the number of study comparisons (n = 3 in the HLA analysis and n = 6 in the combined HLA C- KIR genotype analysis). The level of statistical significance was set at $P < 0.05$ and $P_c < 0.05$ after application of the Bonferroni correction. Odds Ratios were estimated using the approximation of Woolf to avoid problems when critical entries were zero. All statistical analyses were performed using the SAS statistical software package version 9.1.

7.4 RESULTS

Baseline demographics of the unrelated caucasian cohort recruited from a single clinic population at Southampton (104 cases with AMD and 93 normal controls) are described in table 1. AMD grading data from fundus photographs and fluorescein angiograms were available for 58 (55.8%) of the 104 AMD cases (table 1). Genotyped frequencies were distributed in accordance with Hardy-Weinberg equilibrium for all HLA and KIR loci

tested. The results from genotyping repeats performed in 10% of cases and controls were found to be 100% concordant with those obtained during initial genotyping. In total, genotyping data for 23 HLA C principal allele groups was available for analysis after HLA class I genotyping. The allele frequencies for both the HLA and KIR genotypes in the control group were similar to those previously reported for the respective Caucasian populations[126;127;152].

Table 39. Basic available demographic characteristics of the HLA-KIR study population.

		AMD CASES total n = 104 (%)	**CONTROLS total n = 93 (%)**
Age	Mean	76.5	73.1
	Range	55 - 91	55 - 91
	SD	7.8	10.2
Sex	Female	67 (64.4)	58 (62.4)
	Male	37 (35.6)	35 (37.6)
BMI		n = 86	n = 84
	Mean	26.4	26.1
	Range	17.3 – 40.5	17.7 – 36.9
	SD	4.6	4.5
Smoking status		n = 86	n = 86
	Ever smoked	49 (57.0)	34 (39.5)
	Never smoked	37 (43.0)	52 (60.5)
AMD subtypes		n = 58	
	Dry AMD	29 (50.0)	
	Occult CNV	11 (19.0)	
	Minimally classic CNV	09 (15.5)	
	Predominantly classic CNV	09 (15.5)	

HLA-KIR RESULTS

The allele frequencies of HLA C alleles seen in our controls were similar to those previously reported for the UK population.[152] Based on known KIR ligand specificity to a dimorphism on HLA-C allotypes[275], HLA C alleles were initially grouped into C1 and C2 allele groups in the Southampton cohort. The frequency of individuals with two copies of HLA C1 alleles (C1C1), one copy of C1 (C1C2) and two copies of HLA C2 alleles (C2C2) was similar between cases and controls (table 3). HLA C1 and C2 allele distributions and the prevalence of HLA Cw*07 principal allele groups with the exception of Cw*0701 did not show any significant difference between AMD cases and controls. The HLA Cw*0701 allele was found to be strongly associated with the AMD group (*P*=0.03, OR=1.92).

Table 40. HLA C allele groups (C1, C2), Cw*07 and Cw*0701 allele frequencies in Southampton AMD and control groups.

HLA C allele/s	**AMD Group *n*=104 (%)**	**Control Group *n*=93 (%)**	***P* value†**	**Odds Ratio‡**	**95% CI**
C1C1	43 (41.3)	46 (49.4)	0.25	0.72	0.41-1.27
C1C2	50 (48.1)	34 (36.6)	0.10	1.60	0.91-2.84
C2C2	11 (10.6)	13 (14.0)	0.46	0.73	0.31-1.71
C1 alleles	136 (65.4)	126 (67.7)	0.38	1.16	0.82-1.64
C2 alleles	72 (34.6)	60 (32.3)	0.14	1.44	0.88-2.39
Cw*07 allele	63 (60.1)	46 (49.5)	0.11	1.57	0.89 - 2.76
Cw*0701 allele	43 (41.3)	25 (26.9)	0.03*	1.92	1.05 - 3.50

†Two-sided *P* values using chi-squared test (*Significant values). ‡Odds Ratio using the approximation of Woolf.

Of the 16 KIR genes tested (table 4), the frequency of KIR 2DL5 was found to be significantly more in the control group as compared to AMD patients (39.8% vs. 28.8%). This gene is a component of the "B" group of KIR haplotypes. The significant protective

odds ratio for this association was 0.45 (*P*=0.006). None of the remaining 15 KIR genes showed any trend for significant association with either AMD or control groups. No significant associations with AMD were observed for the HLA C1 allele group in combination with its known KIR ligands, KIR2DL2, KIR2DL3 and KIR2DS2 (table 5). The remaining HLA C1, C*07 and C*0701 allele combinations with KIR ligand alleles also did not show any significant association with AMD.

Table 41. KIR genotype and haplotype frequencies in Southampton AMD and control groups.

KIR genotypes	**AMD Group *n*=104 (%)**	**Control Group *n*=93 (%)**	***P* value†**	**Odds Ratio‡**	**95% CI**
2DL1	101 (97.1)	91 (97.8)	0.74	0.74	0.12-4.52
2DL2	50 (48.1)	48 (51.6)	0.24	0.87	0.50-1.52
2DL3	98 (94.2)	90 (96.8)	0.39	0.54	0.13-2.24
2DL4	103 (99.0)	93 (100.0)	0.34	1.90	1.67-2.17
2DL5	38 (28.8)	52 (39.8)	0.006*	0.45	0.26-0.80
2DS1	36 (34.6)	43 (46.2)	0.09	0.61	0.35-1.09
2DS2	50 (48.1)	50 (53.8)	0.42	0.79	0.45-1.39
2DS3	25 (24.0)	32 (34.4)	0.10	0.60	0.32-1.12
2DS4	95 (30.8)	89 (50.5)	0.21	0.47	0.14-1.59
2DS5	27 (26.0)	28 (30.1)	0.51	0.81	0.44-1.52
3DL1	101 (97.1)	90 (96.8)	0.89	1.12	0.22-5.70
3DL2	104 (100.0)	93 (100.0)	-	-	-
3DL3	104 (100.0)	92 (98.9)	0.29	3.38	0.13-84.3
3DS1	30 (28.8)	34 (36.6)	0.24	0.70	0.38-1.28
2DP1	101 (97.1)	91 (97.8)	0.74	0.74	0.12-4.53
3DP1	104 (100.0)	93 (100.0)	-	-	-
AA	35 (33.6)	23 (24.7)	0.17	1.54	0.82-2.87
AB/BB	69 (71.2)	70 (51.6)	0.17	0.65	0.34-1.20

†Two-sided *P* values using chi-squared test (*Significant values). ‡Odds Ratio using the approximation of Woolf.

It has previously been noted that there is association with specific KIR halpotypes in the development of pre-eclampsia where this genotype has been suggested to influence vascular remodelling.[279] As KIR2DL5 is present on the majority of B group haplotypes, we determined if a haplotypic association was relevant for AMD or CNV formation in AMD.

The observed KIR genotypes were grouped into AA, AB, and BB haplotypes based on gene content. The KIR A halotype is "inhibitory" and contains KIR2DS4 and KIR2DL4 as activating KIR and is the most common KIR haplotype being present in about 30% of the Caucasian population. The remaining genotypes were categorised as B haplotypes. The Southampton cohort with KIR2DS4 and KIR2DL4 as the only activating receptors were thus designated "AA" and the remainder "AB/BB".(table 4). There was no significant difference in the distribution of the AA and AB/BB genotypes between the AMD cases and controls.

Table 42. HLA C and KIR receptor paired genotype frequencies in AMD and controls.

HLA + KIR genotypes	**AMD Group *n*=104 (%)**	**Control Group *n*=93 (%)**	***P* value**[†]	**Odds Ratio**[‡]	**95% CI**
C1 + 2DL2	37 (35.6)	43 (46.2)	0.12	0.64	0.36-1.14
C1 + 2DL3	87 (83.7)	77 (82.8)	0.87	1.06	0.50-2.25
C1 + 2DL5	33 (31.7)	47 (50.5)	0.007*	0.45	0.25-0.81
C1 + 2DS2	37 (35.6)	45 (48.4)	0.07	0.59	0.33-1.04
C7 + 2DL2	27 (26.0)	27 (29.0)	0.62	0.86	0.46-1.60
C7 + 2DL3	62 (59.6)	43 (46.2)	0.06	1.71	0.97-3.02
C7 + 2DL5	22 (21.1)	28 (30.1)	0.15	0.62	0.32-1.19
C7 + 2DS2	28 (26.9)	29 (31.2)	0.51	0.81	0.44-1.50
Cw*0701 + 2DL2	19 (18.3)	14 (15.1)	0.54	1.26	0.59-2.68
Cw*0701 + 2DL3	18 (17.3)	24 (25.8)	0.14	0.60	0.30-1.20
Cw*0701 + 2DL5	13 (12.5)	18 (19.3)	0.19	0.59	0.27-1.29
Cw*0701 + 2DS2	20 (19.2)	15 (16.1)	0.57	1.23	0.59-2.59

[†]Two-sided *P* values using chi-squared test. [‡]Odds Ratio using the approximation of Woolf.

To further test the KIR – HLA haplotype model of Hiby et al [279], we performed univariate analyses. Variables tested in this analysis included combined frequencies of the C1 group of HLA alleles, the individual HLA alleles, Cw*07, Cw*0701 and the AA KIR haplotypes (table 6). The combination of total HLA Cw*07 alleles and KIR AA haplotypes correlated significantly with AMD status (odds ratio= 3.36, P=0.003, P_c=0.018). When looking at the individual HLA C*0701 allele and KIR AA haplotypes in combination, a strong association was seen with AMD. The odds ratio risk for AMD was significant at 4.35 (P=0.006, P_c=0.036) for this HLA-KIR allele combination.

In order to distinguish these independent variables, we performed multivariate analysis by logistic regression using the binary logit model with backward elimination (table 7). The significance of the HLA Cw*0701 - KIR AA paired association with AMD did not alter after this analysis. No significance was found with KIR 2DL5 and KIR AA genotypes in the regression model. Interestingly, the effect of HLA Cw*0701 when KIR AA genotypes were removed was found to be non-significant.

Table 43. HLA C and KIR genotype paired frequencies in Southampton AMD and controls.

HLA + KIR genotype	**AMD Group *n*=104 (%)**	**Control Group *n*=93 (%)**	***P* value†**	***P_c* value§**	**Odds Ratio‡**	**95% CI**
C*1 + AA	31 (29.8)	23 (24.7)	0.42	2.52	1.29	0.68-2.43
Cw*07 + AA	25 (24.0)	8 (8.6)	0.003*	0.018*	3.36	1.43-7.89
Cw*0701 + AA	17 (16.3)	4 (4.3)	0.006*	0.036*	4.34	1.40-13.44
C*1 + AB/BB	67 (64.4)	70 (75.3)	0.09	0.54	0.59	0.32-1.10
Cw*07 + AB/BB	38 (36.5)	38 (40.9)	0.54	3.24	0.83	0.47-1.48
Cw*0701 + AB/BB	26 (25.0)	21 (22.6)	0.69	4.14	1.14	0.59-2.21

†Two-sided P values using chi-squared test (*Significant values); §P_c indicates P values after application of Bonferroni correction factor (n=6). ‡Odds Ratio using the approximation of Woolf.

Further categorization was possible in 58 subjects in the Southampton AMD group, who were divided into 4 sub-groups of increasing AMD severity; dry AMD, occult CNV, minimally classic CNV and predominantly classic CNV based on available fluorescein angiographic data. No significant difference or associations could be found in these patient sub-groups when tested for HLA alleles C1, Cw*07, Cw*0701 or their combinations with KIR genes and KIR AB/BB or AA haplotypes (data not shown).

7.5 DISCUSSION

The importance of defining associations between HLA and KIR genes in AMD lies in providing new insights into its basic pathogenesis and to help define an 'at risk' population for preventive measures to become applicable. Due to the critical role of HLA in regulating the immune response, the majority of HLA gene polymorphisms are known to result in functional amino acid substitutions in the expressed HLA molecules. These variant HLA molecules in turn affect the immune response to a vast range of antigens, with inter-individual differences in immune responses to complex or simple antigens[280]. Many HLA gene polymorphisms have been linked to susceptibility in a large number of immunologically mediated diseases affecting the retina and uvea of the eye, the skin, gut, endocrine and joint systems[281]. KIR gene models have now been proposed which genetically control levels of activation or inhibition[275]. Many KIR genes and combinations of HLA class I -KIR genotypes that predispose to activating receptor–ligand interactions have now been linked to autoimmune inflammatory diseases like rheumatoid arthritis[282], psoriatic arthritis[283], psoriasis vulgaris[284], etc.

Significant associations have previously been reported between HLA-DRB1*15 (P_c=0.000001) and DQB1*06 (P_c=0.00001) alleles and the development of CNV in

presumed ocular histoplasmosis syndrome[285]. These findings, along with the data from the current study, support the notion that certain HLA alleles are associated with CNV in various retinal diseases. Hence, further investigation of these three HLA alleles in a larger and/or different cohort would be useful in determining the amount of risk contributed by these alleles towards advanced AMD and CNV formation.

The combined HLA Cw*0701 allele and KIR AA haplotype association with AMD status was highly significant in the Southampton cohort when tested by stepwise logistic regression analysis. The risk for disease was greater than 4 fold when compared to controls for this paired HLA allele and KIR haplotype. Preponderance of inhibiting KIR 2DL5 in controls seems to be protective for AMD while the presence of an appropriate HLA C1 ligand interaction seems to render an individual more susceptible to AMD. This may be because KIR2DL5 is a component of the B group of haplotypes. Therefore its association with protection from AMD could be an inverse correlate of the susceptibility effect of the A group of haplotypes. Alternatively KIR2DL5 itself or the B group of haplotypes which contain the majority of the activating KIR may be protective against this disorder.

Traditionally, activating KIR genotypes have been reported as being important in inflammatory conditions. A weak association of the activating receptor–ligand pair KIR2DS2:HLA-C 1 was observed in diabetes mellitus[286] . A correlation with an 'activating' KIR genotype was also found in the chronic inflammatory idiopathic bronchiectasis[287]. This activating receptor association appears to be involved more in co-stimulation or activation of T cells than of NK cells.[288]

The underlying mechanisms by which KIR and HLA genes determine susceptibility to various ocular diseases has not been fully elucidated yet and it should be stressed that a KIR-HLA association determined by a case-control study does not necessarily confirm KIR-HLA causality, but may reflect linkage disequilibrium (LD) with causal polymorphisms in other genes. The epistatic interactions of KIR with HLA make this less likely though. Although we cannot exclude LD with non KIR-HLA polymorphisms as causal in AMD, our data bears similarities to that observed in pre-eclampsia.[279] In this disease the KIR-HLA genotype of the mother and foetus seems to influence vascular remodelling in the placenta leading to pre-eclampsia. This model could therefore be relevant in AMD or in particular during CNV formation.

It is important to note that the AA haplotype is a minimal KIR haplotype containing the inhibitory receptors for groups 1 and group 2 HLA C allotypes (KIR2DL1 and KIR2DL3) and the activating KIR2DL4 and KIR2DS4, the latter gene being non-functional in many individuals.[289].As the KIR2DL3: HLA-C interaction is considered one of weak inhibition this halplotype is weakly inhibitory. This interaction may confer susceptibility to AMD via either NK cells or via weak inhibition of KIR-positive T cells, which are predominantly of the antigen-experienced effector-memory type.[290]

Due to the extreme polymorphic nature of the KIR and HLA gene regions, sampling variation between cases and controls can easily lead to both false-positive and false-negative associations. Accordingly, a Bonferroni correction was applied in this study. Applying the correction for functionally well characterized KIR alleles permitted a realistic Bonferroni correction, rather than an over-conservative correction for all possible KIR alleles, which may cause even true positive associations to be discarded.

This study had 80% power to detect significant differences in HLA allele frequencies between cases and controls at an alpha level of 0.05. For example, for an allele occurring at a frequency of 10% in the control population, detectable odds ratios are 2.3 for a predisposing allele and 0.5 for a protective allele with 95% confidence levels. The corresponding detectable odds ratio figures for KIR genes was 2.9 for a predisposing genotype and 0.2 for a protective genotype with 95% confidence levels. We may therefore have not detected smaller HLA and KIR associations with our sample sizes.

In summary, this study is the first to establish HLA-C/KIR gene mediated genetic susceptibility in AMD. HLA-AMD associations were reproducible to a major extent in a different population cohort. These could therefore be important for AMD among most western populations. The HLA C*0701 allele in combination with inhibiting KIR haplotypes (AA genotype) is strongly associated with AMD. Following on from this hypothesis-generating study, further larger studies in different ethnic populations are further needed to confirm these HLA-KIR associations with AMD. In addition, where possible these studies should be complemented by functional studies to determine the contribution of these genes to the development of AMD. Dissecting the role of HLA and immune pathways in AMD may ultimately lead to opportunities to modulate these pathways by precise pharmacological means and thus improve the visual outcome in this devastating disease.

8. CHAPTER EIGHT: SUMMARY AND FUTURE RESEARCH.

8.1 SUMMARY

In summary, evidence from my initial study of the HLA system which is essential for the immune regulation of self and foreign peptides[150] points to an important mechanism which may contribute to susceptibility for immune mediated attacks on RPE or endothelial cells in AMD. The HLA study showed that individuals harbouring the HLA Cw*0701 allele had an increased risk of developing AMD[212] and also reported the localization of HLA class I and DR antigens in the choriocapillaris and drusen, respectively[106;212]. These novel HLA-AMD associations were reproducible in a cohort of AMD cases and controls from Michigan. Therefore these HLA associations could predictably play an important role in AMD among most western populations. This research has provided evidence for our hypothesis of genetic predisposition to inflammation as being important in a significant proportion of AMD cases.

Our results confirm previous CRP associations with AMD. Interleukin IL8-251A/T variant may have a pro-inflammatory role in AMD while the ICAM1 12959G/A variant appears to raise ICAM1 levels especially in cigarette smokers and could predict AMD development. The effect of smoking seen in AMD may thus be mediated in part by increased expression of ICAM1. Finally, the strong association of the CFH Y402H coding polymorphism with AMD was confirmed in our UK cohort, while no association could be found between CRP levels and the CFH Y402H polymorphism in AMD. CFH Y402H association was significantly strongest in the predominantly classic CNV group both for CC (OR=17.87, P= < 0.0001) and CT (OR=9.06, P= 0.0002) genotypes. These patients seemed to have poorer visual outcomes following PDT, this was significant both in the CFH CC and CT genotype

groups (P= 0.038; 50% of CC cases, n=13 and 45% of the CT cases, n=12) who lost 15 or more ETDRS letters.

Following this evidence for complement activation in AMD, I tested other epidemiologically similar diseases like coronary heart disease and atherosclerosis (characterized by lipid like deposits similar to drusen in AMD) for possible association with the CFH Y402H polymorphism. No evidence was found in this large UK Caucasian cohort to link this CFH polymorphism with CHD, coronary artery disease severity (CAD), recurrence of myocardial infarction or plasma levels of cholesterol and triglycerides.

In addition to testing various immune linked genetic pathways, I also evaluated critical genes encoding structural membrane proteins which may have functional implications in AMD. Although the fibulin group of genes have been strong associated with AMD and novel fibulin5 missense mutations have been found in our cohort, mutation screening of linked candidate genes responsible for basement membrane integrity and elastinogenesis (LOXL1), and testing for promoter region SNP's in PEDF and CRYAA genes did not yield significant results. These null findings need to be confirmed by studying other population cohorts and also perhaps by LD based studies.

Following the HLA results, further analysis of their interaction with KIR ligands in AMD was undertaken. Healthy cells are protected from spontaneous killing when they express an appropriate HLA class I ligand for an inhibitory KIR receptor expressed on NK cells. Analysis of KIR genotypes in AMD showed that HLA C*0701 allele in combination with inhibiting KIR haplotypes (AA genotype) is strongly associated with AMD status (P=0.006, P_c=0.036, OR= 4.34). HLA-C/KIR gene mediated genetic susceptibility in AMD seems important and needs to be explored in different population studies.

8.2 FUTURE RESEARCH

Following on from this hypothesis-generating research, further larger studies in different ethnic populations are needed to confirm these HLA-KIR associations with AMD. To this end, complete HLA class I genotyping and KIR genotyping is planned to be done in the Michigan cohort. Functional studies need to be undertaken to determine the contribution of these genes to the development of AMD. The effect of these genotypes on outcomes to AMD treatments need to be assessed prospectively. Dissecting the role of other immune pathways in AMD would ultimately lead to ways to modulate these pathways by precise pharmacological means and thus improve the visual outcome in this devastating disease.

PUBLICATIONS

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PRESENTATIONS

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2. "HLA genotypes and Age related macular degeneration: A replication study in a North American cohort". ARVO annual meeting, Florida, May 2007.
3. "Does CFH Y402H Polymorphism Influence Visual Outcomes Following V-PDT Therapy for Age Related Macular Degeneration?" Paper at ARVO annual meeting, Florida, May 2006.
4. "Novel HLA gene polymorphisms in age-related macular Degeneration". Paper at Royal Society of Medicine, London June 2005.
5. "HLA polymorphisms in Age-related macular degeneration". Rapid fire presentation at RCO annual congress, Birmingham, May 2005.
5. "CRP and sICAM-1 as biomarkers in Age-related macular degeneration." Paper at ARVO annual meeting, Florida, May 2005.
6. "Association of HLA class I and class II polymorphisms with age-related macular Degeneration". Presented at the House of Lords parliamentary meeting for researchers, London, Mar 2005.
7. "Association of HLA polymorphisms in Age-related macular degeneration" ARVO meeting, Florida, April 2004.
8. "Current status of nutritional supplementation and smoking in patients with age-related macular degeneration (AMD): implications from AREDS" RCO Annual Congress, May 2004.

9. “Macular degeneration associated with a novel Treacher Collins TCOF1 mutation and evaluation of this mutation in Age-related macular degeneration” European Pediatric Ophthalmological Society meeting, Manchester, Oct 2004.

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