AGE-RELATED MACULOPATHY: A GENETIC AND EPIDEMIOLOGICAL APPROACH

Jacqueline JM Assink

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Chapter 4

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Chapter 6

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The international ABCR Screening Consortium. Statistically significant association of sequence variants in the ABCR gene with age-related macular degeneration. *Submitted*

CCW Klaver, JJM Assink, AAB Bergen, CM van Duijn. ABCR and age-related macular degeneration (technical comment). *Science* 1998; 279:1107.

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PART I

INTRODUCTION

- CHAPTER 1 -

INTRODUCTION

In the 19th century, age-related maculopathy (ARM) was described for the first time as an age-related abnormality of the macula lutea.[1,2] ARM consists of a variety of clinical signs, from the early stages with soft distinct drusen, indistinct drusen and pigment alterations up to the late stages of geographic atrophy and neovascular macular degeneration. These late stages are also referred to as age-related macular degeneration.[3] Nowadays, ARM is the leading cause of blindness among the elderly in the Western world.[4] With the rapidly growing population of the elderly, it can be estimated that in the Netherlands almost 115,000 people will have signs of the end stages in the year 2030.[5] This will have great implications for the quality of life of these patients, since age-related macular degeneration decreases central vision. Furthermore, treatment is only limited to a small group of patients.

Although many investigators have tried to unravel the pathogenesis of ARM, the knowledge of its etiology is still limited. Many environmental factors have been implicated as risk factors in ARM, however limited factors were consistent.[6,7] While already in 1875 the first description of a genetic component in ARM was given by Hutchington and Tay,[2] it was only since the last decade that research on the genetic factors in ARM has gained worldwide attention. Studies on the genetics of ARM have been hampered by the lack of suitable patient material and insight in complex diseases such as ARM.

The aim of this thesis is to obtain more knowledge on the disease frequency and its risk factors. Special attention is addressed to the genetic factors involved in ARM. The first part of this thesis gives an overview of the current knowledge on the genetics of ARM. In the second part the disease frequency and its risk factors are described. The third part covers two genetic-epidemiological approaches to study the genetics of ARM. The fourth part addresses two candidate genes for ARM. The first, TIMP-3, was studied in a large pedigree with Sorsby fundus dystrophy, which is a disease with clinical signs similar to ARM. The second candidate gene, ABCR, was studied in patients with diseases which are related to ARM, as well as in a large combined population with ARM. Finally, a general discussion concerning the main findings and methodological issues is given in the last part, together with suggestions for future research.

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- CHAPTER 2 -

GENETICS OF AGE-RELATED MACULOPATHY
A REVIEW

INTRODUCTION

Age-related maculopathy (ARM) is the leading cause of permanent visual impairment among the elderly in western countries.[1-6] ARM is a degenerative disorder affecting the macula that is characterized clinically by soft drusen, hypo and hyperpigmentations of the retinal pigment epithelium (RPE) and retina in the early stage, and by geographic atrophy, choroidal neovascularisation, pigment epithelium detachment and subsequent fibrous scarring of the macula in the late stage. These changes are all manifestations of the disease ARM, but the term age-related macular degeneration (AMD) is only used for the late stage. Age-related macular degeneration can be divided in two forms, geographic atrophy ("dry" form) and neovascular macular degeneration (also called "wet" form).[7-9] This end stage is not always easily distinguishable from other macular disorders as any chorioretinal inflammation or scar may result in the growth of a subretinal neovascular membrane. Therefore, neovascular macular degeneration sometimes resembles myopic macular degeneration, pseudoxanthoma elasticum, Paget disease, Stargardt disease, Best disease, juxtafoveolar telangiectasia, adult foveomacular dystrophy, pattern dystrophy, presumed ocular histoplasmosis syndrome, ocular toxoplasmosis, central areolar choroidal sclerosis, laser photocoagulation scars as well as more rare traumatic, inflammatory, toxic, and congenital processes.[8,10,11] These disorders have to be excluded before the diagnosis of AMD can be made. Moreover, there are racial differences in clinical expression. In the Japanese drusen accompanying AMD are rarer than in Caucasians.[12] In the Inuit there is often progressive peripapillary geographic atrophy, including the macular area in the later stages of the disease.[13]

Due to leakage from choroidal neovascularisation blurring or distortion of central vision can occur. The loss of vision is irreversible and is the result of degeneration of the photoreceptors in the macular area which occurs when the retinal pigment epithelium cells with which they are associated deteriorate and die.

The prevalence of AMD increases with advancing age. It affects less than one percent of 50 - 60 years olds, and up to 10 percent of those over age 85.[14-24] Studies suggest that racial variation exists in the frequency of ARM. ARM is rare among blacks and Hispanic Americans.[25-29] Incidence increases with aging and is estimated to be 1 per 1000 person years in 50-60 year olds, and 11.2 per 1000 person years in individuals older than 85.[30,31]

The growing population of the elderly and increased life expectancy necessitates research into the causes and risk factors of this disease. In the Rotterdam Study, Klaver et al studied the importance of ARM in relation to visual decline as a function of age. In subjects older than 75 AMD was the most important cause of incurable blindness. The impact grew with further increasing age to a prevalence of 6% of bilateral blindness or visual impairment due to AMD in those aged 85 and over.[5] This fact underscores the pressing need for research into potential causative factors of and treatments for AMD.

Although certain risk factors have been identified, no interventions have been proven effective in preventing AMD. Laser photocoagulation and photodynamic therapy have been established as an effective treatment, but the use of the former is confined to a small proportion of patients with the exudative form of AMD, and the long-term benefits are limited or unknown.[32-43] Besides age, smoking was found in all studies to be associated with AMD.[44-58] Other risk factors for AMD have been identified, but not consistently. These risk factors are cardiovascular factors, such as elevated blood pressure, obesity, history of cardiovascular disease, as well as estrogens and light exposure.[45,46,59-64] Also some ocular factors have been identified as possible risk factors for AMD, such as iris color, refractive error and cataract surgery.[25,45,46,50,65-74] Besides these risk factors, genetic factors play a role in the etiology of AMD.

The aim of this review is to give an overview of the various methods of locating the genes involved in AMD and to summarize the results of studies on genetics and AMD.

METHODOLOGICAL ISSUES

Since ARM develops only after the fifth decade of life, the study of heritability and genetics in ARM is difficult. The molecular genetic analyses have been hampered by the complexity of the diagnosis, multifactorial or polygenic inheritance, and the lack of multigenerational data within families.[75-77] In most cases the parents of the patients are deceased, while in the younger generations the disease has not yet manifested itself on a clinical level. Most families available for genetic studies are therefore limited to sibpairs. Another problem is the uncertainty about unaffected people, because it is never known which subjects might develop ARM in the near future. All apparently unaffected individuals, should be considered as persons with an unknown affection state for the purpose of genetic analysis.

In general, the methods available for genetic dissection of complex traits such as AMD can be divided into four categories: linkage analysis in families, allele sharing methods in sibpairs, association studies in human populations, and genetic and biological analysis of large crosses in model organisms such as mouse and rat.[78-85] An overview of these methods is given in Table 1.

Table 1 *Overview of analysis methods used for genetic diseases.*

	Linkage analysis	Allele sharing methods	Association studies
study population	families	related cases	unrelated cases compared with unrelated controls from a general population
scope of the method	identification of loci or genes causing the disease by positional cloning	applicable as linkage analysis or as association study	contribution of mutations in candidate genes to the disease
a priori knowledge	inheritance pattern	non parametric	non parametric
type of genetic mechanism	monogenic to oligogenic	monogenic tol multifactorial	multifactorial
disadvantage	- no direct conclusions for the general population - families with enough meiosis needed	- no direct conclusions for the general population - many sibpairs and markers are needed for genome screening	- not appropriate for genomic screening - only applicable if candidate genes are known
advantage	- very robust method for mendelian inheritance - applicable in genetic isolates - applicable for genomic screening	- sibpairs are easier to ascertain than families - applicable in genetic isolates	- data collection is relatively easy - direct conclusions for general population

In linkage analysis a transmission model is constructed to explain the observed inheritance of a disease in pedigrees.[86-89] The model is relatively straightforward for monogenic Mendelian inheritance but can become complicated for complex traits, because it may be difficult to construct a precise model that adequately explains the observed inheritance pattern. In linkage analysis the relationship of two or more markers or genes on a chromosome is investigated; the closer together genes or markers are, the lower the probability that they will be separated by recombination during meiosis. Consequently, it is more probable that they will be inherited together (see figure 1). Linkage analysis can be used to find the gene responsable for a disease by observing markers which are cosegregating with the disease in a pedigree. If they cosegregate in a significant number of meioses, then the disease-causing gene is likely to be closely localised to these markers. The likelihood that the observed inheritance of marker alleles and the inheritance of the disease are actually linked, compared with the likelihood that these observed inheritance patterns occur by random chance, is referred to as the LOD score (logarithm of the odds ratio for linkage).[87,88] Linkage analysis should be performed within families, because the specific marker allele linked with the disease generally varies from family to family, in accordance with the allelic distribution of the marker in the population. Such a linkage within a family is unlikely to be attributable to systematic bias, because most genetic markers segregate codominally without any consequences for the phenotype, and marker information is collected by laboratory analysis of biologic samples, independently of disease status.

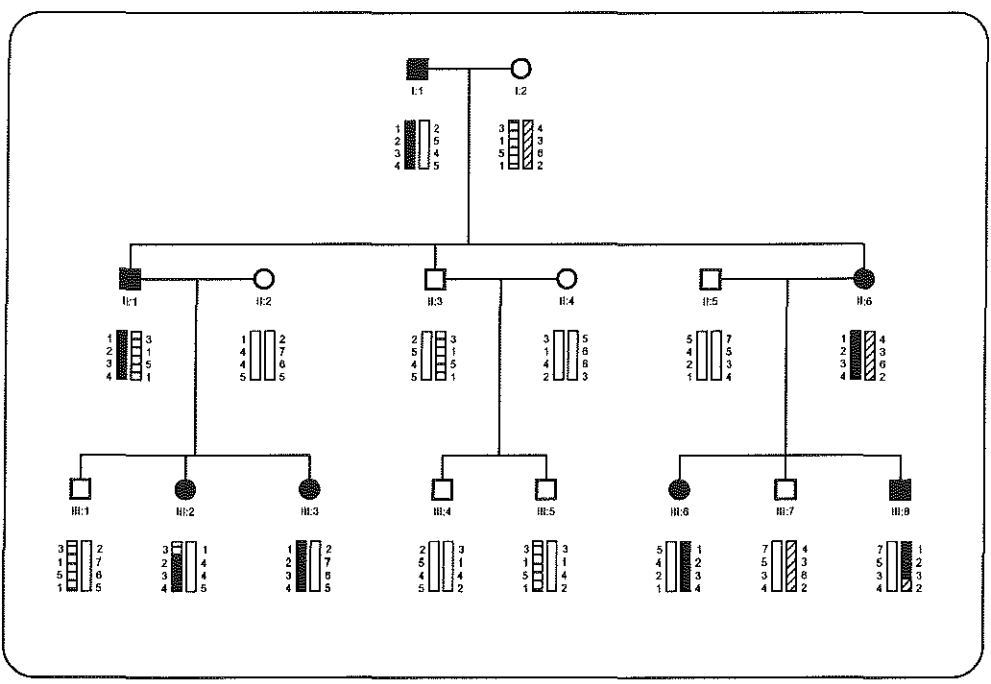

Figure 1 *An example of linkage analysis in a pedigree*

Males are represented by squares and females are represented by circles. The symbols of affected individuals are blackened. The bars beneath represent the haplotypes and the digits the corresponding alleles. Alleles are the natural variation of the DNA sequence at a certain locus (e.g. a marker). All individuals inherit one allele from their father and one from their mother. Therefore, one marker reveals per individual two alleles. The order of well-localized markers is generally known. Alleles from a group of closely linked markers in a known order and derived from one parent are usually inherit as a unit and form a haplotype.

Individuals I:1 and I:2 are the founders of this family, the male is affected with a blackened haplotype with alleles 1,2,3 and 4. This entire haplotype is cosegregating with the disease in his affected son (II:1) and daughter (II:6), both having the blackened haplotype with the alleles 1,2,3 and 4. His other son (II:3) is unaffected and has the other haplotype (with alleles 2,5,4 and 5) derived from his father. In the third generation, the two affected daughters (III:2 and 3) of the affected male (II:1) do have at least a part of the blackened haplotype. This is also the case in individuals III:6 and III:8. The fact that individuals III:2 and III:8 do have a part from both haplotypes from a single parent is caused by recombination. Recombination can occur during meiosis, in which part of the chromosomes (haplotypes) can be exchanged. The part of the chromosome which is cosegregating with the disease in this family is the blackened haplotype with allele 2 for the second marker and allele 3 for the third marker. In this family the disease-causing gene is probably located somewhere between the first and last marker. The recombination events define where the disease-causing gene is probably located. The more recombination events occur the more information can be obtained; however, part of the chromosome should be shared among the affected persons.

If ARM is studied by linkage analysis in one large family, it may be assumed that one or two genes are involved, unless spouses introduce ARM into the pedigree. Unfortunally, for complex traits such as ARM, there are many variables that complicate linkage methods. These include the late age of onset, unknown gene frequency, reduced penetrance, possible multiple genes contributing to the development of the disorder (heterogeneity) and phenocopies (cases of ARM that arise from non-genetic factors). For large family studies, the identification of mild cases might be essential to maximize the informativeness of the family and thereby increase the power. All unaffected individuals should be considered as unknown affection state.

Allele sharing methods are based on the identical by descent (IBD) principle (see figure 2A and 2B) and involve testing whether affected relatives inherit a region IBD more often than expected under random Mendelian segregation.[90] A special type of alle sharing method is the affected sibpair analysis. The alleles of sibpairs, both affected with ARM, are compared with each other. If more than the expected 50% IBD allele sharing is revealed in a region, then the disease-causing gene might be closely linked to this region. In contrast with linkage analysis this method is non-parametric (that is, it assumes no model for the inheritance of the trait).

IBD is less sensitive for genetic complications or clinical misclassification than linkage analysis but is less powerful than a correctly specified linkage model.[85,90] Sibling pairs for studies on ARM are in general easier to recruit than extensive pedigrees with affected family members, but for genomic screening many sibpairs are needed.

Another special type of this method is allele sharing in a family originating from a genetically isolated population. In such a population the number of founders is limited, therefore the number of mutations or genes involved in ARM is decreased compared to the general population. The haplotypes of all affected individuals in such a large family are compared.[91] If a certain haplotype is cosegregating with ARM, then the disease-causing gene might be closely linked to this region. Confirmation of the diagnosis is critical in allele sharing methods, because the presence of confounding clinical subtypes can limit the power of the study to detect a major gene. It might be better to exclude ambiguous mild or subclinical cases of ARM at the cost of reducing the power of the analysis.

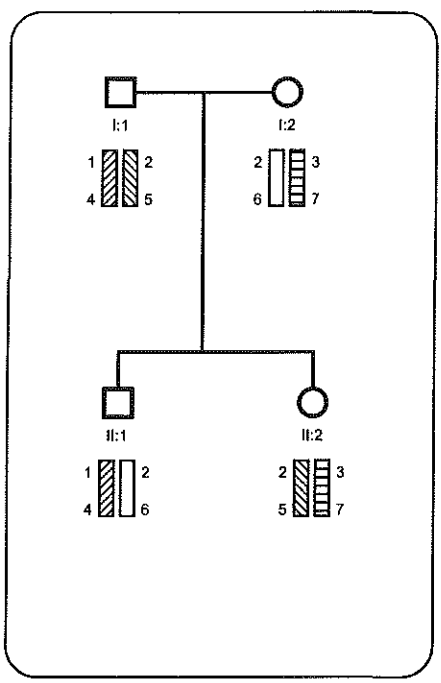

Figure 2a *Principle of identical by state (IBS)*

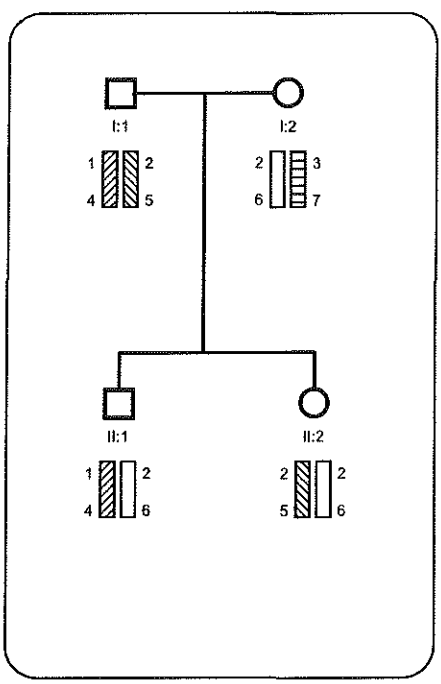

Figure 2b *Principle of identical by descent (IBD)*

Figure 2A shows a small family consiting of two parents (individuals I:1 and I:2) with a son (individual II;1) and a daughter (individual II;2). The son received alleles 1 and 4 from his father and alleles 2 and 6 from his mother.

The daughter also has allele 2, but she received this from her father (together with allele 5). These alleles 2 are identical by state, IBS (they both are allele 2, but received from a different parent). Figure 2B shows the same small family but the daughter has now received a different haplotype from her mother. Brother and sister both have alleles 2 and 6 received from their mother. These alleles are therefore not only identical by state but also identical by descent (IBD).

Association studies are based on a comparison of unrelated affected and unaffected individuals from a population. Association studies test whether a particular allele or mutation is present at a higher frequency among affected as compared to unaffected individuals.[84,85,87] In these genetic association studies, unrelated individuals are studied instead of families to test candidate genes. This facilitates data collection substantially. However, in genetically hetereogenous disorders multiple mutations in different genes may exist. This reduces the statistical power of genomic screens tremendously. Furthermore, for a genome screening one would need many markers close to one another in order to find a locus which would cosegregate among a part of the cases.[92] For purposes of candidate gene screening association studies might be helpful. A problem with association studies in late-onset diseases is the selection of the control population. Unaffected individuals might develop the disease in the near future depending on their genotype which can lead to false negative results. Furthermore, genotypes of at least controls should be in Hardy-Weinberg equilibrium, which means that in the general population mating is random with respect to the gene in question.[87,93]

Experimental crossing and biological analyses of mice or rats offer an ideal setting for genetic dissection of mammalian pathology and physiology. With the opportunity to study hundreds of meioses from a single set of founders, the problem of heterogeneity is reduced, and more genetic interactions can be studied than is possible for human families. Unfortunately, no animal models are available for ARM. One of the problems might be that the experimental animals used for this kind of analysis usually do not become old enough to get ARM or do not have a macula. Such an animal would tremendously stimulate research into AMD. Also mice with knocked-out candidate genes would be very helpful.

Recently, a knock out mouse of the ABCR gene was created.[94] The ABCR gene is involved in Stargardt disease, cone-rod disease and in some types of retinitis pigmentosa, and it appears to be involved in some AMD cases.[95-102] Mice lacking the ABCR gene show delayed darkness adaptation, a transient accumulation of all-trans-retinaldehyde (all-trans-RAL) following light exposure, elevated phosphatidylethanolamine (PE) in outer segments, accumulation of a protonated complex of all-trans-RAL and PE (N-retinylidene-PE), and deposition of a major lipofuscin fluorophore (A2-E) in retinal pigment epithelium (RPE). These data suggest that ABCR functions as a flippase to eleminate N-retinylidene-PE from the disc interior, thus protecting the RPE from accumulation of lipofucsin. Delayed darkness adaptation is likely due to a transient accumulation in discs of the complex between opsin and all-trans-RAL. The authors concluded that ABCR-mediated retinal degeneration may result from "poisoning" of the RPE due to A2-E accumulation, with secondary photoreceptor degeneration due to loss of the RPE support role.[94]

One of the major advantages of experimental animal studies is that the pathogenesis of a disease can be more easily investigated and more genetic interactions can be studied. The major disadvantage is that animal models, if they do not exist in nature, are difficult to obtain. A gene or protein has to be known in order to be able to produce a knock-out animal or a transgenic animal.

As ARM consists of a variety of clinical symptoms and is a complex disease, some of which have to be overcome in order to study the genetics of ARM. Linkage analysis in families is often impossible due to lack of suitable patient material. For sibpair analysis one can estimate that that at least 500-1000 affected sibpairs are needed for such an analysis to be meaningful and significant.[92, 103] Combination of the linkage method with the allele sharing method increases the power of both methods. One point of consideration is the combination of populations. If the families from the linkage analysis and the affected sibpairs with a single defined phenotype are derived from the same population, one might expect that in both cases the disease-causing gene or region is the same. However, when the families and the sibpairs are from genetically different populations, the disease-causing gene or region might be quite different, due to the heterogeneity of the populations. Association studies in populations are not appropriate for genomic screening for ARM. For a candidate gene approach, however, they are useful. If experimental animal models with ARM or with one of its clinical sign exists, they could be useful for studying the pathogenesis of ARM.

EVIDENCE OF A GENETIC CONTRIBUTION TO AMD

The evidence for genetic contribution to AMD in humans can be divided into three parts: twin studies, research into familial aggregation and segregation analysis. These three provide compelling arguments for the role of genetics in AMD. However, they provide only approximate estimates as to the complexity and extent of the genetics of AMD.

Twins are uniquely matched for age and many environmental factors, and therefore form a fixed sample design provided by nature. Monozygotic twin are genetically identical, while dizygotic twins have the genetic similarity of other siblings (on average 50% of their genome is identical). Twins have been used to study the role of genetic factors for a large number of different phenotypes. The aim of a twin study is to compare similarities and/or differences in phenotype in monozygotic and dizygotic twins in order to infer and measure the genetic contribution to their phenotype. Greater concordance in monozygotic twins as compared to dizygotic twins argues in favor of genetic factors. Discordance in monozygotic twins underscores the role of environmental factors. There are relatively few twin studies on AMD. Three case reports exist on identical twins having atrophic and/or neovascular macular degeneration as well as one study of nine monozygotic twin pairs having a high concordance in either extensive drusen or late stage age-related maculopathy.[104-106] Klein and co-workers noted that eight of nine monozygotic twin pairs had similar fundus appearances.[107] In the ninth pair, one twin had advanced neovascular AMD, while the other had large confluent drusen.

Meyers et al compared concordance in the presence or absence of any type of ARM in 98 monozygotic and 38 dizygotic twin pairs, predominantly ascertained through twin organizations.[108] Of all twin pairs affected by ARM, all monozygotic twin pairs were concordant (25/25), while only 42% of dizygotic pairs were concordant. Selection bias or environmental factors may have played a role, but the striking similarity of fundus appearances in each twin pair suggests an increased genetic susceptibility in at least some of the patients.

Although Hutchinson and Tay have observed familial occurence of ARM as early as 1875, the disease has not been the subject of extensive genetic investigations.[109] Familial studies incorporating the late stages of ARM have received comparatively little attention. The aim of a family aggregation study is to ascertain whether there is an increased risk of AMD in relatives of affected persons as compared to relatives of unaffected individuals or to the general population. Hyman et al. were the first to compare on history data the prevalence of ARM among the relatives of affected individuals to that among the relatives of a control population.[45] They found that a positive family history of ARM was reported in 21.6% of affected subjects as compared to 8.6% of unaffected controls. Two clinic-based studies found a positive family history of macular disease in patients with drusen or pigmentary changes and/or atrophic and neovascular macular degeneration. The authors estimated a familial risk of 19,3 and 2,4 respectively.[110-112] Possible explanations for this large difference are the high chance of selection bias with hospital-derived probands, the large range of ARM features that were combined, and the use of family history and self-reported diagnoses. Klaver et al reported on familial aggregation as part of the Rotterdam Study.[113] Independent of other risk factors they found on examination of cases and controls that the prevalence of early and late ARM was significantly higher in relatives of patients with late ARM. The lifetime risk ratio of late ARM was 4.2. First-degree relatives of patients with late ARM developed early ARM at an increased rate at a relatively young age. The authors concluded that almost one fourth of all late ARM cases in the general population may have a genetic component and that genetic susceptibility may play a role in determining the onset of disease.

Observations on familial occurence of ARM indicate a genetic factor in ARM. Francois described brothers and sisters in one family who were suffering from senile macular dystrophy.[114] Bradley stated in 1966 that every patient with ARM he had seen had other members of the family which were similarly affected.[115] Gass performed a study of 200 patients with macular drusen and reported that 10-20% of affected individuals in his study had a positive family history of loss of central vision.[116] Klein et al. performed a linkage analysis in a family consisting of 21 family members of which 10 were affected.[117] Recently, we have identified a large family with 22 affected individuals with ARM, of which nine with early ARM and 13 with late ARM (see Chapter 6 of this thesis).

Segregation analysis tests explicit models of inheritance on family data. The analytic strategy relies heavily on fitting genetic models, along with a few non-genetic ones, and selecting the model that best explains the data. So far only one segregation analysis was performed, on data from the Beaver Dam Eye study.[118]

The Beaver Dam population provided evidence for an autosomal dominant trait for AMD with a major gene effect and rejected the hypothesis of a random environmental major effect .This approach, however, provides no insight into the number of genes involved in all these families. One can only conclude from this that within one family the ARM features are quite similar and within one family probably caused by a single gene.

CANDIDATE LOCI AND GENES

1Q25-1Q31

Klein et al identified a large family consisting of 21 family members in which ARM was segregating as an autosomal dominant trait.[117] In the 10 affected family members ARM was manifested by the presence of large, soft, confluent drusen accompanied by varying degrees of retinal pigment epithelial degeneration and/or geographic atrophy. After an initial pooled genome-wide screen, markers from areas most suggestive of shared chromosomal segments were used to genotype all potentially informative family members. Genetic analysis of this family produced significant evidence for linkage of the disease-gene to a region of approximately 9 cM on chromosome 1q with a LOD score of 3.0. The disease causing gene in this predominantly dry AMD family was located at 1q25-1q31, whereas the ABCR gene, located on 1p21, was excluded as a major gene effect in this family.

ABCR

The photoreceptor cell-specific ATP-binding transporter gene (ABCR), located on chromosome 1p21, is exclusively expressed in the retina.[119, 120] The ABCR protein is identical to the rim protein (RmP), a protein of unknown function, that colocalizes with peripherine/RDS to the rod outer segment.[121] ABCR binds ATP, which is consistent with its role as an active transporter. ABCR is phosphorylated in response to light, which suggests that it plays a role in photo-transduction. Mutations in this gene have been implicated in Stargardt disease, retinitis pigmentosa type 19 and cone-rod dystrophy.[95-102] Recently, Allikmets et al described that variations in ABCR were associated with AMD.[96] Most of these mutations were found in patients with the dry type of AMD. Furthermore, some of these mutations were seen in Stargardt disease as well as in AMD patients. After this publication a scientific debate started; main points of discussion were flaws in study design, analysis as well as potential misclassification of the cases.[122, 123] Moreover, results from two other studies did not confirm these data.[124, 125] Large population studies, animal studies or functional ABCR assays are needed to clarify the involvement of ABCR in AMD. Clinical and histopathological examination of the recently constructed ABCR knock-our mouse will be helpful.

TIMP-3

The tissue inhibitor of metalloproteinases-3 (TIMP-3) is localized on chromosome 22q12.1-q13.2.[126-128] Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of the matrix metalloproteinases, which are involved in the synthesis and degradation of extracellular matrix molecules, thereby continously remodelling this matrix.[129] Mutations in TIMP-3 were demonstrated in affected members of two pedigrees with Sorsby fundus dystrophy.[130, 131] Sorsby fundus dystrophy on chromosome 22 is an autosomal dominant macular dystrophy that phenotypically resembles AMD. Sorsby fundus dystrophy is characterised by choroidal neovascularisation and macular disciform scarring in younger people.[132-137] The demonstration that Sorsby fundus dystrophy is due to mutations in the proteinase inhibitor TIMP3 has potential implications for our understanding of the pathophysiology of choroidal neovascularisation and possibly AMD. De la Paz identified 38 multiplex families with extensive intermediate drusen, any large drusen, geographic atrophy or evidence of exudative maculopathy.[138] These families were used to perform a linkage analysis and an association study with the TIMP3 gene. No evidence of linkage or association or both was found between AMD and TIMP3 in the families. However, the authors could not exclude the possibility that a subset of cases could be caused by TIMP3 locus, due to several difficulties in evaluating candidate genes in complex diseases, such as hetereogeneity, multiple underlying trait loci, misclassification of the phenotype, and potential phenocopies.

APOLIPOPROTEIN E

Apolipoprotein E is the major apolipoprotein of the central nervous system and an important regulator of cholesterol and lipid transport.[139, 140] It appears to be associated with neurodegenerative diseases, such as Alzheimer disease. Apolipoprotein E mobilizes and redistributes lipids for maintenance and repair of neuronal cell membranes. The apolipoprotein E gene (ApoE), located on chromosome 19q13.2, is polymorphic. Klaver et al. performed a genetic association study among 88 AMD cases and 901 controls derived from a population-based cohort.[141] The Apo ϵ4 allele was significantly associated with a decreased risk for AMD (Odds ratio 0.43; 95% CI 0.21-0.88). Furthermore they investigated whether ApoE is involved in the pathogenesis of AMD, using immunoreactivity in 15 AMD and 10 control maculae. They found that ApoE staining was consistently present in drusen and basal laminar deposits of post-mortem human AMD maculae. Souied et al evaluated ApoE alleles among 116 unrelated patients with exudative AMD in one eye and hard or soft drusen in the other eye.[142] They compared the frequency of these alleles with a control population of 168 age- and sex-matched controls. They found a lower frequency of the Apo ϵ4 allele in the case group compared to those in the control group (0.073 vs 0.149; $p<0.006$).

OTHER CANDIDATE GENES

The analysis of candidate genes involves an educated guess about the gene or gene loci that may be involved in the disease. Candidate genes for ARM might be selected on the basis of their known function(s) as well as known involvement in other forms of inherited retinal degeneration or chromosomal position. Potential candidate genes/loci include regions linked to early onset macular degeneration with the same phenotypical features of ARM. For example, isolation of the gene responsible for Best disease or Stargardt disease may lead to elucidation of a role played by these or similar genes in AMD. An overview of candidate genes for AMD is given in Table 2.[143, 144]

Table 2. *Candidate genes for Age-related maculopathy*

Locus	Gene	Disease
1p31	RPE65	Leber congenital amaurosis
1p21-p13	ABCR	STGD, RP, cone-rod dystrophy, ARM
1q25-31	unknown	ARM
2p16-p21	DHRD	Doyne honeycomb retinal degeneration, Malattia Leventinese
6p21.2-cen	RDS	RP, dominant vitelliform MD
6p21.1	COD3	dominant cone dystrophy
6q	CORD7	dominant cone-rod dystrophy
6q11-q15	STGD3	dominant MD, Stargardt like
6q14-q16.2	MCDR1	North Carolina MD
6q25-q26	RCD1	retinal cone dystrophy
7p21-p15	CYMD	dominant cystoid MD
8q24?	VMD1	dominant atypical vitelliform MD
10q24	RBP4	recessive RPE degeneration
11q13	VMD2	dominant MD, Best type
13q34	STGD2	dominant MD, Stargardt-like
17p13-p12	CORD5	dominant cone-rod dystrophy
17p13.1	CORD6	dominant cone-rod dystrophy
17q	CORD4	dominant cone-rod dystrophy
18q21.1-q21.3	CORD1	cone-rod dystrophy, de Grouchy syndrome
19q13.2	APOE	neurodegenerative diseases, ARM
19q13.3	CORD2	dominant cone-rod dystrophy
22q12.1-q13.2	TIMP3	Sorsby fundus dystrophy

With the use of microsatellite markers close to a gene or to a chromosomal locus, given the availability of a large family, linkage analysis can be performed in order to determine whether the disease can be caused by the candidate gene. Another technique based on candidate genes is the screening for mutations in these genes. In contrast to linkage analysis, in which a disease locus can also be used, in candidate gene screening the gene has to be characterized. The nucleotide sequence of the gene is compared between in affected patients and unaffected controls.

CONCLUSIONS AND FUTURE STRATEGIES

Age-related macular degeneration seems to be partially genetically determined. The late onset of the disease, the clinical and genetic heterogeneity, and the multifactorial or polygenic inheritance complicate the study of the genetic causes of the disease.[145] Given the many problems that can hamper genetic dissection of complex traits such as AMD, various different techniques should be combined. Futhermore, by narrowing the definition of a disease, it is often possible to work with a trait that is more nearly Mendelian in its inheritance pattern and more likely to be homogeneous. Four criteria are often used: clinical phenotype, age at onset, family history, and severity.[145, 146] Another way to improve the prospects for genetic dissection is to focus on specific ethnic groups. Population genetic theory and data suggest that there will be greater genetic and allelic homogeneity in a more genetically isolated group than in a large, mixed population.

Which technique should be used to unravel the genetics of ARM depends on the availability of patient material. Linkage analysis does not seem to be a promising technique to detect new genes involved in ARM, as families with ARM patients in multiple generations are rare. However, when a large family with ARM is obtained, linkage analysis is the best method to use. An alternative approach is to examine affected sib-pairs, but this requires at this moment at least 500 sibpairs. Association studies are important to determine the contribution of newly identified mutations in genes to the occurrence of disease. For this purpose, population-based studies are needed in order to overcome referral bias, whereas the problem of survival bias can only be overcome in a follow-up setting. As the Human Genome Project advances further in identifying genes, the opportunities for mutation analysis in candidate-genes will increase.

Further research is into the pathogenesis of AMD is necessary in order to eventually develop effective therapy.

METHOD OF LITERATURE SEARCH

Literature selection for this article was based on a MEDLINE search covering the past 30 years for all articles, using key word combinations including age-related macular degeneration, age-related maculopathy, genetics, genes, segregation, association, aggregation, twins, macular dystrophy, macular degeneration, linkage analysis and (genetic) epidemiology.

For review of topics not yet published, literature selection was also based on references from these articles and on abstracts from the annual meeting of the Association for Research in Vision and Ophthalmology, focusing on the years 1995-1999. We intended to cite a wide range of previous work to achieve an objective and balanced review on the topic. Given the broad scope of the topic, however, we were forced to limit citations to those articles that were most clinically relevant or represented new contributions to the field.

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PART II

DISEASE FREQUENCY

-CHAPTER 3-

RISK FACTORS FOR AGE-RELATED MACULAR DEGENERATION: POOLED FINDINGS FROM THREE CONTINENTS

ABSTRACT

Objective:	To assess the prevalence and potential risk factors for late age-related macular degeneration (AMD) in three racially similar populations from North America, Europe and Australia.
Design:	Combined analysis of population-based eye disease prevalence data.
Participants:	14752 participants with gradable photographs from the Beaver Dam Eye Study (n=4756), Rotterdam Study (n=6411) and Blue Mountains Eye Study (n=3585).
Main Outcome Measures:	AMD diagnosis was made from masked grading of stereo macular photographs. Final classification of AMD cases was agreed byconsensus between study investigators.
Results:	AMD prevalence was strongly age-related. Overall, AMD was present in 0.2% of the combined population aged 55-64 years, rising to 13% of the population aged over 85 years. Prevalence of neovascular AMD (NV) increased from 0.17% among subjects aged 55-64 years to 5.8% for those aged over 85 years. Prevalence of pure geographic atrophy (GA) increased from 0.04% to 4.2% for these age groups. There were no significant gender differences in the prevalence of NV or GA. Subjects in the Rotterdam population had a significantly lower age and smoking adjusted risk of NV than subjects in the Beaver Dam and Blue Mountains populations. Apart from age, tobacco smoking was the only risk factor consistently associated with any form of AMD in all sites separately, and in pooled analyses over the three sites.
Conclusions:	These combined data from racially similar communities across three continents provide strong and consistent evidence that tobacco smoking is the principal known preventable exposure associated with any form of AMD.

The causes of age-related macular degeneration (AMD), the leading cause of blindness in the western world[1-5], remain unknown. Tobacco smoking has now been accepted by most investigators as an important environmental exposure associated with AMD [6-13], but other risk factors apart from age and heredity remain controversial. The situation is further complicated by the repeated suggestion that AMD is not a homogenous diagnosis. Clinicians, pathologists and researchers alike refer to two principal late stage lesions (which possibly have different etiologies) that constitute AMD[14]: (1) "geographic atrophy" (GA) and (2) "neovascular AMD" (NV).

Most studies investigating associations between AMD and potential risk factors are hindered by the relative infrequence of AMD in the population. This lack of statistical power is further aggravated when the investigator examines differences in risk factors between the two subtypes of AMD. Consequently, some studies have reported their findings as associations with AMD as a homogenous identity because they had limited power to make statements about the separate types.

Three population-based studies conducted in Beaver Dam, Wisconsin[15], in Rotterdam, the Netherlands[16] and in the Blue Mountains area, west of Sydney, Australia[17] have provided prevalence estimates for GA and NV. These three studies commenced examinations during 1988, 1990 and 1992, respectively. They used very similar methods, including a standardised technique for grading stereoscopic macular photographs, based on the Wisconsin Age-related Macular Grading System[18]. Although differences in AMD prevalence were found, each study reported approximately a 2:1 ratio of NV to GA[15-17]. This matches the recent 5-year incidence report from the Beaver Dam Eye Study which found that NV incidence (0.4%) was twice the incidence of GA (0.2%)[19]. Besides using very similar outcome measures, these three sites used similar exposure measures for a range of potential risk factors.

Use of the same grading methods and similar risk factor measures in the three population-based reports[15-17] permitted us to pool data on prevalence of, and risk factor associations for AMD and its subtypes. We aim in this report to describe the prevalence of NV and GA, and examine factors associated with these diagnoses, in the combined population of racially similar subjects who attended large population-based epidemiological studies of eye disease in three continents (North America, Europe and Australia).

METHODS

The Beaver Dam Eye Study (BDES) was conducted during the period 1988-1990 in Beaver Dam, Wisconsin and examined 4926/ 5925 eligible residents aged 43-86 years (response 83.1%), identified in a private census. The Rotterdam Study (RS) was conducted during the period 1990-1993 in Rotterdam, the Netherlands and examined 7983/10,275 eligible residents aged 55-106 years (response 77.7%), identified from the municipal registers. The Blue Mountains Eye Study (BMES) was conducted during the period 1992-1994 in two postal codes of the Blue Mountains area,

west of Sydney, Australia and examined 3654/ 4433 eligible residents aged 49-97 years (response 82.4%), identified in a private census. The populations in all three studies were almost entirely white, with most subjects in the two non-European sites having a northern European ethnic origin. All studies employed a similar masked photographic grading technique, based on the Wisconsin Age-related Maculopathy Grading System (WARMGS)[18]. The standard WARMGS grid was modified for grading the Rotterdam photographs, because of the difference in magnification between the Zeiss 30° and Topcon 35° photographs. During grading, one of the stereo-photographs was overlaid by a grid and the stereo pair was viewed by the grader using a Donaldson stereo-viewer with 5× magnification. Among participants examined in the three studies, gradable photographs of at least one eye were available to assess the presence of AMD for 4756 Beaver Dam participants (97.9%), 6411 Rotterdam participants (80.3%) and 3585 Blue Mountains participants (98.1%). These 14,752 subjects provided the data used to assess prevalence of age-related macular degeneration. All analyses of risk factors used data from only those subjects aged 55 to 86 years (total 12,468 subjects), the common age range for the three sites. The proportion of participants with ungradable photographs increased with increasing age. Among subjects aged 55 to 64 years, 0.9% of Beaver Dam participants, 8.9% of Rotterdam participants and 1.3% of Blue Mountains participants had ungradable photographs. For subjects aged 65 to 74 years, corresponding proportions were 1.9%, 14.5% and 0.9% and for those aged 75 to 86 years, the proportions were 6.6%, 27.0% and 3.4%, respectively. All denominator and numerator populations for each site within this age range are presented in the Tables presenting age-adjusted associations with AMD.

The two late stage lesions of age-related maculopathy are now termed "age-related macular degeneration" (AMD), using nomenclature adopted by the International ARM Study Group[14]. One of the two lesions, termed "geographic atrophy" (GA) by Gass[20], was defined as a discrete area of retinal depigmentation, at least 175 μm in diameter, with a sharp border and visible choroidal vessels. The second, more frequent lesion, termed "neovascular AMD" (NV), was defined to include serous or hemorrhagic detachment of either the retinal pigment epithelium (RPE) or sensory retina, the presence of subretinal or sub-RPE haemorrhage or subretinal fibrous tissue. Eyes in which minimal subretinal fibrosis was present as well as widespread surrounding atrophy were also arbitrarily classified as NV as it was considered that this sign indicated a previous neovascular AMD lesion.

Senior investigators from each of the three sites (RK, PTVMdJ & CCWK, PM) adjudicated all cases graded as having NV or GA in either or both eyes. Some AMD cases previously included in earlier prevalence reports, including six from each of the Beaver Dam and Blue Mountains Studies and four from the Rotterdam Study, were excluded from the analysis after adjudication. This was due to either poor photographic quality or because the investigators thought that the signs could represent other disease processes[21], such as adult vitelliform lesions[22], myopic retinopathy[23], retinal dystrophy or infective/ inflammatory lesions.

Four cases initially graded as NV were excluded because their appearance was consistent with the drusenoid type of RPE detachment, resulting from confluence of soft drusen material[24].

Subjects with AMD were classified as having 'Pure GA' when GA was present in one or both eyes and neither eye had signs of NV. Subjects were classified as having 'mixed AMD' when GA was present in one eye and NV was present in the fellow eye. Subjects with NV as the only AMD type present were classified as having 'Pure NV'. If both GA and NV were present in one eye, this eye was classified as having NV.

Data sets from each study site including age, gender, tobacco smoking status and other key variables were merged into a combined dataset for statistical analysis using SAS (Statistical Analysis System, SAS Institute, Cary, NC).,

Age-standardized rate ratios and logistic regression were used to assess differences in prevalence of AMD and its sub-types between study sites, and to investigate associations with major potential risk factors reported for AMD - age, tobacco smoking, gender, high blood pressure and other cardiovascular disease diagnoses, cholesterol, iris colour, age at menopause, and hormone replacement therapy. All sites included a self-reported history of doctor diagnosis of high blood pressure, and measured systemic blood pressure in a clinic. Hypertension was deemed to be present if a subject had a history of doctor-diagnosed hypertension or measured systolic blood pressure of $\geq$160mm Hg, or measured diastolic blood pressure of $\geq$90mm Hg. All sites contained similar questions about current smoking and ever smoking. Smoking was categorized as current smoker (cigarettes, pipe or cigars), ex-smoker and never smoker. All sites measured height and weight. Body mass index (BMI) was calculated from measured height and weight using the formula weight (kg) divided by height (meters) squared. Categories used included underweight (BMI<20), normal BMI ($20 \leq BMI \leq 25$), overweight ($25 < BMI < 30$) and obese ($BMI \geq 30$). All sites included self-reported doctor-diagnosed history of cardiovascular diseases (acute myocardial infarction, angina, stroke), self-reported age at menarche and age at menopause, and self-reported history of ever using hormone replacement therapy. Iris color was graded before pupil dilatation using illuminated standard iris color slides in the Beaver Dam and Blue Mountains sites and using standard color prints at the RS site. Cholesterol and HDL-cholesterol were measured from fasting blood at all sites, and results converted to mmol/L. Associations between exposures and outcomes were analyzed for each site separately, then combined using dummy variables for study site to assess heterogeneity across study sites.

RESULTS

CHARACTERISTICS OF THE COMBINED POPULATION

The combined population with gradable photographs totalled 14,752 participants aged from 43 to 99 years (mean age 66.0 years), and included 8464 women (57.4%) and 6288 men (42.6%) (Table 1).

Table 1. *Prevalence of age-related macular degeneration, including geographic atrophy and neovascular AMD in participants to population-based eye surveys in three continents.*

age group (years)	<55	55-64	65-74	75-84	85+	all ages
Beaver Dam Eye Study	**n=1501**	**n=1300**	**n=1243**	**n=675**	**n=37**	**n=4756**
pure geographic atrophy	0	1 (0.08%)	4 (0.32%)	14 (2.07%)	2 (5.41%)	21 (0.44%)
pure neovascular AMD	0	4 (0.31%)	13 (1.05%)	23 (3.41%)	2 (5.41%)	42 (0.88%)
mixed AMD	0	0	0	8 (1.19%)	1 (2.70%)	9 (0.19%)
any AMD	0	5 (0.38%)	17 (1.37%)	45 (6.67%)	5 (13.51%)	72 (1.51%)
Rotterdam Study	**Nil**	**n=2340**	**n=2358**	**n=1350**	**n=363**	**n=6411**
pure geographic atrophy	-	1 (0.04%)	8 (0.34%)	17 (1.26%)	16 (4.41%)	42 (0.66%)
pure neovascular AMD	-	2 (0.09%)	7 (0.30%)	23 (1.70%)	14 (3.86%)	46 (0.72%)
mixed AMD	-	0	1 (0.04%)	3 (0.22%)	12 (3.31%)	16 (0.25%)
any AMD	-	3 (0.13%)	16 (0.68%)	43 (3.19%)	42 (11.57%)	104 (1.62%)
Blue Mountains Eye Study	**n=478**	**n=1157**	**1=1198**	**n=631**	**n=121**	**n=3585**
pure geographic atrophy	0	0	2 (0.17%)	10 (1.58%)	4 (3.31%)	16 (0.45%)
pure neovascular AMD	0	2 (0.17%)	6 (0.50%)	21 (3.33%)	14 (11.57%)	43 (1.20%)
mixed AMD	0	0	0	3 (0.48%)	3 (2.48%)	6 (0.17%)
any AMD	0	2 (0.17%)	8 (0.67%)	34 (5.39%)	21 (17.36%)	65 (1.81%)
Combined 3-site population	**n=1979**	**n=4797**	**n=4799**	**n=2656**	**n=521**	**n=14752**
pure geographic atrophy	0	2 (0.04%)	14 (0.29%)	41 (1.54%)	22 (4.22%)	79 (0.54%)
pure neovascular AMD	0	8 (0.17%)	26 (0.54%)	67 (2.52%)	30 (5.76%)	131 (0.89%)
mixed AMD	0	0	1 (0.02%)	14 (0.53%)	16 (3.07%)	31 (0.21%)
any AMD	0	10 (0.21%)	41 (0.85%)	122 (4.59%)	68 (13.05%)	241 (1.63%)

The female: male ratio averaged 1.35 for subjects with gradable photos and increased with age. Among the Beaver Dam, Rotterdam and Blue Mountains studies, female: male ratios were 1.25, 1.45 and 1.30, respectively. These ratios reflect age differences between the three studies; in Beaver Dam, the mean age was 61.8 years (range 43-86 years), in Rotterdam, the mean age was 69.0 years (range 55-99 years),and in the Blue Mountains, the mean age was 66.1 years (range 49-97 years). There were 12,468 subjects aged between 55 and 86 years, the age range common to all study sites.

Prevalence of AMD

The distribution of individual and combined AMD lesions by age group and study site is shown in Table 1. AMD lesions (NV or GA) were found in 241 participants (1.63% of the combined population), including 159 women (1.88%) and 82 (1.30%) men. Seventy-two Beaver Dam participants (1.51%), 104 Rotterdam participants (1.62%) and 65 Blue Mountains participants (1.81%) had AMD lesions. AMD prevalence at each site was strongly age-related, with AMD lesions not found in subjects aged less than 55 years, 0.21% of persons aged 55-64 years, 0.85% of persons aged 60-74 years, 4.59% of persons aged 75 to 84 years and 13.05% of persons aged 85 years or older.

Prevalence of Pure GA, Pure NV and Mixed AMD

The prevalence of Pure GA was 0.44%, 0.66% and 0.45%, for the three sites, respectively. Pure NV was almost twice as prevalent as Pure GA in the combined population, with Pure NV prevalence of 0.88%, 0.72% and 1.20%, found for these three sites, respectively. This 2:1 ratio of Pure NV to Pure GA was found in Beaver Dam and the Blue Mountains, with the proportion of AMD due to GA increasing with age. The ratio of NV to GA, however, was lower in Rotterdam (1.1:1), but the relative proportion of GA also increased with age. Mixed AMD was relatively infrequent, and was found only in persons older than 74 years, rapidly increasing as a total proportion of all AMD (Table 1). The age-specific prevalence for any GA lesion (either Pure GA or Mixed AMD) were fairly similar in all study sites. Age-specific prevalence for any GA in the combined population (including both Pure GA and Mixed AMD cases) were very similar in men and women (data not shown).

Table 2 shows the risk of AMD and its subtypes in the Blue Mountains and Beaver Dam populations relative to the Rotterdam population aged from 55 to 86 years, presented as age and smoking adjusted odds ratios. There were significantly lower age-adjusted prevalences for AMD and Pure NV lesions in the Rotterdam compared to both the Beaver Dam and Blue Mountains populations, for this age group. The age standardised rate ratios were only marginally lower than the age-adjusted odds ratios (data not shown). The lower NV prevalence in Rotterdam was almost unchanged after adjusting for age and smoking status. The age-adjusted risk for Pure GA was not significantly lower in Rotterdam than in Beaver Dam or Blue Mountains. In the combined population, there were few differences in age-specific AMD prevalence between men and women (data not shown).

Table 2. *Risk of AMD and its subtypes in the Blue Mountains and Beaver Dam populations relative to the Rotterdam population aged 55 to 86 years, adjusted for age and tobacco smoking.*

	Odds Ratios (95% confidence intervals)*		
Diagnosis	AMD (n)	Pure Neovascular (n)	Pure Geographic Atrophy (n)
†Age-adjusted			
Rotterdam (Index) n=6180	1.00 (74)	1.00 (35)	1.00 (30)
Blue Mountains n=3034	1.51 (1.04-2.19) (49)	2.20 (1.36-3.54) (34)	0.91 (0.46-1.79) (12)
Beaver Dam n=3254	2.15 (1.54-3.01) (71)	2.57 (1.63-4.06) (41)	1.54 (0.88-2.71) (21)
†Age and ‡smoking adjusted			
Rotterdam (Index)	1	1	1
Blue Mountains	1.71 (1.17-2.49)	2.61 (1.60-4.26)	1.01 (0.51-2.00)
Beaver Dam	2.42 (1.72-3.41)	3.07 (1.92-4.90)	1.68 (0.95-2.98)

* *Including all subjects from each study aged 55 to 86, excluding subjects without smoking data in smoking the smoking-adjusted model*

† *Ages from 55 to 86 years (common to all populations), included in the model as a continuous (year) variable*

‡ *Smoking status coded as either never, former or current smokers, included in the model as a categorical variable.*

FACTORS ASSOCIATED WITH NEOVASCULAR AMD, GEOGRAPHIC ATROPHY AND ANY AMD

A total of 12,468 subjects aged 55 to 86 years with gradable retinal photographs were included in the merged dataset used to assess associations between AMD and potential risk factors. Tables 3-5 present associations between a range of exposures and any AMD, Pure GA and Pure NV, respectively. The tables show that, apart from age, the only factor that retained a clear association with either AMD subtype in all sites and pooled over all sites, was tobacco smoking. Pooled analyses showed no significant heterogeneity in the association between tobacco smoking and AMD over the study sites. Compared to never smokers, ex-smokers (OR 1.34, CI 0.96-1.87) and current smokers (OR 3.11, CI 2.10-4.63) were more likely to have AMD. The association with current smoking was stronger with Pure NV (OR 4.55, CI 2.74-7.54) than with Pure GA (OR 2.56, CI 1.26-5.20).

Table 3. *Age-adjusted associations between potential risk factors and AMD : Odds ratios (95% CI) by location*

Potential Risk Factor	Rotterdam	n/N*	Beaver Dam	n/N	Blue Mountains	n/N	All 3 Sites	n/N
Age *(Index=55-69)*	1.00	9/3593	1.00	11/1977	1.00	5/1823	1.00	25/7393
70-79	4.42 (2.02-9.67)	21/1914	6.20 (3.12-12.3)	33/984	8.70 (3.29-23.1)	22/941	6.02 (3.82-9.47)	76/3839
80-86	27.9 (13.5-57.3)	44/673	18.8 (9.26-38.2)	28/294	32.3 (12.1-85.9)	22/270	25.3 (16.2-39.5)	94/1237
Gender *(Index=Female)*		74/6180		72/3255		49/3034		194/12469
Male	1.22 (0.75-1.98)		0.86 (0.52-1.42)		0.74 (0.41-1.36)		0.95 (0.70-1.28)	
Smoking		74/6109		72/3253		48/2936		194/12298
Never *(Index)*	1.00		1.00		1.00		1.00	
Ex-smoker	1.34 (0.75-2.37)		1.47 (0.87-2.49)		1.21 (0.62-2.36)		1.36 (0.97-1.90)	
Current smoker	3.11 (1.72-5.62)		2.39 (1.14-4.96)		4.22 (1.90-2.37)		3.12 (2.10-4.64)	
Body Mass Index		73/6068		72/3229		46/2985		191/12300
Underweight (BMI<20)	1.60 (0.53-4.84)		2.93 (1.02-8.38)		1.24 (0.44-3.48)		1.75 (0.95-3.22)	
Normal *(Index)*	1.00		1.00		1.00		1.00	
Overweight (25<BMI<30)	1.35 (0.79-2.31)		1.08 (0.58-2.01)		1.06 (0.52-2.19)		1.19 (0.85-1.98)	
Obese (BMI≥30)	0.85 (0.38-1.86)		1.42 (0.74-2.72)		1.89 (0.82-4.37)		1.30 (0.85-1.98)	
Cardiovascular & Other Disease *(Index=no disease, normal range)*								
Acute myocardial infarction	0.66 (0.28-1.53)	72/6047	0.61 (0.26-1.43)	71/3244	1.39 (0.63-3.05)	49/3019	0.81 (0.51-1.30)	192/12310
Angina	0.59 (0.21-1.64)	74/6114	0.60 (0.28-1.27)	70/3191	1.25 (0.62-2.50)	49/3021	0.78 (0.50-1.23)	193/12326
Stroke	1.04 (0.44-2.46)	74/6081	0.69 (0.27-1.78)	72/3255	1.25 (0.48-3.26)	47/3018	0.94 (0.55-1.60)	193/12354
Hypertension	0.62 (0.34-1.10)	73/6102	1.65 (0.96-2.82)	72/3254	1.30 (0.73-2.31)	49/3034	1.09 (0.79-1.50)	194/12390
Cholesterol (per mmol/l)	0.88 (0.72-1.09)	58/4877	0.94 (0.80-1.21)	72/3246	1.28 (0.98-1.67)	43/2739	1.00 (0.88-1.14)	173/10862
HDL-cholesterol (per mmol/l)	1.85 (0.95-3.58)	58/4873	0.99 (0.59-1.67)	72/3246	1.36 (0.71-2.59)	43/2740	1.27 (0.90-1.80)	173/10859
Iris colour		74/6157		72/3138		47/2966		193/12333
Grey or Blue *(Index)*	1.00		1.00		1.00		1.00	
Hazel or green	0.42 (0.13-1.37)		0.88 (0.46-1.69)		0.92 (0.45-1.87)		0.79 (0.51-1.22)	
Tan or brown	1.00 (0.57-1.78)		1.18 (0.68-2.07)		0.27 (0.08-0.89)		0.88 (0.61-1.22)	
Age at menopause		41/3442		44/1790		30/1579		115/6811
Age >=45 years *(Index)*	1.00		1.00		1.00		1.00	
Age <45 years	1.78 (0.85-3.73)		0.80 (0.39-1.65)		1.07 (0.47-2.42)		1.13 (0.73-1.76)	
Time from menarche to menopause		40/3402		44/1782		30/1568		114/6752
<30 years *(Index)*	1.00		1.00		1.00		1.00	
30-35 years	0.64 (0.25-1.62)		0.94 (0.39-2.23)		0.69 (0.25-1.93)		0.76 (0.45-1.31)	
≥35 years	0.64 (0.28-1.50)		1.06 (0.49-2.29)		0.79 (0.33-1.91)		0.83 (0.51-1.34)	
Hormone Replacement Therapy		45/3495		45/1825		30/1634		120/6954
(Index=Never)	1.00		1.00		1.00		1.00	
Ever	0.55 (0.13-2.36)		0.82 (0.38-1.75)		0.78 (0.22-2.68)		0.77 (0.43-1.39)	

* n = number of cases of AMD N = number of subjects available for analysis for each potential risk factor

Table 4. *Age-adjusted associations between potential risk factors and Pure Geographic Atrophy : Odds ratios (95% CI) by location*

Potential Risk Factor	Rotterdam	n/N*	Beaver Dam	n/N	Blue Mountains	n/N	All 3 Sites	n/N
Age *(Index=55-69)*	1.00	6/3590	1.00	3/1969	No cases	0/1823	1.00	9/7377
70-79	2.52 (0.88-7.29)	8/1901	6.89 (1.89-25.1)	10/961	Not calculable	6/925	5.25 (2.44-11.3)	24/3787
80-86	15.2 (5.92-39.0)	16/645	19.7 (5.20-74.8)	8/274	Not calculable	6/254	21.8 (10.3-46.0)	30/1173
Gender *(Index=Female)*		30/6136		21/3204		12/2997		63/12337
Male	1.21 (0.58-2.54)		1.48 (0.62-3.53)		1.98 (0.62-6.33)		1.45 (0.88-2.41)	
Smoking		30/6065		21/3202		12/2900		63/12167
Never *(Index)*	1.00		1.00		1.00		1.00	
Ex-smoker	1.38 (0.57-3.30)		2.10 (0.85-5.17)		1.14 (0.30-4.31)		1.58 (0.90-2.79)	
Current smoker	2.62 (1.03-6.62)		0.77 (0.09-6.34)		5.82 (1.27-26.71)		2.54 (1.25-5.17)	
Body Mass Index		30/6043		21/3178		10/2949		61/12170
Underweight (BMI<20)	1.15 (0.14-9.36)		2.13 (0.24-18.80)		1.38 (0.14-13.60)		1.45 (0.41-5.06)	
Normal *(Index)*	1.00		1.00		1.00		1.00	
Overweight (25<BMI<30)	1.56 (0.66-3.66)		1.31 (0.43-3.96)		1.88 (0.41-8.56)		1.52 (0.82-2.82)	
Obese (BMI≥30)	1.32 (0.43-4.05)		1.41 (0.42-4.70)		2.81 (0.45-17.49)		1.51 (0.72-3.18)	
Cardiovascular & Other Disease								
(Index=no disease, normal range)								
Acute myocardial infarction	0.55 (0.13-2.32)	29/6004	1.16 (0.33-4.00)	20/3193	0.63 (0.08-4.92)	12/2982	0.76 (0.33-1.78)	61/12179
Angina	0.74 (0.17-3.14)	30/6070	0.55 (0.13-2.42)	19/3140	0.38 (0.05-2.94)	12/2984	0.56 (0.22-1.42)	61/12194
Stroke	1.42 (0.42-4.77)	30/6037	-	21/3204	0.89 (0.11-7.04)	12/2983	0.72 (0.26-2.00)	63/12244
Hypertension	0.40 (0.14-1.16)	29/6058	2.12 (0.85-5.33)	21/3203	0.40 (0.11-1.50)	12/2997	0.72 (0.39-1.32)	62/12258
Cholesterol (per mmol/l)	0.85 (0.61-1.17)	24/4843	0.70 (0.47-1.04)	21/3195	1.19 (0.69-2.03)	10/2706	0.84 (0.67-1.06)	55/10744
HDL-Cholesterol (per mmol/l)	2.39 (1.02-5.59)	24/4839	0.81 (0.30-2.16)	21/3194	0.82 (0.19-3.46)	10/2708	1.25 (0.68-2.32)	55/10741
Iris colour		30/6113		21/3159		12/2931		63/12203
Grey or Blue *(Index)*	1.00		1.00		1.00		1.00	
Hazel or green	0.33 (0.05-2.48)		0.80 (0.26-2.49)		1.02 (0.26-3.90)		0.71 (0.33-1.54)	
Tan or brown	0.89 (0.36-2.19)		0.70 (0.22-2.16)		-		0.64 (0.32-1.29)	
Age at menopause		17/3418		11/1757		5/1554		33/6729
Age >=45 years *(Index)*	1.00		1.00		1.00		1.00	
Age <45 years	2.32 (0.80-6.75)		0.26 (0.03-2.05)		0.63 (0.07-5.75)		1.00 (0.43-2.33)	
Time from menarche to menopause		13/3379		11/1749		5/1543		33/6671
<30 years *(Index)*	1.00		1.00		1.00		1.00	
30-35 years	0.75 (021-2.63)		3.96 (0.46-34.13)		0.88 (0.05-14.31)		1.31 (0.49-3.49)	
≥35 years	0.38 (0.11-1.39)		2.43 (0.28-20.91)		1.51 (0.15-14.81)		0.85 (0.32-2.24)	
Hormone Replacement Therapy		18/3468		11/1791		5/1609		34/6868
(Index=Never)	0.78 (0.10-6.11)		1.07 (0.27-4.18)		1.77 (0.19-16.82)		1.12 (0.42-3.03)	
Ever								

* n = number of cases of Pure GA N = number of subjects (excluding subjects with Pure NV) available for analysis for each potential risk factor

Table 5. Age-adjusted associations between potential risk factors and Pure Neovascular AMD : Odds ratios (95% CI) by location

Potential Risk Factor	Rotterdam	n/N*	Beaver Dam	n/N	Blue Mountains	n/N	All 3 Sites	n/N
Age *(Index=55-69)*	1.00	3/3587	1.00	8/1974	1.00	5/1823	1.00	16/7384
70-79	7.57 (2.14-26.9)	12/1893	5.69 (2.52-12.8)	22/973	5.54 (1.99-15.4)	14/933	5.96 (3.38-10.5)	48/3811
80-86	38.0 (11.3-128)	20/649	11.1 (4.49-27.4)	12/278	22.0 (7.92-61.0)	15/263	20.0 (11.3-35.5)	47/1190
Gender *(Index=Female)*		35/6141		41/3224		34/3019		110/12384
Male	1.76 (0.90-3.47)		0.91 (0.47-1.73)		0.50 (0.23-1.09)		0.95 (0.64-1.41)	
Smoking		35/6070		41/3222		33/2921		109/12213
Never *(Index)*	1.00		1.00		1.00		1.00	
Ex-smoker	2.53 (0.99-6.44)		1.46 (0.71-2.97)		1.11 (0.50-2.48)		1.54 (0.97-2.44)	
Current smoker	7.07 (2.80-17.84)		3.32 (1.39-7.90)		4.18 (1.66-10.53)		4.55 (2.74-7.54)	
Body Mass Index		34/6047		41/3198		33/2972		108/12217
Underweight (BMI<20)	2.10 (0.58-7.63)		1.84 (0.39-1.59)		1.41 (0.44-4.46)		1.72 (0.81-3.65)	
Normal *(Index)*	1.00		1.00		1.00		1.00	
Overweight (25<BMI<30)	0.97 (0.46-2.04)		0.83 (0.37-1.89)		1.04 (0.45-2.43)		0.94 (0.60-1.49)	
Obese (BMI≥30)	0.33 (0.07-1.45)		1.52 (0.58-3.36)		1.71 (0.63-4.65)		1.19 (0.69-2.03)	
Cardiovascular & Other Disease *(Index=no disease, normal range)*								
Acute myocardial infarction	0.70 (0.21-2.30)	34/6009	0.53 (0.16-1.74)	41/3214	1.84 (0.78-4.34)	34/3004	0.94 (0.52-1.70)	109/12227
Angina	0.63 (0.15-2.63)	35/6075	0.68 (0.26-1.75)	41/3162	1.81 (0.85-8.87)	34/3006	1.03 (0.60-1.75)	110/12243
Stroke	0.71 (0.17-3.02)	35/6042	1.09 (0.38-3.14)	41/3224	1.46 (0.50-4.29)	33/3004	1.07 (0.55-2.08)	109/12270
Hypertension	0.83 (0.38-1.79)	35/6064	1.77 (0.89-3.52)	41/3223	2.02 (1.00-4.08)	34/3019	1.48 (0.99-2.22)	110/12306
Cholesterol (per mmol/l)	0.88 (0.65-1.19)	27/2846	1.13 (0.88-1.47)	42/3216	1.24 (0.90-1.70)	30/2726	1.01 (0.91-1.27)	99/10788
HDL-Cholesterol per mmol/l)	1.14 (0.40-3.25)	27/4842	0.90 (0.46-1.78)	42/3215	1.73 (0.83-3.62)	30/2728	1.19 (0.76-1.86)	99/10785
Iris colour		35/6118		41/3179		32/2951		108/12248
Grey or Blue *(Index)*	1.00		1.00		1.00		1.00	
Hazel or green	0.33 (0.04-2.42)		0.94 (0.39-2.24)		0.85 (0.36-2.04)		0.79 (0.45-1.41)	
Tan or brown	1.44 (0.68-3.04)		1.60 (0.80-3.21)		0.40 (0.12-1.34)		1.15 (0.73-1.81)	
Age at menopause		17/3418		25/1771		23/1572		65/6761
Age >=45 years *(Index)*	1.00		1.00		1.00		1.00	
Age <45 years	1.63 (0.52-5.07)		0.84 (0.33-2.14)		1.35 (0.56-3.30)		1.20 (0.68-2.09)	
Time from menarche to menopause		16/3378		25/1763		23/1561		64/6702
<30 years *(Index)*	1.00		1.00		1.00		1.00	
30-35 years	0.43 (0.09-2.17)		0.64 (0.19-2.15)		0.54 (0.17-1.73)		0.53 (0.25-1.12)	
≥35 years	0.87 (0.24-3.19)		1.13 (0.42-2.99)		0.63 (0.24-1.65)		0.85 (0.45-1.69)	
Hormone Replacement Therapy		18/3468		25/1805		23/1627		66/6900
(Index=Never)	1.00		1.00		1.00		1.00	
Ever	-		0.97 (0.37-2.50)		0.66 (0.15-2.93)		0.71 (0.33-1.54)	

* n = number of cases of Pure NV N = number of subjects (excluding subjects with Pure GA) available for analysis for each potential risk factor

DISCUSSION

This report represents the first attempt to combine unit data from distinct population-based eye studies to investigate AMD and its two subtypes. Strengths of this pooled study include its large sample size and the relatively similar photographic documentation performed at each of the three sites. A further strength is the use of a similar grading protocol by graders who had all spent periods of training at the Fundus Photograph Reading Center in Wisconsin. Also, the inclusion of all cases classified as having AMD was agreed by investigators from each of the three studies. Potential weaknesses, however, include differences in the response rates for the three studies and site differences in photographic technique. Although graders from each of the three sites spent a period of training in Wisconsin, there remains a possibility that systematic grading differences in the three studies could have occurred. It seems likely that this would tend to reduce rather than increase apparent AMD prevalence, as all identified AMD cases had to be agreed by senior investigators from the three sites before inclusion.

The exposures considered here were collected using similar, but not identical, interviewer-administered questionnaires, and coded in a similar manner at each study site. Despite apparent similarities in data collection and coding, there may remain some differential exposure measurement error between sites, either through differences in question wording, interviewer administration or cultural factors. However, it is more likely that non-differential measurement error will be present at all sites, leading to a bias toward the null - that is leading to less likelihood of finding a significant association between exposures and AMD.

The combined study has shown relatively similar age-specific prevalence for both Pure NV and any AMD separately in the Beaver Dam and Blue Mountains Studies. However, substantially lower age-specific NV (and consequently AMD) prevalences were found in the Rotterdam Study. Adjusting for gender and the major known potential confounders, including tobacco smoking, did not greatly alter these differences in population risks. Is this site difference real or could it be due to systematic grading differences or to differences in subject selection? There were some differences in the photographic documentation performed, principally the use of Topcon 35° stereo-photographs in Rotterdam while Zeiss 30° stereo-photographs were taken at the other two sites. It does not seem very likely, however, that this magnification difference or other variations in photographic technique or quality among those with gradable photographs could explain such a large age-specific difference in prevalence. In eyes with reduced photo quality, a small GA lesion might be expected to be less easy to grade than a small neovascular AMD lesion. However, a similar 2:1 ratio of Pure NV to Pure GA was found in Rotterdam as well as in the other two sites, which suggests that reduced photo quality in one site may not have caused the difference observed.

However, there remains a distinct possibility that the lower proportion of Rotterdam participants who had gradable photographs in at least one eye could have contributed to the differences found.

In the overall 55 to 86 year age group, 84% of Rotterdam participants had gradable photographs, compared with 97.4% of Beaver Dam participants and 98.2% of Blue Mountains participants. A lower prevalence of AMD in the Rotterdam population could have been found if subjects with AMD were less likely than others in the community to have gradable photographs.

Differences in the population ascertainment methods (use of Municipal Registers in Rotterdam compared with conduct of a private door-to-door census in Beaver Dam and Blue Mountains) or in the participation rate (78% in Rotterdam, compared to 83% in Beaver Dam and 82% in Blue Mountains) could also have contributed to the differences in prevalence. For example, some non-responders may have decided not to attend the respective Eye Studies because they had previously been diagnosed with eye diseases such as AMD and were already under regular ophthalmic review. Furthermore, different health care systems or attitudes toward health may explain differences in prevalence. Individuals with disease may be more likely to participate in one country (because they believe they can derive benefit from the examination) than in another (because they are more fatalistic about their disease). The results could then be biased by location due to differential response by cases with ARM. However, such differential non-response seems unlikely to explain a large difference given the similar publicity provided to each local community and to local ophthalmologists about the emphasis on AMD by each study.

If the lower age-specific AMD prevalence found in the Rotterdam Study, however, is real, it is possible that there may be differences in the prevalence of major non-genetic (environmental) AMD risk factors found between the three communities. The only environmental exposure clearly associated with any AMD type in both the individual and pooled studies was tobacco smoking. However, smoking is unlikely to have contributed to the differences in prevalence of AMD found, as after including smoking status in the multivariate models, the associated risk for AMD by study site was almost unchanged. The three-fold risk of any AMD associated with current smoking in the pooled data was relatively similar across the three sites, with statistically significant odds ratios of 2.4, 3.1 and 4.2 for the Beaver Dam, Rotterdam and Blue Mountains populations, respectively. The magnitude of risk with current smoking was higher in the pooled dataset for NV (OR 4.6) compared to GA (OR 2.6), although site differences existed in the subset analyses for GA. These findings reflect the consistency of recent population-based and cohort study reports that have linked smoking to AMD[6,8,25,12,26,13,27,28,29].

The finding of only one significant association with AMD in the pooled data (tobacco smoking) may be due to relatively greater measurement error associated with the other exposures investigated (medical history etc). The higher measurement error would lead to larger biases toward the null, decreasing the possibility of finding true associations with AMD. However, this seems unlikely to have been a sufficiently large bias to affect the associations examined.

There was no meaningful heterogeneity across study strata for the association between tobacco smoking and any type of AMD, strengthening the argument that this is likely to be a true association. For some exposures, a weak consistent non-significant association across study sites and in the pooled estimate was found. For example, for the variable 'ever having taken hormone replacement therapy', site-specific associations ranged from 0.55 to 0.84 and the pooled estimate was 0.78 (0.44-1.41), but was clearly not statistically significant. For other exposures, the pooled estimates were virtually uninterpretable because estimates of association were relatively heterogeneous across sites. For either of these reasons, the pooled analyses could not provide any evidence to support any consistent association between AMD and any of the remaining exposures investigated. There were no apparent differences in risk factors for NV compared to GA.

Another possible explanation for the differences in AMD prevalence observed between study sites may be differences in sunlight exposure, as suggested in the Chesapeake Bay Watermen Study[30]. However, recent findings from a large Australian case-control study failed to confirm a sunlight exposure link with AMD[31]. A major criticism of many studies of the association between sunlight exposure and AMD is the difficulty in quantifying exposure differences among people all living in the same location. The three sites are likely to have considerable differences in sun exposure due to their latitude differences (52°in Rotterdam, 44° in Beaver Dam and 34° in the Blue Mountains), as well as differences in average cloud cover. If sun exposure were important, then this could explain a lower AMD prevalence in the Rotterdam participants, but does not explain the lower prevalence in the Blue Mountains, latitude 34° compared to Beaver Dam, latitude 44°. There may be a threshold effect for sunlight exposure, which may be more strongly associated with NV than GA lesions. This could explain why Beaver Dam and Blue Mountains had higher prevalence than Rotterdam while there was no trend across the three sites with latitude. A possible confounder could be individual exposure through lifestyle and occupation. As Beaver Dam is a rural community, participants may be more likely to have worked in outdoors jobs, or spent leisure time outdoors, than in the other two populations. This may have decreased prevalence differences between the Blue Mountains and Beaver Dam populations.

Current research indicates there may be a strong genetic basis for AMD[32], with high familial aggregation rates reported from a number of recent studies[33-35]. Thus, a third possibility is that there may be important genetic differences between the three communities. For example, there may be differences in the prevalence of key mutations in AMD candidate genes. Given the influence of genetic and environmental factors found for this disease, it is also possible that there may be differences between the communities in the extent of gene-environment interaction.

In summary, despite the differences found in age-specific prevalence for AMD and its subtypes between study sites, the characteristics of the lesions and the ratio of NV to GA were relatively consistent across study sites in three continents. The NV differences in age-specific prevalence found comparing Rotterdam with the other two sites cannot readily be explained by differences in age, sex, smoking status, grading or diagnostic procedures.

The differential effects of latitude and genetic factors also could not be ruled out as possible explanations for the observed differences in age-specific prevalence of neovascular AMD lesions.

These pooled data from racially similar communities across three continents provide strong and consistent evidence that tobacco smoking is the principal known preventable exposure associated with any form of AMD. The findings support further health advocacy approaches to reduce the tobacco smoking rate, even among the older elderly in our population, and also support research to identify possible candidate genes that may predispose to age-related macular degeneration.

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- CHAPTER 4 -

INCIDENCE AND PROGRESSION OF AGE-RELATED MACULOPATHY; THE ROTTERDAM STUDY

ABSTRACT

Objective: To describe the incidence of late stages of age-related maculopathy (ARM) and the progression of earlier stages, and to study the hierarchy of fundus features that determine progression.

Design: Population-based prospective cohort study.

Participants: A population of 4948 subjects aged 55 years and older living in Rotterdam, the Netherlands, was studied to determine the incidence of neovascular and atrophic macular degeneration (AMD). A subgroup of 1244 subjects was studied for progression of early stages of ARM.

Methods: At baseline and at 2-year follow-up, fundus transparencies were graded for features of age-related maculopathy using the International Classification System. ARM was stratified in four exclusive stages according to type of drusen and presence of pigmentary irregularities.

Main Outcome Measures: AMD, ARM

Results: The overall 2-year cumulative incidence of AMD was 0.2%, increasing to 1.2% in subjects of 85 years and older. Of the early stages, 22% showed progression to a more severe stage. Most important predictors for progression were more than 10% of macular area covered by drusen (OR 5.7, 95% CI 2.5, 13.1), presence of depigmentation (OR 5.0, 95% CI 3.2, 7.8), and hyperpigmentation (OR 3.1, 95% CI 2.0, 4.7).

Conclusions: The incidence of AMD appears to be lower in the Netherlands than in the United States. Progression of early stages occurs in a distinct pattern at a stable rate with a large area of drusen and pigmentary changes as most important predictors.

INTRODUCTION

A large number of studies have shown that ARM is a frequent eye disorder in the elderly,[1-3] and that its end stages are the most important cause of irreversible blindness in the Western world.[4-6] The design of most of the epidemiologic studies has been cross-sectional, and they have provided information on disease prevalence and prevalence associations.

In etiologic research, however, incidence is commonly preferred over prevalence. Incidence represents the actual disease occurrence, and risk analyses based on incident cases are more suggestive of a causal relation, since exposures are measured before the onset of disease. Incidence data of ARM would improve the knowledge on the etiology, early development and progression of this disease. At present, these data are still scarce.[7,8]

The purpose of this study was to describe the incidence and progression of ARM in the population-based Rotterdam Study in the Netherlands. We studied the incidence of the late stages of ARM in the entire cohort, and investigated progression of early features in specific subgroups. Furthermore, we aimed to assess the prognostic value of the various fundus features that are associated with ARM.

SUBJECTS AND METHODS

POPULATION

The Rotterdam Study is a population-based prospective cohort study conducted in a suburb of Rotterdam, the Netherlands, in which chronic ophthalmologic, neurologic, cardiovascular, and locomotor as well as endocrine disorders are investigated. Methods used to identify and describe the population have appeared in previous reports.[2,9] Baseline interview and screening examinations took place from 1990 to mid 1993, follow up examinations from mid 1993 to the end of 1994.

Of 10,275 eligible subjects aged 55 years and older living in Rotterdam, 7983 (78%) agreed to participate in the baseline phase of the study. Gradable fundus transparencies were available on 6411 subjects, of whom 104 (1.6%) subjects were diagnosed with the late stages of ARM, i.e., atrophic or neovascular AMD. This resulted in a cohort of 6307 subjects at risk for incident AMD.

PROCEDURES AND DEFINITIONS

The screening for presence of ARM followed the same protocol at baseline and at follow up. Procedures have been described in detail elsewhere.[2,9] In brief, during the screening eye examination 35° color transparencies were taken of the macular area (Topcon TRV-50VT fundus camera, Topcon Optical Company, Tokyo, Japan). The diagnosis of ARM features was based on grading of fundus transparencies according to the International Classification System,[10] in which all features of age-related maculopathy are called ARM and the two late stages are called AMD. At baseline, fundus transparencies of the entire cohort were graded in a detailed manner to identify all features of ARM. At follow up, all fundus transparencies of the entire cohort were graded for presence of atrophic or neovascular AMD.

Table 1. *Stratification of ARM in exclusive stages of severity*

Stage of ARM	Criteria	No. at baseline	No. at follow-up	No. selected for analysis
No ARM	No ARM features or only drusen ≤ 63μm	4025	3234	327
Stage 1 (*i*)	Soft distinct drusen	1465	1144	331
(*ii*)	Pigmentary irregularities	332	248	248
Stage 2 (*i*)	Soft indistinct drusen or reticular drusen	180	121	121
(*ii*)	Soft distinct drusen with pigmentary irregularities	222	170	170
	83			
Stage 3	Soft indistinct or reticular drusen with pigmentary irregularities	83	47	47
Stage 4	Atrophic or neovascular macular degeneration (AMD)	104	54	0

To assess the incidence and progression of early ARM features, ARM at baseline was stratified in four exclusive stages of disease (Table 1). On the basis of previous findings,[7,8,11,12] we assumed more clinical severity and a higher risk of development of AMD with each successive stage. The stage classification of a subject was based on the eye with the most severe stage of ARM. ARM stages 1 (*i*) and (*ii*) were considered one stage of clinical severity, as were stages 2 (*i*) and (*ii*). For reasons of feasibility and efficiency, only a randomly selected subset of subjects with no ARM or ARM stage 1 (*i*) at baseline underwent detailed grading of early ARM features at follow up. Of all other stages, the entire group of subjects with gradable fundus transparencies underwent detailed grading at follow up.

Incidence of an ARM lesion was defined as absence of this particular lesion within the grid area of either eye at baseline and presence of this lesion in at least one eye at follow up. Progression of ARM was defined as an increase in one or more stages of ARM; no progression was defined as no change or a decrease in stage.

STATISTICAL METHODS

Subjects with AMD at baseline were excluded from the incidence and progression analyses. The age-specific incidence rate of AMD was obtained per 10-year age-categories by dividing the number of incident cases by the number of person-years per age-category. The latter was calculated by summing each participant's contribution of follow up time per age-category. Confidence intervals of incidence rates were calculated with the exact method. Age at follow up was regarded as age at onset of incident AMD. Cumulative incidences were calculated from the incidence rates with the formula

$$CI(t) = 1 - e^{-IR*t},$$

where CI is the cumulative incidence over a period of t years, IR is the incidence rate, and e is the constant 2.71828, the base of the natural logarithm. Progression of early ARM stages was studied by logistic regression analysis with age, gender, baseline stage of ARM and duration of the follow up period fixed in the model. In an initial 'univariate' analysis with these fixed factors the predictive

power of drusen size and location, proportion of macular grid area covered by drusen, most frequent drusen size, largest drusen size, drusen confluence, presence and area of hyperpigmentation, and presence and area of depigmentation was evaluated. Statistical interaction between macular area of drusen and hyper- or depigmentation, between hyper- and depigmentation, and between area of drusen and drusen confluence was studied by entering the product term of these factors in the model. Fundus features or product terms with a significant odds ratio entered a subsequent 'multivariate' analysis to determine the independence and magnitude of prognostic factors.

RESULTS

INCIDENCE OF AMD

Of the 6307 subjects at risk for incident AMD, 5442 (86%) participated in the 2-year follow up phase of the Rotterdam Study. Of the non-participants, 326 subjects had died before follow up, and 539 (10%) subjects refused to participate in any follow up examination. Of subjects that consented to follow up, 5097 participated in the re-screening eye examination. Gradable fundus transparencies of at least one eye were present in 4948 subjects, 78% (4948/6307) of the total number of subjects at risk for incident AMD, and these subjects were included in the incidence analyses. They significantly differed from other eligibles by age, but, after adjustment for age, not by stage of ARM at baseline (table 2).

Table 2. *Baseline characteristics of subjects at risk of incident AMD*

Characteristics	Eligible subjects (n=6307)		*P*
	In analysis[1] (n=4948)	Not in analysis[2] (n=1359)	
Age at baseline (%)			
55 - 64 y	43.2	24.3	
65 - 74 y	38.1	30.2	<0.001
75 - 84 y	16.2	33	
85 + y	2.5	12.5	
Gender (% women)[3]	58.5	61.3	0.07
Institutionalized (%)[3]	4.4	10.2	<0.001
ARM at baseline (%)[3]			
Stage 1	29.1	28.4	
Stage 2	6.5	5.9	0.15
Stage 3	1.1	2.1	

[1] subjects with gradable fundus transparencies

[2] deceased, non-participants, and subjects with ungradable fundus transparencies

[3] adjusted for age

After an average follow up period of 2.0 (SD 0.6) years, there were 11 cases with incident AMD. Of those, 5 cases were identified with atrophic AMD and 6 with neovascular AMD. Given a total of 9833 person-years, the overall incidence of AMD was 1.1 per 1000 person-years (2-yr cumulative incidence 0.22%). The incidence increased with age (table 3). Figure 1 shows the estimated 5-year cumulative incidence risk in the Rotterdam Study in comparison with the two other population-based incidence studies that report 5-year incidences.

Table 3. *Age-specific incidence rates per 1000 person-years and 2-year cumulative incidences (%) of AMD in the Rotterdam Study*

Age-group	Person-years	N	Rate	95% CI	2-yr incidence
55-64	3530	0	0	(0, 0.001)	0
65-74	4009	1	0.2	(0.01, 1.4)	0.05
75-84	1955	8	4.1	(1.8, 8.1)	0.82
85+	338	2	5.9	(0.7, 21.4)	1.18
Total	9833	11	1.1	(0.6, 2.0)	0.22

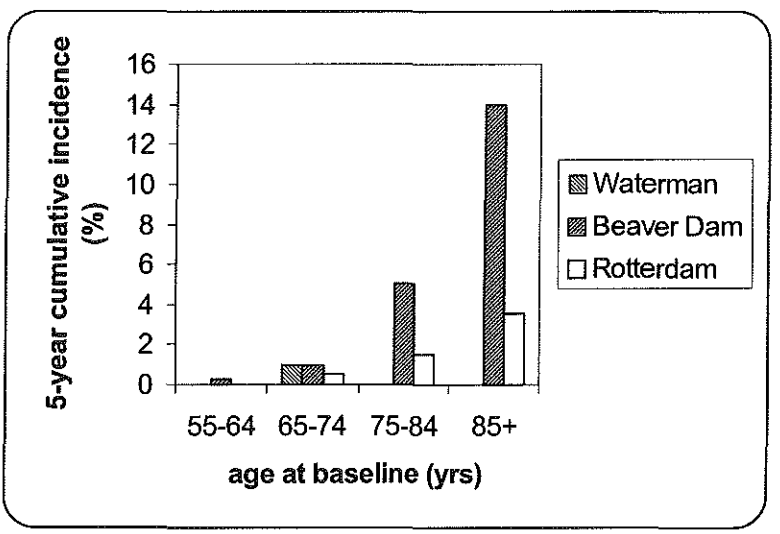

Figure 4. *Comparison of the estimated age-specific 5-year cumulative incidence of AMD in the Rotterdam Study with the 5-year cumulative incidences in the Waterman study[7] and in the Beaver Dam Eye Study[8].*

Overall, there were no statistically significant gender differences in incidence of AMD. Among the 2078 men, the overall incidence was 1.00 per 1000 person-years (2-yr cumulative incidence 0.2%), while among the 2870 women, the overall incidence was 1.20 per 1000 person-years (2-yr incidence 0.2%, $P = 0.24$, adjusted for age and follow up time).

Incident AMD was strongly associated with stage of ARM at baseline. Neither ARM stage 0 nor stage 1 progressed to incident AMD. ARM stage 2 gave rise to 3 subjects with incident atrophic AMD and 4 with incident neovascular AMD. For this stage, the overall incidence rate of AMD was 12.1 per 1000 person-years (2-year cumulative incidence 2.4%), ranging from 4.0 per 1000 person years (2-yr incidence 0.8%) in subjects under 65 years to 35.4 per 1000 person-years (2-yr incidence 6.8%) in subjects aged 85 years and older. Stage 3 at baseline gave rise to 2 subjects with incident atrophic AMD and 2 with incident neovascular AMD. For stage 3, the total incidence rate of AMD was 37.6 per 1000 person-years (2-yr incidence 7.4%), and the age-category in which this occurred was 74-85 years .

Of the 31 subjects with AMD in only one eye at baseline, 3 subjects with atrophic AMD and 4 subjects with neovascular AMD developed incident AMD in the second eye at 2-yr follow up. This resulted in an incidence rate of 109.9 per 1000 person-years (2-yr cumulative incidence 19.7%) for involvement of the second eye. The 3 subjects with unilateral atrophic AMD at baseline developed the same type of AMD in the second eye. Of the 4 subjects with neovascular AMD, 2 developed neovascular AMD and 2 developed atrophic AMD in the other eye. The baseline ARM stages of the second eye were stage 2 (3 subjects) and stage 3 (4 subjects).

PROGRESSION OF EARLY STAGES

Of the 1244 subjects who were selected for the early ARM progression analyses, 315 subjects progressed to a more severe stage of ARM. For the total cohort, this implied a 2-year cumulative progression rate of 21.5%. Table 4 shows the incidence rates of the various stages of ARM at follow up. Age was associated with progression: adjusted for gender, follow up time and baseline stage of ARM, the odds ratio of progression for age per year was 1.02 (95% CI 1.00, 1.03). Gender was not associated with progression: the odds ratio for women versus men was 0.98 (95% CI 0.75, 1.27; adjusted for age, follow up time and baseline stage of ARM).

Table 4. *Incidence rates of the various stages of ARM per 1000 person-years based on 2-year follow-up (2-yr cumulative incidences,%)*

Stage of ARM	*Follow-up Stage 1*	*Follow-up Stage 2*	*Follow-up Stage 3*	*Follow-up Stage 4*
Baseline Stage 0	112.8 (20%)	4.5 (1%)	0	0
Baseline Stage 1		112.5 (20%)	15.8 (3%)	0
Baseline Stage 2			119.9 (21%)	11.7 (2%)
Baseline Stage 3				37.6 (7%)

In the 'univariate' analysis of prognostic factors, macular area covered by drusen, presence and area of hyperpigmentation, presence and area of depigmentation, number of small drusen ($\leq 63\mu$m), number of large drusen ($\geq 125\mu$m), and drusen confluence were significantly associated with progression (data not shown).

In the 'multivariate' analysis with these significant factors in the model, all factors except number of large drusen (≥125μm) remained statistically significant. A large area of drusen was the most important predictor of ARM progression; the odds ratio for >10% of macular area covered by drusen was 5.8 (95% CI 2.5, 13.3) (table 5). The other important independent predictors were presence of depigmentation, hyperpigmentation, 10 or more small drusen, and at least 10% drusen confluence. Area of depigmentation with a total diameter larger than 500 μm had a higher odds ratio than did smaller area's (odds ratio for area >500 μm versus area <175 μm 4.61 (95% CI 2.48, 8.56), indicating that larger area's of depigmentation were more prognostic than smaller area's. Area's of hyperpigmentation larger than 125 μm did not have higher odds ratio's than area's of 125 μm or smaller, indicating that larger area's of hyperpigmentation were not of additional prognostic value. We found no evidence for statistical interaction between area of drusen and pigmentary irregularities, between hyper- and depigmentation, or between area and confluence of drusen (data not shown).

Table 5. *Fundus features prognostic for progression of ARM*

Fundus feature	OR (95% CI)*
Total drusen area ≥10% of grid	5.78 (2.52, 13.30)
Presence of depigmentation	4.95 (3.15, 7.79)
Presence of hyperpigmentation	3.09 (2.02, 4.72)
≥10 small drusen (≤63μm)	3.01 (1.90, 5.11)
≥10% drusen confluence	2.77 (1.81, 4.24)

* based on a model which included these factors, plus age, baseline stage of ARM, and duration of follow-up period

DISCUSSION

In the Rotterdam Study, the incidence of ARM's late stage, AMD, was 1.12 per 1000 persons per year for subjects aged 55 years and over. The incidence of AMD showed a strong relation with age and increased to 5.9 per 1000 persons per year for those aged 85 years and older. The incidence of AMD in the contralateral eye of subjects already affected by unilateral AMD was 109.9 per 1000 persons per year. The most predictive stage for development of incident AMD was ARM stage 3, which comprises the presence of either soft indistinct or reticular drusen, or soft drusen with pigmentary irregularities. Progression of early stages of ARM occurred in a very distinct pattern at a rate of 22% in two years. Most important predictors for progression were more than 10% of macular area covered by drusen, presence of depigmentation, and presence of hyperpigmentation.

A good estimate of the incidence of AMD requires the follow up of many subjects over a long period of time, because the occurrence of this clinical end stage is relatively infrequent.

A large study population with a significant number of elderly is one of the strengths of the Rotterdam Study. However, the length of the follow up period was not long, and the number of subjects who developed incident AMD was low. Therefore, our estimated incidence rate of AMD is rather imprecise. On the other hand, the short follow up period was a benefit for the study of the progression of early ARM stages. This enabled us to register small changes and to determine a pattern of progression, which may add to the understanding of the natural course of this disease.

The age-specific incidences of AMD appeared to be lower in the Rotterdam Study than in the Waterman Study or the Beaver Dam Eye Study (figure 1).[7,8] The American studies took place in different parts of the United States, but show incidences within the same range. We estimated a five-year cumulative incidence by extrapolation of our data in order to allow for a meaningful comparison, and in coming years we will be able to evaluate whether this estimation is correct. Nevertheless, the difference seems considerable, consistent over the age-groups, and in agreement with earlier reports indicating global differences in the occurrence of AMD. Comparison of prevalence data from the Beaver Dam Eye Study, the Blue Mountain Eye Study, and the Rotterdam Study learned that the prevalence of AMD was highest in the United States and lowest in the Netherlands.[1-3] The three studies used very similar methods of diagnosis based on fundus photography, which makes it less likely that the differences were a result of observation bias. Known risk factors such as smoking and cardiovascular disease did not explain the differences ("Smith et al, submitted"), and it remains a key point of interest to identify the environmental and genetic factors that are accountable.

The 2-year incidence of AMD in the fellow eye in subjects with unilateral AMD was 20%, and the type of AMD was not necessarily concordant with the first eye. The Beaver Dam Eye Study found a 5-year incidence of 22% for the second eye,[8] considerably lower than the Rotterdam Study. The lower age-range in Beaver Dam may well account for this difference. Our data are in line with clinic-based studies reporting the rate of fellow eye involvement. The majority of these studies focussed on patients with neovascular AMD, and estimates for annual second eye incidence mostly ranged from 4 to 10%,[11,13-17] although annual incidences up to 15% have been published.[18] Comparison of rates is generally hampered by differences in age, duration of disease, and diagnosis, and long-time follow up of large, well-defined study groups will be needed to provide valid and precise estimates.

An important objective of the study was to describe the progression of early features of ARM. For long it has been known that soft drusen and pigmentary changes are precursor lesions that increase the risk of geographic atrophy and neovascular AMD.[7,8,11-20] After appearing, drusen and pigmentary changes may regress and disappear, but generally this is a result of appearance of more severe lesions.[7,8] In the Rotterdam Study, we did not focus on individual fundus lesions. To enhance clinical relevance, we preferred to study progression of ARM in exclusive stages of disease.

We stratified early features of ARM in three stages based on type of drusen and presence of pigmentary changes, the factors which have been shown to be strong predictors for the development of AMD.[7,8,11,12] The ranking of the stages proved to be in accordance with clinical severity: the risk of AMD increased from virtually no risk for stages 0 and 1, to a 2-year risk of 2.4% and 7.4% for stages 2 and 3, respectively. An interesting finding was that progression predominantly occurred to only one more advanced stage at a rate of approximately 20% in 2 years for the earliest stages (table 4). Progression from stage 3 to 4 was slower and occurred at a rate of 7% in 2 years. Some subjects progressed fast and skipped one stage, but no subjects skipped more than one stage in the 2 years of follow up. These findings add to the view that development of ARM is not a random occurrence of events, but rather seems to follow a well-defined pattern at a stable rate.

In accordance with others,[8] we found that a large area of the macula covered by drusen and pigmentary irregularities were important and independent predictors of ARM progression. Other predictors were number of small drusen, and drusen confluence. The number of intermediate (64-124μm) and large drusen (≥ 125μm) did not have additional predictive power, neither did location of drusen. Although small drusen (≤ 63μm) are not considered an ARM feature in the International Classification System, our data indicate that more than 10 small drusen are predictive of ARM progression independent of other features. This is consistent with findings from the Waterman Study[7] and the Beaver Dam Eye Study,[8] which both reported that many small drusen increased the risk of large and soft indistinct drusen, but not of AMD. From our results and those of others we conclude that progression of early ARM appears to follow a distinct pattern. A large number of small hard drusen or isolated pigmentary changes may indicate the very early start of ARM. Then soft drusen emerge. Subsequently, at a stable rate, multiple drusen of various sizes appear and become confluent, the total area increases and some of the drusen become soft indistinct. Next, the appearance of pigmentary changes at this stage, especially large areas of depigmentation, then further increases the risk of AMD. Subretinal neovascularization or development of geographic atrophy denote the etiologic end stage of ARM.

In conclusion, the 2-year incidence of AMD in the Rotterdam Study was 2.2 per 1000 subjects. Our data provide further evidence that ARM is a progressive disease with a distinct temporal sequence of events ultimately ending in AMD.

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PART III

GENETIC-EPIDEMIOLOGICAL STUDIES ON AGE-RELATED MACULOPATHY

- CHAPTER 5 -

HETEROGENEITY OF THE GENETIC RISK IN AGE-RELATED MACULOPATHY A POPULATION-BASED FAMILIAL RISK STUDY

ABSTRACT

Earlier studies demonstrated an increased risk of age-related maculopathy (ARM) for first degree relatives of affected subjects. We aimed to assess whether the genetic risk of ARM shows heterogeneity among families. Case (n=64) and control (n=100) probands were selected from the population-based Rotterdam Study, and first degree relatives were examined for diagnosis of ARM by fundus photography. A family score method was used to estimate a risk for each family taking into account the risk of disease expected on the basis of age. Families of cases were at a higher risk of ARM than families of controls. Familial heterogeneity of risk was suggested within the case families, with 17% at low familial risk of ARM, 14% at intermediate risk, and 3% at high familial risk of ARM. Family score was significantly associated with late ARM (β 1.28, 95% CI 0.56-1.99; adjusted for age, sex, and smoking). Our results show that the risk of ARM varies among families, and suggest that only a small fraction of all ARM is due to a strong genetic component.

INTRODUCTION

Age-related maculopathy (ARM) is a frequent eye disease among elderly, and currently the leading cause of blindness in developed countries.[1] It has been well recognized that genetic factors are implicated in the etiology of the disease,[2,3] However, estimates of the magnitude of this component vary significantly among family studies. In two clinical studies, the reported odds ratios for first degree relatives of cases were 2.4 to 19.3, respectively.[4,5] In a segregation analysis based on sibships from the Beaver Dam Eye study, it was suggested that ~ 56% of the total ARM variability was compatible with a single major gene.[6] In our previous familial aggregation study based on probands from the Rotterdam Study, we estimated that the life time relative risk of end stage ARM was 4.2 (95% CI 2.6, 6.8) for first degree relatives of cases, and calculated that genetic factors attributed ~23% to all end stage ARM in the population.[7]

Although the ABCR and APOE genes have been associated with ARM, the knowledge of the disease-causing genes is limited.[8-11] Most former study designs regarded ARM families as a genetically homogeneous population. In a complex disease such as ARM, that is rather unlikely. Presumably, there are families with a strong genetic susceptibility, as well as families with only a mild or no genetic risk, and the relative frequencies of these families will determine the overall magnitude. Knowledge of the specific familial risks will enhance genetic research, but is also particularly relevant for clinical counseling of individual families.

In this report, we further explored the data of our previous familial aggregation study with the aim to detect variation in risk among ARM families. For each family, we calculated a risk score based on observed and expected number of affected relatives using demographic and epidemiologic data.[12] This methodology allowed us to discriminate between high and low risk families, to investigate their frequencies, and to assess the association with risk of ARM.

SUBJECTS AND METHODS

COLLECTION OF FAMILIES

Design of the familial aggregation study and methods of data collection have been described previously.[7,13] In brief, all probands were identified from the baseline phase of the Rotterdam Study; case probands (n=101) were all subjects with atrophic or neovascular macular degeneration, and control probands (n=154) were a randomly selected sample of study subjects who did not have any soft drusen (≥63μm), nor any atrophic or neovascular macular degeneration. Probands differed in age (cases mean age 81.9 years vs controls 76.7 years, $P<0.001$), but not in gender (cases 63% women vs controls 56% women, age-adjusted $P = 0.64$). Genealogical data of the last five generations were obtained from probands; no probands were genealogically linked. First degree relatives were subsequently invited for a screening examination at a research center or at home.

DIAGNOSIS OF ARM

The ophthalmologic examination included fundus photography of a 20° and 35° macular field with a stereoscopic (Topcon TRC-SS2 stereoscopic fundus camera, Topcon Optical Company, Tokyo, Japan) and monoscopic camera (Topcon TRV-50VT fundus camera, Topcon Optical Company, Tokyo, Japan). Subjects who were examined at home were photographed with a portable camera (35° field, Kowa RC-2 fundus camera, Kowa Corporation LTD, Tokyo, Japan). Fundus transparencies were graded for presence of ARM features in a masked fashion according to the International Classification System, identical to the protocol that was used for probands.[14] ARM was stratified in two stages of disease. Early ARM was defined as the presence of either soft distinct drusen with pigmentary changes or the presence of soft indistinct or reticular drusen. Late ARM was defined as the presence of atrophic (geographic atrophy) or neovascular AMD.

Table 1. *Age-specific prevalence (%) of ARM in the baseline phase of the Rotterdam Study, 1989-1993*

Age (years)	Early ARM	Late ARM	Total ARM
55 - 59	2.2	0.2	2.4
60 - 64	3.2	0.1	3.3
65 - 69	5.1	0.5	5.6
70 - 74	10.6	0.8	11.4
75 - 79	12.6	1.7	14.3
80 - 84	14.8	6.5	21.3
85 - 89	18.5	9.2	27.7
90+	21.2	17.7	38.9

STATISTICAL ANALYSES

Age-specific prevalences of ARM were determined from the baseline phase of the Rotterdam Study,[12] and they served as the expected outcome of ARM for each relative (Table 1). For each family, the expected number (E_i) of affected relatives for the ith family was compared to the observed number (O_i) to give a family score (FS) for this family as

$$FS = O_i - E_i$$

This formula was originally described by Houwing-Duistermaat et al, who showed that this equation may well be used for incorporation of family data into logistic regression models.[15,16]

Since our study population consisted of first degree relatives only, no weights reflecting the distance between relative and proband were used.

Family scores were subsequently stratified in four risk groups: no increased risk ($FS < 0.5$); low risk ($0.5 \leq FS < 1$); intermediate risk ($1 \leq FS < 2$); and high risk ($FS \geq 2$).[15-17] The relative frequencies of these strata were calculated. The risk of late ARM was estimated for family score using linear regression analysis, adjusting for the possible confounding effect of age, sex, and smoking. An additional analysis was performed to study the potential confounding effect of atherosclerosis.

Table 2. *General characteristics of the study population*

	Case families (n = 64)	Control families (n=100)
Total no. of relatives	186	343
No. of siblings	73	142
Mean age of siblings, yrs, ± SD	76.0 ± 8.7	75.4 ± 9.4
% women among siblings	55	59
No. of offspring	113	201
mean age of offspring, yrs, ± SD	53.7 ± 10.4*	48.8 ± 8.9
% women among offspring	43	46

* P < .001 for the difference with relatives of controls

RESULTS

Of case probands, 87 (86%) subjects consented to participation in the familial aggregation study; of control probands, 135 (88%) responded. Of the relatives of cases, 73 (85%) siblings and 113(86%) children responded. Of relatives of controls, these responses were 142 (80%) and 201(81%), respectively. This resulted in 64 case families and 100 control families available for the family score analyses. Table 2 shows the distribution of age, gender, and composition of the families; table 3 shows the number of affected and unaffected relatives among case and control families.

Table 3. *Frequency of ARM among first degree relatives per family**

No. of relatives affected	Case families† (n = 64)	Control families (n = 100)
0	41 (64)	84 (84)
1	14 (22)	15 (15)
2	7 (11)	0 (0)
3+	2 (3)	1 (1)

* Frequencies in numbers (percentages)

† The difference in distribution of number of affected relatives between cases and controls was statistically significant, P=0.002

The individual family scores ranged from -0.7 to 3.5 in the entire study group. Table 4 shows the distribution of family scores among cases and controls. The family scores in the case families varied from -0.4 to 2.9. Nine families had a family score between 1 and 2, and 2 families had a score above 2. No significant differences were found in distribution of low, medium, and high family scores between probands with atrophic and neovascular AMD (data not shown). The family scores in the control families ranged from -0.7 and 1.0 with an outlier of one family with a family score of 3.5. This family consisted of 12 relatives of whom four were affected. Two affecteds were relatively young and largely determined this high family score. The control proband in question was still unaffected at the time of this analysis.

Table 4. *Distribution of Family Scores in risk categories among case and control families*[*]

Family score		Case families[†] (n=64)	Control families (n=100)
$FS < 0.5$ no risk		43 (67)	90 (90)
$0.5 \leq FS < 1$	low risk	11 (17)	9 (9)
$1 \leq FS < 2$	intermediate risk	8 (13)	0 (0)
$FS \geq 2$	high risk	2 (3)	1 (1)

[*] Frequencies in numbers (percentages)

[†] The difference in distribution of *FS* between cases and controls was statistically significant, $P=0.0004$

In Figure 1A and 1B, the relative frequencies of family scores are plotted. Among cases, there was a peak around zero with a skewed tail composed of families with higher than expected rates of ARM. Among controls, the distribution of the family scores was, apart from the outlier, centered around 0.

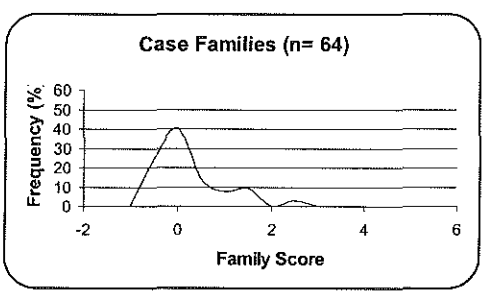

Figure 5a *Frequency (%) of family scores in case families*

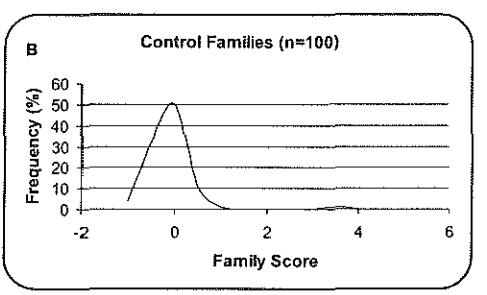

Figure 1b *Frequency (%) of family scores in control families*

The risk β of late ARM for family score was 1.10 (95% CI 0.43, 1.76) when adjusting for age, and gender, and increased to 1.28 (95% CI 0.55, 1.99) when adjusting for age, sex, and smoking. Additional adjustment for atherosclerosis did not alter the risk estimate.

DISCUSSION

In this analysis, we demonstrated familial heterogeneity of the ARM risk using a family score method.[15-17] The majority of ARM families did not appear to have higher frequencies of disease than expected on the basis of their age distribution. However, 17% of families had a low increased risk, 13% an intermediate risk, and 3% a high of disease, much higher than expected by the age distribution of the family members. Subjects with an intermediate or high familial risk were at least 30 times more likely to be affected than subjects with no excess familial risk. This increased risk could not be explained by the known risk factors smoking and atherosclerosis; on the contrary, the risk accrued when the effect of these factors was taken into account.

There are several advantages of the design and method of analysis in this study. In contrast to former familial risk analyses of ARM which pooled relatives from different families, we used a family score method which regarded individual families as the unit of analysis. This strategy was developed earlier by Houwing-Duistermaat et al. for modeling family history in logistic regression models.[15,16] Per family, the observed number of affected relatives was compared with the expected number, resulting in a risk estimate for each family. An important benefit is that this allowed for discrimination of risk in families from the same proband group. Other strengths of the study include the calculation of the expected ARM risk from the age-specific prevalences in the Rotterdam Study, the same source population as where the case and control probands originated from. Moreover, relatives were actually examined and photographed, and the diagnostic criteria for observed and expected number of affected relatives, as well as for cases and controls, were identical.

Among the limitations of our approach is, that consideration of the family as the unit of analysis created loss of statistical power. Due to the relatively small number of families, and the small number of relatives per family, statistical significance between familial risk strata could not be achieved. For the total number of families that were studied, we were limited by the frequency of ARM in the Rotterdam Study. Increasing the number of families and expanding the study population with second degree relatives would improve precision of the risk estimates. Another issue is that our study population consisted of prevalent rather than incident cases, leading to misclassification of family scores due to an unknown age of onset. On the other hand, this will be the situation encountered in clinical practice. By using age-adjusted prevalences for the expected number of relatives, we adjusted the excess familial risk for age at examination. Finally, control probands were younger than case probands, and may have harbored 'subclinical' familial cases of ARM. The effect of this potential misclassification appeared to be small, for observed family scores were close to the expected score among the control families. The exception was one family with four relatives affected, of whom the proband was still unaffected at the age of 87 years.

Given these considerations, what can be learned from this study? Our data emphasize that ARM is a genetically complex disorder with a large variation in the contribution of the genetic component in families. Although possible, it is unlikely that all our data are explained by variation of one single major gene.[6]

Presumably , a strong genetic factor with a Mendelian inheritance determines the risk in high risk families, and this proportion of families appears to be small. In the other families, multiple etiologies may be involved: some genetic, some environmental, and most likely a combination of these.

In summary, the results of this analysis complement our earlier findings.[7] We confirmed that families of cases are at an increased risk of ARM, but now demonstrated that the variation in familial risk is large. The classification of ARM families into high, intermediate, and low risk families may allow molecular genetic studies to focus on the appropriate risk groups in the search for disease-causing genes.

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-CHAPTER 6-

GENOMIC SCREENING FOR AGE-RELATED MACULOPATHY IN AN ISOLATED POPULATION

INTRODUCTION

Age-related maculopathy (ARM) is a complex eye disorder, affecting people of 55 years and over. In this disease the central part of the retina, the macula, is affected, which causes central vision loss.[1-3] Age-related maculopathy consists of a variety of signs varying from drusen to the endstages of the disease, geographic atrophy and neovascular macular degeneration.[4-6]

Among the many risk factors potentially involved in ARM, aging and smoking were consistently found in studies.[7] Other factors possibly involved are cardiovascular factors, light, cataract surgery and diet.[8,9] For the involvement of genetic factors many clues can be found in the literature. Twin studies, familial aggregation and segregation analyses provided evidence for involvement of genetic risk factors in ARM.[10-17] Many genes are possible candidate genes for ARM. Candidate genes may be selected from their known function, chromosomal position, and their known involvement in other forms of inherited retinal degeneration such as Stargardt disease or Sorsby fundus dystrophy.

Recently, in a large pedigree, in which 10 family members were affected with early or late ARM, Klein et al. demonstrated linkage between ARM and a locus on chromsome 1q25-31.[18] Others have showed in association studies that ARM is possibly caused by mutations in the ABCR gene on chromsome 1p.[19] The ABCR gene is a photoreceptor cell specific ATP-binding transporter gene and known to be involved in Stargardt disease. Another candidate gene for ARM, the TIMP-3 gene, known to be involved in Sorsby fundus dystrophy, is located on chromosome 22.[20] La Paz and co-workers excluded TIMP-3 gene to be involved in 38 small families with ARM, it still remains a candidate gene for ARM.[21]

One of the major problems in studies of the genetics in ARM is the late onset of the disease. The parents are usually deceased while the younger generation does not have the disease on a clinical level yet. Individuals available for studies are usually limited to sibships. It is therefore very difficult to find families, which are large enough for linkage analysis. Unrelated individuals, which are generally easier to recruit than large families, are used in association studies. However, in genetically hetereogenous disorders, such as ARM, multiple mutations in different genes may exist or different mutations in one gene derived from several founders may exist. Association studies are therefore not appropriate for genomic screening. Genetically isolated populations are more suitable

for such studies, because the number of founders is limited. The number of mutations cosegregating in such a community is therefore more limited.

In the current study we have examined individuals derived from a genetically isolated population and we have identified a large family with ARM within this population. The aim of this study is to identify genetic loci which are involved in ARM in this family and to exclude other candidate loci.

MATERIALS AND METHODS

The study was approved by the medical ethical committee of the Academic Medical Center in Amsterdam, the Netherlands. Informed consent was obtained from all study participants. Individuals with ARM from a genetically isolated community in the Netherlands were ascertained through the registers of ophthalmologists and general practitioners. Ophthalmological examination was performed at their homes. A questionnaire was used for indicating general health problems.

The ophthalmologic examination included fundus photography of 35° macular field with a portable camera (35° field, Kowa RC-2 fundus camera, Kowa Corporation LTD, Tokyo, Japan). Fundus transparencies were graded for presence of ARM features in a masked fashion according to the International Classification System.[6] ARM was stratified in two stages of disease. Early ARM was defined as the presence of either soft distinct drusen with pigmentary changes or the presence of soft indistinct or reticular drusen. Late ARM was defined as the presence of atrophic (geographic atrophy, GA) of neovascular AMD (NMD).

Genealogical data were obtained at least six generations back. From all individuals blood samples were taken from which DNA was isolated by standard techniques as described elsewhere.[22] If applicable, blood was also taken from spouses as well as children, to construct the haplotypes of the cases and the controls. A genome wide screening with 400 polymorphic markers was performed in this pedigree. The markers were equally spaced with an interval of maximum 10 cM between them.

From all cases the haplotypes were manually constructed, using the alleles from the children and spouses if available. The reconstructed chromosomes of the spouses were used as controls. In the control group 40 chromosomes (18 were tested, 22 reconstructed) could be used for analysis, for the case group 44 chromosomes were identified. Of the 22 affected individuals in this pedigree, the haplotypes of 15 individuals could be constructed by the use of the alleles of children and spouses. The alleles of the other 7 individuals were known, although no haplotype could be constructed. From all 400 markers the alleles were counted among the cases and controls. Chi-squares were calculated to detect differences in allele distribution between the case and control group. The Chi-squares and p-values were calculated and if it was significant an odds ratio was calculated.
Genotypic data were also analysed with a likelihood ratio statistic which tests whether a single marker allele is overrepresented on chromosomes of patients as compared to control chromosomes (Terwilliger, 1995).[23] This approach rests on the assumption that many of the patients may share a

chromosomal segment surrounding a shared disease mutation, which may have been inherited from a single common ancestor. Given the availability of genotypic data of spouses and offspring for many of the patients we employed an implementation of this test statistic which can appropriately analyse data for related individuals, as described in Escamilla et al.[24]

The first aim of this study was to examine three candidate loci, chromosome 1q, ABCR and TIMP3 gene, to see whether excess sharing of marker alleles and/or haplotypes in any of these regions could be observed in patients. For the locus on chromosome 1q we used marker D1S2655, for the ABCR gene markers D1S435, D1S495, D1S221 and D1S252, and for the TIMP3 gene on chromosome 22 markers D22S280, D22S283, and D22S274 from the genomic screening marker set together with some additional markers in these regions (see Table 1).

For the purpose of this initial study, haplotypes of these three regions were constructed in the cases. When more than one solution was found for a haplotype construct, all solutions were taken in the analysis. The haplotypes were compared with each other, to investigate a possible region of haplotype sharing, as well as to investigate cosegration of a haplotype with a specific form for ARM (early ARM, GA or NMD).

The second aim of this study is to identify regions of interest, in which a disease-causing gene might be localised. Markers which revealed a chi-square with a p-value lower than 0.05 with both (manually and by the method of Terwilliger) methods were identified as regions of interest. This part of the data analysis is still in progress.

RESULTS

In total 23 affected individuals with ARM were ascertained in this genetically isolated community. All but one individual were genealogically linked to each other, constructing a large five generation family with multiple loops (figure 1). One individual with ARM was excluded from this study. He was not born in this community, but lived here since he had married. Of the 22 affected individuals in this family, nine individuals were diagnosed with early ARM. The end stages of ARM, age-related macular degeneration (AMD), were seen in 13 individuals, of whom 38,4 % were diagnosed with geographic atrophy and 61,5% were diagnosed with neovascular macular degeneration. In this study 45 % of the affected individuals were male. The age at examination varied from 66 years to 91 years of age.

An overview of the chi-squares, p-values and markers of the three candidate loci is given in Table1. For the region of the 1q locus, markers D1S2655 revealed a chi-square of 0.06. The ABCR gene markers D1S435, D1S495, D1S221, and D1S252 showed a chi-square of 0.30, 1.32, 1.09 and 0.30 respectively. The TIMP-3 gene markers D22S280, D22S283, and D22S274 showed a chi-square of 0.46, 0.84 and 1.36 respectively.

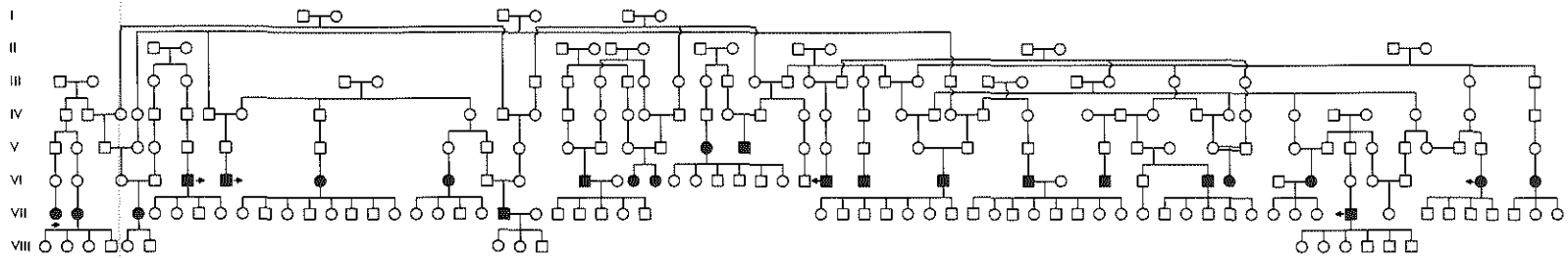

Figure 1. *Pedigree of the affected individuals with ARM in a genetically isolated community.*

Table 1 *The three candidate regions examined in the initial study*

	Marker	cM from pter	Chi-square	p-value
Chromosome 1q	D1S2625	215.6	0.16 *	0.7
	D1S412	216.8	0.13 *	0.7
	D1S413	219.4	2.48 *	0.1
	D1S2655	221.1	0.06	0.4
ABCR gene	D1S435	128.9	0.3	0.3
	D1S206	137.6	0.69 *	0.4
	D1S495	146	1.32	0.2
	D1S221	146.7	1.09	0.2
	D1S252	155.1	0.3	0.3
TIMP3 gene	D22S280	25.9	0.46	0.3
	D22S283	33.4	0.84	0.2
	D22S274	45.5	1.36	0.1

** based on manually constructed haplotypes.*

The haplotypes did not cosegregate with a specific type of ARM (early ARM, geographic atrophy or neovascular macular degeneration) and no haplotype sharing region was found in these three candidate regions (data not shown).

The genomic screening revealed five regions of interest, in which the p-value was in both methods lower than 0.05. The highest chi-square of these two methods is noted. The chi-squares of these five regions were 4.5 (p = 0.03), 4.6 (p=0.03), 7.2 (p=0.007), 8.9 (p= 0.003) and 11.4 (p = 0.0003). In these regions more markers will be tested in the near future. This will lead to exclusion of some regions and probably one or two regions will be candidate regions for the disease-causing gene in this family.

DISCUSSION

In the current study we identified a large family with ARM (early and late ARM) in a genetic isolate. A genetically isolated population has several advantages; for the use of allele sharing methods the number of founders is limited and therefore the mutations in genes or the number of genes are most likely also limited. For this study not only an allele sharing method was performed, the nucleair families makes it possible to perform linkage analysis. The combination of these two approaches provides maximum information and statistical power.

In the first part of this study we focussed on three candidate genes / loci, chromosome 1q, the ABCR gene and TIMP-3 gene. Although significant proof was given by Klein et al.[18] for the involvement of chromosome 1q in a large pedigree with ARM, no evidence of involvement of this locus was found in our pedigree. Equally no involvement of ABCR or TIMP-3 was found. However, we can not exclude these loci to be involved in ARM, since heterogeneity and multiple

underlying trait loci are difficulties in evaluating candidate genes in complex diseases, such as ARM.

The five regions of interest which have been identified will be investigated in the near future. Additional markers in these regions will be tested and analysed. The allele sharing method will be combined with a linkage analysis in closely related affected individuals to obtain maximum statistical power. One might expect that some regions will be excluded and that one or two regions will remain of great interest as possible loci for disease-causing gene.

In the evaluation of the results obtained in a genome wide search for association, a statistical correction should be applied for the fact that manu markers are tested. Each additional marker tested will lead to an increase in the overall possibility to obtain one or more false-positive signals. There is debate over the exact threshold that shoul be applied in a genome wide search for association of the results to accepted as conclusive evidence. In this study we have not included any corrections for multiple testing, yet. The current results should be reviewed more as an attempt to identify and rank-order promising chromosomal regions that deserve futher analysis, than as a final proof for one or more ARM loci. As has been suggested before, many genes may be involved in ARM. A gene in a pedigree from a genetically isolated population may be involved in just a very small proportion of the general population. However, it could provide clues to the pathogenesis of ARM.

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PART IV

CANDIDATE GENES FOR AGE-RELATED MACULOPATHY

-CHAPTER 7 -

SORSBY FUNDUS DYSTROPHY WITHOUT MUTATION IN TIMP-3 GENE

ABSTRACT

Aims: To examine a large family with an autosomal dominant fundus dystrophy and to investigate whether mutations in TIMP-3 gene were involved.

Methods: A large family of 58 individuals with an autosomal dominant fundus dystrophy was ophthalmologically examined. A DNA linkage analysis of the region 22q12.1-q13.2 was performed. The TIMP-3 gene was screened for mutations in all five exons.

Results: In this large family 15 individuals were affected, all other individuals were found to be clinically unaffected. Pisciform flecks in the midperiphery and drusen-like deposits were the most typical ophthalmological finding in this family and were encountered from the fifth decade on. Chorioretinal atrophy and neovascularization with disciform lesions characterized the disease from the sixth decade on. Linkage analysis, in an affected-only analysis, showed a maximum positive lodscore of 3.94 at $\theta = 0.0$ with marker D22S283. No mutations possibly causing Sorsby fundus dystrophy were found in either the exonic sequences, the promotor region or 3'UTR.

Conclusion: The family in this pedigree is suffering from an autosomal dominant fundus dystrophy, which is most likely Sorsby fundus dystrophy. Although, in the linkage analysis significant positive lodscores were found with the region 22q12.1-q13.2, no causative mutations could be identified in the five exons and the promotor region of the TIMP-3 gene.

INTRODUCTION

Sorsby fundus dystrophy (SFD) is an autosomal dominant disorder in which patients lose central vision during the 4th or 5th decade of life.[1] Monogenic maculopathies such as Sorsby fundus dystrophy have been suggested as a possible mendelian model for the study of the genetically complex age-related macular degeneration (AMD). However, de la Paz exlcuded TIMP-3 gene in 38 families as a candidate gene for AMD.[2] Age-related macular degeneration is the leading cause of permanent visual impairment among the elderly in western countries, affecting over 10% of the population aged 75 years and over.[3,4] Identification of molecular defects underlying AMD has been hampered by the late onset of symptoms, as well as the clinical heterogeneity commonly observed in the disorder.

Sorsby described five families with a fundus dystrophy that occurred in several generations in a dominant pedigree pattern.[1] The dystrophy became manifest at about the age of 40 years, the earliest manifestations were colour vision deficits and abnormal yellow-white deposits; followed by a central macular lesion with oedema, heamorrhage and exudates. In the subsequent years, atrophy with pigmentation of the central area and extension peripherally occurred. The choroidal vessels became exposed and appeared somewhat sclerotic. Within about 35 years after onset the entire fundus was involved. The choroidal vessels disappeared by this stage and the terminal picture was one of extensive choroidal atrophy. Night blindness was not a consistent feature.

Sorsby's observations have been extended in the descendants of the original families with SFD.[5-7] Also a genealogic link between these families and an Australian family with SFD have been reported.[8,9] Hamilton et al. described a seven-generation family with Sorsby fundus dystrophy.[10] Later on, Weber et al. demonstrated linkage of the SFD locus with DNA markers on chromosome 22q13-qter.[11]

In parallel, Apte et al. isolated a novel gene-member of the tissue inhibitors of metalloproteinases (TIMPs), TIMP-3 and localized it to human chromosome 22q12.1-q13.2.[12] TIMPs are natural inhibitors of matrix metalloproteinases (MMPs), which are involved in the synthesis and degradation of extracellular matrix (ECM) molecules, thereby remodeling the ECM continously. Because of the colocalization of TIMP-3 and SFD on chromosome 22 and its pivotal physiological role in extracellular matrix remodeling, Weber et al. studied TIMP-3 as a candidate gene for SFD.[13] Point mutations in the TIMP-3 gene were demonstrated in affected members of two SFD pedigrees. These mutations predicted disruption of the tertiary structure and thus the functional properties of the mature protein.

In this study we examined a large family with a Sorsby fundus dystrophy to investigate whether mutations in TIMP-3 gene were involved.

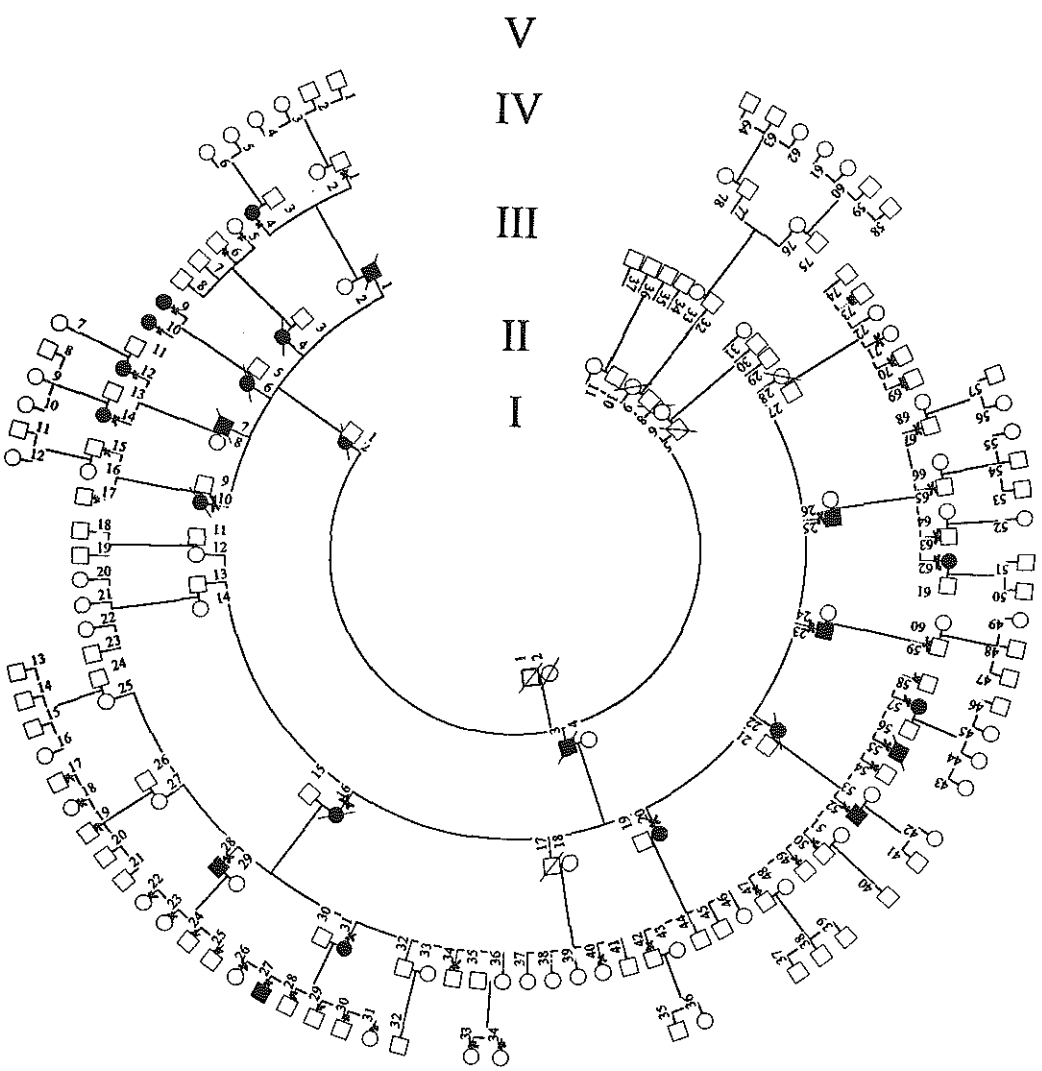


Figure 1 *Pedigree of the family with Sorsby fundus dystrophy. Males are represented by squares and females are represented by circles; blackened symbols represent individuals with Sorsby fundus dystrophy, unblackened symbols represent normal individuals. ✱ represents the individuals who were clinically examined and used for linkage analysis.*

MATERIALS AND METHODS

A large family of 58 individuals with a five generation history of fundus dystrophy was examined by indirect ophthalmoscopy (see figure 1), followed by fluorescein and indocyanine green angiography in case fundus lesions were found on ophthalmoscopy. No genealogic link between this family and the families originally described by Sorsby was established. Informed consent was obtained from all individuals. Two patients (III,10 and III,16) were examined in our department respectively in 1975 and 1984; both died before the start of this study. Another individual (IV,55) died during the examination period and underwent only ophthalmoscopy.

Blood samples from all individuals were obtained. Standard techniques were used to extract DNA, and PCR and CA-repeats analyses were performed as described elsewhere.[14]

Because the clinical data were compatible with a dominant mode of inheritance and the diagnosis of Sorsby fundus dystrophy was proposed after examination of the individuals, a linkage analysis of the region 22q12.1-q13.2 was performed. Markers D22S275, D22S304, D22S283 and D22S274 were used to perform a linkage analysis.[11,15] We have used an affected only analysis. The data were analyzed with the programme package LINKAGE, version 5.1. Two point Z values were calculated with the programme LINKMAP. The fixed order and genetic distances were set as follows D22S275- 0.07 - D22S304 - 0.066 - D22S283 - 0.168 - D22S274.

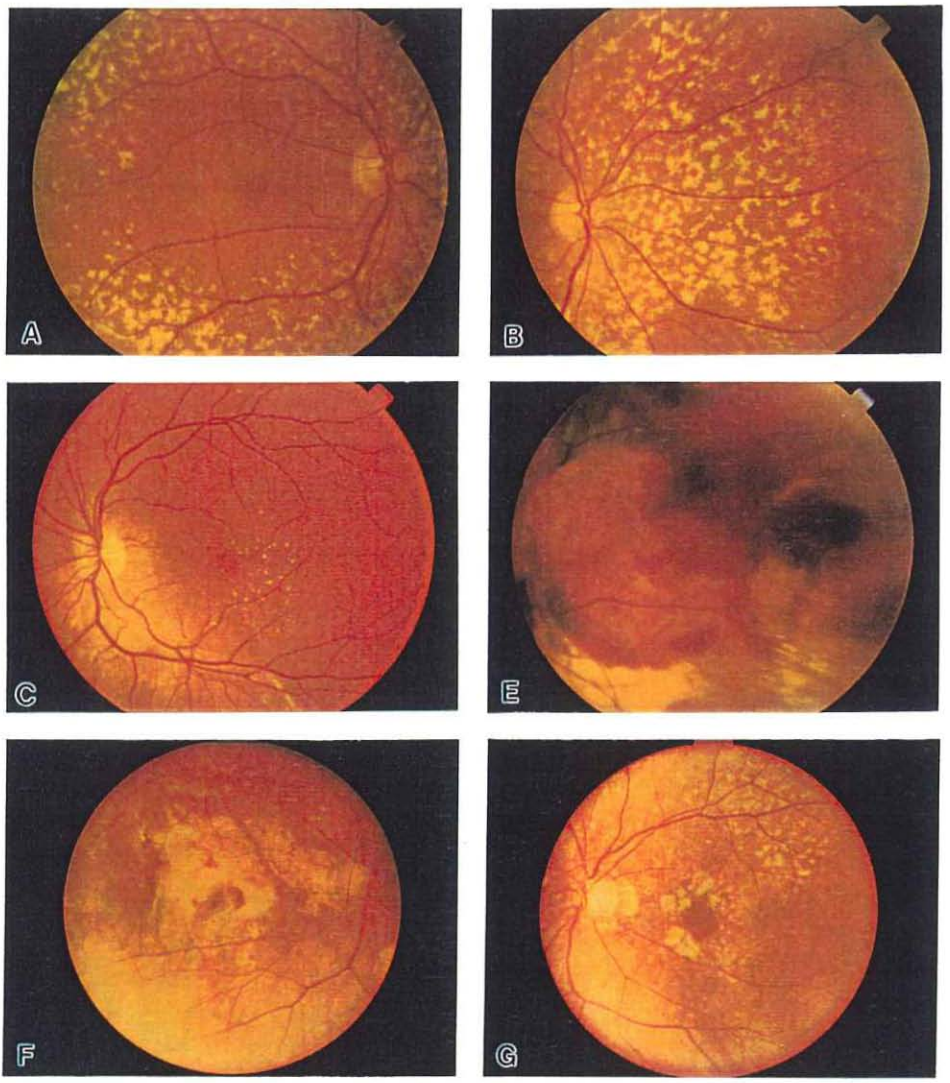
Because the linkage analysis showed significant evidence for linkage to this region, the TIMP-3 gene was screened for mutations. The likely position of TIMP-3 is between D22S275 and D22S283. We used all sequence and primers available to us of the TIMP-3 gene for the single strand conformational polymorphism (SSCP) of the five exons, the promotor region and 3'UTR.[16,17] SSCP analyses were performed as described elsewhere.[18] Also further analysis with sequencing, using the dideoxy nucleotide chain termination method, was performed. The direct sequences of exon 5 could not be analysed beyond doubt, therefore PCR amplified exon 5 fragments were cloned into the pCR vector (Invitrogen) using TA cloning kit and multiple colonies were sequenced.

RESULTS

In this large family 15 individuals were affected with SFD, all other individuals were found to be unaffected. However, some of the healthy individuals have not reached the age of 40 years and therefore they might develop the disease in the coming years.

Figure 2

a. Pisciform flecks at the posterior pole (patient IV,57). b. Flecks nasally to the papil (patient IV,57). c. Drusen-like deposits (patient IV,62). d. Extensive chorioretinal atrophy (patient IV,12).e. Choroidal neo-vascularization with haemorrhages and exudation (patient IV,9). f. Disciform lesion with fibrotic reaction extending far outside the macula (patient III,16 OD). g. Pisciform flecks at the arcades, atrophic patches surrounding the macula (patient III,16 OS).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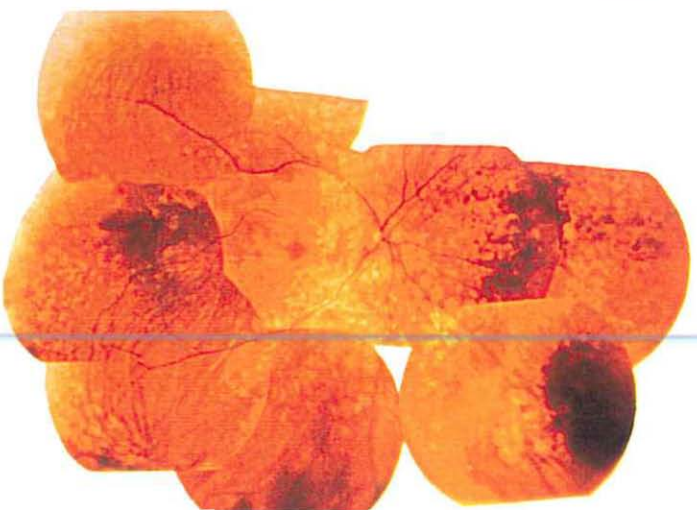
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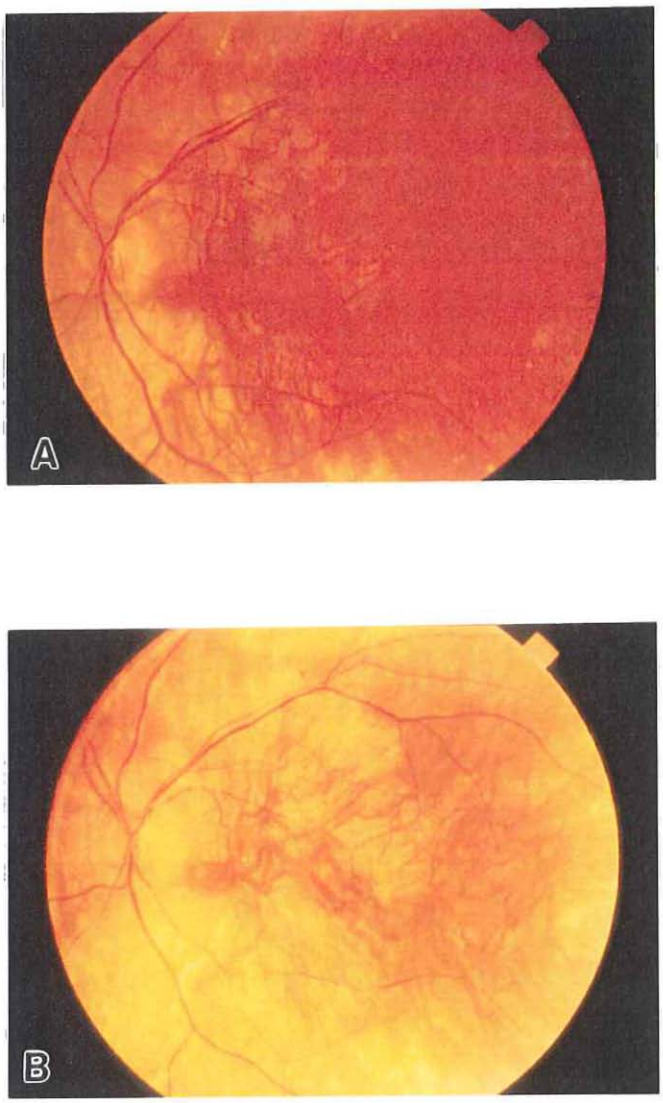


Figure 3

a. Patient IV,12 at the age of 60 years; OS visual acuity 0.2, notice atrophy of the capillary.

b. Same patient (IV,12) at the age of 63; OS visual acuity decreased to LP due to involvement of the macula by the choriocapillary atrophy.

The ophthalmological data of these 15 patients are summarized in Table 1. The results of two patients, who were examined in the past, are also included in this table. The affected individuals had a large variety of ophthalmoscopical findings (see figure 2-3).

Table 1. *Ophthalmological data of patients with Sorsby fundus dystrophy.*

Patient	Age (years)	Pisciform flecks	Drusen-like deposits	Chorioretinal atrophy	Disciform lesion		Visual acuity	
		ODS	ODS	ODS	OD	OS	OD	OS
III,10	60	+	+	+	-	P	1	1
III,16	74	np	np	+	M	-	0.01	0.05
III,20	78	-	-	+	M	M	0.01	0.01
III,23	60	-	+	-	M	M	<0.05	<0.05
III,25	80	-	+	+	-	-	0.4	0.01
IV,4	50	+	-	-	-	-	1	1
IV,9	556063	+	+	+	-	M	1.0	0.01
		-	+	+	P	M	1.0	0.01
		-	+	+	RD	M	LP	0.01
IV,10	55	+	-	-	-	-	1	1
IV,12	4.7e+07	np	np	-	-	-	1.0	1.0
		+	+	+	P	-	0.8	0.8
		+	+	+	P	-	0.1	0.2
		-	-	+	P	-	0.03	LP
IV,14	52	+	+	-	-	-	1	1
IV,28	4960	-	+	-	M	-	0.01	1.0
		-	+	+	M	-	LP	0.5
IV,31	59	+	+	-	-	-	1	1
IV,52	59	+	+	-	-	-	1	1
IV,55	54		+	-	-	-	np	np
IV,57	35	+	-	-	-	-	1	1
IV,62	48	-	+	-	-	-	0.6	1
V,27	31	-	+	-	-	-	1	1

np = not performed
M = macular disciform lesion
P = peripapillary disciform lesion
RD = retinal detachment
IV,62 OD amblyopia

In early stage of the disease, in patients of the fifth and sixth decade, pisciform yellow flecks at the level of the arcades and midperiphery were the most typical findings in this family (fig. 2a,b).

Most patients had additionally round drusen-like deposits (fig. 2c). These flecks became less apparent with age and disappeared when atrophy supervened. In the latest stage of the disease extensive chorioretinal atrophy and sclerosis was observed (fig. 2d). Macular (7 eyes) and peripapillary (3 eyes) choroidal neovascularization occurred. The onset of neovascularization in our family was at the age of 49 years in one patient and after the age of 55 years in the others. Exudative inferior retinal detachment was observed in one patient (IV,9) (fig. 2e). Pisciform flecks fluoresced like window defects when fluorescein angiography was performed in young symptomatic patients, no delayed choroidal filling was observed. Fluorescein angiography in older patients showed chorioretinal atrophy and choroidal neovascularization. Electrophysiology and dark adaptation tests were only performed in a small number of patients (Table 2). In all patients the EOG was abnormal in an early stage of the disease and was indicative for retinal pigment epithelium dysfunction. The dark adaptation showed a slight elevation of rod threshold. Electroretinograms remained normal until chorioretinal atrophy became manifest. Progressive atrophy was reflected by severe impairment of rod function and relatively preserved cone function. The visual acuity in the patients followed the different stages of the disease. Visual acuity abruptly decreased when central maculopathy was observed either of disciform or atrophic nature. Extension of geographic retinal atrophy progressed within 4 years in patient IV,12 with a dramatic deterioration of the visual acuity from 0.8 ODS to 0.03 OD and LP OS when the fovea became involved (fig. 3a,b)

Table 2. *Electrophysiological data of patients with Sorsby fundus dystrophy.*

Patient	Age (years)	Visual acuity		Dark adaptation (threshold log unit)	EOG (arden index)		ERG response	
		OD	OS		OD	OS	photopic	scotopic
III,10	60	1	1	1.5 log U ↑	↓18%	111%	slight↓	severe↓
III,16	74	0	0.1	2 log U ↑	↓00%	↓00%	slight↓	absent
IV,12	4660	1.0	1.0	normal	np	np	normal	normal
		1.0	1.0	1 log U ↑	120%	111%	slight↓	severe↓
IV,14	495659	1.0	1.0	np	118%	120%	normal	normal
		1.0	1.0	1 log U ↑	120%	111%	slight↓	severe↓
		0.8	0.8	1 log U ↑	np	np	severe↓	absent
IV,28	49	0	1	2 log U ↑	↓23%	↓28%	slight↓	severe↓

np = not performed

The linkage analysis with the marker D22S275 revealed a maximum lodscore of 2.14 at $\theta = 0.05$ and with marker D22S274 a maximum lodscore of 0.15 at $\theta = 0.3$ was found. Both markers D22S304 and D22S283 revealed significantly positive lodscores of resp. 3.89 and 3.94 at $\theta = 0.0$. The results of the linkage analysis are given in Table 3. All affected individuals shared a haplotype. Marker D22S275 revealed a recombination in individual IV,57. Marker D22S274 revealed a recombination in individuals IV,10, IV,55 and V,27.

Table 3. *Two point Z values for linkage between markers on chromosome 22 and SFD.*

Locus	Z at θ =						
	0	0.01	0.05	0.1	0.2	0.3	0.4
D22S275	-1.95	1.68	2.14	2.11	1.69	1.08	0.42
D22S304	3.89	3.81	3.48	3.05	2.17	1.28	0.49
D22S283	3.94	3.86	3.54	3.14	2.29	1.42	0.6
D22S274	-6.78	-3.14	-1.16	-0.41	0.1	0.15	0.05

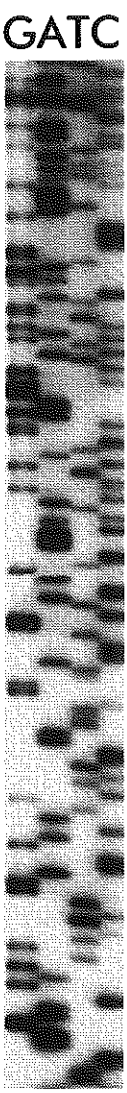
The SSCP of the five exons, promotor region and 3'UTR showed no changes between unaffected and affected individuals. Nevertheless, all exons of the TIMP-3 gene were sequenced in one healthy person and in at least two affected individuals. No mutations possibly causing SFD were found in either the exons or their splice sites. A sequence gel of exon 5 of one of the affected individuals is shown in figure 4a.

DISCUSSION

In this family with Sorsby fundus dystrophy, the presence of the midperipheral yellow pisciform flecks was the most typical finding. They were invariably bilateral and have not been described in previous Sorsby's pedigrees. The yellow flecks may correspond to the subretinal deposits that were reported in SFD. Such deposits were described in Bruch's membrane by Capon et al. after a light and electron microscopic study of the eyes of a descendant of the Kempster family.[19] A localized destruction of the pigment epithelium was the window defect on fluorescein angiography. In later stages of the disease progressive extensive peripheral and in some patients central atrophy occurred. Loss of central vision was due to disciform lesions or central geographic atrophy.

So far, six different mutations have been identified which are implicated in SFD. These are, besides a splice site mutation in the intron4/exon5 junction,[20] Tyr168Cys,[13,17] Ser181Cys,[13,21] Ser156Cys,[22] Gly166Cys[23] and Gly167Cys.[24] It is remarkable that these five mutations were identified in a 80 bp interval at exon 5 of the TIMP-3 gene (see figure 4b). Futhermore, these five mutations lead to the introduction of an additional cysteine residue in the peptide sequence. In this SFD family we could not identify a mutation in this critical region of exon-5 (figure 4a) nor in the splice sites. Although linkage was found with the region 22q12.1-q13.2, no mutations were found in all five exons of the TIMP-3, including the splice sites and also the promotor region and 3'UTR revealed no mutations.

Possible explanations for finding no mutations in these exons could be that the introns or regulatory sequences of the TIMP-3 gene contain mutations, which were not identified yet. Another explanation for this finding could be that the disease in this family might be caused by mutations in another gene located in the chromosomal vicinity of the TIMP-3 gene, similar to the heterogeneity on the X-chromosome where at least two RP genes are located close to each other.[25]




```
124  atc aag tcc tgc tac tac ctg cct tgc ttt
       I   K   S   C   Y   Y   L   P   C   F

134  gtg act tcc aag aac gag tgt ctc tgg acc
       V   T   S   K   N   E   C   L   W   T

144  gac atg ctc tcc aat ttc ggt tac cct ggc
       D   M   L   S   N   F   G   Y   P   G

154  tac`cag tcc aaa cac tac gcc tgc atc cgg
       Y   Q   S   K   H   Y   A   C   I   R
                 C

164  cag aag ggc ggc tac tgc agc tgg tac cga
       Q   K   G   G   Y   C   S   W   Y   R
                 C   C   C

174  gga tgg gcc ccc ccg gat aaa agc atc atc
       G   W   A   P   P   D   K   S   I   I
                                     C

184  aat gcc aca gac ccc tga
       N   A   T   D   P   -
```

Figure 4

a. Sequence of exon 5 in an affected individual with SFD.

b. Nucleotide sequence and sequence of the conceptual translation product of exon 5. For previously described mutations, the replacing amino acid is shown underneath the consensus amino acid.

We also should consider the possibility that the disease, which is described in this paper, is resembling Sorsby fundus dystophy, but in fact is a different disease or a different type of SFD. An argument for this explanation could be that, although these spots may correspond to the subretinal deposits which were reported in SFD previously, in this family the presence of the midperipheral yellow spots was the most typical finding.

In conclusion, the family in this pedigree is suffering from an autosomal dominant fundus dystrophy, which is most likely Sorsby fundus dystrophy. In the linkage analysis significant positive lod-scores were found with the region 22q12.1-q13.2. No causative mutations could be identified in the five exons of the TIMP-3 gene.

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- CHAPTER 8 -

MOLECULAR EVALUATION OF THE ABCR GENE MUTATIONS IN A VARIETY OF RETINAL DISORDERS

ABSTRACT

The photoreceptor cell-specific ATP-binding transporter gene (ABCR), located on chromosome 1p21, is exclusively expressed in the retina. Mutations in this gene have been implicated in Stargardt disease, and, recently, in patients with age-related macular degeneration, retinitis pigmentosa and cone-rod dystrophy.

The ABCR gene is very polymorphic and is involved in several retinal diseases. Recently a model was postulated, which could explain a relation between the residual activity of the ABCR gene product and the severity of the disease phenotype. The clinical expression of the ABCR related disorders apparently depends on different combinations of mutations and alleles of this gene. In order to test this model, 29 unrelated individuals with a variety of retinal disorders were examined. The ABCR gene was screened for mutations using SSCP and direct sequencing. In total, ten mutations were found in six patients with Stargardt disease, cone rod dystrophy and cone dystrophy. Furthermore, several polymorphisms were found. In general our data did support and enhance the proposed model. However, our data indicate that the relationship between ABCR mutations and the resulting phenotype may be much more complex than previously thought.

INTRODUCTION

The photoreceptor cell-specific ATP-binding transporter gene (ABCR), located on chromosome 1p21, is a member of the ATP-binding cassette transporter superfamily and expressed exclusively in the retina.[1-5] The ABCR gene is involved in the active transport of a large variety of bio-molecules across the photoreceptor membrane.[2,6] The ABCR protein is localized to the disc membrane of retinal rod outer segments.[7]

Mutations in the ABCR gene have previously been implicated in Stargardt disease (STGD), a relatively common autosomal recessive monogenic retinal disorder.[6, 8-14] Stargardt disease (STGD) is characterized by juvenile-onset, central visual impairment, as well as atrophic macular lesions sometimes surrounded by orange-yellow flecks; the so called fundus flavimaculatus.[15-17] In more advanced stages of the disease, additional flecks in the midperiphery of the retina appear, which later on develop into atrophic fundus lesions. While the ERG potentials are normal in initial stages of the disease, they may eventually become subnormal or abnormal.[18] In the ABCR gene, about 30 different mutations were so far implicated in STGD. The majority of these represented missense mutations, but also three deletions and four insertions were found.[6,10]

Martinez-Mir et al. demonstrated that a causative homozygously present mutation (1874delA) in a family with retinitis pigmentosa type 19 causes a frame shift in ABCR gene.[19] The heterozygous parents in this family showed no signs of retinitis pigmentosa or age-related macular degeneration. In a consanguineous family with individuals showing either retinitis pigmentosa or cone-rod dystrophy, mutations in splice sites of ABCR gene were demonstrated by Cremers et al.[20] An analysis of ABCR mutations in 70 British patients with recessive retinal dystrophies revealed 31 sequence changes of which 20 were considered to be novel mutations.[21]

The ABCR gene was recently also potentially implicated in the genetically complex disorder age-related macular degeneration (AMD).[22] AMD is the leading cause of visual impairment in the western countries, affecting over 10% of people aged 75 years and over. AMD is characterized by a late onset and progressive central visual impairment, and can be divided in "wet" AMD (neovascular) and "dry" AMD (geographic atrophy).[23] Although the study design, analysis and the conclusions were discussed, in 16% of 167 AMD patients either deletions or amino-acid substitutions were found in at least one allele of the ABCR gene.[24, 25] Most of these mutations were found in patients with the dry type of AMD. Some of the mutations were seen in AMD as well as in STGD patients. In contrast, two other studies did not find any support for allelic variation in ABCR associated with AMD.[26, 27]

The ABCR gene seems to be a very polymorphic gene with involvement in a variety of retinal diseases. Van Driel et al. postulated a model which provides a framework to explain the observed genotypes and phenotypes.[9] They hypothesized that most ABCR mutations could be classified in different classes of severity, and that, depending on the remaining total activity of ABCR, the phenotype can range from AMD at the mild end, to RP at the severe end of the spectrum. Recently, a knock-out mouse of the ABCR mouse was created.[28]

Mice lacking the ABCR gene show delayed dark adaptation. This is likely due to a transient accumulation in discs of the complex between opsin and all-trans-RAL. It would be interesting to investigate mice with different ABCR activity (e.g. homozygous and heterozygous) in order to validate this model.

In order to understand the role of ABCR in retinal disorders and to validate this model, mutational analysis of additional patients is warranted. In this study we examined 29 patients with a variety of retinal diseases, followed by a mutation analysis of the entire ABCR gene.

MATERIAL AND METHODS

PATIENTS AND DNA EXTRACTION

All 29 unrelated patients underwent a complete ophthalmological examination and an electroretinogram (ERG) was made where appropriate. All individuals were treated in accordance with the tenets of the Declaration of Helsinki and informed consent was obtained. Patients were diagnosed with a large variety of retinal disorders with macular involvement, including STGD (2), early onset macular degeneration (2), macular dystrophy (2), autosomal dominant and recessive cone-dystrophy (7), cone-rod dystrophy (12), cystoid macular oedema (1), pattern dystrophy (1), choroidal dystrophy (1) and Bull's eye maculopathy (1). If possible, family members of patients with mutations in the ABCR gene were invited to participate in this study. Blood samples of all individuals were obtained and standard DNA extraction protocols were followed for processing of these.

PCR PRIMERS, SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP) ANALYSIS AND SEQUENCING.

The exon/intron primers of the 50 exons of the ABCR gene used for SSCP analysis are described elsewhere.[8] PCR was essentially performed as described elsewhere.[29] Three different PCR reaction conditions were used for the different primers. SSCP analyses were performed as described elsewhere.[30] If any band shifts were detected in the gel, further analysis with direct sequencing was performed, and additional mutation analysis was performed in at least 130 control chromosomes to exclude potential polymorphism.

RESULTS

Mutational analysis of the ABCR gene in 29 patients with a variety of retinal disorder with macular involvement was carried out. The ophthalmological findings of patients with mutations in the ABCR gene are presented in Table 1. Altogether, 10 apparently functional mutations, not present in at least 130 control chromosomes, were identified in two patients with STGD, three patients with cone-rod dystrophy and in one patient with cone dystrophy.

Table 1. *The results of the ophthalmic examinations.*

Family number	Diagnosis	Age of onset	Visual Acuity		Ophthalmoscopy	ERG
			OD	OS		
25902	Stargardt disease	7 years	0.05	0.05	macular alterations	scotopic decreased photopic residual activity
25797	Stargardt disease	7 years	0.01	0.01	macular alterations, flavimaculatus pigment clumping in the periphery	not performed
25532	Cone-rod dystrophy	7 years	0.02	0.02	sclerotic macular alterations, RPE spiculae	scotopic as well as photopic absent
25479	Cone-rod dystrophy	3 years	0.1	0.25	dystrophy of the macula, pale optic nerve, narrow vessels	scotopic as well as photopic decreased
25815	Cone-rod dystrophy	32 years	0.05	0.05	macular alterations, bull's eye maculopathy	scotopic: 85% of normal values photopic: 12% of normal values
20681	Cone dystrophy	8 years	0.25	0.25	pigmentary in changes in the macula	cone dystrophy

Table 2. *Mutation analysis of patients with several eye diseases with macular involvement.*

P-number	diagnosis	exon	mutation aminoacid	nucleotide change	control chromosomes	diagnosis with same mutation, described in earlier reports
25902	Stargardt disease	17	G863A	G2588C	2/172	STGD[(Allikmets et al, Cremers et al)] AMD[(Allikmets et al.)]
		43	G1977S	G5929A	0/184	STGD[(Rozet et al.)]
		46	Splice site	6385+2 C->G		(a)
25797	Stargardt disease	22	E1087K	G3259A	0/162	-
		48		6543del 36 bp	5/170	(b)
25532	Cone-rod dystrophy	33	I1562T	T4685C	0/130	AMD[(Allikmets et al.)]
		35	R1640W	C4918T	0/186	STGD[(Rozet et al.)]
25479	Cone-rod dystrophy	44	L2027F	C6079T	2/194	STGD[(Nasonkin et al. Allikmets et al)]
25815	Cone-rod dystrophy	17	Splice site	2588-12 C->G		(c)
20681	Cone dystrophy	6	R212H	G635A	6/140	-

(cc) Splice score of consensus sequence: 0.4 ; with 8385+2 C->G it remains 0.4

(dd) In the same region Allikmets et al. found a deletion of one nucleotide (6568ΔC) in a patient with AMD. And in the same exon (48) they found an 11 bp deletion at position 6519 in one AMD patient.

(ee) Splice score of consensus sequence: 8.0; with 2588-12 C->G splice score: 6.1

The majority of the mutations were single nucleotide alterations at conserved amino acids positions. The summarized results of the mutation analysis are presented in Table 2.

MUTATION ANALYSIS OF ABCR IN STARGARDT DISEASE.

Ophthalmoscopy of the affected members in pedigree (P)25902 (Figure 1) showed macular alterations compatible with Stargardt disease. The electroretinogram of the proband showed a residual photopic activity as well as a reduced scotopic activity. Besides the maculopathy, diffuse flecks were seen in the posterior pole of the fundus. Mutation analysis of the entire ABCR gene in patient III,4 of P25902 revealed two heterozygously present mutations (G863A and G1977S) and a mutation in the splice site of exon 46.

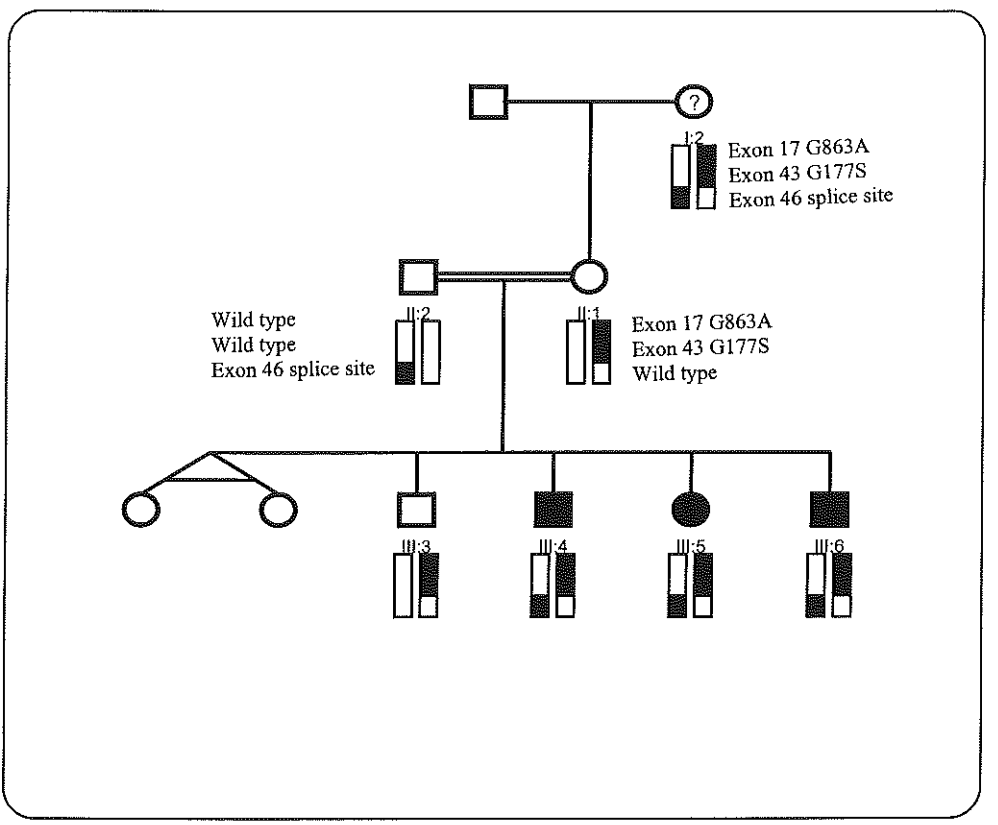

Figure 7 *The pedigree of family 25902 with STGD with the segregation analysis of respectively mutations G863A, G1977S and 6385+3 C->G. Males are represented by squares and females are represented by circles; blackened symbols represent affected individuals. The black bar indicates the mutations found in the proband of this family (III,4)*

The G863A mutation was previously described by Allikmets (1997) in patients with STGD and AMD (Table 2) as well as in a small number of healthy individuals. We found this sequence change also in two out of 172 control chromosomes. Amplification of exon 43 revealed a nucleotide substitution, with a predicted replacement of serine for glycine at amino residue 1977 (G1977S) which was not present in 184 control chromosomes. In the same patient an additional (heterozygously present) mutation was found in the splice site of exon 46 (6385 + 2 C->G). The splice site potential score, according to Shapiro and Senapathy did not change; it remains 77.1.[31] Segregation analysis in the family 25902 showed that mutation G863A and G1977S were inherited from the mother, while the potential splice site mutation was derived from paternal side (Figure 1).

In patient 25797 with STGD, ophthalmological examination showed macular alterations compatible with STGD, as well as flavimaculatus flecks in the posterior pole. An ERG was performed in 1982, which showed a decrease in photopic and scotopic activity. Mutational analysis of the entire ABCR gene revealed one mutation (E1078K) and a 36 bp deletion. Exon 22 revealed a sequence substitution, which would replace a glutamate at amino-residue 1078 with lysine (E1078K), and would possibly introduce a change in polarity in the predicted protein. This mutation was not seen in the 162 control chromosomes. Furthermore, a deletion of 36 bp (6543Δ36bp) was found in the analysis of exon 48, which does not alter the reading frame, but apparently will cause a predicted protein of reduced size. This deletion, however, was also found in five out of 170 control chromosomes and might therefore be a polymorphism. The observed nucleotide change and the deletion were found only in one allele of the ABCR gene, while the other allele revealed the wild-type sequence. DNA of additional family members was not available for further studies to formally prove potential compound heterozygosity in this patient.

MUTATION ANALYSIS OF ABCR IN CONE-ROD DYSTROPHY.

The proband of family 25532 showed sclerotic macular alterations at ophthalmoscopy. The findings of the ERG were consistent with cone-rod dystrophy. Mutational analysis of the ABCR gene in this patient revealed mutation I1562T in exon 33 which was described before by Allikmets (1997), but, in contrast with our finding, only in patients with AMD (Table 2). Upon further analysis of ABCR in this patient exon 35 revealed a heterozygously present nucleotide substitution which would replace in the predicted protein a positively charged arginine with the neutral tryptophan at amino-residue 1640 (R1640W). This could affect the folding of the predicted protein. Both mutations were not seen in at least 130 control chromsomes. Mutation analysis of the ABCR gene in the family members is shown in Figure 2. The I1562T mutation in exon 33 is maternal where the mutation in exon 35 should be paternal (DNA of the father was not available). The proband in this family is therefore compound heterozygous.

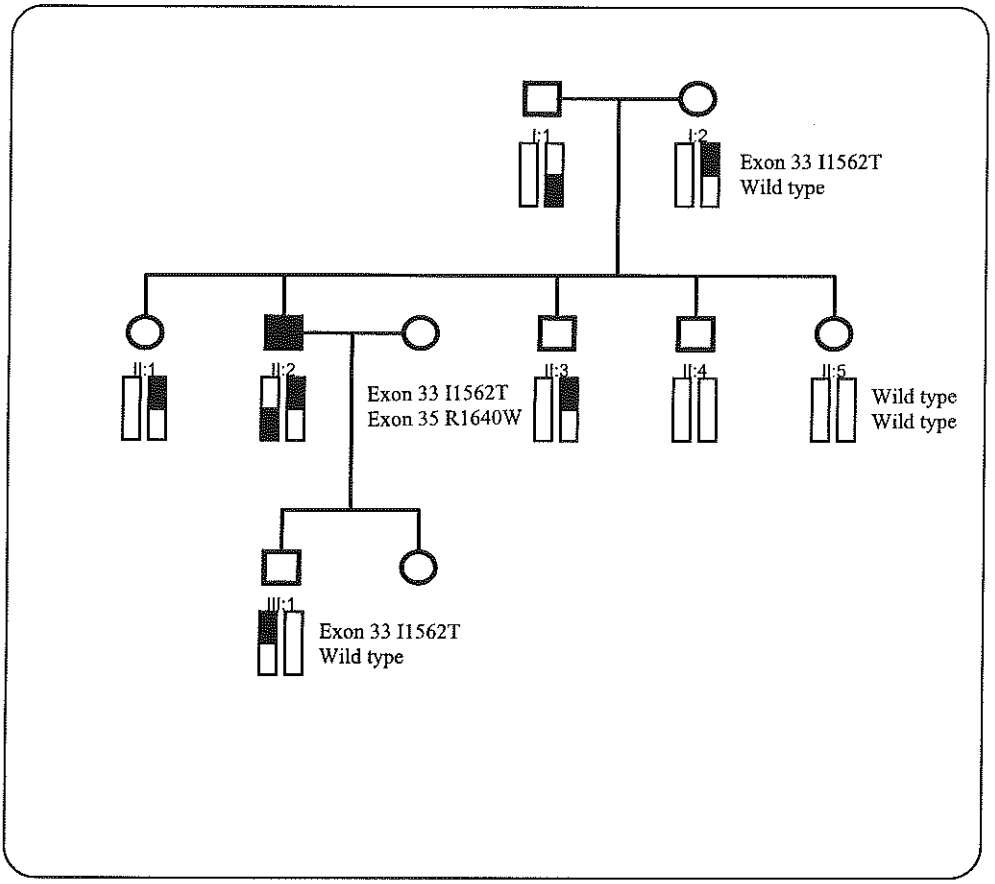

Figure 8 *The pedigree of family 25532 with cone-rod dystrophy with the segregation analysis of respectively mutations I1562T and R1640W. Males are represented by squares and females are represented by circles; blackened symbols represent affected individuals.*

Fundus examination of the proband of family 25479 showed dystrophy of the macula with a pale optic nerve and narrow vessels. The ERG findings confirmed a cone-rod dystrophy. DNA analysis of the ABCR gene revealed a nucleotide substitution in exon 44, which would replace a leucine at amino residue 2027 with phenylalanine (L2027T) in the predicted protein. This mutation was also described before, but only in patients with STGD by both Allikmets and Nasonkin (Table 2). In two out of 194 control chromosomes this sequence change was also present. No family members were available for segregation analysis.

Ophthalmological examination of proband P25815 revealed a decreased visual acuity with macular alterations in both fundi. ERG showed a decrease in scotopic activity (70% of normal values) as well as in photopic activity (20% of normal values). Mutation analysis of the ABCR revealed a splice site mutation at exon 17 (2588-12 C->G).

The splice potential score of the consensus sequence was 79.9, whereas the splice potential score with this mutation was 77.4 according to Shapiro and Senapathy. No family members were available for segregation analysis.

MUTATION ANALYSIS OF ABCR IN CONE-DYSTROPHY.

In P20861 an autosomal dominant cone-dystrophy is segregating in the family. Fundus examinations of the proband showed pigmentary changes in the macula, pale optic nerve and normal blood vessels. No pigmentary changes were seen in the periphery. The ERG showed normal scotopic responses but reduced photopic responses, conform the diagnosis cone dystrophy. Mutational analysis of the ABCR gene in the proband revealed the heterozygously presence of a nucleotide substitution in exon 6. This would replace an arginine with a histidine at amino residue 212 (R212H). This mutation was found in six out of 140 control chromosomes. Interestingly, none of the other affected family members revealed this mutation, nor any other mutation.

Table 3. *Apparently non-functional changes.*

nucleotide change	amino acid change	exon
C6069T	I2023I	44
C6249T	I2083I	45
T6285C	D2095D	46
G2829A	R943Q	19
sequence changes	intron	cryptic splice site
4668-58 C-> T	IVS33	no
4773+48 C->T	IVS33	no
4774-15 GT ins	IVS34	no
6282+7 G->A	IVS45	no

POLYMORPHISMS

Besides the functional sequence alterations described above, a number of apparently non-functional changes were observed (See Table 3). In two patients with cone-rod dystrophy, the analysis of exon 46 revealed a T6285C nucleotide substitution, which would not yield a different amino acid (D2095D). Moreover, this sequence change was also found in eight out of 80 control chromosomes. This suggests the presence of a neutral polymorphism.

Furthermore, two missense mutations (C6069T; C6249T) were found in at least one control individual, suggesting that they are infrequent neutral polymorphisms, and four, apparently non-functional, sequence changes were found in introns. These sequence changes did not introduce a cryptic splice site according to splice site calculations and were apparently not present in any potential regulatory sequences. The analysis of exon 19 revealed a G2829A nucleotide substitution (R943Q) in three patients (with STDG, cone-rod dystophy and Bull's eye maculopathy), but this nucleotide change was found in 10% of the control chromosomes. Allikmets et al reported the latter variant in 24 out of 167 AMD patients and in 24 out of 220 control persons.

DISCUSSION

In 29 unrelated patients with a variety of retinal disorders, we identified mutations in five probands. In total ten different mutations were found. The majority of these mutations were single nucleotide alterations at conserved amino acids positions, but also a 36 bp deletion was found. Both STGD patients included in this study did carry mutations in the ABCR gene. In three patients with cone-rod dystrophy mutations were found in the ABCR gene as well as in one patient with cone dystrophy. In the other patients no mutations could be identified in the ABCR-gene.

MUTATION ANALYSIS OF ABCR IN STARGARDT DISEASE

The mutational segregation patterns in family P25902 are of interest, since the complex allele segregating in this pedigree carries, besides a previous described nucleotide change (G863A) a novel mutation (G5929A) on a single allele and on the other allele a mutation in a splice site. This result indicates, first of all that in mutation studies of the ABCR gene, all of the exons of the gene should be checked carefully for the presence of multiple (additional) sequence alterations on a single allele. Secondly, given the unaltered splice site potential score of 77.1, one expects the splice mutation to be only a mild mutation.

We applied our data to the model proposed by van Driel et al. If one considers the G1977S or the G863A to be a severe mutation on one allele with the mild splice site mutation on the other allele, then these mutations would fit in this model and the phenotype-genotype correlation model would be justified. Moreover, if only one allele is affected, one would assume that the STGD in this family would be an autosomal dominant disease, while, in contrast, the parents were consanguinous and unaffected. Another option is that the combination of mutations G863A and G1977S in one allele would cause a strong reduced ABCR activity, with a mildly affected other allele caused by the splice site mutation. According to van Driel et al. mutation G863A is a mild mutation, which seems to be quite often involved in STGD patients in the Netherlands.[12] However, G863A is not only found in patients with AMD and STGD but also in controls. Therefore, it is more likely that the G177S would cause a more severe effect. Moreover, the G1977S mutation is located in the second ATP binding site. So, most likely the G1977S mutation is a severe mutation, which strongly reduces the ABCR activity.

In the ABCR gene of the other Stargardt disease patient (P25797), a 36 bp deletion was found in one allele. Although this deletion was also found in some of the control chromosomes, it is interesting that it occured in the same protein domain as a deletion reported by Allikmets et al. (1997) in a patient with AMD (6568ΔC). Moreover, in the same exon Allikmets et al. described an 11 bp deletion at position 6519 in a patient with AMD as well as in a STGD patient. According to their location, these deletions might influence the second nucleotide binding fold. The exact pathogeneity of this deletion prone region remains to be established.

MUTATION ANALYSIS OF ABCR IN CONE-ROD DYSTROPHY.

In patient 25532 with cone-rod dystrophy, we found the I1562T mutation in combination with R1640W. Interestingly, the first mutation was described by Allikmets et al. in patients with AMD while the latter is found in STGD patients. Apparently the combination of mutations causes cone-rod dystrophy. According to the proposed model of van Driel et al, these mutations could fit in the model: the mutations are located in different alleles.

In patient 25815 with cone-rod dystrophy, only a heterozygously present potential mutation in the splice site could be identified, with a small reduction of the splice site potential score. This would not fit in the proposed model, in which is suggested that two alleles should be affected.

The L2027F mutation, which was the only mutation we found in patient 25974 with cone-rod dystrophy, was also found heterozygously in patients with STGD (Nasonkin et al). This would not fit in the model and it remains unclear why the same mutation without any other sequence alteration in ABCR would cause different phenotypes.

MUTATION ANALYSIS OF ABCR IN CONE-DYSTROPHY.

In one patient with cone-dystrophy (20681) a sequence change (R212H) was found in exon 6. This change was also found in control chromosomes and not found in any of the other affected family members. This implies that this change might be only a polymorphism and that it is not involved in the development of cone dystrophy.

CONCLUSIONS

Combining our data with those of the literature, the (clinical) expression of the ABCR related disorders apparently depends on different combinations of mutations or sequence substitutions of this gene. Our data show that a number of sequence changes can occur on a single allele. Consequently, all exons of ABCR should be checked carefully for the presence of multiple (additional) sequence alterations on a single or both allele(s). Specific combinations of mutations may lead to specific phenotypes; however, futher mutational analysis of ABCR and genotype-phenotype studies are needed to elucidate the reason why a mutation, apparently, without any other sequence alteration in ABCR would cause different phenotypes.

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- CHAPTER 9 -

VARIANTS IN THE ABCR GENE ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION

ABSTRACT

Age-related macular degeneration (AMD) accounts for over 50% of the registered visual disability among the elderly Caucasians in the Western World, and has been associated both with environmental factors such as smoking and genetic factors. Previously we have reported disease-associated variants in the ABCR gene in a subset of patients affected with this complex disorder. We have now tested our original hypothesis, that ABCR is a dominant susceptibility locus for AMD, by screening 1385 unrelated AMD patients of Caucasian origin and 1478 comparison individuals from 15 centers in N. America and Europe for the two most frequent variants found in ABCR. These two sequence changes, G1961E and D2177N, were found in one allele of ABCR in 53 patients (~4%), and in 13 controls (~0.9%). Fisher's two-sided exact test confirmed that these two variants are associated with AMD at a statistically significant level ($P < 0.0001$), and the results remain significant on an independent sample, after exclusion of the data from the previous study ($P < 0.0001$). The risk of AMD is elevated about 3-fold in D2177N carriers and about 7-fold in G1961E carriers; the two variants together account for about 3% of AMD. Mutations were found more commonly in subjects diagnosed as having the dry (non-neovascular) form than in those with the exudative form of the disorder ($P = 0.003$). The identification of a gene that confers risk of AMD is an important step in unraveling this complex disorder.

Age-related macular degeneration (AMD) is a complex human disorder estimated to affect at least 11 million individuals worldwide, and is the most common cause of acquired visual impairment in people over the age 60 years.[1-3] Prevalence increases with age; among persons 75 years and older, mild forms occur in nearly 30% and advanced forms in about 7%.[2] Visual loss in AMD is due to either choroidal neovascularization or geographic atrophy, which occur as a reaction to age-changes at the level of the retinal pigment epithelium and Bruch's membrane.[4-5] The clinical heterogeneity, late onset, and complex etiology of the disorder render genetic studies in AMD extremely difficult. Recently, we identified the rod photoreceptor-specific, ATP-binding cassette transporter gene, ABCR, as the causative gene for recessive juvenile macular dystrophy, Stargardt disease (STGD1).[6] We subsequently reported several heterozygous amino acid-changing, disease-associated, alterations in ABCR in patients diagnosed with AMD.[7] However, several have challenged the statistical analyses,[8] and one investigation did not find an association between ABCR alterations and AMD.[9]

In the absence of a functional assay for ABCR activity, a good strategy to evaluate the role of ABCR in AMD is to assess the association of DNA sequence variants of ABCR with the AMD phenotype in case-control studies. Application of the family-based transmission disequilibrium test (TDT)[10] to control for population stratification is difficult because typically one cannot genotype parents of patients with this late onset disease, although sibling-based studies would be possible. Previous case-control association studies of rare ABCR variants in AMD patients have been problematic because of relatively small sample sizes, differences in ascertainment of both cases and controls, and differences in methods of data analysis in each individual study.[7, 9] To overcome these limitations, we assembled an International Consortium to screen for variants of the ABCR gene in large, regionally diverse populations of both subjects and control populations. The International Consortium for ABCR screening includes 15 ophthalmic centers and laboratories from eight countries (Table 1). Each center was tasked: (i) to diagnose independently and to collect a new and independent set of patients with AMD; (ii) to examine and to collect a control population; and (iii) to screen all collected DNA samples for the presence of three selected variants – G1961E, D2177N and R943Q by the screening method of its choice.[11] To assure proper genotyping, each center was provided with positive controls for each of the three variants. A comparison group was matched for ethnicity, and, as indicated in Table 1, some centers selected a population above 64 years of age ("age-matched" controls, AM) whereas other centers selected from the general population (GP). The two variants G1961E and D2177N, which lead to non-conservative missense amino acid substitutions, were detected in about 4% of patients each in our previous study and were deemed associated with the AMD phenotype.[7] The R943Q polymorphism was used to assess comparability of case and control populations, to compare populations between centers, and to decide if pooling of data was appropriate. In our earlier report, the R943Q variant had been found in excess in the control population,[7, 8] a discrepancy explained by incomplete screening of all cohorts.[12]

The results are summarized in Table 1. The D2177N variant was detected in 28 (2.1%) out of the total 1356 AMD patients, and in 9/1478 (0.61%) of the age-matched and general population controls (two-sided Fisher's exact P = 0.0007).

Table 1. *Association of G1961E and D2177N variants with AMD.*

Center	Population	AMD samples			Controls			G1961E		D2177N		R943Q	
		Dry	Wet	Total	GP	AM	Total	AMD (D/W)	Cont. (GP/AM)	AMD (D/W)	Cont. (GP/AM)	AMD	Cont.
Science study (7)	Caucasian American	134	33	**167**	220		**220**	6 (6/0)	0	7 (7/0)	1 (1/0)	14/167 (8.4%)	24/220 (10.9%)
Boston Utah Baltimore	Caucasian American	185	44	**229**		200	**200**	3 (3/0)	0	6 (6/0)	1 (0/1)	10/180 (5.6%)	16/200 (8%)
Houston	Caucasian American	55	46	**101**		100	**100**	1 (1/0)	0	1 (0/1)	0	9/101 (9%)	10/100 (10%)
Los Angeles	Caucasian American	53	50	**103**	158		**158**	5 (3/2)	1 (1/0)	4 (2/2)	0	7/105 (7.6%)	6/71 (8.4%)
Wuerzburg	German	100	100	**200**		100	**100**	4 (1/3)	1 (0/1)	3 (2/1)	2 (0/2)	18/200 (9%)	4/100 (4%)
Nijmegen	Dutch	24	59	**83**	168		**168**	1 (0/1)	0	1 (1/0)	3 (3/0)	8/83 (9.6%)	33/308 (10.7%)
Rotterdam Amsterdam	Dutch	36	79	**115**		100	**100**	0	1 (0/1)	0	1 (0/1)	5/63 (7.9%)	11/93 (11.8%)
Naples Milan	Italian	31	61	**92**	120	51	**171**	3 (2/1)	0	0	0	5/68 (7.4%)	8/171 (4.7%)
Barcelona	Spanish	10	26	**36**		34	**34**	0	0	1 (0/1)	0	4/36 (11.1%)	4/34 (11.8%)
Uppsala	Swedish	20	82	**102**	100		**100**	1 (1/0)	1 (1/0)	2 (1/1)	0	12/98 (12.2%)	7/90 (7.8%)
London	British	15	75	**90**		20	**20**	1 (0/1)	0	1 (0/1)	0	4/90 (4.4%)	0/20 (0%)
Paris	French	4	63	**67**	107		**107**	0	0	2 (0/2)	1 (1/0)	4/72 (5.6%)	6/107 (5.6%)
Total		**667**	**718**	**1385**	**873**	**585**	**1478**	25/1385 (1.8%)	4/1478 (0.27%)	28/1356 (2.1%)	9/1478 (0.61%)	100/1263 (7.9%)	129/1514 (8.5%)
*P**								***P* < 0.0001**		***P* = 0.00072**		***P* = 0.5866**	
Both variants								53/1356 (3.91%)	13/1478 (0.88%)	***P* < 0.0001**			
Without Science study								19/1218 (1.56%)	4/1258 (0.32%)	21/1189 (1.77%)	8/1258 (0.64%)		
*P**								***P* = 0.0013**		***P* = 0.014**			
Both variants								40/1189 (3.36%)	12/1258 (0.95%)	***P* < 0.0001**			

GP, general population

AM, age-matched

D, dry form of AMD

W, wet form of AMD

*Statistical analysis was in all cases performed with two-sided Fisher's exact test.

Unless otherwise stated, all p values are based on the two-sided Fisher's exact test.[13] The corresponding odds ratio, calculated from exact conditional methods[13] was 3.3 with 95% confidence interval (1.5, 8.0). Thus, carriers of the D2177N mutation have 3.3 times the risk of developing AMD as non-carriers. Similar results were obtained when the analysis was stratified on type of control (age matched versus general population) or on study center, and there was no statistically significant evidence of heterogeneity of odds ratios across centers. The proportion of AMD attributable to this mutation, the attributable risk, [14] is estimated as only 1.5% (0.6%, 2.3%), because the allele is rare in the general population.

Twenty-five (1.8%) of 1385 AMD cases carried the G1961E variant, compared to 4/1478 (0.27%) of controls, ($P < 0.0001$). The relative odds ratio was 6.8 (2.3, 27), and the attributable risk was 1.5% (0.8%, 2.3%). Despite that fact that carriers of G1961E have 6.8 times the risk of non-carriers, this allele explains no more AMD than does D2177N, because G1961E is rarer (0.27%). Similar odds ratios were obtained if the analysis was stratified on type of control or on study center, and there was no statistically significant evidence of heterogeneity across centers. Together, the two variants were detected in 53/1356 (3.91%) of individuals with AMD and in 13/1478 (0.88%) of controls, ($P < 0.0001$), and the proportion of disease attributable to having either variant is 3.1% (1.9%, 4.2%). No case or control possessed both variants.

In Table 1 we present the data from the current study as well as previously published results.[7] In order to test our earlier hypothesis that ABCR variants are statistically significantly associated with AMD, we excluded our earlier results[7] from these analyses. Without those data, the association of the two variants with AMD is still significant: $P = 0.001$ for the G1961E variant and $P = 0.01$ for the D2177N amino acid change. Together, these two variants are found in 40/1189 patients and in 12/1258 controls ($P < 0.0001$). Therefore the data from this expanded study independently support our earlier conclusions about the association of pathogenic variants in patients with AMD.

Although it is possible that the association of AMD with variants of ABCR is due to other unrelated gene(s) that cause AMD and that are in disequilibrium with these variants of ABCR, there are several reasons why this is unlikely. Whereas a single uncommon mutation, such as G1961E, might be in linkage disequilibrium with a putative disease-causing gene, it is unlikely that two rare variants within the same ABCR gene would be in linkage disequilibrium with that gene. This is especially so because there is no evidence that G1961E and D2177N tend to occur together. More probable explanations are that these two variants are both causally related to AMD through their action on the ABCR protein, or in linkage disequilibrium with other, currently unknown, ABCR mutations. Preliminary data from photoaffinity labeling experiments indicate that at least one of these variants, G1961E, is indeed a pathogenic mutation. The mutant G1961E protein, produced following the transfection of 293 cells with cloned cDNA, exhibits several fold lower binding of 8-azido-ATP than the wild-type ABCR protein (Y. Sun and J. Nathans, personal communication). The D2177N variant had no effect on 8-azido-ATP binding, suggesting that this change may go undetected in this in vitro assay system or that D2177N is in linkage disequilibrium with a pathogenic ABCR mutation. It is of interest to note that, unlike G1961E, the D2177N variant so far

has not been detected in patients with Stargardt disease.[7, 15-17] Data for the R943Q polymorphism also provides evidence against the possibility that another gene causes the association of AMD with G1961E and D2177N. There is no association between R943Q and AMD in these data (p= 0.58 with odds ratio 0.92 (.70, 1.00), nor is there evidence of heterogeneity of odds ratios across centers (Breslow-Day test p=.75).[13] If a nearby mutation in another gene were the cause of AMD, one might expect it to be in disequilibrium with one or more polymorphisms of ABCR such as R943Q. Another possibility is that the association of AMD with G1961E and with D2177N stems from improper choice of controls ("population stratification"), as can occur when cases originate from one subpopulation and controls from another subpopulation of an admixed population. In Table 1, however, higher levels of G1961E and D2177N are found in AMD cases than controls in practically each study center. It is unlikely that population stratification would occur consistently in such diverse populations. These data thus provide the strongest evidence to date that some variants in ABCR are associated with phenotypes of age-related macular degeneration.

In this study, we analyzed the two most prevalent disease-associated variants described in our earlier study.[7] The reported genetic heterogeneity of ABCR alleles in various eye diseases, including STGD1, is remarkable.[6, 9, 15, 16] The vast majority of disease-associated alleles are rare and often reported only in single cases.[7, 9, 15] At the same time the reported mutation detection efficiency in ABCR even in STGD1 patients has ranged from 60-70% of alleles in some studies,[15, 16] to 20-30% in the others.[9, 17] Together, these data indicate that both the numbers of affected individuals analyzed and the efficiency of mutation detection techniques must be increased significantly to correctly assess the fraction of ABCR variants involved in AMD cases. We have reported previously that ABCR variants are more prevalent among subjects manifesting the dry (non-neovascular) form of the disease.[7] Distinction between the dry and wet phenotypes is not unambiguous.[9] This relates to lack of uniformity in clinical classification, in which the term AMD may be used to denote any age-change whatever the vision or to those with visual loss as a consequence. In the first case the term dry refers to those with drusen only as well as those with visual loss so that patients classified as dry represent 90% of cases.[4] In the second, the term dry refers to those with geographic atrophy only that represent a minority of cases. Whether or not these two clinical manifestations have different genetic risk factors remains to be elucidated. Occasionally, individuals with the dry form in one eye may develop choroidal neovascularization later in life either in the same or fellow eye.[18] Each participating clinician in this Consortium segregated its patient population by phenotype, between those with the dry form, characterized by drusen, RPE pigmentary abnormalities, and/or geographic atrophy; and those manifesting the wet, or exudative (neovascular), stage.[19, 20] For the purposes of these analyses, when different phenotypes appeared in the two eyes, the assignment of the disease status was based on the more severely affected eye.

Of the 1385 patients analyzed, 667 had the dry phenotype and 718 had the exudative form (Table 1). The proportion of patients diagnosed with the exudative complication was much higher in European centers compared to those from North America (Table 1). If analyzed separately, association of the two sequence changes with AMD was more prominent in N. American subset

(data not shown). Altogether, the G1961E variant was detected in 17/667 (2.55%) patients with the dry form, compared to 8/718 (1.1%) of those with the wet form (p = 0.044). The corresponding odds ratio, 2.4 (1.0, 6.4), indicates that G1961E was found 2.4 times more commonly in those designated as having dry lesions as with wet AMD. Similarly, the D2177N mutation was found in 19/648 (2.93%) of patients with the dry and in 9/708 (1.27%) of patients with the wet phenotype (p= 0.036), and the corresponding odds ratio was 2.3 (1.0, 5.9). Together, these two variants were present in 36/648 patients with non-exudative disease and in 17/708 with exudative lesions (p = 0.0030). This observation may explain the difference between N. American and European centers and the lack of association between ABCR variants and AMD found in another study,[9] where more than 60% of the 182 enrolled patients were reported to manifest the exudative phenotype. The difference in the ratio of wet and dry between centers may reflect different criteria used to select cases or genuine variance in the prevalence of difference phenotypes in diverse populations subjected to a variety of environmental pressures. Although we have demonstrated that variants in ABCR are associated predominantly with non-exudative AMD, the clinical relevance of this finding remains to be determined. Future studies, with larger numbers of affected individuals and controls, may be able to link allelic variants in ABCR to specific disease phenotypes.

Knowledge of the relationship of individual ABCR alterations to the AMD phenotype(s) will permit a better understanding of this complex disorder, and will facilitate detection of other genetic and environmental risk factors. The influence of each ABCR variant on the protein function and on pathogenesis of AMD has yet to be evaluated. Functional studies of this ABC transporter have implicated all-trans retinal or its conjugates with phosphatidylethanolamine (PE), N-retinylidene-PE and N-retinylidene-N-retinylethanolamine (A2E), as potential substrates [21, 22] for the protein. Mice homozygous for a null mutation in ABCR accumulate higher levels of a major fluorophore of lipofuscin, A2E, in the retinal pigment epithelium (RPE). [22] Additional supportive evidence for the potential role of ABCR variants in AMD is being sought from studies on segregation of ABCR variants in Stargardt pedigrees in which elderly members manifest AMD. Several reports have indicated an increased rate of AMD in parents and grandparents of STGD patients, harboring ABCR variants.[15, 23-27]

In summary, we have tested our hypothesis that some ABCR variants are associated with AMD, on a large and diverse collection of subjects and controls. Overall this association is statistically significant for at least these two known mutations. The G1961E and D2177N variants alone are detected in about 4% of these geographically diverse subjects with AMD. These variants in the ABCR gene are associated predominantly with the dry, non-neovascular, disease phenotype. The risk of AMD is increased about three-fold in carriers of D2177N and about seven-fold in carriers of G1961E, and these two variants account for about 3% of AMD cases, which in the context of common complex disorders represents an important contribution to the disease load. Since AMD affects millions of people worldwide and the described mutations represent only two out of thirteen reported earlier,[7] the number of people at increased risk of developing age-related maculopathy, as carriers for variant ABCR alleles, is substantial. As more genes are identified the true contribution of ABCR to the disorder will become clearer. Our experience indicates that very

large numbers of cases will be required to accomplish the objectives, and it is likely that success can only be achieved by formation of large International Consortia using carefully planned and unified protocols.

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PART V

GENERAL DISCUSSION AND SUMMARY

-CHAPTER 10-

GENERAL DISCUSSION

In this general discussion a brief overview will be given of the major findings, together with methodological considerations. Special attention is addressed to some issues of consideration when studying the genetics of a genetically complex disease such as age-related maculopathy (ARM). Finally, I will provide recommendations for future research.

DISEASE FREQUENCY AND RISK FACTORS

Prevalences of ARM have been studied in several epidemiological studies.[1-5] In Chapter 3, we have combined the data of three studies from three different continents; Australia, North America and Europe. The prevalence of AMD was consistently found to increase with age. Besides age, smoking was the only risk factor found in all three studies. Remarkably, the prevalence of AMD in the Rotterdam Study was lower than in the other two studies. In Rotterdam, where we studied the incidence of AMD at 2-year follow-up, we also found a lower incidence of AMD at all ages compared to the Beaver Dam Eye study.[6]

Selection bias can be a reason for the lower prevalences and incidences in Rotterdam. The overall response rate was reasonably good at base-line (78%), as well as after 2 years of follow-up (79%). It is, however, lower than in the other studies. The non-participants were older and more likely to be diseased. This would mean that the Rotterdam population was healthier, which would decrease the ascertained prevalence and incidence of the disease. In reality these percentages may be higher than our studies indicate.

Information bias could occur due to misclassification. In these studies we have made a great effort to classify ARM as correctly as possible. We have used the international classification and grading system and all available ophthalmological information to diagnose ARM.[7] We have tested interobserver and intraobserver variation, using weighted kappa values. Furthermore, all diagnoses of AMD were confirmed by the principal investigators on all three continents.

GENETIC EPIDEMIOLOGY OF AGE-RELATED MACULOPATHY

Many investigators have described that genetic factors are involved in ARM.[8-14] Genetic studies of ARM have, however, been hampered by the lack of suitable patient material due to the late age of onset, the clinical heterogeneity of the disorder and phenocopies. In chapter 5 a simple method is described which facilitates identification of families with a high genetic risk.

This is done by using age-specific prevalences of ARM in the general population and observed frequencies of ARM in a family. In this study we used a case-control design; families of cases with AMD were compared with families of controls without any signs of ARM. In contrast to familial aggregation studies, the family unit was respected. For small families sizes, the family score method is more unreliable as it is uncertain whether the family score is the result of genetic risk or just coincidence. This method is more useful for studies in molecular genetics than it is for clinicians, since no effective treatment for ARM is known.

In chapter 6 a large family derived from an isolated community is described. Genetic isolates are advantageous for the study of genetically complex diseases: the number of founders is limited which reduces the number of mutations residing in the population considerably. In this family we performed a genomic screening for ARM by using about 400 marker loci. In the initial analysis we investigated three candidate loci or genes, chromosome 1q, ABCR gene and TIMP-3 gene.[15-17] Although these three loci are apparently not cosegregating with ARM in this family, they can not be excluded on the basis of our results as a candidate gene for ARM, since it is, of course, possible that due to the relatively small sample size these results are falsely negative. It will be of great interest to see which locus or loci are associated with ARM in this pedigree. Currently, further research is being performed to test the five regions with a high chi-square for a disease locus. Additional markers will be tested in these regions. Since in this analysis multiple markers with multiple alleles are used, a higher statistical significance level will be required. The height of this significance level is debatable.[18] Since a genetic isolate is not a good reflection of the general population, it is possible that genes which are associated with ARM in this pedigree are not associated with ARM in the general population. These genes could, however, give clues to the pathogenesis of ARM.

CANDIDATE GENES FOR AGE-RELATED MACULAR DEGENERATION

Many candidate genes were suggested to be involved in ARM. Candidate genes may be selected from their known function, their chromosomal position or their known involvement in other forms of inherited retinal degeneration. Two known candidate genes for ARM are TIMP-3, which is involved in Sorsby fundus dystrophy (SFD), and ABCR, which is known to be involved in Stargardt disease.[16, 17]

In chapter 7 of this thesis we examined a large family with SFD. SFD is thought to be a monogenic disease due to mutations in the TIMP-3 gene on chromosome 22. In this family, SFD was linked to the region of chromosome 22 in which TIMP-3 is located. However, no mutations could be identified in TIMP-3. There may be mutations in regions of the gene, like intronic sequences, which are not investigated in the current study. Another possible explanation is that a different gene is located in the vicinity of TIMP-3, which is involved in this pedigree.

The ABCR gene is a photoreceptor cell-specific ATP-binding transporter gene, located on chromosome 1p21 and is exclusively expressed in the retina.[19,20] ABCR is phosphorylated in response to light, which suggests that it plays a role in phototransduction.

Mutations in this gene have been implicated in Stargardt disease and cone-rod dystrophy as well as in retinitis pigmentosa type 19.[16,21-27] We have investigated the ABCR gene in a variety of retinal disorders in order to investigate the mutations in ABCR in relation to the phenotype. Since ABCR exhibits a large genetic variability associated with a number of retinal disorders, it is important to describe the pheno- genotype relation accurately. Why and how the same mutation without any other sequence alteration in ABCR would cause different phenotypes has yet to be solved.

In chapter 9 a large collaborative effort involving several study groups was initiated. The first publication of Allikmets et al., in which he claimed an association of ABCR variants in unrelated AMD cases in comparison to a control group, resulted in a major scientific debate.[28] Major points of discussion were flaws in study design and analysis as well as potential misclassification of the cases. Moreover, the overall frequency of variations did not significantly differ between the case and control group (p=0.3).[29, 30] Two other studies could not confirm these data.[31, 32] In our collaborative study, a total of 1385 cases with ARM and 1478 controls were examined for two mutations in ABCR. These mutations, G1951E and D2177N, were the ones most often associated with ARM in the previous study. In the current study, single strand conformational polymorphism (SSCP) techniques to identify mutations in ABCR and additional sequence analyses were performed. Positive controls with the two mutations were used to prevent false negative results; a negative control was also used to prevent false positive results. To prevent systematic errors, the reading of the gels was done masked to the disease status. A different frequent variation, R943Q, was used to test whether the different populations could be compared to each other. Population admixture might lead to a false positive association. No significant difference in the frequency of R943Q could be observed between the different populations. The other two mutations were found to occur at a statistically significant higher rate in ARM cases than in the controls. Although the ABCR gene is unlikely to alter over time, it may be useful to analyse mutations in the ABCR gene in incidental cases.

STRATEGIES FOR FUTURE RESEARCH

In most epidemiologic studies a cross-sectional design is used, and information on disease prevalence is provided. For research on the etiology of diseases, data based on incidence is preferred over prevalence. Incidence represents the actual occurrence of the disease. The exposure to potential risk factors is measured before the onset of disease, and identified risk factors are therefore more likely to be causal. Incidence data on ARM would improve our knowledge of the etiology and the progression of the disease. More incidence studies with a large follow-up are still needed to provide more accurate estimates of the incidence of ARM and to identify its risk factors.

One of the major problems in the genetic study of a late onset disease such as ARM is the lack of suitable patient material. Parents are often deceased, while the younger generation does not yet have the disease on a clinical level. Most families are limited to sibships. Linkage analyses in families are therefore often impossible. Large families with multiple affected individuals are scarce.

A different method based on family relations of the cases is the affected sibpair method, in which affected sibpairs are examined and the haplotypes of both sibs are compared. This technique is based on the principle of inherited alleles which are identical by decent. If at a certain locus more alleles are inherited identical by descent than expected, this would indicate a candidate locus for the disease. For genomic screening, many affected sibpairs would be needed for such a complex disease as ARM. Association studies are also not well-suited for a genomic screening in large, mixed populations. One would need tens of thousands of markers to screen the whole genome, because linkage disequilibrium extends over very short distances. Moreover, testing many markers would elevate the required significance level tremendously. Genomic search may be more feasible in a genetically isolated population in which fewer genes and mutations are involved in the disease, due to common founders among the cases.

In contrast to linkage analysis and allele sharing methods, association studies do not concern familial inheritance patterns at all. Unrelated cases are compared with a control group. This facilitates data collection remarkably. If candidate genes are identified, association studies are used to demonstrate a correlation between functionally relevant allelic variation and the risk of disease in humans.

In the human genome project, whole chromosomal regions will be sequenced in order to identify genes in these regions. Some of these genes will be candidates and may eventually prove to be disease-causing genes for ARM.

For complex diseases, like ARM, the most powerful strategy may prove to be the allele sharing method in a genetically isolated population. The idea is to find many affected individuals who have inherited the same disease-causing gene from a common ancestor. The affected inidividuals will tend to have a shared haplotype in the region of the disease-causing gene. Since data on many historical meisoses are incorporated in this method, the number of recombinations will be higher, therefore mapping the disease causing-gene to a smaller region.

FINAL REMARKS

The aim of this thesis was to provide more insight into the frequency of ARM and its risk factors, in particular the genetic factors. I feel that this thesis has advanced our knowledge about the pathogenesis of ARM. Concerning the genetic factors, many studies and collaborations are needed to investigate all candidate loci and genes. With the identification of the molecular defects underlying ARM, intervention in the processes leading to ARM may come within reach.

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-CHAPTER 11-

SUMMARY

This thesis describes a genetic and epidemiologic approach to age-related maculopathy. Age-related maculopathy (ARM) is a disorder of the yellow spot in the eye (the macula), and it affects people over 55 years of age. ARM consists of a variety of clinical signs, from the early stages with soft distinct drusen, indistinct drusen and pigment alterations up to the late stages, characterized by geographic atrophy and neovascular macular degeneration. These late stages are also referred to as age-related macular degeneration (AMD). Several risk factors are reported to be associated with ARM. Aging and smoking are the two most important and were consistently found. Besides these and others, genetic factors play a role in ARM.

In Chapter 2 an overview is given of the genetic factors involved in ARM, which are described in the literature. One of the main problems in the genetic studies on ARM is the lack of suitable patient material. Parents are often deceased while in the younger generations the disease has not yet manifested itself on a clinical level. Families available for genetic studies are therefore mostly limited to sibships with ARM. Moreover, ARM is a genetically complex disorder in which several genes and mutations are involved. Cases with ARM might also arise from non-genetic risk factors; these are called phenocopies. In this chapter methods are discussed which can be used in the research of genetic complex diseases such as ARM.

In chapter 3 and 4 the disease frequency of ARM is described. The prevalence (chapter 3) and the incidence (chapter 4) were investigated in the Rotterdam Study. In this study 7983 individuals were examined for ophthalmological, neurological, cardiovascular and locomotor diseases. Gradable fundus photographs were available for 6411 individuals. The diagnosis AMD was made in 104 cases (1,6%). In Chapter 3 this is compared with two other studies, the Beaver Dam Eye study and the Blue Mountain study. The age-specific prevalence of neovascular macular degeneration was lower in the Netherlands compared to the other two studies. Age-specific incidences of AMD also appeared to be lower in Rotterdam than in Beaver Dam. In 2 years, 2 per 100 subjects of our total study population developed AMD. The course of the disease was examined. Subjects with indistinct drusen with pigmentary alterations in the macular area were most at risk.

Chapter 5 shows a method to identify families with a high genetic risk of AMD. Families of AMD-affected individuals can be divided into three groups of genetic risk; low, intermediate and high.

In Chapter 6 a large family with ARM originating from a genetic isolate is identified. A whole genome screening with 400 markers was carried out. Three candidate loci/genes, the ABCR gene, chromosome 1q25-31 and TIMP3 gene, were tested and excluded form involvement in ARM

in this pedigree. In the genomic screening five interesting regions with a high chi-square were identified. These regions will be investigated in more detail in the near future.

Chapter 7 describes a pedigree with Sorsby fundus dystrophy in which the TIMP3 gene is investigated. In the literature mutations in exon 5 of this gene are associated with this disorder. In our study, Sorsby fundus dystrophy was linked to the region on chromosome 22 in which TIMP3 is located, however we could not identify any mutation in TIMP3 gene. Especially, no mutations in exon 5 were found.

The potential role of the ABCR gene is described in Chapters 8 and 9. The ABCR gene is exclusively expressed in the retina and is associated with Stargardt disease, retinitis pigmentosa and cone-rod dystrophy. ABCR is also potentially implicated in AMD. In Chapter 8 several retinal disorders were investigated for mutations in ABCR. In five of the 29 patients examined we found mutations in the ABCR gene. In Chapter 9 the results of a large collaborative effort are given. Fifteen study groups worldwide examined two mutations, D2177N and G1951E, in AMD patients compared to controls. These two mutations were significantly more frequent in the case group.

Chapter 10 provides a general discussion on all the studies described in this thesis, and makes recommendations for future research.

SAMENVATTING

In dit proefschrift wordt een genetisch epidemiologische studie naar ouderdoms maculopathie beschreven. Dit is een aandoening van de gele vlek in het oog die met name op oudere leeftijd voorkomt. De gele vlek in het oog moet intact zijn om scherp te kunnen zien, gezichten te herkennen en te lezen. Bij de eindstadia van ouderdoms maculopathie, dat zijn geografische atrofie en neovasculaire maculadegeneratie, is dit functioneren verstoord en ontstaat een visuele handicap. Deze eindstadia noemt men ouderdomsmaculadegeneratie (OMD). Van OMD is reeds een aantal risicofactoren beschreven; toenemende leeftijd en roken zijn tot dusver de belangrijkste en worden in elk onderzoek ook geassocieerd met OMD. Naast deze en andere risicofactoren spelen ook genetische oorzaken een rol.

In hoofdstuk 2 wordt een overzicht gegeven van de genetische factoren, die een rol spelen bij OMD. Eén van de grote problemen van het genetische onderzoek naar OMD is het gebrek aan patiëntenmateriaal. Door de late leeftijd waarop OMD ontstaat zijn de ouders van een persoon met OMD vaak overleden, terwijl de kinderen de ziekte nog niet hebben. Hierdoor is het familie-onderzoek vaak beperkt tot broers en zussen met OMD. Verder zijn er waarschijnlijk diverse genen en mutaties betrokken bij het ontstaan van OMD. Bovendien kan OMD bij een persoon natuurlijk ook het gevolg zijn van een niet genetische oorzaak (bijvoorbeeld roken). In het hoofdstuk worden tevens methoden besproken die toepasbaar zijn voor het onderzoek naar genetisch complexe ziekten zoals OMD.

In de hoofdstukken 3 en 4 wordt het voorkomen van de ziekte beschreven. De prevalentie (hoofdstuk 3) en de incidentie (hoofdstuk 4) zijn bepaald in het ERGO bevolkingsonderzoek. ERGO staat voor Erasmus Rotterdam Gezondheid en Ouderen. Hierin zijn 7983 personen onderzocht op oogziekten, aandoeningen van het bewegingsapparaat, neurologische en cardiovasculaire aandoeningen. Van 6411 personen zijn er scherpe, goed gradeerbare foto's beschikbaar. Bij 104 personen (1,6%) is de diagnose eindstadia OMD gesteld. In hoofdstuk 3 wordt dit vergeleken met twee andere studies, de Beaver Dam studie en de Blue Mountain studie. Daaruit bleek dat in Nederland de oogaandoening pas op latere leeftijd in dezelfde frequentie voorkomt. Dit wordt ook waargenomen in hoofdstuk 4 waarin de incidentie wordt vergeleken met de Beaver Dam studie. In twee jaar onwikkelden twee op de 1000 personen in onze studie een eindstadium. Tevens is het natuurlijk verloop van de aandoening bestudeerd en daaruit bleek dat met name personen met onscherp begrensde witte vlekjes op de retina (drusen) met veranderingen in het retinaal pigmentepitheel de grootste kans hebben op het krijgen van een eindstadia.

In hoofdstuk 5 wordt een methode beschreven waarmee families met een hoog genetisch risico voor OMD geïdentificeerd kunnen worden. Families met personen met OMD kunnen worden opgedeeld naar laag, middelmatig verhoogd en sterk verhoogd genetisch risico. Elke stijging van familie-score geeft een risico (β) van 1.3 gecorrigeerd voor leeftijd, geslacht en roken.

In hoofdstuk 6 wordt een methode toegepast die goed bruikbaar is voor genetisch complexe aandoeningen, namelijk "genome screening" in een genetisch isolaat. In een genetisch isolaat kunnen veel personen door middel van stamboomonderzoek aan elkaar verbonden worden. In het onderzoek kon van 21 aangedane personen met zowel voor- als eindstadia van OMD één grote familie worden herleid. In deze familie zijn bijna 400 markers getest, die verspreid over het gehele genoom liggen. Drie kandidaat regio's c.q. genen, te weten het ABCR-gen, chromosoom 1q25-31, en het TIMP3-gen konden worden uitgesloten van betrokkenheid bij OMD in deze stamboom. Momenteel zijn er over het hele genoom vijf interesante gebieden gevonden die mogelijk betrokken kunnen zijn bij OMD in deze familie. Verder onderzoek naar deze gebieden zal nog worden uitgevoerd.

In hoofdstuk 7 wordt het TIMP3-gen, dat verantwoordelijk is voor Sorsby fundus dystrofie, nader bekeken in een stamboom waarin deze aandoening voorkomt. Tot nu toe zijn in de literatuur alleen mutaties in exon 5 van dit gen geassocieerd met deze aandoening. In deze studie is aangetoond dat Sorsby fundus dystrofie in deze familie gekoppeld is aan het gebied op chromosoom 22 waar het TIMP3-gen zich bevindt. Er zijn echter geen mutaties gevonden die verantwoordelijk kunnen zijn voor deze dystrofie in deze stamboom, met name zijn er geen mutaties in exon 5 van het TIMP3-gen gevonden.

In hoofdstuk 8 en 9 wordt gekeken naar het ABCR-gen. Het ABCR-gen komt specifiek in de retina voor en is geassocieerd met Stargardt fundus dystrofie, retinitis pigmentosa en cone-rod dystrophy. Er zijn aanwijzingen dat ABCR-mutaties geassocieerd zijn met OMD. In hoofdstuk 8 worden verschillende retinale aandoeningen beschreven met de ABCR mutaties die daarbij gevonden zijn. Bij vijf van de 29 onderzochte patienten zijn mutaties in het ABCR-gen gevonden. In hoofdstuk 9 wordt een samenwerking tussen 15 verschillende centra over de wereld beschreven, waarbij naar twee specifieke mutaties is gekeken in een grote groep personen met OMD en een grote controle-groep. Het is gebleken dat deze mutaties, D2177N en G1951E, relatief vaak voorkomen bij de groep met OMD in vergelijking tot de controle-groep.

In hoofdstuk 10 worden alle bevindingen van de voorgaande hoofdstukken bediscussieerd en worden aanbevelingen gedaan voor verder onderzoek.

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Ikram MK, Borger PH, Assink JJM, Hofman A, de Jong PTVM. Comparison between ophthalmoscopy, Topcon imagnet system and Heidelberg retina tomograph in measuring the optic disc. *Submitted*

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