Genetic studies of age-related macular degeneration

Baas, D.C.

Citation for published version (APA):
Baas, D. C. (2012). Genetic studies of age-related macular degeneration
CHAPTER 9

General Discussion
In the developed world the average life expectancy is constantly increasing. It is one of modern medicine’s aims, that a high quality of life is preserved during these additional days of old age and that these will not be crippled by disease. Age-related macular degeneration (AMD) is the leading cause of irreversible visual loss in the industrialized countries and one of the major threats for independence and activity in old age. Many factors, including environmental and genetic factors, are associated with the disease. Previous studies have generated strong hypotheses on genes and pathways potentially involved in the etiology of AMD. These include oxidative stress and inflammatory pathways. In addition, a role for the extracellular matrix (ECM) as a barrier for choroidal neovascularization (CNV) has been proposed. The aim of this thesis was to further enlighten the genetic background of AMD, based on these formerly implicated molecular pathways. This chapter discusses the main findings of this thesis in the light of the current literature, discusses strengths and weaknesses of the studies performed, and suggests future directions.

**The role of oxidative stress**

Oxidative stress refers to cellular or molecular damage caused by reactive oxygen species (ROS) and has been implicated in aging and many age-related diseases [1,2]. The retina, and especially the retinal pigment epithelium (RPE), is exposed to high levels of oxidative stress [3]. Sources of local oxidative stress include the high metabolic rate of photoreceptors required to sustain their normal function and structural renewal, the exposure to light, the high local oxygen pressure and the high metabolic rate of the RPE due to the processing of photoreceptor outer segments (POS) [4]. All these processes may lead to molecular (DNA, RNA, protein) and cellular damage, which may be susceptibility factors for the development of AMD. Indeed, a number of studies in the literature showed that human donor eyes affected by AMD contain higher levels of oxidatively modified proteins, carbohydrates and lipids compared with non-AMD eyes, which implicates that oxidative stress is involved in AMD [5,6].

Oxidative stress may be caused by both environmental and genetic factors. Inside the cell, mitochondria produce the majority of ROS. Genetic variation in genes related to mitochondrial function may thus influence oxidative damage mediated AMD pathology. In this context, it is of interest that a major susceptibility locus for AMD has been mapped to the chromosome 10q26 region by several genome-wide linkage studies [7-9]. This locus contains two genes. Since the physical distance between these genes is about 6 kb, they are in strong linkage disequilibrium (LD). Consequently, it is not yet clear which of these (or both) genes is involved in
AMD. The two genes are age-related maculopathy susceptibility 2 (ARMS2), also known as LOC387715 [10,11] and high temperature requirement factor A1 (HTRA1) [12-14].

One of these genes, the ARMS2 gene, may play a role in mitochondrial function [13]. The ARMS2 protein was initially localized at the mitochondrial outer membrane [15]. However, this finding is not undisputed: Wang and coworkers localized the protein to the cytosol [16] and the group of Kortvely described ARMS2 as a constituent of the ECM [17]. Genetically, association odds ratios up to 8.21 for homozygous cases, were observed between the A69S single nucleotide polymorphism (SNP) in exon 1 of the ARMS2 gene and AMD [11]. Several subsequent studies suggested that also other SNPs in the ARMS2 gene increase susceptibility to oxidative damage, possibly in association with smoking [10,18].

Besides autosomal ARMS2 SNPs, combinations of neutral polymorphisms in the mitochondrial DNA genome, defined as mtDNA haplogroups, may also contribute to AMD onset or progression [19]. Mitochondrial haplogroups (H, J, U and T) were previously associated with AMD in case-control studies from the United States and Australia [19-23]. Haplogroup H appears to be protective. It was associated with a reduced (early and late) AMD prevalence and large distinct and indistinct soft drusen. In contrast, haplogroup J was associated with increased risk of large soft distinct drusen. Haplogroup U was associated with higher prevalence of retinal pigment abnormalities [20-22]. Finally, A4917G, a defining polymorphism for the T haplogroup, was reported as an independent predictor of AMD [23].

Antioxidants are bio-molecules that protect the cell against oxidative damage. So far, studies, which investigated the potential human genetic association between variants in antioxidant genes (CAT, CYP, GPX1, PON1, SOD2 and SOD1) and AMD, were inconclusive. Esfandiary and coworkers investigated the potential role of SNPs in antioxidant genes such as catalase (CAT), several cytochrome P450 gene superfamily members (CYPs), glutathione peroxidase 1 (GPX1) and paraoxonase 1 (PON1) but reported no association with AMD [24]. In contrast, Ikeda and coworkers did find an association between SNPs in the PON1 gene and CNV [25]. Kimura et al. [26] reported a significant association between SNPs in SOD2 and CNV in a Japanese population. However, two additional studies, one also in a Japanese population [27] and one in a Northern Irish cohort [24], failed to corroborate this evidence. Interestingly, Sod1 (-/-) knock-out mice [28] showed pathological features resembling AMD. These features included drusen, thickened Bruch's membrane (BM) and CNV [29]. Obviously, these observations in mice strongly suggest that oxidative stress does play a role in AMD.
General Discussion

Environmental factors, such as smoking and diet, also point at the role of oxidative stress in AMD. Numerous studies robustly confirm tobacco smoking as one of the major risk factors for AMD: by itself, it increases the risk for AMD from 1.7 to 3.2-fold in ever smokers and from 1.9 to 4.5-fold in current smokers [30-32]. Indeed, oxidants present in cigarette smoke have numerous relevant effects: they reduce the plasma concentration of antioxidants and the choroidal blood flow, and increase platelet adhesiveness, hypoxia, and the release of free radicals [33]. Epidemiological evidence further suggested that dietary antioxidant supplementation (vitamins C and E, beta-carotene and zinc) decreases the incidence of AMD [34]. Van Leeuwen et al. [35] described that the risk of developing advanced AMD is greatly reduced by high dietary intake of nutrients with antioxidant properties. In 2011, the same group reported that antioxidants reduce the risk of early AMD in those with a high genetic risk [36].

Yet other evidence for an important role of oxidative stress in the pathogenesis of AMD comes from in vitro studies. Lipofuscin is a photoinducible free radical generator and plays an important role in the oxidative stress theory. Lipofuscin accumulates in many metabolically active post-mitotic cells during aging. The lipofuscin present in the RPE is composed of a complex heterogeneous mixture of oxidized proteins and lipids and probably derives, at least in part, from oxidatively damaged photoreceptor outer segments [3]. RPE lipofuscin contains A2E, the bis-retinoid N-retinylidene-N-retinylethanolamine, which arises as toxic byproduct of phototransduction. A2E impairs RPE phagocytosis, induces complement activation and confers susceptibility to blue light induced generation of ROS [37-40]. Indeed, blue light mediated accumulation of lipofuscin has been implicated in oxidative stress related RPE cytotoxicity [41] and the development of AMD [42].

To further investigate the causative role of oxidative stress in the pathogenesis of AMD we focused in this thesis on two potentially relevant genes: the excision repair cross-complementation group 6 (ERCC6) (chapter 2) and the solute carrier family 2 (facilitated glucose transporter) (SLC2A1) (chapter 3). Both are potentially involved in oxidative stress and oxidative damage related pathways.

In chapter 2, we studied the potential association between SNPs in the DNA repair gene ERCC6 and AMD. Previously, Tuo and colleagues had reported that the C6530G SNP was associated with AMD [43]. They also found enhanced ERCC6 expression in lymphocytes from healthy donors bearing the ERCC6 C6530G alleles [43]. We did not find an association between ERCC6 variants and AMD. Using GWAS, Chen and coworkers recently confirmed our results: they did not find an association between the C6530G, or other ERCC6 SNPs and AMD [44]. In addition, no associations were
found between copy number variations (CNVs) in the ERCC6 gene and (neovascular) AMD [45]. Although a genetic association between ERCC6 and AMD is unlikely, our study did suggest a functional role for ERCC6 in AMD: We found that RPE expression levels of ERCC6 were significantly reduced in early AMD affected RPE, independent of genotype. In addition, Ercc6 knock-out mice are very sensitive to oxidative stress and develop retinal degeneration [46]. Further functional studies are needed to determine the exact role of ERCC6 in the etiology of AMD.

As a second target, we tested the role of SNPs in the SLC2A1 gene, the main glucose transporter in the retina. SLC2A1 could potentially be involved in oxidative stress mediated AMD pathology by changing the bioavailability of glucose in the RPE and neural retina and thereby altering the local oxidative burden. Expression of SLC2A1 in retina and brain is altered in several pathophysiological conditions related to oxidative stress like hypoxia [47,48], Alzheimer’s disease [49] and epilepsy [50]. In a multi-cohort association analysis, we could not find a consistent genetic association between SLC2A1 SNPs and AMD. This was possibly due to significant heterogeneity of effect across the study populations. Interestingly, Kortvely and coworkers (2012) very recently genotyped nine SLC2A1 SNPs in three independent cohorts and detected a significant correlation of SLC2A1 SNP rs3768029 and AMD [51]. Further studies regarding the role of SLC2A1 SNPS and AMD are therefore needed.

In summary, we investigated two oxidative stress genes (ERCC6 and SLC2A1). No consistent genetic association between SNPs in these genes and AMD was found. Nevertheless, our findings on ERCC6 expression in the RPE did suggest that this gene might be functionally involved in AMD. More studies are required to further assess the genetic contribution to the oxidative stress component of AMD.

**THE ROLE OF THE IMMUNE RESPONSE**

Strong indications that inflammation plays an important role in the pathogenesis of AMD came initially from immunocytochemical studies of the composition of drusen. Several studies illustrated the presence of complement components and complement regulatory proteins in drusen and nearby RPE of AMD patients [5,52-57]. Final proof for the inflammatory pathogenesis of AMD came from recent genetic studies. Genetic association studies for at least five genes (complement factors H (CFH), B (CFB), I (CFI), 2 (C2), 3 (C3)) consistently suggested that the complement system plays a major role in the molecular pathology of AMD. Genetic variation in the complement regulator CFH accounts for the strongest association [58-64] (Figure 1).
Figure 1. The complement pathway.
The components of the complement system that have been genetically associated with increased or decreased risk of drusen, geographic atrophy (GA), and choroidal neovascularization (CNV), are circled. Reprinted with permission from Donoso et al. [65]. A color version of this figure can be found in the color figures section.

The complement system is part of the host innate immune system and enables a number of essential functions, including (1) opsonization and lysis of microorganisms, (2) recruitment of inflammatory cells, (3) removal of dead cells, (4) regulation of antibody production and (5) removal of immune complexes [66]. The complement system can be activated by three pathways, i.e., the classical, the mannose-binding lectin, and the alternative pathway. In short, the classical pathway is triggered by antigen-antibody complexes and surfaced-bound C-reactive protein (CRP). The lectin pathway is triggered when mannose-binding lectins or ficolin interact with surface sugar molecules on microorganisms. The alternative pathway is triggered by non-self antigens, such as intermediates of the lipofuscin biosynthesis
or oxidized lipids or proteins. The pathway subsequently sustains a continuous low-level state of activation through the spontaneous hydrolysis of C3 into C3a and C3b. C3 is abundantly present in the blood plasma [66,67]. All complement pathways come together at component C3, and lead eventually to the formation of the lytic membrane attack complex (MAC), consisting of C5b-C9 [68,69] (Figure 1).

As mentioned above, genetic association studies revealed that the Y402H polymorphism of the *CFH* gene was associated with greatly altered susceptibility to all forms of AMD. This SNP results in the substitution of histidine for tyrosine at codon 402 in a domain of CFH that contains binding sites for CRP, heparin, and streptococcal M6 protein [70]. Since *CFH* is a negative regulator of the alternative complement pathway [71], the risk allele leads to inadequate inhibition of the complement cascade and eventually induces chronic damage to the RPE, BM and/or choroid via the MAC complex. This is consistent with the observation that the CFH protein is present in drusen [72]. Very recently, a potentially important functional clarification for the strong association of the Y402H polymorphism with AMD was provided: Weisman *et al.* present evidence that malondialdehyde (MDA), a naturally occurring product of lipid peroxidation, is an important ligand for CFH present on apoptotic cells. MDA epitopes allow CFH to bind to the surface of the apoptotic cells and neutralize the pro-inflammatory properties of the MDA epitopes. This occurs through iC3b generation and closing down complement activation. The Y402H polymorphism reduces the capacity of CFH to bind MDA and thereby decreasing the generation of anti-inflammatory iC3b fragments. MDA epitopes were shown to be present on the surface of in vitro-generated necrotic RPE cells and throughout the choroid and BM including drusen of AMD lesions [73].

Another complement component genetically linked to AMD is *C3*. C3 is an important plasma protein, a common component of all three complement pathways and critical for the downstream formation of the terminal MAC that leads to cell lysis. Initially, association between SNPs in the *C3* gene and AMD was found in two independent AMD case-control cohorts with English (discovery) and Scottish (replication) origin. The associated *C3* SNP rs2230199 resulted in an amino acid change with at least some functional consequences: The change influenced binding to pathogenic cell surfaces and enhanced complement activation, which, in the end, leads to RPE cell lysis and AMD [58]. Multiple subsequent studies replicated these initial findings [59,74]. A Dutch study by Despriet *et al.* (2009) showed that not only rs2230199, but also another variant, rs1047286, significantly increased the risk of all subtypes of AMD [75].
In 2006, association was found between AMD and genetic variants in yet two other complement components: C2 and CFB, respectively activators of the classic and alternative complement pathways, which exhibited a protective instead of risk increasing effect [62]. Gold and his colleagues hypothesized that the significance is largely due to the CFB variants, which are in strong LD with C2. Structural variants in CFB may lead to impairment in the complement activating function of CFB and may thus provide a lower risk of chronic complement activation. Combined analysis of the C2/CFB haplotypes and CFH variants showed that variation in the two loci can predict the clinical outcome in 74% of all AMD cases in European and North American populations [62,67]. Subsequently, Fagerness et al. (2009) provided yet additional evidence for the involvement of the complement system in AMD. They found association between common variants near the CFI gene on chromosome 4q25 and advanced AMD [63]. A SNP located 2781 bp upstream of the 3' UTR of the CFI gene, rs10033900, yielded the statistically most significant evidence. CFI is a serine protease that inhibits all complement pathways by degrading activated complement components C3b and C4b. To complicate matters, C3b inactivation by CFI is regulated by CFH. CFH inhibits C3 activation by binding to C3b and acting as a co-factor for CFI-mediated cleavage of C3b and has decay-accelerating activity for the alternative pathway C3 convertase, C3bBb [63].

Multiple complement genes are locally expressed by the RPE and choroid, which, in theory, would be sufficient to invoke and sustain local chronic inflammation. However, Scholl et al. (2008) showed that markers of chronic complement activation, like Ba and C3d, and regulator of the alternative pathway of complement, factor D, are abundantly present in the plasma of AMD patients. This finding implied that AMD is, at least in part, a systemic disease with a local disease manifestation [76]. Next to complement genes, yet another immune system-related gene, CX3CR1, may be associated with AMD. The function of the chemokine receptor protein CX3CR1 is macrophage and microglia recruitment. Three independent studies showed that CX3CR1 variants were moderately associated with AMD [77-79]. However, a recent GWAS study and a recent study in a large population in France could not corroborate this finding [80-81].

Based on the crucial role of the immune system in AMD (Figure 1, Donoso et al.), we further tested the contribution of other complement system players to AMD by extensively genotyping the complement component 5 (C5) gene (chapter 4) and a member of the classical complement pathway, serpin peptidase inhibitor; clade G, member 1 (SERPING1) (chapter 5). Our C5 study showed that, in spite of the
crucial role of C5 in the complement cascade and the presence of C5 protein in drusen, no consistent significant association between C5 SNPs and AMD was found. The negative outcome of our association analysis may depend on genetic or clinical differences between our study populations. C5a is, among many other alternative complement activation molecules, elevated in peripheral blood of AMD patients [76]. In 2006, Nozaki provided evidence that bioactive fragments of complement components C3 and C5 (C3a and C5a) accumulate in drusen of patients with AMD and promote CNV [57].

Initially, Ennis et al. (2008) found an association between AMD and genetic variants in the SERPING1 gene, encoding the complement component 1 inhibitor (C1inh) protein [82]. We tested the rs2511989 SNP in the SERPING1 gene in seven large case-control studies. We found no evidence that this variant plays a role in AMD. Park et al. in 2009 and Carter and Churchill in 2011 also failed to find an association between SERPING1 SNPs and AMD [83,84]. Yet, a study in 2012 by Gibson et al. shows significantly higher plasma levels of C1inh, the protein product of the SERPING1 gene, in AMD cases versus controls. This group also shows evidence that one SNP (rs2649663), located 7.7 kb of SERPING1 or its proxies may influence C1inh levels by differential expression of SERPING1 [85]. This SNP was not associated with AMD susceptibility by Ennis et al. [82].

In this thesis (chapter 6) we furthermore tested the role of toll-like receptor 3 (TLR3), known to be involved in the innate immune system, in geographic atrophy (GA) cases and controls in eight well-known cohorts. Yang and coworkers (2008) found that variation in TLR3 (rs3775291) was associated with protection against GA, possibly through suppression of RPE apoptosis [86]. However, in this thesis, we illustrate that it is unlikely that TLR3 variant rs3775291 has a major effect in the etiology of AMD. Our findings were confirmed by several other studies [87-89]. Interestingly, for another member of the TLR family, TLR4, also conflicting results exist. A study by Zareparsi et al. reported that the D299G polymorphism leads to increased AMD risk [90] whereas Despriet et al. could not find a significant association [91]. Perhaps SNPs in TLR3 and TLR4 interact, and need to be tested together in future studies.

In conclusion, we tested genetic variation in C5, SERPING1 and TLR3 for association with AMD. The relationship between these genes and AMD is controversial to date and requires additional studies in diverse and larger populations.
The role of the extracellular matrix

BM is a multi-layered ECM complex that functions as a molecular sieve. BM partly regulates the reciprocal exchange of biomolecules, nutrients, oxygen, fluids and metabolic waste products between the retina and the general circulation. In chapter 7 of this thesis we provided a review of BM. With age, many functional properties of BM change, leading not only to normal age-related changes in the RPE and photoreceptor cells, but also to the onset and/or progression of diseases like AMD. We showed that the changes occurring in BM with age include increased calcification of elastic fibres, increased cross-linkage of collagen fibres and an alteration in the turnover of glycosaminoglycans. In addition, lipids and advanced glycation end products (AGEs) accumulate in BM. In particular, diffuse thickening and progressive deposition of material in BM may result in a barrier to normal metabolic exchange between the choroid and the RPE or vice versa [92]. Extracellular deposits slowly but surely appear in BM. They include drusen and basal deposits (flat and diffuse deposits beneath the RPE that include basal laminar deposits and basal linear deposits) [93]. Undoubtedly, BM is the primary site of drusen development in the early stage of AMD [93,94]. Biochemically, drusen contain (oxidation products of) proteins, lipids and carbohydrates [5,94-96]. The oxidized components in drusen may be recognized as non-self epitopes by the human body and trigger inflammatory and immune responses [97]. Besides the complement system, also multiple immune-related cells, including dendritic cells, macrophages and lymphocytes, as well as fibroblasts have been implicated in RPE atrophy, the breakdown of BM, and, ultimately, neovascularization in AMD [98]. In chapter 7, we provided a list of genes that are expressed at higher levels in the RPE than in the choroid with the functional annotation “angiogenesis”. These data indicate that at least 23 genes may be involved in this process, which clearly illustrates the complexity of this matter. Next to the aging changes and its relation to AMD pathology, one could hypothesize that mutations in genes encoding ECM molecules result in disturbance of the transport or the barrier function of BM (and the RPE), which may also contribute to AMD. Genetic variants in ECM genes such as TIMP3, COL8A1, COL10A1, FBLN5, VEGFA and possibly HTRA1 support involvement of the ECM in the pathogenesis of AMD. As illustrated below, variation in these genes was linked to the pathology of AMD. The tissue inhibitor of metalloproteinases-3 (TIMP3) is an inhibitor of MMP activity, i.e. an inhibitor of enzymatic degradation of the ECM. Mutations in this gene cause Sorsby fundus dystrophy (SFD) [99]. The clinical picture of SFD strikingly resembles early onset AMD. Although initial investigations [99,100] did not find an association between SNPs in TIMP3 and AMD, recently a SNP near this gene was associated with
Two other ECM genes, the collagen matrix pathway genes \textit{COL8A1} and \textit{COL10A1}, were recently implicated in advanced AMD using GWAS studies [101,102]. As early as 2004, Stone et al. described rare missense mutations in fibulin 5 (\textit{FBLN5}) in patients with AMD [103]. Besides mutations, several other lines of evidence support a role of \textit{FBLN5} in AMD: \textit{FBLN5} is located in an AMD associated GWAS locus on chromosome 14q32.1 [104]. \textit{FBLN5} encodes an ECM protein that plays a role in maintaining the integrity of elastic lamina, such as that found in BM. Mullins et al. [105] examined fibulin 5 expression in human donor eyes and localized it to BM (deposits) and choriocapillaris in normal eyes and to pathologic basal deposits as well as some small drusen in AMD eyes. These data suggested a role for \textit{FBLN5} in extracellular deposit formation in AMD [105]. \textit{FBLN5} has also similarities with \textit{FBLN3}. Mutations in \textit{FBLN3} cause a rare, early-onset form of macular degeneration known as malattia leventinese (Doyne’s honeycomb dystrophy), a disease characterized by the presence of BM deposits with similarities to drusen in AMD [106]. Additionally, the \textit{Fbln5} (-/-) knock-out mice showed abnormalities in normal elastinogenesis [107], which may increase susceptibility to accumulate abnormal extracellular deposits and/or CNV later in life. Finally, Albig and Schiemann [108], showed interaction between vascular endothelial growth factor (VEGF) and \textit{FBLN5}. VEGFA is a member of the VEGF family and functions to increase vascular permeability, angiogenesis, cell growth and migration of endothelial cells. Activation of VEGFA mediates CNV [109,110]. While association between VEGFA and AMD remains controversial [111,112], two SNPs, one in the VEGFA promoter region (rs2010963) and one (rs4711751) near VEGFA, may be associated with AMD [113,102].

In \textit{chapter 8} we replicated the study of Stone et al. (2004) and revealed the identification of two novel heterozygous \textit{FBLN5} missense mutations possibly implicated in AMD. We further demonstrated \textit{in vitro} that these genetic variants may lead to AMD susceptibility due to reduced fibulin 5 secretion resulting in a corresponding reduction in elastinogenesis. Mullins et al. (2007) hypothesized that the \textit{FBLN5} missense mutations described in our study [114] could indeed potentially lead to reduced fibulin 5 secretion or to decreased affinity between \textit{FBLN5} and VEGF. Both mechanisms could possibly modify the regulation of VEGF [105]. Bhutto et al. suggested that if the local balance between pro- and anti-angiogenic factors is disturbed substantially in favor of VEGF, BM may become more vulnerable, thereby creating an environment permissive for CNV [115,116]. Additionally, a very recent paper describes an important functional role for \textit{HTRA1} in altered elastogenesis in BM through fibulin 5 cleavage [117]. \textit{HTRA1} is a key modulator of proteoglycans degradation in the ECM and one of the two genes located on the major AMD
susceptibility locus on 10q26. The HTRA1 protein was localized in drusen of AMD patients [118]. Accumulation of the protein in drusen could possibly compromise the integrity of the BM and so allow growth of choroidal capillaries and result in CNV. Vierkotten et al. (2011) showed that overexpression of HTRA1 in the mouse leads to AMD like features by fragmentation of the elastic layer in BM due to decreased fibulin 5 [117].

In conclusion, we conducted an extended review of BM, which showed that many aging processes affect BM, such as thickening of its layers due to lipid deposition, calcification of the elastin layer, oxidative stress and drusen formation. Clearly, these normal (subclinical) aging events may predispose BM and the RPE to disease. Furthermore, mutations in genes encoding ECM molecules might result in disturbance of the structural and/or functional properties of the BM, which may ultimately lead to the devastating clinical manifestations of AMD.

**Strengths and Weaknesses of Our Studies; Non-replication**

Both genome-wide linkage analysis and genome-wide association studies (GWAS) have been proven useful in detecting susceptibility genes of AMD. Numerous genome-wide scans repeatedly showed linkage between AMD and markers on the long arms of chromosomes 1 and 10 [9,119-124]. Later, association studies discovered two major risk variants, the Y402H polymorphism of the CFH gene on 1q32, and, subsequently, the ARMS2/HTRA1 locus on 10q26. Beside genome wide linkage and GWAS, an additional approach to find disease genes for complex disorders is to screen SNPs in functional candidate genes for a disease. For the studies performed in this thesis, candidate disease genes were selected in two ways: First, based on their previously reported association with AMD in the literature or second, based on the outcome of an Illumina SNP genotyping assay containing 1,536 SNPs. Potential AMD disease genes for this genotyping assay were selected on the basis of AMD associated gene expression studies of the RPE carried out by our research group. Subsequently, the results of the genetic association studies found in the initial discovery cohorts were replicated in a study population from the University of Southampton, Southampton, United Kingdom and/or (a subset of) populations of the International Age-related Macular Degeneration Genetics Consortium which includes eight international cohorts (see introduction section of this thesis) of patients and controls matched by age and ethnicity (all of European descent). One of the greatest challenges in interpreting genetic association studies (not only in this thesis), is the lack of consistent reproducibility. The association between AMD and CFH Y402H is an example of the common disease-common variant
hypothesis, which means that a relatively common allele consistently predisposes to a common disease [125,126]. This can be understood because of the vital role of the complement system in innate immunity and the critical role of \textit{CFH} in protecting host cells from the excessive attack of complement. The role of \textit{CFH} and the complement system is fundamental and wide-spread (common) in many pathological processes, including AMD. Yet, these “strong” common risk factors are rather an exception than rule. Initial associations between putative “weaker” AMD risk factors, such as genetic variants in \textit{ERCC6}, \textit{TLR3} and \textit{SERPING1} could not be consistently replicated in several other case-control cohorts [127-129]. Replication has become the gold standard for evaluating statistical results from genetic association studies. In general, the results of many reported studies can neither be replicated nor corroborated. Lohmueller \textit{et al}. [130] and Hirschhorn \textit{et al}. [131] reviewed large numbers of reported associations between common gene variants and disease and found that the majority is not robust. Inconsistent replication in genetic association studies can be caused by numerous factors including chance, variation in study design, phenotypic AMD (grading) differences, genotype errors, population substructure (stratification), environmental interactions and inadequate sample size [132-134].

Many of these factors were already discussed in previous chapters. The effect of these factors is stronger in small cohorts, such as defined by clinical subtypes of AMD. A limitation of our studies is that we selected for relatively common SNPs, with MAFs >10%. Consequently, we could have missed variants with MAFs <10% which might influence the disease phenotype. Also, for this thesis, 2 different study designs (prospective and case-control) were used, which may have affected the outcomes. Conversely, phenotypic grading differences may not have accounted for a large variability in our studies since all study subjects underwent clinical examination and stereoscopic color fundus photography after pharmacologic mydriasis, and the grading criteria were very similar for the studies. Study subjects were either graded according to the International Classification and Grading System for AMD (Reykjavik, Melbourne and Würzburg sites), Rotterdam modification of the International classification (Columbia, Iowa, Amsterdam and Rotterdam sites), or according to the classification established by the AREDS study (AREDS and Southampton). An issue that possibly may have played a crucial role in the outcomes of our studies is that in genetic association studies the allele frequencies of SNPs may differ due to sampling error or population differences. Several studies showed that extensive differences in genetic variation across different ethnic populations exist in terms of allele frequency, LD and haplotype structure [135-137]. Even across Europe, subtle geographic variation in allele frequencies exist [138-140]. Indeed, although all our
cohorts were from European descent (which can be considered a strength), in almost all of our studies we observed extensive differences in MAFs of SNPs from the tested genes between the studied populations. In our studies, this probably originated from varied ethnic composition within the populations of European descent. The Columbia study population originates mainly from Eastern Europe, whereas that from Iowa from Western Europe and Scandinavia. Replication failure of statistically significant independent genetic effects might occur when allele frequencies among populations differ and when the polymorphism under study interacts with one or more other polymorphisms at another locus [141,142]. For example, Greene et al. (2009) illustrated that under an epistatic model (gene-gene interaction), successful replication of a significantly associated SNP can decrease from 80% to 20% with a MAF change of less than 0.1 of an interacting SNP (with heritability’s ranging from 0.025 to 0.4)[141]. They also showed that allele frequency differences under an epistatic model could reverse the allelic outcomes (i.e., a risk increasing effect becomes a protective effect after replication). In chapters 3 and 5 of this thesis, we were not able to replicate initial findings and we found such opposite associations in highly comparable populations. This pattern of allelic reversal has been noted in replication studies of other candidate SNPs [130]. In a recent study investigating the potential causes of this “flip-flop” phenomenon, Lin et al. [142] suggested that flip-flop associations can occur due to variation in LD, or to unaccounted multi-locus effects and variation in inter-locus correlations.

**Future directions**

Our knowledge of the genetics of AMD has increased significantly in the past years. We expect even more advances within the next couple of years. GWAS and whole genome sequencing will allow us to find other loci and genes associated with disease susceptibility and offer us a better understanding of the molecular basis of AMD.

As mentioned above, one of the pitfalls in genetic association studies is lack of consistent reproducibility. We need to interpret associations in the perspective of differences in allele frequencies, LD and haplotype structure that occur in different populations or as a result of sample heterogeneity. The effect of one locus on disease risk may be inconsistent or missed entirely if we fail to examine it together with other known disease variants. Hence, we propose that SNPs that fail to replicate should be tested for interactions with other polymorphisms, predominantly when samples are collected from groups with distinct ethnic backgrounds or different geographic regions. Yet, the power to identify the interactions is limited particularly in the case of GWAS where the number of potential comparisons is enormous. Epistasis analysis
would also require adjustment for multiple comparisons to avoid the introduction of false-positives. Recently, Marchini et al. (2005) [143] and Evans et al. (2006) [144], demonstrated that, even considering a conservative multiple testing burden, it was possible to identify interacting loci that increased odds of disease using realistic sample sizes (approximately 1500 individuals).

Other strategies to elucidate the genetic influence on AMD may include the study of gene-environment interactions. To achieve this objective, large cohorts would be required, with the emphasis on harmonization of descriptions (e.g., primary features, age of onset, environmental factors) of AMD cases. This would allow for stratification into well-characterized phenotypes, which need to be standardized across multiple populations, such as GA and CNV or primary features, such as soft drusen. Environmental factors for stratification might include cigarette smoking and body mass index. Since AMD is such a complex disease, individual gene effects may only be exposed within patient subgroups with specific environmental exposures.

GWAS was designed to detect associations between disease and common SNPs (e.g., MAF>5%). Recent studies have revealed that common variants explain only a small fraction of the heritability [145]. Some studies suggest that not common, but rare variants are more likely to be pathological variants [146,147]. In order to capture rare variants, it will be necessary to sequence whole genome sections of cases. An effective approach seems to be next-generation sequencing, which holds great promise in cost-effectively detecting rare variants.

Genetic epidemiology provided us with the names of major molecular players in AMD and will reveal more in the next couple of years. Although this methodology paves the way to population and individual (genetic) risk assessments, it does not provide detailed mechanistic insight. Further functional studies are needed to specify the role of the identified players and reveal the pathophysiological process itself. Hopefully this will lead to intervention in the course of this devastating disease and improve the quality of life for AMD patients.
REFERENCES


