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

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Download date: 12 Dec 2018
CHAPTER 1

General Introduction
THE RETINA

The retina is the light sensitive neural tissue lining the inner surface of the eye. It is as thin as 0.4 mm and finds its embryologic origin in the developing brain. Therefore it may be considered a part of the central nervous system. The retina can be divided in the neural retina, adjacent to the vitreous, and the retinal pigment epithelium (RPE), which is attached to the vascular layer (the choroid) by Bruch's membrane (BM) (Figure 1). The neural retina is composed of multiple layers and contains many different cell types including the photoreceptor cells. Photoreceptors are the light-sensitive cells in the retina. They transform light into electrical and neurochemical signals that are transmitted through the optic nerve to the brain. The two main types of photoreceptors are cones and rods. Cones function in photopic conditions (bright light) and provide high spatial resolution and mediate color vision. Rods enable vision in scotopic conditions (dim light) and are used for contrast, brightness and motion perception.

The retinal pigment epithelium and Bruch's membrane

The RPE is a polarized monolayer of pigmented neuroepithelial cells, attached to the photoreceptors on the apical side, and to the BM and choroid on the basal side (Figure 1). RPE cells are post-mitotic cells, hexagonally shaped, densely packed and joined by tight junctions. The RPE constitutes the outer blood-retinal barrier and regulates the transport of ions, nutrients and waste products between retinal photoreceptors and choroid. The RPE performs several crucial functions in preservation of sight, such as phagocytosis and degradation of photoreceptor outer segments (POS), recycling of visual pigments, light absorption and regulation of the ion balance in the subretinal (between the neural retina and RPE) space [1].

BM is a five-layered sheath of connective tissue consisting of 1) the basement membrane of the RPE; 2) the inner collagenous layer; 3) the elastin layer; 4) the outer collagenous layer; 5) the basement membrane of the choriocapillaris. Together, the RPE and BM play an essential role as a barrier between the subretinal space and the choroid and in the selective transport of molecules between the neural retina and choroidal circulation [2].

The macula

The macula is an oval, yellow-colored area of approximately 5 mm in diameter located in the center of the posterior pole of the human eye (Figure 1). The yellow appearance is due to a high concentration of carotenoids in this area. The macula is
the most important part of the eye for high-resolution daylight vision due to the high concentration of cones. The central area of the macular region is represented by the fovea centralis (1.85 mm in diameter), which has a central pit, the foveola (diameter 0.35 mm). Outside the fovea is the parafovea (radius 0.5 mm), and the perifovea (radius 1.5 mm). The highest density of cone photoreceptor cells is found in the fovea. The relative highest density of rod photoreceptor cells in the retina is present in a circular perifoveal ring [3,4].

Figure 1. Normal Macular Anatomy (left) and Normal Macular Fundus Appearance (right).
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AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a late-onset, degenerative and progressive disorder of the macula with a multifactorial etiology [5]. It is the leading cause of severe visual impairment in the elderly in the Western world. As the elderly population is rising in number, AMD is becoming an important public health issue [6]. In the early stages of the disease, AMD causes no or hardly any visual impairment; however, in late or end stages it leads to irreversible loss of central vision. Important functions such as driving a car, reading and face recognition are lost. Individuals who have advanced stages of AMD experience a 60% reduction in the quality of life [7]. Persons suffering from low vision are prone to depression and social isolation [8,9].
AMD classification

A universal and uniform description of the disease phenotype is essential in order to study the genetics of a complex disease, such as AMD. The diagnosis of AMD can be made by visual inspection of the retina (fundoscopy or fundus photography). An international and uniform fundus grading system for AMD was established in 1995 by Bird and coworkers (The International Classification and Grading System for AMD)[10]. Since that time, other definitions, classifications and modifications have been proposed for grading AMD. The most commonly used classification is the one proposed by the Age-Related Eye Disease Study (AREDS) [11], which is comparable to the grading system described by Bird et al., but uses slightly different criteria for classification of early AMD and geographic atrophy (GA). Throughout this thesis, only studies using (a modification of) The International Classification and Grading System for AMD (Figure 2) or a similar protocol, like AREDS, were considered.

Yellow extracellular deposits between the basal membrane of the RPE and the inner collagenous layer of BM, called drusen, are the first hallmark of AMD [12-14]. Clinically, two main drusen types can be distinguished: hard and soft. Hard drusen are small deposits characterized by a light yellow color, round shape and distinct/well defined borders. Soft drusen may vary in size and shape, but are usually flatter and larger than hard drusen. They are characterized by pale yellow color and indistinct/ill-defined borders. Hard drusen sometimes develop into soft drusen. A subtype of soft drusen is reticular drusen. Klein et al. defined reticular drusen as ill-defined networks of broad, interlacing ribbons [15]. They have been shown to be associated with a high risk of progression to late stages of AMD.

The early stage of AMD (early AMD) is characterized by the presence of either soft distinct drusen with pigmentary irregularities or soft indistinct drusen without pigmentary irregularities and soft indistinct drusen with pigmentary irregularities (Figure 2; stages 2 and 3). At this stage, blurred vision and abnormal dark adaptation may occur; but the visual loss is relatively mild. Later stages of the disease (late AMD) involve two forms: the dry form (geographic atrophy; GA), and the wet (exudative) form, choroidal neovascularization (CNV). Combinations of both forms also exists (mixed AMD). GA is characterized by progressive atrophy of the RPE, choriocapillaris, and photoreceptors (Figure 2; stage 4) and patients start to lose central vision and experience visual scotomas (localized areas of decreased or missing vision) in or near the center of the visual field. GA is the most common end stage and accounts for 80-90% of AMD cases. CNV is characterized by abnormal growth of choroidal blood vessels through BM into the retina (Figure 2; stage 4). These new blood vessels, that grow in between the RPE and BM (sub-RPE space)
or into the subretinal space, are very fragile and tend to leak or bleed. This leads to accumulation of fluid and exudates or even hemorrhage in the sub-RPE and subretinal spaces. Vessel growth into the subretinal space causes damage to the photoreceptors and eventually results in detachment of the neural retina or the RPE. Consequently, vision abnormalities or loss of vision ensues [16]. Although CNV accounts for less than 15% of late AMD prevalence, it is responsible for more than 80% of cases of severe vision loss or legal blindness resulting from AMD [3].

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No signs of AMD</td>
</tr>
<tr>
<td></td>
<td>Hard drusen (&lt;63 µm) only</td>
</tr>
<tr>
<td>1</td>
<td>Soft distinct drusen (≤63 µm) only</td>
</tr>
<tr>
<td></td>
<td>Pigmentary abnormalities only, no soft drusen (≤63 µm)</td>
</tr>
<tr>
<td>2</td>
<td>Soft indistinct drusen (≤125 µm) or reticular drusen only</td>
</tr>
<tr>
<td></td>
<td>Soft distinct drusen (≤63 µm) with pigmentary abnormalities</td>
</tr>
<tr>
<td>3</td>
<td>Soft indistinct drusen (≤125 µm) or reticular drusen with pigmentary abnormalities</td>
</tr>
<tr>
<td>4</td>
<td>Geographic atrophy or choroidal neovascularization</td>
</tr>
</tbody>
</table>

Figure 2. Fundus Photographs Illustrating The Signs of AMD Graded According to a Modification of The International Classification and Grading System for AMD. (A). Stage 0; (B). Stage 1; (C). Stage 2; (D). Stage 3; (E). Stage 4 (geographic atrophy); (F). Stage 4 (choroidal neovascularization). Definitions of the stages are given in the text. Fundus photographs reprinted with permission from Redmer van Leeuwen et al. [17]. A color version of this figure can be found in the color figures section.
Prevalence of AMD

Globally, AMD may currently affect as many as 50 million people. Several population-based studies like the Beaver Dam Eye Study (BDES), The Blue Mountains Eye Study (BMES) and The Rotterdam Study [18-20] described a higher population prevalence of early AMD (7.2%-15.6%) in comparison with late AMD (1.6%-1.9%). A sharp increase can be seen after 75 years of age in the prevalence of late AMD, with percentages in age groups being: 0.2% (55-64 yrs), 0.9% (65-74 yrs), 4.6% (75-84 yrs) and 13.1% (>85 yrs) [21]. Given the overall aging of the population, it is estimated that by the year 2020 at least 80 million people will be affected by AMD [22]. However, the overall AMD prevalence is different across various ethnic populations. For example, the prevalence of AMD is relatively high in the Western (Caucasian) populations compared with Asian populations. Nonetheless, AMD is becoming a public health issue worldwide due to senescence, life-style factors and the quick shift in demographics [23,24].

Risk factors

AMD has a multifactorial etiology. Both environmental and genetic risk factors contribute to AMD pathogenesis [25].

Environmental risk factors

Multiple environmental factors have been described as potential risk factors for AMD, among which age and smoking are the most consistent [26].

Aging

Aging is the most important risk factor for AMD [21,27]. The mechanism by which age affects retinal health is unclear. It may relate to the accumulation of free radical related oxidative damage in the RPE and BM over the years. Indeed, oxidative damage may be the main driving force limiting longevity according to the free radical theory of aging [28,29].

Smoking

Numerous case-control, population-based, and prospective studies robustly point at tobacco smoking as a strong risk factor for AMD susceptibility. Tobacco smoking 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]. The number of cigarettes smoked is directly linked with the risk of developing advanced AMD [32]. Indeed, cigarette smoking has
numerous pathobiologically relevant effects: it reduces the plasma concentration
of antioxidants and the choroidal blood flow, and increases platelet adhesiveness,
hypoxia, and the release of free radicals [33].

Other, less consistent AMD risk factors include:

**Dietary antioxidants**
The harmful effects of oxidative stress in the retina may be reduced by dietary
antioxidants. High dietary intake of zinc, the carotenoids, lutein and zeaxanthin,
and of vitamins C and E, is associated with a reduced risk of AMD [34-36]. Vitamin
C is found in the retina and protects the eye from light damage [37]. Vitamin E is
found in the membranes of POS and is a powerful antioxidant that helps to protect
cell membranes against damage by free radicals [38]. Lutein and zeaxanthin (found
mostly in green leafy vegetables) are the two major components of the macular
pigment of the retina. These carotenoids also hold antioxidant properties and may
decrease the risk of AMD by absorption of short-wavelength, potentially phototoxic,
light [39,40].

**Female sex**
Female gender may be an AMD risk factor in persons aged over 75 years, with a
relative risk for CNV possibly twice as high in women compared to age-matched men
[21]. However, this issue is controversial, since regression models adjusting for age
as a continuous variable, rather than by decade, showed no statistically significant
effect of gender on the risk of AMD [41,42].

**Ethnicity**
Several studies have shown that AMD occurs more frequently in Caucasians than
in persons of other ethnic origins. AREDS, the National Health and Nutrition
Examination Survey III (NHANES-III), the Baltimore Eye Study (BES) and a
population-based study in Barbados found that rates of early AMD in black people
were similar to those found in white people. In contrast, the rates of CNV were lower
in black populations [27,43]. Klein *et al.* recently described AMD prevalence in 4
ethnic groups aged 45-85 years [44]. The prevalence in African-Americans was the
lowest (2.4%) whereas the prevalence in Hispanics (4.6%), Chinese (4.6%) and
Caucasians (5.4%) was higher.
Cardiovascular factors
Cardiovascular disease factors may also play a role in the development of AMD. However, so far, results of different studies have been inconsistent. Hypertension was shown to be associated with AMD in some studies [45-49], while other studies found no association [50,51]. The same holds for body mass index and the presence of cardiovascular disease (stroke, myocardial infarction or angina). The association between high cholesterol levels and AMD is also not clear: Tomany et al. described in 2004 that elevated total serum cholesterol levels were associated with a higher risk of GA, but not CNV [52], while Tan et al. described in 2007 that an increased total/high density lipoprotein (HDL) ratio predicted both CNV and GA [50]. Increased serum levels of HDL cholesterol may lower the risk of late AMD [47,52]. Some studies suggest a direct association between elevated serum and diet cholesterol concentrations and AMD [52,53].

Sunlight
Finally, sunlight exposure was suggested as a risk factor in the development of AMD [52]. Whether or not sunlight exposure is involved in AMD is not clear. The BDES implied that persons exposed to sun more than 5 hours a day (during their whole life) had a higher 10-year incidence of early AMD than those exposed less than two hours [54]. So far, other studies could not confirm this [45].

Genetic predisposition
In addition to environmental factors, genetic factors greatly influence the susceptibility to AMD. Genetic predisposition for AMD was first demonstrated already 30 years ago: Twin studies showed a clearly greater concordance of AMD among monozygotic twins than dizygotic twins [55]. Estimates of heritability ranged from 46% to 71%, the highest rates being for advanced AMD [56]. Familial aggregation studies also provided evidence that genetic factors may play a key role in AMD pathogenesis by showing a higher disease risk among the first-degree relatives of AMD probands [57].

Over the last years, enormous progress has been made in the dissection of the genetic susceptibility for AMD. Progress has primarily come from the outcome of genetic association studies, in combination with data from gene expression and functional studies. Association studies are based on a comparison of unrelated affected and unaffected individuals from a population. Apart from the (disease) trait(s) under investigation, these populations must be highly similar to avoid misinterpretations. Genetic association studies test whether a particular allele or mutation is present at
a higher frequency among affected as compared to unaffected individuals [58,59]. Below, we describe the most important genetic susceptibility genes in AMD one by one (APOE, CFH and other complement factors, ARMS2/HTRA1, LIPC and TIMP3). As early as 1998, Klaver and coworkers discovered the first susceptibility gene for AMD: apolipoprotein E (APOE) [60]. APOE is central to the metabolism of low density lipoprotein (LDL) cholesterol and triglycerides, and has been associated with increased risk of a number of age-related disorders [61]. The gene for APOE is located on chromosome 19q13.2, displaying three major allelic variants (ε2, ε3, and ε4). Klaver et al. showed a statistically significant protective effect of the APOE ε4 allele against AMD, whereas the ε2 allele may be associated with a slightly increased risk of AMD [60].

In 2005, a major susceptibility gene for AMD on chromosome 1q32 [62-64] was independently found in three different cohorts: The finding of the association between the Y402H polymorphism in the complement factor H (CFH) gene and AMD was a major breakthrough in ophthalmic research. The complement system is an important component of the innate immunity and plays amongst others a role in chronic low-level inflammation. Besides the CFH gene, four other genes of the complement system (complement factors B (CFB), I (CFI), 2 (C2), 3 (C3)) were found to be consistently associated with AMD. This indicates that the immune system plays a major role in the etiology of AMD [65-68]. Together, these associations account for a large part of the genetic contribution to AMD susceptibility.

Another major susceptibility locus for AMD has been mapped to the chromosome 10q26 region by several genome-wide linkage studies [69-71]. This locus contains two genes. As yet it is not clear which one of these (or both) genes is involved in AMD. The genes are: age-related maculopathy susceptibility 2 (ARMS2), also known as LOC387715 [72,73] and the high-temperature requirement factor A1 gene (HTRA1) [74-76]. Rivera et al. concluded that the A69S SNP in exon1 of the ARMS2 gene was the most likely susceptibility allele of AMD [73]. The ARMS2 protein was previously immunolocalized to the mitochondrial outer membrane in mammalian cells and to the cytosol [77-79]. Until now, the functional characterization of this gene has been incomplete, but variation in the ARMS2 gene may increase vulnerability for oxidative damage, possibly in association with smoking [72]. The HTRA1 gene encodes a heat shock serine protease which regulates the degradation of extracellular matrix (ECM) proteoglycans. Proteolytic cleavage of proteoglycans by HTRA1 enables access of additional degradative matrix enzymes, such as collagenases and matrix metalloproteinases, to their substrates [80]. HTRA1 is expressed in retina and RPE and was also found in drusen by immunohistochemical staining [74].
In summary, although progress has been made, the precise involvement of *ARMS2* and/or *HTRA1* in AMD remains to be elucidated. In 2010, a genome-wide association study (GWAS) showed an association between late AMD cases and a variant in the hepatic lipase gene (*LIPC*) [81]. This finding was replicated in eight additional cohorts [81,82]. *LIPC* is involved in triglyceride hydrolysis and it affects serum HDL cholesterol levels [83]. The T allele of the *LIPC* SNP rs493258 consistently increases HDL serum levels and decreases AMD risk across all study populations [81,82]. At the same time, Chen and co-workers identified yet another AMD susceptible locus 100 kb upstream of the metalloproteinase inhibitor 3 gene (*TIMP3*). The protein encoded by this gene is part of a family of inhibitors of matrix metalloproteinases, a group of peptidases involved in degradation of the ECM [82]. Interestingly, mutations in *TIMP3* were previously also found in patients with Sorsby fundus dystrophy, an early onset retinal disorder strongly resembling AMD [84]. Yet other candidate genes previously implicated in AMD are: *ABCA1, ABCA4, ABCR, ACE, C5, CETP, CFHR1-5, CST3, CX3CR1, ELOVL4, ERCC6, FBLN5, HMCN1, LPL, LRP6, PON1, SERPING1, SOD1-2, SYN3, TLR3-4, VEGF* and *VLDLR* [85-87]. The individual contribution of these genes as a risk factor for AMD appears to be heterogeneous or non-consistent across populations and relatively minor.

**AIM AND APPROACH OF THIS THESIS**

Altogether, the (major) AMD susceptibility genes described above as well as a number of environmental risk factors suggest an important role for lipid metabolism, inflammatory and oxidative stress pathways, and a role for the ECM in the etiology of AMD. Notwithstanding the recent discovery of the major genetic risks factors associated with genetic variants in *CFH* and *ARMS2/HTRA1*, a substantial part of the heritability of AMD still awaits clarification. The aim of this thesis was to further enlighten the genetic background of AMD based on these formerly implicated molecular pathways. In order to do so, we selected candidate genes based on their previously reported association with AMD and involvement in these AMD molecular pathways (oxidative stress, inflammation and ECM). In addition, we based our selection on the outcomes of our SNP genotyping assays that were done with 1.536 SNPs chosen from AMD gene expression studies carried out by our research group.
Microarray analysis and SNP genotyping assays

A part of our candidate gene identification was based on gene expression analyses (22k microarray, Agilent technologies) of in vivo [88] and in vitro RPE cells (Baas, unpublished data). For our in vivo studies, macular RPE cells from healthy human donor eyes (aged 63-78 years) were isolated by laser dissection microscopy and used for microarray studies. Gene expression was analyzed and functional annotation was performed in order to gain insight into the functional properties of the human RPE, as described by Booij et al. [88].

For our in vitro studies, human ARPE-19 cells were cultured for 84 days with and without the addition of bovine photoreceptor outer segments (POS) to the culture medium. Gene expression patterns were determined on several time points: 56 days (T0; start POS addition), 70 days (T1; 2 weeks POS addition) and 84 days (T2; 1 month POS) post-plating. Next, we performed functional annotation analysis in order to gain insight into the functional properties of the human ARPE-19 cell line (Baas et al.; unpublished results).

The above described microarray experiments showed amongst others a role for oxidative stress, ECM and inflammation pathways. Based on these results we selected a total of 45 AMD candidate genes and screened all known common genetic variants of these genes in the Amsterdam-Rotterdam-Netherlands (AMRO-NL) study population. All subjects were Caucasian and recruited from the Erasmus University Medical Centre, Rotterdam and the Netherlands Institute of Neuroscience, Amsterdam, the Netherlands. The analysis was carried out with the Illumina GoldenGate SNP genotyping assay, capable of multiplexing 1.536 SNPs in a single reaction.

Study populations for testing genetic association

In this thesis, 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. This consortium includes eight international cohorts (Columbia University, New York, USA; University of Iowa, Iowa City, USA; AREDS cohort, NEI/NIH, USA; Rotterdam Study, Erasmus University, Rotterdam, The Netherlands; The AMRO-NL study, The Netherlands Institute for Neurosciences (NIN), Amsterdam, The Netherlands; University Eye Clinic, Reykjavik, Iceland; University of Würzburg, Würzburg, Germany; and the Center for Eye Research Australia, University of Melbourne,
Chapter 1

Royal Victorian Eye and Ear Hospital (RVEEH), Melbourne, Australia. All cohorts contained patients and controls of European descent.

Outline of this thesis

In this thesis, we describe seven studies related to three pathobiological AMD mechanisms (oxidative stress, immunological aspects and ECM turnover):

Part 1. Aspects of oxidative stress

Oxidative stress was previously implicated in AMD. Indeed, the retina, and especially the RPE is exposed to high levels of oxidative stress. In this respect, a recent manuscript of Tuo and co-workers (2006) is of interest. They reported that a SNP (rs3793784) in the promoter region of the ERCC6 gene was associated with AMD. ERCC6 is involved in repair of oxidative DNA damage. In chapter 2, we analyzed the potential association of rs3793784 with AMD in four study populations. In addition, to examine a possible functional relationship, we determined ERCC6 mRNA expression levels in healthy and early AMD affected human RPE, isolated from human donor eyes. In chapter 3, we tested the hypothesis whether genetic variation in the glucose transporter 1 (SLC2A1) is associated with AMD. SLC2A1 regulates the bio-availability of glucose in the RPE, which, as we hypothesized, may influence oxidative stress mediated AMD pathology.

Part 2. Aspects of the immune system

Complement activation appears to play a key role in the pathogenesis of AMD. The complement component 5 (C5) protein was previously localized in drusen. C5 plays a role as a chemoattractant regulating local inflammatory processes, and plays a crucial role in the propagation of the complement reaction toward the membrane attack complex. In chapter 4, we describe an extensive association analysis to test the hypothesis whether complement C5 gene variants (independently) mediate AMD susceptibility. Chapter 5 portrays the involvement of a variant (rs2511989) in intron 6 of serpin peptidase inhibitor, clade G, member 1 (SERPING1) in the development of AMD. As a member of the classical complement pathway, SERPING1 is a plausible candidate gene. Two recent studies provided conflicting data about the association between this gene and AMD. To resolve this issue, we genotyped rs2511989, in seven large case-control studies involving individuals from European descent. Yet another gene involved in the innate immune system is toll-like receptor 3 (TLR3). Also for genetic variants in this gene, conflicting association results have
been described. In chapter 6, we, and our collaborators from the International AMD Genetics Consortium describe that the association between TLR3 and (dry) AMD is non-consistent between populations.

Part 3. Aspects of the extracellular matrix

Bruch’s membrane is a complex extracellular matrix located between the RPE and the endothelial cells of the choriocapillaris. Chapter 7 consists of a review of BM. The chapter starts out with an introduction about the normal structure and function of BM and the changes that occur with age. We subsequently describe the formation and composition of drusen and illustrate that abnormalities in the BM play a key role in the pathogenesis of AMD. Amongst others, fibulin proteins are widespread components of ECM. Previously, rare missense mutations in fibulin 5 (FBLN5) were reported in association with AMD. For that reason, we investigated the role of FBLN5 in AMD (chapter 8) and determined the functional effects of missense mutations on fibulin 5 expression. We also attempted to correlate the FBLN5 genotype with the AMD phenotype.

In the final chapter, chapter 9, we discuss the main findings of the thesis in the light of the current literature, we discuss strengths and weaknesses of the studies performed, and suggest future directions.
REFERENCES


