# GENETICS OF AGE-RELATED MACULAR DEGENERATION

## New Insights and Perspectives

**DOMINIEK DESPRIET**

## ACKNOWLEDGEMENTS

The work presented in this thesis was conducted at the Department of Epidemiology & Biostatistics in close collaboration with the Departments of Ophthalmology and Clinical Genetics of the Erasmus Medical Centre in Rotterdam and the Department of Clinical and Molecular Ophthalmogenetics, Netherlands Institute for Neuroscience, an institute of the Royal Netherlands Academy of Arts and Sciences (KNAW) in Amsterdam.

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# GENETICS OF AGE-RELATED MACULAR DEGENERATION

New insights and perspectives

# GENETICA VAN LEEFTIJDSGEBONDEN MACULADEGENERATIE

Nieuwe inzichten en vooruitzichten

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Voor Mijn Ouders

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## PUBLICATIONS AND MANUSCRIPTS ON WHICH THIS THESIS IS BASED

1. Despriet D. D., Klaver C. C., Witteman J. C., Bergen A. A., Kardys I., de Maat M. P., Boekhoorn S. S., Vingerling J. R., Hofman A., Oostra B. A., Uitterlinden A. G., Stijnen T., van Duijn C. M. and de Jong P. T. *Complement Factor H* Polymorphism, Complement Activators, and Risk of Age-related Macular Degeneration. *Jama.* Jul 19 2006;296(3):301-309.

2. Despriet D. D., Weber B. H., Houwing-Duistermaat J. J., Bakker A., Fritsche L., Isaacs A., De Jong P. T., Klaver C. C. and Bergen A. A. *CFH* Gene and Age-related Macular Degeneration: Separating Culprits from Innocent Bystanders. *(submitted).*

3. Despriet D. D., Van Duijn C. M., Oostra B. A., Uitterlinden A. G., Hofman A., Wright A. F., Ten Brink J. B., de Jong P. T., Vingerling J. R., Bergen A. A. and Klaver C. C. *Complement Component C3* and Risk of Age-related Macular Degeneration. *(submitted).*

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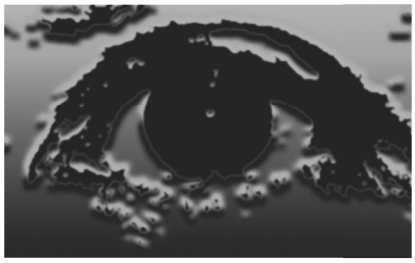
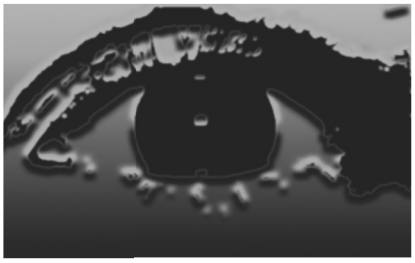
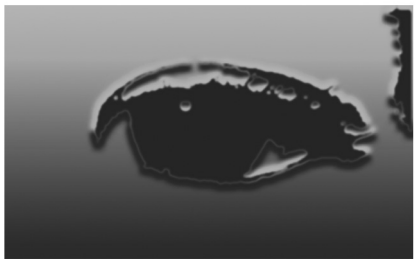


# GENERAL INTRODUCTION





## GENERAL INTRODUCTION



Approximately 30.5 million people aged 50 years and older are blind worldwide.[1] Visual impairment, or low vision that cannot be corrected with glasses, leads to a significant decrease in quality of life irrespective of its underlying cause. The effect on the psychosocial and emotional aspects of life and the functional independence of patients is devastating.[2] Individuals with low vision have difficulty driving a car, recognizing faces, watching television, and reading. In addition, they are susceptible to depression, social isolation, as well as feelings of frustration and sadness.[3,4] The leading cause of severe visual impairment in the elderly of the Western world is age-related macular degeneration (AMD).

AMD is a progressive disorder affecting the macula, the central part of the retina, which is responsible for high-resolution visual acuity. Early signs of the disease include depositions of extracellular material (drusen) underneath the retinal pigment epithelium (RPE), and areas of pigment alterations (early AMD). These early characteristics rarely cause clinical symptoms, and are therefore often unnoticed. Nevertheless, patients with early AMD are at increased risk of developing late or end-stage disease. Late AMD can be subdivided into geographic atrophy (i.e atrophic or dry AMD), which is characterized by well-defined areas of atrophy of the RPE and neural retina; or subretinal neovascularization (i.e. neovascular or wet AMD), in which new blood vessels arise from the underlying choriocapillaris, leading to a haemorrhagic retinal and/or RPE detachment with subsequent fibrovascular scarring of the macular area. These end-stages of AMD are associated with severe central visual loss.[5,6]

AMD mainly affects people aged 60 years and older. Statistics of the World Health Organization (WHO) revealed that 8.7% of worldwide blindness is due to AMD, making it the third leading cause of blindness behind cataract and glaucoma.[7] The disease is the primary cause of visual impairment in industrialized countries and affects approximately 2.5 million people in Europe.[8] However, as life expectancies increase, the prevalence of AMD will also rise. It is likely that the number of blind persons due to AMD will double in the next decade, unless better prevention and therapy becomes available.[9]

Etiological research has shown that AMD results from the interplay of multiple environmental and genetic factors. Important environmental risk factors include smoking, atherosclerosis, diet, sunlight and cataract extraction.[10,11] Strong evidence for a genetic component was provided by twin studies, familial aggregation studies, and a segregation analysis. Twin studies described a greater concordance in monozygotic twins compared to dizygotic twins, and reported heritability estimates for AMD ranging from 45% to 71%. This indicated that 45 to 71% of the disease occurrence may be due to genetic factors.[12,13] Familial aggregation studies demonstrated that first-degree relatives of patients with AMD were at increased risk for the disease, were affected at a younger age, and had an increased lifetime risk of late AMD compared to first-degree relatives of participants without AMD.[14-16] Additional evidence for the important role of genes in the etiology of AMD came from a segregation analysis, which suggested that a major gene may account for 55-57% of the total disease variability of AMD.[17]

Given this substantial evidence on an underlying genetic component to AMD, researchers have carried out both linkage studies and candidate gene association studies in an attempt to identify the genetic susceptibility loci for AMD. This proved to be a challenging task. Nearly every chromosome in the human genome has been implicated by one or more linkage studies for AMD.[18-30] In addition, at least at the start of this research project, candidate gene association studies had largely yielded disappointing and inconclusive results due to a lack of consistent replication.[31]





## Objective and outline of this thesis

The main objective of the research presented in this thesis is to further unravel the genetic background of AMD.

Since genetic heterogeneity in outbred populations is likely to play an important role in the disappointing results of linkage studies achieved at the beginning of the study in 2003, we initially designed a study within the framework of a recently founded Dutch genetically isolated population. The small number of founders (< 400) and genetic drift increased the genetic homogeneity in this isolate, creating a powerful setting to study the genetics of complex diseases, such as AMD. This study was part of the Genetic

Research in Isolated Populations (GRIP) research program, and eligibility for participation in the study was determined by genealogical background, not by any phenotype of interest. However, the disappointing number of individuals with an AMD phenotype (57/2939 with early AMD, 4/2939 with late AMD) stimulated us to use other research settings to obtain our goal.

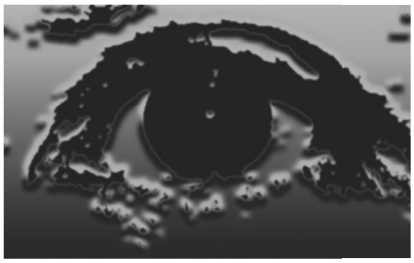
We subsequently performed candidate gene studies in the Rotterdam Study and in an independent clinic-based case-control study. The Rotterdam Study is a large population-based prospective cohort study among 7983 participants aged 55 years and older living in a suburb of Rotterdam. The case-control study consisted of 357 unrelated AMD patients and 173 control individuals which were recruited from the Netherlands Institute of Neuroscience Amsterdam, and Erasmus University Medical Center Rotterdam, and through newsletters and patient organizations. In both study populations, we assessed the risk of AMD for different genetic variants, and carefully studied gene-gene and gene-environment interactions.

This work is divided into three sections:

**Part 1** Genetic risk factors of AMD and their interaction with environmental factors

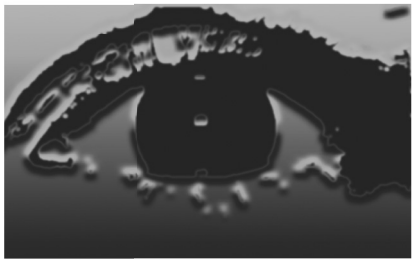
**Part 2** Predictive value of multiple genetic testing for AMD and usefulness of genetic testing in clinical practice

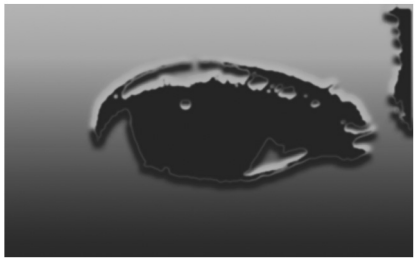
**Part 3** General discussion



# PART I

## GENETIC RISK FACTORS OF AMD





# 1. *COMPLEMENT FACTOR H* POLYMORPHISM, COMPLEMENT ACTIVATORS, AND RISK OF AGE-RELATED MACULAR DEGENERATION

## ABSTRACT

**Context:** The evidence that inflammation is an important pathway in age-related macular degeneration (AMD) is growing. Recent case-control studies demonstrated an association between the *complement factor H* (*CFH*) gene, a regulator of complement, and AMD. **Objective:** To assess the associations between the *CFH* gene and AMD in the general population, and to investigate the modifying effect of smoking, serum inflammatory markers, and genetic variation of C-reactive protein (CRP). **Design, Setting, and Participants:** Population-based, prospective cohort study of individuals aged 55 years or older (enrollment between March 20, 1990, and July 31, 1993, and 3 follow-up examinations that were performed between September 1, 1993, and December 31, 2004) in Rotterdam, the Netherlands. The *CFH* Y402H polymorphism was determined in a total of 5681 individuals. Information on smoking, erythrocyte sedimentation rate, CRP serum levels and haplotypes of the *CRP* gene were assessed at baseline. **Main Outcome Measures:** All severity stages of prevalent and incident AMD, graded according to the International Classification and Grading System for AMD. **Results:** The frequency of *CFH* Y402H was 36.2% (4116/11,362 alleles). At baseline, there were 2062 persons (36.3%) with any type of AMD (prevalent cases), including 78 (1.4%) with late AMD (stage 4). During follow-up (mean, 8 years; median, 10 years), 1649 (35.5%) of 4642 participants progressed to a higher stage of AMD (incident cases), including 93 (5.6%) who developed late AMD. The odds ratio (OR) of AMD increased in an allele-dose manner with 2.00 (95% confidence interval [CI], 1.56–2.55) for stage 2 AMD, 4.58 (95% CI, 2.82–7.44) for stage 3 AMD, and 11.02 (95% CI, 6.82-11.81) for stage 4 (late, vision threatening) AMD for homozygous persons. Cumulative risks calculated by Kaplan-Meier analysis of late AMD by age 95 years were 48.3% for homozygotes, 42.6% for heterozygotes, and 21.9% for noncarriers. The population-attributable risk for *CFH* Y402H was 54.0%. Elevated erythrocyte sedimentation rates further increased the OR to 20.2 (95% CI 9.5–43.0), elevated serum *CRP* to 27.7 (95% CI, 10.7–72.0), and smoking to 34.0 (95% CI, 13.0–88.6) for homozygotes compared with noncarriers without these determinants. The *CRP* haplotypes conferring high levels of CRP significantly increased the effect of CFH Y402H. ($P < 0.01$) **Conclusion:** The *CFH* Y402H polymorphism may account for a substantial proportion of AMD in individuals similar to those in the Rotterdam Study and may confer particular risk in the presence of environmental and genetic stimulators of the complement cascade.

## INTRODUCTION

Age-related macular degeneration (AMD) is the most important cause of irreversible visual loss in the elderly of the Western World.[9] This late-onset disorder causes focal deposition of extracellular material (drusen) underneath the retinal pigment epithelium, ultimately leading to geographic atrophy or subretinal neovascularization. Recent studies provide increasing evidence that inflammation is an important disease mechanism. Drusen were shown to contain complement components and regulators, immunoglobulins, and anaphylatoxins;[32] C-reactive protein (CRP) was associated with AMD;[33] and a mouse model lacking the gene for monocyte chemoattractant protein appeared to develop hallmarks of AMD.[34]

It has long been recognized that hereditary factors play a role in AMD. First-degree relatives were shown to have an increased risk,[14,15] and segregation analysis suggested the presence of a major gene.[17] Genome-wide linkage analyses identified a disease locus on 1q25 - q31,[21,22,25,26,29,35,36] and case-control studies recently identified *complement factor H (CFH)* as the responsible gene.[37-43] This gene has many frequent polymorphic variants that relate to AMD.[40] The *CFH* Y402H variant, located within a binding site for CRP, was consistently shown to have the strongest association in the coding region.[37-43]

Complement factor H is an important inhibitor of the complement pathway. Activation of this pathway initiates a proteolytic cascade that releases pro-inflammatory anaphylatoxins and causes formation of a membrane-attack complex ultimately leading to cell lysis. CFH preferentially binds and inactivates complement component C3b, and prevents the production of C3 convertase, and progression of the cascade.[44] The association between *CFH* and AMD emphasizes the inflammatory pathogenesis of AMD and suggests that triggering the complement cascade in genetically predisposed individuals promotes development of AMD.

The purpose of this study was 3-fold. First, we examined the associations between the *CFH* Y402H polymorphism and early (less severe) as well as late (vision threatening) AMD in a general population. Second, we investigated whether smoking and other pro-inflammatory markers may modify the relationship between *CFH* and AMD. And third, we assessed whether genetic variants of *CRP* interact with this *CFH* polymorphism. We investigated these issues in the population-based Rotterdam Study. The large study sample, the variety of risk factors determined at baseline, and the unbiased diagnosis of

AMD during a long follow-up particularly addressed the multifactorial origin of AMD, and facilitated the study of gene-environment interaction.

## METHODS

### Study population

The Rotterdam Study is a prospective, population-based cohort study of chronic diseases in the elderly. The eligible population comprised all 10,275 inhabitants aged 55 years or older living in Ommoord, a suburb of Rotterdam, the Netherlands. Inhabitants were ascertained from the municipal register, were invited by mail, and contacted by telephone for a home interview and examinations at the research center. Of the eligible population, 7983 (78%) individuals participated (58% female, 98% white).[45] The ophthalmologic part of the study became operational after the pilot phase of the study had started, and consisted of 9774 eligible individuals, of whom 7598 (78%) participated. The investigation was approved by the medical ethics committee of Erasmus University (Rotterdam, the Netherlands), and all participants provided signed informed consent for participation in the study, publication of obtained data, retrieval of medical records, and the use of blood and DNA for scientific purposes.

Baseline examinations took place from March 20, 1990 through July 31, 1993; One follow-up examination was performed in September 1, 1993-December 31, 1994, and had a mean (SD) time between baseline and follow-up of 1.98 (0.64) years; another examination was performed between April 15, 1997, and December 31, 2000, and had a mean (SD) time of 6.50 (0.35) years; and the third examination was performed between April 23, 2002, and December 31, 2004, and had a mean (SD) time of 11.08 (0.53) years. At baseline, 6418 participants underwent an eye examination and had gradable fundus photographs; 5681 of these had a successful assessment of the *CFH* gene polymorphism (88.6% of persons with AMD and 88.4% of those without AMD) and were therefore available for prevalence analyses. Seventy-eight persons with prevalent late AMD (stage 4) were excluded from further incidence analyses. At first follow-up examination, 270 persons had died and 691 were not included in the analyses due to refusal, lost to follow-up, or ungradable fundus photographs, resulting in 4642 individuals with complete data of whom 12 had late AMD. At the second follow-up examination, 663 persons had died and 561 were

not included due to refusal, lost to follow-up, or ungradable photographs, leaving 3406 with complete data of whom 32 had late AMD. At third follow-up examination, 738 persons had died, 249 were not included due to refusal, lost to follow-up, or ungradable photographs, and 2387 individuals had complete data of whom 49 had late AMD. The total number of person-years on which incidence analyses were based was 30 621. Data-analysis took place from May 10, 2005, to May 30, 2006.

**Genotyping**

All participants were genotyped for the *CFH* Y402H polymorphism (1277 T>C, rs1061170) in 2-ng genomic DNA, extracted from leukocytes, with the Taqman assay (Applied Biosystems, Foster City, Calif).

To assess variation in the *CRP* gene, we genotyped single-nucleotide polymorphisms (rs1130864 C→T, rs1205 C→T, rs3093068 C→G) enabling stratification into the 4 haplotypes that are present in persons of European descent (SeattleSNPs, http://pga.gs.washington.edu).[46] Overlap between these 3 tagging single-nucleotide polymorphisms was only present in 9 of 10,800 alleles (<0.001%). Haplotypes were estimated with the PHASE program[47] and the probability of the estimated haplotypes was 0.999 or higher in all individuals. Haplotypes were coded as 1 to 4 in order of decreasing frequency in the population (coding from rs1130864 C→T, rs1205 C→T and rs3093068 C→G: haplotype 1 = C-T-C, haplotype 2=T-C-C, haplotype 3=C-C-C, and haplotype 4=C-C-G). In the analysis of interaction between *CRP* and *CFH*, not all strata had sufficient cases for haplotype 4. These analyses were restricted to the 3 most common haplotypes, which describe 94.2% of the study population.

**Inflammatory mediators**

The inflammatory mediators erythrocyte sedimentation rate (ESR), CRP serum level, and leucocyte count were evaluated at baseline. Blood was drawn by venous puncture and the ESR was read after 60 minutes. Leukocyte count was directly assessed using a Coulter Counter T540 (Coulter Electronics, Luton, England). Serum samples were initially stored at -20ºC, and were thawed and assayed for CRP by Rate Near Infrared Particle Immunoassay method method (IMMAGE high sensitive CRP, Beckman Coulter, Fullerton, Calif). Information on cigarette smoking (never, past, and current) was obtained by interview at baseline.[45]

**Diagnosis of AMD**

Fundus photographs covering a 35° field centered on the macula were taken at each visit (Topcon TRV-50VT fundus camera, Topcon Optical Co, Tokyo, Japan) after pharmacologic mydriasis. All signs of AMD were graded according to the International Classification and Grading System for AMD under the supervision of a senior ophthalmologist (JRV and PTVMdJ), and graders were tested regularly for interrater and intrarater agreement.[5,27] We categorized these signs into 5 exclusive stages that represent an increasing severity of AMD. In short, stage 0 contains no or only small hard drusen; stage 1, either small soft distinct drusen or pigmentary irregularities; stage 2, either soft distinct drusen with pigmentary irregularities, or soft indistinct drusen without pigmentary irregularities; stage 3, soft indistinct drusen with pigmentary irregularities; and stage 4, geographic atrophy (dry AMD), neovascular AMD (wet AMD), or mixed AMD (both dry and wet AMD). Persons were classified in stage 0 to 4 based on the eye with the more severe stage. Unaffected participants were defined as those who remained in stage 0 throughout the follow-up period. Incident cases were defined as the absence of a stage in both eyes at baseline and its first appearance of AMD in at least 1 eye at follow-up.

**Statistical analysis**

Baseline characteristics of participants were compared among *CFH* genotypes with analysis of covariance for continuous variables, and with logistic regression analysis for discrete variables and were adjusted for age and sex. Natural-log transformed values of CRP levels, ESRs, and leukocyte counts were used to normalize a skewed distribution. $P < .05$ was considered statistically significant along with 95% confidence intervals (CIs) excluding the null hypothesis.

Prevalences of AMD stages were calculated as percentages of the study population at baseline and incidence rates were calculated by dividing the number of incident cases by the number of person-years of follow-up. Duration of follow-up was calculated from the date of fundus photography at baseline until diagnosis of AMD, lost to follow-up, last visit before death, or end of follow-up, whichever came first.

Genotype distributions for *CFH* were tested for Hardy-Weinberg equilibrium using a $\chi^2$ test. Prevalence odds ratios (ORs) were estimated with logistic regression analysis and incidence relative risks were estimated with Cox proportional hazards analysis. Both analyses were adjusted for age and

sex. The proportional hazards assumption was tested by drawing log minus log plots of the survival function, which was verified by visual inspection. The ORs based on both prevalent and incident cases were estimated with logistic regression analyses with age, sex, and follow-up time in the model. Cumulative risks of late AMD were calculated using Kaplan Meier product-limit analysis in the presence of competing risks. Participants who died, and those who were free of AMD and were lost to follow-up, were censored at the time of their last visit. *P*-values from logrank tests of equality were used to test significant differences between CFH genotypes. The formula to calculate population attributable risk is:

population attributable risk = (relative risk −1/ relative risk)-proportion of exposed.

Relative risk of late AMD in this formula is estimated by the OR. The proportion exposed is the proportion of participants with late AMD carrying the Y402H allele.

To maximize contrast between affected and healthy individuals, the disease outcome for the interaction analyses was defined as late AMD (stage 4) vs. no AMD (stage 0). Risk assessments were initially performed for prevalent and incident cases separately. However, because risk estimates were similar, interaction analyses were subsequently performed using pooled data of prevalent and incident cases to increase statistical power. For the pooled data, ORs were used as representative for relative risks because pooling allows a case-control analysis. The ESRs were evaluated using categories of normal and elevated rates (cutoff of 12 mm per hour for men and 20 mm per hour for women). Because CRP levels and leukocyte counts were within normal limits (CRP < 8 mg/L; leukocyte count 3.5–10.0 * $10^9$/L) for all participants, we evaluated these mediators in tertiles. Smoking was stratified as never, past, and current. Biological interaction with CFH was assessed by calculating the synergy index.[48] The synergy index measures deviation from additivity of 2 risk factors, and is based on the ratio of the combined effect to the sum of the separate effects. A synergy index exceeding 1.00 suggests the presence of at least 1 pathway in the pathogenesis of the disease for which both risk factors are required.

Interaction with the *CRP* gene was studied with homozygous haplotype 1 carriers (1–1) as the reference category because this genotype was associated with the lowest CRP levels. We pooled all haplotype 2 carriers (2*) in 1 group, and all haplotype 3 carriers in another group (3*). Individuals with haplotypes 2 and 3 (2–3) were present in both groups 2* and 3*.

## RESULTS

At baseline, we identified 2062/5681 (36.3%) of 5681 persons with any type of AMD, and 78 (1.4%) with late AMD (stage 4). During follow-up (mean, 7.85 years; median, 10.31 years), we identified 1858 (40.0%) of 4642 persons who had remained in stage 0, and 1649 (35.5%) who progressed to a higher stage of AMD. Of these 1649, 93 persons (5.6%) had late AMD. The baseline characteristics and frequency of AMD, stratified by CFH genotypes are presented in **Table 1**.

Genotype frequencies of *CFH* were analogous for participants and non-participants and were in Hardy-Weinberg equilibrium. The risks of AMD for the *CFH* Y402H genotypes appear in **Table 2**. The prevalence of the *CFH* Y402H allele was 36.2% (4116/11 362). Risks were consistently higher for homozygous than for heterozygous persons, representing an allele-dose effect. Overall, carriers of the *CFH* Y402H allele had higher risks of all types of AMD than noncarriers and risks increased with each stage of AMD.
The ORs of bilateral late AMD (17.93; 95% CI, 9.00–35.70) were higher than of unilateral late AMD (6.58; 95% CI, 3.47–12.48) in homozygotes and the same trend was seen in heterozygotes. Within AMD subtypes adjusted for bilaterality, homozygotes had higher ORs of geographic atrophy (17.2; 95% CI, 6.7–44.2) and mixed AMD (19.2; 95% CI, 6.1–60.2) than of neovascular AMD (12.4; 95% CI, 2.4–65.4), although differences between subtypes did not reach statistical significance (geographic atrophy *vs.* neovascular AMD: $P = 0.63$; mixed vs. neovascular AMD: $P = 0.66$; geographic atrophy *vs.* mixed AMD: $P = 0.56$). The population attributable risk of late AMD for *CFH Y402H* was 54.0% (*i.e.*, 23.2% for those carrying 1 allele and 30.8% for those carrying 2 alleles).

The cumulative incidence of late AMD for the CFH genotypes appears in **Figure 1**. Risk differences between carriers and noncarriers became statistically significant after age 75 years (log rank $P < .01$). At age 95 years, risks had increased to 48.3% (95% CI, 28.8%–67.7%) for homozygous and 42.6% (95% CI, 24.3%–60.8%) for heterozygous carriers compared to 21.9 (95% CI, 5.4%–38.4%) for noncarriers.

**Table 1:** Baseline characteristics stratified by *CFH* Y402H genotype*

| | Noncarrier (n = 2346) | Heterozygous (n = 2554) | Homozygous (n = 781) |
|---|---|---|---|
| Age, mean (SD), y | | | |
| At baseline | 68.71 (8.59) | 68.75 (8.72) | 68.81 (8.87) |
| At diagnose | 73.05 (8.20) | 72.94 (8.15) | 72.59 (8.41) |
| Women, No. (%) | 1366 (58.2) | 1519 (59.5) | 449 (57.5) |
| CRP | | | |
| median (IQR), mg/L | 1.83 (0.88-3.58) | 1.87 (0.89-3.55) | 1.86 (0.90-3.64) |
| Erythrocyte sedimentation rate | | | |
| median (IQR), mm/h | 10 (6-16) | 10 (6-17) | 10 (6-18) |
| Leukocyte count | | | |
| median (IQR), $10^9$/L | 6.44 (5.33-7.67) | 6.33 (5.44-7.67) | 6.44 (5.39-7.56) |
| Smoking status, No. (%) | | | |
| Never | 780 (33.8) | 878 (34.8) | 267 (34.7) |
| Former | 1012 (43.9) | 1058 (41.9) | 314 (40.8) |
| Current | 515 (22.3) | 590 (23.4) | 188 (24.4) |
| CRP haplotypes, No. (%) | | | |
| 1-1 | 213 (9.6) | 249 (10.4) | 74 (10.0) |
| 2-2 | 225 (10.2) | 241 (10.1) | 64 (8.6) |
| 3-3 | 202 (9.1) | 187 (7.8) | 65 (8.8) |
| 1-2 | 449 (20.3) | 489 (20.5) | 169 (22.8) |
| 1-3 | 436 (19.7) | 492 (20.6) | 160 (21.6) |
| 2-3 | 440 (19.9) | 449 (18.8) | 125 (16.9) |
| AMD prevalence, No. (%) | | | |
| Stage 0 | 1558 (66.4) | 1644 (64.4) | 417 (53.4) |
| Stage 1 | 649 (27.7) | 688 (26.9) | 219 (28.0)* |
| Stage 2 | 114 (4.9) | 151 (5.9) | 92 (11.8)† |
| Stage 3 | 13 (0.6) | 36 (1.4)* | 22 (2.8)† |
| Stage 4 | 12 (0.5) | 35 (1.4)* | 31 (4.0)† |
| AMD incidence, rates per 1,000 pyrs | | | |
| Stage 1 | 55.83 (n = 436) | 59.94 (n = 468) | 70.96 (n = 131) |
| Stage 2 | 16.82 (n = 180) | 18.47 (n = 205) | 21.02 (n = 60)* |
| Stage 3 | 2.23 (n = 26) | 2.71 (n = 33) | 5.36 (n = 17)† |
| Stage 4 | 1.60 (n = 19) | 3.35 (n = 42)* | 9.34 (n = 32)† |

Abbreviations: AMD, age-related macular degeneration; *CFH*, complement factor H; CRP, C-reactive proteine; IQR, interquartile range

*Baseline characteristics of participants were compared among *CFH* genotypes with analysis of covariance for continuous variables and with logistic regression analyses for discrete variables, and adjusted for age and sex.

* *P* value < 0.05 for the difference with noncarriers

† *P* value < 0.001 for the difference with noncarriers

**Table 2A:** Risks of AMD for *CFH* genotypes

a. prevalence analyses[†]

| | ***no AMD*** | | ***Early AMD*** | | | | | ***Late AMD*** | |
|---|---|---|---|---|---|---|---|---|---|
| *CFH* Y402H (thv n=3619) | Stage 0 (n=3619) | | Stage 1 (n=1556) | | Stage 2 (n=357) | | Stage 3 (n=71) | | Stage 4 (n=78) |
| *genotype* | *%* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* |
| Noncarrier | *43.1* | *41.7* | 1 | *31.9* | 1 | *18.3* | 1 | *15.4* | 1 |
| Heterozygous | *45.4* | *44.2* | 1.01 (0.89-1.15) | *42.3* | 1.23 (0.96-1.59) | *50.7* | **2.59 (1.36-4.92)** | *44.9* | **2.91 (1.47-5.75)** |
| Homozygous | *11.5* | *14.1* | **1.32 (1.10-1.60)** | *25.8* | **2.67 (1.99-3.57)** | *31.0* | **5.23 (2.60-10.52)** | *39.7* | **12.32 (5.98–25.36)** |
| Frequency of Y402H (%) | *34.0* | *36.2* | | *46.9* | | *56.4* | | *62.2* | |

b. incidence analyses[‡]

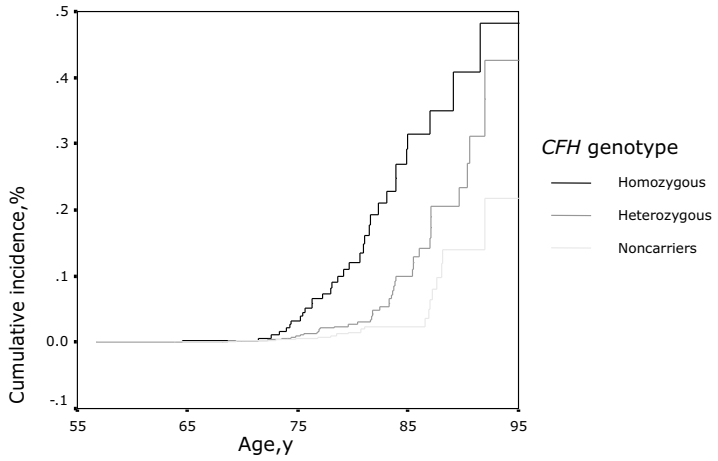
| | ***Early AMD*** | | | | | | ***Late AMD*** | |
|---|---|---|---|---|---|---|---|---|
| *CFH* Y402H | Stage 1 (n=1035) | | Stage 2 (n=445) | | Stage 3 (n=76) | | Stage 4 (n=93) | |
| | *%* | *HR(95%CI)* | *%* | *HR(95%CI)* | *%* | *HR(95%CI)* | *%* | *HR(95%CI)* |
| Noncarrier | *42.1* | 1 | *40.4* | 1 | *34.2* | 1 | *20.4* | 1 |
| Heterozygous | *45.2* | 1.09 (0.95-1.24) | *46.1* | 1.17 (0.96-1.43) | *43.4* | 1.38 (0.83-2.31) | *45.2* | **2.52 (1.46-4.34)** |
| Homozygous | *12.7* | **1.29 (1.06-1.57)** | *13.5* | **1.46 (1.09-1.96)** | *22.4* | **3.00 (1.62-5.53)** | *34.4* | **7.61 (4.30-13.46)** |
| Frequency of Y402H (%) | *35.3* | | *36.5* | | *44.1* | | *57.0* | |

c. analysis based on combined prevalent and incident cases[$]

| | ***no AMD*** | | ***Early AMD*** | | | | | ***Late AMD*** | |
|---|---|---|---|---|---|---|---|---|---|
| *CFH* Y402H | Stage 0 | | Stage 1 | | Stage 2 | | Stage 3 | | Stage 4 |
| | (n=2392) | | (n=2221) | | (n=764) | | (n=133) | | (n=171) |
| | *%* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* | *%* | *OR(95%CI)* |
| Noncarrier | *43.9* | *42.5* | 1 | *37.4* | 1 | *27.1* | 1 | *18.1* | 1 |
| Heterozygous | *45.4* | *44.5* | 0.98 (0.87-1.12) | *44.6* | **1.16 (0.97-1.38)** | *45.1* | **1.67 (1.09-2.54)** | *45.0* | **2.66 (1.72-4.13)** |
| Homozygous | *10.7* | *13.0* | **1.16 (0.96-1.41)** | *17.9* | **2.00 (1.56-2.55)** | *27.8* | **4.58 (2.82-7.44)** | *36.8* | **11.02 (6.82-17.81)** |
| Frequency of Y402H (%) | *33.4* | *35.2* | | *40.2* | | *49.6* | | *59.4* | |

Abbreviations: AMD, age-related macular degeneration; CFH, complement factor H; CI, confidence interval; HR, hazard ratio; OR, odds ratio.
* The ORs and HRs are estimates of the relative risk of AMD, and represent the risk of disease (AMD *vs* stage 0) in the genetic risk group divided by the risk of disease (AMD vs stage 0) in the non-risk group (noncarriers).
[†]Estimated with logistic regression analyses and included age and sex in the model.
[‡]Estimated with Cox proportional hazards analyses and included age and sex in the model
[$]Estimated with logistic regression analyses and included age, sex, and follow-up time in the model.
The numbers in this section do not equal the sum of numbers in the "prevalence analyses" and "incidence analyses" sections because prevalence analyses and incidence analysis were performed on the same cohort of 5681 persons. In the combined analysis, we used the diagnosis of AMD at the last visit to the study center or at baseline when no follow-up examination took place.

**Figure 1:** Cumulative incidence of late AMD for homozygous and heterozygous carriers versus noncarriers of the Y402H polymorphism




Numbers at risk

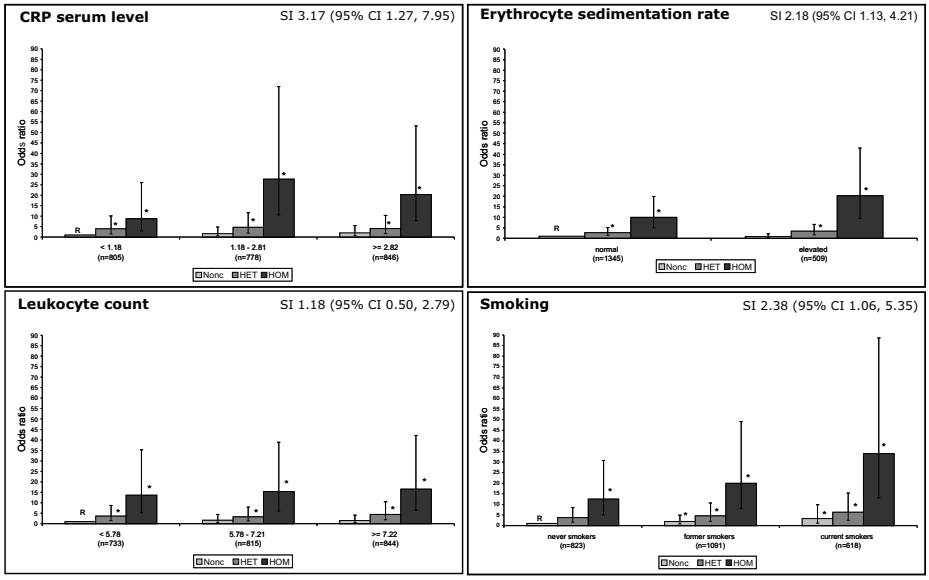
| | | | | | |
|---|---|---|---|---|---|
| Noncarriers | 1493 | 1308 | 623 | 117 | 3 |
| Heterozygous | 1580 | 1396 | 617 | 95 | 3 |
| Homozygous | 434 | 368 | 163 | 29 | 3 |

CFH indicates complement factor H. Cumulative risks of late AMD were calculated using Kaplan-Meier product-limit analysis in the presence of competing risks. Differences in risks between the *CFH* genotypes were statistically significant (log-rank $P < .001$).

Interaction between ESR, CRP, leukocyte count, smoking, and *CFH* Y402H is demonstrated in **Figure 2**. The joint effect of each determinant with *CFH* Y402H was significantly greater than the sum of the independent effects except for leukocyte count. An elevated ESR augmented the association to an OR of 20.2 (95% CI, 9.5–43.0). Higher serum CRP levels increased the association for the second tertile to an OR of 27.7 (95% CI, 10.7–72.0) while the third tertile did not further increase the OR. Neither ESR nor CRP levels were significantly associated with AMD in noncarriers.

Smoking had a large influence on the risk of AMD related to *CFH*. Current smokers homozygous for *CFH* Y402H had an OR of 34.0 (95% CI, 13.0–88.6) for late AMD compared with individuals who never smoked without the risk allele. In the absence of *CFH* Y402H, current smoking increased the OR for AMD to 3.36 (95% CI,1.14–9.86).

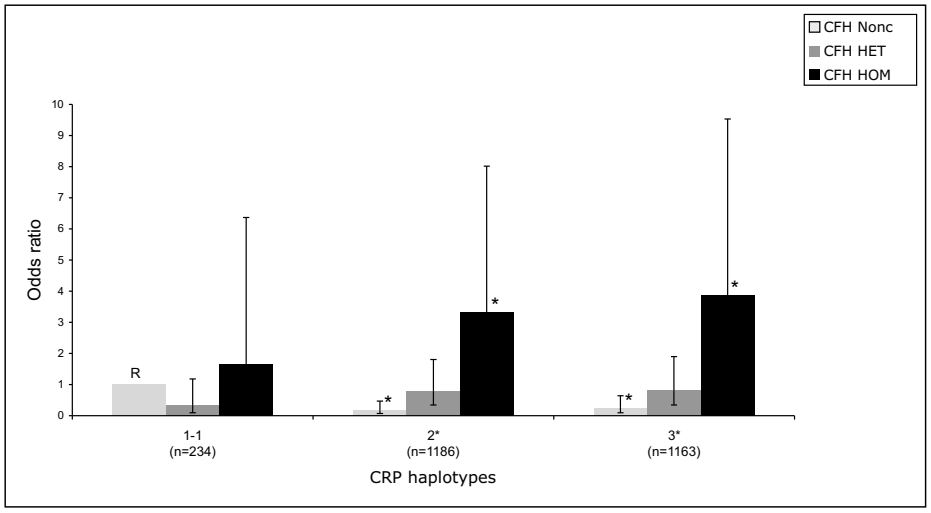
**FIGURE 2:** RISK OF LATE AMD FOR *CFH* Y402H GENOTYPES, STRATIFIED BY CRP SERUM LEVEL, ERYTHROCYTE SEDIMENTATION RATE, LEUKOCYTE COUNT AND SMOKING




AMD indicates age-related macular degeneration; *CFH*, Complement Factor H; SI, synergy index; Nonc, noncarrier; HET, heterozygous; HOM, homozygous
* *P* value < 0.05 for comparison with reference category

Serum levels of CRP varied among the *CRP* haplotypes in this study population. Haplotype 1 (the most common) had a frequency of 32.7% and the lowest CRP levels; haplotype 2 had a frequency of 31.6% and the highest CRP levels; and haplotype 3 had a frequency of 29.9% and intermediate levels of CRP.[29] The *CRP* haplotypes per se were not related to AMD. We tested the hypothesis whether these haplotypes influenced the effect of *CFH* Y402H on AMD (**Figure 3**). Compared with noncarriers of *CFH* Y402H with *CRP* haplotype 1, noncarriers with *CRP* haplotype 2 had an OR of AMD of 0.17 (95% CI, 0.06–0.46); $P < 0.001$) and noncarriers with *CRP* haplotype 3 had an OR of 0.25 (95% CI,0.09–0. ; $P = .004$). In contrast, in homozygous *CFH* Y402H carriers, the OR of AMD was 3.32 (95% CI, 1.38–8.01; $P = .007$) for haplotype 2 and 3.86 (95% CI, 1.56–9.53); $P = 003$) for haplotype 3. The highest difference in ORs between homozygous carriers and noncarriers was observed for *CRP* haplotype 2, which is the haplotype with the highest CRP levels. Our results show that those participants homozygous for *CFH* Y402H with an additional genetic predisposition to high serum CRP levels were at higher risk of developing AMD.

**Figure 3:** Risk of late AMD for *CRP* haplotypes stratified by *CFH* genotypes




AMD indicates age-related macular degeneration; CI, confidence interval; Nonc, noncarrier; HET, heterozygous; HOM, homozygous. Risk of late AMD estimated by logistic regression analysis and adjusted for age and sex. Haplotype 2 carriers (2*) were grouped, as were haplotype 3 carriers (3*). Individuals with haplotypes 2 and 3 (2-3) were present in both the 2* and 3* group. $P < .01$ for comparison with homozygous haplotype 1 carriers (1-1). [R] reference category; * $P$ value < 0.05 for comparison with reference category

## COMMENT

In this prospective study, which was based on an older, white population in the middle socioeconomic class in the Netherlands, we find that the *CFH* gene is a major risk factor for AMD. The gene was implicated in all stages of AMD from early hallmarks such as drusen to vision-disabling late AMD and the risks increased with each successive stage to a high of 11 for late AMD. We calculated that individuals homozygous for the *CFH* Y402H polymorphism had a 48% risk of developing late AMD by age 95 years while this risk did not exceed 22% for noncarriers. These data suggest that *CFH* Y402H may be a causal factor in more than 50% of all AMD cases in the general population.

Previous reports on the association between *CFH* and AMD were from clinic or family-based case-control studies with cross-sectional designs. This hampers extrapolation of the role of *CFH* in AMD development for the population at large. Strengths of our current study are the population-based prospective design, the large study sample, and the use of standardized

procedures for AMD diagnosis by experienced graders.[6] We restricted the analyses of potential modifiers to individuals with late AMD (stage 4) and those who remained free of any type of AMD (stage 0) throughout the study. To maximize statistical power and enable precise risk estimates, we pooled prevalent and incident cases with late AMD. Prevalent cases showed similar risk estimates as incident cases so the exposures to inflammation and smoking likely preceded AMD and pooling did not jeopardize causal inference. Although not negligible, our a priori hypothesis and assessment of only 1 single-nucleotide polymorphism in the *CFH* gene make it unlikely that our findings are falsely positive.

Complement factor H was associated with both late AMD subtypes in this study. Homozygous *CFH* Y402H carriers had a higher risk of bilateral than of unilateral late AMD, and risks of geographic atrophy and mixed AMD were slightly but not significantly higher than neovascular AMD. This is in agreement with other studies that reported higher frequencies of *CFH* Y402H carriers in persons with geographic atrophy,[41,49] and 1 study that suggested a lower risk of geographic atrophy for a *CFH* haplotype containing the non-risk allele.[40] Nevertheless, the high risk for both subtypes signifies a common inflammatory pathogenesis.

Complement factor H is an important regulator of the complement system. Three enzyme cascades exist: the classical complement pathway, initiated by antigen-antibody complexes and surface-bound CRP; the lectin, turned on by mannose groups of microbial carbohydrates; and the alternative complement pathway, activated by surface-bound C3b. The pathways converge at the point in which C3 is cleaved into C3a and C3b by C3 convertase, which initiates C5 convertase, resulting in the formation of the membrane-attack complex with the terminal components (C5b – C9). CFH specifically inhibits the alternative complement cascade but also regulates the common pathway. It binds C3b and acts as a cofactor in the proteolysis of C3b by factor I, resulting in an inactive C3b molecule. This prevents the production of C3 convertase in the alternative cascade, as well as the production of C5 convertase in the common pathway. As a result, CFH interferes with progression of the entire cascade.[44,50]

Genetic predisposition to a malfunctioning CFH can only be of importance when the complement system is switched on. Our data provide strong evidence that onset of this cascade leads to AMD in persons with the *CFH* Y402H polymorphism. This is demonstrated by the significant interaction between chronic as well as acute inflammation and *CFH* Y402H. Elevated

baseline ESRs considerably increased the risk of AMD in carriers, and a similar trend was observed for serum CRP levels. Neither ESR nor CRP levels increased the risk significantly in noncarriers. Earlier studies reported a relationship between serum CRP level and progression of AMD.[33] However, our results imply that this relationship is mostly determined by the *CFH* polymorphism. Increased leukocyte counts did not contribute to an additional effect, possibly due to the absence of clinically elevated levels in our study.

Smoking was considered the highest risk factor for AMD prior to the introduction of *CFH* in AMD. Our data show that the combined effect of both exposures exceeds the sum of the independent effects. Compared with no exposure, smoking increased the risk of AMD 3.3 times, the presence of 2 *CFH* Y402H alleles increased the risk 12.5 times, while the combination of both determinants increased this risk 34-fold. Smoking increases cytokines and inflammatory cells and has been shown to activate the complement pathway by weakening the susceptibility of C3 to CFH and factor I.[51,52] When CFH function is genetically impaired, progression may be further accelerated. However, the elevated risk of smoking in noncarriers suggests that smoking may have an alternative mechanism in AMD pathogenesis.

We further explored the relationship with CRP for 2 reasons. First, the *CFH* Y402H variant represents an amino acid change in the SCR7 domain, which contains a binding site for CRP, heparin, and M-protein, prompting a functional interaction with these proteins.[44] Second, CRP not only triggers the classical complement cascade by binding to C1q, it also limits the amount of complement activation by its ability to interact with CFH, thereby reducing the complement-associated damaging effect.[53] Because serum CRP levels are known to fluctuate, a single measurement of CRP may not accurately reflect a continuous baseline level nor adequately represent the possible response after an inflammatory stimulus. This motivated us to examine the *CRP* gene. Our data suggest that *CRP* haplotypes, which increase serum levels, modify the effect of *CFH*. These haplotypes decrease the risk of AMD in noncarriers but increase the risk in persons homozygous for *CFH* Y402H. We propose the following as a plausible biological mechanism. Genetic variants of *CRP* have been shown to determine serum levels especially in response to inflammatory stimuli.[54] The *CFH* Y402H polymorphism may impair CRP binding, decrease CFH inhibition, and lead to destruction of host cells in particular in those individuals who are genetically predisposed to high CRP levels. By contrast, normal binding of CFH with CRP may increase inhibition

and decelerate complement activation in those who are hyperresponsive to inflammation.[50]

In conclusion, *CFH*, an inhibitory gene of the complement pathway, is a major risk factor for AMD in this population. It is involved in early as well as late disease pathogenesis and markedly increases risk of late AMD in the very old. The effect of *CFH* is significantly influenced by environmental and genetic factors that determine the inflammatory response and activate the complement pathway. Future research on therapeutic modalities that help regulate the terminal complement pathway, thereby sparing host tissue, may provide an approach for preventing sight-threatening AMD in genetically predisposed individuals.

# 2. *CFH* GENE AND AGE-RELATED MACULAR DEGENERATION: SEPARATING CULPRITS FROM INNOCENT BYSTANDERS

## ABSTRACT

Many studies have provided evidence that the *Complement Factor H* (*CFH*) gene, a regulator of complement, plays a major role in the pathogenesis of AMD. Initially, Y402H was launched as the risk variant, but recent studies showed that multiple alleles in *CFH* are highly associated with AMD. The extensive linkage disequilibrium (LD) across the region complicates identification of the true susceptibility alleles. The aim of the present study was to investigate which *CFH* variants describe the risk of AMD most accurately. We screened the coding and flanking regions of *CFH* in 360 AMD cases and 183 age-matched controls from the Netherlands. Univariate analysis revealed 24 sequence variants of which nine were significantly associated with AMD. The strongest association was observed for Y402H, followed by IVS1 (rs35507625) and V62I. An independent German study consisting of 335 cases and 373 controls showed a slightly different hierarchy: IVS10 (rs203674), Y402H, and IVS14 (rs1410996). Two LD blocks were identified, and both were independently associated with AMD. The first block included V62I, the second block Y402H, and each block comprised one protective and one causative haplotype. Conditional regression analysis showed that variants within each block independently influenced the risk of AMD, as did variant IVS18 (rs16840522), located outside the blocks. In conclusion, our data show that, apart from Y402H, other variants including IVS1 (rs35507625), V62I and A473A appear to be true susceptibility alleles and not merely risk indicators resulting from linkage disequilibrium.

## INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of severe visual impairment in the elderly of the Western world.[9] Early signs of the disease are depositions of extracellular material (drusen) underneath the retinal pigment epithelium, and areas of hyper- and depigmentation. At this stage, patients rarely suffer from clinical symptoms, but are at increased risk of developing severe visual loss. This late stage is characterized by either subretinal neovascularization (*i.e.* wet AMD) or geographic atrophy (*i.e.* dry AMD).

Recent progress has emphasized the importance of genetic predisposition in the etiology of the disease. The *Complement Factor H (CFH)* gene and the *LOC38771/HTRA1* locus have been identified as the most prominent susceptibility genes.[37-40,49,55-57] While the functional contribution of the *LOC387715/HTRA1* locus to AMD pathology is still under debate, *CFH* is known to be a key regulator of the alternative and common complement cascade. It inhibits unrestricted progression of the pathway, resulting in decreased formation of the membrane-attack complex and diminished cell lysis. The most studied variant in the *CFH* gene is the non-synonymous polymorphism rs1061170, which causes a tyrosine-to-histidine substitution at amino acid position 402 (Y402H) located within a binding site for C-reactive protein (CRP). The Y402H allele has been associated with all stages of AMD with odds ratios up to 11 for homozygous persons.[55]

Several studies suggest that Y402H may not be the most important *CFH* variant for AMD.[58,59] Investigation of single nucleotide polymorphisms (SNPs) across the entire *CFH* region by Li et al. showed that at least 20 other SNPs were more strongly associated than Y402H, of which rs2274700 (A473A) and rs1410996 (IVS14) were most significant. Functional studies investigating the biochemical consequences of the variants other than Y402H are scarce. Therefore, it is still unclear which variants truly determine susceptibility to AMD.

We sought to determine which variant or combination of variants describes the risk between *CFH* and AMD most accurately. We screened all exons and flanking introns for sequence variations in a case-control study from the Netherlands. We calculated univariate risks, estimated linkage disequilibrium (LD), determined risk haplotypes, and investigated the independent effects of risk variants. To compare ranking order of the most associated SNPs, we genotyped selected variants in a separate German study.

## MATERIALS AND METHODS

### Study population

The Dutch study consisted of 360 unrelated AMD patients and 183 control individuals from the Netherlands. Subjects were all Caucasian and recruited from the Netherlands Institute of Neuroscience Amsterdam, Erasmus University Medical Centre Rotterdam, and through newsletters, patient organizations, and nursery homes. Controls were aged 65 years and older, and were mostly unaffected spouses or non-related acquaintances of cases, or individuals who attended the ophthalmology department for reasons other than retinal pathology. The German study included 373 unrelated individuals with AMD and 335 unrelated controls recruited from the University Eye Clinics of Tübingen (area of Swabia) and Munich (area of Upper Bavaria).[49]

The study was approved by the Ethics Committee of Academic Medical Centre Amsterdam, and the Ethics Committee of the University of Würzburg, and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for AMD research.

### Diagnosis of AMD

All participants of both studies underwent fundus photography after pharmacologic mydriasis. Fundus transparencies were subsequently graded according to a modification of the International Classification and Grading System for AMD under the supervision of senior retinal specialists (PTVMdJ, CCWK, CNK).[5] Grading criteria were identical for both studies. AMD was categorized into early and late AMD according to methods described earlier.[6] In short, early AMD (stage 2 and 3) was defined as the presence of either soft distinct drusen with pigmentary irregularities, or soft indistinct drusen with or without pigmentary irregularities; and late AMD (stage 4) as geographic atrophy (dry AMD), neovascular AMD (wet AMD), or mixed AMD (wet AMD in one eye and dry AMD in the other eye, or both types in one eye). Persons were classified based on the eye with the more severe diagnosis. Control persons had no AMD (stage 0: no or only small hard drusen) in either eye, and no other macular pathology.

**Genotyping**

DNA was extracted from peripheral blood leukocytes after venous puncture. In the study from the Netherlands, all exons and flanking intronic regions of the *CFH* gene were amplified by polymerase chain reaction (PCR) except for exon 1 and exon 8. The samples were analyzed for sequence variations using denaturing high-performance liquid chromatography (DHPLC) on an automated system (Wave; Transgenomic, Santa Clara, California, USA). To identify homozygous variants in amplicons with frequent heterozygous SNPs, aliquots of a known wild-type sample were added to the DNA prior to the re-annealing step. Variants were subsequently identified by direct sequencing using the ABI-310 (Applied Biosystems, Foster City, California, USA). Variant rs1410996 was genotyped with the Taqman assay (Applied Biosystems, Foster City, California, USA).

In the German study, participants were genotyped with the Taqman assay (Applied Biosystems, Foster City, California, USA) for rs800292, rs1061170, rs203674 and rs1410996. Primers were checked against mispriming in the neighbouring *CFH*-like genes with in silico PCR, and primer sequences are available upon request.

**Statistical analysis**

Baseline characteristics of cases and controls were compared with analysis of covariance for continuous variables, and with logistic regression analysis for discrete variables, adjusting for age and sex. Fisher's Exact test was used to test genotype distributions for Hardy-Weinberg equilibrium and single SNP association. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with logistic regression analysis, assuming an allele-dose effect model. Haploview software (http://www.broad.mit.edu/mpg/haploview/) was used to perform linkage disequilibrium (LD) analysis and delineate haplotype blocks based on the confidence interval method that is integrated in the program.[60] The risk of AMD for each haplotype was determined with HAPLO.STATS 1.3.0. (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm). We performed a conditional regression analysis to assess independent effects of SNPs. The likelihood ratio statistic was used to asses significant increase in goodness of fit of the model. $P < 0.05$ was considered to be significant.

## RESULTS

**Table 1** shows the distribution of baseline characteristics of the study populations. Cases were slightly older than controls in both studies. The distribution of gender was not significantly different.

**Table 1:** Baseline characteristics of the study population

| | Study from the Netherlands | | | Study from Germany | | |
|---|---|---|---|---|---|---|
| | **Controls** | **Cases** | ***P*** | **Controls** | **Cases** | ***P*** |
| | ($N_{tot}$ = 183) | ($N_{tot}$ = 360) | | ($N_{tot}$ = 335) | ($N_{tot}$ = 373) | |
| Diagnosis | | | | | | |
| No AMD | 183 (100.0) | | | 335 (100.0) | | |
| Early AMD | | 89 (24.7) | | | 3 (0.8) | |
| Neovascular AMD | | 181 (50.3) | | | 135 (36.2) | |
| Geographic atrophy | | 54 (15.0) | | | 102 (27.3) | |
| Mixed AMD | | 36 (10.0) | | | 133 (35.7) | |
| Age, y | 74.3 (6.3) | 78.1 (7.6) | < 0.001 | 72.3 (8.2) | 79.0 (7.45) | < 0.001 |
| < 65 | 5/183 (2.7) | 20/360 (5.6) | | 60/331 (18.1) | 16/367 (4.4) | |
| 65–74 | 102/183 (55.7) | 88/360 (24.4) | | 158/331 (47.7) | 87/367 (23.7) | |
| 75–84 | 67/183 (36.6) | 186/360 (51.7) | | 94/331 (28.4) | 206/367 (56.1) | |
| > = 85 | 9/183 (4.9) | 66/360 (18.3) | | 19/331 (5.7) | 58/367 (15.8) | |
| Sex | | | 0.14 | | | 0.08 |
| Women | 98 (53.6) | 215 (59.7) | | 185(55.2) | 242(64.9) | |
| Men | 85 (46.4) | 145 (40.3) | | 150(44.8) | 131(35.1) | |

Data are unadjusted mean ± SD for continuous variables and percentages for dichotomous variables
Ntot = total number of persons

Single SNP analysis revealed 24 sequence variants in the 543 tested individuals from the Netherlands: two SNPs in the promoter region; nine SNPs in the coding region including 5 synonymous and 4 non-synonymous substitutions; and 13 intronic SNPs (**Figure 1**). Genotype frequencies of all variants were in Hardy-Weinberg Equilibrium. Nine SNPs were significantly associated with AMD (**Table 2a**). The strongest association in terms of statistical significance was observed for Y402H ($P = 8.07*10^{-7}$) followed by IVS1 ($P = 1.04*10^{-6}$), and V62I ($P = 1.12*10^{-6}$). Three variants increased the risk of AMD: Y402H [OR 2.27 (95%CI 1.69–3.04)], IVS10 [OR 2.04 (95%CI 1.54–2.71)], and A307A [OR 1.69 (95%CI 1.27–2.26)]; all other minor alleles were associated with a decreased risk of AMD. The recently

established IVS14 variant was sixth in ranking with OR 0.50 (95%CI 0.38–0.66). Single SNP analysis of the variants genotyped in 708 persons of the German study showed the strongest association for IVS10 ($P$ = $3.72*10^{-22}$), followed by Y402H ($P$ = $1.22*10^{-19}$), IVS14 ($P$ = $9.79*10^{-18}$), A473A ($P$ = $1.15*10^{-15}$) and V62I ($P$ = $2.49*10^{-6}$) (**Table 2b**). The minor alleles of these SNPs were protective excluding IVS10 and Y402H. The calculated ORs of these SNPs were OR 3.12 (95%CI 2.43–4.00) for IVS10, OR 2.98 (95%CI 2.32–3.83) for Y402H, OR 0.33 (95%CI 0.25–0.43) for IVS14, OR 0.36 (95%CI 0.28–0.47) for IVS10 and OR 0.45 (95%CI 0.33–0.61) for V62I.

**FIGURE 1:** STRUCTURE OF THE *CFH* GENE WITH THE RELATIVE POSITION OF EACH SNP FOUND IN THE NETHERLANDS

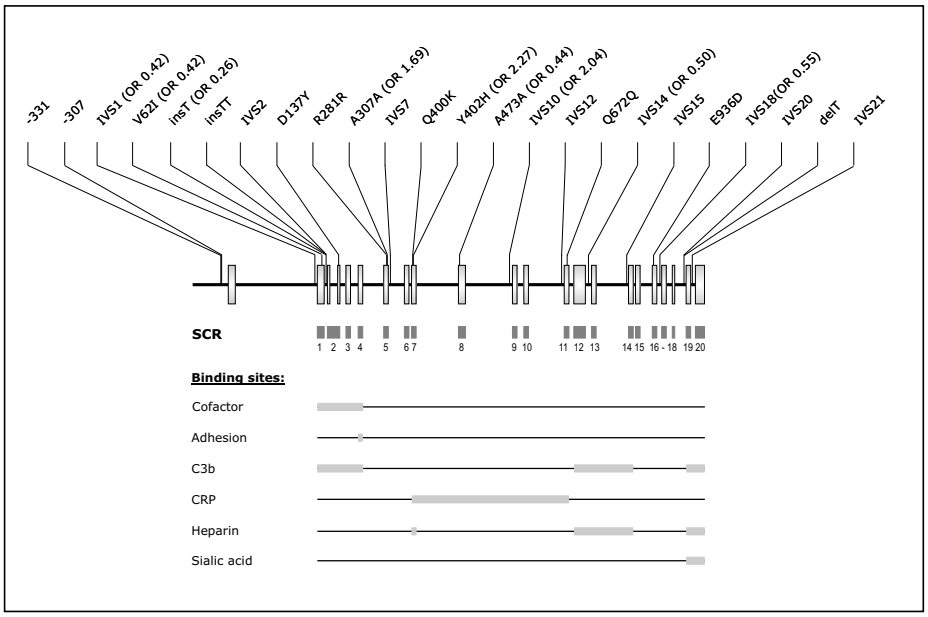


**Table 2:** *CFH* single SNP association with AMD
a. study from the Netherlands

| SNP location | Designation | | rs-number | LD block | Controls Frequency | Cases Frequency | *P* | Effect of minor allele |
|---|---|---|---|---|---|---|---|---|
| **Exon 9** | **Y402H** | **Y402H** | rs1061170 | 2 | 0.376 | 0.541 | 8.067E-07 | Causative |
| **IVS 1** | **IVS1** | **IVS1 –36 C>T** | rs551397 | 1 | 0.269 | 0.142 | 1.036E-06 | Protective |
| **Exon 2** | **V62I** | **V62I** | rs800292 | 1 | 0.269 | 0.140 | 1.115E-06 | Protective |
| **Exon 10** | **A473A** | **A473A** | rs2274700 | 2 | 0.415 | 0.259 | 1.662E-06 | Protective |
| **IVS 10** | **IVS10** | **IVS10 –98 G>T** | rs203674 | 2 | 0.437 | 0.586 | 2.179E-05 | Causative |
| **IVS 14** | **IVS14** | **IVS14 -543 C>T** | rs1410996 | 2 | 0.404 | 0.267 | 2.871E-05 | Protective |
| **IVS 2** | **Ins T** | **IVS2 -(9-17) ins T** | rs35507625 | 1 | 0.324 | 0.144 | 6.643E-05 | Protective |
| **Exon 7** | **A307A** | **A307A** | rs1061147 | | 0.432 | 0.538 | 0.005 | Causative |
| **IVS 18** | **IVS18** | **IVS18 -87 T>C** | rs16840522 | | 0.186 | 0.119 | 0.017 | Protective |
| IVS 15 | IVS15 | IVS15 -28 C>A | rs375046 | | 0.000 | 0.018 | 0.246 | |
| IVS 20 | delT | IVS 20 -(59-61) del T | - | | 0.006 | 0.002 | 0.275 | |
| IVS 2 | Ins TT | IVS2 -(9-17) ins 2T | rs35507625 | | 0.034 | 0.011 | 0.278 | |
| Exon 9 | Q400K | Q400K | - | | 0.000 | 0.006 | 0.303 | |
| IVS 7 | IVS7 | IVS7 +72 T>C | - | | 0.003 | 0.000 | 0.342 | |
| IVS 12 | IVS12 | IVS12 -69 C>T | - | 2 | 0.006 | 0.000 | 0.476 | |
| Exon 4 | D137Y | D137Y | - | | 0.000 | 0.012 | 0.497 | |
| Exon 13 | Q672Q | Q672Q | rs3753396 | 2 | 0.095 | 0.115 | 0.572 | |
| Exon 18 | E936D | E936D | rs1065489 | | 0.136 | 0.144 | 0.583 | |
| Promotor | -307 | -307 C>T | - | | 0.009 | 0.012 | 0.756 | |
| Promotor | -331 | -331 C>T | rs3753394 | | 0.260 | 0.249 | 0.931 | |

*(continued)*

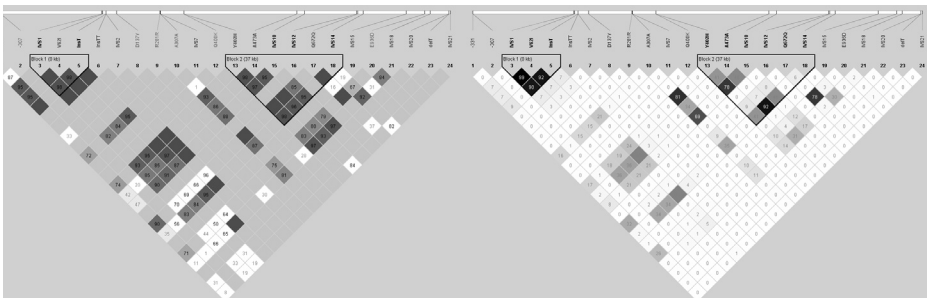
| SNP location | Designation | | rs-number | LD block | Controls Frequency | Cases | *P* | Effect of minor allele |
|---|---|---|---|---|---|---|---|---|
| Exon 7 | R281R | R281R | - | | 0.000 | 0.002 | 1.000 | |
| Exon 21 | IVS21 | IVS 21 +36 C>A | - | | 0.003 | 0.002 | 1.000 | |
| Exon 21 | IVS20 | IVS 20 -73 G>C | - | | 0.006 | 0.007 | 1.000 | |
| Exon 3 | IVS2 | IVS2 -7 G>A | rs35814900 | | 0.011 | 0.011 | 1.000 | |

b. Study from Germany

| SNP location | Designation | | rs-number | Controls Frequency | Cases Frequency | *P* | Effect of minor allele |
|---|---|---|---|---|---|---|---|
| **IVS 10** | **IVS10** | **IVS10 -98 G>T** | rs203674 | 0.378 | 0.656 | 1.28E-22 | Causative |
| **Exon 9** | **Y402H** | **Y402H** | rs1061170 | 0.358 | 0.617 | 1.222E-19 | Causative |
| **IVS 14** | **IVS14** | **IVS14 -543 C>T** | rs1410996 | 0.446 | 0.216 | 9.79E-18 | Protective |
| **Exon 10** | **A473A** | **A473A** | rs2274700 | 0.434 | 0.220 | 1.147E-15 | Protective |
| **Exon 2** | **V62I** | **V62I** | rs800292 | 0.221 | 0.117 | 2.493E-06 | Protective |

LD analysis was confined to the study from the Netherlands because this study had screened the entire coding region. Two major LD blocks were identified (**Figure 2**). Block 1 comprised of three SNPs (IVS1, V62I and insT). Haplotype analysis of this block revealed two haplotypes with frequency > 1%, which were both significantly associated with AMD (**Table 3a**). The global *P*-value for association was $1.45*10^{-7}$. Haplotype $H_A$ consisted of all major alleles, and was more frequent in cases (86% in cases *vs* 72% in controls; $P = 4.24*10^{-7}$). Haplotype $H_B$ consisted of all minor alleles, and was more frequent in controls (12% in cases *vs* 27% in controls, $P = 3.76*10^{-8}$). Using conditional regression analysis to evaluate models with two SNPs, we found that when V62I was included, insT showed significant evidence for association (**Table 3b**). The second haplotype block consisted of six SNPs: Y402H, A473A, IVS10, IVS12, Q672Q and IVS14. Haplotype analysis of this block revealed five haplotypes with frequency > 1% (**Table 4a**). The global *P*-value for association was $2.50*10^{-5}$. Individual haplotype analysis revealed a significant association between the H1 and H2 haplotypes and AMD. H1 contained the minor alleles of A473A, IVS10, and IVS14, and was most frequent in the control group (25% in cases vs 39% in controls, $P$ $6.99*10^{-6}$). H2 contained the minor allele of Y402H and major alleles of all other SNPs, and was more frequent in cases (53% in cases *vs* 37% in controls, $P$ $1.08*10^{-6}$). **Table 4b** shows the results of the conditional regression analyses which evaluated models with two SNPs. When Y402H was included in the model, A473A still showed significant evidence for association. In contrast, adding IVS14 did not show a significant improvement of the model.

**Figure 2:** Haplotype block structure of the *CFH* gene



Haploview plot depicting the haplotype block structure of the *CFH* gene. Regions of LD were defined based on the confidence interval method that is integrated in the program and were estimated in 543 individuals (360 AMD cases and 183 controls). The figure represents the pairwise values of D' (upper panel) and $r^2$ (lower panel).

**TABLE 3:** LD BLOCK1

a. Haplotype analysis

| | IVS1 | V62I | insT | Hap freq in controls (n=178) | Hap freq in cases (n=344) | *P* |
|---|---|---|---|---|---|---|
| $H_A$ | 1 | 1 | 1 | 0.724 | 0.859 | $4.24^{E-7}$ |
| $H_B$ | 2 | 2 | 2 | 0.270 | 0.120 | $3.76^{E-8}$ |

1 major allele; 2 minor allele

b. Conditional regression analysis

| | Conditional on V62I |
|---|---|
| **IVS1** | *P* = 0.36 |
| **V62I** | - |
| **InsT** | $P = 2.27^{E-4}$ |

**TABLE 4:** LD BLOCK2

a. Haplotype analysis

| | Y402H | A473A | IVS10 | IVS12 | Q672Q | IVS14 | Hap freq in controls (n=183) | Hap freq in cases (n=380) | *P* |
|---|---|---|---|---|---|---|---|---|---|
| H1 | 1 | 2 | 2 | 1 | 1 | 2 | 0.387 | 0.251 | $6.99^{E-6}$ |
| H2 | 2 | 1 | 1 | 1 | 1 | 1 | 0.367 | 0.527 | $1.08^{E-6}$ |
| H3 | 1 | 1 | 2 | 1 | 2 | 1 | 0.088 | 0.109 | 0.597 |
| H4 | 1 | 1 | 2 | 1 | 1 | 1 | 0.069 | 0.044 | 0.591 |
| H5 | 1 | 1 | 1 | 1 | 1 | 1 | 0.038 | 0.043 | 0.453 |

1 major allele; 2 minor allele

b. Conditional regression analysis

| | Conditional on Y402H |
|---|---|
| **Y402H** | - |
| **A473A** | $P = 3.12^{E-3}$ |
| **IVS10** | *P* = 0.60 |
| **IVS12** | *P* = 0.09 |
| **Q672Q** | *P* = 0.10 |
| **IVS14** | *P* = 0.07 |

To investigate whether the two LD blocks had independent value on the risk of AMD, we perfored a haplotype analysis combining the SNPs in both LD blocks (**Table 5**). Haplotype combination $H_A$-$H_2$ was more significantly associated than combination $H_A$-$H_1$ ($P = 9.78*10^{-7}$ *vs.* $P = 8.61*10^{-2}$),

Similarly, $H_B$-$H_1$ was more significantly associated than $H_A$-$H_1$. In addition, conditional regression analysis showed that adding V62I to a model containing Y402H significantly improved the model. Thus, both LD blocks appear to have an independent risk of AMD. Moreover, adding SNPs located outside both LD blocks, in particular IVS18, to a model containing V62I and Y402H significantly improved the model and still showed significant evidence for association. This signifies that these blocks do not fully describe the risk between the *CFH* gene and AMD.

**Table 5:** Combination of block1 and block2

| | Block1 | Block2 | Hap freq in controls (n=183) | Hap freq in cases (n=380) | *P* |
|---|---|---|---|---|---|
| $H_A$-$H_2$ | 1 1 1 | 2 1 1 1 1 1 | 0.367 | 0.521 | $9.78^{E-7}$ |
| $H_B$-$H_1$ | 2 2 2 | 1 2 2 1 1 2 | 0.217 | 0.103 | $4.71^{E-6}$ |
| $H_B$-$H_3$ | 2 2 2 | 1 1 2 1 2 1 | 0.022 | 0.004 | $8.35^{E-3}$ |
| $H_A$-$H_1$ | 1 1 1 | 1 2 2 1 1 2 | 0.162 | 0.125 | $8.61^{E-2}$ |
| $H_A$-$H_3$ | 1 1 1 | 1 1 2 1 2 1 | 0.072 | 0.105 | 0.317 |
| $H_A$-$H_5$ | 1 1 1 | 1 1 1 1 1 1 | 0.037 | 0.043 | 0.652 |
| $H_A$-$H_4$ | 1 1 1 | 1 1 2 1 1 1 | 0.064 | 0.049 | 0.782 |

1 major allele; 2 minor allele

## DISCUSSION

Many variants in the *CFH* gene have been related to AMD, and which SNP is most associated varies considerably among studies. This is partly due to the extensive LD in the cluster of complement genes that harbours *CFH* (RCA locus), and it hampers identification of the variants that truly increase or decrease susceptibility to AMD. We focused on this dilemma and performed a comprehensive genetic analysis of the *CFH* coding region.

In the single SNP analysis, we found nine SNPs which were significantly associated with AMD: two non-synonymous SNPs (Y402H, V62I), two synonymous SNPs (A473A, A307A), and 5 SNPs in intronic regions (IVS1, IVS10, IVS14, IVS2, IVS18). Y402H appeared to be the most significantly associated SNP in our study, followed by IVS1 and V62I. The minor allele frequency (MAF) of Y402H was higher in cases (cases 54.1%; controls 37.6%) while the MAF of IVS1 (cases 14.2%; controls 26.9%) and V62I (cases 14.0%; controls 26.9%) was lower in cases. It is noteworthy that

variants V62I, Y402H, and A473A have been shown to increase susceptibility to membranoproliferative glomerulonephritis type II,[40,61] while A473A has also been related to atypical haemolytic uraemic syndrome.[62]

Our findings confirm observations from the Caucasian AMD studies,[40,58,63] which all show that Y402H is the most prominent non-synonymous SNP in the coding region. However, in the German cohort as well as in several other studies, synonymous and intronic SNPs had higher associations than Y402H.[40,58] This variation in ranking order is likely to result from the divergence in genetic background. Comparison to Asian studies demonstrates even larger differences in the hierarchy of SNPs.[64-66] In particular, V62I was ranked higher than Y402H in Japanese, Chinese, and Korean populations, but also had a much higher population frequency, with rates varying from 24–29% in cases to 38–47% in controls. In contrast, Y402H was uncommon in these Asian populations, occurring in ~6–10% of cases versus ~4–7% of controls. Despite the frequency differences, the associations of these SNPs with AMD appeared to be in the same direction.

Given these associations, can we statistically differentiate causal variants from those related only due to LD? To tackle this issue, we first investigated the extent of LD across the gene. We identified two LD blocks, which were both highly associated with AMD. With respect to the first block, the haplotype containing all major alleles of the variants IVS1, V62I and insT increased the risk of AMD, while the haplotype with all minor alleles had a protective effect. The second block comprised of Y402H, A473A, IVS10, IVS12, Q672Q and IVS14, and was predominated by two haplotypes. The haplotype with the minor allele of Y402H and the major alleles of the other variants increased the risk of AMD, while the haplotype with the major allele of Y402H and the minor alleles of A473A, IVS10, and IVS14 conferred a significant protective effect. Our findings are in line with the previous report of Hageman *et al.* We cannot draw any conclusions regarding the haplotype containing IVS6 since we only screened the coding region and did not type IVS6.

The identification of these blocks and haplotypes raises several questions: (1) Can the association of each block be explained by one single variant? Variants within each block were not exchangeable with respect to risk of AMD. In the first block, the variants V62I and insT independently contributed to the risk of AMD. Likewise, in the second block, adding A473A to a model containing only Y402H significantly improved the goodness-of-fit. Thus, the answer to this question is negative. (2) Do the blocks have an independent

effect on the risk of AMD? Haplotypes of the first block determined association with AMD independent of the second block. This is in line with previous studies showing an additional association for variants located in the first LD block.[40,67,68] The answer to this question is affirmative. (3) Do variants located outside these blocks contribute to AMD? The conditional regression analyses showed that the IVS18 variant significantly improved a model containing SNPs of either block. Similarly, Li *et al.* found that adding variants outside their LD blocks improved prediction of AMD.[58] Hence, this question can be answered positive as well. Whereas the LD in the *CFH* gene is high, it is clear that multiple SNPs appear to influence the risk of AMD in an independent manner. This may suggest that other independently associated variants on the tagged haplotypes differentiate more appropriately between the risk and non-risk individuals than Y402H. In this light, neighboring copy number variations and/or the previously described deletion of *CFHR1* and *CFHR3*[69,70] could be the true causal variants.

CFH is an important regulator of the alternative and common pathway of the complement system. It acts as a cofactor in the proteolysis of C3b by factor I, resulting in an inactive C3b molecule and inhibition of the cascade. This cofactor activity is controlled by other molecules, such as C-reactive protein (CRP), heparin and sialic acid (**Figure 1**).[44] The CFH protein is ubiquitously expressed, and most abundant in the liver. In the eye, it has been detected in the retina, RPE/choroid complex, lens, sclera, and ciliary body. Recently, functional studies in mice showed that *CFH* knock-out animals had reduced rod responses, increased autofluorescent subretinal deposits, accumulation of complement C3 in the neuroretina, thinning of Bruch's membrane, and disorganization of photoreceptor outer segments. Although other mice models have displayed more typical hallmarks of AMD,[34] these results imply that the CFH protein is necessary for maintenance of normal retinal physiology. Regarding the effect of individual variants, a recent in vitro study on Y402H showed that this variant reduced the binding to CRP, heparin, and RPE cells.[71] This effect may jeopardize the negative feedback mechanism, and result in uncontrolled progression of the complement cascade. Unfortunately, functional data on other AMD risk variants are not yet available.

In conclusion, although the initially proposed Y402H variant was the most significant SNP, our study clearly demonstrates that multiple variants in the *CFH* gene carry an independent risk of AMD. Comparison of large study populations with different ethnic origin should further disentangle

individual allelic effects. Functional studies on expression profiles and binding properties of the altered gene product will merit insight into the consequences of AMD risk haplotypes.

# 3. *COMPLEMENT COMPONENT C3* AND RISK OF AGE-RELATED MACULAR DEGENERATION

## ABSTRACT

**Objective:** To explore the association between polymorphisms in the *Complement Component 3* (*C3*) gene and age-related macular degeneration (AMD), and to investigate the modifying effect of *CFH* Y402H, *LOC387715* A69S and smoking. **Design and participants:** Pooled data from the prospective, population-based Rotterdam Study (enrolment between 1990 and 1993, and 3 follow-up examinations between September 1, 1993, and December 31, 2004; N = 6418) and an independent case-control study from the Netherlands (357 cases; 173 controls). **Main outcome measures:** Early and late stages of prevalent and incident AMD, graded according to the International Classification and Grading System for AMD. **Methods:** The variants R102G and P314L of the *C3* gene, *CFH* Y402H and *LOC387715* A69S were genotyped in all study participants. Information on cigarette smoking was obtained by interview at baseline. **Results and conclusions:** We found a population frequency of 0.217 for R102G and 0.211 for P314L in the Rotterdam Study. Both alleles significantly increased the risk of early AMD and all subtypes of late AMD, and this risk appeared independent of *CFH* Y402H, *LOC387715* A69S, and smoking. Detailed analysis showed that the haplotype carrying both alleles had the highest frequency difference between cases and controls ($P$ = 0.006). We estimated a total population-attributable risk of 14.6%. Meta-analysis on all currently available data yielded a pooled odds ratio (OR) 1.61 (95%CI 1.46–1.78) for the R102G allele, and OR 1.50 (95%CI 1.31–1.71) for the P314L allele. These findings further highlight the crucial role of the complement pathway in the etiology of AMD.

## INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of severe visual impairment in industrialized countries. The early stages, characterized by subretinal deposits (drusen) and pigment changes, affect 15.4% of those aged 65 years and older, while the late stages, *i.e.*, subretinal neovascularization (wet AMD) and atrophy of the retinal pigment epithelium (dry AMD), occur in 3.3% of those individuals.[8] The prevailing view on AMD etiology has been that the disease is genetically complex with family history, race, smoking, and dietary factors as important risk factors.

Dissection of the genetic background of AMD has undergone tremendous progress the last two years. Different polymorphisms in at least five genes are currently known to be highly associated with the disease, and appear to explain more than 50% of all cases.[37-40,55-57,72 73] Identification of these genes has significantly enhanced our understanding of the disease pathogenesis. Discovery of genetic risk factors in components of the complement pathway, *i.e.*, *complement factor H (CFH), factor B (FB), and complement component 2 (C2)*, together with the finding that drusen contain complement components, regulators and immunoglobulins,[32] point to the important role of local inflammation and activation of the complement system in the pathogenesis of AMD. Revelation of two variants in the *HTRA1/LOC387715* region launched the hypothesis that regulation of transforming growth factor TGF-ß, a pleiotropic cytokine with a key role in inflammation, is involved in neovascular AMD.[74-76]

The central element of the complement cascade, complement component C3 (C3), has been a plausible candidate since its cleavage product C3a was found in drusen.[32,77] The demonstration that C3a can induce vascular endothelial growth factor (VEGF) expression and promote choroidal neovascularization in both *in vitro* as well as *in vivo* models of AMD provided additional clues.[78] Two recent studies suggested that genetic variants in the *C3* gene may alter the risk of AMD.[79,80] We aimed to explore this association in a population-based study and an independent case-control study from the Netherlands. We assessed the population frequency of the *C3* variants rs2230199 (R102G) and rs1047286 (P314L), calculated the risk for early and late AMD, determined whether the association varied among types of late AMD, and studied interaction with other risk factors. To evaluate the magnitude of the genetic association in a larger context, we performed a meta-analysis on the currently available data.

## MATERIALS AND METHODS

### Study populations

*Population-based study*

The Rotterdam Study is a prospective cohort study aimed at investigation of chronic diseases in the elderly. All inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands, were invited to participate in the study.[45,81] Of the initial cohort of 10,275 eligible individuals, 7,983 (78%) participated (98% Caucasian). The ophthalmologic part of the study became operational after the pilot phase of the study had started and consisted of 9,774 eligible individuals, of whom 7,598 (78%) participated. Baseline examinations took place from 1990 to 1993; three follow-up examinations were performed in 1993–1994, 1997–1999, and 2000–2005.[55] At baseline, 6,418 participants had gradable fundus photographs; 5,771 of these had a successful assessment of rs2230199, and 5,717 had a successful assessment of rs1047286.

*Case-control study*

This study consisted of 357 unrelated AMD patients and 173 control individuals. Subjects were all Caucasian and recruited from the Erasmus University Medical Centre Rotterdam and the Netherlands Institute of Neuroscience Amsterdam, through newsletters, via patient organizations, and nursing home visits. Controls were aged 65 years and older, and were mostly unaffected spouses or non-related acquaintances of cases, or individuals who attended the ophthalmology department for reasons other than retinal pathology.

The study was approved by the Ethics Committees of Erasmus Medical Center and Academic Medical Centre Amsterdam, and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for AMD research.

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes. All study participants were genotyped with the Taqman assay (Applied Biosystems, foster City, California, USA) for rs2230199 (R102G) and rs1047286 (P314L) in the *C3* gene. Rs1061170 (Y402H) in the *CFH* gene and rs10490924

(A69S) in the *LOC387715* gene were analyzed with the Taqman assay in the Rotterdam Study, and with denaturing high-performance liquid chromatography (DHPLC) in the case-control study (Wave; Transgenomic, Santa Clara, California, USA). Variants on DHPLC were graded by two researchers, and subsequently identified by direct sequencing using the ABI-310 (Applied Biosystems, Foster City, California, USA).

**Smoking**

Information on cigarette smoking was obtained by interview, and categorized as never, former, and current smoking.

**Diagnosis of AMD**

All participants underwent fundus photography after pharmacologic mydriasis. Fundus transparencies of both studies were graded according to a modification of the International Classification and Grading System for AMD by the same well-trained graders under the supervision of senior retinal specialists (PTVMdJ, JRV, CCWK). AMD was categorized into early and late AMD according to methods described earlier.[5,6] In short, early AMD (stage 2 and 3) was defined as the presence of either soft distinct drusen with pigmentary irregularities, or soft indistinct drusen with or without pigmentary irregularities; and late AMD (stage 4) as geographic atrophy (dry AMD), neovascular AMD (wet AMD), or mixed AMD (wet AMD in one eye and dry AMD in the other eye, or both types in one eye). Persons were classified based on the eye with the more severe diagnosis. Control persons had no AMD (stage 0: no or only small hard drusen) in either eye, and no other macular pathology.

In the Rotterdam study, incident cases were defined as the absence of AMD in both eyes at baseline and its first appearance in at least 1 eye at follow-up. Unaffected participants remained in stage 0 throughout the follow-up period.

**Statistical analysis**

Characteristics of participants were compared among those affected and non-affected with analysis of covariance for continuous variables, and with logistic regression analysis for discrete variables adjusting for age and sex. Hardy-Weinberg equilibrium of the *C3*, *CFH* and *LOC387715* genotype distributions were tested using a $\chi^2$ test.

Associations were initially analyzed for each study separately. In the Rotterdam Study, odds ratios for prevalent AMD were estimated with logistic regression analyses, and relative risks for incident AMD were estimated with Cox proportional hazards analyses. In the case-control study, odds ratios were estimated with logistic regression analysis. We performed subsequent risk analyses on the pooled data using dummy variables for the studies to assess heterogeneity across study populations. All analyses were adjusted for age and sex. Haplotypes were based on the combination of the two C3 variants, and were estimated using the expectation-maximization algorithm. The risk of AMD for each haplotype was determined with HAPLO.STATS 1.3.0 (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm). Effect modification was determined for smoking, *CFH Y402H,* and *LOC387715 A69S* using late AMD (stage 4) and no AMD (stage 0) as disease outcomes. Association analyses were initially performed on separate data sets (prevalent AMD Rotterdam Study, incident AMD Rotterdam Study; case-control study), and subsequently on the pooled set. Statistical significance for biological interaction was determined by calculating the synergy index (SI), which measures deviation from additivity of two risk factors, and is based on the ratio of the combined effect to the sum of the separate effects.[48,55,82]

Meta-analysis was performed using Review Manager, version 4.2 (Cochrane Collaboration, Oxford, UK). ORs and 95% confidence intervals (CI) were calculated using the random-effects model of the DerSimonian and Laird method.[83] The population attributable risk (PAR) was calculated according to the formula: PAR=(relative risk −1/ relative risk) * proportion of exposed. Relative risk of late AMD in this formula was estimated by the OR. The proportion exposed was the proportion of participants with late AMD carrying the *C3* allele.

## RESULTS

In the Rotterdam Study, we identified 476 of 6418 persons with early AMD, and 106 with late AMD at baseline. During follow-up (mean 7.85 years, median 10.31 years), we identified 586 (15.0%) of 3897 persons who had progressed to early AMD, 99 persons (2.5%) who had progressed to late AMD, and 2078 (53.3%) who had remained in stage 0. The case-control study consisted of 357 AMD patients and 173 control individuals. Baseline characteristics of the study participants are shown in **Table 1**.

The distributions of age, smoking, *CFH Y402H*, and *LOC387715 A69S* were significantly different between cases and controls. Genotype frequencies of the *C3* polymorphisms were in Hardy Weinberg equilibrium in both studies.

**Table 1:** Baseline characteristics of the study populations

| | | The Rotterdam study | | |
|---|---|---|---|---|
| | | **No AMD (N = 4055)** | **Early AMD (N = 476)** | **Late AMD (N = 106)** |
| Age, mean (sd), yrs | | | | |
| Age, No (%) | | 67.52 (8.31) | 75.05(8.70)$ | 81.99(8.20)$ |
| | < 65 yrs | 1814(44.7) | 65(13.7)$ | 4(3.8)$ |
| | 65-74 yrs | 1451(35.8) | 172(36.1)$ | 17(16.0)* |
| | 75-84 yrs | 657(16.2) | 175(36.8)$ | 46(43.4)$ |
| | ≥ 85 yrs | 133(3.3) | 64(13.4)$ | 39(36.8)$ |
| Women, No (%) | | 2358(58.2) | 285(59.9) | 70(66.0) |
| Smoking status, No/Total (%) | | | | |
| | Never | 1327/4008(33.1) | 182/465(39.1) | 40/102(39.2)$ |
| | Past | 1759/4008(43.9) | 187/465(40.2) | 31/102(30.4) |
| | Current | 922/4008(23.0) | 96/465(20.6) | 31/102(30.4)$ |
| CFH Y402H, minor allele frequency | | 0.342 | 0.485$ | 0.622$ |
| LOC387715 A69S, minor allele frequency | | 0.188 | 0.276$ | 0.378$ |

| | | The Case-control study | | |
|---|---|---|---|---|
| | | **No AMD (N = 173)** | **Early AMD (N = 89)** | **Late AMD (N = 268)** |
| Age, mean (sd), yrs | | | | |
| Age, No (%) | | 74.11(6.34) | 76.50(7.21)* | 78.73(7.69)$ |
| | < 65 yrs | 5(2.9) | 4(4.5)* | 15(5.6)$ |
| | 65-74 yrs | 98(56.6) | 33(37.1) | 55(20.5)* |
| | 75-84 yrs | 61(35.3) | 41(46.1) | 143(53.4) |
| | ≥ 85 yrs | 9(5.2) | 11(12.4) | 55(20.5) |
| Women, No (%) | | 90(52.0) | 60(67.4)* | 154(57.5) |
| Smoking status, No/Total (%) | | | | |
| | Never | 54/138(39.1) | 28/83(33.7) | 72/232(31.0)* |
| | Past | 68/138(49.3) | 46/83(55.4)* | 116/232(50.0)* |
| | Current | 16/138(11.6) | 9/83(10.8) | 44/232(19.0)* |
| CFH Y402H, minor allele frequency | | 0.383 | 0.575$ | 0.530$ |
| LOC387715 A69S, minor allele frequency | | 0.186 | 0.388$ | 0.405$ |

AMD = age-related macular degeneration; Data are unadjusted mean ± SD for continuous variables and

percentages for dichotomous variables; *$P$ < 0.05 compared to participants with no AMD; $^{\$}P$ < 0.001 compared to participants with no AMD.

**TABLE 2:** RISK OF AMD FOR *C3* GENOTYPES*

| | No AMD (n=2605) | Early AMD (n=989) | | Late AMD (n=417) | | GA (n=117) | | NMD (n=225) | | MIX (n=75) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **R102G** | | | | | | | | | | | |
| Noncarrier | 1652(63.4) | 572(57.8) | 1.00 | 236(56.6) | 1.00 | 65(55.6) | 1.00 | 133(59.1) | 1.00 | 38(50.7) | 1.00 |
| Heterozygous | 835(32.1) | 365(36.9) | **1.27(1.09-1.49)** | 150(36.0) | **1.46(1.11-1.92)** | 44(37.6) | 1.52(0.99-2.33) | 77(34.2) | **1.54(1.07-2.22)** | 29(38.7) | **1.77(1.03-3.02)** |
| Homozygous | 118(4.5) | 52(5.3) | 1.27(0.90-1.78) | 31(7.4) | **1.93(1.14-3.28)** | 8(6.8) | 2.00 (0.89-4.50) | 15(6.7) | 1.63(0.79-3.35) | 8(10.7) | **3.32(1.38-7.98)** |
| Allele freq | 0.206 | 0.237 | | 0.254 | | 0.256 | | 0.238 | | 0.300 | |

| | No AMD (n=2585) | Early AMD (n=975) | | Late AMD (n=419) | | GA (n=119) | | NMD (n=225) | | MIX (n=75) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **P314L** | | | | | | | | | | | |
| Noncarrier | 1650(63.8) | 569(58.4) | 1.00 | 225(53.7) | 1.00 | 62(52.1) | 1.00 | 125(55.6) | 1.00 | 38(50.7) | 1.00 |
| Heterozygous | 819(31.7) | 359(36.8) | **1.28(1.09-1.49)** | 163(38.9) | **1.49(1.14-1.95)** | 48(40.3) | **1.57(1.03-2.39)** | 85(37.8) | **1.55(1.08-2.23)** | 30(40.0) | **1.70(1.01-2.87)** |
| Homozygous | 116(4.5) | 47(4.8) | 1.15(0.81-1.64) | 31(7.4) | 1.58(0.92-2.71) | 9(7.6) | 1.73(0.77-3.89) | 15(6.7) | 1.29(0.62-2.69) | 7(9.3) | 2.28(0.91-5.72) |
| Allele freq | 0.203 | 0.232 | | 0.268 | | 0.277 | | 0.256 | | 0.293 | |

Abbreviations: AMD, age-related macular degeneration; OR, odds ratio; GA, geographic atrophy; NMD, neovascular macular degeneration; MIX, mixed type of late AMD

* Pooled data from the Rotterdam Study (prevalent and incident cases) and the case-control study.

The disease in each person was classified according to the highest stage of AMD in either eye. Controls were defined as those who were diagnosed with stage 0 and no other macular pathology in both eyes. Early AMD was defined as stage 2 or stage 3 AMD. Late AMD was defined as stage 4 AMD in the eye with the more severe stage.

The ORs are estimates of the relative risk of AMD, and represent the risk of disease (AMD vs stage 0) in the genetic risk group divided by the risk of disease (AMD *vs* stage 0) in the non-risk group (noncarriers).

[a] adjusted for sex, age

The risks of AMD for the R102G and P314L genotypes are summarized in **Table 2**. We found a significant association between these polymorphisms and AMD in both studies (see online **supplemental material**). The pooled data set showed a higher risk of AMD for carriers, and risks increased with severity of AMD to odds ratio (OR) 1.93 (95%CI 1.14–3.28) of late AMD for R102G, and to OR 1.58 (95%CI 0.92–2.71) of late AMD for P314L. Detailed analysis of late AMD revealed that cases with mixed AMD had higher risks than those with only geographic atrophy or neovascular AMD.

The two C3 variants were in high linkage disequilibrium (D' = 0.90, $r^2$ = 0.80) and the combination of both variants yielded four haplotypes: H1 comprised the major alleles of both variants; H2 the major allele of R102G and the minor allele of P314L; H3 both minor alleles; and H4 the minor allele of R102G and the major allele of P314L. The global *P*-value for association was $4.94*10^{-10}$. H1, H3 and H4 were significantly associated with AMD (**Table 3**). The association with H1 was protective, while those with the H3 and H4 haplotypes were associated with an increased risk of AMD. The OR of late AMD was 1.25 (95%CI 1.03–1.52) for those with both minor alleles (H3) compared to those with both major alleles (H1). The haplotype H4 was only present in cases.

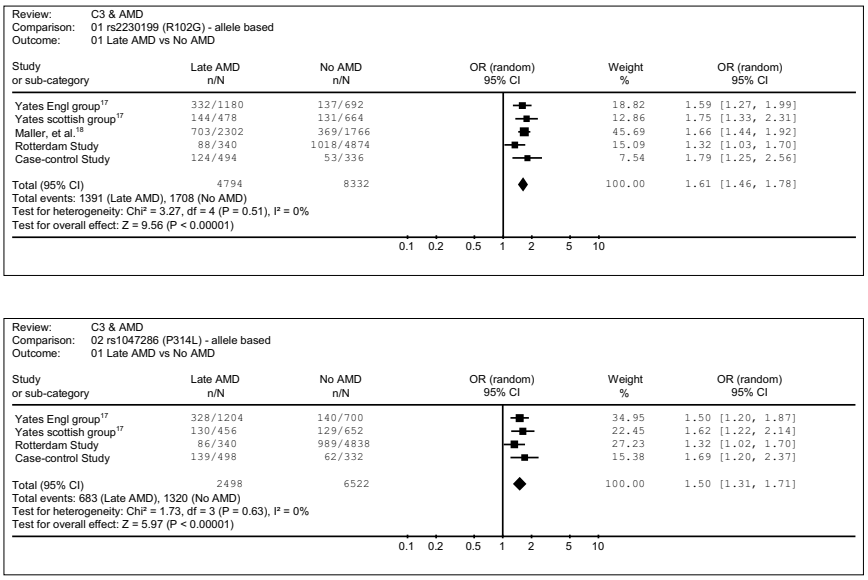
**TABLE 3:** HAPLOTYPE ANALYSES OF R102G AND P314L

| | R102G | P314L | Freq in cases | Freq in controls | *P*-value |
|---|---|---|---|---|---|
| H1 | 1 | 1 | 0.643 | 0.696 | **0.002** |
| H2 | 1 | 2 | 0.101 | 0.097 | 0.448 |
| H3 | 2 | 2 | 0.245 | 0.207 | **0.006** |
| H4 | 2 | 1 | 0.011 | - | **$1.26e^{-10}$** |

* Pooled data from the Rotterdam Study (prevalent and incident cases) and the case-control study.
[1] major allele; [2] minor allele

We did not find significant synergy indices for *CFH Y402H*, *LOC387715 A69S*, and smoking, implying that these factors do not modify the relationship between C3 and AMD. The meta-analysis yielded a pooled OR of 1.61 (95%CI 1.46–1.78) for R102G, and a pooled OR of 1.50 (95%CI 1.31–1.71) for P314L. (**Figure 1**) The PAR of late AMD was 9.7% for those carrying the R102G allele, and 4.9% for those carrying P314L but not R102G, resulting in a total PAR of 14.6%.

**Figure 1:** Meta-analysis of all currently available studies investigating *C3* variants and risk of AMD







## DISCUSSION

Our results show that variants in the C3 gene, R102G and P314L, are significantly associated with AMD. These SNPs were implicated in early as well as late AMD, and had an almost two times increased risk of the latter stage. Stratification of AMD subtypes indicated that the risk was not confined to geographic atrophy or subretinal neovascularization, but was most prominent in those with a mix of both subtypes. The effect of the C3 alleles was independent from the established genetic and environmental risk factors *CFH* Y402H, *LOC387715* A69S, and smoking.

This investigation was performed in a prospective population-based study as well as an independent case-control study from the Netherlands. We found a positive association in both settings, although we detected slightly higher odds ratios in the case-control study. However, the relationship sustained in a setting based on an unselected group of participants from the general population. This indicates that the association is likely to be true and not the result of ascertainment bias, a frequently encountered problem in case-control studies.

Our results confirm the findings of two recently published case-control studies.[79,80] These studies had investigated the association with *C3* by testing multiple SNPs across the gene, and found only evidence for an association with the R102G allele. Yates et al. found that R102G determined the association in the logistic regression analysis, and thus argued that P314L did not carry additional risk. We investigated the independent effects of these alleles by haplotype analysis. The haplotype (H3) carrying the minor alleles of both variants was much more common than those with only one minor allele, and this haplotype was significantly associated with AMD. The haplotype carrying only P314L (H2) was slightly but not statistically significantly more frequent in cases, which suggests that this SNP is indeed less important for AMD. The haplotype carrying only R102G (H4) was rare, which may signify that this SNP was founded on a background of P314L. This haplotype occurred only in cases.

To create a perspective for the overall magnitude of the association between *C3* and AMD, we performed a meta-analysis on the current studies. Genotype data on R102G were available on a total of 2397 cases and 4166 controls, while data on P314L were available on 1249 cases and 3261 controls. The analysis yielded a significant OR of 1.6 for the R102G allele, and OR 1.5 for P314L. In comparison, the risk of the CFH Y402H allele was 2.5.[84] What is the contribution of the *C3* gene to the occurrence of late AMD? We considered the combined effect of both alleles in the calculation of the PAR and estimated a total PAR of 14.6%, which was somewhat lower than the 22% estimated by Yates et al. When we compared this PAR with the estimate for *CFH* Y402H (55.6%) and *LOC387715* A69S (41.1%,) in our data set, we found that *C3* had a smaller contribution to the total disease occurrence. This results from the lower frequency of the risk allele in the *C3* gene (*C3* 25.4% *vs CFH Y402H* 55.6% and *LOC387715 A69S* 38.9%) and a lesser association.

Complement component C3 plays a pivotal role in the complement cascade and is the most abundant of its proteins. Activation of C3 can be initiated by all three pathways (the classical, lectin, and alternative pathway), and leads to cleavage of C3 into the anaphylatoxin C3a and the major fragment C3b. This fragment subsequently binds foreign structures, and forms a complex with factor B that cleaves C5 (C3bBb, C5 convertase). This complex amplifies the complement response, resulting in the formation of lytic pores in the cell membrane (membrane-attack complex).[85]

The gene for C3 is located on chromosome 19p13.3-13.2 and consists of 41 exons. The sequence deduced from the cDNA encodes for a total of 1,663 amino acids – including a 22-amino acid signal peptide, a 645-amino acid ß chain, and a 992-amino acid $\alpha$ chain – that form 13 functional domains. Native C3 is biologically inactive, but undergoes important structural rearrangements upon cleavage. These conformational changes expose an intramolecular thioester-containing domain (TED) which can bind pathogens, and reveal binding sites for complement components, such as C5, Properdin, Factor H, B and I, complement receptor 1, and membrane co-factor protein.[85,86] R102G and P314L are located in the first ring of macroglobulin domains, which are key elements for correct orientation of the TED.[87] The amino acid changes introduced by the genetic variants may alter the configuration of the macroglobulin ring. In addition, the R102G variant may alter the net charge of the molecule, thereby influencing the position of the TED. The end result of these changes may be altered binding to pathogenic cell surfaces or other complement proteins. It should be noted that the *C3* alleles R102G and P314L are not located in the binding sites for CFH. Similarly, the *CFH* alleles Y402H and IVS14 (rs1410996), which confer a high risk of AMD, are not positioned in the binding sites for C3b. Although the risk alleles may still interact via alteration of the proteins' three-dimensional structure, this supports the statistical evidence that these genes cause AMD in an independent manner.

In summary, our study showed a significant association between variants in the *C3* gene with AMD. These findings further accentuate the key role of the complement pathway in the aetiology of AMD. To help solve the pathogenic puzzle, the focus of future research should be on identification of the functional consequences of the risk alleles.

## SUPPLEMENTAL MATERIAL

### Rotterdam study – prevalent analyses

| | No AMD (n=3687) | Early AMD (n=433) | | Late AMD (n=77) | | GA (n=32) | | NMD (n=28) | | MIX (n=17) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **R102G** | | | | | | | | | | | |
| Noncarrier | 2290(62.1) | 238(55.0) | 1.00 | 42(54.5) | 1.00 | 20(62.5) | 1.00 | 14(50.0) | 1.00 | 8(47.1) | 1.00 |
| Heterozygous | 1221(33.1) | 171(39.5) | **1.34(1.08-1.67)** | 30(39.0) | 1.22(0.74-2.02) | 10(31.3) | 0.88(0.40-1.92) | 13(46.4) | 1.68(0.78-3.63) | 7(41.2) | 1.38(0.46-4.10) |
| Homozygous | 176(4.8) | 24(5.5) | 1.40(0.88-2.24) | 5(6.5) | 1.68(0.62-4.54) | 2(6.3) | 1.41(0.32-6.28) | 1(3.6) | 1.05(0.14-8.19) | 2(11.8) | 4.53(0.87-23.69) |
| Allele freq | 0.213 | 0.253 | | 0.260 | | 0.219 | | 0.268 | | 0.324 | |

| | No AMD (n=3653) | Early AMD (n=426) | | Late AMD (n=77) | | GA (n=32) | | NMD (n=28) | | MIX (n=17) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **P314L** | | | | | | | | | | | |
| Noncarrier | 2298(62.9) | 237(55.6) | 1.00 | 43(55.8) | 1.00 | 20(62.5) | 1.00 | 15(53.6) | 1.00 | 8(47.1) | 1.00 |
| Heterozygous | 1190(32.6) | 165(38.7) | **1.37(1.10-1.71)** | 30(39.0) | 1.23(0.75-2.03) | 10(31.3) | 0.91(0.41-1.98) | 12(42.9) | 1.50(0.69-3.24) | 8(47.1) | 1.66(0.58-4.74) |
| Homozygous | 165(4.5) | 24(5.6) | 1.41(0.88-2.27) | 4(5.2) | 1.33(0.45-3.93) | 2(6.3) | 1.42(0.32-6.34) | 1(3.6) | 0.99(0.13-7.62) | 1(5.9) | 2.26(0.26-19.57) |
| Allele freq | 0.208 | 0.250 | | 0.247 | | 0.219 | | 0.25 | | 0.294 | |

Abbreviations: AMD, age-related macular degeneration; OR, odds ratio
[a] adjusted for sex, age

## Rotterdam study – incident analyses

| | No AMD (n=1894) | Early AMD (n=525) | | Late AMD (n=93) | | GA (n=36) | | NMD (n=41) | | MIX (n=16) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] |
| **R102G** | | | | | | | | | | | |
| Noncarrier | 1208(63.8) | 311(59.2) | 1.00 | 52(55.9) | 1.00 | 19(52.8) | 1.00 | 25(61.0) | 1.00 | 8(50.0) | 1.00 |
| Heterozygous | 602(31.8) | 188(35.8) | 1.16(0.97-1.39) | 34(36.6) | 1.34(0.87-2.07) | 14(38.9) | 1.55(0.78-3.10) | 13(31.7) | 1.09(0.56-2.13) | 7(43.8) | 1.83(0.66-5.07) |
| Homozygous | 84(4.4) | 26(5.0) | 1.07(0.72-1.60) | 7(7.5) | 1.90(0.86-4.19) | 3(8.3) | 2.33(0.69-7.91) | 3(7.3) | 1.78(0.54-5.92) | 1(6.3) | 1.80(0.22-14.46) |
| Allele freq | 0.203 | 0.229 | | 0.258 | | 0.278 | | 0.232 | | 0.281 | |

| | No AMD (n=1876) | Early AMD (n=519) | | Late AMD (n=93) | | GA (n=36) | | NMD (n=41) | | MIX (n=16) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] | N(%) | HR[a] |
| **P314L** | | | | | | | | | | | |
| Noncarrier | 1209(64.4) | 315(60.7) | 1.00 | 52(55.9) | 1.00 | 20(55.6) | 1.00 | 25(61.0) | 1.00 | 7(43.8) | 1.00 |
| Heterozygous | 586(31.2) | 183(35.3) | 1.16(0.96-1.39) | 34(36.6) | 1.45(0.94-2.24) | 13(36.1) | 1.52(0.75-3.08) | 13(31.7) | 1.16(0.59-2.28) | 8(50.0) | 2.67(0.96-7.42) |
| Homozygous | 81(4.3) | 21(4.0) | 0.90(0.58-1.40) | 7(7.5) | 1.93(0.88-4.26) | 3(8.3) | 2.28(0.68-7.71) | 3(7.3) | 1.81(0.55-6.02) | 1(6.3) | 2.06(0.25-16.78) |
| Allele freq | 0.199 | 0.217 | | 0.258 | | 0.264 | | 0.232 | | 0.313 | |

Abbreviations: AMD, age-related macular degeneration; HR, hazard ratio
[a] adjusted for sex, age

## Case-control study

| | Stage 0 (n=168) | Stage 2 +3 (n=4) | | Stage 4 (n=247) | | GA (n=51) | | NMD (n=162) | | MIX (n=34) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **R102G** | | | | | | | | | | | |
| Noncarrier | 123(73.2) | 55(65.5) | 1.00 | 142(57.5) | 1.00 | 28(54.9) | 1.00 | 97(59.9) | 1.00 | 17(50.0) | 1.00 |
| Heterozygous | 37(22.0) | 24(28.6) | 1.47(0.78-2.75) | 86(34.8) | **2.08(1.28-3.37)** | 20(39.2) | **2.56(1.20-5.50)** | 53(32.7) | **1.89(1.13-3.17)** | 13(38.2) | **2.93(1.17-7.37)** |
| Homozygous | 8(4.8) | 5(6.0) | 1.48(0.45-4.92) | 19(7.7) | 2.20(0.90-5.37) | 3(5.9) | 2.46(0.57-10.60) | 12(7.4) | 2.03(0.77-5.30) | 4(11.8) | 3.93(0.96-16.00) |
| Allele freq | 0.158 | 0.202 | | 0.251 | | 0.255 | | 0.238 | | 0.309 | |

| | Stage 0 (n=166) | Stage 2 +3 (n=82) | | Stage 4 (n=249) | | GA (n=53) | | NMD (n=163) | | MIX (n=33) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] | N(%) | OR[a] |
| **P314L** | | | | | | | | | | | |
| Noncarrier | 114(68.7) | 49(59.8) | 1.00 | 130(52.2) | 1.00 | 24(45.3) | 1.00 | 89(54.6) | 1.00 | 17(51.5) | 1.00 |
| Heterozygous | 42(25.3) | 28(34.1) | 1.55(0.85-2.84) | 99(39.8) | **2.06(1.29-3.29)** | 25(47.2) | **2.80(1.34-5.84)** | 62(38.0) | **1.91(1.16-3.15)** | 12(36.4) | 1.84(0.75-4.54) |
| Homozygous | 10(6.0) | 5(6.1) | 1.09(0.34-3.49) | 20(8.0) | 1.54(0.67-3.56) | 4(7.5) | 1.70(0.43-6.72) | 12(7.4) | 1.36(0.54-3.41) | 4(12.1) | 2.43(0.62-9.53) |
| Allele freq | 0.187 | 0.232 | | 0.279 | | 0.311 | | 0.264 | | 0.303 | |

Abbreviations: AMD, age-related macular degeneration; OR, odds ratio
[a] adjusted for sex, age

# 4. COMPREHENSIVE ANALYSIS OF THE CANDIDATE GENES *CCL2, CCR2* AND *TLR4* IN AGE-RELATED MACULAR DEGENERATION

## ABSTRACT

**Purpose:** To determine whether variants in the candidate genes *TLR4, CCL2,* and *CCR2* are associated with age-related macular degeneration (AMD). **Methods:** This study was performed in two independent Caucasian populations that included 357 cases and 173 controls from the Netherlands and 368 cases and 368 controls from the United States. Exon 4 of the *TLR4* gene and the promoter, all exons, and flanking intronic regions of the *CCL2* and *CCR2* genes were analyzed in the Dutch study and common variants were validated in the U.S. study. Quantitative (q)PCR reactions were performed to evaluate expression of these genes in laser-dissected retinal pigment epithelium from 13 donor AMD eyes and 13 controls. **Results:** Analysis of single nucleotide polymorphisms (SNPs) in the *TLR4* gene did not show a significant association between D299G or T399I and AMD, nor did haplotypes containing these variants. Univariate analyses of the SNPs in *CCL2* and *CCR2* did not demonstrate an association with AMD. For *CCR2*, haplotype frequencies were not significantly different between cases and controls. For *CCL2*, one haplotype containing the minor allele of C35C was significantly associated with AMD ($P$ = 0.03), but this did not sustain after adjustment for multiple testing (q = 0.30). Expression analysis did not demonstrate altered RNA expression of *CCL2* and *CCR2* in the retinal pigment epithelium from AMD eyes (for *CCL2* $P$ = 0.62; for *CCR2* $P$ = 0.97). **Conclusions:** No evidence was found of an association between *TLR4*, *CCR2, CCL2,* and AMD, which implies that the common genetic variation in these genes does not play a significant role in the etiology of AMD.

## INTRODUCTION

Accumulating evidence demonstrates that disregulation of the local inflammatory and immunologic response is an important causal pathway in age-related macular degeneration. Initial proof of this insight was provided by histopathology studies which showed that drusen contain complement components, complement regulators, immunoglobulins and anaphylatoxins.[32] Recently, the role of inflammation in AMD was further established by multiple genetic studies. Genes involved in the complement pathway, such as the *complement factor H (CFH)* gene, the *complement factor B (FB)* gene, and the *complement component 2 (C2)* gene have repetitively been associated with AMD.[37-40,55,59,72] The general hypothesis is that dysfunction of these genes may lead to an increase in complement activation and a high release of proinflammatory proteins, which results in an augmentation of the local inflammatory response. It is currently unclear whether inflammatory pathways other than complement regulation are involved in AMD pathogenesis.

The immune system detects and responds to infection mainly through a family of pattern recognition receptors called toll-like receptors (TLRs).[88] These receptors recognize a wide range of microbial molecules (e.g. lipopolysaccharide, peptidoglycan, lipopeptide) and induce phagocytosis after binding. They are expressed by many immune cells, as well as by corneal cells and retinal pigment epithelium (RPE) cells.[89] TLRs trigger a signal transduction cascade that results in activation of transcription factor NF-κB, which leads to increased expression of proinflammatory genes.[90,91] A significant association between a common single nucleotide polymorphism (SNP; rs4986790, D299G) in the *TLR4* gene and AMD was reported by Zareparsi *et al*.[92] This genetic variant alters the extracellular domain of the receptor, which interrupts the signaling transduction cascade,[93] and interferes with the expression of genes such as *TNF-α, IL-1, IL-6, monocyte chemo-attractant protein (MCP-1or CCL2)* and its cognate receptor *C-C chemokine receptor-2 (CCR2)*.[94] Although biologically plausible, the reported association between *TLR4* and AMD awaits confirmation.[95]

CCL2 and CCR2 are key mediators in the infiltration of monocytes from blood into foci of inflammation. The CCL2 protein is ubiquitously expressed and exerts its effect after binding to its receptor CCR2, which leads to actin rearrangement, shape change, and movement of monocytes.[96] Evidence of a potential role of CCL2 and CCR2 in AMD was provided by Ambati *et al*.,[18]

who showed that aging mice deficient of these genes developed hallmarks of AMD (*i.e.*, accumulation of drusen and lipofuscin, photoreceptor atrophy, and choroidal neovascularization).[34] Similar to human AMD, complement-associated proteins as C5, IgG, vitronectin, CD46, and serum amyloid P component were also present in the RPE of these mice. The occurrence of AMD-like disease in these knockout mice raises the question of whether CCL2 and CCR2 play a role in human AMD.

In this study, we assessed the association with the D299G allele of *TLR4* in independent case-control studies from the Netherlands and the United States. Furthermore, we performed a comprehensive genetic analysis of the *CCL2* and *CCR2* genes in the Dutch study and validated common variants of these genes in the U.S. study. We also performed quantitative (q)PCR experiments to investigate whether mRNA expression of these genes in the retinal pigment epithelium was different between individuals with AMD and healthy control subjects.

## METHODS

### Study population

This study consisted of two independent populations of AMD cases and age-matched control subjects. The first set consisted of 357 unrelated patients with AMD and 173 unrelated control individuals from the Netherlands. Subjects were all Caucasian and were recruited from the Netherlands Institute of Neuroscience Amsterdam, and Erasmus University Medical Center Rotterdam, and through newsletters and patient organizations. Controls were 65 years of age and older and were mostly unaffected spouses or non-related acquaintances of cases or individuals who attended the ophthalmology department for reasons other than retinal disease.

The second set consisted of 368 unrelated individuals with AMD and 368 unrelated controls of American-European descent recruited at Columbia University as previously described.[40] Cases and controls of both studies were examined by trained ophthalmologists before diagnosis (described later).

The study was approved by the Ethics Committee of Academic Medical Centre Amsterdam, and the Institutional Review Board of Columbia University and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, and for

the publication of the data obtained, retrieval of medical records, and use of blood and DNA for AMD research.

**Diagnosis of AMD**

All participants underwent fundus photography after pharmacologic mydriasis. Fundus transparencies were subsequently graded according to a modification of the International Classification and Grading System for AMD under the supervision of senior retinal specialists (CCWK, PTVMdJ, IAB, RTS, GRB).[5,6] Grading criteria were identical for both studies. Cases were stratified according to the eye with the most severe disease: early AMD (soft indistinct drusen with or without pigmentary changes, or soft distinct drusen with pigmentary changes, *i.e.*, stages 2 and 3), or end-stage AMD (stage 4). The latter was subclassified into geographic atrophy, neovascular macular degeneration, or a mixed type of end-stage AMD. Controls had or only a few small hard drusen and no other macular disease (stage 0) in both eyes.

**Genotyping**

DNA was extracted from peripheral blood leukocytes after venous puncture. Exon 4 of the *TLR4* gene, as well as the promoter region, all exons and flanking intronic regions of *CCR2* and *CCL2,* and exon 9 of *CFH* were amplified by PCR. In the Dutch study, the samples were analyzed for sequence variations using denaturing high-performance liquid chromatography (DHPLC) on an automated system (Wave; Transgenomic, Santa Clara, CA). For indentification of homozygous variants in amplicons with frequent heterozygous SNPs, aliquots of a known wild-type sample were added to the DNA before the reannealing step. Variants on DHPLC were graded by two researchers and subsequently identified by direct sequencing (model 310;Applied Biosystems,Inc.[ABI], Foster City, CA). Discrepancies between DHPLC grading were also analyzed using direct sequencing.

In the U.S. study, participants were genotyped for common sequence variations in the *CCR2, CCL2* and *TLR4* genes (*Taq*man assay; ABI). Primer sequences are available upon request.

**Human postmortem eyes and evaluation of RNA expression**

Human bulbi from 26 donors were provided by the Corneabank Amsterdam. Histopathology was evaluated on 8-µm sections of the maculae that were stained with the periodic-acid-Schiff reaction. Maculae with drusen and/or

a continuous layer of basal laminar deposit were classified as cases (mean age, 76.31 ± 2.72 [SD] years; *N* = 13); maculae with no disease were classified as age-matched controls (mean age, 75.43 ± 2.07 years; *N* = 7) and young controls (mean age, 24.83 ± 6.91 years; *N* = 6)]. We collected RPE cells from retinal sections using a laser dissection microscope (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) and isolated and amplified RNA according to Agilent protocols (Agilent Technologies, Palo Alto, CA). Amplified RNA (200 ng) was transcribed into cDNA by reverse transcriptase (Superscript III, Invitrogen, Carlsbad, CA). We performed qPCR reactions and detected levels of amplified product by real-time monitoring of SYBR Green I dye fluorescence (Prism 7300; ABI), according to methods described earlier by van Soest, *et al.*[97]

**Statistical analysis**

Baseline characteristics of cases and controls were compared by using analysis of covariance for continuous variables and logistic regression analysis for discrete variables, and were adjusted for age and sex. Genotype distributions were tested for Hardy-Weinberg equilibrium using the $\chi^2$ test. Haploview software (http://www.broad.mit.edu/mpg/haploview/ provided in the public domain by The Broad Institute, Massachusetts Institute of Technology, Cambridge, MA) was used to estimate allele frequencies and allele-based risk of AMD. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CI) for risk of AMD adjusted for age and sex, with major alleles used as the reference. Haplotypes were estimated by using the expectation-maximization algorithm, and the risk of AMD for each haplotype was determined with Haplo.stats 1.2.2 (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm/ provided in the public domain by Mayo Clinic, Rochester, MN). To account for multiple comparisons, we estimated the q statistic to determine the approximate false-discovery rate (FDR), which is defined as the proportion of statistical tests called significant that are actually false positive.[98,99] The q statistic, also known as FDR-adjusted probabilities, was calculated incorporating all probabilities from the 54 tests performed for SNPs and haplotypes in this study. Mean gene expression levels between cases and controls were compared by Mann-Whitney U test and were adjusted for expression of housekeeping genes (RBLP0, CYCLOP, and EF1a) to correct for differences in cDNA load.[100]

## RESULTS

**Table 1** shows the characteristics of cases and controls. Cases were, on average, 4 years older than controls in both studies. The distribution of gender was not significantly different between cases and controls.

**TABLE 1:** BASELINE CHARACTERISTICS OF THE STUDY POPULATION

| | **Study from the Netherlands** | | | **Study from the United States** | | |
|---|---|---|---|---|---|---|
| | **Cases ($n_{tot}$=357)** | **Controls ($n_{tot}$=173)** | ***P*** | **Cases ($n_{tot}$=368)** | **Controls ($n_{tot}$=368)** | ***P*** |
| Diagnosis | | | | | | |
| No AMD | | 173 (100.0) | | | 368 (100.0) | |
| Early AMD | 89 (24.9) | | | - | | |
| Neovascular AMD | 180 (50.4) | | | 276 (75.0) | | |
| Geographic atrophy | 54 (15.1) | | | 92 (25.0) | | |
| Mixed AMD | 34 (9.5) | | | - | | |
| Age, y | 78.2 (7.6) | 74.1 (6.3) | < 0.001 | 78.7 (6.9) | 74.6 (5.8) | < 0.001 |
| <65 | 19 (5.3) | 5 (2.9) | | 18 (4.9) | 28 (7.6) | |
| 65-74 | 88 (24.6) | 98 (56.6) | | 70 (19.0) | 150 (40.8) | |
| 75-84 | 184 (51.5) | 61 (35.3) | | 180 (48.9) | 160 (43.5) | |
| >=85 | 66 (18.5) | 9 (5.2) | | 100 (27.2) | 30 (8.2) | |
| Sex | | | 0.07 | | | 0.06 |
| Men | 143 (40.1) | 83 (48.0) | | 139 (37.8) | 164 (44.6) | |
| Women | 214 (59.9) | 90 (52.0) | | 229 (62.2) | 204 (55.4) | |

Data are unadjusted mean ± SD for continuous variables and percentages for dichotomous variables. $n_{tot}$, total number of participants.

SNP analysis in the *TLR4* gene did not show a significant association with D299G or T399I in the *TLR4* gene and AMD. We identified a previously unknown rare variant (*i.e.*, K354K) in the amplified region of exon 4 **(Table 2)**. Haplotype analysis of the three SNPs in *TLR4* did not convey a risk of AMD. We determined the potential additive effect of the hetero- and homozygous genotypes of D299G and T399I in *TLR4*, and did not find evidence for such an effect (D299G *P* = 0.74; T399I *P* = 0.50). The frequency of D299G was within the same range in the U.S. study, and no significant frequency differences between cases and controls were found. Pooling studies did not alter results **(Table 2)**, nor did adjustment or stratification for the *CFH* Y402H allele (data not provided). Analysis of RNA expression of TLR4 in the

RPE was low and did not reveal any significant differences between cases and controls.

**Table 2:** Frequency of the single nucleotide polymorphisms in the TLR4 gene

a. Study from the Netherlands

| | rs-numbers | Nucleotide | Frequency in | | | |
|---|---|---|---|---|---|---|
| **SNP** | | **Change** | **Cases** | **Controls** | **OR (95%CI)*** | ***P*** |
| *Genotype* | | | | | | |
| D299G | rs4986790 | AA | 0.903 | 0.893 | 1.00 | |
| | | AG | 0.094 | 0.107 | 0.85 (0.45-1.60) | 0.61 |
| | | GG | 0.003 | - | - | |
| K354K | | AA | 0.980 | 0.988 | 1.00 | |
| | | AG | 0.020 | 0.012 | 2.04 (0.39-10.71) | 0.40 |
| | | GG | - | - | - | |
| T399I | rs4986791 | CC | 0.897 | 0.877 | 1.00 | |
| | | CT | 0.100 | 0.123 | 0.72 (0.40-1.32) | 0.29 |
| | | TT | 0.003 | - | - | |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| D299G | rs4986790 | G | 0.050 | 0.053 | 0.05 | 0.83 |
| K354K | | G | 0.010 | 0.006 | 0.47 | 0.49 |
| T399I | rs4986791 | T | 0.050 | 0.061 | 0.57 | 0.45 |

*adjusted for age & sex

b. Study from the United States

| | Rs-numbers | Nucleotide | Frequency in | | | |
|---|---|---|---|---|---|---|
| **SNP** | | **Change** | **Cases** | **Controls** | **OR (95%CI)** | ***P*** |
| *Genotype* | | | | | | |
| D299G | rs4986790 | AA | 0.885 | 0.907 | 1.00 | |
| | | AG | 0.107 | 0.090 | 1.21 (0.74-1.97) | 0.44 |
| | | GG | 0.008 | 0.003 | 3.07 (0.32-29.71) | 0.33 |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| D299G | rs4986790 | G | 0.058 | 0.048 | 1.32 | 0.25 |

c. Both studies combined

| SNP | Rs-numbers | Nucleotide Change | Frequency in Cases | Frequency in Controls | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| D299G | rs4986790 | AA | 0.895 | 0.903 | 1.00 | |
| | | AG | 0.101 | 0.096 | 1.07 (0.73-1.56) | 0.74 |
| | | GG | 0.004 | 0.002 | 2.30 (0.24-22.13) | 0.47 |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| D299G | rs4986790 | G | 0.055 | 0.050 | 0.31 | 0.58 |

In the Netherlands study, we found five different variants in the *CCL2* gene: two localized in the promoter region (-2578 A>G ; -2136 A>T), one intronic variant (IVS1 +50 A>T), one previously described synonymous SNP (C35C), and a newly identified missense variant in exon 3 (A71T). We observed six variants in the *CCR2* gene: two synonymous (V52V; N260N) and three non-synonymous (V64I; R233Q; I318T) substitutions, and one intronic SNP (IVS1 +103G>A). Genotype frequencies of all SNPs were in Hardy-Weinberg equilibrium. No statistically significant association was found between any of the sequence variations in these genes and AMD in the univariate analysis. The frequencies of C35C of *CCL2* and V64I of the *CCR2* gene were within the same range in the U.S. study, and did not show any significant differences between cases and controls. Pooling did not alter these results **(Tables 3 and 4)**, nor did stratification or adjustment for Y402H of *CFH* (data not provided). We generated haplotypes using all identified SNPs in the Dutch study. For *CCR2*, the estimated haplotype frequencies were not significantly different. For *CCL2*, one haplotype containing the minor allele of C35C and the major alleles of all other SNPs was significantly associated with AMD ($P$ = 0.03). This difference did not remain significant after adjustment for multiple testing (q = 0.30; **Table 5).**

**Table 3:** Frequency of the single nucleotide polymorphisms in the CCL2 gene

a. Study from the Netherlands

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Frequency in Controls | OR (95%CI)* | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| -2578 A>G | | AA | 0.569 | 0.519 | 1.00 | |
| | | AG | 0.350 | 0.442 | 0.71 (0.45-1.13) | 0.15 |
| | | GG | 0.081 | 0.039 | 1.96 (0.67-5.73) | 0.22 |
| -2136 A>T | | AA | 0.663 | 0.589 | 1.00 | |
| | | AT | 0.287 | 0.397 | 0.67 (0.43-1.06) | 0.09 |
| | | TT | 0.050 | 0.014 | 3.13 (0.67-14.57) | 0.15 |
| IVS1 +50 A>T | rs28730833 | AA | 0.975 | 0.992 | 1.00 | |
| | | AT | 0.008 | 0.025 | 0.25 (0.04-1.65) | 0.15 |
| | | TT | - | - | - | |
| C35C | rs4586 | TT | 0.388 | 0.451 | 1.00 | |
| | | TC | 0.473 | 0.459 | 1.31 (0.81-2.13) | 0.27 |
| | | CC | 0.139 | 0.090 | 1.67 (0.75-3.73) | 0.21 |
| A71T | | GG | 0.996 | 1.000 | | |
| | | GA | 0.004 | - | - | |
| | | AA | - | - | - | |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| -2578 A>G | | G | 0.256 | 0.221 | 0.059 | 0.81 |
| -2136 A>T | | T | 0.193 | 0.213 | 0.544 | 0.46 |
| IVS1 +50 A>T | rs28730833 | T | 0.004 | 0.012 | 1.704 | 0.19 |
| C35C | rs4586 | C | 0.376 | 0.320 | 2.213 | 0.14 |
| A71T | | A | 0.002 | - | 0.491 | 0.48 |

*adjusted for age & sex

b. Study from the United States

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Controls | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| C35C | rs4586 | TT | 0.343 | 0.367 | 1.00 | |
| | | TC | 0.480 | 0.481 | 1.07 (0.77-1.47) | 0.70 |
| | | CC | 0.177 | 0.152 | 1.24 (0.81-1.92) | 0.32 |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| C35C | rs4586 | C | 0.417 | 0.393 | 0.90 | 0.34 |

c. Both studies combined

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Controls | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| C35C | rs4586 | TT | 0.361 | 0.388 | 1.00 | |
| | | TC | 0.477 | 0.476 | 1.08 (0.83-1.40) | 0.57 |
| | | CC | 0.162 | 0.137 | 1.27 (0.88-1.83) | 0.20 |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| C35C | rs4586 | C | 0.400 | 0.374 | 1.53 | 0.22 |

**Table 4**: Frequency of the single nucleotide polymorphisms in the *CCR2* gene

a. Study from the Netherlands

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Controls | OR (95%CI)* | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| V52V | rs3918367 | GG | 0.980 | 0.975 | 1.00 | |
| | | GT | 0.020 | 0.025 | 0.73 (0.16-3.21) | 0.67 |
| | | TT | - | - | - | |
| V64I | rs1799864 | GG | 0.855 | 0.810 | 1.00 | |
| | | GA | 0.141 | 0.182 | 0.61 (0.33-1.13) | 0.11 |
| | | AA | 0.004 | 0.008 | 0.38 (0.02-6.20) | 0.50 |
| R233Q | | GG | 1.000 | 0.992 | | |
| | | GA | - | 0.008 | - | |
| | | AA | - | - | - | |
| N260N | rs1799865 | TT | 0.453 | 0.492 | 1.00 | |
| | | TC | 0.449 | 0.443 | 1.04 (0.65-1.68) | 0.86 |
| | | CC | 0.097 | 0.066 | 1.47 (0.60-3.61) | 0.40 |
| IVS1 +103 G>A | rs3092960 | GG | 0.992 | 0.992 | 1.00 | |
| | | GA | 0.008 | 0.008 | 1.11 (0.10-12.96) | 0.93 |
| | | AA | - | - | - | |
| I318T | | TT | 0.992 | 1.000 | | |
| | | TC | 0.008 | - | - | |
| | | CC | - | - | - | |
| *Allele* | | | | | $\chi^2$ | ***P*** |
| V52V | rs3918367 | T | 0.010 | 0.012 | 0.08 | 0.78 |
| V64I | rs1799864 | A | 0.153 | 0.099 | 1.26 | 0.26 |
| R233Q | | A | - | 0.004 | 2.00 | 0.16 |
| N260N | rs1799865 | C | 0.322 | 0.287 | 0.93 | 0.33 |
| IVS1 +103 G>A | rs3092960 | A | 0.004 | 0.004 | 0 | 0.99 |
| I318T | | C | 0.004 | - | 1.00 | 0.32 |

*adjusted for age & sex

b. Study from the United States

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Frequency in Controls | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| V64I | rs1799864 | GG | 0.796 | 0.826 | 1.00 | |
| | | GA | 0.183 | 0.166 | 1.14 (0.78-1.67) | 0.50 |
| | | AA | 0.022 | 0.008 | 2.77 (0.43-10.53) | 0.14 |
| *Allele* | | | | | $\chi^2$ | *P* |
| V64I | rs1799864 | A | 0.113 | 0.091 | 1.90 | 0.17 |

c. Both studies combined

| SNP | Rs-number | Nucleotide Change | Frequency in Cases | Frequency in Controls | OR (95%CI) | *P* |
|---|---|---|---|---|---|---|
| *Genotype* | | | | | | |
| V64I | rs1799864 | GG | 0.820 | 0.822 | 1.00 | |
| | | GA | 0.166 | 0.170 | 0.98 (0.71-1.34) | 0.88 |
| | | AA | 0.015 | 0.008 | 1.79 (0.55-5.84) | 0.34 |
| *Allele* | | | | | $\chi^2$ | *P* |
| V64I | rs1799864 | A | 0.097 | 0.093 | 0.11 | 0.74 |

**TABLE 5:** HAPLOTYPE ANALYSES IN THE DUTCH STUDY

a. TLR4 gene

| | D299G | K354K | T399IT | Freq in Cases | Freq in Controls | OR (95%CI)* | *P* |
|---|---|---|---|---|---|---|---|
| H1 | 1 | 1 | 2 | 0.009 | 0.013 | 0.61 (0.16-2.31) | 0.47 |
| H2 | 2 | 1 | 2 | 0.041 | 0.048 | 0.75 (0.38-1.46) | 0.40 |
| H3 | 1 | 1 | 1 | 0.939 | 0.924 | ref | |

*adjusted for age & sex

b. CCL2 gene

| | -2518 A>G | -2076 A>T | IVS1 +50 A>T | C35C | A71T | Freq in Cases | Freq in Controls | OR (95%CI)* | *P* |
|---|---|---|---|---|---|---|---|---|---|
| H1 | 1 | 2 | 1 | 1 | 1 | 0.190 | 0.197 | 1.11 (0.72-1.71) | 0.65 |
| H2 | 2 | 1 | 1 | 2 | 1 | 0.248 | 0.247 | 1.15 (0.76-1.72) | 0.51 |
| H3 | 1 | 1 | 1 | 2 | 1 | 0.125 | 0.072 | 1.99 (1.07-3.73) | 0.03 |
| H4 | 1 | 1 | 1 | 1 | 1 | 0.424 | 0.458 | Ref | |

*adjusted for age & sex

c. CCR2 gene

| | V52V | V64I | R233Q | N260N | IVS1 +103 G>A | I308T | Freq in Cases | Freq in Controls | OR (95%CI)* | *P* |
|---|---|---|---|---|---|---|---|---|---|---|
| H1 | 1 | 2 | 1 | 1 | 1 | 1 | 0.073 | 0.099 | 0.61 (0.34-1.09) | 0.10 |
| H2 | 1 | 1 | 1 | 2 | 1 | 1 | 0.313 | 0.274 | 1.10 (0.75-1.61) | 0.63 |
| H3 | 1 | 1 | 1 | 1 | 1 | 1 | 0.597 | 0.606 | Ref | |

*adjusted for age & sex

Results from the gene expression study did not reveal any significant differences between cases and controls. Gene expression levels of *CCL2* and *CCR2* in the human RPE decreased with age. The expression level of *CCL2* was, on average, 2.6 times lower in the old controls eyes than in young non-AMD eyes *(P* = 0.15 for difference). The expression level of *CCR2* was on average 1.3 times lower (*P* = 0.81 for difference). Expression levels were highly variable in the entire group, and showed no significant differences between the AMD and the old controls (*CCL2*: *P* = 0.62; *CCR2*: *P* = 0.97).

## DISCUSSION

We could not confirm the association between the D299G variant of the *TLR4* gene and AMD in two large, independent case-control studies. In addition, we did not find a significant relationship with genetic variants in the coding region of the *CCR2* and *CCL2* genes. The qPCR experiments did not reveal any significant differences in expression levels in these genes. The lack of positive results implies that these genes do not play an important role in the etiology of AMD.

Strengths of our study include the use of two independent case-control studies, both employing the same method of diagnosis. Although the genetic approach was different, the studies had very similar findings. The Dutch study was designed to detect known and unknown variants by using DHPLC; the U.S. study validated known variants with a genotyping assay (Taqman; Invitrogen). A limitation was that the statistical power to establish significant associations of rare alleles was still relatively low. We detected ORs of at least 1.47 with a power of 80% and a significance level of 0.05 for allele frequencies of 0.20, whereas we were able to detect odds ratios of 1.90 or higher for allele frequencies of 0.05. Therefore, we cannot exclude that infrequent alleles of these genes carry a low risk of AMD.

The association of *TLR4* with AMD was initially proposed by Zareparsi, *et al.*[13] in a study of Caucasians consisting of 667 cases and 439 controls, showing an increased risk for those with the D299G allele (OR = 2.65, 95% CI 1.13–6.25).[92] Kaur, *et al.*[16] could not replicate this finding in a study consisting of 100 cases and 120 controls from India; on the contrary, they found a slightly lower risk of AMD for the haplotype containing D299G.[95] Our Caucasian study from two continents consisted of 725 cases and 541 controls and yielded results in line with those of Kaur et al. The allele frequency of D299G was very similar in both our case groups (5%), which approached the frequency in the cases of Zareparsi *et al.* (6%).[13] However, we found a similar frequency in controls (5%), whereas Zareparsi *et al.* found a frequency of 3% in the control group.

The *CCL2* and *CCR2* genes were initially proposed as candidate genes in animal studies.[34] We analyzed the genetic variation of these genes in all exons and flanking intronic regions in the Dutch study, and validated common variants in the U.S. study. The allele frequencies were very similar in both study populations and were within the same range as reported for other Caucasian populations.[101] Our analyses revealed no significant associations with single SNPs. In particular, we did not find altered risks for the -2518 and -2076 alleles in the promoter of *CCL2*, which are known to increase the risk of coronary artery disease, HIV infection and AIDS dementia.[102,103] We also failed to detect an association with the V64I allele in *CCR2,* which reduces the risk of HIV progression and coronary artery disease.[104,105] Contrary to the univariate analyses, haplotype analysis revealed one statistically significant haplotype in *CCL2*. However, this association did not remain significant after correction for false-discovery rate, suggesting a false- positive finding.

RNA expression of CCL2 and CCR2 in the RPE showed high variation among individuals, but was within the same range in cases and controls. Thus, as opposed to mice, in which deficiency of the *CCL2* or *CCR2* genes leads to a prominent AMD-like phenotype, we did not find evidence for decreased RNA-expression of *CCL2* and *CCR2* in humans with AMD, nor did we find any association with genetic variants. The opposite appears to be true of the *CFH* gene: whereas genetic variations show high association with AMD in humans, *CFH*-deficient mice do not develop a significant AMD phenotype. Taken together, these data suggest a different pathogenesis in mice and humans leading to similar pathologic features. What are possible explanations? First, the sequences of these genes are not fully

identical, which could lead to differences in protein function between mice and humans. Second, biological pathways generally contain many proteins with equivalent function, and this functional redundancy may differ across species.

In summary, the findings in our study do not support a role for common genetic variation in the *TLR4*, *CCL2*, and *CCR2* genes in the etiology of AMD. These results, however, do not exclude the possibility that immune response and/or inflammatory pathways other than the alternative complement cascade are involved in the disease. The broad spectrum of inflammatory proteins found in AMD eyes warrants further research in this domain.

# 5. *ERCC6* AND THE RISK OF AGE-RELATED MACULAR DEGENERATION

## ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment in the developed countries, affecting 4% of the population over the age of 60 years. The onset and progression of the disease is triggered by both environmental and genetic risk factors, such as smoking and genetic variation in *CFH, LOC387715*, *BF*, *C2* and *C3*. Despite recent progress, a large proportion of the genetic origin of AMD remains to be elucidated. One of the additional genes involved in AMD may be *ERCC6*, previously implicated in transcription-coupled DNA repair and Cockayne syndrome. We investigated the association between the polymorphism *ERCC6* rs3793784:C>G (c.-6530C>G) and AMD in two independent Dutch study populations: the population-based Rotterdam Study and an independent case-control study. We also determined *ERCC6* expression levels in retinal pigment epithelium cells of healthy and AMD affected donor eyes. We found that the *ERCC6* promoter SNP rs3793784:C>G confers no significant association in the prevalent analyses of the Rotterdam study and the case control study. A small but significant increase in risk for late AMD was seen in the incident analyses. The pooled data yielded an odds ratio of 1.39 (95%CI 1.02–1.89) for homozygous carriers. Calculation of the synergy index revealed no significant interaction between *ERCC6* and smoking, *CFH* Y402H or *LOC387715* A69S. In contrast with previous findings, we did not measure increased *ERCC6* expression in AMD affected retinal cells. In conclusion, we do not find a consistent relationship between a promoter variant in the *ERCC6* gene and AMD, nor do we find evidence for differential expression of the gene. Larger studies incorporating more variants will be needed to reveal whether *ERCC6* has a true role in the pathogenesis of AMD.

## INTRODUCTION

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in the Western world. The prevalence of AMD rises sharply with age, affecting 4% of the population over the age of 60 and more than 10 % of individuals older than 75.[9,106] AMD affects the macula, the central part of the retina. This specialized area accounts for high visual acuity, which is indispensable for reading, face recognition and moving freely.[107,108] Consequently, the loss of central vision in AMD severely affects the quality of life.

The early stages of AMD are characterized by drusen, which are focal depositions of waste material underneath the retinal pigment epithelium (RPE). This early stage progresses over time into late AMD, which presents itself in two forms: geographic atrophy (GA or dry AMD) and neovascular AMD (wet AMD). GA is characterized by atrophy of RPE and photoreceptors; the essential features of neovascular AMD are pathologic growth of new blood vessels from the choroid into the retina. In either case, a severe loss of central vision ensues.

AMD is a complex disease with environmental as well as genetic determinants. Environmental risk factors include age, smoking, and diet. Genetic risk factors that have been elucidated recently are the *Complement Factor H (CFH)* gene, the *LOC387715* gene, *C2/FB*, and the *C3* gene.[37-40,49,56,57,72,73 79,80] *CFH, C2, FB*, and *C3* are all genes encoding key proteins of the complement cascade, while *LOC387715* is a gene of unknown function which was recently localized to the mitochondrial membrane. Identification of these genes helped to decipher the disease pathogenesis. It is now clear that two major pathways are involved: local inflammation and oxidative stress.[72,79,80]

In the retina, a high concentration of oxygen combined with intense light exposure in the presence of photosensitizers, such as lipofuscin, may easily lead to excessive DNA damage. In this context, the recent paper of Tuo *et al.* is of interest which reports that a SNP (rs3793784) in the promoter region of the *ERCC6* gene is associated with AMD.[109] This gene is important for DNA repair, and loss-of-function mutations in *ERCC6* cause Cockayne syndrome (CS). CS is an autosomal recessive progeroid disorder characterized by severely impaired physical and intellectual development. Among the many clinical features, photosensitivity and retinopathy are hallmarks of CS.[110] Targeted ablation of *ERCC6* in the mouse resulted in a mouse

model for CS showing spontaneous retinal degeneration characterized by a gradual photoreceptor loss.[111,112] Interestingly, the retina of this mouse is hypersensitive to X rays, which indeed suggests that oxidative DNA damage is involved in CS retinal pathology.

In order to further explore the role of DNA damage in AMD, we examined the association of the *ERCC6* SNP rs3793784 with AMD in two independent study populations in the Netherlands, the population-based prospective Rotterdam Study and a hospital-based case-control study. In addition, we studied interactions with smoking, *CFH* and *LOC387715*. To investigate functional consequences, we determined *ERCC6* expression in healthy and AMD affected human retinas.

## MATERIALS AND METHODS

### Study populations

Genetic association was analyzed in two study groups from the Netherlands: the population-based Rotterdam Study and an independent case-control study.

In the Rotterdam Study all inhabitants of 55 years or older living in a suburb of Rotterdam, the Netherlands, were invited to participate.[45,81] The initial cohort consisted of 10,275 eligible individuals, of whom 7,983 (78%) participated (98% Caucasian). The ophthalmologic part of the study became operational after the pilot phase of the study had started and consisted of 9,774 eligible individuals, of whom 7,598 (78%) participated. Baseline examinations took place from 1990 to 1993; three follow-up examinations were performed in 1993-1994, 1997-1999, and 2000-2005. At baseline, 6,418 participants had gradable fundus photographs; 5679 had a successful assessment of rs3793784 (*ERCC6* c.-6530C>G), 5681 had a successful assessment of rs1061170 (*CFH* Y402H), and 5766 had a successful assessment of rs10490924 (*LOC387715* A69S).

The case-control study consisted of 357 unrelated AMD patients and 172 control individuals. Subjects were all Caucasian and recruited from the Netherlands Institute of Neuroscience Amsterdam and Erasmus University Medical Centre Rotterdam, through newsletters, via patient organizations, and nursing home visits. Controls were aged 65 years and older, and were unaffected spouses or non-related acquaintances of cases, or individuals who attended the ophthalmology department for reasons other than retinal

pathology. Of all subjects, 501 had a successful assessment of rs3793784 (*ERCC6* c.-6530C>G), 494 had a successful assessment of rs1061170 (*CFH* Y402H), and 508 had a successful assessment of rs10490924 (*LOC387715* A69S).

The study was approved by the Ethics Committees of Erasmus Medical Center Rotterdam and Academic Medical Center Amsterdam, and adhered to the tenets of the Declaration of Helsinki. All participants provided signed informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for AMD research.

**Genotyping**

Genomic DNA was isolated from peripheral leucocytes. Genotyping for the polymorphisms rs3793784 (*ERCC6* c.-6530C>G), rs1061170 (*CFH* p.Y402H) and rs10490924 (*LOC387715* p.A69S) was performed with the Taqman assay (Applied Biosystems, Foster City, CA).

**Diagnosis of AMD**

Fundus photographs were taken of all participants after pupil dilatation. Fundus transparencies were graded according to a modification of the International Classification and Grading System for AMD by well-trained graders under supervision of senior retinal specialists (PTVMdJ, JRV, CCWK). AMD was categorized into early and late AMD according to methods described earlier.[5,6] In short, early AMD (stage 2 and 3) was defined as the presence of either soft distinct drusen with pigmentary irregularities, or soft indistinct drusen with or without pigmentary irregularities; and late AMD (stage 4) as geographic atrophy (dry AMD), neovascular AMD (wet AMD), or mixed AMD (wet AMD in one eye and dry AMD in the other eye, or both types in one eye). Persons were classified based on the eye with the more severe diagnosis. Control persons had no AMD (stage 0: no or only small hard drusen) in either eye, and no other macular pathology.

In the Rotterdam study, incident cases were defined as the absence of AMD in both eyes at baseline and its first appearance in at least 1 eye at follow-up. Unaffected participants remained in stage 0 throughout the follow-up period.

**Human postmortem eyes and mRNA expression**

Studies on human eye tissue were carried out in accordance with the Declaration of Helsinki on the use of human material for research. Donor

eyes were obtained from the Corneabank Amsterdam. Medical history of the donors revealed no pre-existing disorders, prolonged medication, or other prolonged agonal states that could possibly influence RPE gene expression or mRNA quality. Ten mm sections of the macula were stained with periodic acid Schiff's reagens to identify and quantify drusen. Donor eyes were categorized into (1) "Young" control, if the donor was between 20 and 40 years of age, (2) "Old" control, if the donor was older than 70 and histology revealed no drusen, and (3) "AMD", if the donor was older than 70 years and histology showed 30 or more drusen per 10 sections.

We measured *ERCC6* mRNA expression in the RPE cells since this cell type occupies a central position in AMD pathology. We measured expression with real-time PCR, as described previously.[97] Human donor eyes were snap-frozen in isopentane and stored at $-80^0$C. Cryosections of 20 µm from the macula and RPE cells were dissected using a PALM laser dissection microscope (P.A.L.M. Micro Laser Technologies AG). Total RNA was isolated with RNeasy mini (Qiagen) and amplified with the MessageAmp aRNA kit (Ambion). Template cDNA for the real-time PCR was made by reverse transcription of 200 ng aRNA with Superscript III (Invitrogen). Real-time PCR reactions were carried out in a 20 µl volume using qPCR Core Kit Sybr Green I (Eurogentec) and the following primer set: 5'- 5'AAATCTGTGCACTTTCCATAGAACTTC-3' and 5'- Reverse: TATTCTGGCTTGAGTTTCCAAATTC-3'. The levels of amplified product were detected by real time monitoring of SYBR Green I dye fluorescence in the ABI Prism 7300 (Applied Biosystems). Expression levels of *ERCC6* were normalized using the geo-mean of the expression of internal control genes *RBLP0*, *CYCLOP*, and *EF1a.*[100] The non-parametric Kruskal-Wallis test was used to calculate the statistical significance.

**Smoking**

Information on cigarette smoking was obtained by interview, and registered as never, former, and current smoking.

**Statistical analysis**

Characteristics of participants were compared among those affected and non-affected with analysis of covariance for continuous variables, and with logistic regression analysis for discrete variables adjusting for age and sex. Hardy-Weinberg equilibrium of the *ERCC6*, *CFH* and *LOC387715* genotype distributions were tested using a $\chi^2$ test.

In the Rotterdam study, odds ratios for prevalent AMD were estimated with logistic regression analysis, and relative risks for incident AMD were estimated with Cox proportional hazards analysis. Association analyses in the case-control study were estimated with logistic regression analysis. All analyses were adjusted for age and sex.

Interaction with *ERCC6* c.-6530C>G on AMD was determined for smoking, *CFH* Y402H, and *LOC387715* A69S contrasting late AMD (stage 4) with no AMD (stage 0). Analyses were initially performed on separate data sets (prevalent AMD Rotterdam Study, incident AMD Rotterdam Study, and case-control study), and subsequently performed on all data combined. Statistical significance for biological interaction was determined by calculating the synergy index (SI), which measures deviation from additivity of two risk factors.[55,82]

## RESULTS

### Association analysis

In the Rotterdam Study, 427 were diagnosed with prevalent early AMD, and 78 with prevalent late AMD at baseline. After a mean follow up of 7.85 years, 509 persons were diagnosed with incident early AMD and 93 with incident late AMD. The case-control study consisted of 85 persons with early AMD, 264 persons with late AMDs, and 170 controls. Genotype frequencies were in Hardy Weinberg Equilibrium in both studies. Baseline characteristics stratified for the *ERCC6* c.-6530C>G genotype are shown in **Table 1**.

The risk of AMD for the *ERCC6* c.-6530C>G allele is summarized in **Table 2**. In the Rotterdam study, we only found a significant association between *ERCC6* and late AMD in the incident analyses, although an increasing trend was seen in the prevalent analyses as well. In the case-control study, the disease OR was not significantly increased for persons carrying *ERCC6* risk alleles. To increase the statistical power, we combined the data from the Rotterdam Study and the case-control study. This yielded a borderline significant increased risk of late AMD for persons homozygous for *ERCC6* c.-6530C>G (OR 1.39, 95%CI 1.02–1.89). Analyzing subtypes of late AMD separately (dry, wet or mixed) did not yield statistical significant results (data not shown).

**Table 1:** Baseline characteristics of the study populations

a. The Rotterdam Study

| | ***ERCC6* c.-6530C>G Noncarrier (N = 1872)** | ***ERCC6* c.-6530C>G Heterozygous (N = 2790)** | ***ERCC6*c.-6530C>G Homozygous (N = 1062)** |
|---|---|---|---|
| Age, mean (sd), y | | | |
| At baseline | 68.87 (8.60) | 68.63 (8.66) | 68.92 (8.70) |
| At diagnose | 73.05 (8.08) | 72.83 (8.22) | 73.10 (8.14) |
| Women, No (%) | 1071 (58.6) | 1620 (58.1) | 638 (60.1) |
| Smoking status, No/Total (%) | | | |
| Never | 617/1793 (34.4) | 921/2757 (33.4) | 377/1048 (36.0) |
| Past | 751/1793 (41.9) | 1173/2757 (42.5) | 459/1048 (43.8) |
| Current | 425/1793 (23.7) | 663/2757 (24.0) | 212/1048 (20.2) |
| CFH Y402H | | | |
| Noncarrier | 750/1789(41.9) | 1130/2727(41.4) | 416/1036(40.2) |
| Heterozygous | 802/1789(44.8) | 1215/2727(44.6) | 473/1036(45.7) |
| Homozygous | 237/1789(13.2) | 382/2727(14.0) | 147/1036(14.2) |
| LOC387715 A69S | | | |
| Noncarrier | 1147/1805(63.5) | 1733/2771(62.5) | 666/1058(62.9) |
| Heterozygous | 584/1805(32.4) | 928/2771(33.5) | 368/1058(34.8) |
| Homozygous | 74/1805(4.1) | 110/2771(4.0) | 24/1058(2.3)* |

* $P < .05$ compaired to *ERCC6* c.-6530 C>G noncarrier

b. The Case-control Study

| | ***ERCC6* c.-6530C>G Noncarrier (N = 157)** | ***ERCC6* c.-6530C>G Heterozygous (N = 257)** | ***ERCC6* c.-6530C>G Homozygous (N = 87)** |
|---|---|---|---|
| Age, mean (sd), y | 76.22(7.17) | 77.26(7.20) | 76.71(8.79) |
| Women, No (%) | 90(57.3) | 150(48.4) | 44(50.6) |
| Smoking status, No/Total (%) | | | |
| Never | 52/131(39.7) | 68/219(31.1) | 26/79(32.9) |
| Past | 64/131(48.9) | 105/219(47.9) | 45/79(57.0) |
| Current | 15/131(11.5) | 46/219(21.0)* | 8/79(10.1) |
| CFH Y402H, No/Total (%) | | | |
| Noncarrier | 37/150(24.7) | 65/241(27.0) | 21/81(25.9) |
| Heterozygous | 74/150(49.3) | 127/241(52.7) | 39/81(48.1) |
| Homozygous | 39/150(26.0) | 49/241(20.3) | 21/81(25.9) |
| LOC387715 A69S | | | |
| Noncarrier | 72/151(47.7) | 122/250(48.8) | 41/86(47.7) |
| Heterozygous | 59/151(39.1) | 91/250(36.4) | 35/86(40.7) |
| Homozygous | 20/151(13.2) | 37/250(14.8) | 10/86(11.6) |

* $P < .05$ compared to *ERCC6* c.-6530 C>G noncarrier

**TABLE 2:** RISK OF AMD FOR *ERCC6* C.-6530C>G GENOTYPES

**1. The Rotterdam study**

a. Prevalent analyses

| | Stage 0 (n=3629) | Early AMD (n=427) | | Late AMD (n=78) | |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| *ERCC6* c.-6530C>G | | | | | |
| Noncarrier | 1157(31.9) | 138(32.3) | 1.00 | 22(28.2) | 1.00 |
| Heterozygous | 1795(49.5) | 204(47.8) | 0.99(0.78-1.26) | 35(44.9) | 1.01(0.57-1.77) |
| Homozygous | 677(18.7) | 85(19.9) | 1.12(0.83-1.50) | 21(26.9) | 1.71(0.90-3.25) |
| Allele freq | 0.434 | 0.438 | | 0.494 | |

b. Incident analyses

| | Early AMD (n=509) | | Late AMD (n=93) | |
|---|---|---|---|---|
| | N(%) | HR[a] | N(%) | HR[a] |
| *ERCC6* c.-6530C>G | | | | |
| Noncarrier | 162(31.8) | 1.00 | 21(22.6) | 1.00 |
| Heterozygous | 257(50.5) | 1.12(0.92-1.37) | 50(53.8) | **1.70(1.02-2.82)** |
| Homozygous | 90(17.7) | 0.99(0.77-1.29) | 22(23.7) | **1.92(1.05-3.49)** |
| Allele freq | 0.429 | | 0.505 | |

2. The case-control study

| | Stage 0 (n=170) | Early AMD (n=84) | | Late AMD (n=247) | |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| *ERCC6* c.-6530C>G | | | | | |
| Noncarrier | 53(31.2) | 31(36.9) | 1.00 | 73(29.6) | 1.00 |
| Heterozygous | 92(54.1) | 38(45.2) | 0.60(0.32-1.09) | 127(51.4) | 0.90(0.56-1.44) |
| Homozygous | 25(14.7) | 15(17.9) | 0.93(0.42-2.09) | 47(19.0) | 1.37(0.72-2.59) |
| Allele freq | 0.418 | 0.405 | | 0.447 | |

Abbreviations: AMD, age-related macular degeneration; OR, odds ratio; HR, hazard ratio
The ORs and HRs are estimates of the relative risk of AMD, and represent the risk of disease (AMD *vs.* stage 0) in the genetic risk group divided by the risk of disease (AMD vs stage 0) in the non-risk group (noncarriers).
[a] adjusted for sex, age

3. Pooled data from the Rotterdam study and the case-control study (pooled data from prevalent and incident cases)

| | Stage 0 (n=2567) | Early AMD (n=967) | | Late AMD (n=418) | |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| *ERCC6* c.-6530C>G | | | | | |
| Noncarrier | 840(32.7) | 319(33.0) | 1.00 | 116(27.8) | 1.00 |
| Heterozygous | 1254(48.9) | 470(48.6) | 0.99(0.84-1.17) | 212(50.7) | 1.22(0.95-1.57) |
| Homozygous | 473(18.4) | 178(18.4) | 1.00(0.80-1.24) | 90(21.5) | **1.39(1.02-1.89)** |
| Allele freq | 0.429 | 0.427 | | 0.469 | |

Abbreviations: AMD, age-related macular degeneration; OR, odds ratio; HR, hazard ratio
The ORs and HRs are estimates of the relative risk of AMD, and represent the risk of disease (AMD *vs.* stage 0) in the genetic risk group divided by the risk of disease (AMD vs stage 0) in the non-risk group (noncarriers).
[a] adjusted for sex, age

## Analysis of interaction

We studied the interactions of *ERCC6* c.-6530C>G with three prominent AMD risk factors: the most important environmental risk factor smoking, and the two most important genetic risk factors *CFH* Y402H and *LOC387715 A69S*. As assessed by calculation of the SI, no significant interaction between smoking and the ERCC6 variant was found. Neither did we found a significant SI for the interaction with *CFH* Y402H or *LOC387715* A69S. This implies that these risk factors did not modify the relation of *ERCC6* with AMD.

## Expression in human retina

We compare mRNA expression levels in donor eyes with early AMD with age-matched and young healthy eyes. We did not find a significant difference in *ERCC6* gene expression levels between eyes with early AMD and old healthy eyes, nor did we find a difference between young and old non-AMD eyes, signifying no regulation of *ERCC6* expression in the RPE with age (**Table 3**).

**Table 3:** *ERCC6* mRNA expression in human RPE

| Donor eye class | No of eyes | expression (normalized) ± S.E.M. | P-value tested against "Old" |
|---|---|---|---|
| AMD (early) | 13 | 5.25 ± 0.98 | 0.39 |
| Old | 7 | 5.81 ± 0.75 | Non applicable |
| Young | 5 | 6.14 ± 1.28 | 0.88 |

## DISCUSSION

Our results fail to show consistent statistical significance regarding the association between AMD and *ERCC6* rs3793784. We only detected a significant risk in the incident analysis of the Rotterdam study, and not in the prevalent analysis. Neither did we find a significant association in the case-control study. Increasing the power by combining all data showed a marginal increased effect of homozygous *ERCC6* carriers for late AMD. We did not find evidence for effect modification by the three major AMD risk factors smoking, *CFH* Y402H and *LOC387715* A69S. There were no significant differences in expression levels of the gene. These results imply that this SNP has a minor role, if any, in the pathogenesis of AMD.

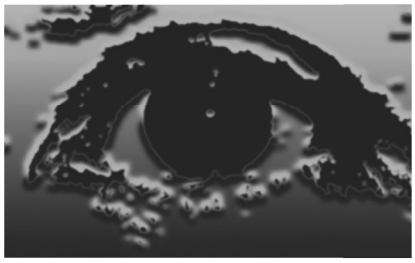
This study attempted to replicate the initially reported association between *ERCC6* and AMD by Tuo and co-workers.[109] The minor allele frequency of rs3793784:C>G was higher in our study than that reported by Tuo et al (0.429 *vs.* 0.353), which is most likely due to genetic differences between the study populations in the Netherlands and the U.S.A. Furthermore, we could not confirm the reported increased risk for heterozygotes and our ORs for homozygotes were smaller than those reported earlier. Although we found a small increase in ORs in the prevalent and incident analysis in the Rotterdam study as well as in the case-control study, only the incident analysis yielded a significant result. This prompted us to specifically analyze whether selective mortality may be involved. Persons with two copies of *ERCC6* rs3793784 have a higher risk of AMD, and if these persons also die earlier, it could explain the discrepancy between prevalent and incident analyses. However, in the Rotterdam study, the *ERCC6* variant was not associated with increased mortality, neither did we find age-related changes in genotype distribution. Thus, the fact that incident data yielded significant results and prevalent data not, probably occurred by chance. Increasing the power of the analysis by combining all data yielded a small but significant association between *ERCC6* and AMD. Overall, the small magnitude of the association, the inconclusiveness in our different analyses and the borderline significance do not provide evidence that the *ERCC6* gene is involved in AMD. Nevertheless, taking into account the data of Tuo et al. we cannot exclude a marginal effect.

ERCC6 plays a role in transcription-coupled repair of DNA damage.[113] Mutations in *ERCC6* lead to CS, a progeroid disorder characterized with severe growth retardation, retinal degeneration, neurological symptoms,

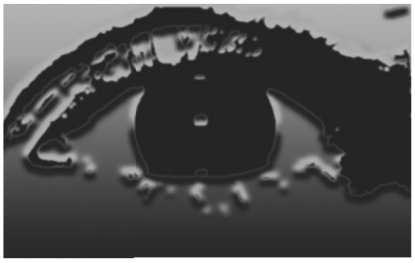
and early death.[110] *ERCC6* deficiency in mice leads to retinal degeneration and hypersensitivity of the retina for ionizing radiation, which suggest that oxidative DNA damage is involved in ERCC6 related retinal pathology.[112] The retina is particularly sensitive for oxidative stress given its high consumption of oxygen, its high proportion of polyunsaturated fatty acids, and its exposure to visible light.[114] Recently high quantities of oxidative protein modifications were found in drusen and Bruch's membrane and oxidized lipoproteins were detected in choroidal neovascular membranes of AMD eyes.[115,116] An appealing hypothesis is that *ERCC6* polymorphisms impair the efficiency of transcription-coupled DNA repair, and thus affect defense of the retinal cells against oxidative stress or other genotoxic agents. According to this reasoning, an *increase* in repair activity of *ERCC6* would lead to a *decrease* of retinal damage, and vice versa.

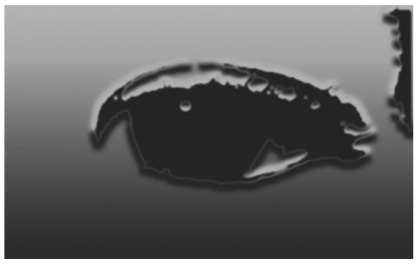
Intriguingly, the data currently available for the particular *ERCC6* rs3793784:C>G (c.-6530C>G) SNP do not fully support this hypothesis. Tuo et al. showed that the SNP is located in the 5' UTR of the gene and influences ERCC6 expression level: the G allele resulted in 2-3 times higher *ERCC6* expression in the RPE than the C variant.[109] In addition the *C variant* was associated with *increased* lung cancer susceptibility, suggesting that lower *ERCC6* expression leads to *decreased* DNA repair function, and thus *increased* cellular damage.[117] In contrast, our data as well as the data of Tuo et al. indicated that *the G allele and not the C allele* is associated with *increased* AMD susceptibility. A higher ERCC6 expression in the RPE (G allele) would thus be correlated with increased AMD risk. However, we could not confirm a difference in *ERCC6* expression in AMD eyes, neither did we found any indications for a compromised function of ERCC6: we did not find an indication that the polymorphism is related with mortality and age, nor did we find a significant interaction with smoking, *CFH* and *LOC387715*.

In conclusion, our results do not provide enough evidence for a consistent association between *ERCC6* and AMD. Further functional and epidemiological studies in larger populations are needed to further unravel its role in AMD pathogenesis and clarify whether this is a true contributor to AMD susceptibility.



# PART II
# PREDICTIVE VALUE OF GENETIC PROFILING FOR AMD





# 6. PREDICTIVE VALUE OF MULTIPLE GENETIC TESTING FOR AGE-RELATED MACULAR DEGENERATION

The unraveling of the genetic background of age-related macular degeneration (AMD) is occurring at a tremendously fast pace. Researchers have shown that single nucleotide polymorphisms (SNPs) in the *CFH*, *LOC387715, C2/FB* and *HTRA1* genes are highly associated with AMD,[37-40,49,55-58,72] and have estimated that they explain more than 50% of all cases.[39,55,57,72] These genetic discoveries are a major breakthrough for understanding the pathogenesis of the disease, but whether they can be used to improve the prediction of end-stage AMD in individuals at risk is still open to question.[118] This requires that testing of multiple SNPs, or genetic profiling, be a better predictor than classical risk factors.

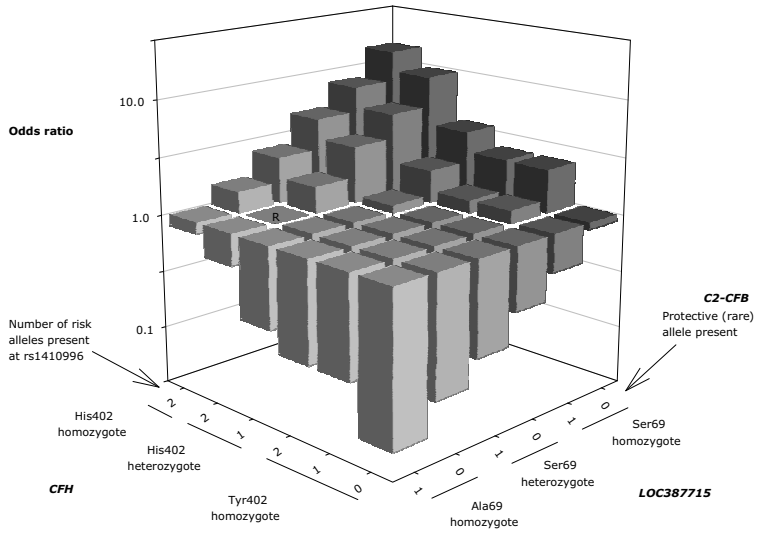
An important step in this direction has been made by Maller *et al*.,[59] who investigated the association between 5 variants in the *CFH, LOC387715* and *C2/FB* genes and AMD in a large case-control study. Simultaneous testing of these 5 SNPs showed that 10% of the study population had a 40-fold increased risk of AMD, and that persons homozygous for all risk variants had a 285-fold greater risk than the lowest risk group. These findings are promising, and suggest that these genes should be considered important determinants of the risk of AMD. However, several issues hamper a direct translation of the reported risks to clinical practice.

The first issue concerns the validity of the reported risk estimates. The authors estimated the odds ratios for each combination of the genotypes using a regression model. However, these odds ratios can also be calculated from the observed genotype frequencies provided in their supplementary tables. Comparison of these calculations showed that the odds ratios based on observed frequencies were systematically lower (approximately 2-fold) than those predicted by the model. For instance, persons homozygous for all risk variants had a 285-fold increased risk of AMD according to the model, while they had a 118-fold increased risk based on observed genotype frequencies. This indicates that there is room for improvement of the risk model.

A second important issue is whether the odds ratios estimated by Maller *et al*.[59] are informative for clinical practice. Maller and colleagues[59] calculated all odds ratios relative to the lowest risk group, *i.e.*, individuals who carry low-risk genotypes at all loci. This reference group concerned approximately 3% of the study population and, consequently, nearly the entire population (~97%) was at increased risk of AMD. This is a valid statistical comparison, but not useful for individuals in whom testing would be applied in a clinical or public health setting. Before testing, all will have

the same AMD risk, i.e. the average population risk. After testing, some will have risks that are higher than average, while others will have risks that are lower than average. Therefore, it would have been more useful if Maller and colleagues[59] had chosen a reference group whose risk equals the average risk in the population. In the **Figure**, we present odds ratios relative to the mean prevalence of end-stage AMD (geographic atrophy and/or neovascular AMD; 3% in those older than 65 years.[8,119]) from data provided by the Three Continent Study[119] and EUREYE (European Eye) Study.[8] The odds ratios of most genetic profiles were lower than 1, which means that most profiles were associated with a lower than average risk of AMD. Individuals homozygous for all risk variants, who were reported to have a 285-fold increased risk compared to the lowest risk group in the paper of Maller *et al.* now had a 14-fold higher risk of AMD compared to the population risk. Individuals who carried only low-risk genotypes had a 20-fold decreased risk. While our estimates still indicate a considerable deviation from the general population risk, this interpretation of the data provides a perspective that is more relevant for practical use. Assuming a population risk of 3%, the findings of Maller *et al*.[12] translate to absolute AMD risks of approximately 35% for those carrying all risk genotypes and 0.17% for those carrying none.

**FIGURE:** RISK OF AMD AS A FUNCTION OF THE RISK VARIANTS




Odds ratios based on the logistic regression model by Maller *et al.*[59] are calculated relative to a reference group (R), which has a post-test AMD risk equal to the population risk of AMD in persons aged 65 years or older (3%)

Third, a test that identifies high- and low-risk profiles is not necessarily a useful test. When high- and low-risk profiles concern only small subgroups, the test bears little relevance to the majority of the population. A measure that indicates the usefulness is the discriminative accuracy of the test results, *i.e.*, the extent to which the test results can discriminate between persons who will develop the disease and those who will not.[120,121] This discriminative accuracy is calculated using the *concordance*-statistic, and varies between 50% (no discrimination, as accurate as tossing a coin) and 100% (perfect discrimination). Using the data of Maller *et al*.,[59] we calculated the discriminative accuracy of testing the SNPs in the *CFH, LOC387715* and *C2/FB* genes for the prediction of end-stage AMD, and found a score of 80%. This high value is similar to the discriminative accuracy of high serum cholesterol for the prediction of cardiovascular disease (77%).[122] Of course, the discriminative accuracy may further increase with the identification of additional SNPs, especially when they are found in independent loci. In this regard, testing the newly identified variant (rs11200638) in the *HTRA1* gene may not increase the discriminative accuracy, because this SNP is in complete linkage disequilibrium (D'>0.99) with rs10490924 of *LOC387715,* a variant which was already present in these risk calculations.[56]

The final and most important limitation for using the reported risks in AMD prediction is that Maller *et al*.[12] investigated the predictive value of the 5 SNPs in a population that is not representative for clinical practice, as was mentioned by the authors in their discussion. They did not include the entire range of patients with AMD but only those from the extreme tails of the clinical spectrum, *i.e.*, those with end-stage AMD (3% of those aged ≥ 65 years) and those with no or fewer than 10 small drusen without pigment abnormalities (57% of those aged ≥ 65 years). The authors did not consider most patients with early features of AMD in their study. Because the data of Maller *et al.* only showed to what extent the 2 most extreme groups can be disentangled, it is still unclear whether genetic testing can also separate patients with early stages who will develop end-stage AMD from those who will remain stable.

Studies like those by Maller *et al.*,[59] which investigate the combined effects of multiple genetic variants in extreme group comparisons, are a significant starting point towards application of predictive genetic testing for end-stage AMD. Yet, to demonstrate the true predictive value of genetic profiling, these studies need to be replicated in populations that are representative for the settings in which the genetic testing will be applied. Only such studies can

reveal whether multiple genetic testing will be a better or earlier predictor of end-stage AMD than currently known risk factors, such as a phenotype of soft drusen and pigment changes, smoking, and familial aggregation.[10,16]

# 7. GENETIC DIAGNOSIS OF AGE-RELATED MACULAR DEGENERATION: THE ROLE OF MOLECULAR GENETICS IN THE IDENTIFICATION OF HIGH RISK EYES

## ABSTRACT

**Purpose:** Five genes have been highly associated with age-related macular degeneration (AMD): *Complement Factor H, LOC387715/HTRA1, Complement component C3*, *Complement component C2,* and *Factor B*. We calculated the predictive value of multiple genetic testing to assess whether genetic testing will be useful for clinical practice. **Methods:** We investigated these genes in three different settings: the population-based Rotterdam Study, a case-control study, and a study from a genetic isolate. The analyses were based on 4871 persons with no AMD, 653 with early AMD, and 460 with late AMD. **Results:** All risk alleles were independently associated with AMD in a multivariate analysis including age, sex and smoking, except for alleles from *C3*. The predictive value of genetic testing (86%) exceeded that of age and smoking (77%) in a model which included all subjects, indicating that multiple genetic testing can be used to discriminate those who will develop late AMD from those who will not in a general population. The predictive value of genetic testing (72%) also exceeded that of age and smoking (61%) in a model including late AMD and older subjects with early AMD, signifying that genetic testing helps to distinguish those who will progress to late AMD from those who will remain stable. Absolute risks of late AMD by age 85 yrs altered after testing to 60% for persons homozygous for high risk alleles, and to < 5% for persons carrying no risk alleles. **Conclusions:** Genetic testing has a greater predictive value than currently known risk factors such as age, presence of early AMD, and smoking, and may become a useful application when protective therapies become available.

## INTRODUCTION

Age-related macular degeneration (AMD) is a common eye disorder in the elderly leading to severe visual impairment. Dissection of the genetic background of this disease has undergone tremendous progress in recent years. There is now ample evidence that common single nucleotide polymorphisms (SNPs) in the *CFH*, *LOC387715/HTRA1*, *C2/FB*, and *C3* genes are highly associated with AMD.[23,37-40,49,55,72,79,80] The contribution of these SNPs to the disease occurrence is remarkably large, and it has become apparent that they explain more than 50% of cases.[9,55] The genetic discoveries are a major breakthrough in understanding the pathogenesis. The prominence of genes involved in the complement cascade has signified that inflammation is an important cause of AMD.

While it is clear that risk alleles should be further investigated for research purposes, it is still open to question whether it is useful to determine these in a clinical setting. Clinical parameters which are currently used to predict the development of the visually disabling end-stages (late AMD) are age, the presence of early stages, and smoking. For example, an individual aged 80 years with indistinct drusen and pigmentary changes in the macular area has an absolute risk of 42% to develop late AMD within 5 years, and this risk is doubled when it concerns a smoker.[6] By contrast, a non-smoking person aged 60 with only small drusen has a less than 1% chance to develop late AMD in 5 years. Hence, determination of genetic risk alleles will only be appealing when testing proves to be a better predictor of late AMD than the conventional risk factors.

The principle question addressed in this study is whether multiple genetic testing for age-related macular degeneration can be used to improve the prediction of the course of AMD for individuals at risk. We investigated three study populations, each with a unique design regarding ascertainment of study subjects, but with very similar methods for data collection. This setting allowed for internal validation of study results, but also increased potential extrapolation of the findings.

## MATERIALS AND METHODS

### Study Populations

*Population-based study*

The Rotterdam Study is a prospective cohort study aimed at studying chronic diseases in elderly patients. All inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands, were invited to participate in the study.[45,81] Of the initial cohort of 10,275 eligible individuals, 7,983 (78%) participated (98% Caucasian). The ophthalmologic part of the study became operational after the pilot phase of the study had started and consisted of 9,774 eligible individuals, of whom 7,598 (78%) participated. Baseline examinations took place from 1990 to 1993; three follow-up examinations were performed in 1993-1994, 1997-1999, and 2000-2005.[55] At baseline, 6,418 participants had gradable fundus photographs.

*Case-control study*

This study consisted of 360 unrelated AMD patients and 183 control individuals. Subjects were all Caucasian and recruited from the Netherlands Institute of Neuroscience Amsterdam and Erasmus University Medical Centre Rotterdam, through newsletters, via patient organizations, and nursing home visits. Controls were aged 65 years and older, and were mostly unaffected spouses or non-related acquaintances of cases, or individuals who attended the ophthalmology department for reasons other than retinal pathology.

*Genetic isolate study*

The Erasmus Rucphen Family (ERF) study is part of the Genetic Research in Isolated Populations Study (GRIP) and is a family-based cohort study conducted in a genetically isolated population located in the southwest of the Netherlands. Characterization of this population has been presented elsewhere.[123-125] In short, twenty-two families who had at least six children baptized in the community church between 1800 and 1900 were selected for the ERF study, and all their living descendants and spouses were invited for examination. In addition, individuals with late AMD were recruited via ophthalmologists from the catchments area of GRIP. The inclusion criteria for the current analyses was age 55 years or older, resulting in a total of 88 participants with AMD and 83 with no AMD.

The studies were approved by the Ethics Committees of Erasmus Medical Center and Academic Medical Centre Amsterdam, and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for AMD research.

**Genotyping**

Genomic DNA was extracted from peripheral blood leukocytes. All study participants were genotyped with the Taqman assay (Applied Biosystems, foster City, California, USA) for rs2230199 (R102G) and rs1047286 (P314L) in the *C3* gene, and for rs4151667 (L9H) and rs541862 (IVS8) in the *FB* gene. The R32Q SNP, which was reported to be protective of AMD,[72] could not be genotyped with the Taqman assay. Since rs541862 is in complete LD with this SNP (Hapmap r2=1, D'=1), we genotyped rs541862 as a proxy for the R32Q SNP. Rs1061170 (Y402H) and rs1410996 (IVS14) in the *CFH* gene and rs10490924 (A69S) in the *LOC387715* gene were analyzed with the Taqman assay in the Rotterdam and ERF studies, and with denaturing high-performance liquid chromatography (DHPLC) in the case-control study (Wave; Transgenomic, Santa Clara, California, USA). Variants on DHPLC were graded by two researchers, and subsequently identified by direct sequencing using the ABI-310 (Applied Biosystems, Foster City, California, USA).

**Smoking**

Information on cigarette smoking was obtained by interview, and categorized as never, former, and current smoking.

**Diagnosis of AMD**

All participants underwent fundus photography after pharmacologic mydriasis. Fundus transparencies of both studies were graded according to a modification of the International Classification and Grading System for AMD by the same well-trained graders under the supervision of senior retinal specialists (PTVMdJ, JRV, CCWK). AMD was categorized into early and late AMD according to methods described earlier.[5,6] Early AMD (stage 2 and 3) was defined as the presence of either soft distinct drusen with pigmentary irregularities, or soft indistinct drusen with or without pigmentary irregularities; and late AMD (stage 4) as geographic atrophy (dry AMD), neovascular AMD (wet AMD), or mixed AMD (wet AMD in one eye and dry

AMD in the other eye, or both types in one eye). Persons were classified based on the eye with the more severe diagnosis. Control persons had no AMD (stage 0: no or only small hard drusen) in either eye, and no other macular pathology.

In the Rotterdam study, incident cases were defined as the absence of AMD in both eyes at baseline and its first appearance in at least 1 eye at follow-up. Unaffected participants remained in stage 0 throughout the follow-up period.

**Statistical analysis**

Characteristics of participants were compared among those affected and non-affected with analysis of covariance for continuous variables, and with logistic regression analysis for discrete variables, adjusting for age and sex. Hardy-Weinberg equilibrium of the genotype distributions were tested using a Fisher's exact test.

Associations were initially analyzed for each study separately. In the Rotterdam Study, odds ratios for prevalent AMD were estimated with logistic regression analysis, and relative risks for incident AMD were estimated with Cox proportional hazards analyses. In the case-control and genetic isolate studies, odds ratios were estimated with logistic regression analysis. We performed subsequent risk analyses on the pooled data using dummy variables for the studies to assess heterogeneity across study populations. All analyses were adjusted for age and sex.

Using the logistic regression model, we computed probabilities of AMD for each subject as predicted by different disease risk functions: (1) age and sex, (2) age, sex and smoking, (3) age, sex, smoking and genetic risk alleles. Using each risk function as a diagnostic test, we constructed receiver operating characteristic (ROC) curves and assessed the area under the ROC curve as a measure of the accuracy of discrimination between cases and controls for each of the risk functions. The ROC curve indicates the probability of a true-positive result (sensitivity) as a function of the probability of a false-positive result (1-specificity).

Cumulative risks of late AMD were calculated using Kaplan-Meier product-limit analysis in the presence of competing risks. Participants who died, and those who were free of AMD and were lost to follow-up, were censored at the time of their last visit. The population attributable risk (PAR) was calculated according to the formula:

PAR=(relative risk −1/ relative risk) * proportion of exposed.

Relative risk of late AMD in this formula was estimated by the OR. The proportion exposed was the proportion of participants with late AMD carrying the *C3* allele.

## RESULTS

Overall, the pooled studies comprised 1185 participants with early AMD, 559 with late AMD, and 3523 with no AMD. Regarding the separate studies, the Rotterdam Study contributed 476 individuals with early AMD, 106 with late AMD, and 4055 without AMD at baseline; and 586 with incident early AMD, 99 with incident late AMD, and 2078 persons who remained free of any AMD. The case-control study provided 89 participants with early AMD, 271 with late AMD, and 183 individuals without AMD. The GRIP study contributed 88 persons with early AMD, 83 with late AMD, and 633 persons without AMD. Baseline characteristics of the study participants are shown in **Table 1**.

All genotype frequencies were in Hardy Weinberg equilibrium in the controls. In the univariate analyses, all variants showed a significant association with AMD (**Supplementary Tables and Table 2**). In the multivariate risk analyses which included age, sex, and smoking, significance remained for all SNPs, except for the *C3* variants (**Table 2**). Ranking of the variants according to highest OR for homozygous persons yielded a sequence of *LOC387715* A69S and *CFH* Y402H for causative minor alleles, and a sequence of *CFH* IVS14, *FB* rs541862 and *FB* L9H for protective minor alleles. Stratification of late AMD in subtypes revealed that risks were not significantly different between geographic atrophy and neovascular AMD, although all risks appeared more pronounced for mixed AMD (data not shown).

We tested for interaction between risk alleles, and found a significant synergy index for *CFH* Y402H and *LOC387715* A69S (SI 12.83(3.53-46.55)). This interaction was not statistically significant in the multiplicative analyses. In addition, we analyzed the potentially modifying effect of smoking, and found no significant interaction between any of the risk alleles and smoking.

**Table 1:** Baseline characteristics of the study populations

| | | Rotterdam Study | | |
|---|---|---|---|---|
| | | No AMD (N=4055) | Early AMD (N=476) | Late AMD (N=106) |
| Age, mean (sd), y | | 67.52 (8.31) | 75.05(8.70)$ | 81.99(8.20)$ |
| | < 65 | 1814(44.7) | 65(13.7)$ | 4 (3.8)$ |
| | 65-74 | 1451(35.8) | 172(36.1)$ | 17 (16.0)* |
| | 75-84 | 657(16.2) | 175(36.8)$ | 46 (43.4)$ |
| | ≥ 85 | 133(3.3) | 64(13.4)$ | 39 (36.8)$ |
| Women, No (%) | | 2358(58.2) | 285(59.9) | 70(66.0) |
| Smoking status, No/Total (%) | | | | |
| | Never | 1327/4008(33.1) | 182/465(39.1) | 40/102(39.2)$ |
| | Past | 1759/4008(43.9) | 187/465(40.2) | 31/102(30.4) |
| | Current | 922/4008(23.0) | 96/465(20.6) | 31/102(30.4)$ |
| | | **Case-control study** | | |
| | | No AMD (N=183) | Early AMD (N=89) | Late AMD (N=271) |
| Age, mean (sd), y | | 74.27(6.27) | 76.50(7.21)* | 78.66(7.70)$ |
| | < 65 | 5 (2.7) | 4 (4.5) | 16 (5.9)$ |
| | 65 – 74 | 102 (55.7) | 33 (37.1) | 55 (20.3)* |
| | 75 – 84 | 67 (36.6) | 41 (46.1) | 145 (53.5) |
| | ≥ 85 | 9 (4.9) | 11 (12.4) | 55 (20.3) |
| Women, No (%) | | 98(53.6) | 60(67.4)* | 155(57.2) |
| Smoking status, No/Total (%) | | | | |
| | Never | 59/147(40.1) | 28/83(33.7) | 74/235(31.5)* |
| | Past | 71/147(48.3) | 46/83(55.4)* | 117/235(49.8)* |
| | Current | 17/147(11.6) | 9/83(10.8) | 44/235(18.7)* |

| | Genetic isolate study | | |
|---|---|---|---|
| | No AMD (N=633) | Early AMD (N=88) | Late AMD (N=83) |
| Age, mean (sd), y | 63.34(6.05) | 69.14(9.11) $ | 81.35(6.54) $ |
| < 65 | 418(66.0) | 34(38.6)* | 1(1.2)* |
| 65 – 74 | 181(28.6) | 28(31.8) $ | 12(14.5) $ |
| 75 – 84 | 34(5.4) | 22(25.0)* | 44(53.0)* |
| ≥ 85 | - | 4(4.5) | 26(31.3) |
| Women, No (%) | 337(53.2) | 42(47.7) | 48(57.8) |
| Smoking status, No/Total (%) | | | |
| Never | 141(22.3) | 28(31.8) | 32(38.6) |
| Past | 260(41.1) | 34(38.6) | 36(43.4) |
| Current | 232(36.7) | 26(29.5) | 15(18.1) |

AMD = age-related macular degeneration; Data are unadjusted mean ± SD for continuous variables and percentages for dichotomous variables; *$P$ < 0.05 compared to participants with no AMD; $$P$ < 0.001 compared to participants with no AMD.

**Table 2:** Associations between risk alleles and AMD*

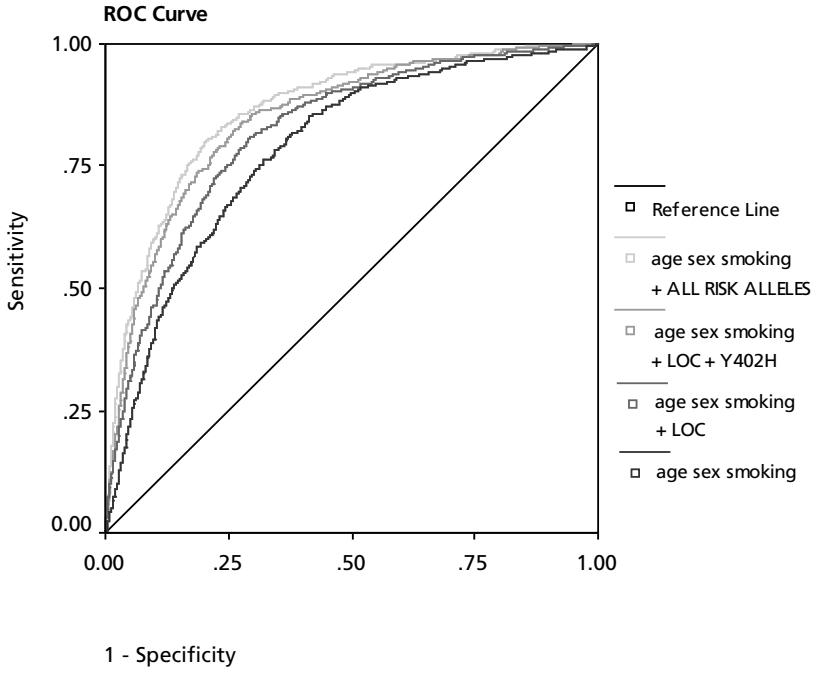
| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | **Early AMD** OR**(95%CI) | **Late AMD** OR*(95%CI) | **Early AMD** OR**(95%CI) | **Late AMD** OR**(95%CI) |
| Smoking | | | | |
| Past | 0.93(0.79-1.10) | **1.44(1.09-1.91)** | 0.98(0.81-1.19) | **1.70(1.19-2.44)** |
| Current | 0.88(0.72-1.08) | **2.15(1.54-3.01)** | 1.00(0.80-1.24) | **2.49(1.63-3.81)** |
| CFH Y402H | | | | |
| Heterozygous | **1.22(1.04-1.42)** | **2.42(1.80-3.27)** | 0.99(0.81-1.21) | **1.85(1.21-2.84)** |
| Homozygous | **2.42(1.96-2.97)** | **8.75(6.14-12.49)** | **1.59(1.19-2.13)** | **5.82(3.37-10.03)** |
| CFH IVS14 | | | | |
| Heterozygous | **0.62(0.53-0.72)** | **0.31(0.24-0.40)** | **0.72(0.59-0.89)** | **0.47(0.33-0.69)** |
| Homozygous | **0.50(0.41-0.62)** | **0.16(0.10-0.24)** | **0.58(0.43-0.78)** | **0.39(0.21-0.74)** |
| LOC387715 A69S | | | | |
| Heterozygous | **1.66(1.43-1.93)** | **2.61(2.01-3.38)** | **1.71(1.46-2.01)** | **3.11(2.27-4.25)** |
| Homozygous | **2.03(1.44-2.88)** | **9.91(6.46-15.20)** | **1.96(1.34-2.88)** | **13.46(8.01-22.65)** |
| FB L9H | | | | |
| Carriers | 0.90(0.71-1.15) | **0.34(0.20-0.56)** | 0.86(0.67-1.12) | **0.20(0.10-0.39)** |
| FB rs541862 | | | | |
| Carriers | **0.79(0.64-0.97)** | **0.32(0.20-0.49)** | 0.80(0.64-1.00) | **0.23(0.13-0.40)** |
| C3 R102G | | | | |
| Heterozygous | 1.28(1.10-1.49) | **1.50(1.16-1.94)** | 1.12(0.79-1.60) | 1.36(0.74-2.51) |
| Homozygous | 1.36(0.98-1.88) | **2.52(1.57-4.04)** | 0.95(0.44-2.05) | 1.60(0.41-6.23) |
| C3 P314L | | | | |
| Heterozygous | **1.29(1.11-1.50)** | **1.53(1.19-1.98)** | 1.19(0.84-1.69) | 1.28(0.69-2.39) |
| Homozygous | 1.20(0.85-1.68) | **2.11(1.30-3.44)** | 1.55(0.73-3.30) | 2.05(0.55-7.62) |

* Pooled data from the Rotterdam Study (prevalent and incident cases), the case-control study, and the Genetic isolate study.
**adjusted for age and sex

To investigate whether multiple genetic testing can accurately discriminate those who will develop late AMD from those who will not, we calculated the AUC as a measure for discriminative accuracy. The first question we addressed was: is genetic testing of the population useful for the prediction of late AMD? The ROC curve (**Figure 1**) showed that genetic testing had a significantly higher AUC than age, sex and smoking alone. The highest AUC was achieved by testing all genes and yielded a score up to 0.86. The second question concerned the use of genetic testing in clinical practice: can genetic testing be used to discriminate those who will remain early AMD from those who will progress to late AMD? The ROC curve (**Figure 2**)

showed that genetic testing greatly improved the accuracy of prediction beyond age, sex, and smoking. The model with age, sex and smoking yielded an AUC of 0.63; this model improved significantly to an AUC of 0.72 when all genetic variants were determined.

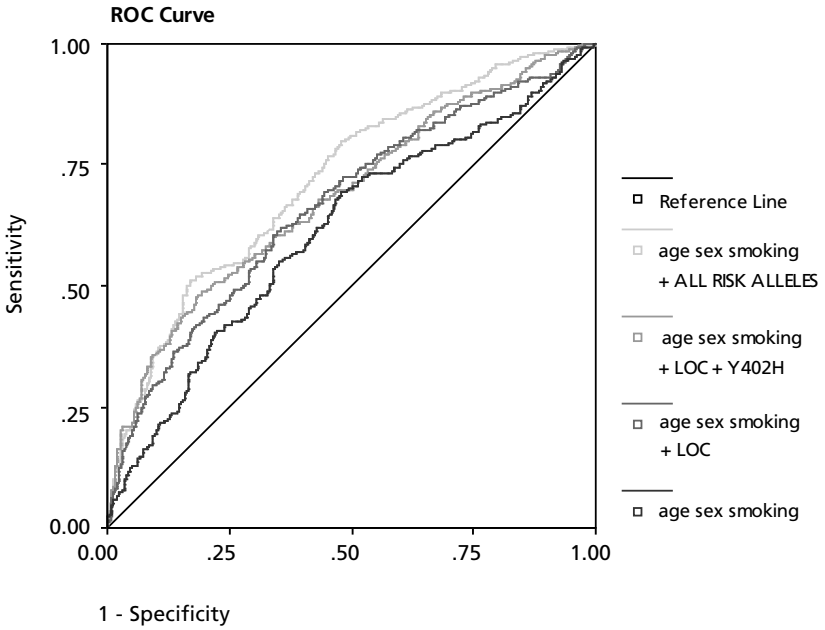
**FIGURE 1:** RECEIVER OPERATING CHARACTERISTIC CURVES AND AREAS UNDER THE CURVE FOR PREDICTION OF AMD IN A POPULATION OF ASYMPTOMATIC PERSONS*




| Risk function | AUC (95% CI) |
|---|---|
| age+sex | 0.766(0.746-0.785) |
| age+sex+smoking | 0.775(0.755-0.796) |
| age+sex+smoking+LOC | 0.815(0.795-0.834) |
| age+sex+smoking+LOC+Y402H | 0.843(0.825-0.862) |
| age+sex+smoking+Y402H+IVS14+LOC+FB SNPS+C3 SNPS | 0.864(0.847-0.883) |

Separate plots are depicted for four different risk functions. The diagonal line is the reference and depicts a discriminative accuracy of 50% (non-discrimination, as accurate as tossing a coin)
***** Pooled data from the Rotterdam Study (prevalent and incident cases), the case-control study, and the Genetic isolate study.

**Figure 2:** Receiver operating characteristic curves and areas under the curve for prediction of AMD comparing those with late AMD to older subjects* with early AMD. **




| Risk function | AUC (95% CI) |
|---|---|
| age+sex | 0.606(0.571-0.641) |
| age+sex+smoking | 0.636(0.601-0.672) |
| age+sex+smoking+LOC | 0.676(0.640-0.712) |
| age+sex+smoking+Y402H+LOC | 0.696(0.660-0.731) |
| age+sex+smoking+Y402H+IVS14+LOC+FB SNPS+C3 SNPS | 0.720(0.684-0.756) |

Separate plots are depicted for four different risk functions. The diagonal line is the reference and depicts a discriminative accuracy of 50% (non-discrimination, as accurate as tossing a coin)
***** older subjects with early AMD are those persons who are 80 yrs and older, and still remain with early AMD. (80 yrs is the mean age of onset of late AMD in our population)
****** Pooled data from the Rotterdam Study (prevalent and incident cases), the case-control study, and the Genetic isolate study.



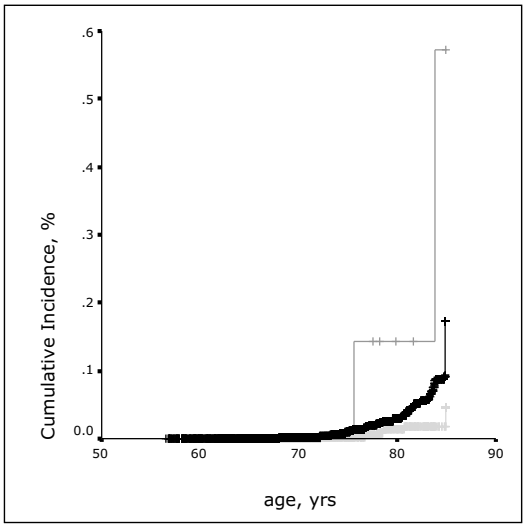




Kaplan-Meier product-limit analyses (**Figure 3**) indicated that the absolute lifetime risk of developing late AMD by the age of 85 years was 17.24% for the total population. This risk can be regarded as the a priori or pre-test risk. After testing for *LOC387715* A69S, and *CFH* Y402H, the absolute lifetime risk augmented to 57.17% for individuals who were homozygous for all risk alleles, but decreased to a mere 4.49% for those homozygous for

the non-risk alleles. The frequency of those carrying additional protective alleles was very low, and could not be included in this analysis.

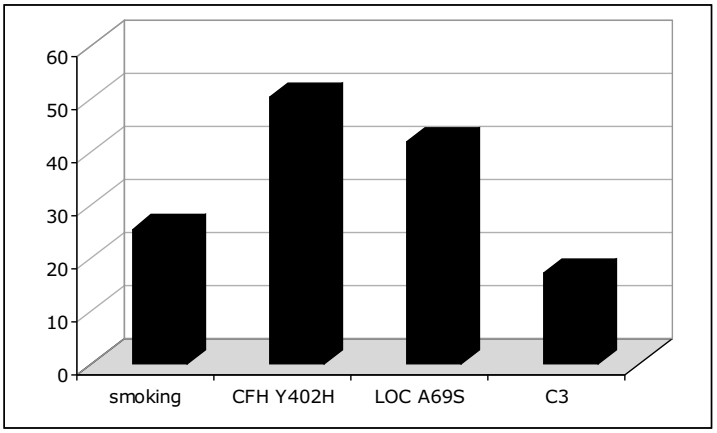
**FIGURE 3:** CUMULATIVE INCIDENCE OF LATE AMD




Absolute risks of late AMD by age 85 yrs are depicted. The middle curve represents the pre-test risk, i.e., the risk in the general population before genetic testing. The lower and upper curves represent the risks after testing of *CFH* Y402H and *LOC387715* A69S. The absolute risks post testing altered after genetic testing to 57% for persons homozygous for high risk alleles (upper curve), and to < 5% for persons carrying no risk alleles (lower curve).

**Figure 4** illustrates the PAR, or excess case load, caused by the individual risk alleles as well as by smoking. Variant *CFH* Y402H provided the highest PAR (50.4%), and smoking had a PAR of 25.4%.

**FIGURE 4:** POPULATION ATTRIBUTABLE RISK FOR THE MAJOR RISK FACTORS OF AMD



## DISCUSSION

We investigated the most established high-risk alleles for AMD in three independent study populations. All variants appeared to carry an independent risk of AMD, except for the *C3* alleles. In our study, *LOC387715* had the highest association, followed by *CFH* Y402H, *FB/C2*, and *CFH* IVS14. Since the allele frequency of Y402H (36%) was higher than the risk allele of the *LOC387715* gene (17%), the *CFH* gene had the highest contribution to the overall disease occurrence (see **Figure 4**). These genetic discoveries have had an enormous impact on the dissection of the pathogenesis of AMD, and have initiated many new lines of research. Nevertheless, these findings do not necessarily imply that genetic testing is relevant for the clinical setting.

In a population of asymptomatic individuals, genetic profiling can predict who will develop late AMD and who will not with an accuracy of 86%. The AUC, or discriminative accuracy, can be interpreted as the probability that the test correctly identifies the diseased subjects from a pair of whom one is affected and one is not. An AUC of 0.86 means that 86% of the pairs is correctly classified whereas a test with an AUC of 0.50 is non discriminative – as accurate as tossing a coin.[126] The AUC for genetic testing for AMD is considerable, *e.g.*, it is higher than that of total serum cholesterol for the prediction of coronary heart disease (77%), and higher than that of neuropsychological testing for the prediction of Alzheimer's disease (81%).[122,127] One can imagine that, if personalized medicine becomes available in the future, simple DNA tests may be used to predict the development of late AMD already at a young age.

In current clinical practice, risk assessments for late AMD are mainly based on the presence of early disease. Our data show that genetic testing can improve the prediction of late AMD. When comparing subjects with late AMD to those aged 80 years with early AMD, the combination of the conventional risk factors age, sex, and smoking had a discriminative accuracy of 63%. Adding genetic testing to the model increased this score to 72%, a significant improvement. Subjects without risk alleles had a lifetime cumulative risk of late AMD that is virtually naught (**Figure 3**), therefore, the specificity (true negatives) of genetic testing appeared to be very high. Individuals with early disease who tested positive for risk alleles may still develop late AMD at a later age. This results in a relatively high proportion of false positives, and thus decreases the AUC. We expect

that our score is considerably lower than the discriminative accuracy, which would be assessed in a population of very old.

What may be the advantages of genetic profiling for patients? We can already use this information to distinguish those who will benefit most from measures such as anti-oxidant therapy and omega-3 supplements. The benefits will be greatly enhanced when more adequate preventive therapies become available. Whether genetic profiling will have a true potential in the future depends on the costs of testing and treatment, adverse effects of therapy, and the social, psychological, and financial burden of disease. Nonetheless, our findings indicate that multiple genetic testing for AMD has a higher predictive value than conventional risk factors, and may become a new clinical assessment for AMD.

## SUPPLEMENTAL MATERIAL

### Rotterdam study – prevalent analyses

| | controls | | Early AMD | | Late AMD |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| **CFH Y402H** | | | | | |
| Noncarrier | 1558 | 127 | 1.00 | 12 | 1.00 |
| Heterozygous | 1644 | 187 | **1.42(1.11-1.82)** | 35 | **2.91(1.47-5.75)** |
| Homozygous | 417 | 114 | **3.66(2.73-4.90)** | 31 | **12.32(5.98-25.36)** |
| *Allele frequency* | *0.340* | *0.485* | | 0.622 | |
| **CFH IVS14** | | | | | |
| Noncarrier | 1069 | 201 | 1.00 | 49 | 1.00 |
| Heterozygous | 1820 | 170 | **0.47(0.37-0.59)** | 25 | **0.26(0.16-0.44)** |
| Homozygous | 773 | 60 | **0.38(0.28-0.52)** | 4 | **0.09(0.03-0.26)** |
| *Allele frequency* | *0.460* | *0.336* | | 0.212 | |
| **LOC387715 A69S** | | | | | |
| Noncarrier | 2408 | 217 | 1.00 | 31 | 1.00 |
| Heterozygous | 1156 | 194 | **2.02(1.62-2.51)** | 35 | **3.03(1.79-5.12)** |
| Homozygous | 115 | 23 | **2.54(1.55-4.18)** | 12 | **11.84(5.24-26.76)** |
| *Allele frequency* | *0.188* | *0.276* | | 0.378 | |
| **FB L9H** | | | | | |
| Noncarrier | 3330 | 392 | 1.00 | 74 | 1.00 |
| Carrier | 331 | 39 | 1.00(0.70-1.45) | 3 | 0.39(0.12-1.27) |
| *Allele frequency* | *0.047* | *0.046* | | 0.019 | |
| **FB rs541862** | | | | | |
| Noncarrier | 3068 | 372 | 1.00 | 73 | 1.00 |
| Carrier | 582 | 49 | **0.67(0.48-0.92)** | 6 | **0.35(0.15-0.84)** |
| *Allele frequency* | *0.083* | *0.058* | | 0.038 | |
| **C3 R102G** | | | | | |
| Noncarrier | 2290 | 238 | 1.00 | 42 | 1.00 |
| Heterozygous | 1221 | 171 | **1.34(1.08-1.67)** | 30 | 1.22(0.74-2.02) |
| Homozygous | 176 | 24 | 1.40(0.88-2.24) | 5 | 1.68(0.62-4.54) |
| *Allele frequency* | *0.213* | *0.253* | | *0.260* | |
| **C3 P314L** | | | | | |
| Noncarrier | 2298 | 237 | 1.00 | 43 | 1.00 |
| Heterozygous | 1190 | 165 | **1.37(1.10-1.71)** | 30 | 1.23(0.75-2.03) |
| Homozygous | 165 | 24 | 1.41(0.88-2.27) | 4 | 1.33(0.45-3.93) |
| *Allele frequency* | *0.208* | *0.250* | | *0.247* | |

[a] adjusted for sex, age

**Rotterdam study – incident analyses**

| | controls | | Early AMD | | Late AMD |
|---|---|---|---|---|---|
| | N(%) | N(%) | HR[a] | N(%) | HR[a] |
| **CFH Y402H** | | | | | |
| Noncarrier | 832 | 206 | 1.00 | 19 | 1.00 |
| Heterozygous | 832 | 238 | 1.18(0.98-1.42) | 42 | **2.52(1.46-4.34)** |
| Homozygous | 194 | 77 | **1.59(1.22-2.07)** | 32 | **7.61(4.30-13.46)** |
| *Allele frequency* | *0.328* | *0.376* | | *0.570* | |
| **CFH IVS14** | | | | | |
| Noncarrier | 530 | 180 | 1.00 | 55 | 1.00 |
| Heterozygous | 925 | 250 | **0.81(0.67-0.98)** | 33 | **0.37(0.24-0.57)** |
| Homozygous | 420 | 91 | **0.64(0.50-0.82)** | 7 | **0.15(0.07-0.32)** |
| *Allele frequency* | *0.471* | *0.415* | | *0.247* | |
| **LOC387715 A69S** | | | | | |
| Noncarrier | 1266 | 324 | 1.00 | 37 | 1.00 |
| Heterozygous | 562 | 186 | **1.20(1.00-1.44)** | 47 | **2.45(1.59-3.77)** |
| Homozygous | 60 | 18 | 1.18(0.74-1.90) | 10 | **6.39(3.16-12.92)** |
| *Allele frequency* | *0.181* | *0.210* | | *0.356* | |
| **FB L9H** | | | | | |
| Noncarrier | 1699 | 478 | 1.00 | 87 | 1.00 |
| Carrier | 175 | 46 | 0.91(0.67-1.23) | 5 | 0.49(0.20-1.22) |
| *Allele frequency* | *0.049* | *0.044* | | *0.027* | |
| **FB rs541862** | | | | | |
| Noncarrier | 1586 | 438 | 1.00 | 86 | 1.00 |
| Carrier | 290 | 77 | 0.95(0.74-1.20) | 7 | **0.42(0.19-0.90)** |
| *Allele frequency* | *0.081* | *0.080* | | *0.038* | |
| **C3 R102G** | | | | | |
| Noncarrier | 1208 | 311 | 1.00 | 52 | 1.00 |
| Heterozygous | 602 | 188 | 1.16(0.97-1.39) | 34 | 1.34(0.87-2.07) |
| Homozygous | 84 | 26 | 1.07(0.72-1.60) | 7 | 1.90(0.86-4.19) |
| *Allele frequency* | *0.203* | *0.229* | | *0.258* | |
| **C3 P314L** | | | | | |
| Noncarrier | 1209 | 315 | 1.00 | 52 | 1.00 |
| Heterozygous | 586 | 183 | 1.16(0.96-1.39) | 34 | 1.45(0.94-2.24) |
| Homozygous | 81 | 21 | 0.90(0.58-1.40) | 7 | 1.93(0.88-4.26) |
| *Allele frequency* | *0.199* | *0.217* | | *0.258* | |

[a] adjusted for sex, age

**Case-control study**

| | controls | | Early AMD | | Late AMD |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| **CFH Y402H** | | | | | |
| Noncarrier | 62 | 16 | 1.00 | 54 | 1.00 |
| Heterozygous | 93 | 35 | 1.49(0.75-2.96) | 124 | **1.66(1.03-2.69)** |
| Homozygous | 19 | 28 | **6.37(2.75-14.73)** | 69 | **5.37(2.75-10.48)** |
| *Allele frequency* | *0.376* | *0.576* | | *0.530* | |
| **CFH IVS14** | | | | | |
| Noncarrier | 65 | 51 | 1.00 | 148 | 1.00 |
| Heterozygous | 88 | 30 | **0.43(0.24-0.75)** | 97 | **0.42(0.27-0.64)** |
| Homozygous | 30 | 7 | **0.29(0.12-0.73)** | 25 | **0.32(0.17-0.60)** |
| *Allele frequency* | *0.404* | *0.250* | | *0.272* | |
| **LOC387715 A69S** | | | | | |
| Noncarrier | 116 | 31 | 1.00 | 102 | 1.00 |
| Heterozygous | 52 | 47 | **3.81(2.12-6.85)** | 97 | **2.05(1.30-3.23)** |
| Homozygous | 6 | 11 | **8.87(2.93-26.87)** | 54 | **13.09(5.14-33.33)** |
| *Allele frequency* | *0.181* | *0.210* | | *0.356* | |
| **FB L9H** | | | | | |
| Noncarrier | 157 | 82 | 1.00 | 263 | 1.00 |
| Carrier | 26 | 6 | 0.47(0.18-1.19) | 7 | **0.18(0.07-0.43)** |
| *Allele frequency* | *0.071* | *0.034* | | *0.013* | |
| **FB rs541862** | | | | | |
| Noncarrier | 144 | 82 | 1.00 | 239 | 1.00 |
| Carrier | 27 | 5 | **0.36(0.13-0.99)** | 16 | **0.35(0.18-0.70)** |
| *Allele frequency* | *0.079* | *0.029* | | *0.037* | |
| **C3 R102G** | | | | | |
| Noncarrier | 123 | 55 | 1.00 | 142 | 1.00 |
| Heterozygous | 37 | 24 | 1.47(0.78-2.75) | 86 | **2.08(1.28-3.37)** |
| Homozygous | 8 | 5 | 1.48(0.45-4.92) | 19 | 2.20(0.90-5.37) |
| *Allele frequency* | *0.158* | *0.202* | | *0.251* | |
| **C3 P314L** | | | | | |
| Noncarrier | 114 | 49 | 1.00 | 130 | 1.00 |
| Heterozygous | 42 | 28 | 1.55(0.85-2.84) | 99 | **2.06(1.29-3.29)** |
| Homozygous | 10 | 5 | 1.09(0.34-3.49) | 20 | 1.54(0.67-3.56) |
| *Allele frequency* | *0.187* | *0.232* | | *0.279* | |

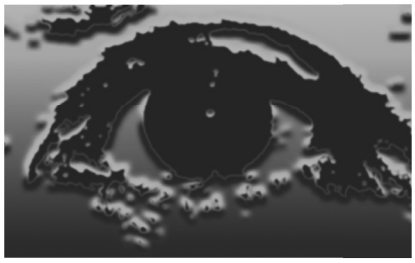
[a] adjusted for sex, age

## GRIP study

| | controls | | Early AMD | | Late AMD |
|---|---|---|---|---|---|
| | N(%) | N(%) | OR[a] | N(%) | OR[a] |
| **CFH Y402H** | | | | | |
| Noncarrier | 228 | 28 | 1.00 | 15 | 1.00 |
| Heterozygous | 222 | 32 | 1.23(0.70-2.17) | 40 | **9.02(2.86-28.42)** |
| Homozygous | 79 | 20 | **2.06(1.06-4.00)** | 27 | **15.92(4.29-59.11)** |
| *Allele frequency* | *0.359* | *0.450* | | *0.573* | |
| **CFH IVS14** | | | | | |
| Noncarrier | 173 | 39 | 1.00 | 50 | 1.00 |
| Heterozygous | 255 | 32 | **0.55(0.32-0.94)** | 28 | **0.17(0.07-0.43)** |
| Homozygous | 98 | 9 | **0.43(0.19-0.96)** | 4 | **0.05(0.01-0.31)** |
| *Allele frequency* | *0.429* | *0.313* | | *0.220* | |
| **LOC387715 A69S** | | | | | |
| Noncarrier | 382 | 47 | 1.00 | 33 | 1.00 |
| Heterozygous | 132 | 24 | 1.24(0.71-2.18) | 33 | **3.20(1.32-7.76)** |
| Homozygous | 15 | 10 | **4.77(1.87-12.13)** | 14 | **15.81(2.73-91.44)** |
| *Allele frequency* | *0.153* | *0.272* | | *0.381* | |
| **FB L9H** | | | | | |
| Noncarrier | 425 | 66 | 1.00 | 71 | 1.00 |
| Carrier | 99 | 11 | 0.88(0.43-1.78) | 6 | 0.54(0.14-2.02) |
| *Allele frequency* | *0.097* | *0.078* | | *0.039* | |
| **FB rs541862** | | | | | |
| Noncarrier | 457 | 72 | 1.00 | 79 | 1.00 |
| Carrier | 69 | 8 | 0.54(0.23-1.25) | 2 | **0.03(0.003-0.23)** |
| *Allele frequency* | *0.068* | *0.050* | | *0.012* | |
| **C3 R102G** | | | | | |
| Noncarrier | 385 | 50 | 1.00 | 40 | 1.00 |
| Heterozygous | 130 | 24 | 1.27(0.73-2.22) | 30 | 1.87(0.75-4.65) |
| Homozygous | 17 | 6 | 2.86(0.98-8.29) | 11 | **12.66(2.57-62.43)** |
| *Allele frequency* | *0.154* | *0.225* | | *0.321* | |
| **C3 P314L** | | | | | |
| Noncarrier | 385 | 52 | 1.00 | 41 | 1.00 |
| Heterozygous | 119 | 23 | 1.24(0.70-2.18) | 29 | 1.84(0.73-4.64) |
| Homozygous | 14 | 4 | 2.28(0.64-8.10) | 10 | **21.40(3.07-149.09)** |
| *Allele frequency* | *0.142* | *0.392* | | *0.306* | |

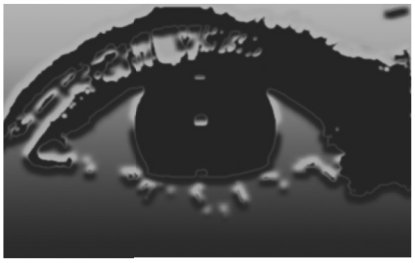
[a] adjusted for sex, age

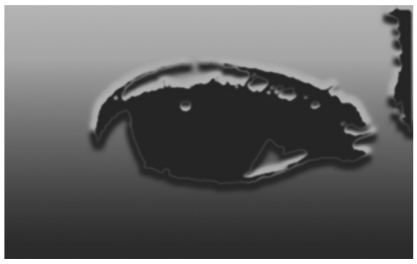
The disease in each person was classified according to the highest stage of AMD in either eye. Controls were defined as those who were diagnosed with stage 0 and no other macular pathology in both eyes. Early AMD was defined as stage 2 or stage 3 AMD. Late AMD was defined as stage 4 AMD in the eye with the more severe stage. The ORs and HRs are estimates of the relative risk of AMD, and represent the risk of disease (AMD vs. stage 0) in the genetic risk group divided by the risk of disease (AMD vs. stage 0) in the non-risk group (noncarriers).



# PART III

## GENERAL DISCUSSION





## GENERAL DISCUSSION

The work presented in this thesis aimed at further enlightening the genetic background of AMD. We studied the influence of variations in genes that may affect AMD, and analyzed the value of genetic profiling to predict the outcome of patients. In the previous chapters, the merits and limitations of each study have been described in detail. In the current chapter, the main findings of this thesis are recapitulated and placed in a broader perspective. Special emphasis will be given on the role of genetic research in unraveling the pathobiology of AMD, and the implications of our results for clinical practice and future research will be discussed.

### Which Genes are Implicated in AMD Etiology?

During the past two decades, much effort has been made to identify genes in complex human genetic disorders like AMD. Complex diseases are not caused by single genes, but are the result of the interplay between several interacting genes and environmental factors. Dissecting the genetics of AMD has been difficult due to the late age of onset of the disease and identification of criteria for disease that go beyond normal aging. Nevertheless, despite these challenges, investigators have made tremendous progress. Candidate gene studies, linkage studies and genome wide association studies have been utilized to elucidate the genetic background of AMD. **Table 1** lists the genes that have been found associated with the disease. The role of each of these genes will be discussed in the following paragraphs.

**Table 1:** Important genes in AMD etiology

| Gene | Location | Mechanism/rationale | Risk allele | Minor allele frequency | Population attributable risk | Year of first publication |
|---|---|---|---|---|---|---|
| *CFH* | 1q32 | inhibits activation of the complement pathway by binding and inactivating complement component C3b | Y402H<br>A473A<br>V62I<br>IVS14 | 0.36<br>0.42<br>0.27<br>0.46 | 54.0%<br>protective<br>protective<br>protective | 2005 |
| *LOC387715* | 10q26 | LOC387715: oxidative stress | A69S | 0.17 | 41.9% | 2005 |
| *C3* | 19p13 | Central enzyme in the complement cascade, where the classical, alternative, and lectin pathways converge | R102G/P314L | 0.21 | 14.6% | 2007 |
| *ABCA4* | 1p21-p13 | ATP-binding protein transports vitamin A derivatives | G1961E<br>D2177N | 0.003<br>0.006 | 1.3%<br>1.1% | 1997 |
| *C2/FB* | 6q21 | C2: activator of the classical complement pathway, FB: activator of the alternative complement pathway | L9H<br>R32Q | 0.04<br>0.11 | protective | 2006 |
| *APOE* | 19q13 | transports lipids and cholesterol in the central nervous system | ε4 | 0.13 | protective | 1998 |

***CFH***

In the same issue of *Science* in March 2005, three separate case-control studies simultaneously described a strong association between the *CFH* Y402H variant and AMD.[37-39] This first major risk gene for AMD was found by Klein *et al.* using a genome-wide testing strategy with an Affymetrix GeneChip Mapping 100K Set of microarrays.[37] The other two reports found this association by screening several SNPs in the 1q25-31 region which was previously associated with AMD in several linkage studies.[38,39] The *CFH* gene is a key regulator of the complement cascade inhibiting amplification of the cascade. The Y402H variant leads to a tyrosine-to-histidine substitution which is located in a binding site for CRP, heparin, and M-protein. In the population-based Rotterdam study,[55] we found this polymorphism to be highly associated with early as well as all subtypes of late AMD. The risks increased with each successive stage, up to an OR of 11.02 for vision-disabling disease. Individuals homozygous for the *CFH* Y402H polymorphism had an absolute lifetime risk of 48% to develop late AMD, while for noncarriers this risk did not exceed 22%. The effect of *CFH* Y402H was significantly influenced by smoking and environmental factors of chronic as well as acute inflammation. Furthermore, our data suggested a gene-gene interaction between *CFH* and *CRP*: we found a significant effect modification between Y402H and *CRP* haplotypes that determine CRP serum levels after an inflammatory stimulus.

Although all AMD studies in Caucasians demonstrated that Y402H is the most prominent non-synonymous SNP in the coding region,[40,58,63] several studies suggested that Y402H may not be the most important *CFH* variant for AMD.[58,59] A few synonymous and intronic SNPs had higher associations, and several protective *CFH* haplotypes have been associated with the disease.[40,58,59] To determine which variant or combination of variants described the risk between *CFH* and AMD most accurately, we did a comprehensive analysis of the coding region of *CFH* in a Dutch case-control study. Univariately the strongest association was observed for Y402H. An independent German study showed the highest association in the univariate analysis for IVS10 (rs203674). We identified two highly associated LD blocks in the gene, which comprised both one protective and one causative haplotype. Variants within each block independently influenced the risk of AMD, as did variants located outside the blocks. In line with other reports, our data showed that, apart from Y402H, other variants including IVS1, V62I and A473A appear as independent susceptibility alleles in the coding region of the gene.

### *LOC387715*

The 10q26 locus is the second major risk locus contributing to AMD pathogenesis. This second region was again repetitively shown to be associated with AMD in linkage studies. The region was narrowed down to a region containing the *PLEKHA1, LOC387715,* and HTRA1/*PRSS11* genes by Jakobsdottir *et al.* who performed a focused SNP genotyping study of 594 families and an additional case-control study.[23] In addition, Rivera *et al.* screened 95 SNPs in this region in two independent case-control studies and found the highest association for A69S, an alanine-to-serine substitution in the hypothetical *LOC387715* gene, with odds ratios up to 8.21 for homozygous persons.[49] Several studies suggested that this *LOC387715* locus contributed to AMD independent of Y402H,[49,128] and some studies suggested a synergistic effect between *LOC387715* and smoking.[128,130] In the pooled data-analysis of the Rotterdam Study, the case-control study and the GRIP study, we also found a significant association between the *LOC387715* A69S variant and AMD and the ORs increased in an allele-dose manner with OR 2.03(1.44–2.88) for early AMD, and OR 9.91(6.46–15.20) for late AMD for homozygous persons. In our study, we detected a significant interaction between *LOC387715* and *CFH* at the additive model, but not at the multiplicative level. In contrast to previous studies, we did not find an interaction between *LOC387715* and smoking.

Two recent studies, one in a Chinese population[56] and one in a Caucasian population,[57] located a second SNP in the promoter region of the neighboring *HTRA1* gene. This promoter SNP was in almost complete linkage with A69S and had a higher association with AMD than the A69S variant. The SNP potentially modulates expression levels of the *HTRA1* gene, influencing extracellular matrix degradation. Unfortunately, the study of Dewan et al. compared only persons with neovascular AMD with controls. The study of Yang *et al.*, comprised persons with neovascular AMD, early AMD and controls. Since they did not include geographic atrophy, it cannot be concluded from these data that *HTRA1* is a gene for neovascularization, as has been proposed in the literature.

In October 2007, a case-control study comprising 466 cases and 280 controls evaluated 45 SNPs across the 10q26 region, including the *LOC387715* A69S variant and the promoter SNP in *HTRA1*.[131] Using conditional statistical analysis, they demonstrate that primary association can be explained by the A69S SNP, and that the strong association for the promoter SNP in *HTRA1* can be explained by the statistical correlation with A69S. These data

suggest that *LOC387715,* and not *HTRA1,* is the AMD gene in the 10q26 locus. Further research is needed to clarify these conflicting findings.





### *C2/FB*

Gold and colleagues,[72] reported a strong association with two important activators of the complement pathway, factor B (FB) and complement component 2 (C2). They identified two protective haplotypes, one containing the *FB* L9H variant, and the other containing the *FB* R32Q variant. In the pooled data analysis of the Rotterdam study, the case-control study and the GRIP study, we confirmed this association and found an OR of 0.34(0.20–0.56) for *FB* L9H, and an OR of 0.32(0.20–0.49) for the *FB* rs541862 variant – a SNP reported to be in complete linkage disequilibrium (D'=1.00, $r^2$=1.00) with R32Q in Hapmap. These results extend the magnitude of the complement pathway in the pathobiology of AMD.

### *C3*

Complement component C3 is the central element of the complement cascade and exerts its effect where the three activation pathways of the complement cascade converge. An association between two variants in the *C3* gene and AMD was recently detected using candidate gene analysis in a British case control study and an American case-control study.[79,80] We sought to confirm this finding in the population-based Rotterdam Study as well as in an independent Dutch case-control study. Our findings verify an association with the R102G allele and the P314L allele and AMD. Meta-analysis on all currently available data yielded a pooled OR of 1.61 (95%CI 1.46–1.78) for the R102G allele, and OR 1.50 (95%CI 1.31–1.71) for the P314L allele. Both alleles significantly increased the risk of early AMD and all subtypes of late AMD, and this risk appeared independent of *CFH* Y402H, *LOC387715* A69S, and smoking. These findings further highlight the crucial role of the complement pathway in the etiology of AMD.

### *APOE*

Apolipoprotein E (APOE) is a lipid transport protein that acts as a ligand for the low density lipoprotein (LDL) receptor. It is also involved in the repair and maintenance of neuronal cell membranes in the peripheral and central nervous system, playing a pivotal role in the reinnervation process following injury.[132-134] The *APOE2* gene is polymorphic and has three common alleles: ŋ2, ŋ3 and ŋ4, in which the ŋ3 is considered to be the ancestral

allele. In 1998 two studies demonstrated a protective effect of the ŋ4 allele and a detrimental effect of the ŋ2 allele on AMD: Klaver *et al.* described this association in a nested case-control study in the population-based Rotterdam study[135] and Souied *et al*. found a similar effect in a French case-control study.[136] In 2006, Thakkinstian *et al*. published a meta-analysis on all available data reported and confirmed the protective effect of the ŋ4 allele on AMD.[137] This finding is in contrast to other complex diseases such as Alzheimer's disease, atherosclerosis, multiple sclerosis, and stroke, which show a strong positive association of APOE4 allele with disease.[138-141] In addition, contrary to the expectation, eyes of aged, targeted replacement mice expressing human APOE4 and maintained on a high fat cholesterol-rich diet show a constellation of changes that mimic the pathology associated with human AMD. This difference between mice and men could arise from the effect of diet as a modifier of the APOE allele effect which was not investigated in human studies, or there might be a difference in mouse and human physiology. In any case, more functional studies are necessary to asses the exact role of APOE in the pathophysiology of AMD.

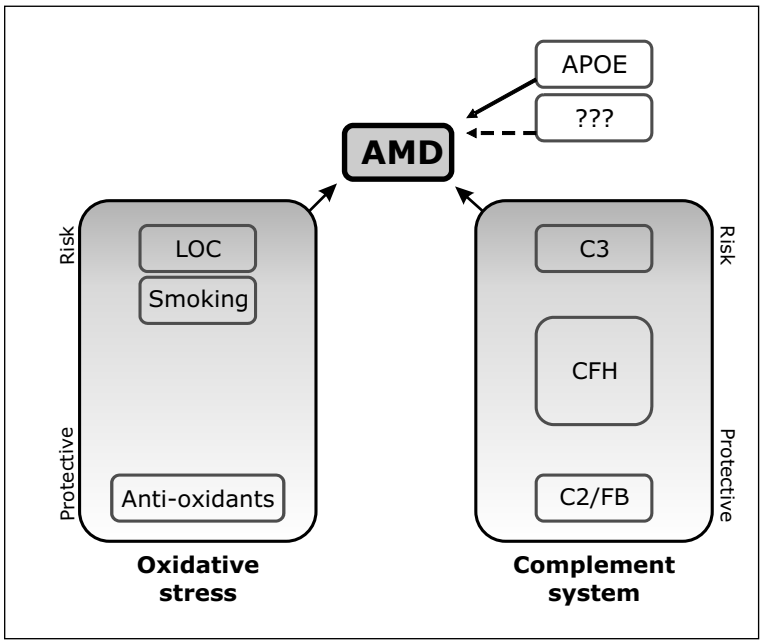
***ABCA4***

*ABCA4 (ABCR)* has long been a popular candidate gene for AMD research because this gene is critical in Stargardt disease, the most common form of hereditary, recessive macular degeneration. *ABCA4* encodes an ATP-binding transporter protein.[142] In the absence of a functional *ABCA4* gene, N-retinylidine-PE accumulates within the outer segment disks followed by formation of N-retinylidine-N-retinylethanolamine (A2-E), the major component of lipofuscin.[143] Consequently, abnormally high levels of lipofuscin accumulate in the RPE, triggering RPE-cell death and cause secondary photoreceptor degradation. Allikmets *et al*. first described an association between *ABCA4* and AMD in 1997,[144] however, a small number of studies could not replicate the initial findings. A consortium study led by Allikmets, consisting of subjects from seven centers in the USA and eight centers in Europe, screened 1218 AMD patients and 1258 controls for the G1961E and D2177N variants in the ABCA4 gene.[145] They reported an OR of 5.0 (1.6–20) for G1961E and an OR of 2.8 (1.2–7.4) for D2177N, indicating a possible role for *ABCA4* in AMD. Due to the low frequency of the risk allele (**Table 1**) this gene is not a major contributor to the total disease occurence.

### How do these genes give insight into the pathobiology of AMD?

The dream of a genetic epidemiologist is that identifying the genetic basis of a complex disease will provide a detailed view at the underlying pathogenesis of a condition and suggest possibilities for therapeutic intervention. During the course of my research project, the major genetic risk factors for AMD were elucidated. The discovery of these genes has greatly contributed to the understanding of the pathophysiology of AMD. Two main pathways play a crucial role in AMD pathobiology:

**Figure 1:** Genes have been implicated in AMD susceptibility – 2 major pathways play a pivotal role




## AMD is an inflammatory disease

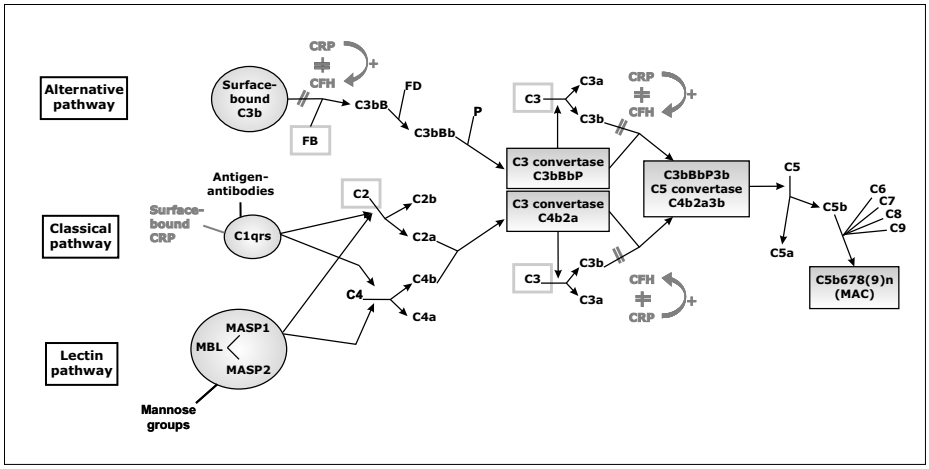
From a histopathologic point of view, the earliest detectable changes associated with AMD are focal deposition of extracellular material, *i.e.* drusen, which occur at the interface between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane. Extensive immunocytochemical analysis of these extracellular deposits revealed

several components of the inflammatory process, in particular late stage, activated complement components, including the C5b-9 complex, and MHC class II antigens, immunoglobulin lambda chains and anaphylatoxins.[32,146,147] The identification of the *CFH* gene, a regulator of the complement pathway, as the first major genetic risk factor in AMD supported the inflammatory pathogenesis for AMD. More recent genetic studies also implicated *C2/FB*, and *C3* in the etiology of AMD. This further corroborated the role of inflammation, in particular the complement system in AMD pathophysiology.

All organisms are continuously challenged by a variety of infectious microbial agents. Therefore, the simplest up to the most complex organisms have developed defense mechanisms, to block assaults from hostile micro-organisms. In higher vertebrates this resulted in the development of an immune system consisting of an innate and an adaptive arm. The innate arm, an evolutionary ancient system, is designed to respond immediately to invading pathogens. In contrast, the adaptive arm, which is specific to vertebrates, responds in a highly specific way to a microbial challenge and produces T and B lymphocytes in order to control an infection.

The complement system is part of the innate immunity and recognizes, attacks and kills invading micro-organisms. It consists of the classical, lectin and alternative activation pathway, which converge on a final common or terminal pathway. The classical pathway is initiated by antigen-antibody complexes and surfaced-bound CRP; the lectin pathway is turned on by mannose groups of microbial carbohydrates; and the alternative pathway, the most rudimentary and non-specific pathway, is activated by C3b bound to self as well as microbial cells. The pathways converge at the point in which C3 is cleaved into C3a and C3b by C3 convertase, which initiates C5 convertase, resulting in the formation of the membrane-attack complex with the terminal components (C5b-C9).

**Figure 2:** The complement system




Complement factor H specifically inhibits the alternative complement cascade but also regulates the common pathway. It binds C3b and acts as a cofactor in the proteolysis of C3b by factor I resulting in an inactive C3b molecule. This inhibits the production of C3 convertase in the alternative cascade as well as the production of C5 convertase in the common pathway. As a result, CFH interferes with progression of the entire cascade.[44,50]

CFH contains 20 short-consensus repeats that contain binding sites for C3b, heparin, sialic acid, and CRP. These short-consensus repeats (SCR) are composed of ~ 60 amino acids each.[50] The repetitively associated Y402H variant of *CFH* is located in SCR7, which has been implicated in the binding of heparin, CRP and streptococcal M protein. Binding of heparin and CRP increases the affinity of CFH for C3b, enhancing CFHs ability to inhibit complement.[31,148]

The CFH protein was shown to accumulate within drusen and is synthesized by the retinal pigment epithelium.[40] Recently, functional studies in mice showed that *CFH* knock-out animals had reduced rod responses, increased autofluorescent subretinal deposits, accumulation of complement C3 in the neuroretina, thinning of Bruch's membrane, and disorganization of photoreceptor outer segments.[149] Although other mice models have displayed more typical hallmarks of AMD,[34] these results imply that the CFH protein is necessary for maintenance of normal retinal physiology. Regarding the effect of the Y402H polymorphism, a study on 28 donor eyes showed that individuals homozygous for Y402H had elevated levels

of CRP in the choroid, but no differences were found in CFH-levels.[150] Two recent in vitro study showed that this variant reduced the binding to CRP, heparin, and retinal pigment epithelium cells.[71,151] This finding is supported by our data describing gene-gene interaction between *CFH* and *CRP.* The reduced CFH-CRP binding may decrease CFH inhibition, jeopardize the negative feedback mechanism, and result in uncontrolled progression of the complement cascade. Evidence for increased complement activation in patients with AMD is obtained since Scholl *et al.* found increased levels of the activated FB, C3 and the membrane-attack complex in plasma (personal communication).

The environmental risk factor smoking shows a significant interaction with the CFH gene.[55,152,153] This can be explained by the fact that smoking increases cytokines and inflammatory cells and has been shown to activate the complement pathway by weakening the susceptibility of C3 to CFH and factor I.[51,52] Complement progression may be further accelerated in smokers particularly when CFH inhibition is already genetically impaired.

Not all SNPs in the CFH gene result in an increased risk for AMD, *i.e.* V62I and IVS14. Moreover, several haplotypes have been identified in the *CFH* gene that have a protective effect on AMD.[40,58,63,154] In addition, deletions of *CFHR1* and *CFHR3* seem to be protective against AMD and independent of Y402H.[69,70] Hughes and coworkers suggested that this protective effect might be due to elimination of the misregulation of the complement pathway by the many *CFH*-related transcripts which reduced the competition for the binding of CFH to C3b. The functional implications of the additional variants in the *CFH* gene, and the speculation concerning the *CFH*-related genes should be further investigated in functional studies.

Subsequent screening of other complement components identified variants in the *FB* and *C2* genes that are protective.[72] Gold et al hypothesize that the variants may reduce the enzymatic activator activity of the proteins and subsequently lead to a lower risk for chronic complement response. As previously described, variants in the *C3* gene were also associated with AMD. It is hypothesized that the variants in this gene introduce conformational changes in the C3 protein, influencing binding to pathogens and other cell surfaces, and therefore increase complement activation, ultimately leading to cell lysis and AMD.

This evidence strongly supports the inflammatory origin of AMD. However, a few questions remain to be answered:

1. Are drusen the consequence of complement activation – or alternatively – do drusen induce complement activation?
2. The currently identified complement genes all encode regulatory enzymes. The initiator of AMD remains unknown. Maybe there is no exact initiator of AMD. Does a lifelong exposure to complement activation – activated as a consequence of anomalous infections such as respiratory infections – eventually lead to an excessive immunological response attacking host cells?
3. Why is the disease limited to the eye? The complement cascade and especially the amplification loop are very efficient in producing a large number of complement activation products on the target surfaces. Therefore, it is critical that this system is tightly regulated and directed only against foreign structures and not against viable host cells. The complement activation on host cells is tightly regulated by the combined action of membrane-bound and soluble regulators. When the function of CFH, a soluble complement regulator, is jeopardized, complement inhibition is generally taken over by other membrane-bound regulators. One could speculate that the eye, and especially the macula, a highly specialized region which is very different in structure from the rest of the retina, lacks membrane-bound complement regulators. Thus, CFH might be the critical regulator of complement activation in the macula. Although impaired CFH function only causes clinical significant disease in the eye, increased serum levels of activated complement products indicate that systemic complement activation is present. Yet this doesn't appear to cause overt clinical disease in other organs.

**Oxidative stress is important in AMD**

There is a general consensus that cumulative oxidative damage is responsible for aging, and may, therefore, play an important role in the pathogenesis of AMD. Oxygen-derived metabolites are known to cause oxidative damage to cytoplasmic and nuclear elements of cells and cause changes in the extra-cellular membrane.

According to Beatty and colleagues,[114] the retina is an ideal environment for the generation of free radicals and other reactive oxygen intermediates for the following reasons:

1. Oxygen consumption by the retina is much higher than by any other tissue.
2. The retina is subject to high levels of cumulative irradiation.
3. Photoreceptor outer segments membranes are rich in polyunsaturated fatty acids, which are readily oxidized and which can initiate a cytotoxic chain-reaction.
4. The retina and the RPE contain an abundance of photosensitizers.
5. The process of phagocytosis by the RPE is itself an oxidative stress and results in the generation of reactive oxygen intermediates.

*In vitro* studies indicated that lipofuscin in the RPE, which is continuously exposed to visible light and high oxygen tension, is a photoinducible generator of reactive oxygen species that can compromise lysosmal integrity, induce lipid peroxidation, reduce phagocytic capacity, and cause RPE cell death.[155,156] Lipofuscin is derived in part from vitamine A metabolites and lipid peroxides, and A2-E is the major photosensitizing chromophore in lipofuscin that causes reactive oxygen species production.[157] When RPE cells are exposed to light, A2-E conjugates to low-density lipoprotein, which accumulates in RPE lysosomes. RPE cells with excessive A2-E exhibit membrane blebbing and extrusion of cytoplasmic material into the Bruch's membrane, *i.e.* drusen. In AMD oxidative stress eventually results in RPE and possibly choriocapillaris injury, which in turn elicits an inflammatory response in the Bruch's membrane and the choroid.

Smoking is considered the largest environmental risk factor for AMD. Besides its previously described modifying effect on the complement pathway, smoking has an additional alternative mechanism in AMD pathogenesis. Smoking induces oxidative stress: it depresses antioxidants (e.g. decreases plasma vitamin C and carotenoids), induces hypoxia and free radicals, and alters choroidal blood flow. Recent longitudinal studies showed that smoking is in particular positively associated with geographic atrophy [OR 10.3 (2.7–39.1) for current smokers] suggesting a destructive effect of smoking on the retinal pigment epithelium.[158] Additionally several studies showed that risks decreased in those who ceased smoking more than 20 years ago to a risk similar to that of never smokers.[158,159] The pivotal role of the oxidative stress pathway in the etiology of AMD is further emphasized by the findings that the risk of developing advanced AMD is greatly reduced by supplementation with high doses of antioxidant vitamins and minerals[160] or a high dietary intake of antioxidants.[11]

Current genetic data appear to support this hypothesis. Recent evidence showed that *LOC387715*, the second major genetic risk factor for AMD, encodes a protein which is highly expressed in human placenta tissue, and moderately expressed in the retina. By means of extensive experiments the protein was located to the outer membrane of the mitochondria. No significant differences in the expression stability or localization of the A69S variant LOC387715 protein were observed in mammalian cells, but Kanda et al. speculate that it is plausible that the A69S variant modifies the function of the LOC387715 protein by affecting its conformation and/or interaction. These results demonstrate how A69S may influence AMD susceptibility. Mitochondrial dysfunction associated with aging can result in impairment of energy metabolism and homeostasis, generation of free radicals and activation of the apoptotic pathway.[161,162] A decreased number and size of mitochondria, and aberrant mitochondrial morphology has been observed in AMD retina compared to control, suggesting a role for LOC387715 in AMD via this pathway. Additional analysis of LOC387715 and the A69S variant and its function in vivo should further enlighten its contribution to AMD pathogenesis. Considering this, it is remarkable that no associations have been found between known oxidative stress genes such as PON1 and SOD2, and AMD.[163-166]

## What Are the Implications of Genetic Research for Clinical Practice?

The exceptionally high odds ratios found for genes involved in AMD bear the promise of the benefit and usefulness of genetic knowledge in preventive therapy. However, skepticism exists regarding the value of genetic testing for complex diseases in which multiple genes interact with environmental risk factors.

We hypothesized based on the data described in chapter 7, that there might be merit in genomic profiling for AMD in the future. First, we calculated the additional discriminative accuracy of multiple genetic testing in a general population. Our data showed that, for instance, in a person aged 20 years, the first predictor to discriminate whether he/she will develop end-stage AMD is increasing age (AUC = 77%). Smoking has no significant additional value for the prediction of late AMD, but multiple genetic testing increases the discriminative accuracy to a height of 86%.

Nevertheless, predictive genetic testing is only useful in clinical practice when it has an additional value to the existing risk predictors for AMD: age, smoking and a phenotype of early AMD. We therefore compared the discriminative accuracy of genetic profiling comparing patients with late AMD with older subjects (> 80 yrs) with early AMD. Again, the predictive value of multiple genetic testing (72%) greatly exceeded that of age, and smoking (61%).

These results are very promising, and this information can already be used to specifically target those who will benefit most from current preventive therapies such as anti-oxidant vitamins. However, the benefits will be greatly enhanced when preventative or curative treatments targeting specific pathways become available, *e.g.* treatments specifically targeting complement activation may only be of particular benefit in those with genetic variations in the complement genes. Whether genetic profiling will have a true potential in the future greatly depends on the financial burden of disease, treatment and genomic profiling, as well as on the adverse effects of future therapeutic interventions. Studies exploring the interactions between specific treatment regimens, environmental risk factors, and patient genotype are needed and will have great potential for future patient care.

## Future Research

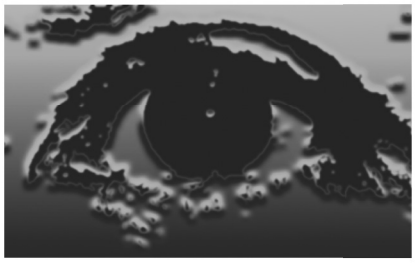
The progress made in unraveling the genetic basis of AMD over the last few years, reveals a great spectrum of new challenges.

1. Further research is needed to determine additional susceptibility genes. The in-depth evaluation of the linkage regions have been proven to be an effective approach to find AMD genes. Besides the 1q and 10q regions, the genome-scan meta-analysis[30] also found evidence for linkage on chromosomes 2p, 3p, 4q, 12q and 16q, which are still open for future genetic epidemiologic research. Another potentially successful approach might be to perform a genome wide association study in a population-based study like the Rotterdam Study, which holds a great promise in detecting other genes important in AMD etiology. Moreover, both meta-analyses of published studies and association studies pooling together patients from multiple sources should be conducted to achieve large study samples. The increased power of such studies, would allow us to

analyze differences in the genetic background of the different subtypes of late AMD. These studies can also provide enough power to further analyze gene-gene and gene-environment interactions, which will provide additional knowledge on the complex nature of AMD.

2. In addition, there will be great value in protein expression studies and functional studies to examine the role of the currently found genes, and to determine the implications of the different polymorphisms and how the protein structure and function is changed.
3. And last but certainly not least, although the new anti-VEGF therapy is very promising in neovascular endstage AMD, there is need for new therapeutic agents. Preferably agents that focus on the currently unapplied knowledge that inflammation plays a pivotal role in AMD. Such therapies may improve the preventive measures available to date and create...

new hope for old eyes!



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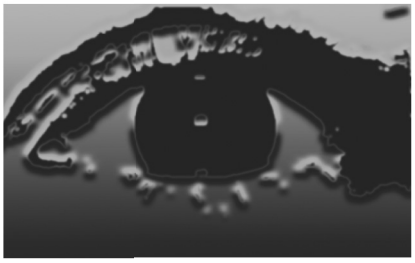

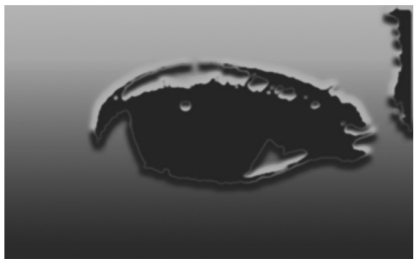

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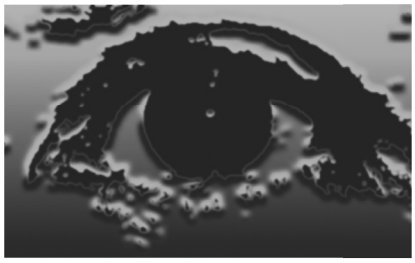
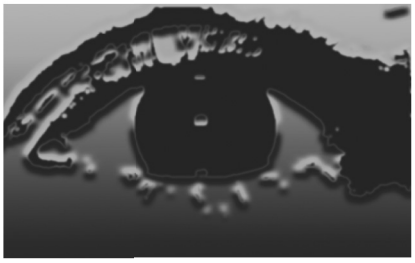
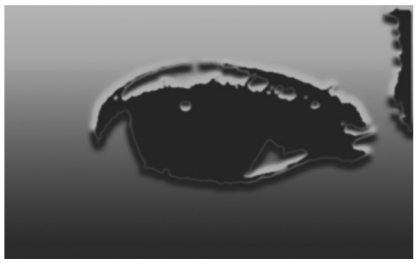


# SAMENVATTING





## SAMENVATTING

Leeftijdsgebonden maculadegeneratie is een ingrijpende aandoening van het netvlies en veroorzaakt een ernstig verlies van het centrale zien waardoor patiënten niet meer kunnen autorijden, geen gezichten herkennen, moeilijk TV kunnen kijken en niet kunnen lezen. Het is de meest voorkomende oorzaak van blindheid in de Westerse Wereld. Ondanks recente wetenschappelijke doorbraken is slechts voor een klein deel van de patiënten een behandeling voorhanden en ook daarvan zijn de lange termijn effecten nog niet duidelijk. Veel studies hebben aangetoond dat leeftijdsgebonden maculadegeneratie erfelijk is. Het doel van dit proefschrift was om de kennis omtrent de genetische oorzaak van leeftijdsgebonden macula degeneratie te vergroten. Hiertoe maakten wij gebruik van drie grote studies:

1. de ERF/GRIP studie: een studie in een genetisch isolaat
2. de ERGO studie: een algemeen bevolkingsonderzoek waarbij alle personen van 55 jaar en ouder uit een deelgemeente van Rotterdam werden uitgenodigd en gedurende 15 jaar werden gevolgd
3. een patiënt-controle studie: een studie waarbij patienten via de kliniek of via voorlichtingsdagen werden uitgenodigd voor deelname en vergeleken met mensen die geen enkel teken hadden van maculadegeneratie (controle personen)

### Deel I: Genetisch risico op leeftijdsgebonden maculadegeneratie

HOOFDSTUK 1 levert bewijs voor een cruciale rol van het *CFH* gen in leeftijdsgebonden maculadegeneratie. Dit gen heeft een belangrijke functie in ons afweersysteem, dat ons normaal gezien beschermt tegen virussen en bacteriën. Soms valt ons afweersysteem echter de cellen van de mens zelf aan. Om dit te vermijden zijn er allerlei eiwitten die dit systeem in bedwang houden, waaronder CFH. Wij toonden aan dat personen die een afwijking hadden in dit gen een 11x hoger risico hadden om leeftijdsgebonden maculadegeneratie te ontwikkelen. Als mensen daarbij rookten steeg hun risico tot 40x. Als je de risico verhogende variant in het *CFH* gen hebt, dan heb je op de leeftijd van 95 jaar ~50% kans om ernstige maculadegeneratie te hebben.

In HOOFDSTUK 2 bekeken we het *CFH* gen wat meer gedetailleerd. Er zijn veel variaties in dit gen. Sommigen zorgen voor een verhoging van het risico

op leeftijdsgebonden maculadegeneratie. Anderen zijn weer beschermend. Uit dit onderzoek bleek dat het genetisch risico van het *CFH* gen op AMD niet door één variant bepaald wordt, maar dat meerdere varianten hiervoor verantwoordelijk zijn.

HOOFDSTUK 3 beschrijft het risico van het *C3* gen, een ander gen dat belangrijk is voor ons afweersysteem. In deze analyse lieten we zien dat ook afwijkingen in dit gen een verhoogd risico op leeftijdsgebonden maculadegeneratie teweegbrengen. Dit onderzoek bevestigt de rol van het afweersysteem in deze oogziekte.

In HOOFDSTUK 4 onderzochten we of genen die belangrijk zijn bij het aanzwengelen van het immuunsysteem (*TLR4*, *CCL2* en *CCR2*) ook een rol speelden bij leeftijdsgebonden maculadegeneratie. Wij konden geen relatie vinden tussen deze genen en de oogziekte. Verder waren er geen veranderingen in de expressie van deze genen te vinden. Het lijkt er dus op dat zij geen rol spelen in leeftijdsgebonden maculadegeneratie.

HOOFDSTUK 5 laat zien dat ook het *ERCC6* gen, belangrijk in het herstel van DNA-schade, geen grote rol speelt in het ontstaan van leeftijdsgebonden maculadegeneratie. Dit gen is belangrijk in Cockayne syndroom, een syndroom waarbij mensen heel vroeg oud worden en waar ook afwijkingen worden gezien aan het netvlies. In muizen veroorzaakten defecten in dit gen ook netvliesafwijkingen. Wij konden echter geen consistente relatie aantonen met leeftijdsgebonden maculadegeneratie.



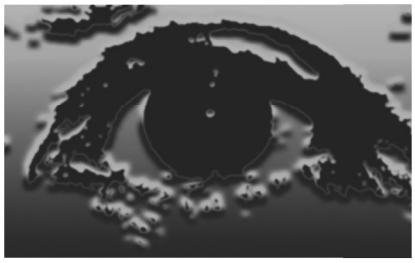


**Deel II: Voorspellende waarde van het testen van genen voor leeftijdsgebonden maculadegeneratie**

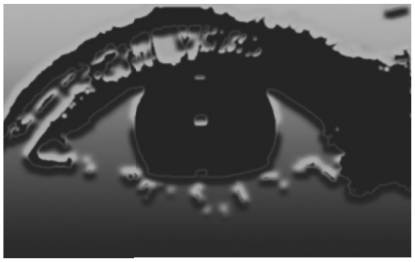
HOOFDSTUK 6 opent de discussie over de rol van genen in de klinische praktijk. Het vinden van de genen die een rol spelen in het ontstaan van een ziekte betekent een grote stap voorwaarts in het begrijpen van de ziekte. Zeker genen die een groot risico hebben op de ziekte zijn enorm waardevol. Echter, als de afwijkingen in die genen enkel voorkomen in een beperkt aantal personen, betekent dit niet persé dat ze ook waardevol zijn om de gehele populatie te screenen en gebruikt kunnen worden om te voorspellen wie ziek wordt en wie niet.

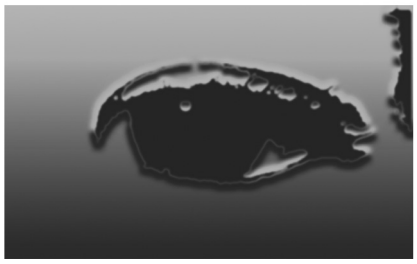
HOOFDSTUK 7 schetst de rol van genetisch testen in de voorspelling van leeftijdsgebonden maculadegeneratie. In onze studies hebben we alle bekende en belangrijke genen voor leeftijdsgebonden maculadegeneratie getest. We konden aantonen dat bepaling van het genenprofiel van een 20-jarige heel goed het ontwikkelen van leeftijdsgebonden maculadegeneratie op latere leeftijd kan voorspellen. Het zou zelfs mogelijk zijn om bij vroege stadia van de ziekte door middel van het screenen van genen aan te duiden wie uiteindelijk de meest ernstige vorm van maculadegeneratie zal krijgen. Deze ontdekking opent nieuwe wegen voor klinische toepassingen. Het screenen van genen in de klinische praktijk zal zeker een plaats hebben als in de toekomst goede preventieve behandeling voor leeftijdsgebonden maculadegeneratie beschikbaar komt.

Tenslotte werden in de ALGEMENE DISCUSSIE alle bevindingen van dit proefschrift samengebracht en werd beschreven hoe de ontdekking van de genen en de resultaten in dit proefschrift hebben geleid tot het begrijpen van de ziekte. Daarnaast worden in dit hoofdstuk aanbevelingen gedaan voor verder onderzoek.



# SUMMARY





## SUMMARY

AMD is a devastating disease of the retina and causes a severe loss of central visual function. Patients have difficulty driving a car, recognizing faces, watching television and reading. AMD is the leading cause of severe visual impairment in the elderly of the Western World. Despite intensive research in the last decades, only in a minority of the cases treatment strategies are available, and the long-term effects of this treatment is not yet clear. Many studies have provided strong evidence for a genetic component of AMD. The ultimate goal of this thesis was to gain more insight into the genetic background of AMD. For this purpose, we used three study populations:

1. the ERF/GRIP study: a genetic isolate study
2. the Rotterdam study: a prospective cohort study among all inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands. Participants were examined at baseline and during 15 years of follow-up
3. a case-control study: a study in which AMD patients who were recruited from hospitals and patient organizations, were compared with individuals who had no AMD (controls)

### Part I: Genetic risk factors of AMD

CHAPTER 1 provides evidence for a crucial role of the *CFH* gene in AMD. This gene has an important function in our immune system, which protects us in normal circumstances against viruses and bacteria. However, sometimes the immune system attacks our own cells. To avoid this, the system is tightly regulated by numerous proteins including CFH. We demonstrated that persons with a variation in the *CFH* gene had an 11-times higher risk to develop AMD. If those individuals also smoked, risks increased to a high of 40. At age 95 years, individuals with the causal genetic variation in the CFH gene had an almost 50% risk to have vision-disabling macular degeneration.

CHAPTER 2 describes the *CFH* gene in greater detail. The gene has many variants. Some are associated with an increased risk of AMD; others are protective. Our data suggest that the genetic risk of *CFH* on AMD is not determined by only one variation, but that different variants are involved.

CHAPTER 3 reports the risk of the C3 gene, which is also important in our immune system. In this analysis, we showed that variations in this gene also increase the risk of AMD. These results support the role of the immune system in this eye disease.

In CHAPTER 4 we investigated whether genes which activate the immune system (TLR4, CCL2 and CCR2) play a role in AMD. We could not find any relation between those genes and the eye disease. Neither could we detect any differences in expression of these genes. These results imply that these genes do not play a role in AMD.

CHAPTER 5 shows that the *ERCC6* gene, a gene which is important in DNA repair, does not play a significant role in the etiology of AMD. This gene causes Cockayne syndrome, a syndrome in which patients age rapidly and which is associated with retinal degeneration. In mice, *ERCC6* genetic defects also caused retinal degeneration. However, we could not demonstrate any consistent relationship with AMD.

**Part II: Predictive value of genetic profiling for AMD**

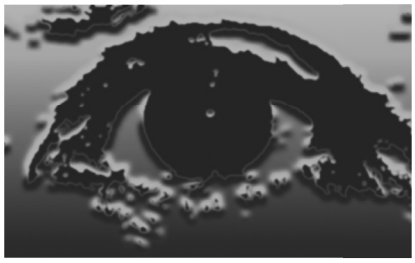
CHAPTER 6 discusses the role of genes in clinical practice. The discovery of genes that play an important role in the development of a disease is a major breakthrough for understanding the pathogenesis of the disease. Particularly revelation of genes that carry a high population risk has shed new light on the possible causes of AMD. However, a genetic variant which only occurs in a small amount of individuals does not necessarily imply a valuable tool to screen an entire population and to predict who will develop the disease and who will not.



CHAPTER 7 outlines the role of genetic screening in the prediction of AMD. We tested all currently known genes with a considerable impact on AMD in our studies. We demonstrated that multiple genetic testing could accurately predict the development of AMD in persons who are not yet affected, e.g. in 20-year old persons. We also found that genetic testing can accurately predict who will develop the most severe forms of AMD in persons with early stages of disease. These discoveries open up new alleys for clinical applications. In the future, genetic screening will most certainly play a role

in clinical practice when good preventive therapeutic strategies for AMD become available.

Finally, in the GENERAL DISCUSSION the main findings of this thesis were recapitulated and special emphasis was given on how the discovery of genes and the results described in this thesis have lead to a better understanding of the disease pathogenesis. In this chapter, strategies for future research are discussed.



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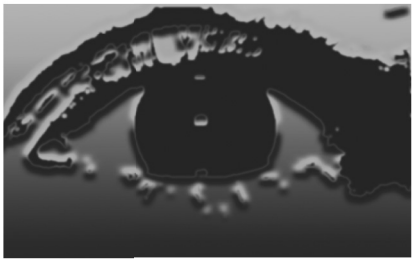

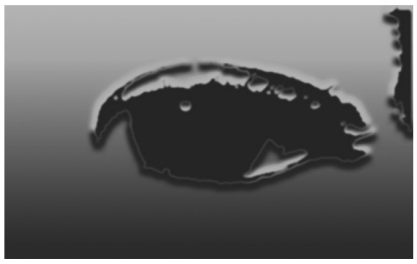

## MERCI... BEDANKT...THANKS...





Dit onderzoek is voor mij een wonderbaarlijke reis geweest, een ontdekking van de genetica van AMD en daarnaast ook van het leven in Nederland… In alle opzichten was het een eye opener… Natuurlijk heb ik dit onderzoek niet alleen gedaan… velen hebben mij hierbij gesteund en geholpen. Allen wil ik op deze plaats bedanken.

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Epidemiologisch onderzoek kan niet plaatsvinden zonder de inzet van alle deelnemers. Jullie bereidheid om mee te werken aan het ERF/GRIP onderzoek, het ERGO onderzoek en het case-controle onderzoek waardeer ik enorm.

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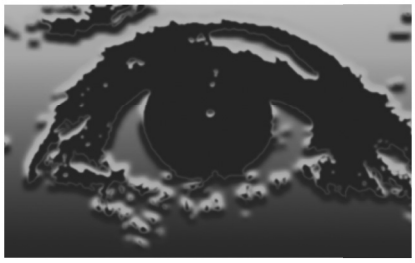
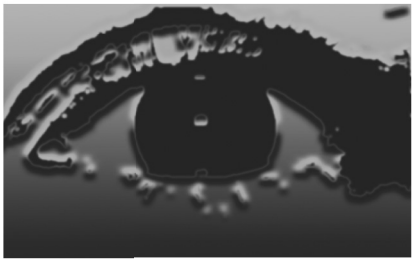
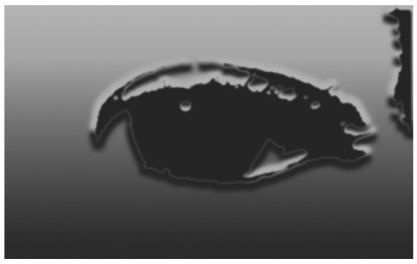
Dominiek



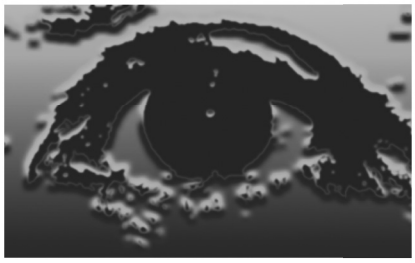
# ABOUT THE AUTHOR



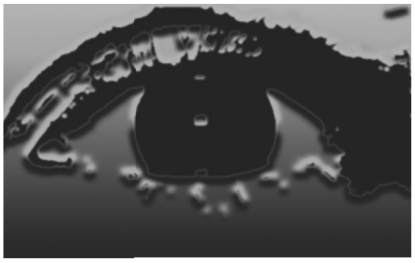


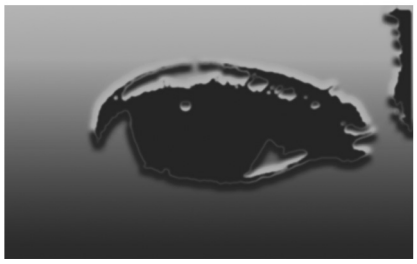
## ABOUT THE AUTHOR

Dominiek Denise Gasparine Despriet was born on December 3rd, 1978 in Kortrijk, Belgium. In 1996 she graduated from the Visitatiehumaniora, Ghent, Belgium. That same year she started her medical study at the Rijksuniversiteit Ghent, Belgium. During her studies she assisted at the practical courses of the Histology Department. During medical school she spent a six-month clinical exchange at the Humboldt University Hospital and Benjamin Franklin University Hospital, Berlin, Germany. She obtained her medical degree magna cum laude in 2003. After this she started the research described in this thesis at the ErasmusMC, Rotterdam, the Netherlands. The research project was a collaboration between the Genetic Epidemiology Unit of the Department of Epidemiology & Biostatistics, and the Department of Ophthalmology. In August 2005, she obtained a Master's Degree in Genetic Epidemiology from the Netherlands Institute of Health Sciences. In July 2007, she started a residency in ophthalmology at the Department of Ophtalmology of the ErasmusMC, headed by Prof. dr. G. van Rij.



# LIST OF PUBLICATIONS





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1. de Jong F. J., Ikram M. K., Despriet D. D., Uitterlinden A. G., Hofman A., Breteler M. M. and de Jong P. T. Complement factor h polymorphism, inflammatory mediators, and retinal vessel diameters: the rotterdam study. *Invest Ophthalmol Vis Sci.* Jul 2007;48(7):3014-3018.

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