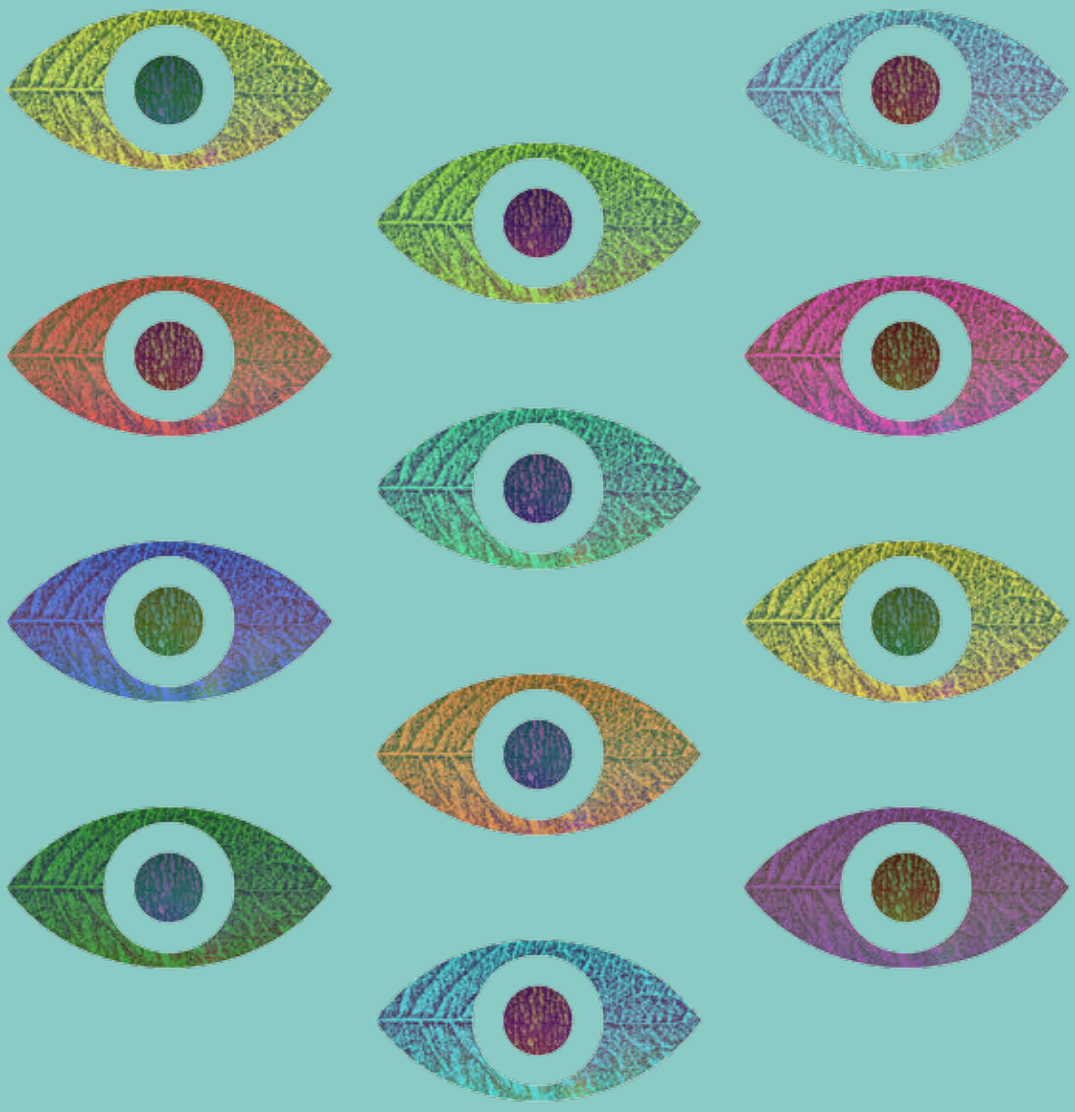
TOWARD PERSONALIZED MEDICINE IN AGE-RELATED MACULAR DEGENERATION



Yara Lechanteur

TOWARD PERSONALIZED MEDICINE IN AGE-RELATED MACULAR DEGENERATION

Yara Terefech Esther Lechanteur

TOWARD PERSONALIZED MEDICINE IN AGE-RELATED MACULAR DEGENERATION

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 9 december 2016
om 10.30 uur precies

door
Yara Terefech Esther Lechanteur
geboren op 28 november 1986 te Addis Abeba (Ethiopië)

COLOFON

ISBN: 9789462955257
Lay-out and cover design: Jet Westbroek
Printing: Proefschriftmaken.nl

The research presented in this thesis was funded by MD Fonds, Oogfonds, and Algemene Nederlandse Vereniging ter Voorkoming van Blindheid.
Publication of this thesis was financially supported by the Radboud University Nijmegen, Stichting AF Deutman oogheelkunde researchfonds, Rotterdamse Stichting Blindenbelangen and Landelijke Stichting voor Blinden en Slechtzienden.

Promotoren:
Prof. dr. Carel B. Hoyng
Prof. dr. B. Jeroen Klevering

Co-promotor:
Prof. dr. Anneke I. den Hollander

Manuscriptcommissie:
Prof. dr. Leo A.B. Joosten (voorzitter)
Prof. dr. Maroeska M. Rovers
Prof. dr. Anneke J.M.M. Hooymans (Rijksuniversiteit Groningen)

LIST OF ABBREVIATIONS

3CC	Three Continent AMD Consortium
AMD	age-related macular degeneration
ANOVA	analysis of variance
AP	alternative pathway
AREDS	Age-Related Eye Disease Study
ARMS2	age-related maculopathy susceptibility 2
AUC	area under the curve
BM	bruch's membrane
BMI	body mass index
C2	complement component C2
C3	complement component C3
CAD	computer aided diagnosis
CAPT	Complications of Age-related Macular Degeneration Prevention Trial
CATT	Comparison of AMD Treatments Trials
CFB	complement factor B
CFH	complement factor H
CFI	complement factor I
CI	confidence interval
CIRCL	Cologne Image Reading Center and Laboratory
CNV	choroidal neovascularization
CP	classical pathway
CRP	C-reactive protrein
DNA	deoxyribonucleic acid
DR	diabetic retinopathy
ECM	extracellular matrix
ETDRS	Early Treatment Diabetic Retinopathy Study
EUGENDA	European Genetic Database
FA	fluorescein angiography
FB	factor B
FD	factor D
FH	factor H
FI	factor I
FP	color fundus photography
FROC	free receiver operating characteristic
GA	geographic atrophy
GST	glutathione S-transferase
GWAS	genome wide association study
HDL	high-density lipoprotein
HR	hazard ratio
HSI	hue-saturation-intensity
HTRA1	Htra serine peptidase 1
ICC	intraclass correlation coefficients
IQR	interquartile range
LDA	linear discriminant
LP	lectin pathway
LPL	lipoprotein lipase
Luv	luminescence-saturation-hue angle
OCT	optical coherence tomography
OR	odds ratio
P	properdin
PCR	polymerase chain reaction
Pix	pixel
PY	pack years
RF	random forest
RGB	red-green-blue
ROC	receiver operating characteristic
RPE	retinal pigment epithelium
RS	Rotterdam Study
SD	standard deviation
SD-OCT	spectral domain optical coherence tomography
SNP	single nucleotide polymorphism
SST	Submacular Surgery Trials
VEGF	vascular endothelial growth factor
WMO	Wet medisch-wetenschappelijk onderzoek met mensen
κ	kappa

TABLE OF CONTENTS

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1. BACKGROUND

Age-related macular degeneration (AMD) is the leading cause of blindness among elderly individuals in Western society.[1,2] AMD affects the central retina and is characterized by progressive changes of the Bruch's membrane (BM) - retinal pigment epithelium (RPE) interface in the macular area ultimately leading to a progressive loss of central visual acuity. With the rapid growth of the aging population the projected number of patients with a form of AMD worldwide is expected to increase from 170 million in 2014 to 288 million in 2040. The number of patients with late AMD (geographic atrophy or neovascular AMD), associated with severe visual loss, is projected to almost double from 10 million to 19 million over the same time period.[3] These numbers underline the massive burden of AMD on global health services in the near future.

2. CLASSIFICATION OF AMD

Non-advanced AMD

Non-advanced AMD is characterized by a spectrum of changes in de macula. Visual acuity, although not used to define the presence of AMD, is still relatively preserved in this stage of the disease. The most characteristic sign of non-advanced AMD is the presence of drusen: deposits of extracellular material between Bruch's membrane and the RPE, considered to be composed of cellular remnants and debris from degenerated RPE cells.[4-8] Different drusen phenotypes can be distinguished. Small (< 63 µm), hard drusen are well-demarcated and are considered normal changes related to aging. However, when present in large numbers, they confer an increased risk for the development of soft drusen.[9] Soft drusen are typically larger in size (≥ 63 µm) and irregular in shape with indistinct edges. These drusen tend to enlarge and fuse into large drusen (≥ 125 µm) and clusters drusen may eventually coalesce to form pigment epithelial detachments.[10] Results from the Beaver Dam Eye Study show that eyes with soft drusen have a 17.8% chance to develop advanced AMD within 15 years, compared to 1.2% in eyes without these lesions.[9] In addition to drusen, hyper- and hypopigmentation of the RPE are other frequently observed findings in non-advanced AMD.

Late AMD

Two distinct phenotypes are regarded as the end-stages of AMD. Geographic atrophy (GA), also referred to as 'dry AMD', is characterized by the presence of sharply delineated areas of depigmentation where choroidal vessels become increasingly visible. These areas correspond to focal loss of RPE, outer layers of the neurosensory retina and the choriocapillaris.[11,12] Over the years the areas of atrophy will enlarge and creep toward the fovea, causing a gradual decline in the visual acuity of patients with GA. In the neovascular stage of the disease, on the other hand, leakage of newly formed blood vessels that arise primarily from

≥≥

the choroidal vessels, causes exudation, hemorrhaging and ultimately the formation of a fibrovascular, or disciform, scar. This will lead to a rapid decline in visual acuity. Although only ten percent of patients suffer from neovascular AMD, it accounts for the majority of blindness due to AMD.[2,13]

AMD Classification Systems

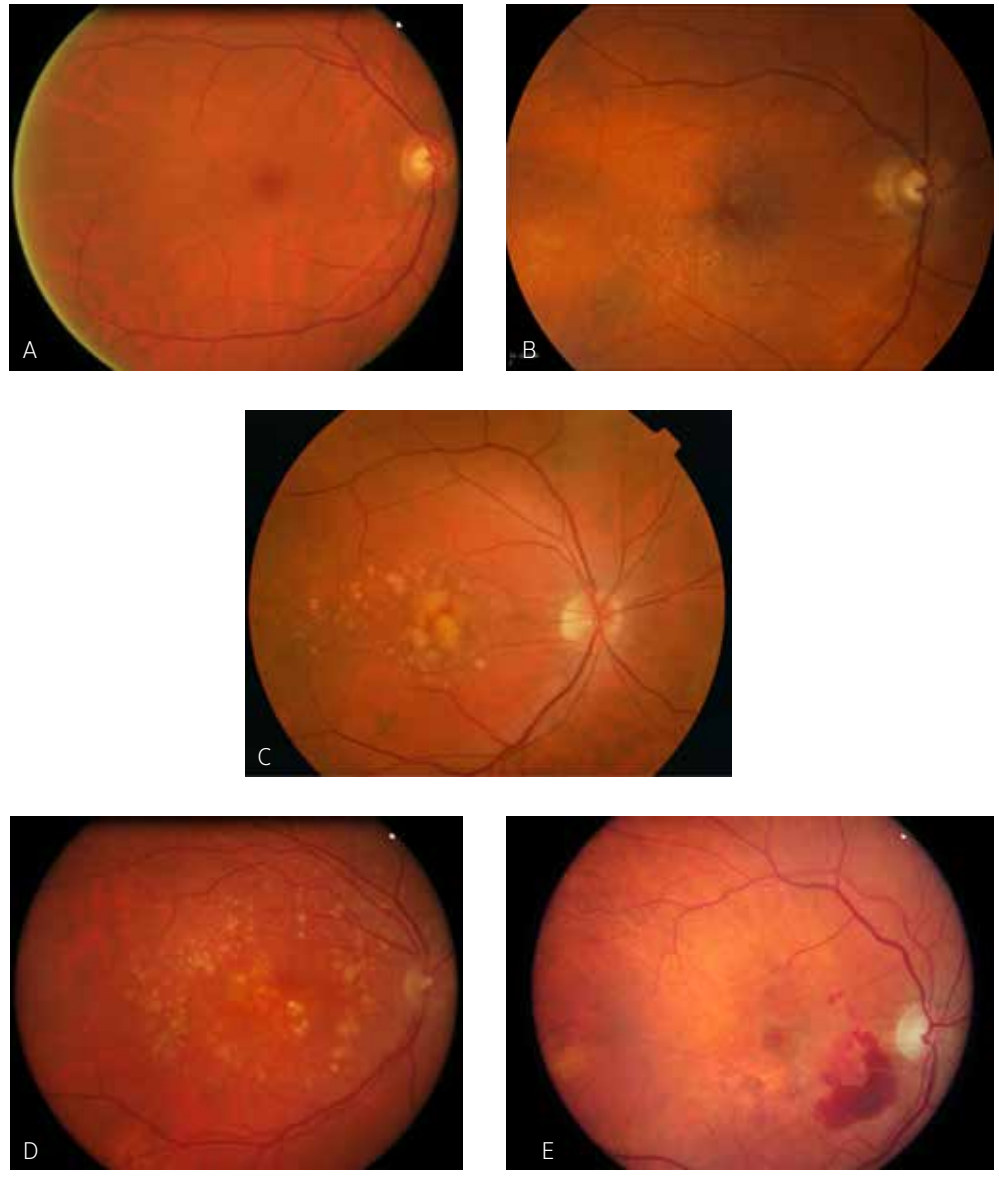
Already in 1875 the first description of the disciform stage of AMD was reported and the term 'senile macular degeneration' was introduced by Haab in 1885. Despite this early introduction it would still take over 100 years before an international classification and grading system was proposed.[4] The International ARM Epidemiological Study Group proposed the prefixes 'early' and 'late' to distinguish drusen and pigmentary changes from end-stage disease (GA or CNV).[4] Over the years several classification protocols have been developed and adapted for various clinical trials and epidemiological studies.[14-18] Most of these schemes require detailed evaluation of fundus photographs and focus on the number, area and size of drusen as well as on the presence or absence of pigmentary changes. In this thesis, AMD is graded according to the standard grading protocol of the Cologne Image Reading Center and Laboratory (CIRCL; https://augenklinik.uk-koeln.de/forschung/arbeitsgruppen-labore/circl/), a grading protocol adopted from the Doheny Image Reading Center grading protocol. A detailed description of the AMD stages according to the CIRCL protocol, and corresponding examples of fundus photographs are shown in Table 1 and Figure 1, respectively.

TABLE 1. Classification of AMD according to the CIRCL protocol

Stage	Description
No AMD	no drusen or only small drusen (< 63 µm) or only pigmentary abnormalites or < 10 small drusen + pigmentary abnormalities
Early AMD	≥ 10 small drusen + pigmentary abnormalities or 1-14 intermediate drusen (≥ 63 and < 125 µm)
Intermediate AMD	≥ 1 large drusen (≥ 125 µm diameter) or ≥ 15 intermediate drusen or geographic atrophy (RPE atrophy ≥ 175 µm) not in the central circle of the ETDRS grid
Late AMD	Any or all of the following: central GA, or evidence of neovascular AMD
GA	GA (RPE atrophy ≥ 175 µm) secondary to AMD involving the central subfield of the ETDRS grid
CNV	CNV within the ETDRS grid secondary to AMD with evidence for fluid, blood, or fibrovascular tissue on FP, signs of active or previous CNV on FA and/or retinal or subRPE fluid and/or tissue secondary to AMD on SD-OCT.

AMD, age-related macular degeneration; CIRCL, Cologne Image and Reading Center Laboratory; RPE, retinal pigment epithelium; ETDRS, Early Treatment Diabetic Retinopathy Study; GA, geographic atrophy; CNV, choroidal neovascularization; FP, color fundus photography; FA, fluorescein angioigraphy; SD-OCT, spectral domain optical coherence tomography.

FIGURE 1. Examples of AMD grades classified by the CIRCL protocol



A, No AMD. Only a few hard drusen are present in the macula. B, Early AMD. Several hard and intermediate drusen and hyper- and hypopigmentation of the retinal pigment epithelium. C, Intermediate AMD. Extensive intermediate and large drusen. Large drusen have merged into pigment epithelium detachtments. D, Advanced AMD, geographic atrophy. An area of central geographic atrophy is surrounded by extensive soft drusen. E, Advanced AMD, choroidal neovascularization. Central exudation and hemorrhaging.
AMD, age-related macular degeneration; CIRCL, Cologne Image and Reading Center Laboratory.

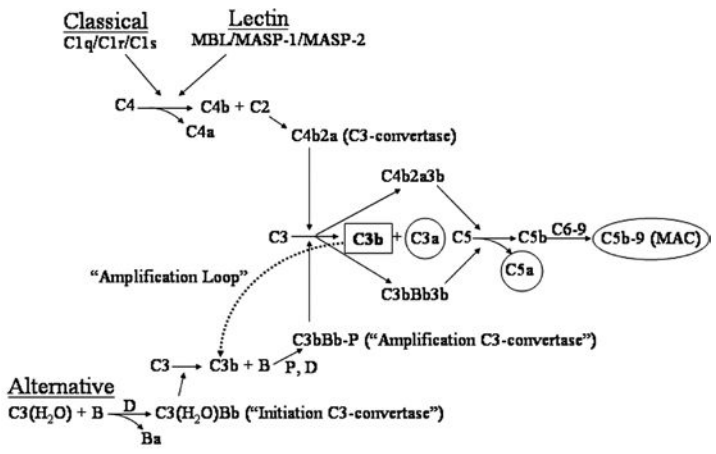
3. PATHOGENESIS OF AMD

The pathogenesis of AMD is complex and multifactorial. Although there is still much to discover, several pathways have been implicated in the development and progression of the disease. The alternative pathway of the complement system is thought to be the most important pathway involved in AMD, and will be discussed in detail in the next paragraph. Associations of cardiovascular risk factors with AMD and the accumulation of cholesterol and lipids in drusen suggest a role for the high-density lipoprotein pathway.[7,19] Several genes involved in this pathway have also been associated with AMD (section 4). Genetic associations also point toward a role for the extracellular matrix (ECM) pathway in AMD (section 4).[20-23] An important ECM complex in the retina is Bruch's membrane, a histologically very important site in the pathogenesis of AMD.[24] A fourth pathway that is implied in AMD is the angiogenesis signaling pathway. The VEGF-A protein is a key factor in the promotion of angiogenesis and an increased expression of VEGF-A has been found in the RPE in post mortem maculae of AMD patients.[25-27]

The Complement System

The complement system is a part of the innate immune system and consists of several plasma proteins that upon activation act in a cascade to induce inflammatory responses in the defense against pathogens and host homeostasis. Activation of the complement system can be initiated through one of three pathways: the classical pathway, the lectin pathway and the alternative pathway. All pathways merge into one final pathway that ends in the formation of the membrane attack complex, resulting in cell lysis.[28] Figure 2 gives an schematic overview of the components of the complement system.

FIGURE 2. Overview of the complement system



The alternative pathway is continuously activated at a low level by a process called tick-over in which there is spontaneous hydrolysis of complement component 3 (C3) into C3(H_2O). C3(H_2O) then binds factor B (FB) under the influence of factor D (FD) Subsequent steps lead to the formation of an initiation C3 convertase (C3(H_2O)Bb) that constantly generates small amounts of C3b by cleaving C3 into C3a and C3b. These C3b molecules also bind FB to form the amplification C3-convertase C3bBb that is stabilized by properdin (P). This C3-convertase is much more potent in cleaving C3 and the C3b molecules that are formed act in the amplification-feedback loop, resulting in an exponential increase in complement activation. Several molecules act as tight regulators of the complement system and are necessary to maintain host homeostasis. An important fluid-phase regulator is factor H (FH), which exerts an inhibitory effect on the alternative pathway through three mechanisms of action: by effecting the decay of C3-convertase C3bBb, by competing with FB in binding to C3b, and by functioning as a cofactor for Factor I (FI) to allow inactivation of C3b.[29] Dysregulation of the complement system is thought to be one of the key elements in AMD pathogenesis.

4. RISK FACTORS FOR AMD

Non-genetic Risk Factors

Age is the strongest risk factor for AMD. All population-based studies on AMD have consistently reported that the prevalence of AMD increases with each age category. Pooled data from the Rotterdam Study, the Beaver Dam Eye Study and the Blue Mountains Eye study showed that 0.2% of those aged 55-64 years had advanced AMD, and this number increased to 13% in the group aged 85 years or older.[30]

Smoking is the strongest modifiable risk factor for AMD and is associated with both development and progression of AMD.[30-40] Even after years of cessation, a past smoker still has a higher risk compared to an individual who never smoked a cigarette.[40] Smoking may exert its effect on AMD through several mechanisms. Smoking adds to oxidative stress, which leads to the formation of reactive oxygen species.[41] In addition, smoking is known to induce VEGF expression and RPE dysfunction and diminishes RPE cell survival; these factors may affect angiogenic homeostasis in the retina.[42,43] Smoking is also thought to directly increase the activity of the alternative pathway of the complement system, further adding to AMD pathogenesis.[44,45]

Other risk factors have not consistently been reported in literature. It has been suggested that obesity, female sex, sunlight exposure and several cardiovascular risk factors such as hypertension confer a higher risk for AMD.[46] Some nutritional factors may be protective for the development of AMD. These include omega-3 fatty acids, lutein, zeaxanthin, and antioxidant vitamins C and E.[47,48]

Genetic Risk Factors

Based on studies on twins and families we know that genetics play an important role in AMD development.[49-52] In the last decade significant progress has been made in the identification of genetic variants that influence AMD risk. Associations have been found for several single nucleotide polymorphisms (SNPs) in complement genes, of which the complement factor H (*CFH*) gene is the most important.[53-56] This gene encodes for FH, the most important inhibitor of the alternative complement pathway. Disruption of the delicate balance of suppression and activation of the alternative pathway, as is thought to be the result of single nucleotide changes in the *CFH* gene, may lead to uncontrolled inflammation and tissue damage, ultimately leading to disease. Smaller effect sizes have been reported for SNPs in other genes of the complement system: complement component 2 (*C2*),[57-59] complement component 3 (*C3*),[57,60-63] complement factor I (*CFI*),[64,65] and complement factor B (*CFB*).[57,58]

The second major genetic risk factor for AMD, next to *CFH*, is the *ARMS2/HTRA1* locus.[66-69] Currently it is not clear which gene is causal in AMD pathogenesis: *ARMS2*, *HTRA1* or both.[69] *HTRA1* encodes a serine protease that targets several ECM proteins. The precise localization and function of the ARMS2 protein are currently not known. Some suggest that it is a mitochondrial protein involved in oxidative stress responses,[66,70] whereas others claim that the protein interacts with ECM proteins,[71] yet another publication suggested that ARMS2 is involved in the phagocytosis of photoreceptor outer segments.[72] Recently, a direct relationship between a variant at the *ARMS2/HTRA1* locus and increased complement activation in AMD patients was reported.[73]

Other associations with AMD have been found for genes involved in several AMD-related pathways. These include genes in the high-density lipoprotein cholesterol pathway (e.g. *APOE*,[74-76] *LIPC*,[77,78] *CETP*[77-79], *ABCA1*,[77] *FADS1_3*,[77,78] *LPL*[77-79] genes), the angiogenesis signaling pathway (e.g. *VEGFA*,[80,81] *TGFBR1*[80]) and the extracellular matrix pathway (e.g. *COL8A1*,[78] *COL10A1*,[81] *TIMP3*,[77] *MMP9*[23] genes).

Table 2 gives an overview of 34 loci for advanced AMD, with the associated odds ratios and allele frequencies in patients and controls. These results were derived from a recent genome wide association study (GWAS) including 16,144 cases and 17,832 controls, and are estimated to explain 27.2% of disease variability, which is almost 60% of the total heritability in AMD.[23]

TABLE 2. Overview of 34 loci for advanced AMD as identified in a large GWAS study[23]

		MAF		Association	
Lead Variant	**Gene**	**Cases**	**Controls**	**OR**	**P value**
rs10922109	*CFH*	0.223	0.426	0.38	9.6×10^{-618}
rs62247658	*ADAMTS9-AS2*	0.466	0.433	1.14	1.8×10^{-14}
rs140647181	*COL8A1*	0.023	0.016	1.59	1.4×10^{-11}
rs10033900	*CFI*	0.511	0.477	1.15	5.4×10^{-17}
rs62358361	*C9*	0.016	0.009	1.80	1.3×10^{-14}
rs116503776	*C2/CFB/SKIV2L*	0.090	0.148	0.57	1.2×10^{-103}
rs943080	*VEGFA*	0.465	0.497	0.88	1.1×10^{-14}
rs79037040	*TNFRSF10A*	0.451	0.479	0.90	4.5×10^{-11}
rs1626340	*TGFBR1*	0.189	0.209	0.88	3.8×10^{-10}
rs3750846	*ARMS2/HTRA1*	0.436	0.208	2.81	6.5×10^{-735}
rs9564692	*B3GALTL*	0.277	0.299	0.89	3.3×10^{-10}
rs61985136	*RAD51B*	0.360	0.384	0.90	1.6×10^{-10}
rs2043085	*LIPC*	0.350	0.381	0.87	4.3×10^{-15}
rs5817082	*CETP*	0.232	0.264	0.84	3.6×10^{-19}
rs2230199	*C3*	0.266	0.208	1.43	3.8×10^{-69}
rs429358	*APOE*	0.099	0.135	0.70	2.4×10^{-42}
rs5754227	*SYN3/TIMP3*	0.109	0.137	0.77	1.1×10^{-24}
rs8135665	*SLC16A8*	0.217	0.195	1.14	5.5×10^{-11}
rs11884770	*COL4A3*	0.258	0.278	0.90	2.9×10^{-8}
rs114092250	*PRLR/SPEF2*	0.016	0.022	0.70	2.1×10^{-8}
rs7803454	*PILRB/PILRA*	0.209	0.190	1.13	4.8×10^{-9}
rs1142	*KMT2E/SRPK2*	0.370	0.346	1.11	1.4×10^{-9}
rs71507014	*TRPM3*	0.427	0.405	1.10	3.0×10^{-8}
rs10781182	*MIR6130/RORB*	0.328	0.306	1.11	2.6×10^{-9}
rs2740488	*ABCA1*	0.255	0.275	0.90	1.2×10^{-8}
rs12357257	*ARHGAP21*	0.243	0.223	1.11	4.4×10^{-8}
rs3138141	*RDH5/CD63*	0.222	0.207	1.16	4.3×10^{-9}
rs61941274	*ACAD10*	0.024	0.018	1.51	1.1×10^{-9}
rs72802342	*CTRB2/CTRB1*	0.067	0.080	0.79	5.0×10^{-12}
rs11080055	*TMEM97/VTN*	0.463	0.486	0.91	1.0×10^{-8}
rs6565597	*NPLOC4/TSPAN10*	0.400	0.381	1.13	1.5×10^{-11}
rs67538026	*CNN2*	0.460	0.498	0.90	2.6×10^{-8}
rs142450006	*MMP9*	0.124	0.141	0.85	2.4×10^{-10}
rs201459901	*C20orf85*	0.054	0.070	0.76	3.1×10^{-16}

AMD, age-related macular degeneration; GWAS, genome wide association study; MAF, minor allele frequency; OR, odds ratio. (Table derived and adapted from Fritsche et al.[23])

5. TREATMENT OF AMD

Prevention

Nutritional supplements can be used to slow down development and progression of advanced AMD in selected patient groups. Data from AREDS show that in patients with intermediate and unilateral advanced AMD, the use of the specific AREDS formulation (consisting of 500 mg vitamin C, 400 IU vitamin E, 15 mg beta-carotene and 80 mg zinc oxide) reduced the risk of development of advanced AMD with 25%.[15] Evidence from other studies suggested the beneficial role of carotenoids (lutein and zeaxanthin) and omega-3 long-chain polyunsaturated fatty acids for AMD. Therefore, the AREDS2 study was initiated to study the effects of these factors in a prospective setting. None of the tested supplements further reduced the risk of progression when added to the original AREDS formulation. However, substitution of beta-carotene with lutein and zeaxanthin was recommended because of the increased risk of lung cancer in former smokers who received beta-carotene.[47]

In a recent study we have shown that oral zinc supplementation decreased systemic complement activation levels, suggesting a direct influence of zinc on the complement system.[82] This may open new doors for treatment of AMD, and based on these results it would seem worthwhile to explore the role of antixodants in the treatment of AMD.

Neovascular AMD

Thermal laser photocoagulation was the first treatment available for neovascular AMD and resulted in a reduction in vision loss. However, because of the risk of laser-induced retinal damage, the high rate of recurrences and the introduction of other therapeutic options, this treatment modality has now been abandoned.[46,83,84] Photodynamic therapy with verteporfin was able to stabilize visual acuity in 61% of patients, a first major breakthrough in the treatment of AMD.[46,85] However, a great leap forward for patients with neovascular AMD was achieved with the introduction of intravitreal injections with drugs targeting vascular endothelial growth factor (VEGF). Ranibizumab was approved for the treatment of neovascular AMD in 2006 and this was the first drug to improve visual acuity.[86] Other anti-VEGF drugs that are currently used as a treatment for AMD are bevacizumab and aflibercept.[87-89]

Complement Inhibitors

As of yet, there are no clinical therapies available for GA. Targeted complement inhibition is a potential treatment of interest and several clinical trials evaluating the effect of complement inhibitors on AMD progression have been initiated. So far, one trial has shown promising results using an antibody directed against factor D. Phase III trials are ongoing.[90,91]

6. AIMS AND OUTLINES OF THIS THESIS

Personalized Medicine in AMD

The goal of personalized medicine is to separate individuals into different groups – for example based on disease etiology, progression, or treatment response – in order to tailor specific medical decisions or interventions to the individual patient. All this is based on the individuals predicted risk of disease or predicted response to treatments. It is also known as P4 medicine, with the four P's standing for predictive, preventive, personalized and participatory.[92-94] With the recent developments in genetic technologies and molecular approaches (e.g. genomics and proteomics), progression can be made toward personalized medicine in AMD. In this thesis we present our research that focuses on different aspects that are required in order to move forward toward personalized medicine in AMD. These include studies on risk factors for development and progression of AMD – both for AMD families as well as sporadic cases – and studies focussing on one of the key pathophysiological pathways involved in AMD, the complement system. We also present a computer aided diagnosis system that can help to further classify subsets of patients.

Risk factors for age-related macular degeneration

Chapter 2 provides insight in risk factors for an earlier age at onset of AMD. The age at which the first signs of age-related macular degeneration (AMD) manifest is highly variable. Better insight in factors that influence disease onset has direct implications for preventive measures and patient counseling.

Chapter 3 concerns patients with end-stage AMD in one eye. These patients are at high risk of progressing toward end-stage disease in the fellow eye. In this chapter we have focused on genetic and environmental risk factors that influence this second eye progression.

Chapter 4 discusses the possible role of genes associated with antioxidant metabolism and AMD. Antioxidant defense mechanisms are important in prevention of AMD. Because of the antioxidant role of GST enzymes it has been suggested that loss of function of these enzymes could lead to an increased risk for AMD.

Familial age-related macular degeneration

Chapter 5 evaluates the differences between familial and sporadic AMD. Certain lifestyle factors are shown to be significantly associated with AMD in sporadic cases, but not in familial cases. This chapter investigates whether the contribution of common genetic variants and complement activation levels differs between familial and sporadic cases with AMD.

Chapter 6 focuses on prediction of AMD in families. Prediction models have been established for AMD based on demographic, environmental and common genetic risk factors. In AMD families, these risk factors may be distributed differently.

Computer aided diagnosis of age-related macular degeneration
Chapter 7 describes the development of a computer-aided diagnosis system that is able to detect and quantify drusen location, area and size on color fundus images.

Complement activation and age-related macular degeneration
Chapter 8 looks into the relationship between systemic complement activation and different AMD stages. In addition, we studied the effect of genes and treatment on this relationship. *Chapter 9* describes the identification of a novel complotype combination that is associated with both AMD and complement activation levels in vivo. A thorough evaluation of complement activation and AMD can provide us with better insight of who might benefit most from complement-lowering treatments.

General discussion
Chapter 10 further discusses the studies described in this thesis and places them in a broader perspective.

REFERENCES

1. Klaver CC, Wolfs RC, Vingerling JR, Hofman A, de Jong PT. Age-specific prevalence and causes of blindness and visual impairment in an older population: the Rotterdam Study. *Arch Ophthalmol* 1998;116:653-658.
2. Klein R, Wang Q, Klein BE, Moss SE, Meuer SM. The relationship of age-related maculopathy, cataract, and glaucoma to visual acuity. *Invest Ophthalmol Vis Sci* 1995;36:182-191.
3. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health* 2014;2:e106-116.
4. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol* 1995;39:367-374.
5. Rudolf M, Clark ME, Chimento MF, Li CM, Medeiros NE, Curcio CA. Prevalence and morphology of druse types in the macula and periphery of eyes with age-related maculopathy. *Invest Ophthalmol Vis Sci* 2008;49:1200-1209.
6. Al-Hussaini H, Schneiders M, Lundh P, Jeffery G. Drusen are associated with local and distant disruptions to human retinal pigment epithelium cells. *Exp Eye Res* 2009;88:610-612.
7. Curcio CA, Presley JB, Malek G, Medeiros NE, Avery DV, Kruth HS. Esterified and unesterified cholesterol in drusen and basal deposits of eyes with age-related maculopathy. *Exp Eye Res* 2005;81:731-741.
8. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* 2002;134:411-431.
9. Klein R, Klein BE, Knudtson MD, Meuer SM, Swift M, Gangnon RE. Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. *Ophthalmology* 2007;114:253-262.
10. Sarks JP, Sarks SH, Killingsworth MC. Evolution of soft drusen in age-related macular degeneration. *Eye (Lond)* 1994;8 (Pt 3):269-283.
11. Sarks JP, Sarks SH, Killingsworth MC. Evolution of geographic atrophy of the retinal pigment epithelium. *Eye (Lond)* 1988;2 (Pt 5):552-577.
12. Green WR, Key SN 3rd. Senile macular degeneration: a histopathologic study. *Trans Am Ophthalmol Soc* 1977;75:180-254.
13. Ferris FL 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* 1984;102:1640-1642.
14. Klein R, Davis MD, Magli YL, Segal P, Klein BE, Hubbard L. The Wisconsin age-related maculopathy grading system. *Ophthalmology* 1991;98:1128-1134.
15. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* 2001;119:1417-1436.
16. Davis MD, Gangnon RE, Lee LY, et al. The Age-Related Eye Disease Study severity scale for age-related macular degeneration: AREDS Report No. 17. *Arch Ophthalmol* 2005;123:1484-1498.
17. Ferris FL, Davis MD, Clemons TE, et al. A simplified severity scale for age-related macular degeneration: AREDS Report No. 18. *Arch Ophthalmol* 2005;123:1570-1574.
18. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. The risk and natural course of age-related maculopathy: follow-up at 6 1/2 years in the Rotterdam study. *Arch Ophthalmol* 2003;121:519-526.
19. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *FASEB J* 2000;14:835-846.
20. Fiotti N, Pedio M, Battaglia Parodi M, et al. MMP-9 microsatellite polymorphism and susceptibility to exudative form of age-related macular degeneration. *Genet Med* 2005;7:272-277.
21. Liutkeviciene R, Lesauskaite V, Sinkunaite-Marsalkiene G, et al. The Role of Matrix Metalloproteinases Polymorphisms in Age-Related Macular Degeneration. *Ophthalmic Genet* 2015;36:149-155.
22. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proceedings of the National Academy of Sciences of the United States of America* 2010;107:7401-7406.
23. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet* 2016;48:134-143.
24. Johnson LV, Anderson DH. Age-related macular degeneration and the extracellular matrix. *N Engl J Med* 2004;351:320-322.
25. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029-1039.

26. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983-985.
27. Kliffen M, Sharma HS, Mooy CM, Kerkvliet S, de Jong PT. Increased expression of angiogenic growth factors in age-related maculopathy. *Br J Ophthalmol* 1997;81:154-162.
28. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* 2015;6:262.
29. Wu J, Wu YQ, Ricklin D, Janssen BJ, Lambris JD, Gros P. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat Immunol* 2009;10:728-733.
30. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. *Ophthalmology* 2001;108:697-704.
31. Risk factors for neovascular age-related macular degeneration. The Eye Disease Case-Control Study Group. *Arch Ophthal* 1992;110:1701-1708.
32. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology* 2000;107:2224-2232.
33. Christen WG, Glynn RJ, Manson JE, Ajani UA, Buring JE. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 1996;276:1147-1151.
34. Delcourt C, Diaz JL, Ponton-Sanchez A, Papoz L. Smoking and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. *Arch Ophthal* 1998;116:1031-1035.
35. Hyman LG, Lilienfeld AM, Ferris FL 3rd, Fine SL. Senile macular degeneration: a case-control study. *Am J Epidemiol* 1983;118:213-227.
36. Klein R, Klein BE, Linton KL, DeMets DL. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. *Am J Epidemiol* 1993;137:190-200.
37. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA* 1996;276:1141-1146.
38. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. The Blue Mountains Eye Study. *Arch Ophthal* 1996;114:1518-1523.
39. Vinding T, Appleyard M, Nyboe J, Jensen G. Risk factor analysis for atrophic and exudative age-related macular degeneration. An epidemiological study of 1000 aged individuals. *Acta Ophthalmol* 1992;70:66-72.
40. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. *Arch Ophthalmol* 1996;114:1193-1196.
41. Bertram KM, Baglole CJ, Phipps RP, Libby RT. Molecular regulation of cigarette smoke induced-oxidative stress in human retinal pigment epithelial cells: implications for age-related macular degeneration. *Am J Physiol Cell Physiol* 2009;297:C1200-1210.
42. Chu YK, Lee SC, Byeon SH. VEGF rescues cigarette smoking-induced human RPE cell death by increasing autophagic flux: implications of the role of autophagy in advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2013;54:7329-37.
43. Pons M, Marin-Castano ME. Cigarette smoke-related hydroquinone dysregulates MCP-1, VEGF and PEDF expression in retinal pigment epithelium in vitro and in vivo. *PLoS One* 2011;6:e16722.
44. Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. *JAMA* 2006;296:301-309.
45. Kew RR, Ghebrehiwet B, Janoff A. Cigarette smoke can activate the alternative pathway of complement in vitro by modifying the third component of complement. *J Clin Invest* 1985;75:1000-1007.
46. Coleman HR, Chan CC, Ferris FL 3rd, Chew EY. Age-related macular degeneration. *Lancet* 2008;372:1835-1845.
47. Age-Related Eye Disease Study 2 Research Group. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. *Jama* 2013;309:2005-2015.
48. Seddon JM, Rosner B, Sperduto RD, et al. Dietary fat and risk for advanced age-related macular degeneration. *Arch Ophthalmol* 2001;119:1191-1199.
49. Klaver CC, Wolfs RC, Assink JJ, van Duijn CM, Hofman A, de Jong PT. Genetic risk of age-related maculopathy. Population-based familial aggregation study. *Arch Ophthalmol* 1998;116:1646-1651.
50. Melrose MA, Magargal LE, Lucier AC. Identical twins with subretinal neovascularization complicating senile macular degeneration. *Ophthalmic Surg* 1985;16:648-651.
51. Seddon JM, Ajani UA, Mitchell BD. Familial aggregation of age-related maculopathy. *Am J Ophthalmol* 1997;123:199-206.
52. Klein ML, Mauldin WM, Stoumbos VD. Heredity and age-related macular degeneration. Observations in monozygotic twins. *Arch Ophthalmol* 1994;112:932-937.

53. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
54. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
55. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
56. Scholl HP, Weber BH, Nothen MM, Wienker T, Holz FG. [Y402H polymorphism in complement factor H and age-related macular degeneration (AMD)]. *Ophthalmologe* 2005;102:1029-1035.
57. Francis PJ, Hamon SC, Ott J, Weleber RG, Klein ML. Polymorphisms in C2, CFB and C3 are associated with progression to advanced age related macular degeneration associated with visual loss. *J Med Genet* 2009;46:300-307.
58. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
59. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. *Hum Mol Genet* 2007;16:1986-1992.
60. Despriet DD, van Duijn CM, Oostra BA, et al. Complement component C3 and risk of age-related macular degeneration. *Ophthalmology* 2009;116:474-480.
61. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat Genet* 2007;39:1200-1201.
62. Park KH, Fridley BL, Ryu E, Tosakulwong N, Edwards AO. Complement component 3 (C3) haplotypes and risk of advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2009;50:3386-3393.
63. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
64. Ennis S, Gibson J, Cree AJ, Collins A, Lotery AJ. Support for the involvement of complement factor I in age-related macular degeneration. *Eur J Hum Genet* 2010;18:15-16.
65. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
66. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* 2008;40:892-896.
67. Lee SJ, Kim NR, Chin HS. LOC387715/HTRA1 polymorphisms , smoking, and combined effects on exudative age-related macular degeneration in a Korean population. *Clin Experiment Ophthalmol* 2010.
68. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
69. Yang Z, Tong Z, Chen Y, et al. Genetic and functional dissection of HTRA1 and LOC387715 in age-related macular degeneration. *PLoS Genet* 2010;6:e1000836.
70. Kanda A, Chen W, Othman M, et al. A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. *Proc Natl Acad Sci U S A* 2007;104:16227-16232.
71. Kortvely E, Hauck SM, Duetsch G, et al. ARMS2 is a constituent of the extracellular matrix providing a link between familial and sporadic age-related macular degenerations. *Invest Ophthalmol Vis Sci* 2010;51:79-88.
72. Xu YT, Wang Y, Chen P, Xu HF. Age-related maculopathy susceptibility 2 participates in the phagocytosis functions of the retinal pigment epithelium. *Int J Ophthalmol* 2012;5:125-132.
73. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
74. Baird PN, Guida E, Chu DT, Vu HT, Guymer RH. The epsilon2 and epsilon4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2004;45:1311-1315.
75. Fritsche LG, Freitag-Wolf S, Bettecken T, et al. Age-related macular degeneration and functional promoter and coding variants of the apolipoprotein E gene. *Hum Mutat* 2009;30:1048-1053.
76. Klaver CC, Kliffen M, van Duijn CM, et al. Genetic association of apolipoprotein E with age-related macular degeneration. *Am J Hum Genet* 1998;63:200-206.
77. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2010;107:7401-7406.
78. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A* 2010;107:7395-7400.
79. Peter I, Huggins GS, Ordovas JM, Haan M, Seddon JM. Evaluation of new and established age-related macular degeneration susceptibility genes in the Women's Health Initiative Sight Exam (WHI-SE) Study. *Am J Ophthalmol* 2011;152:1005-1013 e1001.

80. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet* 2013;45:433-439, 439e431-432.
81. Yu Y, Bhangale TR, Fagerness J, et al. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. *Hum Mol Genet* 2011;20:3699-3709.
82. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One* 2014;9:e112682.
83. Macular Photocoagulation Study Group. Argon laser photocoagulation for senile macular degeneration. Results of a randomized clinical trial. *Arch Ophthalmol* 1982;100:912-918.
84. Macular Photocuagulation Study Group. Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials. *Arch Ophthalmol* 1986;104:694-701.
85. Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials-TAP report 2. *Arch Ophthalmol* 2001;119:198-207.
86. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 2006;355:1419-1431.
87. Catt Research Group, Martin DF, Maguire MG, et al. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N Engl J Med* 2011;364:1897-1908.
88. Heier JS, Brown DM, Chong V, et al. Intravitreal aflibercept (VEGF trap-eye) in wet age-related macular degeneration. *Ophthalmology* 2012;119:2537-2548.
89. Schmidt-Erfurth U, Kaiser PK, Korobelnik JF, et al. Intravitreal aflibercept injection for neovascular age-related macular degeneration: ninety-six-week results of the VIEW studies. *Ophthalmology* 2014;121:193-201.
90. Hoffmann-La Roche. A Study Investigating the Efficacy and Safety of Lampalizumab Intravitreal Injections in Patients With Geographic Atrophy Secondary to Age-Related Macular Degeneration (CHROMA). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 Nov 29]. Available from: https://clinicaltrials.gov/show/NCT02247479 NLM Identifier: NCT02247479.
91. Hoffmann-La Roche. A Study Investigating the Safety and Efficacy of Lampalizumab Intravitreal Injections in Patients With Geographic Atrophy Secondary to Age-Related Macular Degeneration (SPECTRI). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 Nov 29]. Available from: https://clinicaltrials.gov/show/NCT02247531 NLM Identifier: NCT02247531.
92. Evers AW, Rovers MM, Kremer JA, et al. An integrated framework of personalized medicine: from individual genomes to participatory health care. *Croat Med J* 2012;53:301-303.
93. Hamburg MA, Collins FS. The path to personalized medicine. *N Engl J Med* 2010;363:301-304.
94. Younesi E, Hofmann-Apitius M. From integrative disease modeling to predictive, preventive, personalized and participatory (P4) medicine. EPMA J 2013;4:23.

RISK FACTORS FOR AGE-RELATED MACULAR DEGENERATION

CHAPTER 2

ASSOCIATION OF SMOKING AND *CFH* AND *ARMS2* RISK VARIANTS WITH YOUNGER AGE AT ONSET OF NEOVASCULAR AGE-RELATED MACULAR DEGENERATION

Yara T.E. Lechanteur,
Patrick L. van de Camp,
Dzenita Smailhodzic,
Johannes P.H. van de Ven,
Gabriëlle H.S. Buitendijk,
Caroline C.W. Klaver,
Joannes M.M. Groenewoud,
Anneke I. den Hollander,
Carel B. Hoyng,
B. Jeroen Klevering.

JAMA Ophthalmol 2015;133(5):533-541

IMPORTANCE The age at which the first signs of age-related macular degeneration (AMD) manifest is variable. Better insight in factors that influence disease onset has direct implications for preventive measures and patient counseling.

OBJECTIVE To identify risk factors for an earlier age at onset of neovascular AMD.

DESIGN, SETTING, AND PARTICIPANTS Retrospective cohort study, including patient data from the European Genetic Database collected between April 2006 and July 2010. All patients had at least 1 documented visit to the outpatient AMD clinic of the Radboud university medical center, Nijmegen, the Netherlands, a tertiary referral center for retinal disorders. In total, 275 patients with a known age at onset of neovascular AMD and a genetic risk analysis were included.

MAIN OUTCOMES AND MEASURES Effects of several genetic, sociodemographic, behavioral, and ocular factors on the age at onset of neovascular AMD. The mean differences in the age at onset were determined using general linear models with the age at onset as the dependent variable.

RESULTS Past smokers and current smokers developed neovascular AMD on average 4.9 (95% CI 3.0-6.8) and 7.7 (95% CI 5.3-10.0) years earlier, respectively, than never smokers ($P < .001$ for both). Compared with the reference group, the age at onset was 5.2 (95% CI 2.8-7.7) years earlier for homozygous carriers of the A69S risk allele in the age-related maculopathy susceptibility 2 (*ARMS2*) gene ($P < .001$). Homozygous carriers of the Y402H risk variant in the complement factor H (*CFH*) gene developed neovascular AMD 2.8 (95% CI 0.5-5.0) years earlier ($P = .02$). Patients carrying 4 risk alleles in *CFH* and *ARMS2* developed neovascular AMD 12.2 (95% CI 6.2-18.3) years earlier than patients with zero risk alleles ($P < .001$).

CONCLUSIONS AND RELEVANCE Genetic and environmental risk factors influence the age at onset of neovascular AMD. Individuals at risk could be identified at an early age if and when preventive or therapeutic options become available. Insight into individual risk profiles might influence patients' consideration of interventions to increase their chance of avoiding vision loss from AMD.

Age-related macular degeneration (AMD) is the most common cause of severe visual impairment among elderly in developed countries. Typically, 2 forms of end-stage disease that can be distinguished are geographic atrophy (GA) (or dry AMD) and neovascular AMD characterized by choroidal neovascularization (CNV). Although only 10% of patients experience neovascular AMD, it accounts for most blindness due to AMD.[1,2]

Smoking is the strongest modifiable risk factor for AMD.[3-12] Other inconsistent environmental and demographic risk factors include obesity,[4,13-17] sunlight exposure,[18-20] and sex.[4,21,22] A healthy diet containing omega-3 fatty acids, lutein, zeaxanthin, and antioxidant vitamins C and E may be protective against the development of AMD.[23-26] Nutritional supplements containing the Age-Related Eye Disease Study 2 formula[27] can further reduce risk of progression to advanced AMD in patients at risk. Apart from environmental factors, several genetic variants have been associated with AMD. The strongest associations have been found for single-nucleotide polymorphisms (SNPs) in the complement factor H (*CFH*) gene (OMIM 134370)[28-30] and in the age-related macular susceptibility 2 (*ARMS2)* gene (OMIM 611313).[31-33] Besides these variants, other SNPs have been associated with AMD, including variants in genes involved in the complement pathway,[34-37] the high-density lipoprotein cholesterol pathway,[38,39] and the atherosclerosis signaling pathway.[40,41]

To date, much research has focused on the identification of risk factors for the development and worsening of AMD. In contrast, few studies have been conducted to investigate the age at onset of AMD. The age at which the first signs of AMD manifest is variable and it is important to gain insight into contributing factors. A few studies have reported an influence of genetic and environmental risk factors on the age at onset of AMD. Smoking, male sex, triglyceride levels, and variants in the *CFH*, *ARMS2*, *GSTM1*, and *VEGFA* genes have been implicated to be associated with a younger age at onset.[42-48] Statin and aspirin use seemed to postpone the time to CNV development in one study,[42] but another study[48] showed an earlier onset of AMD for statin users. Differences in study results and the magnitude of the effects on the age at onset may arise because of different definitions of the age at onset, inclusion of patients with nonadvanced stages of AMD, and few study participants in some of these studies.

The objectives of this study were to identify additional risk factors that may influence the age at onset and to determine whether conventional risk factors for AMD are also involved in an earlier age at onset of the disease. To define the age at onset, we limited inclusion to patients with neovascular AMD. This approach enabled us to identify the type and influence of genetic and environmental risk factors important in accelerating the development of neovascular AMD.

METHODS

Study Population

This study was conducted in accord with the tenets of the Declaration of Helsinki and was approved by the local ethics committee (Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen). All members of the study population provided written informed consent and participated between April 2006 and July 2010. We included patients who had neovascular AMD with at least 1 documented visit to the outpatient AMD clinic of the Radboud university medical center, Nijmegen, the Netherlands, and who were genotyped for at least 1 of the SNPs studied. In total, 385 eligible patients were selected from the European Genetic Database (EUGENDA; www.eugenda.org), a multicenter database for clinical and molecular analysis of AMD.

The age at onset of neovascular AMD was determined by reviewing historical patient data. *Age at onset* was defined as when a patient experienced the first visual complaints related to CNV occurrence in either eye, with a diagnosis of CNV by an ophthalmologist within the next 6 months.[49] Clinical diagnosis by an ophthalmologist was by slitlamp examination or by evaluation of fundus photography or fluorescein angiography. In case of scarring or other signs indicating that CNV had already been present for a longer time, the patient was excluded from the analysis.

Participants were asked to complete a questionnaire on lifestyle and other environmental variables (Table 1). For classification of smoking habits, patients were asked whether and how long they smoked. For past smokers, we also obtained the duration of smoking cessation in years. These data were adjusted to match the status at the time of CNV development. For example, a patient who had quit smoking 7 years before the interview and had developed CNV 10 years before the interview was classified as a current smoker. Body mass index was calculated from self-reported weight and height. For sunlight exposure, participants were asked how much time they had spent outside before their retirement. Exposure was classified as low for patients who indicated avoidance of the sun or spending most of the time indoors, moderate for patients who were regularly outside but no longer than 8 hours a day, and high for patients who were outside for more than 8 hours a day. If available, data on refractive error were copied from the patients' medical records.

Genetic Analysis

Venous blood samples were collected for extraction of DNA. The DNA was analyzed for SNPs in the following 9 genes previously associated with AMD: *CFH*, *ARMS2*, *CFB*, *C3*, *CFI*, *APOE*, *LPL*, *CETP* and *ABCA1* (Table 2).[30,33-40] The SNPs were genotyped using competitive allele-specific polymerase chain reaction assays (KASPar SNP Genotyping System, KBiosciences).

Statistical Analysis

All variables were analyzed using 1-way analysis of variance (ANOVA) without post hoc tests. For some variables, different categories were merged to create subgroups of sufficient size for statistical analysis. Variables with $P < .15$ were selected for inclusion in a general linear model with the age at onset as the dependent variable. For this model, multiple imputation of missing data values was used. Variables with $P \geq .05$ were removed from the model in a stepwise fashion. For the final variables included in the model, parameter estimates were calculated to derive the mean differences in the age at onset for each risk category. All analyses were performed using statistical software (SPSS, version, 20.0; IBM Corporation).

Multiple imputation of missing data values was performed under the assumption that observations were missing at random. Only the 7 variables that were included in the multivariable model were imputed. For imputation of each variable we used the 6 other variables, the outcomes, and the unselected variables from the 1-way ANOVA as predictors. Imputation was performed using the default settings in SPSS (version 20.0). From Tables 1 and 2, rates of missing data for each variable imputed can be calculated. In total, 69.1% of patients had complete data for all 7 variables. Only 9.1% of patients had missing data for more than 1 of the 7 variables.

Data from the Rotterdam Study I (RS-I), a population-based study with more than 20 years of follow-up, were used to plot survival curves for the risk factors that were identified in the multivariable model to estimate the effect of possible survival bias on our results. Details on the RS-I have been described previously.[50]

RESULTS

Of 385 eligible patients, 275 were included in the study. Complete information on the age at onset was available for 214 participants. For 38 patients, there was no exact information on the duration of visual complaints; in this group, the age at onset was defined as when CNV was diagnosed by an ophthalmologist. In a small subgroup (n = 23), we could only retrieve the year of diagnosis by an ophthalmologist. For this group, July 1 of that year was considered the date of onset. One hundred ten patients were excluded from the study because of incomplete medical records (n = 66), other macular pathology that could interfere with the diagnosis of AMD (n = 36,) and treatment in the macular region for reasons other than AMD-related CNV (n = 8).

The mean (SD) age at onset of CNV was 74.8 (7.7) years (range, 53.1-90.7 years) and 56.7% of participants were female. The patients with 1 or more missing variables did not differ significantly from the patients with complete data with respect to the age at onset. Sex, smoking, alcohol consumption, and exercise level were associated with the age at onset of neovascular AMD in the 1-way ANOVA (Table 1).

TABLE 1. Effects of nongenetic risk factors on the age at onset of neovascular AMD (one-way ANOVA)

Variable	No. (%) n = 275	Age at Onset Mean (SD), y	*P* value
Sex			.006
Male	119 (43.3)	73.3 (7.7)	
Female	156 (56.7)	75.9 (7.5)	
Smoking	(n = 268)		< .001
Never	89 (33.2)	78.5 (6.6)	
Past	127 (47.4)	73.9 (7.4)	
Current	52 (19.4)	70.4 (7.7)	
BMI	(n = 268)		.59
< 25	123 (45.9)	75.2 (7.8)	
25-30	110 (41.0)	74.6 (7.2)	
≥ 30	35 (13.1)	73.7 (9.2)	
Diabetes	(n = 268)		.36
No	237 (88.4)	74.6 (7.6)	
Yes	31 (11.6)	75.9 (9.0)	
Family history of AMD	(n = 225)		.24
No	162 (72.0)	75.2 (7.9)	
Yes	63 (28.0)	73.9 (7.4)	
Iris color	(n = 265)		.95
Light, blue/grey	165 (62.3)	74.8 (7.7)	
Medium, green/hazel	45 (17.0)	74.4 (8.6)	
Dark, brown	55 (20.8)	74.8 (7.0)	
Refractive error	(n = 134)		.19
Emmetropic	46 (34.3)	73.7 (6.3)	
Hyperopic	75 (56.0)	71.6 (7.1)	
Myopic	13 (9.7)	70.7 (8.0)	
Fish consumption	(n = 206)		.63
≥ 2 Times per week	48 (23.3)	75.0 (8.0)	
Once a week	96 (46.6)	74.3 (8.1)	
Almost never	62 (30.1)	75.6 (8.0)	
Red meat consumption	(n = 210)		.67
Every day	25 (11.9)	75.2 (5.4)	
2-6 Times per week	152 (72.4)	75.1 (8.6)	
Once per week or less	33 (15.7)	73.8 (6.9)	
Fruit consumption	(n = 210)		.78
Every day	171 (81.4)	74.8 (7.8)	
2-6 Times per week	25 (11.9)	75.1 (9.2)	
Once per week or less	14 (6.7)	76.3 (9.4)	
Vegetable consumption	(n = 211)		.17
Every day	183 (86.7)	75.2 (7.7)	
≤ 6 Times per week	28 (13.3)	73.0 (9.9)	
Alcohol consumption, U/wk	(n = 243)		.002
Never	83 (34.2)	75.9 (6.6)	
< 14	109 (44.9)	74.4 (7.9)	
14-21	35 (14.4)	72.9 (7.5)	
≥ 21	16 (6.6)	68.3 (8.4)	

Exercise level	(n = 239)		.05
Never	30 (12.6)	76.0 (6.0)	
Almost never	99 (41.4)	76.1 (7.5)	
1-2 Times per week	73 (30.5)	72.9 (8.5)	
≥ 3 Times per week	37 (15.5)	74.5 (7.8)	
Sunlight exposure	(n = 257)		.78
Low, mostly indoors	73 (28.4)	74.9 (8.2)	
Moderate, < 8 h/d outside	100 (38.9)	75.4 (7.9)	
High, ≥ 8 h/d outside	84 (32.7)	74.7 (6.1)	

AMD, age-related macular degeneration; ANOVA, analysis of variance; BMI, body mass index. Some totals do not sum to heading totals because of missing data.

Of the genetic factors analyzed, rs10490924 (*ARMS2* A69S), rs1061170 (*CFH* Y402H), and rs3764261 (*CETP*) were selected for inclusion in the multivariable model (Table 2).

Of the 7 variables that were selected from the 1-way ANOVA, only 3 remained associated with the age at onset in the multivariable model (Table 3). Compared with never smokers, past smokers developed CNV on average 4.9 (95% CI 3.0-6.8) years earlier ($P < .001$). For current smokers, this difference was even larger: they developed CNV 7.7 (95% CI 5.3-10.0) years earlier ($P < .001$). Homozygous carriers of the Y402H risk allele in *CFH* had an earlier disease onset of 2.8 (95% CI 0.5-5.0) years compared with the reference group ($P = .02$). For the *ARMS2* A69S variant, homozygous carriers developed CNV 5.2 (95% CI 2.8-7.7) years earlier ($P < .001$).

TABLE 2. Genetic influences on the age at onset of neovascular AMD (one-way ANOVA)

Variable	No. (%)	Age at Onset Mean (SD), y	*P* value
***CFH* Y402H / rs1061170**	(n = 259)		.14
TT	62 (23.9)	76.0 (7.5)	
TC	100 (38.6)	75.2 (7.6)	
CC	97 (37.5)	73.6 (7.8)	
***ARMS2* A69S / rs10490924**	(n = 260)		.001
GG	57 (21.9)	76.9 (7.8)	
GT	135 (51.9)	75.2 (7.7)	
TT	68 (26.2)	72.0 (7.1)	
***CFB* L9H / rs4151667**	(n = 258)		
TT	246 (95.3)	74.8 (7.5)	.77
TA / AA	12 (4.7)	75.4 (11.3)	
***C3* R102G / rs2230199**	(n = 260)		.29
CC	144 (55.4)	74.3 (7.1)	
CG	95 (36.5)	75.8 (8.1)	
GG	21 (8.1)	73.9 (8.9)	
***CFH* / rs1410996**	(n = 208)		.85
CC	117 (56.3)	74.7 (7.3)	
CT / TT	91 (43.8)	74.9 (8.2)	
***CFB* R32Q / rs641153**	(n = 171)		.31
GG	158 (92.4)	75.0 (8.1)	
GA / AA	13 (7.6)	77.3 (7.7)	
***APOE2* / rs7412**	(n = 195)		.81
CC	158 (81.0)	75.1 (7.6)	
CT/TT	37 (19.0)	74.8 (7.3)	
***APOE4* / rs429358**	(n = 171)		.88
TT	139 (81.3)	75.3 (7.5)	
TC	32 (18.7)	75.1 (7.9)	
***CFI* / rs10033900**	(n = 256)		.41
CC	68 (26.6)	75.3 (7.9)	
CT	130 (50.8)	74.2 (8.1)	
TT	58 (22.7)	75.6 (6.4)	
***LPL* / rs12678919**	(n = 257)		.44
AA	214 (83.3)	74.8 (7.6)	
AG / GG	43 (16.7)	75.8 (8.2)	
***CETP* / rs3764261**	(n = 262)		.08
GG	100 (38.2)	75.9 (8.1)	
GT	120 (45.8)	73.7 (7.8)	
TT	42 (16.0)	75.7 (6.0)	
***ABCA1* / rs1883025**	(n = 202)		.99
GG	110 (54.5)	75.0 (7.6)	
GA / AA	92 (45.5)	75.0 (8.1)	

AMD, age-related macular degeneration; ANOVA, analysis of variance. Some totals do not sum to heading totals because of missing data.

TABLE 3. Multivariable model showing associations of smoking, *CFH* and *ARMS2* on the age at onset of neovascular AMD

Variable	Difference in the Age at Onset, Mean (95% CI), y	*P* value
Smoking		
Never	0	
Past	-4.9 (-6.8 to -3.0)	< .001
Current	-7.7 (-10.0 to -5.3)	< .001
***CFH* Y402H / rs1061170**		
TT	0	
TC	-1.9 (-4.1 to 0.3)	.07
CC	-2.8 (-5.0 to -0.5)	.02
***ARMS2* A69S / rs10490924**		
GG	0	
GT	-2.0 (-4.1 to 0.2)	.07
TT	-5.2 (-7.7 to -2.8)	< .001

AMD, age-related macular degeneration; CFH, complement factor H; ARMS2, age-related macular susceptibility 2.

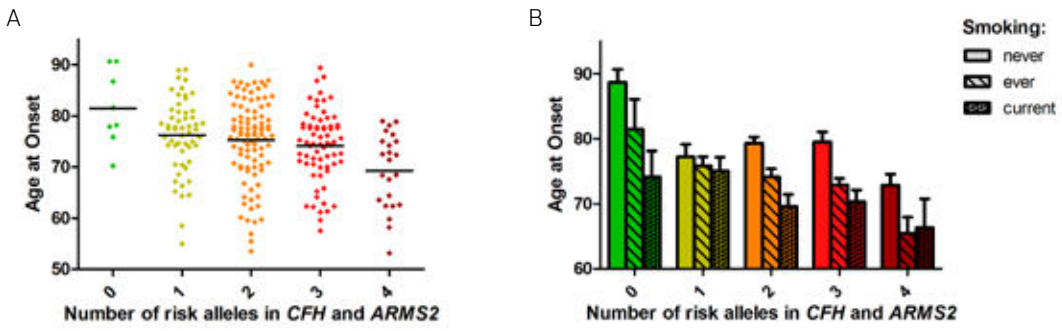
To explore the combined effect of *CFH* and *ARMS2* on the age at onset, we delineated patient groups based on the number of risk alleles of these 2 variants. There was a difference in the age at onset of 12.2 (95% CI 6.2-18.3) years when we compared individuals having zero risk alleles in *CFH* and *ARMS2* with those carrying all 4 risk alleles ($P < .001$) (Table 4 and Figure, A). Panel B in the Figure shows the substantial effect of smoking the age at onset in addition to the genetic risk. Current smokers with zero risk alleles developed CNV 14.5 years earlier compared to never smokers with the same genetic risk profile ($P = .08$). The sole effect of smoking resulted in an age at onset comparable to that of never smokers carrying all 4 risk alleles. When we compared the most extreme groups in panel B in the Figure, there was an observed absolute difference in the age at onset of 22.4 years ($P = .03$). Some of these subgroups were small (range, 2-40 patients).

TABLE 4. Cumulative effect of the number of risk alleles in *CFH* and *ARMS2* on the age at onset of neovascular AMD

No. of risk alleles in *CFH* and *ARMS2*	No. (%) (n = 251)	Difference in the Age at Onset, Mean (95% CI), y	*P* value
0 risk alleles	8 (3.2)	0	
1 risk allele	55 (21.9)	-5.2 (-10.0 to 0.4)	.07
2 risk alleles	96 (38.2)	-6.2 (-11.7 to -0.8)	.03
3 risk alleles	69 (27.5)	-7.4 (-12.9 to -1.9)	.009
4 risk alleles	23 (9.2)	-12.2 (-18.3 to -6.2)	< .001

AMD, age-related macular degeneration; CFH, complement factor H; ARMS2, age-related macular susceptibility 2.

FIGURE 1. Cumulative effect of *CFH*, *ARMS2* and smoking on the age at onset of neovascular age-related macular degeneration



A, Age at onset for each individual based on number of risk alleles in CFH and ARMS2. B, Effect of smoking on mean age at onset for each genetic risk stratum.
CFH, complement factor H; ARMS2, age-related macular susceptibility 2.

The exact age at onset was sometimes difficult to determine. To exclude the possibility of introducing bias, we reran the analyses in a subgroup, selecting only those patients for whom the age at onset was most accurately defined and diagnosed (duration of visual complaints < 6 months, exact date of diagnosis known, and diagnosis confirmed in the Radboud university medical center). These results did not differ significantly; to maintain statistical power, we chose to adhere to the analyses of the complete dataset.

Analyses with data from the RS-I revealed that there was no difference in survival for the different genotypes in *CFH* and *ARMS2*. Survival curves for smoking showed that current smokers had a shorter survival than past smokers and never smokers. Before reaching age 70 years, 7.9% of smokers vs 3.2% of never smokers had died. In total, 79.5% of smokers reached age 75 years compared with 90.2% of never smokers.

DISCUSSION

The development of CNV accounts for most legal blindness in patients with AMD.[1,2] However, the range in the age at onset of CNV is broad, with some patients developing neovascularization as early as age 50 years, whereas others are well past age 90 years before this occurs. On average, the patients in this study were age 74.8 years when their first CNV developed. Many potential risk factors that might influence the age at onset of neovascular complications in AMD were examined. In the presence of homozygous *CFH* Y402H and *ARMS2* A69S risk alleles, the age at onset was advanced by 12.2 years. In addition, smoking was also associated with the development of neovascular AMD at a younger age. When we added smoking to the genetic risk factors, the difference in the age at onset between the most extreme groups increased to 22.4 years. Although these results should be interpreted with caution because of small group sizes in the combination models, no other study has reported effect sizes of this magnitude, to our knowledge. The substantial effect of smoking, *CFH*, and *ARMS2* on the age at onset of CNV has consequences for patient counseling. Based on our results, we hypothesize that many disease-free years may be attained if a patient stops smoking, and it seems likely that other preventive measures (eg, the intake of dietary supplements) could be of benefit as well. Because the development of CNV may occur well before age 70 years in high-risk patients, these countermeasures have to be initiated in a timely manner to be of significance.

The risk factors that remained in the multivariable model accelerate the age at onset probably by increasing complement activity and oxidative stress levels in the retina. Decreased functionality of the CFH protein, for instance in persons with the *CFH* Y402H polymorphism, leads to a continuous state of low-level complement activation.[51,52] Complement activation is known to lead to increased vascular endothelial growth factor (VEGF) expression and CNV formation.[52-54] For ARMS2, some investigators suggest that it is a mitochondrial protein involved in oxidative stress responses,[31,55] although this was disputed by others.[56] One study[57] found that the protein interacts with proteins from the extracellular matrix, while another study[58] demonstrated that ARMS2 is involved in the phagocytosis of photoreceptor outer segments. In 2012, it was reported that the *ARMS2* risk allele is independently associated with increased complement activation in patients with AMD.[59] Cigarette smoke adds to oxidative stress[60] and is known to induce VEGF expression, as well as retinal pigment epithelium (RPE) dysfunction and diminished RPE cell survival that may effect angiogenic homeostasis.[61,62] Moreover, cigarette smoke increases the activity of the alternative complement pathway by weakening the susceptibility of C3 to CFH and factor I.[51,63] Smoking, *CFH* Y402H, and *ARMS2* A69S have been associated with CNV development in large independent cohorts.[3,4,44,64-66] We hypothesize that increased oxidative stress, a continuous state of complement activation, and upregulation of VEGF expression not only increase risk of CNV development but may accelerate the age at which neovascularization occurs.

The association of *CFH* variants with early onset of neovascular AMD was suggested in a 2011 study,[45] in which a rare penetrant mutation in *CFH* was associated with a 6-year earlier onset of disease in patients with advanced AMD. Recently, another study[48] confirmed the association of *CFH* and *ARMS2* with an earlier onset of AMD and showed that heavy smokers are also affected with AMD at a younger age. In that study, the largest difference in the age at onset reported was only 2.2 years for heavy smokers versus the reference group, while for *CFH* and *ARMS2* there was a less than 2-year difference in onset between homozygous carriers of the risk variants compared with those carrying the low-risk variants. These differences are small compared with our findings. However, it is unclear how the age at onset was defined in the study by Keilhauer et al,[48] and their study included patients with CNV and patients with early AMD. Inclusion of different AMD stages makes it impossible to uniformly determine the age at onset in all participants, and this could be reflected in the study outcomes. Several other studies[42,46,67] evaluating the age at onset restricted inclusion to patients with CNV, as described above. However, these study populations were small (≤ 131 patients with CNV), and racial/ethnic differences hinder comparison with the present study.

This study identified much larger effect sizes of *CFH*, *ARMS2*, and smoking on the age at onset than previously reported by including only individuals with neovascular AMD and using a clear definition of the onset of neovascular complications. Because of the nature of the EUGENDA database, we were able to study the effect of multiple genetic and environmental risk factors on the age at onset of neovascular AMD, whereas other studies[42,46,48,67] limited their design to few variables. This enabled us to also study the combined effect of genetic and environmental risk factors.

However, a retrospective study design has its limitations. A possible confounding factor is the subjective nature of the duration of visual complaints and the decision whether these complaints are in fact related to CNV development. However, subgroup analyses that included only those patients with a well-defined age at onset yielded results similar to those of the main analyses. Another possible confounder is the interference of survival bias. Survival curves from the RS-I showed that 79.5% of smokers reached age 75 years (the mean age at onset in this study) compared with 90.2% of never smokers. This makes it unlikely that survival bias accounts for the effect of smoking on the age at onset that we reported. Furthermore, smoking can be seen as an accelerator of aging directly (through oxidative stress) and indirectly (through smoking-associated diseases).[68,69] In this light, it may be that smokers have the same chance of developing neovascular AMD as never smokers, although their survival is shorter.

The current study investigated potential risk factors for the age at CNV onset and did not include patients with GA. Because there is a strong overlap in risk factors for CNV and GA development,[65,66,70-72] we hypothesize that the same risk factors that contribute to the development of GA also lead to an earlier age at onset of GA. It would be difficult to analyze this in a retrospective study because the exact onset of GA in patients with AMD is difficult to define.

CONCLUSIONS

In individuals who are homozygous for *CFH* and *ARMS2* risk alleles and who smoke cigarettes, the onset of neovascular AMD may be accelerated by as much as 1 or 2 decades. To allow countermeasures the time to take effect, these individuals need to be identified at an early age. Providing individuals with a personalized prognosis may give them the incentive to stop smoking. Additional preventive measures such as a healthy diet, use of nutritional supplements, frequent monitoring, and possible future therapeutic options aimed at lowering complement activity could postpone severe visual loss associated with the development of neovascular membranes.

REFERENCES

1. Ferris FL 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* 1984;102:1640-1642.
2. Klein R, Wang Q, Klein BE, Moss SE, Meuer SM. The relationship of age-related maculopathy, cataract, and glaucoma to visual acuity. *Invest Ophthalmol Vis Sci* 1995;36:182-191.
3. Risk factors for neovascular age-related macular degeneration. The Eye Disease Case-Control Study Group. *Arch Ophthal* 1992;110:1701-1708.
4. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology* 2000;107:2224-2232.
5. Christen WG, Glynn RJ, Manson JE, Ajani UA, Buring JE. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 1996;276:1147-1151.
6. Delcourt C, Diaz JL, Ponton-Sanchez A, Papoz L. Smoking and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. *Arch Ophthal* 1998;116:1031-1035.
7. Klein R, Klein BE, Linton KL, DeMets DL. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. *Am J Epidemiol* 1993;137:190-200.
8. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA* 1996;276:1141-1146.
9. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. The Blue Mountains Eye Study. *Arch Ophthal* 1996;114:1518-1523.
10. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. *Arch Ophthalmol* 1996;114:1193-1196.
11. Vinding T, Appleyard M, Nyboe J, Jensen G. Risk factor analysis for atrophic and exudative age-related macular degeneration. An epidemiological study of 1000 aged individuals. *Acta Ophthalmol* 1992;70:66-72.
12. Hyman LG, Lilienfeld AM, Ferris FL 3rd, Fine SL. Senile macular degeneration: a case-control study. *Am J Epidemiol* 1983;118:213-227.
13. Delcourt C, Michel F, Colvez A, et al. Associations of cardiovascular disease and its risk factors with age-related macular degeneration: the POLA study. *Ophthalmic Epidemiol* 2001;8:237-249.
14. Klein BE, Klein R, Lee KE, Jensen SC. Measures of obesity and age-related eye diseases. *Ophthalmic Epidemiol* 2001;8:251-262.
15. Schaumberg DA, Christen WG, Hankinson SE, Glynn RJ. Body mass index and the incidence of visually significant age-related maculopathy in men. *Arch Ophthal* 2001;119:1259-1265.
16. Seddon JM, Cote J, Davis N, Rosner B. Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio. *Arch Ophthal* 2003;121:785-792.
17. Smith W, Mitchell P, Leeder SR, Wang JJ. Plasma fibrinogen levels, other cardiovascular risk factors, and age-related maculopathy: the Blue Mountains Eye Study. *Arch Ophthal* 1998;116:583-587.
18. Cruickshanks KJ, Klein R, Klein BE. Sunlight and age-related macular degeneration. The Beaver Dam Eye Study. *Arch Ophthal* 1993;111:514-518.
19. Mitchell P, Smith W, Wang JJ. Iris color, skin sun sensitivity, and age-related maculopathy. The Blue Mountains Eye Study. *Ophthalmology* 1998;105:1359-1363.
20. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. The long-term effects of visible light on the eye. *Arch Ophthal* 1992;110:99-104.
21. Mitchell P, Wang JJ, Foran S, Smith W. Five-year incidence of age-related maculopathy lesions: the Blue Mountains Eye Study. *Ophthalmology* 2002;109:1092-1097.
22. Rudnicka AR, Jarrar Z, Wormald R, Cook DG, Fletcher A, Owen CG. Age and Gender Variations in Age-related Macular Degeneration Prevalence in Populations of European Ancestry: A Meta-analysis. *Ophthalmology* 2012;119:571-580.
23. Mares-Perlman JA, Brady WE, Klein R, VandenLangenberg GM, Klein BE, Palta M. Dietary fat and age-related maculopathy. *Arch Ophthal* 1995;113:743-748.
24. Seddon JM, Ajani UA, Sperduto RD, et al. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. *JAMA* 1994;272:1413-1420.
25. Seddon JM, Rosner B, Sperduto RD, et al. Dietary fat and risk for advanced age-related macular degeneration. *Arch Ophthal* 2001;119:1191-1199.
26. VandenLangenberg GM, Mares-Perlman JA, Klein R, Klein BE, Brady WE, Palta M. Associations between antioxidant and zinc intake and the 5-year incidence of early age-related maculopathy in the Beaver Dam Eye Study. *Am J Epidemiol* 1998;148:204-214.

27. Age-Related Eye Disease Study 2 Research Group. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. *JAMA* 2013;309:2005-2015.
28. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
29. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
30. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
31. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* 2008;40:892-896.
32. Jakobsdottir J, Conley YP, Weeks DE, Mah TS, Ferrell RE, Gorin MB. Susceptibility genes for age-related maculopathy on chromosome 10q26. *Am J Hum Genet* 2005;77:389-407.
33. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
34. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
35. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
36. Li M, Atmaca-Sonmez P, Othman M, et al. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat Genet* 2006;38:1049-1054.
37. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
38. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2010;107:7401-7406.
39. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A* 2010;107:7395-7400.
40. Klaver CC, Kliffen M, van Duijn CM, et al. Genetic association of apolipoprotein E with age-related macular degeneration. *Am J Hum Genet* 1998;63:200-206.
41. Yu Y, Bhangale TR, Fagerness J, et al. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. *Hum Mol Genet* 2011;20:3699-3709.
42. Wilson HL, Schwartz DM, Bhatt HR, McCulloch CE, Duncan JL. Statin and aspirin therapy are associated with decreased rates of choroidal neovascularization among patients with age-related macular degeneration. *Am J Ophthalmol* 2004;137:615-624.
43. Shuler RK, Jr., Hauser MA, Caldwell J, et al. Neovascular age-related macular degeneration and its association with LOC387715 and complement factor H polymorphism. *Arch Ophthal* 2007;125:63-67.
44. Shuler RK, Jr., Schmidt S, Gallins P, et al. Phenotype analysis of patients with the risk variant LOC387715 (A69S) in age-related macular degeneration. *Am J Ophthalmol* 2008;145:303-307.
45. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet* 2011;43:1232-1236.
46. Othman H, Gholampour AR, Saadat I, Farvardin-Jahromoi M, Saadat M. Age-related macular degeneration and genetic polymorphisms of glutathione S-transferases M1 (GSTM1) and T1 (GSTT1). *Mol Biol Rep* 2012;39:3299-3303.
47. Smailhodzic D, Muether PS, Chen J, et al. Cumulative Effect of Risk Alleles in CFH, ARMS2, and VEGFA on the Response to Ranibizumab Treatment in Age-Related Macular Degeneration. *Ophthalmology* 2012;119:2304-2311.
48. Keilhauer CN, Fritsche LG, Guthoff R, Haubitz I, Weber BH. Age-related macular degeneration and coronary heart disease: evaluation of genetic and environmental associations. *Eur J Med Genet* 2013;56:72-79.
49. Schalnus R, Meyer CH, Kuhli-Hattenbach C, Luchtenberg M. Time between symptom onset and assessment in age-related macular degeneration with subfoveal choroidal neovascularization. *Ophthalmologica* 2010;224:176-182.
50. Hofman A, Darwish Murad S, van Duijn CM, et al. The Rotterdam Study: 2014 objectives and design update. *Eur J Epidemiol* 2013;28:889-926.
51. Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. *JAMA* 2006;296:301-309.
52. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One* 2008;3:e2593.

53. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. *Proc Natl Acad Sci U S A* 2006;103:2328-2333.
54. Rohrer B, Long Q, Coughlin B, et al. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2009;50:3056-3064.
55. Kanda A, Chen W, Othman M, et al. A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. *Proc Natl Acad Sci U S A* 2007;104:16227-16232.
56. Wang G, Spencer KL, Court BL, et al. Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria. *Invest Ophthalmol Vis Sci* 2009;50:3084-3090.
57. Kortvely E, Hauck SM, Duetsch G, et al. ARMS2 is a constituent of the extracellular matrix providing a link between familial and sporadic age-related macular degenerations. *Invest Ophthalmol Vis Sci* 2010;51:79-88.
58. Xu YT, Wang Y, Chen P, Xu HF. Age-related maculopathy susceptibility 2 participates in the phagocytosis functions of the retinal pigment epithelium. *Int J Ophthalmol* 2012;5:125-132.
59. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
60. Bertram KM, Baglole CJ, Phipps RP, Libby RT. Molecular regulation of cigarette smoke induced-oxidative stress in human retinal pigment epithelial cells: implications for age-related macular degeneration. *Am J Physiol Cell Physiol* 2009;297:C1200-1210.
61. Chu YK, Lee SC, Byeon SH. VEGF rescues cigarette smoking-induced human RPE cell death by increasing autophagic flux: implications of the role of autophagy in advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2013;54:7329-37.
62. Pons M, Marin-Castano ME. Cigarette smoke-related hydroquinone dysregulates MCP-1, VEGF and PEDF expression in retinal pigment epithelium in vitro and in vivo. *PLoS One* 2011;6:e16722.
63. Kew RR, Ghebrehiwet B, Janoff A. Cigarette smoke can activate the alternative pathway of complement in vitro by modifying the third component of complement. *J Clin Invest* 1985;75:1000-1007.
64. Magnusson KP, Duan S, Sigurdsson H, et al. CFH Y402H confers similar risk of soft drusen and both forms of advanced AMD. *PLoS Med* 2006;3:e5.
65. Seddon JM, Francis PJ, George S, Schultz DW, Rosner B, Klein ML. Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. *JAMA* 2007;297:1793-1800.
66. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. *Ophthalmology* 2001;108:697-704.
67. Chowers I, Cohen Y, Goldenberg-Cohen N, et al. Association of complement factor H Y402H polymorphism with phenotype of neovascular age related macular degeneration in Israel. *Mol Vis* 2008;14:1829-1834.
68. Bernhard D, Moser C, Backovic A, Wick G. Cigarette smoke--an aging accelerator? *Exp Gerontol* 2007;42:160-165.
69. Nicita-Mauro V, Lo Balbo C, Mento A, Nicita-Mauro C, Maltese G, Basile G. Smoking, aging and the centenarians. *Exp Gerontol* 2008;43:95-101.
70. Francis PJ, George S, Schultz DW, et al. The LOC387715 gene, smoking, body mass index, environmental associations with advanced age-related macular degeneration. *Hum Hered* 2007;63:212-218.
71. Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat Genet* 2006;38:1055-1059.
72. Seddon JM, George S, Rosner B, Klein ML. CFH gene variant, Y402H, and smoking, body mass index, environmental associations with advanced age-related macular degeneration. *Hum Hered* 2006;61:157-165.

CHAPTER 3

GENETIC, BEHAVIORAL AND SOCIODEMOGRAPHIC RISK FACTORS FOR SECOND EYE PROGRESSION IN AGE-RELATED MACULAR DEGENERATION.

Yara T.E. Lechanteur,
Johannes P.H. van de Ven,
Dzenita Smailhodzic,
Camiel J.F. Boon,
B. Jeroen Klevering,
Sascha Fauser,
Joannes M.M. Groenewoud,
Gert-Jan van der Wilt,
Anneke I. den Hollander,
Carel B. Hoyng.

Invest Ophthalmol Vis Sci 2012;53(9):5846-5852

PURPOSE This study was conducted to investigate the correlation of genetic, sociodemographic and behavioral risk factors with second eye progression to end-stage age-related macular degeneration (AMD).

METHODS One hundred-eight patients with end-stage AMD in one or both eyes were included in a retrospective time-to-event analysis of the onset of end-stage AMD in the second eye. Multivariate Cox regression survival analysis was performed for sex, age, smoking, body mass index (BMI), education and sixteen single nucleotide polymorphisms (SNPs) associated with AMD.

RESULTS Except for education, all sociodemographic and behavioral risk factors analyzed were significantly associated with a more rapid progression toward second eye involvement. Hazard ratios (HR) were 2.6 (95% confidence interval [CI] 1.4-5.0) for female sex, 5.0 (95% CI 2.0-12.5) for age > 80, 2.2 (95% CI 1.1-4.1) for BMI > 30, and 4.4 (95% CI 1.4-14.3) for > 40 pack years, compared with the referent groups. Carriers of the lipoprotein lipase (*LPL*; rs12678919) risk alleles were at risk for more rapid progression to end-stage AMD in the second eye compared to the referent wild-type genotype (HR 2.0; 95% CI 1.0-3.6). For complement factor I (*CFI*; rs10033900), homozygous carriers of the risk allele progressed faster than wild-type individuals (HR 2.2; 95% CI 1.1-4.3).

CONCLUSIONS Sociodemographic, behavioral and genetic risk factors are associated with the rate of second eye progression toward end-stage AMD. The findings of this study underline the importance of lifestyle factors and the complement pathway in AMD progression and suggest a role of the high-density-lipoprotein-metabolism in second eye progression.

Age-related macular degeneration (AMD) is a multifactorial disease of the central retina and the most prevalent cause of progressive vision loss in the elderly in the developed world, with a prevalence of 30% after the age of 75 years.[1] With the rapid increase of the elderly population, AMD is considered a major and growing health problem.

Several studies have investigated the contribution of behavioral, genetic, sociodemographic and ocular risk factors to the incidence and progression of AMD, as well as the development of choroidal neovascularization (CNV) or foveal geographic atrophy (GA), viewed as the end-stages of AMD.[2-10]

Patients with unilateral advanced AMD are at high risk of developing end-stage AMD in their fellow eye.[11] Cumulative incidence rates have been reported to be 10% to 14%, 28% to 31% and 36% to 37% after 1, 3, and 4 years of follow-up respectively.[12,13] Some studies have tried to identify risk factors for progression to advanced AMD in the second eye, but results in the different studies are not unanimous. Smoking, age, body mass index (BMI), and systemic hypertension – as well as ocular characteristics such as drusen size, presence of ≥ 5 drusen, focal hyperpigmentation, and non-foveal GA – have been suggested as possible risk factors.[12,14-17]

A variety of single nucleotide polymorphisms (SNPs) have been reported to be associated with AMD.[18] It has been shown that complement factor H (*CFH*) Y402H, age-related maculopathy susceptibility 2 (*ARMS2*) A69S, complement component 2 (*C2*) E318D and complement component 3 (*C3*) R102G risk alleles are associated with progression toward bilateral advanced AMD.[15,16] Carrying all these genetic risk factors together with modifiable risk factors such as smoking and high BMI, increases the risk of developing advanced AMD by a factor of 19.[15]

The purpose of this study is to determine the correlation of genetic, sociodemographic, and behavioral risk factors with second eye progression to end-stage AMD. This will not only allow the selection of patients with a high risk for development and progression of AMD in the second eye, but also provide more insight into the development of AMD. Furthermore, it will provide patients with additional and more accurate information regarding their individual risk profile.

METHODS

Study Population

All 108 subjects were selected by means of chart review from the European Genetic Database (EUGENDA, www.eugenda.org [in the public domain]) and were entered into the database between January 1997 and December 2006. EUGENDA is a multicenter database of AMD patients and control subjects founded by the Radboud University Nijmegen Medical Center and the University of Cologne Medical Center. Before enrollment in the database, written informed consent was obtained from all participants after receiving information about the study objectives and methods.

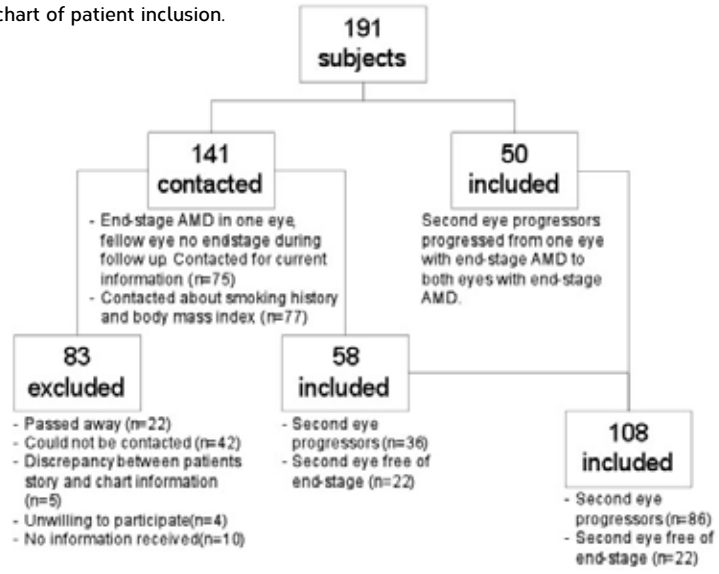
Information about smoking, BMI and education were obtained by a questionnaire. For smoking, patients were asked if they had ever smoked, how long they had smoked, if and how long they had quit, and how many cigarettes a day were smoked on average. For each patient, the number of pack years was calculated, where one pack year was the equivalent of smoking 20 cigarettes a day for 1 year. BMI was calculated from self-reported weight and height of patients. Education was classified into four levels: primary school, high school, higher professional education and university. For statistical analysis the four categories were collapsed into high school or lower and higher than high school.

Color fundus photographs and fluorescein angiography images were taken with a digital fundus camera (Topcon TRC 50IX; Topcon Corporation, Tokyo, Japan). For inclusion, end-stage AMD had to be present in at least one eye. End-stage AMD was defined as either choroidal neovascularization within the central 6 mm ETDRS grid or geographic atrophy of an area of at least 175 µm including the fovea.[19,20] Development of advanced AMD in the first eye was taken as starting-point (T[0]) and had to be known with an accuracy range of 1 month; an accuracy range of 6 months was accepted if the second eye did not develop end-stage AMD within 4 years. Progression time until the development of end-stage AMD in the fellow eye was calculated in months after T(0). The following exclusion criteria were used: no end-stage AMD in both eyes; unknown or unclear time of end-stage AMD in one or both eyes; other retinal diseases that interfered with the diagnosis of end-stage AMD, such as central serous chorioretinopathy; laser treatment or radiotherapy for a retinal disease or treatment for AMD in a stage that could not be determined as end-stage (e.g., laser therapy for extensive drusen). Patients were excluded at baseline. If patients met one of the exclusion criteria during follow-up, they were included in the study until the moment of exclusion and data were entered as censored values.

A group of 191 patients was selected according to the in- and exclusion criteria. Seventy-five patients with end-stage AMD in one eye remained free of CNV or foveal GA in their second eye during the period of follow-up at our department. Patients lost to follow-up for a period of more than 4 months were contacted by phone to determine if the second eye had developed advanced AMD during this period. The status of the second eye was verified either by inviting the patients to our outpatient clinic for clinical evaluation (if the patient reported that the second eye still had clear vision) or by contacting the patient's current ophthalmologist to request conformational data (if the patient was unable to visit our outpatient clinic or if the patient reported that the second eye was also lost to AMD). Only if patients reported that they had developed neovascular AMD for which they had received intraocular injections with anti-VEGF medication, was this information considered reliable and were the patient-reported data used without further supportive evidence. An additional 77 patients were contacted for additional information about smoking history and BMI. In total, 83 patients were excluded because they could not be contacted, had passed away, were not willing to participate or because the patients' information was inconsistent with the information

available from the charts. A total of 86 patients were classified as "second eye progressors," whereas 22 patients did not develop end-stage AMD in the fellow eye during follow up. Data of this last group were entered as censored values. Figure 1 represents a flow chart of patient inclusion. All individuals in the current study were from the Nijmegen (the Netherlands) area. This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Center (Nijmegen, the Netherlands) and University Eye Clinic of Cologne (Cologne, Germany).

FIGURE 1. Flow chart of patient inclusion.



AMD, age-related macular degeneration.

Genetic Analysis

DNA was isolated from venous blood leukocytes and analyzed for 16 SNPs known to be associated with AMD: rs1061170/*CFH* Y402H,[6,21-23] rs10490924/*ARMS2* A69S,[7,24-26] rs4151667/*CFB* H9L,[27,28] rs9332739/*C2* E318D,[27-29] rs2230199/*C3* R102G,[27,30-33] rs10033900/*CFI*,[34,35] rs1410996/*CFH* IVS14,[16,36,37] rs2511989/*SERPING1*,[38,39] rs1883025/*ABCA1*,[40-42] rs3775291/*TLR3* L412F,[43,44] rs7412/*APOE*, E2 allele,[45-47] rs429358/*APOE*, E4 allele,[45-47] rs3764261/*CETP*,[40,41,48] rs12678919/*LPL*,[40,41,48] rs10468017/*LIPC*,[40,41] and rs174547/*FADS1_3*.[41,42] Genotyping of SNPs in all the genes analyzed except for the *CFH* variant Y402H was carried out as described elsewhere.[49] The *CFH* variant Y402H (rs1061170) was analyzed by direct sequencing of PCR products using forward primer TCATTGTTATGGTCCTTAGG and reverse primer AAAGACATGAACATGCTAGG. Polymerase chain reaction (PCR) amplification was conducted following standard protocols

(primers sequences and PCR conditions available upon request). All genotyping was performed in the same lab.

Statistical Analysis

Variables were entered in a Cox regression model for survival analysis and were first analyzed in a univariate model. Statistically significant variables ($P < .05$) were analyzed in a multivariate model. To correct for possible confounding, important nonsignificant variables were also included in the multivariate model. For each AMD-associated SNP, a Cox regression model was made, controlling for the statistically significant baseline variables. Survival timing started at T(0), the moment of end-stage AMD in the first eye. The event was defined as the development of advanced AMD in the second eye. A two-sided *P* value of .05 was considered to be statistically significant. For smoking we analyzed the total number of pack years up to T(0) and BMI was calculated at T(0). All analyses were conducted using statistical analysis software (SPSS 16.0; IBM Corporation, Armonk, NY).

RESULTS

Analysis of Sociodemographic Factors

Mean age was 74.3 years (range 54.3-93.4; standard deviation ± 7.2) in our studied cohort. There were 37 males (34.3%) and 71 females (65.7%). The type of end-stage AMD in the first eye was CNV in 82.4% and GA in 3.7% of cases. Of those who progressed toward end-stage in the fellow eye, 68.5% had a CNV and 9.3% had GA in the second eye.

Sex, age, and BMI were significantly associated with second eye progression in the univariate analysis. For pack years and education no association could be observed. Since both factors have been implied to be involved in (second eye) progression in AMD, they were also added to the multivariate model. Table 1 shows the results from the multivariate analysis. Sex, age, pack years, and BMI were all associated with the risk of developing end-stage AMD in the second eye. For sex and pack years, these findings reached statistical significance. For age, the risk increases with each age category. Only for the two highest age categories was significance reached. Obese patients (BMI ≥ 30) are more at risk for second eye progression as compared with patients with normal weight (BMI 18-25; $P = .020$). For overweight patients (BMI 25-30) there was a trend toward an increased risk, but this did not reach a conventional level of statistical significance. This study used self-reported data on weight and height. It has been shown that people may overstate their height and underreport their weight.[50-53] This would result in an underestimation of the prevalence of obesity. It has therefore been suggested to evaluate BMI as a continuous variable rather than using predefined BMI categories.[52,53] We repeated our analyses with this modification and this did not affect our results in any way. Information about education was available for all study subjects, except for 11 patients. In this study, education was not related to second eye progression, but a trend for

higher education being protective was observed. Additional analyses revealed that there were more females and more older persons in the group with 0 to 1 pack years. This explains why smoking was only significant in the multivariate analysis. With the inclusion of smoking in the multivariate analysis, the effects of age and sex also became more pronounced.

TABLE 1. Multivariate association between sociodemographic risk factors and progression toward end-stage AMD in the fellow eye of patients with unilateral advanced AMD

Variable	No. (%) (n = 108)	Hazard Ratio* (95% CI)	*P* value
Sex			
Male	37 (34)	1	
Female	71 (66)	2.6 (1.4-5.0)	.004
Age†			
< 65	17 (16)	1	
65-70	15 (14)	1.2 (0.5-2.7)	.704
70-75	23 (21)	1.5 (0.7-3.1)	.255
75-80	37 (34)	2.6 (1.3-5.3)	.010
≥ 80	16 (15)	5.0 (2.0-12.5)	.001
BMI			
Normal weight (18-25)	52 (48)	1	
Overweight 25-30	40 (37)	1.3 (0.8-2.1)	.375
Obese (≥ 30)	16 (15)	2.2 (1.1-4.1)	.020
PY‡			
0-1	45 (42)	1	
1-40	54 (50)	2.4 (1.3-4.5)	.005
≥ 40	9 (8)	4.4 (1.4-14.3)	.014
Education§			
≤ High school	59 (61)	1	
> High school	38 (39)	0.6 (0.4-1.1)	.128

AMD, age-related macular degeneration; CI, confidence interval; BMI, body mass index; PY, pack years.
** Corrected for sex, age, BMI and PY.*
† Age at moment of end-stage AMD in the first eye.
‡ Total amount of pack years smoked.
§ Analysis restricted to 97 patients.

Analysis of Genetic Variants

Genotype data of the SNPs were available for at least 87% of all patients. Because of the clear stepwise increase in hazard ratios (HR) with each age category, age was entered as a continuous variable. Results are shown in Table 2. There was an increased risk for homozygous carriers of the *CFI* risk allele with a HR of 2.2 ($P = .028$). Heterozygous and homozygous carriers of the *LPL* risk allele had a higher HR compared with homozygous individuals for the nonrisk alleles (HR 2.0; $P = .036$). Other evaluated risk alleles did not make a significant contribution to development of end-stage AMD in the second eye.

To test for gene-gene interaction of the *CFH* Y402H and *ARMS2* A69S genotypes, separate analyses for each possible allele combination of these two SNPs were performed. Comparison of subjects who were homozygous for both risk alleles with subjects carrying the wild-type alleles for both genotypes yielded no significant results (results not shown here). Since the *LPL* and *CFI* SNPs were significantly related to second eye progression, gene-gene interaction between these two genotypes was also analyzed. Subjects with two or more risk alleles had a higher risk of second eye progression (HR 2.7; 95% CI 1.3-5.6; $P = .008$) when compared with subjects with no risk alleles. No interaction was found between the *LPL* and *CFI* genotypes.

TABLE 2. Multivariate association between 21 SNPs and progression toward end-stage AMD in the fellow eye of patients with unilateral advanced AMD

Variable	No. (%) (n = 108)	Hazard Ratio* (95% CI)	*P* value
***CFI*/rs10033900**			
CC	24 (24.5)	1	
CT	46 (46.9)	1.2 (0.6-2.4)	.519
TT	28 (28.6)	2.2 (1.1-4.3)	.028
***LPL*/rs12678919**			
AA	88 (84.6)	1	
AG/GG	16 (15.4)	2.0 (1.0-3.6)	.036
***CFH* IVS14/rs1410996**			
TT	5 (4.7)	1	
CT	35 (32.7)	2.2 (0.7-6.7)	.177
CC	67 (62.6)	2.6 (0.9-7.6)	.081
***LIPC*/rs10468017**			
CC	49 (49.5)	1	
CT	42 (42.4)	1.5 (0.9-2.4)	.125
TT	8 (8.1)	0.9 (0.3-2.3)	.760
***ARMS2* S69 A/rs10490924**			
GG	24 (23.5)	1	
GT	49 (48.0)	0.8 (0.5-1.5)	.565
TT	29 (28.4)	0.7 (0.4-1.4)	.339
***CFH* Y402H/rs1061170**			
TT	15 (14.6)	1	
TC	44 (42.7)	1.2 (0.6-2.6)	.571
CC	44 (42.7)	1.3 (0.6-2.6)	.476
***CFB* H9L/rs4151667**			
TT	91 (94.8)	1	
TA/AA	5 (5.2)	1.0 (0.4-2.6)	.966
***C3* R102G/rs2230199**			
CC	53 (52.0)	1	
CG	40 (39.2)	1.3 (0.8-2.0)	.363
GG	9 (8.8)	1.4 (0.6-3.1)	.477

***C2* E318D/rs9332739**			
GG	101 (95.3)	1	
GC/CC	5 (4.7)	1.1 (0.4-2.9)	.873
***SERPING1*/rs2511989**			
GG	36 (37.5)	1	
GA	49 (51.0)	1.1 (0.7-1.9)	.697
AA	11 (11.5)	0.9 (0.4-2.0)	.801
***TLR3* L412F/rs3775291**			
CC	49 (48.0)	1	
CT	40 (39.2)	1.0 (0.6-1.6)	.878
TT	13 (12.7)	0.8 (0.4-1.8)	.658
***APOE4*/rs429358**			
TT	75 (79.8)	1	
TC/CC	19 (20.2)	1.1 (0.6-1.9)	.736
***APOE2*/rs7412**			
CC	82 (82.8)	1	
CT/TT	17 (17.2)	0.8 (0.4-1.6)	.596
***CETP*/rs3764261**			
CC	42 (39.6)	1	
CA	46 (43.4)	1.1 (0.7-1.8)	.680
AA	18 (17.0)	0.9 (0.5-1.7)	.724
***FADS1_3*/rs174547**			
TT	50 (47.2)	1	
TC	48 (45.3)	1.2 (0.8-2.0)	.343
CC	8 (7.5)	1.4 (0.6-3.2)	.490
***ABCA1*/rs1883025**			
CC	67 (63.2)	1	
CT/TT	39 (36.8)	0.8 (0.5-1.3)	.355

SNP, single nucleotide polymorphism; AMD, age-related macular degeneration; CI, confidence interval.
Genotype data of the analyzed SNPs were available for at least 87% of all patients.
** Corrected for sex, age, body mass index and pack years.*

DISCUSSION

The primary goal of our study was to determine sociodemographic, behavioral and genetic risk factors that contribute to the progression toward end-stage AMD in the fellow eyes of patients who already have developed end-stage AMD in their first eye. Our results show that the evaluated baseline characteristics sex, age, smoking status, and to a lesser extent BMI, all individually contribute to second eye progression. Of the genetic risk alleles that were analyzed, *CFI* (rs10033900) and *LPL* (rs12678919) were found to confer an increased risk for second eye progression.

There is only one other study that reported a significantly higher risk of progression to bilateral advanced AMD for higher age, current smoking and higher BMI.[16] Other studies have

not reported these associations, although trends have been observed.[12,15,17] The *CFH* Y402H (rs1061170), *ARMS2* A69S (rs10490924), *C3* R102G (rs2230199), and *C2* E318D (rs9332739) risk alleles were also reported to have an influence on the progression of the fellow eye to end-stage AMD.[15,16] Sex has not previously been associated with second eye progression to advanced AMD.

One possible explanation for the differences between the studies previously performed as well as between these studies and our study might be found in the different criteria used for inclusion. The submacular surgery trials (SST) group could not find any significant contribution of age, sex or smoking history.[17] Only patients with a CNV in their first eye were included in the SST study, regardless of the presence of foveal GA. This could have resulted in the inclusion of patients who already had preexisting foveal GA before the CNV occurred. In our study, these patients would have been included at the time foveal GA developed and therefore would have a longer progression time. Another study did not only include patients with new CNV, but also patients with recurrent CNV, which also influences the progression time.[12] In this study no statistically significant differences or trends were found for sex and cigarette smoking.[12] However, this study showed a strong trend for age to influence the incidence of CNV in the fellow eye.[12]

Remarkably, this study demonstrates a relation between sex and second eye progression not previously observed. However, a recent meta-analysis on AMD prevalence shows that the prevalence of late AMD is higher in women compared with men.[54] More specifically, the diagnosis of neovascular AMD occurred more frequently in women compared with men, while for GA no specific sex difference was observed.[54] We are not the first to observe a possible effect of sex on progression in AMD, despite the fact that we are dealing with a relatively small sample size, which may affect the validity of this outcome. Moreover, population differences may further contribute to the observed inconsistencies between studies.

Education was not related to second eye progression in the current study. However, there appears to be a trend for higher education to be protective. This trend is in line with other studies that have associated education with AMD and AMD progression.[2,16]

Two recent studies showed an association of the *CFH* Y402H, *ARMS2* A69S, *C3* R102G, and *C2* E318D risk alleles with progression toward bilateral end-stage AMD.[15,16] We could not confirm this, although for the *C3* R102G (rs2230199) risk allele, our HRs are comparable to the odds ratios found by Seddon in 2009.[16] Table 3 compares the results of our SNP analyses and the two studies by Seddon et al.

A possible explanation for these differences may be due to different definitions used. In both studies, progressors were defined as patients with early or intermediate AMD at baseline who progressed toward end-stage AMD as well as patients with end-stage AMD in one eye at baseline who progressed toward end-stage AMD in the fellow eye.[15,16] Only the latter group is comparable to our second eye progressors. The subcategory "bilateral progressors" included patients without advanced AMD at baseline, progressing toward end-stage AMD in both eyes, as well as those with end-stage AMD in one eye at baseline who progressed

TABLE 3. Comparison of multivariate associations between genetic risk factors and second eye progression to end-stage AMD or incident bilateral advanced AMD

	Our Study		Seddon (2009)		Seddon (2007)	
Genetic risk factors	HR* (95% CI)	*P* value	OR† (95% CI)	*P* value	OR† (95% CI)	*P* value‡
***CFH* Y402H/rs1061170**						
TT	1		1		1	
TC	1.2 (0.6-2.6)	.571	1.1 (0.6-2.1)	.77	1.5 (0.8-2.7)	
CC	1.3 (0.6-2.6)	.476	1.5 (0.7-3.1)	.33	2.3 (1.3-4.2)	
***ARMS2* S69A/rs10490924**						
GG	1		1		1	
GT	0.8 (0.5-1.5)	.565	2.0 (1.2-3.3)	.007	2.5 (1.5-4.1)	
TT	0.7 (0.4-1.4)	.339	4.6 (2.6-8.2)	>.001	5.4 (3.0-9.7)	
***C3* R102G/rs2230199**						
CC	1		1			
CG	1.3 (0.8-2.0)	.363	1.6 (1.0-2.4)	.044		
GG	1.4 (0.6-3.1)	.477	1.6 (0.7-3.6)	.24		
***C2* E318D/rs9332739**						
GG	1		1			
GC/CC	1.1 (0.4-2.9)	.873	0.2 (0.1-0.8)	.021		
***CFI*/rs10033900**						
CC	1					
CT	1.2 (0.6-2.4)	.519				
TT	2.2 (1.1-4.3)	.028				
***LPL*/rs12678919**						
AA	1					
AG	2.1 (1.1-3.9)	.027				
GG	1.1 (0.1-8.7)	.914				

AMD, age-related macular degeneration; HR, hazard ratio; CI, confidence interval; OR, odds ratio
** Corrected for sex, age, body mass index (BMI) and pack years.*
† Adjusted for age, sex, education, smoking, baseline AMD grade, BMI, treatment groups and six genetic variants and associated genotypes.
‡ P-values not mentioned in article.

toward end-stage AMD in the fellow eye. Again, this definition is broader than the definition of second eye progressors employed in the current study. Another difference is that in these studies, the progressors were compared with a group of nonprogressors with regard to genotype and sociodemographic risk factors,[15,16] whereas we started with a group of progressors (the first eye had already progressed toward end-stage AMD) and searched for second eye progressors within this high-risk group.

The implications of these differences become clear if we look at the distribution of the risk alleles of the genotypes across the study groups. In the aforementioned studies, 43% to 44% of the progressors carried the *CFH* Y402H risk allele homozygously, compared with 23% to 24% of the nonprogressors. For *ARMS2* A69S, this was 25% to 27% vs. 9%; and for *C3* R102G,

9% vs. 5%.[15,16] Since all of the subjects in our study are progressors (they all have end-stage AMD in at least one eye), they have the same genotype distribution (see Table 2) as those in the progressor groups in the studies above, showing the homogeneous character of our study group. This suggests that these SNPs influence disease progression only at an earlier stage of the disease. Furthermore, only one paper reported an association between the *CFH* Y402H genotype and the incidence of bilateral advanced AMD[15]; in the other paper this association could not be confirmed.[16] We do realize, however, that we have a relatively small sample size, and that we cannot exclude that the *CFH* Y402H and *ARMS2* A69S genotypes have an influence on second eye progression. To test this hypothesis, these findings should be replicated in a larger and independent cohort.

In our study, we also looked at SNPs that have not previously been investigated for their relationship with second eye progression toward end-stage AMD. By exploring new candidate SNPs, we found new and interesting associations. The *CFI* (rs10033900) and *LPL* (rs12678919) risk alleles were associated with second eye progression. The *CFI* gene encodes complement factor I, one of the complement pathway regulatory proteins involved in the cleavage of C3b.[34,55,56] The complement system plays a major role in the pathogenesis of AMD and these findings suggest that the complement system may also play a role in the progression of earlier-stage AMD toward end-stage AMD in the second eye.[55] The lipoprotein lipase (*LPL*) gene is involved in the high-density lipoprotein metabolism (HDL metabolism) and is associated with a decrease in HDL-c levels in blood.[40,48] Besides its role in the pathogenesis and progression of AMD,[40,48] our findings suggest that HDL metabolism may also play a role in second eye progression in AMD.

Limitations

Because of the relatively small sample size, we were not able to correct for multiple testing and cannot rule out that some of the correlations we found are based on coincidence. Therefore, our findings need to be confirmed in future studies with a larger sample size.

Because of the small number of patients it was not possible to perform additional subanalyses with regard to different phenotypic characteristics. It would, for example, be interesting to evaluate the influence of the type of end-stage in the first eye (GA or CNV) on second eye progression. The presence of reticular macular disease would also be an interesting feature to look at. Reticular pseudodrusen are best visible on autofluorescence and infrared images[57]; however, most patients were collected in a time when these imaging modalities were not used in a standard examination setting. These points will be addressed in future studies with larger cohort sizes and more advanced imaging modalities.

A relatively large group of patients was excluded from this study after initial selection. To exclude the possibility that we were dealing with a biased sample, we used multiple imputation analysis for missing values to compare the included and excluded groups. Pooled results showed that the patients in the excluded group were slightly older and that there were slightly more nonsmokers as well as heavy smokers in the excluded group. For all sig-

nificant variables, we therefore repeated our analyses with the excluded and included group combined using the imputated data for the excluded group. This did not lead to any significant changes in the results, suggesting that our findings are not affected by selection bias.

CONCLUSIONS

The findings of this study give us further insight into the progression of AMD, and they can be a guideline for preventive measures to decrease the risk of second eye progression toward end-stage AMD. By reducing modifiable risk factors such as smoking and BMI, patients may be able to influence their individual risk for progression, thereby preserving their remaining eyesight for a longer period. It would also be possible to select those patients who are at high risk for second eye progression, based on their genetic profile, for future therapeutic trials in research.

The goal of this study was to identify risk factors for the progression of AMD toward end-stage in the fellow eye of patients who had already developed end-stage AMD in their first eye. We found that female sex, age, pack years, and BMI ≥ 30 contribute to this second eye progression. In addition, we have comprehensively investigated the most important AMD-associated SNPs for their relationship with second eye progression to end-stage AMD. In this study, the *CFH* Y402H and *ARMS2* polymorphisms do not play a role in the second eye progression of AMD. The *LPL* and *CFI* risk alleles turned out to be genetic predictors for second eye progression. However, these results were observed in the context of a relatively small sample size, and should therefore be replicated in a larger and independent cohort.

REFERENCES

1. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. *Ophthalmology* 1992;99:933-943.
2. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology* 2000;107:2224-2232.
3. Eye Disease Case-Control Study Group. Risk factors for neovascular age-related macular degeneration. *Arch Ophthalmol* 1992;110:1701-1708.
4. Eye Disease Case-Control Study Group. Antioxidant status and neovascular age-related macular degeneration. *Arch Ophthalmol* 1993;111:104-109.
5. Clemons TE, Milton RC, Klein R, Seddon JM, Ferris FL 3rd. Risk factors for the incidence of Advanced Age-Related Macular Degeneration in the Age-Related Eye Disease Study (AREDS) AREDS report no. 19. *Ophthalmology* 2005;112:533-539.
6. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
7. Lee SJ, Kim NR, Chin HS. LOC387715/HTRA1 polymorphisms , smoking, and combined effects on exudative age-related macular degeneration in a Korean population. *Clin Experiment Ophthalmol* 2010;38:698-704.
8. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. The Blue Mountains Eye Study. *Arch Ophthalmol* 1996;114:1518-1523.
9. Thakkinstian A, Han P, McEvoy M, et al. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. *Hum Mol Genet* 2006;15:2784-2790.
10. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. *Arch Ophthalmol* 1996;114:1193-1196.
11. Macular Photocoagulation Study Group. Five-year follow-up of fellow eyes of patients with age-related macular degeneration and unilateral extrafoveal choroidal neovascularization. *Arch Ophthalmol* 1993;111:1189-1199.
12. Macular Photocoagulation Study Group. Risk factors for choroidal neovascularization in the second eye of patients with juxtafoveal or subfoveal choroidal neovascularization secondary to age-related macular degeneration. *Arch Ophthalmol* 1997;115:741-747.
13. Solomon SD, Jefferys JL, Hawkins BS, Bressler NM. Incident choroidal neovascularization in fellow eyes of patients with unilateral subfoveal choroidal neovascularization secondary to age-related macular degeneration: SST report No. 20 from the Submacular Surgery Trials Research Group. *Arch Ophthalmol* 2007;125:1323-1330.
14. Prenner JL, Rosenblatt BJ, Tolentino MJ, et al. Risk factors for choroidal neovascularization and vision loss in the fellow eye study of CNVPT. *Retina* 2003;23:307-314.
15. Seddon JM, Francis PJ, George S, Schultz DW, Rosner B, Klein ML. Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. *JAMA* 2007;297:1793-1800.
16. Seddon JM, Reynolds R, Maller J, Fagerness JA, Daly MJ, Rosner B. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. *Invest Ophthalmol Vis Sci* 2009;50:2044-2053.
17. Solomon SD, Jefferys JL, Hawkins BS, Bressler NM, Bressler SB. Risk factors for second eye progression to advanced age-related macular degeneration: SST report No. 21 Submacular Surgery Trials Research Group. *Retina* 2009;29:1080-1090.
18. Swaroop A, Branham KE, Chen W, Abecasis G. Genetic susceptibility to age-related macular degeneration: a paradigm for dissecting complex disease traits. *Hum Mol Genet* 2007;16 Spec No. 2:R174-82.
19. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol* 1995;39:367-374.
20. Bressler NM, Bressler SB, Congdon NG, et al. Potential public health impact of Age-Related Eye Disease Study results: AREDS report no. 11. *Arch Ophthalmol* 2003;121:1621-1624.
21. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
22. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
23. Scholl HP, Weber BH, Nothen MM, Wienker T, Holz FG. [Y402H polymorphism in complement factor H and age-related macular degeneration (AMD)]. *Ophthalmologe* 2005;102:1029-1035.

24. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* 2008;40:892-896.
25. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
26. Yang Z, Tong Z, Chen Y, et al. Genetic and functional dissection of HTRA1 and LOC387715 in age-related macular degeneration. *PLoS Genet* 2010;6:e1000836.
27. Francis PJ, Hamon SC, Ott J, Weleber RG, Klein ML. Polymorphisms in C2, CFB and C3 are associated with progression to advanced age related macular degeneration associated with visual loss. *J Med Genet* 2009;46:300-307.
28. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
29. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. *Hum Mol Genet* 2007;16:1986-1992.
30. Despriet DD, van Duijn CM, Oostra BA, et al. Complement component C3 and risk of age-related macular degeneration. *Ophthalmology* 2009;116:474-480.
31. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat Genet* 2007;39:1200-1201.
32. Park KH, Fridley BL, Ryu E, Tosakulwong N, Edwards AO. Complement component 3 (C3) haplotypes and risk of advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2009;50:3386-3393.
33. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
34. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
35. Ennis S, Gibson J, Cree AJ, Collins A, Lotery AJ. Support for the involvement of complement factor I in age-related macular degeneration. *Eur J Hum Genet* 2010;18:15-16.
36. Li M, Atmaca-Sonmez P, Othman M, et al. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat Genet* 2006;38:1049-1054.
37. Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat Genet* 2006;38:1055-1059.
38. Ennis S, Jomary C, Mullins R, et al. Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. *Lancet* 2008;372:1828-1834.
39. Lee AY, Kulkarni M, Fang AM, Edelstein S, Osborn MP, Brantley MA. The effect of genetic variants in SERPING1 on the risk of neovascular age-related macular degeneration. *Br J Ophthalmol* 2010;94:915-917.
40. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2010;107:7401-7406.
41. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A* 2010;107:7395-7400.
42. Fauser S, Smailhodzic D, Caramoy A, et al. Evaluation of serum lipid concentrations and genetic variants at high-density lipoprotein metabolism loci and TIMP3 in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:5525-5528.
43. Yang Z, Stratton C, Francis PJ, et al. Toll-like receptor 3 and geographic atrophy in age-related macular degeneration. *N Engl J Med* 2008;359:1456-1463.
44. Zhou P, Fan L, Yu KD, Zhao MW, Li XX. Toll-like receptor 3 C1234T may protect against geographic atrophy through decreased dsRNA binding capacity. *FASEB J* 2011;25:3489-3495.
45. Baird PN, Guida E, Chu DT, Vu HT, Guymer RH. The epsilon2 and epsilon4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2004;45:1311-1315.
46. Fritsche LG, Freitag-Wolf S, Bettecken T, et al. Age-related macular degeneration and functional promoter and coding variants of the apolipoprotein E gene. *Hum Mutat* 2009;30:1048-1053.
47. Klaver CC, Kliffen M, van Duijn CM, et al. Genetic association of apolipoprotein E with age-related macular degeneration. *Am J Hum Genet* 1998;63:200-206.
48. Peter I, Huggins GS, Ordovas JM, Haan M, Seddon JM. Evaluation of new and established age-related macular degeneration susceptibility genes in the Women's Health Initiative Sight Exam (WHI-SE) Study. *Am J Ophthalmol* 2011;152:1005-1013 e1001.
49. Hawkins JR, Khripin Y, Valdes AM, Weaver TA. Miniaturized sealed-tube allele-specific PCR. *Hum Mutat* 2002;19:543-553.

50. Kuczmarski MF, Kuczmarski RJ, Najjar M. Effects of age on validity of self-reported height, weight, and body mass index: findings from the Third National Health and Nutrition Examination Survey, 1988-1994. *J Am Diet Assoc* 2001;101:28-34.
51. Shields M, Gorber SC, Tremblay MS. Estimates of obesity based on self-report versus direct measures. *Health Rep* 2008;19:61-76.
52. Vailas LI, Nitzke SA. Self-reported versus measured weight and height in an older adult meal program population. *J Gerontol A Biol Sci Med Sci* 1998;53:M481-M483.
53. Meng H, He XZ, Dixon D. Self-reported versus measured height and weight in the health and retirement study. *J Am Geriatr Soc* 2010;58:412-413.
54. Rudnicka AR, Jarrar Z, Wormald R, Cook DG, Fletcher A, Owen CG. Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis. *Ophthalmology* 2012;119:571-580.
55. Boon CJ, van de Kar NC, Klevering BJ, et al. The spectrum of phenotypes caused by variants in the CFH gene. *Mol Immunol* 2009;46:1573-1594.
56. Jha P, Bora PS, Bora NS. The role of complement system in ocular diseases including uveitis and macular degeneration. *Mol Immunol* 2007;44:3901-3908.
57. Schmitz-Valckenberg S, Alten F, Steinberg JS, et al. Reticular drusen associated with geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:5009-5015.

CHAPTER 4

GENOTYPES IN GLUTATHIONE S-TRANSFERASE ARE NOT ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION IN A CAUCASIAN POPULATION

Yara T.E. Lechanteur,
Wilbert H.M. Peters,
Rene H.M. te Morsche,
Carel B. Hoyng,
B. Jeroen Klevering,
Anneke I. den Hollander.

Submitted

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population in the western world. Reactive oxygen species are known to play a role in the pathogenesis of the disease. Glutathione S-transferases (GST) are a family of enzymes that neutralize the oxidative damage caused by these reactive oxygen spiecies. This study was conducted to determine whether polymorphisms in GST genes are associated with AMD in a large case-control dataset.
We included 477 patients with AMD and 359 control subjects selected from the European Genetic Database. Participants were genotyped for deletion polymorphisms in *GSTM1* and *GSTT1* and the *GSTP1* p.Ile105Val polymorphism. No association was detected between the GST genes studied and AMD (*P* values > .05 for all analyses). Also, combined genotype analyses did not reveal any associations. In conclusion, our data indicate that the *GSTM1*, *GSTT1* and *GSTP1* genotypes studied here do not confer a risk for the development of AMD.

Age-related macular degeneration (AMD) is a complex multifactorial disorder, and cellular and tissue damage by reactive oxygen species has long been established as one of the pathogenic factors.[1,2] An important family of enzymes neutralizing oxidative stress are the glutathione S-transferases (GSTs).[3,4] The genes coding for the isoenzymes GSTM1, GSTT1 and GSTP1 contain polymorphisms that alter the critical function of these enzymes in defending against reactive oxygen species.[3,4] Several studies focused on the relation between GST polymorphisms and AMD. Some studies identified a direct relationship,[5,6] whereas others were not able to find any association or only for specific combinations of GST polymorphisms or age at onset of AMD.[7-9] Interpretation of these results is difficult, mainly because of the small study populations. To provide clarity on this issue, we studied the association between GST polymorphisms and AMD in a large cohort of AMD patients and controls. Our data indicate that the *GSTM1*, *GSTT1* and *GSTP1* genotypes do not confer a risk for the development of AMD.

METHODS

A total of 477 patients with AMD and 359 population-matched controls with gradable fundus images and available genotyping of *GST* polymorphisms were selected from the European Genetic Database (EUGENDA). All participants were collected at the Radboud university medical center in Nijmegen, the Netherlands between 2006 and 2011. This research was conducted in accordance with the tenets of the Declaration of Helsinki. The local institutional review board approved this study and we obtained written informed consent from all participants. Controls had no drusen or only small drusen (< 63 µm diameter) or < 10 small drusen in combination with pigmentary abnormalities. Patients with dry AMD were classified by the presence of pigmentary changes together with at least 10 small drusen or the presence of intermediate (63-125 µm) or large drusen (≥ 125 µm diameter) or AMD with geographic atrophy. The subgroup of neovascular AMD was defined as choroidal neovascularisation in at least one eye secondary to AMD. Participants were genotyped for deletion polymorphisms in *GSTM1* and *GSTT1* (resulting in no activity of the corresponding enzymes) and the *GSTP1* p.Ile105Val polymorphism that lowers enzyme activity. Genotyping was carried out as described elsewhere.[10]

Associations of the GST polymorphisms and AMD were studied using multivariable logistic analysis with AMD status as the dependent variable. Analyses were stratified by AMD subtype and adjusted for age, sex, smoking, and the *CFH* Y402H and *ARMS2* A69S gene polymorphisms, the two most important genetic risk variants associated with AMD. In addition, combinations of GST genotypes were analyzed with logistic regression analysis for their association with AMD to investigate additive effects. The reference group consisted of individuals with at least one functional allele for the genotypes studied. Odds ratios (ORs) and 95% confidence intervals were computed for all associations. Subjects with missing data for any of the variables studied, were excluded from the analysis. Data analysis was performed using SPSS software version 20.0 (IBM Corp., Armonk, NY). A two-sided *P* value < 0.5 was considered statistically significant.

RESULTS

Patient characteristics and results from multivariable logistic regression analysis are presented in Table 1. No direct associations were observed between the *GSTM1*, *GSTT1*, or *GSTP1* genotypes and AMD. Also, combined genotype analyses did not reveal any associations (Table 2).

DISCUSSION

Oxidative stress is known to play a key role in AMD development. Antioxidant defense mechanisms are important in prevention of AMD. Because of the antioxidant role of GST enzymes it has been suggested that loss of function of these enzymes could lead to an increased risk for AMD.[5-8] In the current study individual or combined functional polymorphisms in the *GSTM1*, *GSTT1*, and *GSTP1* genes were not associated with AMD. This is in accordance with a previous report by Liu and co-workers,[9] but several other studies reported an increased risk for the development of AMD or an earlier age at onset in the presence of these polymorphisms.[5-8] However, all these studies were hampered by small sample sizes, with the number of AMD patients ranging from 35 to 131.[5-9] This has implications for the power of these studies, especially when evaluating combinations of genotypes. One study reported an increased risk of AMD for patients carrying both the *GSTM1* and *GSTT1* null genotypes as well as for patients carrying the *GSTM1* null variant in combination with the homozygous *GSTP1* risk variant, with ORs of 3.88 and 7.70, respectively, which would imply that GST polymorphisms are important risk factors for AMD development.[8] However, ORs of this magnitude are not frequently reported in genetic association studies in the field of AMD.[11] Importantly, the composition of control subjects in the referred study raises some concerns and may have introduced severe bias. Only 39.6% of controls were reported to carry the *GSTM1* null variant. However, pooled results of more than 10,000 control subjects from 50 different studies show an expected frequency of 53%, while none of the included studies had a frequency of less than 42%.[12] This suggests that the control subjects in the referred study may have been incorrectly genotyped, or might not be a representative control cohort.

TABLE 1. Demographic and clinical information of study participants, including GST genotype distribution

Variables	Controls (n = 359)	All AMD (n = 477)	Controls / All AMD OR (95% CI)*	*P* value	Dry AMD (n = 184)	Controls / Dry AMD OR (95% CI)*	*P* value	NV AMD (n = 293)	Controls / NV AMD OR (95% CI)*	*P* value
Mean age (SD)	71.3 (5.9)	76.0 (7.7)			73.2 (6.8)			77.8 (7.7)		
Male (%)	161 (44.8)	192 (40.3)			78 (42.4)			114 (38.9)		
GSTM1										
Present (%)	161 (44.8)	245 (51.4)	1		100 (54.3)	1		145 (49.5)	1	
Absent (%)	198 (55.2)	232 (48.6)	0.8 (0.6-1.1)	.19	84 (45.7)	0.7 (0.5-1.1)	.10	148 (50.5)	0.9 (0.6-1.4)	.62
GSTT1										
Present (%)	301 (83.8)	383 (80.3)	1		151 (82.1)	1		232 (79.2)	1	
Absent (%)	58 (16.2)	94 (19.7)	1.1 (0.7-1.7)	.63	33 (17.9)	1.1 (0.7-1.9)	.66	61 (20.8)	1.0 (0.6-1.8)	.89
GSTP1										
AA (Ile/Ile) (%)	159 (44.8)	199 (42.2)	1		79 (43.9)	1		120 (41.1)	1	
AG (Ile/Val) (%)	147 (41.4)	211 (44.7)	1.0 (0.7-1.5)	.92	85 (47.2)	1.0 (0.7-1.5)	.99	126 (43.2)	1.1 (0.7-1.7)	.70
GG (Val/Val) (%)	49 (13.8)	62 (13.1)	1.0 (0.6-1.6)	.90	16 (8.9)	0.6 (0.3-1.3)	.21	46 (15.8)	1.5 (0.8-2.7)	.24

AMD, age-related macular degeneration ; OR, odds ratio ; CI, confidence interval; NV AMD, neovascular AMD
Genotype data of the GST polymorphisms analyzed were available for at least 98.3% of all participants.
** Adjusted for age, sex, smoking, complement factor H Y402H and age-related maculopathy susceptibility 2 A69S gene polymorphisms and all other GST polymorphisms analyzed*

TABLE 2. Association analysis of combinations of GST genotypes in AMD

Variables	Controls (n = 362)	All AMD (n = 479)	Controls vs All AMD OR (95% CI)*	*P* value	Dry AMD (n = 184)	Controls vs Dry AMD OR (95% CI)*	*P* value	NV AMD (n = 295)	Controls vs NV AMD OR (95% CI)*	*P* value
GSTM1 - GSTT1										
Present - Present (%)	143 (39.8)	193 (40.5)	1		82 (44.6)	1		111 (37.9)	1	
Absent - Present (%)	158 (44.0)	190 (39.8)	0.9 (0.6-1.3)	.52	69 (37.5)	0.7 (0.5-1.1)	.15	121 (41.3)	1.1 (0.7-1.7)	.75
Present - Absent (%)	18 (5.0)	52 (10.9)	1.7 (0.9-3.3)	.14	18 (9.8)	1.3 (0.6-2.9)	.49	34 (11.6)	2.1 (0.9-4.8)	.09
Absent - Absent (%)	40 (11.1)	42 (8.8)	0.7 (0.4-1.3)	.27	15 (8.2)	0.7 (0.3-1.4)	.33	27 (9.2)	0.6 (0.3-1.4)	.26
GSTM1 - GSTP1										
Present - AA/AG (%)	134 (37.7)	206 (43.6)	1		89 (49.4)	1		117 (40.1)	1	
Absent - AA/AG (%)	172 (48.5)	204 (43.2)	0.8 (0.6-1.2)	.25	75 (41.7)	0.7 (0.5-1.0)	.08	129 (44.2)	1.0 (0.6-1.5)	.87
Present - GG (%)	26 (7.3)	35 (7.4)	1.0 (0.5-1.9)	.98	7 (3.9)	0.5 (0.2-1.3)	.17	28 (9.6)	1.7 (0.8-3.8)	.19
Absent - GG (%)	23 (6.5)	27 (5.7)	0.8 (0.4-1.5)	.43	9 (5.0)	0.6 (0.2-1.4)	.21	18 (6.2)	1.0 (0.4-2.5)	.92
GSTT1 - GSTP1										
Present - AA/AG (%)	257 (72.4)	327 (69.3)	1		134 (74.4)	1		193 (66.1)	1	
Absent - AA/AG (%)	49 (13.8)	83 (17.6)	1.2 (0.7-1.9)	.47	30 (16.7)	1.1 (0.6-1.9)	.76	53 (18.2)	1.2 (0.7-2.2)	.55
Present - GG (%)	40 (11.3)	51 (10.8)	1.1 (0.6-1.8)	.80	13 (7.2)	0.7 (0.3-1.3)	.25	38 (13.0)	1.7 (0.9-3.2)	.12
Absent - GG (%)	9 (2.5)	11 (2.3)	0.8 (0.3-2.2)	.62	3 (1.7)	0.7 (0.2-2.8)	.62	8 (2.7)	0.8 (0.2-2.9)	.71

AMD, age-related macular degeneration ; OR, odds ratio ; CI, confidence interval; NV AMD, neovascular AMD
** Adjusted for age, sex, smoking, complement factor H Y402H and age-related maculopathy susceptibility 2 A69S gene polymorphisms*

CONCLUSIONS

In conclusion, our data indicate that the reported GST genotypes do not confer a risk on the development of AMD. In view of personalized healthcare, it is essential to know which genes play an important role in AMD development and progression. This will allow high risk individuals to be identified at an early phase of the disease and will contribute to the understanding of underlying disease mechanisms and subsequent development of novel therapeutic approaches.

REFERENCES

1. Jarrett SG, Boulton ME. Consequences of oxidative stress in age-related macular degeneration. *Mol Aspects Med* 2012;33:399-417.
2. Ferris FL 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* 1984;102:1640-1642.
3. Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci* 1999;49:156-164.
4. Strange RC, Spiteri MA, Ramachandran S, Fryer AA. Glutathione-S-transferase family of enzymes. *Mutat Res* 2001;482:21-26.
5. Gu H, Sun E, Cui L, et al. Association of glutathione s-transferase pi isoform single-nucleotide polymorphisms with exudative age-related macular degeneration in a chinese population. *Retina* 2012;32:1967-1972.
6. Guven M, Gorgun E, Unal M, et al. Glutathione S-transferase M1, GSTT1 and GSTP1 genetic polymorphisms and the risk of age-related macular degeneration. *Ophthalmic Res* 2011;46:31-37.
7. Othman H, Gholampour AR, Saadat I, Farvardin-Jahromoi M, Saadat M. Age-related macular degeneration and genetic polymorphisms of glutathione S-transferases M1 (GSTM1) and T1 (GSTT1). *Mol Biol Rep* 2012;39:3299-3303.
8. Oz O, Aras Ates N, Tamer L, Yildirim O, Adiguzel U. Glutathione S-transferase M1, T1, and P1 gene polymorphism in exudative age-related macular degeneration: a preliminary report. *Eur J Ophthalmol* 2006;16:105-110.
9. Liu MM, Agron E, Chew E, et al. Copy number variations in candidate genes in neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:3129-3135.
10. Dura P, Salomon J, Te Morsche RH, et al. No role for glutathione S-transferase genotypes in Caucasian esophageal squamous cell or adenocarcinoma etiology: an European case-control study. *BMC Gastroenterol* 2013;13:97.
11. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet* 2013;45:433-439, 439e431-432.
12. Garte S, Gaspari L, Alexandrie AK, et al. Metabolic gene polymorphism frequencies in control populations. *Cancer Epidemiol Biomarkers Prev* 2001;10:1239-1248.

FAMILIAL AGE-RELATED MACULAR DEGENERATION

CHAPTER 5

ANALYSIS OF RISK ALLELES AND COMPLEMENT ACTIVATION LEVELS IN FAMILIAL AND NON-FAMILIAL AGE-RELATED MACULAR DEGENERATION

Nicole T.M. Saksens,
Yara T.E. Lechanteur,
Sanne K. Verbakel,
Joannes M.M. Groenewoud,
Mohamed R. Daha,
Tina Schick,
Sascha Fauser,
Camiel J.F. Boon,
Carel B. Hoyng,
Anneke I. den Hollander.

***PLoS One* 2016;11:e0144367**

PURPOSE Age-related macular degeneration (AMD) is a multifactorial disease, in which complement-mediated inflammation plays a pivotal role. A positive family history is an important risk factor for developing AMD. Certain lifestyle factors are shown to be significantly associated with AMD in non-familial cases, but not in familial cases. This study aimed to investigate whether the contribution of common genetic variants and complement activation levels differs between familial and sporadic cases with AMD.

METHODS 1216 AMD patients (281 familial and 935 sporadic) and 1043 controls (143 unaffected members with a family history of AMD and 900 unrelated controls without a family history of AMD) were included in this study. Ophthalmic examinations were performed, and lifestyle and family history were documented with a questionnaire. Nine single nucleotide polymorphisms (SNPs) known to be associated with AMD were genotyped, and serum concentrations of complement components C3 and C3d were measured. Associations were assessed in familial and sporadic individuals.

RESULTS The association with risk alleles of the age-related maculopathy susceptibility 2 (*ARMS2*) gene was significantly stronger in sporadic AMD patients compared to familial cases (P = .017 for all AMD stages and P = .003 for advanced AMD, respectively). *ARMS2* risk alleles had the largest effect in sporadic cases but were not significantly associated with AMD in densely affected families. The C3d/C3 ratio was a significant risk factor for AMD in sporadic cases and may also be associated with familial cases. In patients with a densely affected family this effect was particularly strong with ORs of 5.37 and 4.99 for all AMD and advanced AMD respectively.

CONCLUSIONS This study suggests that in familial AMD patients, the common genetic risk variant in *ARMS2* is less important compared to sporadic AMD. In contrast, factors leading to increased complement activation appear to play a larger role in patients with a positive family history compared to sporadic patients. A better understanding of the different contributions of risk factors in familial compared to non-familial AMD will aid the development of reliable prediction models for AMD, and may provide individuals with more accurate information regarding their individual risk for AMD. This information is especially important for individuals who have a positive family history for AMD.

Age-related macular degeneration (AMD) is a multifactorial disease and the leading cause of blindness among the elderly in developed countries.[1] With an ageing population, AMD is considered a major and growing health problem.[2] The disease, in its early stages, is characterized by drusen deposits and pigmentary abnormalities. Vision loss mainly occurs when the disease progresses to late AMD, which can be subdivided into geographic atrophy (GA) and choroidal neovascularization (CNV).[3]

Both environmental and genetic risk factors have been associated with the development and progression of AMD. The most consistently reported demographic and environmental risk factors are advanced age, high body mass index (BMI) and current cigarette smoking.[4-8]

Population-based analysis and twin studies have shown a strong genetic contribution to the development of AMD.[2,9-12] Major associations were reported for genetic variants in the complement factor H (*CFH*) and age-related maculopathy susceptibility 2 (*ARMS2*) genes.[3,13-17] Several pathways have been described to be implicated in the development of AMD, including the alternative complement pathway.[18,19] Genetic variants in several complement genes have been associated with AMD, including the *CFH*,[13-15,20] complement factor 3 (*C3*),[21-25] complement factor B (*CFB*),[24,26,27] and complement factor I (*CFI*) genes.[28]. Besides genetic variants in the complement genes, also systemic levels of complement components have been associated with AMD.[24,29,30]

Approximately 20% of AMD patients have a positive family history,[9-11,31] and first-degree relatives of AMD patients have an increased risk of developing AMD.[9,10,32] It has been suggested that the familial component of AMD may be explained by shared genetic or environmental factors.[10] However, the contribution of such factors in familial compared to non-familial AMD patients has not been studied comprehensively. We recently demonstrated that certain lifestyle factors, such as physical activity and red meat consumption, are significantly associated with AMD in sporadic cases but not in familial cases.[33] A recent study showed that the mean genotypic load of common AMD risk alleles in AMD families did not deviate significantly from genotypic loads predicted by simulation models.[34] However, the mean genotypic load in densely affected families was significantly lower than expected, suggesting such families may carry rare, highly penetrant genetic variants.[34] The purpose of this study is to investigate whether the contribution of common genetic variants differs between familial and non-familial AMD cases by interaction analyses. This will support the development of reliable prediction models for AMD, and may provide more accurate information regarding the individual risk for AMD, in particular for individuals who have family members with AMD and for whom this question is most urgent.

METHODS

Study Population

In this study, we evaluated 2259 subjects, including 1216 AMD patients and 1043 control individuals from the Netherlands and Germany. All participants were derived from the European Genetic Database (EUGENDA, www.eugenda.org), an international database for molecular and clinical analysis of AMD. Subjects 50 years of age or older were included when information about sex, BMI, smoking behavior, and family history was available. In case subjects were related, only the first derived AMD patient and control subject of the family were included. Clinical data of their relatives were available in 68 families and were only used to determine the degree of reliability of the self-reported questionnaire. This study was approved by the local ethics committee on Research Involving Human Subjects of the RadboudUMC "Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen" and met the criteria of the Declaration of Helsinki.

Before enrollment in the EUGENDA database, all subjects provided written informed consent and completed a detailed questionnaire on their medical history, family history of AMD, BMI, and lifestyle factors, such as smoking behavior. The study cohort was split into familial and sporadic subjects, based on the self-reported family history. A positive family history was defined as at least two first-degree relatives (parents and/or siblings) with AMD or possible AMD in a family. Participants with a positive family history were labeled as familial and participants without a positive family history were labeled as sporadic. Based on diagnosis and family history, the participants in this retrospective study were divided into four groups: unaffected individuals with a family history of AMD (referred to as familial controls) (n = 143), familial AMD cases (n = 281), unaffected individuals without a family history of AMD (referred to as sporadic controls) (n = 900), and sporadic AMD cases (n = 935). Familial cases were subdivided in patients with a mild (n = 184) or dense (n = 97) positive family history, where the latter group meets one of next 3 criteria: (1) both parents have (possible) AMD, or (2) one affected parent and at least 25% of the siblings are affected, or (3) at least 50% of the siblings are affected. Subjects with a mild positive family history did not meet any of these criteria. The BMI was subdivided in three groups: < 25, 25-30 and > 30 and smoking behavior was categorized into never, past and current smoking.

Each participant underwent digital color fundus photography performed after pupillary dilatation with topical 1.0% tropicamide and 2.5% phenylephrine. Both patients and controls also received spectral-domain optical coherence tomography (SD-OCT). Color fundus photographs and OCT scans of both eyes of all individuals were evaluated by two independent certified reading center graders according to the standard protocol of the Cologne Image Reading Center and Laboratory (CIRCL).[35] The diagnosis of AMD was defined as described previously,[36] based on the grading of the worst affected eye. AMD was classified by the presence of pigmentary changes together with at least 10 small drusen (< 63µm) or the presence of intermediate (63-124 µm) or large drusen (≥ 125 µm diameter) in the Early Treatment Diabetic Retinopathy Study (ETDRS) grid. The subgroup of advanced AMD was defined as either AMD with subfoveal GA and/or CNV in at least one eye. Controls were classified as no abnormalities or only small drusen or pigmentary abnormalities._

Genetic Analysis

Venous blood was obtained for genetic analysis and the measurement of the complement components C3 and C3d. Complement component C3 and the activation fragment C3d were measured in serum samples as described previously.[29] The C3d/C3 ratio was calculated as a measure of complement activation. Genomic DNA was extracted from peripheral blood samples using standard procedures. Genotyping of nine single nucleotide polymorphisms (SNPs) known to be associated with AMD, in the *ARMS2* (rs10490924), *CFH* (rs1061170, rs800292, and rs12144939), *C3* (rs2230199 and rs1047286), *CFB* (rs4151667 and rs641153), and *CFI* (rs10033900) genes was performed in at least 85% of the included subjects with KASP™ genotyping assays (LGC Genomics) according to the manufacturer's instructions. Genotype frequencies in the control individuals were tested for Hardy-Weinberg equilibrium.

Statistical Analysis

Standard descriptive statistics were used to describe baseline and clinical characteristics. To study differences in age (at participation), sex, BMI, smoking status, risk allele frequencies for AMD-associated SNPs, and complement levels between AMD patients and controls, multivariable logistic regression analyses were performed adjusted for the covariates age, sex, BMI and smoking status. Differences in association of AMD-associated SNPs and complement levels in familial compared to sporadic AMD were analyzed with a multivariable logistic regression analysis, with correction for the covariates age, sex, BMI and smoking status. Statistical analyses were also performed with subdivision into mildly and densely affected families for factors which were significantly associated with familial AMD, to study the effect of AMD-associated SNPs and complement levels on the density of AMD in affected families. Due to the skewed nature of the data, log-transformed values of the C3d/C3 ratios were used for analysis. Histograms of the distribution of the C3d/C3 ratio before and after log-transformation are shown in supplementary Figure 1.

Two-sided *P* values of less than .05 were considered statistically significant. Because multiple SNPs were analyzed and many tests of significance were performed in our study, Bonferroni correction was performed for the risk and interaction analysis of environmental and genetic factors. Data were analyzed using SPSS Software version 20.0 (SPSS Inc., Chicago, IL).

RESULTS

Baseline demographic data are depicted in Table 1. Increased age was a significant risk factor for AMD, in sporadic (odds ratio [OR] 1.10; 95% confidence interval [CI] 1.09-1.11; $P < .001$) and familial patients (OR 1.17; 95% CI 1.13-1.21; $P < .001$). Female sex was not significantly associated with AMD in sporadic nor in familial cases. In sporadic patients the risk for AMD increased with increasing BMI (OR 1.45; 95% CI 1.05-1.99; $P = .023$), while BMI was not associated with AMD in familial patients. Current smoking was a significant risk factor for developing AMD

TABLE 1. Demographics in familial and sporadic individuals

	Total	Familial				Sporadic			
		AMD	Controls			AMD	Controls		
	(n = 2259)	(n = 281)	(n = 143)	OR (95% CI)*	*P* value*	(n = 935)	(n = 900)	OR (95% CI)*	*P* value*
Mean Age (SD)	73.7 (8.2)	75.5 (7.9)	66.7 (6.8)	1.17 (1.13-1.21)	< .001	76.6 (8.5)	71.3 (6.7)	1.10 (1.09-1.11)	< .001
Sex									
Male (%)	931 (41.2)	99 (35.2)	56 (39.2)	1		379 (40.5)	397 (44.1)	1	
Female (%)	1328 (58.8)	182 (64.8)	87 (60.8)	1.06 (0.67-1.67)	.801	556 (59.5)	503 (55.9)	1.20 (0.97-1.48)	.086
BMI									
< 25 (%)	1033 (45.7)	137 (48.8)	67 (46.9)	1		426 (45.6)	403 (44.8)	1	
25-30 (%)	948 (42.0)	110 (39.1)	58 (40.6)	0.91 (0.57-1.46)	.701	386 (41.3)	394 (43.8)	1.06 (0.86-1.32)	.572
≥ 30 (%)	278 (12.3)	34 (12.1)	18 (12.6)	1.01 (0.51-1.99)	.983	123 (13.2)	103 (11.4)	1.45 (1.05-1.99)	.023
Smoking									
Never (%)	993 (44.0)	105 (37.4)	62 (43.4)	1		427 (45.7)	399 (44.3)	1	
Past (%)	1075 (47.6)	147 (52.3)	70 (49.0)	1.42 (0.89-2.26)	.142	415 (44.4)	443 (49.2)	0.98 (0.79-1.22)	.868
Current (%)	191 (8.5)	29 (10.3)	11 (7.7)	1.97 (0.87-4.24)	.103	93 (9.9)	58 (6.4)	2.12 (1.44-3.12)	< .001

AMD, age-related macular degeneration; Familial, positive family history for AMD (confirmed or possible AMD in at least one close relative [parent, sibling or child]); Sporadic, negative family history for AMD; OR, odds ratio; CI, confidence interval; BMI, body mass index.
** Adjusted for age, sex, body mass index, and smoking*

in sporadic patients (OR 2.12; 95% CI 1.44-3.12; $P < .001$), but was not significantly associated with AMD in familial patients.

In a subset of 68 families, clinical examination data of the siblings and parents were available. The self-reported family history of the probands was correct in 93% of these families. Only in 1 out of 68 subjects (1.5%) who reported in the questionnaire to have close relatives with (possible) AMD, none of the examined family members seemed to be affected on ophthalmological examination and therefore he was incorrectly classified as familial. In addition, 4 out of 68 subjects (6%) were incorrectly classified as sporadic. 56 probands reported a positive family history. Of those, 30 reported a densely positive family history, which was correct in 29 probands (97%). Only in one proband who reported AMD in one parent and in 1 out of 4 sibs, the densely positive family history was incorrect since no siblings had AMD at ophthalmic examination. The number of affected family members was correct in 66%, and an underestimation or overestimation of the number affected family members was reported in 27% and 7%, respectively.

The allele frequencies of AMD-associated SNPs and the differences in association with AMD (all stages) between familial and sporadic subjects are shown in Table 2. The *ARMS2* risk allele was a significant risk factor for AMD in sporadic cases (OR 2.49; 95% CI 2.12-2.93; $P < .001$). In familial cases this effect was also observed, albeit with a weaker effect (OR 1.60; 95% CI 1.16-2.22; $P = .005$). This difference in association was significant ($P = .017$). The *CFH* Y402H allele was significantly associated with AMD in both sporadic and familial cases (OR 1.81; 95% CI 1.57-2.09; and OR 2.20; 95% CI 1.58-3.06, respectively; $P < .001$), and contrary to the *ARMS2* SNP, this association did not significantly differ between familial and sporadic patients. Other genetic variants in the *CFH, C3, CFB* and *CFI* genes were not significantly associated with AMD in both sporadic and familial cases. The serum C3d/C3 ratio, as a measure of the systemic activity of the complement system, was a significant risk factor for AMD among sporadic patients (OR 1.84; 95% CI 1.40-2.4; $P < .001$) but did not reach significance among familial patients (OR 2.10; 95% CI 1.14-3.87; $P = .017$) after correction for multiple testing. The difference in serum C3d/C3 levels between familial and sporadic subjects was not significant ($P = .669$).

The allele frequencies of AMD-associated SNPs and the differences in association with advanced AMD between familial and sporadic subjects are shown in Table 3. The findings for advanced AMD were similar as for all AMD stages, although the ORs of the common variants were stronger than for all AMD stages. Also, the difference in association of the *ARMS2* allele in subjects with a positive family history compared to those with a negative family history was even stronger for the development of advanced AMD ($P = .003$). No other SNPs differed in association between familial and sporadic subjects with advanced AMD and neither did the C3d/C3 ratio.

TABLE 2. Risk estimates and risk differences of allele frequencies of AMD-associated SNPs and serum complement activation levels for all AMD grades based on family history

	Total	Familial / Sporadic	Familial				Sporadic			
	No. (%) (n = 2259)	*P* value*	AMD (n = 281)	Controls (n = 143)	OR (95% CI)*	*P* value*	AMD (n = 935)	Controls (n = 900)	OR (95% CI)*	*P* value*
SNP / risk allele										
ARMS2 rs10490924 / T	2259 (100)	0.017‡	46.6	33.2	1.60 (1.16-2.22)	.005‡	39.4	21.0	2.49 (2.12-2.93)	< .001‡
CFH Y402H rs1061170 / C	2259 (100)	0.288	60.0	40.9	2.20 (1.58-3.06)	< .001‡	50.6	35.4	1.81 (1.57-2.09)	< .001‡
CFH rs800292 / A	1936 (85.7)	0.478	16.9	19.0	0.82 (0.53-1.28)	.385	18.8	25.5	0.70 (0.58-0.83)	< .001‡
CFH rs12144939 / T	1947 (86.2)	0.896	9.2	16.4	0.62 (0.38-1.01)	.052	13.9	20.3	0.60 (0.49-0.73)	< .001‡
C3 rs2230199 / G	2254 (99.8)	0.848	28.1	23.5	1.31 (0.91-1.88)	.148	23.5	20.4	1.26 (1.06-1.49)	.007
C3 rs1047286 / A	1952 (86.4)	0.556	28.6	21.1	1.48 (1.00-2.21)	.052	22.8	19.6	1.30 (1.08-1.56)	.005‡
CFB rs4151667 / A	2241 (99.2)	0.574	3.1	3.2	0.88 (0.36-2.15)	.781	3.5	4.9	0.67 (0.47-0.96)	.027
CFB rs641153 / A	1944 (86.1)	0.728	5.3	8.2	0.64 (0.32-1.28)	.210	6.4	8.2	0.74 (0.55-0.99)	.044
CFI rs10033900 / T	2227 (98.6)	0.260	49.1	44.7	1.25 (0.90-1.73)	.193	50.7	49.2	1.01 (0.88-1.16)	0.851
C3d/C3 ratio	1840 (81.5)	0.669	4.47 (3.48-6.10)†	4.04 (3.16-5.43)†	2.10 (1.14-3.87)	.017	4.46 (3.39-5.72)†	3.95 (3.01-5.21)†	1.84 (1.40-2.43)	< .001‡

AMD, age-related macular degeneration; Familial, positive family history for AMD (confirmed or possible AMD in at least one close relative [parent, sibling or child]); Sporadic, negative family history for AMD; OR, odds ratio; CI, confidence interval.
Missing genotypes were < 15%.
** Adjusted for age, sex, body mass index, and smoking.*
† Median (interquartile range).
‡ Significant associations after correction for multiple testing

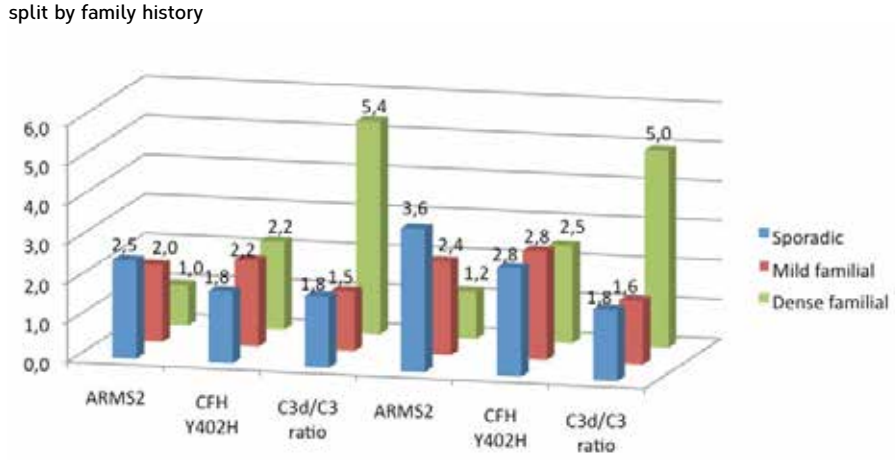
TABLE 3. Risk estimates and risk differences of allele frequencies of AMD-associated SNPs and serum complement activation levels for advanced AMD based on family history

	Total	Familial / Sporadic	Familial			Sporadic		
	No. (%) (n = 1815)	*P* value	AMD (n = 201)	Controls (n = 143)	OR (95% CI)*	AMD (n = 571)	Controls (n = 900)	OR (95% CI)*	*P* value*
SNP / risk allele									
ARMS2 rs10490924 / T	1815 (100)	.003‡	50.3	33.2	1.92 (1.33-2.79)	46.6	21.0	3.63 (2.98-4.42)	< .001‡
CFH Y402H rs1061170 / C	1815 (100)	.875	64.2	40.9	2.66 (1.79-3.95)	58.3	35.4	2.75 (2.30-3.30)	< .001‡
CFH rs800292 / A	1522 (83.9)	.373	12.8	19.0	0.60 (0.34-1.08)	13.9	25.5	0.45 (0.35-0.59)	< .001‡
CFH rs1214939 / T	1532 (84.4)	.824	7.2	16.4	0.44 (0.23-0.83)	11.6	20.3	0.40 (0.30-0.54)	< .001‡
C3 rs2230199 / G	1810 (99.7)	.928	28.3	23.5	1.32 (0.87-2.00)	23.8	20.4	1.30 (1.06-1.59)	.012
C3 rs1047286 / A	1537 (84.7)	.882	27.7	21.1	1.32 (0.82-2.12)	23.1	19.6	1.37 (1.09-1.73)	.008
CFB rs4151667 / A	1799 (99.1)	.466	3.1	3.2	0.81 (0.29-2.26)	3.1	4.9	0.53 (0.34-0.85)	.008
CFB rs641153 / A	1529 (84.2)	.949	5.2	8.2	0.64 (0.27-1.48)	5.5	8.2	0.62 (0.41-0.93)	.022
CFI rs10033900 / T	1791 (98.7)	.578	48.8	44.7	1.15 (0.79-1.66)	51.6	49.2	1.02 (0.87-1.21)	.784
C3d/C3 ratio	1478 (81.4)	.532	4.29 (3.52-5.77)†	4.04 (3.16-5.43)†	2.23 (1.15-4.30)	4.37 (3.38-5.70)†	3.95 (3.01-5.21)†	1.76 (1.26-2.46)	.001‡

AMD, age-related macular degeneration; Familial, positive family history for AMD (confirmed or possible AMD in at least one close relative [parent, sibling or child]); Sporadic, negative family history for AMD; OR, odds ratio; CI, confidence interval. Missing genotypes were < 17%.
** Adjusted for age, sex, body mass index, and smoking.*
† Median (interquartile range).
‡ Significant associations after correction for multiple testing

97 of the 281 familial AMD patients and 34 of the 143 familial controls reported a densely affected family history. The *ARMS2* SNP was not associated with AMD in patients from densely affected families, and this was significantly different from the association with sporadic AMD (*P* = .010 for all AMD stages and *P* = .002 for advanced AMD) (Table 4 and Figure 1). The association of the *CFH* Y402H allele with familial and sporadic AMD again did not differ. The C3d/C3 ratio showed the largest risk effect in patients with a densely affected family for all AMD (OR 5.37; 95% CI 1.54-18.69; *P* = .008) and advanced AMD (OR 4.99; 95% CI 1.41-17.68; *P* = .013) but this was not significantly different from the association with sporadic AMD.

FIGURE 1. Odds ratios for risk variants in *ARMS2* and *CFH* and the C3d/C3 ratio for development of AMD split by family history



The risk variant in ARMS2 confers a strong risk for AMD in the sporadic group. In the group with a dense family history there is no effect of this SNP. The CFH Y402H risk allele is associated with AMD in all subgroups, irrespective of family history. The C3d/C3 ratio is associated with AMD development in sporadic individuals. For patients with a mild family history the odds ratio for the C3d/C3 ratio is comparable. In the subgroup with a dense family history, the C3d/C3 ratio confers a much higher risk for AMD.
ARMS2, age-related macular susceptibility 2; CFH, complement factor H; AMD, age-related macular degeneration; OR, odds ratio; Sporadic, negative family history for AMD; Familial, positive family history for AMD; Dense familial, a positive family history for AMD satisfying 1 out of 3 criteria: (1) both parents have (possible) AMD, or (2) one affected parent and at least 25% of number of the sibs are affected, or (3) at least 50% of the number of sibs is affected; Mild familial, a positive family history for AMD but in a lesser extent, not meeting one of the 3 criteria.

TABLE 4. Risk estimates and risk differences of allele frequencies of *ARMS2* and *CFH* SNPs and serum complement activation levels in mild and densely affected AMD families

	All AMD grades					Advanced AMD				
	Familial / Sporadic	Familial		Sporadic		Familial / Sporadic	Familial		Sporadic	
	P value	OR (95% CI)*	*P* value*	OR (95% CI)*	*P* value*	*P* value	OR (95% CI)*	*P* value*	OR (95% CI)*	*P* value*
ARMS2										
Mild familial	.010	1.95 (1.31-2.92)	.001	2.49 (2.12-2.93)	< .001	.002	2.38 (1.49-3.80)	< .001	3.63 (2.98-4.43)	< .001
Dens familial		1.02 (0.58-1.81)	.946				1.19 (0.63-2.24)	.595		
***CFH* Y402H**										
Mild familial	.575	2.18 (1.48-3.22)	< .001	1.81 (1.57-2.09)	< .001	.959	2.76 (1.71-4.41)	< .001	2.75 (2.30-3.31)	< .001
Dens familial		2.23 (1.18-4.23)	.014				2.47 (1.19-5.12)	.015		
C3d/C3 ratio										
Mild familial	.199	1.46 (0.72-2.97)	.296	1.84 (1.40-2.43)	< .001	.268	1.57 (0.71-3.45)	.264	1.76 (1.26-2.45)	.001
Dens familial		5.37 (1.54-18.69)	.008				4.99 (1.41-17.68)	.013		

AMD, age-related macular degeneration; Familial, positive family history for AMD (confirmed or possible AMD in at least one close relative [parent, sibling or child]); Sporadic, negative family history for AMD; Dense familial, a positive family history for AMD satisfying 1 out of 3 criteria: (1) both parents have (possible) AMD, or (2) one affected parent and at least 25% of number of the sibs are affected, or (3) at least 50% of the number of sibs is affected; Mild familial, a positive family history for AMD but in a lesser extent, not meeting one of the 3 criteria; OR, odds ratio; CI, confidence interval.
** Adjusted for age, sex, body mass index, and smoking.*

DISCUSSION

In addition to environmental and genetic risk factors, a positive family history for AMD is an important risk factor for the development of AMD.[9,10,32] For a proper risk assessment it is therefore important to determine an individual's family history for AMD. In this study we investigated whether the contribution of AMD-associated SNPs and C3d/C3 ratio differs between familial and non-familial AMD cases.

Our results show that the association of the *ARMS2* A69S genotype differed between familial and sporadic subjects. Within the group of cases and controls with a dense family history, *ARMS2* was not associated with AMD, whereas it was a strong risk factor for sporadic individuals. For the C3d/C3 ratio no significant difference was found between familial and sporadic subjects. However, in the subgroup with a dense family history, complement activation was most strongly associated with the presence of all AMD stages and advanced AMD.

The *ARMS2* A69S variant is one of the strongest genetic risk factors for AMD.[37] However, in densely affected families this risk variant seems to have less effect, and a high *ARMS2* risk allele frequency was found in controls with a positive family history. Testing the *ARMS2* SNP to estimate an individual's AMD risk is thus more informative in patients without a positive family history. However, since both family history and SNPs are important factors in the development of AMD, and some discordance exists between risk estimates based on genetic testing and that based on family history analysis,[38] they should be used to complement one another in risk assessment. The fact that the family history for AMD affects the risk of the *ARMS2* genotype, suggests that there are other, unknown factors that increase the risk for AMD in the patients from densely affected families. This supports the theory that densely affected families may harbor rare, more penetrant genetic variants for AMD.[34,39,40] Even though no statistically significant difference was observed between familial and sporadic subjects concerning the association of the C3d/C3 ratio with AMD, the very high ORs that we reported for the patients from densely affected families can point toward a more important role for systemic complement activation in families with AMD compared to sporadic AMD patients. Risk alleles of *CFH* and *ARMS2* are independently associated with an increased C3d/C3 ratio,[29] and the higher complement level in familial AMD patients may (partly) be explained by the higher number of risk alleles of those SNPs in familial patients compared to sporadic patients. However, after additional adjustment for the *ARMS2* and complement SNPs, we determined that the estimated OR and corresponding CI for the C3d/C3 ratio did not significantly change. This further supports the hypothesis that rare, highly penetrant variants may contribute to the higher complement activation in familial AMD. Interestingly, several rare, highly penetrant AMD alleles have been described in several genes of the complement system,[39,41-44] and in densely affected families, mutations in the *CFH* gene have been identified.[40,43]

In this study no difference for the role of the *CFH* Y402H risk variant was observed between familial and sporadic subjects. Unlike *ARMS2*, the *CFH* Y402H risk SNP seems to be of equal

importance for the development of AMD in both sporadic and familial individuals. This finding further underlines the important role of the complement system in familial AMD, both through common SNPs as well as rare genetic variants.

Four SNPs in the *ARMS2* and *CFH* genes were associated with AMD in sporadic cases in our study, but only the 2 major SNPs, *ARMS2* rs10490924 and *CFH* rs1061170, were also significantly associated with AMD in familial cases. The lack of association with the remaining SNPs may be due to the limited number of available subjects, and did not differ between familial and sporadic subjects. Stronger associations for advanced AMD compared to all AMD stages in sporadic cases indicate these risk SNPs play a more important role in the development of advanced stages of AMD than in the development of small and intermediate drusen. In sporadic AMD, an increased BMI and current smoking status showed a significant association with AMD in our study, which is in agreement with previous studies.[6-8,45] As these factors were not significantly associated with AMD in familial cases, environmental factors like smoking behavior and BMI may play a more important role in the development of AMD in sporadic patients than in familial cases. However, it should be noted that the absence of significant associations with AMD among familial subjects may be due to the limited number of available familial subjects.

The relatively low number of familial cases and controls is the main limitation of our study. This reduces the power of our analyses. However, after subdividing our familial dataset into mild and densely affected families we found that the differences in association between familial and sporadic cases were more pronounced, and this further underlines our findings. Nonetheless, our results should be interpreted with care and should be replicated in additional familial AMD cohorts in order to confirm our hypothesis.

CONCLUSIONS

This study demonstrates that the association of the *ARMS2* risk allele and complement activation levels in serum with AMD differs between familial and sporadic subjects. Our study suggests that *ARMS2* risk alleles have less effect in familial AMD patients than in sporadic AMD. In contrast, increased complement activation levels seem to play a larger role in patients with a dense positive family history compared to sporadic patients, which cannot be explained by known, common SNPs in the complement genes. A better understanding of factors that differ between individuals with and without a family history will aid the development of reliable prediction models for AMD, and may provide individuals with more accurate information regarding their individual risk for AMD. This information is especially important for individuals who have a dense positive family history for AMD.

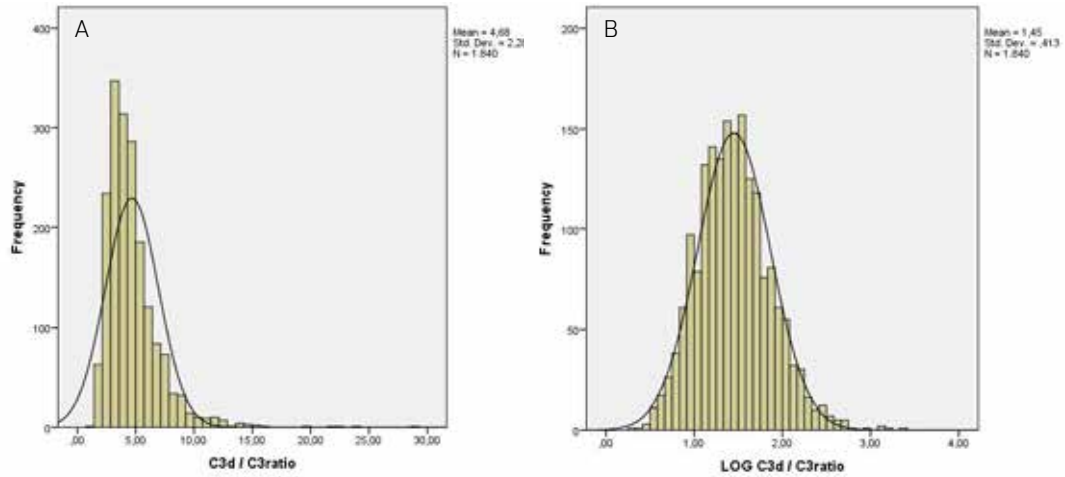
REFERENCES

1. Coleman HR, Chan CC, Ferris FL 3rd, Chew EY. Age-related macular degeneration. *Lancet* 2008;372:1835-1845.
2. Lim LS, Mitchell P, Seddon JM, Holz FG, Wong TY. Age-related macular degeneration. *Lancet* 2012;379:1728-1738.
3. Chakravarthy U, McKay GJ, de Jong PT, et al. ARMS2 increases the risk of early and late age-related macular degeneration in the European Eye Study. *Ophthalmology* 2013;120:342-348.
4. Rudnicka AR, Jarrar Z, Wormald R, Cook DG, Fletcher A, Owen CG. Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis. *Ophthalmology* 2012;119:571-580.
5. Klein BE, Klein R, Lee KE, Jensen SC. Measures of obesity and age-related eye diseases. *Ophthalmic Epidemiol* 2001;8:251-262.
6. Thornton J, Edwards R, Mitchell P, Harrison RA, Buchan I, Kelly SP. Smoking and age-related macular degeneration: a review of association. *Eye (Lond)* 2005;19:935-944.
7. van de Ven JP, Smailhodzic D, Boon CJ, et al. Association analysis of genetic and environmental risk factors in the cuticular drusen subtype of age-related macular degeneration. *Mol Vis* 2012;18:2271-2278.
8. Khan JC, Thurlby DA, Shahid H, et al. Smoking and age related macular degeneration: the number of pack years of cigarette smoking is a major determinant of risk for both geographic atrophy and choroidal neovascularisation. *Br J Ophthalmol* 2006;90:75-80.
9. Klaver CC, Wolfs RC, Assink JJ, van Duijn CM, Hofman A, de Jong PT. Genetic risk of age-related maculopathy. Population-based familial aggregation study. *Arch Ophthalmol* 1998;116:1646-1651.
10. Seddon JM, Ajani UA, Mitchell BD. Familial aggregation of age-related maculopathy. *Am J Ophthalmol* 1997;123:199-206.
11. Meyers SM, Greene T, Gutman FA. A twin study of age-related macular degeneration. *Am J Ophthalmol* 1995;120:757-766.
12. Klein ML, Mauldin WM, Stoumbos VD. Heredity and age-related macular degeneration. Observations in monozygotic twins. *Arch Ophthalmol* 1994;112:932-937.
13. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
14. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
15. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
16. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* 2008;40:892-896.
17. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
18. Johnson LV, Leitner WP, Staples MK, Anderson DH. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Exp Eye Res* 2001;73:887-896.
19. Zipfel PF, Lauer N, Skerka C. The role of complement in AMD. *Adv Exp Med Biol* 2010;703:9-24.
20. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2005;102:7227-7232.
21. Park KH, Fridley BL, Ryu E, Tosakulwong N, Edwards AO. Complement component 3 (C3) haplotypes and risk of advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2009;50:3386-3393.
22. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat Genet* 2007;39:1200-1201.
23. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
24. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci* 2009;50:5818-5827.
25. Thakkinstian A, McKay GJ, McEvoy M, et al. Systematic review and meta-analysis of the association between complement component 3 and age-related macular degeneration: a HuGE review and meta-analysis. *Am J Epidemiol* 2011;173:1365-1379.

26. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
27. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. *Hum Mol Genet* 2007;16:1986-1992.
28. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
29. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
30. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. *Proc Natl Acad Sci U S A* 2006;103:2328-2333.
31. Saksens NT, Fleckenstein M, Schmitz-Valckenberg S, et al. Macular dystrophies mimicking age-related macular degeneration. *Prog Retin Eye Res* 2014;39:23-57.
32. Shahid H, Khan JC, Cipriani V, et al. Age-related macular degeneration: the importance of family history as a risk factor. *Br J Ophthalmol* 2012;96:427-431.
33. Saksens NT, Kersten E, Groenewoud JM, et al. Clinical characteristics of familial and sporadic age-related macular degeneration: differences and similarities. *Invest Ophthalmol Vis Sci* 2014;55:7085-7092.
34. Sobrin L, Maller JB, Neale BM, et al. Genetic profile for five common variants associated with age-related macular degeneration in densely affected families: a novel analytic approach. *Eur J Hum Genet* 2010;18:496-501.
35. Mokwa NF, Ristau T, Keane PA, Kirchhof B, Sadda SR, Liakopoulos S. Grading of Age-Related Macular Degeneration: Comparison between Color Fundus Photography, Fluorescein Angiography, and Spectral Domain Optical Coherence Tomography. *J Ophthalmol* 2013;2013:385915.
36. Ristau T, Ersoy L, Lechanteur Y, et al. Allergy is a protective factor against age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:210-214.
37. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet* 2013;45:433-439, 439e431-432.
38. Aiyar L, Shuman C, Hayeems R, et al. Risk estimates for complex disorders: comparing personal genome testing and family history. *Genet Med* 2014;16:231-237.
39. Duvvari MR, Paun CC, Buitendijk GH, et al. Analysis of rare variants in the C3 gene in patients with age-related macular degeneration. *PLoS One* 2014;9:e94165.
40. Yu Y, Triebwasser MP, Wong EK, et al. Whole-exome sequencing identifies rare, functional CFH variants in families with macular degeneration. *Hum Mol Genet* 2014;23:5283-5293.
41. van de Ven JP, Boon CJ, Fauser S, et al. Clinical evaluation of 3 families with basal laminar drusen caused by novel mutations in the complement factor H gene. *Arch Ophthalmol* 2012;130:1038-1047.
42. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet* 2011;43:1232-1236.
43. Boon CJ, Klevering BJ, Hoyng CB, et al. Basal laminar drusen caused by compound heterozygous variants in the CFH gene. *Am J Hum Genet* 2008;82:516-523.
44. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet* 2013;45:813-817.
45. Howard KP, Klein BE, Lee KE, Klein R. Measures of body shape and adiposity as related to incidence of age-related eye diseases: observations from the Beaver Dam Eye Study. *Invest Ophthalmol Vis Sci* 2014;55:2592-2598.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURE 1. Histograms showing distribution of C3d / C3 ratio before (A) and after (B) log-transformation.



CHAPTER 6

PREDICTION IN FAMILIES WITH AGE-RELATED MACULAR DEGENERATION: CLUSTERING OF RISK FACTORS OR RARE GENETIC VARIANTS?

Eveline Kersten,*
Yara T.E. Lechanteur,*
Nicole T.M. Saksens,
Maartje J. Geerlings,
Tina Schick,
Sascha Fauser,
Camiel J.F. Boon,
Anneke I. den Hollander,
Carel B. Hoyng.
* These authors contributed equally to this study and share first authorship.

Submitted

PURPOSE Age-related macular degeneration (AMD) is known to cluster in families. Prediction models have been established for AMD based on demographic, environmental and common genetic risk factors. In AMD families, these risk factors may be distributed differently, and rare highly penetrant variants have been identified in some families. The aim of this study was to evaluate the prediction of AMD in a family dataset.

DESIGN Case-control study.

PARTICIPANTS Two datasets were extracted from the European Genetic Database (EUGENDA). The first dataset contained 1037 patients with advanced AMD and 1290 control individuals. These individuals were randomly subdivided into a training set (816 cases, 984 controls) and a validation set (221 cases, 306 controls). The second dataset contained 119 advanced AMD cases and 103 controls from 96 AMD families, referred to as family set.

METHODS A prediction model for development of advanced AMD was created in the training set using logistic regression analyses. Subsequently, we validated this model in the validation and family set using receiver operating characteristic curves.

MAIN OUTCOME MEASURES Performance of the prediction model in the family set, expressed as the area under the curve (AUC).

RESULTS The final model included the factors age, smoking, physical exercise, education, family history and seven single nucleotide polymorphisms. This model showed an AUC of 0.873 (95% CI 0.851-0.895) in the training set. The AUCs in the validation and family set were 0.854 (95% CI 0.808-0.900) and 0.842 (95% CI 0.787-0.897), respectively. Further analyses of individual families showed clustering of risk factors in most families, with predicted values above 0.7 for all affected individuals. However, we also identified two densely affected families with low predicted risk scores (≤ 0.4) for all affected family members.

CONCLUSIONS This study demonstrated that a prediction model based on common genetic and environmental risk factors can be applied to AMD families, due to clustering of these factors in most families. In a subset of families the risk of advanced AMD was not explained by these factors. This suggests that other factors, such as rare genetic variants, play a role in the development of advanced AMD in these families.

Age-related macular degeneration (AMD) is a multifactorial, progressive retinal disorder resulting in severe visual impairment. Advanced age is one of the most important risk factors for AMD,[1-3] resulting in a rapidly growing burden of disease due to the aging population.[4,5] To date, no curative treatment is available. Prediction models can aid the identification of individuals with a high risk for disease, enabling personalized healthcare and early detection of disease. Available prediction models for AMD are based on demographic, environmental and common genetic risk factors and usually have been developed in unrelated patients and controls.[6-12] Aggregation of AMD in families has been reported.[13-15] In clinical practice, particularly individuals with affected family members are interested in their individual risk of AMD. It has been suggested that risk factors may be distributed differently in these families.[13,15-17] Also, rare genetic variants with a high risk for disease have been identified in the *CFH*,[18-21] *CFI*,[22,23] *C3*,[22,24-26] *C9*,[22] and the *FBN2* genes.[27] This raises the question if these models, based on common environmental and genetic risk factors, can be applied to individuals deriving from families affected with AMD. Therefore, the aim of this study was to evaluate the prediction of advanced AMD in an AMD family dataset. For this purpose we created a prediction model based on a case-control dataset and validated this model in our family cohort.

METHODS

Study Population

For this study, two datasets were extracted from the European Genetic Database (EUGENDA, www.eugenda.org), a large multicenter database for clinical and molecular analysis of AMD. The first was a group of 1037 patients with advanced AMD in at least one eye and 1290 control individuals who were randomly divided into a training set (816 cases, 984 controls) and a validation set (221 cases, 306 controls). All individuals were unrelated. The second was a set of 119 advanced AMD cases and 103 control individuals derived from 96 AMD families. In order to be considered an AMD family at least two first-degree relatives had to have any stage of AMD confirmed by fundus photography. A densely affected family was defined as two or more advanced AMD cases in a nuclear family. Age-criteria for all datasets were ≤ 50 years for patients and ≥ 65 years for controls. This study was approved by the local ethical committees at the Radboud university medical center and the University Hospital of Cologne and was performed in accordance with the tenets of the Declaration of Helsinki. All individuals provided written informed consent before participation.

Information on lifestyle and other environmental factors was obtained through detailed interviewer-assisted questionnaires. Variables included in this study were: age at participation, sex (male / female), smoking status (never / past / current), body mass index (BMI) calculated as weight in kilograms divided by height in meters squared (normal [< 25] / overweight [25-30] / obese [>30]), physical activity (no or almost never / 1-2 times per week / 3 or more times per week), education level (high school or less / more than high school) and family history of AMD (yes / no). The diagnosis and grading of AMD was based on color fundus photographs of the more severe affected eye and was performed by independent certified reading center graders as described previously.[28]

Genetic Analysis

Genomic DNA was isolated from venous blood leukocytes and genotyping was performed for single nucleotide polymorphisms (SNPs) in the following AMD-associated genes: *CFH* (rs800292,[29] rs1061170,[30-32] rs12144939[33]), *ARMS2* (rs10490924),[34] *CFI* (rs10033900),[35] *CFB* (rs4151667,[36] rs641153[36]), *C3* (rs433594,[37] rs1047286[38,39], rs2230199[38,39]), *COL8A1* (rs13081855),[40] *COL10A1* (rs3812111),[40] *LIPC* (rs10468017),[41,42] *SLC16A8* (rs8135665),[40] *CETP* (rs3764261),[41,42] *LPL* (rs12678919),[41,42] *FADS1* (rs174547),[42] *ADAMTS9* (rs6795735),[40] IER3DDR (rs3130783),[40] *TGFBR1* (rs334353),[40] *SKIV2L* (rs429608),[43] *VEGFA* (rs943080),[44] *MYRIP* (rs2679798),[43] *RAD51B* (rs8017304),[40] *HSPH1* (rs9542236),[40] *TNFRSF10A* (rs13278062),[40] *TIMP3* (rs9621532),[41] *APOE* (rs2075650,[45] rs4420638[40]), *GLI3* (rs2049622),[45] *GLI2* (rs6721654),[45] *TYR* (rs621313),[45] *PON1* (rs705381),[46] *CYP24A1* (rs1570669)[47] and *IGFR1* (rs2872060).[48] SNPs were genotyped using competitive allele-specific PCR assays (KASP SNP Genotyping System, LGC).

Statistical Analysis

A prediction model for the development of advanced AMD was constructed in the training set using logistic regression analyses. First, univariable logistic regression analysis was performed for 7 nongenetic and 35 genetic variables to study associations between each single risk factor and AMD. All variables with a *P* value ≤ .15 were selected for inclusion in the multivariable model. For this model missing data were imputed and backward multivariable regression analysis was performed in each imputation set. Our final prediction model was based on variables with a *P* value less than .10 in all imputation sets. Risk scores and the probability of advanced AMD (P) were calculated as follows:

$$risk\ score = \alpha + \sum_{i=1}^{n} \beta_i X_i$$

$$P = \frac{\exp(risk\ score)}{1 + \exp(risk\ score)}$$

where α is the intercept, n the number of variables included in the model, and β_i the regression coefficient of the corresponding variable (X_i).

The accuracy of the prediction model to discriminate between cases and controls was evaluated using a receiver operating characteristic (ROC) curve and calculation of the area under the curve (AUC). The Hosmer-Lemeshow goodness of fit test was performed to check the

calibration of the model. Probabilities of disease were then calculated for the individuals in the validation and family sets and corresponding ROC curves and AUCs were obtained. In the next step, we examined the individual prediction scores for patients from densely affected families in order to determine how well the model performed for each family.

All statistical analyses were performed using IBM SPSS Statistics software, version 20.0 (IBM Corp., Armonk, NY, USA). For multiple imputation of missing data default settings of SPSS were used. Complete data for all 22 variables were available for 40.6% of cases. Two SNPs in *C3* (rs1047286, rs2230199) were only genotyped in a subset of participants (60%). None of the other variables had missing data for more than 25% of the participants.

RESULTS

Basic demographic characteristics of all three datasets are shown in Table 1. From the univariable analysis 22 variables were selected for inclusion in the multivariable model. Sex and BMI were not significantly associated with advanced AMD in the univariable analysis, but were nevertheless included for further analysis since these variables have been associated with the development of AMD in multiple studies.[1,2,49,50]

The final model included the factors age, smoking, physical exercise, education, family history and seven SNPs in *CFH* (rs800292, rs1061170, rs12144939), *ARMS2* (rs10490924), *CETP* (rs3764261), *VEGFA* (rs943080) and *TIMP3* (rs9621532). A prediction algorithm with these variables was developed as described in the method section using the values as illustrated in Table 2.

The area under the ROC curve (Figure 1, A) was 0.873 (95% CI 0.851-0.895), indicating good discrimination between cases and controls. The Hosmer-Lemeshow test was not statistically significant, indicating adequate calibration of the model (Hosmer-Lemeshow chi-square = 12.284; *P* = .139). ROC curves of the validation and family set are shown in Figure 1, B and C, and the corresponding AUCs are 0.854; 95% CI 0.808-0.900 and 0.842; 95% CI 0.787-0.897, respectively.

TABLE 1. Basic demographic characteristics of the training, validation and family datasets

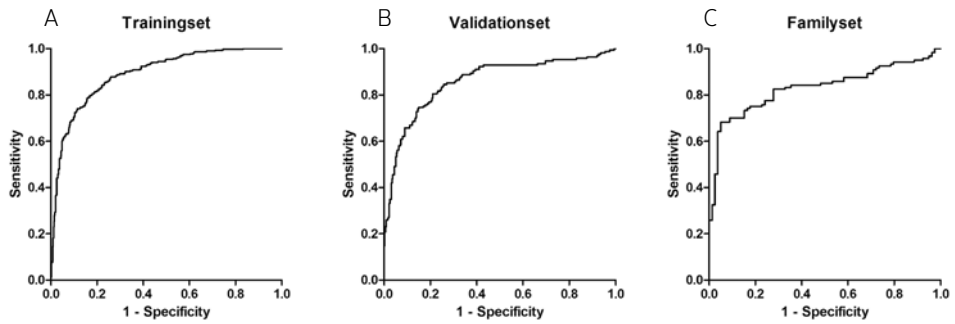
	Training set		Validation set		Family set	
	Case (n = 816)	Control (n = 984)	Case (n = 221)	Control (n = 306)	Case (n = 103)	Control (n = 96)
Mean Age (SD)	76.8 (8.2)	71.7 (6.3)	77.2 (8.5)	72.3 (6.5)	75.9 (7.9)	70.0 (4.1)
Sex						
Male (%)	330 (40.4)	421 (42.8)	95 (43.0)	132 (43.1)	42 (35.3)	50 (48.5)
Female (%)	486 (59.6)	563 (57.2)	126 (57.0)	174 (56.9)	77 (64.7)	53 (51.5)

TABLE 2. Variables included in the prediction model for advanced AMD

i	Variable (X_i)	Regression Coefficient (β_i)*	*P* value*
	Intercept (α)	-8.7793	< .001
1	Age of participation	0.1032	< .001
2	Past smoking	0.3531	.028
3	Current smoking	1.2425	< .001
4	Physical activity: 1 or 2 times a week	-0.5245	.001
5	Physical activity: 3 or more times a week	-1.2050	< .001
6	Education	-0.5675	< .001
7	Family history of AMD	1.2670	< .001
8	*CFH* (I62V), rs800292 G:A	-0.6851	< .001
9	*CFH* (I62V), rs800292 A:A	0.0004	.999
10	*CFH* (Y402H), rs1061170 T:C	0.6504	.001
11	*CFH* (Y402H), rs1061170 C:C	1.4076	< .001
12	*CFH*, rs12144939 G:T	-0.5050	.028
13	*CFH*, rs12144939 T:T	-0.4439	.562
14	*ARMS2* (A69S), rs10490924 G:T	0.8987	< .001
15	*ARMS2* (A69S), rs10490924 T:T	2.4130	< .001
16	*CETP*, rs3764261 C:A	0.5144	.001
17	*CETP*, rs3764261 A:A	0.3367	.161
18	*VEGFA*, rs943080 T:C	-0.2458	.153
19	*VEGFA*, rs943080 C:C	-0.3820	.091
20	*TIMP3*, rs9621532 A:C or C:C	-0.4483	.061

AMD, age-related macular degeneration
** Regression coefficients and P values of pooled data from training set*

FIGURE 1. Receiver operating characteristic (ROC) curves of the training, validation and family set



The area under the curve was obtained for all three ROC curves; 0.873 (95% CI 0.851-0.895) for the training set (A), 0.854 (95% CI 0.808-0.900) for the validation set (B) and 0.842 (95% CI 0.787-0.897) for the family set (C).

Further analyses of a subset of 25 densely affected families showed clustering of risk factors in the majority of families (n = 16), with predicted values above 0.7 for all affected individuals. Examples are shown in Figure 2, A and B. In contrast, we also identified two densely affected families with a low probability of advanced AMD (≤ 0.4) for all individual affected family members (Figure 2, C and D). For the remaining 7 families the probability of advanced AMD varied widely between the affected family members (0.4-0.99; examples shown in Figure 2, E and F).

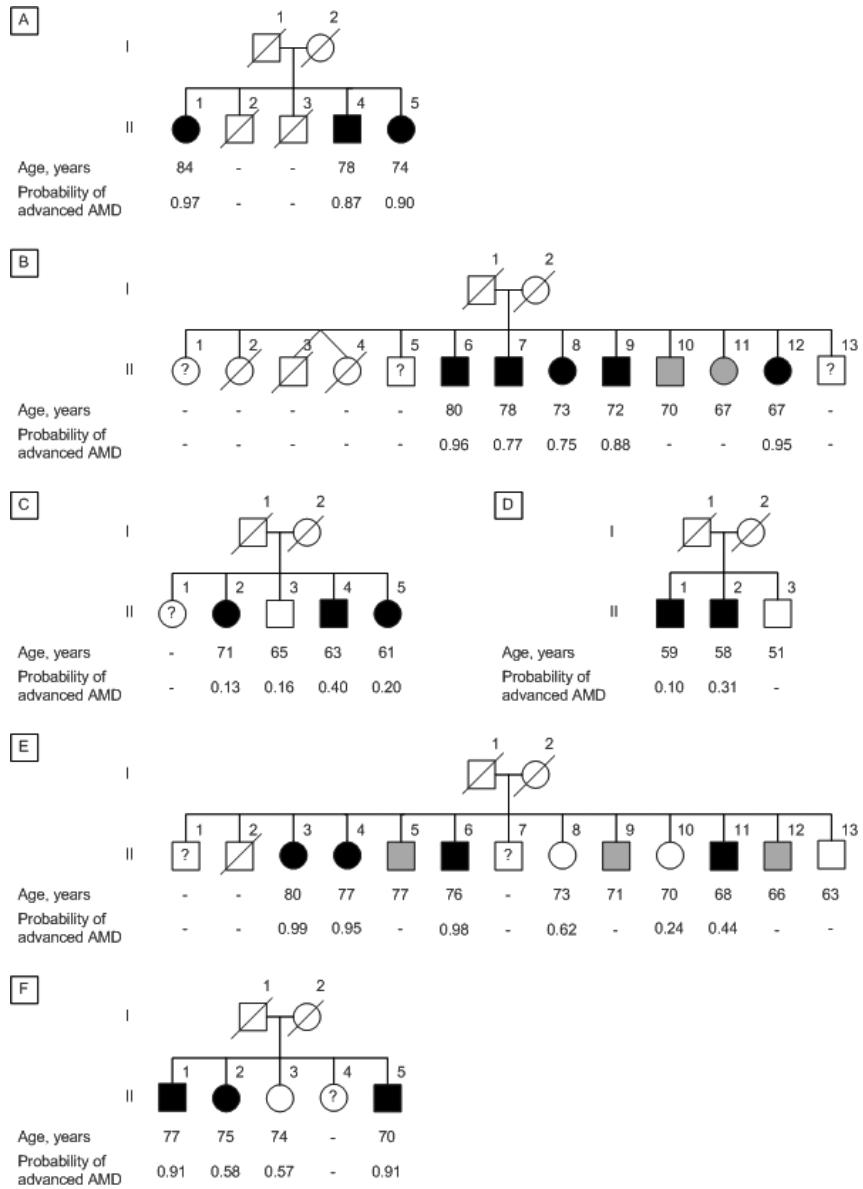
DISCUSSION

In this study, we demonstrated that a prediction model based on demographic, common genetic and environmental risk factors can be applied to AMD families. The model performed similarly in the validation set and family set (AUC 0.854 and 0.842, respectively). Further evaluation of the family dataset showed clustering of risk factors in the affected individuals of the majority of families.

Despite this observation, family history remains significantly associated with the development of advanced AMD in the multivariable model. This indicates that clustering of known risk factors cannot fully explain the aggregation of AMD in families.[13] Therefore, family history might be a substitute for risk factors that are not included in this study, for instance dietary factors, or yet undiscovered environmental or genetic risk factors.

In agreement with our findings, Sobrin et al. previously reported that the genotypic load for five common SNPs in most AMD families did not significantly differ from the expected load.[17] For some densely affected families however, the authors reported a genotypic load that

FIGURE 2. Examples of families with high (A,B), low (C,D) and varying (E,F) probabilities of advanced AMD for the affected individuals



Squares, men; Circles, women; Slashes, deceased family members (no data available); Black symbols, patients with advanced AMD; Shaded symbols, patients with non-advanced AMD (not included in this study); Question mark, AMD status unknown (non-participants).

was lower than expected, suggesting that clustering of common environmental risk factors or rare genetic variants may explain aggregation of AMD in these families. Like Sobrin et al., we identified some AMD families with low predicted risk scores for the affected individuals. Since we also included environmental factors in our model, these factors cannot explain the aggregation of AMD in these particular families. We hypothesize that such families may carry rare genetic risk variants, and are thus of particular interest for additional genetic testing, such as whole exome sequencing.

Based on previous studies that describe different distributions of risk factors and the identification of rare variants in AMD families,[13,15-18,20,21] it may be surprising that our prediction model performs well in the family dataset. However, it must be emphasized that the patients included in this study are all aged 50 years or older, because of the EUGENDA inclusion criteria. It is known that patients carrying rare variants often have an earlier onset of AMD,[18-21] and a small number of patients may therefore have been excluded in this study. In addition, the phenotype in families with rare genetic variants can differ from typical AMD cases. Previously, our group identified rare variants in the *CFH* gene in families with the cuticular drusen phenotype.[18,20] In this study we only included families with a typical AMD phenotype and only evaluated advanced AMD cases. We hypothesize that rare variants predominantly occur in families with a younger, and more atypical presentation of AMD. Research focused on the identification of new rare variants should ideally focus on such families. It is therefore very important to perform detailed phenotyping of AMD, including for example fluorescein angiography to distinguish patients with cuticular drusen.

CONCLUSIONS

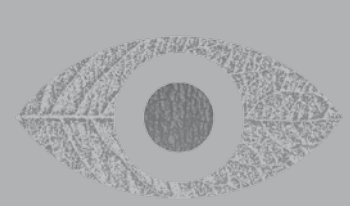
We have shown that common genetic and environmental risk factors can be used for prediction of AMD in AMD families based on clustering of these common risk factors in the majority of families. However, in a small subset of families the risk of advanced AMD is not explained by these common risk factors. This suggests that other factors, such as rare genetic variants, play a key role in the development of advanced AMD in these families.

REFERENCES

1. Chakravarthy U, Wong TY, Fletcher A, et al. Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. *BMC Ophthalmol* 2010;10:31.
2. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology* 2000;107:2224-2232.
3. Evans JR. Risk factors for age-related macular degeneration. *Prog Retin Eye Res* 2001;20:227-253.
4. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob health* 2014;2:e106-116.
5. Boyers LN, Karimkhani C, Hilton J, Richheimer W, Dellavalle RP. Global burden of eye and vision disease as reflected in the Cochrane Database of Systematic Reviews. *JAMA ophthalmology* 2015;133:25-31.
6. Buitendijk GH, Rochtchina E, Myers C, et al. Prediction of age-related macular degeneration in the general population: the Three Continent AMD Consortium. *Ophthalmology* 2013;120:2644-2655.
7. Chen Y, Zeng J, Zhao C, et al. Assessing Susceptibility to Age-Related Macular Degeneration With Genetic Markers and Environmental Factors. *Arch Ophthal* 2011;129:344-351.
8. Klein ML, Francis PJ, Ferris FL 3rd, Hamon SC, Clemons TE. Risk assessment model for development of advanced age-related macular degeneration. *Arch Ophthalmol* 2011;129:1543-1550.
9. Ristau T, Ersoy L, Hahn M, et al. Nongenetic risk factors for neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:5228-5232.
10. Seddon JM, Reynolds R, Maller J, Fagerness JA, Daly MJ, Rosner B. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. *Invest Ophthalmol Vis Sci* 2009;50:2044-2053.
11. Seddon JM, Reynolds R, Yu Y, Daly MJ, Rosner B. Risk models for progression to advanced age-related macular degeneration using demographic, environmental, genetic, and ocular factors. *Ophthalmology* 2011;118:2203-2211.
12. Spencer KL, Olson LM, Schnetz-Boutaud N, et al. Using genetic variation and environmental risk factor data to identify individuals at high risk for age-related macular degeneration. *PLoS One* 2011;6:e17784.
13. Klaver CC, Wolfs RC, Assink JJ, van Duijn CM, Hofman A, de Jong PT. Genetic risk of age-related maculopathy. Population-based familial aggregation study. *Arch Ophthalmol* 1998;116:1646-1651.
14. Luo L, Harmon J, Yang X, et al. Familial aggregation of age-related macular degeneration in the Utah population. *Vision Res* 2008;48:494-500.
15. Seddon JM, Ajani UA, Mitchell BD. Familial aggregation of age-related maculopathy. *Am J Ophthalmol* 1997;123:199-206.
16. Saksens NT, Kersten E, Groenewoud JM, et al. Clinical characteristics of familial and sporadic age-related macular degeneration: differences and similarities. *Invest Ophthalmol Vis Sci* 2014;55:7085-7092.
17. Sobrin L, Maller JB, Neale BM, et al. Genetic profile for five common variants associated with age-related macular degeneration in densely affected families: a novel analytic approach. *Eur J Hum Genet* 2010;18:496-501.
18. Boon CJ, Klevering BJ, Hoyng CB, et al. Basal laminar drusen caused by compound heterozygous variants in the CFH gene. *Am J Hum Genet* 2008;82:516-523.
19. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet* 2011;43:1232-1236.
20. van de Ven JP, Boon CJ, Fauser S, et al. Clinical evaluation of 3 families with basal laminar drusen caused by novel mutations in the complement factor H gene. *Arch Ophthalmol* 2012;130:1038-1047.
21. Yu Y, Triebwasser MP, Wong EK, et al. Whole-exome sequencing identifies rare, functional CFH variants in families with macular degeneration. *Hum Mol Genet* 2014;23:5283-5293.
22. Seddon JM, Yu Y, Miller EC, et al. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat Genet* 2013;45:1366-1370.
23. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet* 2013;45:813-817.
24. Duvvari MR, Paun CC, Buitendijk GH, et al. Analysis of rare variants in the C3 gene in patients with age-related macular degeneration. *PLoS One* 2014;9:e94165.
25. Helgason H, Sulem P, Duvvari MR, et al. A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nat Genet* 2013;45:1371-1374.

26. Zhan X, Larson DE, Wang C, et al. Identification of a rare coding variant in complement 3 associated with age-related macular degeneration. *Nat Genet* 2013;45:1375-1379.
27. Ratnapriya R, Zhan X, Fariss RN, et al. Rare and common variants in extracellular matrix gene Fibrillin 2 (FBN2) are associated with macular degeneration. *Hum Mol Genet* 2014;23:5827-5837.
28. Ristau T, Ersoy L, Lechanteur Y, et al. Allergy is a protective factor against age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:210-214.
29. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2005;102:7227-7232.
30. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
31. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
32. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
33. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One* 2014;9:e93459.
34. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
35. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
36. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
37. Ryu E, Fridley BL, Tosakulwong N, Bailey KR, Edwards AO. Genome-wide association analyses of genetic, phenotypic, and environmental risks in the age-related eye disease study. *Mol Vis* 2010;16:2811-2821.
38. Maller JB, Fagerness JA, Reynolds RC, Neale BM, Daly MJ, Seddon JM. Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat Genet* 2007;39:1200-1201.
39. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
40. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet* 2013;45:433-439, 439e431-432.
41. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2010;107:7401-7406.
42. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A* 2010;107:7395-7400.
43. Kopplin LJ, Igo RP, Jr., Wang Y, et al. Genome-wide association identifies SKIV2L and MYRIP as protective factors for age-related macular degeneration. *Genes Immun* 2010;11:609-621.
44. Yu Y, Bhangale TR, Fagerness J, et al. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. *Hum Mol Genet* 2011;20:3699-3709.
45. Holliday EG, Smith AV, Cornes BK, et al. Insights into the genetic architecture of early stage age-related macular degeneration: a genome-wide association study meta-analysis. *PLoS One* 2013;8:e53830.
46. Oczos J, Grimm C, Barthelmes D, et al. Regulatory regions of the paraoxonase 1 (PON1) gene are associated with neovascular age-related macular degeneration (AMD). *Age (Dordr)* 2013;35:1651-1662.
47. Morrison MA, Silveira AC, Huynh N, et al. Systems biology-based analysis implicates a novel role for vitamin D metabolism in the pathogenesis of age-related macular degeneration. *Hum Genomics* 2011;5:538-568.
48. Chiu CJ, Conley YP, Gorin MB, et al. Associations between genetic polymorphisms of insulin-like growth factor axis genes and risk for age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:9099-9107.
49. Clemons TE, Milton RC, Klein R, Seddon JM, Ferris FL 3RD. Risk factors for the incidence of Advanced Age-Related Macular Degeneration in the Age-Related Eye Disease Study (AREDS) AREDS report no. 19. *Ophthalmology* 2005;112:533-539.
50. Seddon JM, Cote J, Davis N, Rosner B. Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio. *Arch Ophthal* 2003;121:785-792.

COMPUTER AIDED DIAGNOSIS IN AGE-RELATED MACULAR DEGENERATION



CHAPTER 7

AUTOMATIC DRUSEN QUANTIFICATION AND RISK ASSESSMENT OF AGE-RELATED MACULAR DEGENERATION ON COLOR FUNDUS IMAGES

Mark J. J. P. van Grinsven,*
Yara T. E. Lechanteur,*
Johannes P. H. van de Ven,
Bram van Ginneken,
Carel B. Hoyng,
Thomas Theelen,†
Clara I. Sánchez.†
* These authors contributed equally to this study and share first authorship.
† These authors contributed equally to this study and share last authorship.

Invest Ophthalmol Vis Sci. 2013;54(4):3019-27.

PURPOSE To evaluate a machine learning algorithm that allows for computer aided diagnosis (CAD) of nonadvanced age-related macular degeneration (AMD) by providing an accurate detection and quantification of drusen location, area and size.

METHODS Color fundus photographs of 407 eyes without AMD or with early to moderate AMD were randomly selected from a large European multicenter database. A machine learning system was developed to automatically detect and quantify drusen on each image. Based on detected drusen, the CAD software provided a risk assessment to develop advanced AMD. Evaluation of the CAD system was performed using annotations made by two blinded human graders.

RESULTS Free-response receiver operating characteristics analysis showed that the proposed system approaches the performance of human observers in detecting drusen. The estimated drusen area showed excellent agreement with both observers, with mean intraclass correlation coefficients (ICC) larger than 0.85. Maximum druse diameter agreement was lower with a maximum ICC of 0.69 but comparable to the interobserver agreement (ICC = 0.79). For automatic AMD risk assessment, the system achieved areas under the receiver operating characteristic curve of 0.948 and 0.954, reaching similar performance as human observers.

CONCLUSIONS A machine learning system, capable of separating high-risk from low-risk patients with nonadvanced AMD by providing accurate detection and quantification of drusen, was developed. The proposed method allows for quick and reliable diagnosis of AMD, opening the way for large dataset analysis within population studies and genotype-phenotype correlation analysis.

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in developed countries among individuals older than 50 years.[1] AMD is a gradual progressive disease that evolves from early and intermediate stages, with no or subtle visual changes, to an advanced stage, where the loss of central vision can occur. Patients with intermediate AMD are at higher risk of developing advanced AMD and thus suffering from severe visual loss, and they should undergo routine- and self-monitoring for a timely diagnosis.[2] Lifestyle changes such as cessation of smoking and prophylactic regimen like vitamin supplementation are recommended for patients at risk in order to slow progression of the disease.[3-6]

Deposits of extracellular material localized between the inner collagenous layer of Bruch's membrane and the basal lamina of the RPE, known as drusen, are considered the hallmark feature of AMD.[7] Macular drusen are important in the context of AMD grading and certain drusen characteristics are associated with progressing toward end-stage AMD.[8-14] On fundus photography they appear as yellowish-white spots and different drusen phenotypes can be distinguished. Hard drusen are defined as small (< 63 µm) nodular lesions with well defined borders. Soft drusen, on the other hand, tend to be larger and are generally characterized by poorly demarcated boundaries.[7-9,12,15]

Identification and classification of eyes with AMD are performed mainly using color fundus images by manually determining the size and extension of drusen.[8,12,16-19] However, other imaging modalities such as optical coherence tomography, are gaining traction as well.[20,21] Human observer classification is time-consuming and prone to interobserver variations.[22] Aside from speed, objectivity and reproducibility, implementation of an automatic drusen detection and quantification system could prove useful in many ways. It may allow for a cost-efficient screening program for patients at risk and help identify and classify AMD patients in large cohort studies. Additionally, accurate quantitative measurements can help in large clinical studies for the evaluation and progression of drusen area, for example in clinical trials concerning new therapeutic strategies for dry AMD, and it could help in applying inclusion criteria for large-scale clinical studies and genotype-phenotype correlation analysis.[23]

Previously proposed methods automatically assessed the presence of drusen on color fundus photographs.[24,25] However, the presence of drusen alone is not directly correlated with the risk of progression to advanced AMD.[2] Other works focused on the automatic quantification of drusen without identifying patients at high risk or the AMD stage.[23,26-32] Here, we describe and evaluate a machine learning algorithm that automatically distinguishes between images from low-risk and high-risk AMD patients by providing an accurate quantification of drusen location, area and size.

METHODS

Study Dataset

A total of 407 images of different eyes with nonadvanced stages of AMD (i.e., stages 1, 2, and 3 according to the criteria shown in Table 1), with sufficient grading quality for human graders, was selected in consecutive fashion from the European Genetic Database (EUGENDA, http://www.eugenda.org), a large multicenter database for clinical and molecular analysis of AMD.[33,34] For each subject, images of both eyes were eligible for inclusion, but we did not select multiple images of the same eye. Images with presence of reticular pseudodrusen were excluded from analyses. Number of drusen, age or ethnicity was not taken into account for the selection of data. Written informed consent was obtained before enrolling patients in EUGENDA. The study was performed according to the tenets set forth in the Declaration of Helsinki and Investigational Review Board approval was obtained.

Digital nonstereoscopic color fundus photographs were acquired with a TRC 501X model digital fundus camera at 50° (Topcon Corp., Tokyo, Japan) or with a CR-DGi model nonmydriatic retinal camera at 45° (Canon, Inc., Tokyo, Japan), and pupil dilation was achieved with topical 1.0% tropicamide and 2.5% phenylephrine. All images were macula-centered. Image size varied from 1360 X 1024 to 3504 X 2336 pixels. Before analysis, images were resized in a preprocessing step to have a field of view with a standardized diameter of 630 pixels independently of the image resolution. The data were divided randomly into two sets: set A, consisting of 52 images, for the evaluation of automatic drusen quantification and set B, consisting of 355 images, for the evaluation of automatic risk assessment. Images from the same patients were kept in the same set.

TABLE 1. Criteria for grading AMD according to the CIRCL grading protocol

AMD stage	Criteria
1: No AMD	no drusen or only small drusen (< 63 µm) or only pigmentary abnormalites or < 10 small drusen + pigmentary abnormalities
2: Early AMD	≥ 10 small drusen + pigmentary abnormalities or 1-14 intermediate drusen (≥ 63 and < 125 µm)
3: Intermediate AMD	≥ 1 large drusen (≥ 125 µm diameter) or ≥ 15 intermediate drusen or geographic atrophy (RPE atrophy ≥ 175 µm) not in the central circle of the ETDRS grid
4: Advanced AMD (GA)	GA (RPE atrophy ≥ 175 µm) secondary to AMD involving the central subfield of the ETDRS grid
5: Advanced AMD (CNV)	CNV within the ETDRS grid secondary to AMD with evidence for fluid, blood, or fibrovascular tissue on FP
6: CNV without signs for AMD	CNV is present but no drusen of any size are present within the Field2.
7: Cannot grade	Image is regarded as not gradable.

AMD, age-related macular degeneration; CIRCL, Cologne Image and Reading Center Laboratory; RPE, retinal pigment epithelium; ETDRS, Early Treatment Diabetic Retinopathy Study; GA, geographic atrophy; CNV, choroidal neovascularization; FP, color fundus photography.

Observer Annotations

Resampled images were displayed on an LCD monitor similar to those used in ophthalmology practice and with the ability to zoom and pan. All visible drusen were manually outlined in set A by both observers using a specifically developed annotation tool. Whether confluent drusen were annotated as separate drusen or as one large drusenoid patch was left to the judgment of the observers. Two trained graders (JPHV, designated Observer 1, and YTEL, designated Observer 2) manually performed a risk assessment to develop late-stage AMD in all images of sets A and B. No AMD and early AMD were defined as low-risk stages, and intermediate AMD was considered high-risk stage. AMD was defined according to the standard protocol of the Cologne Image Reading Center and Laboratory (CIRCL) (Table 1),[33] an AMD classification that was adopted specifically for EUGENDA based on different international staging systems.[8,16-19] Both observers performed one session of AMD grading for each study eye in set B and performed one session of drusen annotation for each study eye in set A on a different day.

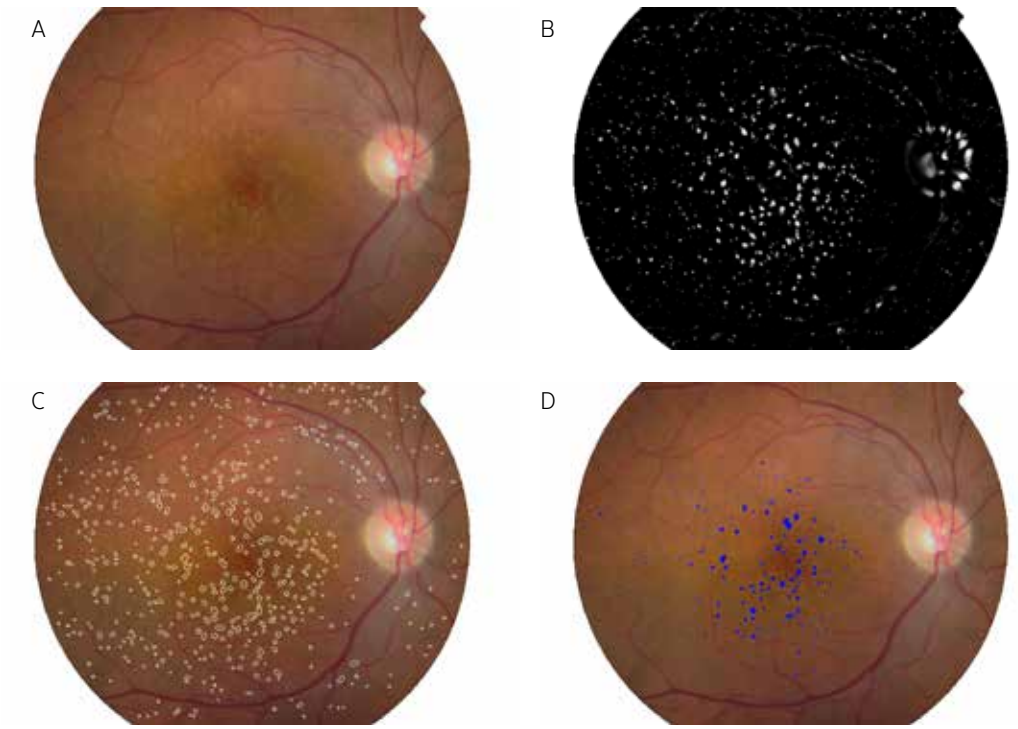
Machine Learning Algorithm

The proposed computer aided diagnosis (CAD) system analyzed all color fundus images to automatically quantify the visible drusen and assigned each image a probability between 0 and 1, with 1 indicating a high risk of developing advanced AMD and 0 indicating low risk. To accomplish this, the system performed the following steps:

1. Druse candidate extraction: Each pixel in the image was assigned a probability that the pixel was part of a bright lesion structure using a supervised pixel classification method. Supervised classification is a machine learning technique where manually labeled training examples are used to infer the classification rule.[30] Neighboring pixels with similar probability, not located close to the automatically detected optic disc,[30] were grouped into druse candidates.
2. Druse candidate segmentation: The boundary of each druse candidate was automatically delineated using intensity and contrast characteristics.
3. Druse candidate classification: Druse characteristics and a supervised lesion classification method were used to assign a probability to each segmented candidate which indicated the likelihood of being a true druse, creating a so-called drusen probability map.
4. AMD risk assessment: Based on the drusen probability map, a supervised image classification method assigned each image a probability to be at high risk of developing advanced AMD.

Figure 1 shows the steps of the CAD algorithm. The classification steps in the system were performed using statistical classifiers that could differentiate between different types of pixels, candidates, or images by using a training set of labeled examples and extracting numerical characteristics (features).[35] Several supervised classifiers were tested for each step and the classifiers that performed best were chosen, namely a k-nearest neighbor (kNN) classifier for step 1, a linear discriminant (LDA) classifier for druse candidate classification (step 3), and

FIGURE 1. Example of the outputs obtained in each step of the proposed CAD system.



A, Original color fundus image. B, Each pixel was assigned a probability of being part of a bright structure after the druse candidate extraction step. A higher intensity indicates a higher probability. C, The boundary of each druse candidate (shown overlaid on the original image) was delineated during the drusen candidate segmentation step. D, Candidates were classified as true drusen in the drusen candidate classification step. The final detected drusen are shown overlaid on the original image. Brighter color represents a higher probability of being a true druse.
CAD, computer aided diagnosis.

a random forest (RF) classifier for step 4.[35,36] A more detailed description of the CAD system can be found in Appendix A. Given an image, the CAD system provides two outputs: (1) detection of all visible drusen in the image and quantification of the drusen area and maximum druse diameter; and (2) a probability indicating the likelihood that the patient was at high risk of developing advanced AMD based on the drusen probability map.

Data Analysis

To evaluate the proposed CAD system, two types of analyses were performed: (1) evaluation of automatic drusen quantification; and (2) evaluation of automatic AMD risk assessment.

Due to the lack of a single gold standard, each evaluation was performed twice, taking Observer 1 as reference standard and comparing the CAD results with those obtained by Observer 2, and vice versa.

For drusen quantification, a fivefold cross-validation approach was performed to train and test the CAD system by using data from set A. Cross-validation analysis allows to determination of the system performance in an unbiased manner.[37] Using the test folds, the lesion sensitivity (fraction of drusen marked in the reference standard that were detected as drusen by the CAD system) and the number of false positives per image were calculated after setting a threshold for the druse probabilities obtained in step 3. Varying this threshold, different lesion sensitivity-false positives pairs were calculated and summarized in a free receiver operating characteristic (FROC) to evaluate the CAD performance on the detection of drusen.[38] The observer performance compared to the reference standard, which corresponds with one lesion sensitivity-false positives pair, was also calculated and included in the obtained FROC curve.

Total drusen area and maximum druse diameter obtained by the CAD system were calculated using the distance between the fovea and the border of the optic disc as a reference distance of 3000 µm.[8,12] After thresholding the drusen probability map, total drusen area and maximum druse diameter were measured and compared to the observers' opinions using intraclass correlation coefficient (ICC) analysis.[39] This threshold was set at the same false-positives rate as Observer 1, as this observer had fewer false positives than Observer 2. During the analysis, the performance of the CAD system and the observers of drusen quantification was evaluated inside and outside the Early Treatment Diabetic Retinopathy Study (ETDRS) grid, which was manually set before the analysis.[40]

For AMD risk assessment, a leave-one-out cross-validation approach was performed to train and test the CAD system, using data from set B.[37] The leave-one-out cross-validation allows measurement of the predictive performance measure of a statistical model by testing a single sample while training with the remaining samples. This is repeated such that each sample is used once as test data. Using the test folds, image sensitivity (fraction of images correctly classified by the CAD system in the high-risk stage) and image specificity (fraction of images correctly classified by the CAD system in the low-risk stage) were calculated after setting a threshold for the estimated risk obtained in step 4. Varying this threshold, different image sensitivity-image specificity pairs were calculated and summarized in a receiver operating characteristic (ROC) curve to evaluate the CAD performance of distinguishing between low-risk and high-risk patients.[38] The area (A_z) under the ROC was used as a measure of performance. The observer performance compared to the reference standard, which corresponds with one image sensitivity-image specificity pair, was also calculated and included in the obtained ROC curve. Overall agreement on risk assessment between the observers was calculated using kappa (κ) statistics (version 17.0.0 software; SPSS, Chicago, IL).

RESULTS

Of 407 images, 145 were captured with the Topcon camera, and 262 were captured with the Canon camera. Table 2 shows some statistics of the performed observer annotations for AMD risk assessment and drusen quantification.

TABLE 2. Summary of the manual annotations performed on sets A and B by the two observers

	Set A		Set B	
Annotation	**Observer 1**	**Observer 2**	**Observer 1**	**Observer2**
Risk assessment				
No AMD	17	20	216	218
Early AMD	13	9	64	64
Intermediate AMD	22	23	75	76
Drusen quantification				
Average number of drusen*	130.4 ± 178.1	198.5 ± 243.1	-	-
Average size of drusen, µm²†	5.87 ± 10.03	5.12 ± 8.26	-	-

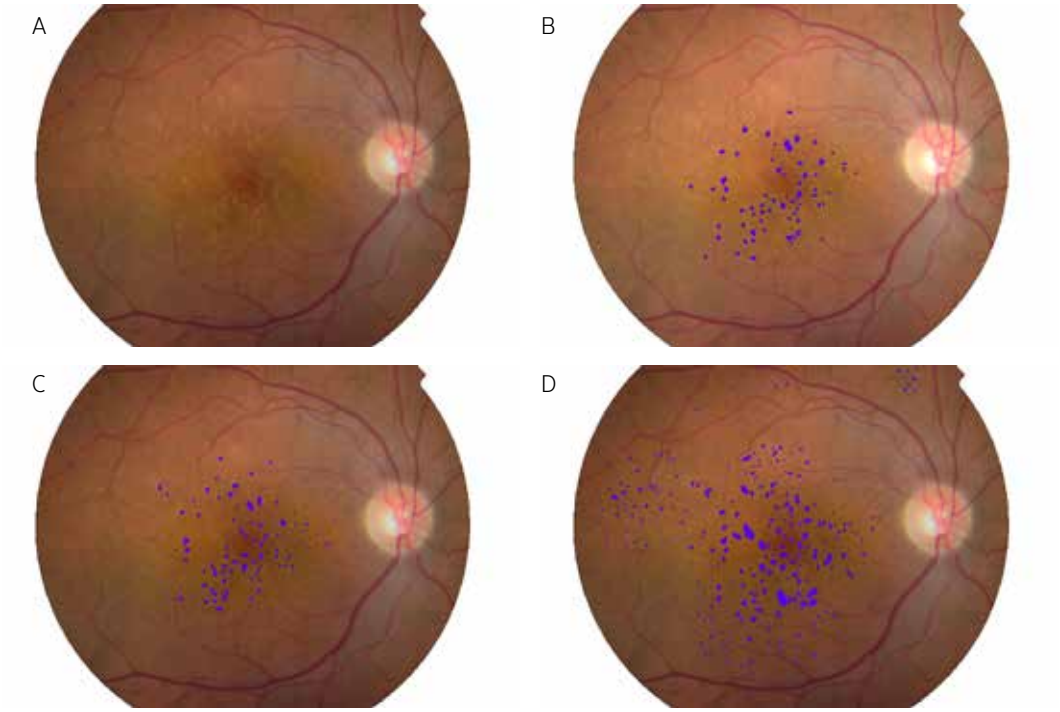
AMD, age-related macular degeneration.
** Average number of annotated drusen per image.*
† Average size of annotated drusen.

Drusen Quantification

Figure 2 shows the drusen automatically detected by the CAD system from a sample image and shows the annotations of the observers. Figures 3A and 3B show the FROC curves for the CAD system inside and outside the ETDRS grid, using Observer 1 and Observer 2 as reference standards, respectively.

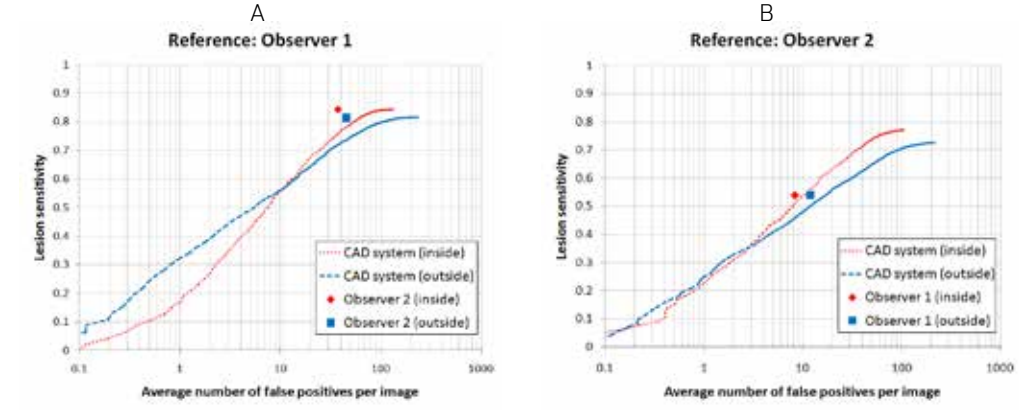
Tables 3 and 4 summarize the mean drusen area and maximum druse diameter obtained by the CAD system and the observers. The corresponding ICCs are shown in Figures 4A and 4B. For estimated drusen area, ICCs of 0.91 and 0.86 were obtained for the CAD system compared to Observer 1 and Observer 2, respectively, whereas the interobserver agreement reached an ICC equal to 0.87. The CAD system showed similar agreement with the observers independently of the camera used for the acquisition, reaching ICC values of 0.80 and 0.88 on images acquired with the Topcon digital fundus camera at 50° and the Canon nonmydriatic retinal camera at 45°, respectively. For the estimation of maximum druse diameter, defined as the diameter of the smallest enclosing circle of a druse, the agreement with the observers was lower with a maximum ICC of 0.69, whereas observers had an agreement with an ICC of 0.79.

FIGURE 2. Example of automatic detection of drusen by the CAD system and annotations by the Observers



A, Original color fundus image. B, Drusen detected by the CAD system overlaid on the original image. C, Drusen annotated by Observer 1 on the original image. D, Drusen annotated by Observer 2 on the original image. CAD, computer aided diagnosis.

FIGURE 3. FROC curves for the CAD system inside and outside the ETDRS grid



A, Observer 1 and B, Observer 2 as reference standard. The corresponding observer performance compared to the reference standard is also plotted as a point in the graph.
FROC, free receiver operating characteristic; CAD, computer aided diagnosis; ETDRS, Early Treatment Diabetic Retinopathy Study.

TABLE 3. Mean area and percentage covered by drusen inside and outside the ETDRS grid and in the total image.

	CAD system	Observer 1	Observer2
Inside grid			
Mean area, mm^2	0.43 ± 0.57	0.44 ± 0.68	0.56 ± 0.73
Area, %	1.52 ± 2.01	1.55 ± 2.40	1.98 ± 2.58
Outside grid			
Mean area, mm^2	0.35 ± 0.70	0.33 ± 0.72	0.46 ± 0.81
Area, %	0.36 ± 0.73	0.34 ± 0.75	0.49 ± 0.87
Total image			
Mean area, mm^2	0.78 ± 1.00	0.77 ± 1.07	1.01 ± 1.21
Area, %	0.67 ± 0.86	0.65 ± 0.96	0.89 ± 1.16

ETDRS, Early Treatment Diabetic Retinopathy Study; CAD, computer aided diagnosis.

TABLE 4. Mean maximum druse diameter pixels inside and outsite the ETDRS grid and in the total image.

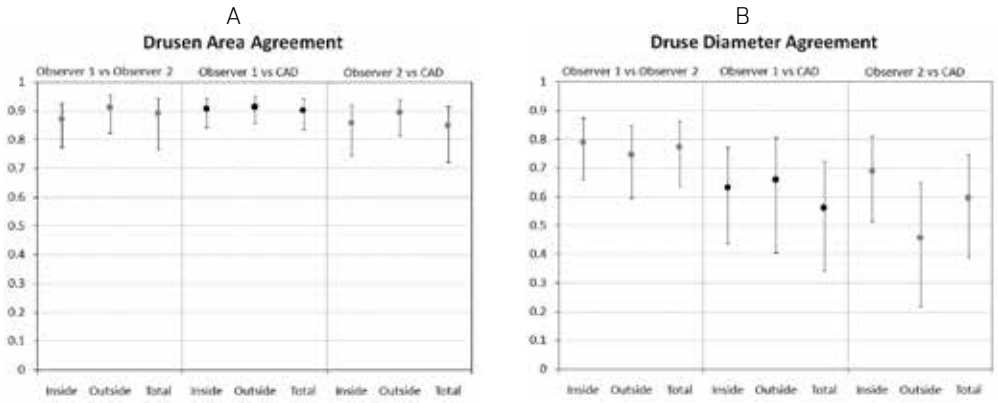
Coverage	CAD system	Observer 1	Observer2
Inside grid			
Maximum diameter, pix	11.54 ± 6.74	13.00 ± 12.53	11.79 ± 10.06
Maximum diameter, mm	0.21 ± 0.12	0.23 ± 0.23	0.21 ± 0.17
Outside grid			
Maximum diameter, pix	10.16 ± 6.30	7.15 ± 7.22	8.91 ± 10.06
Maximum diameter, mm	0.18 ± 0.12	0.13 ± 0.14	0.16 ± 0.15
Total image			
Maximum diameter, pix	13.88 ± 6.27	14.67 ± 12.33	13.74 ± 10.21
Maximum diameter, mm	0.25 ± 0.12	0.27 ± 0.23	0.25 ± 0.18

ETDRS, Early Treatment Diabetic Retinopathy Study; CAD, computer aided diagnosis; Pix, pixel.

AMD Risk Assessment

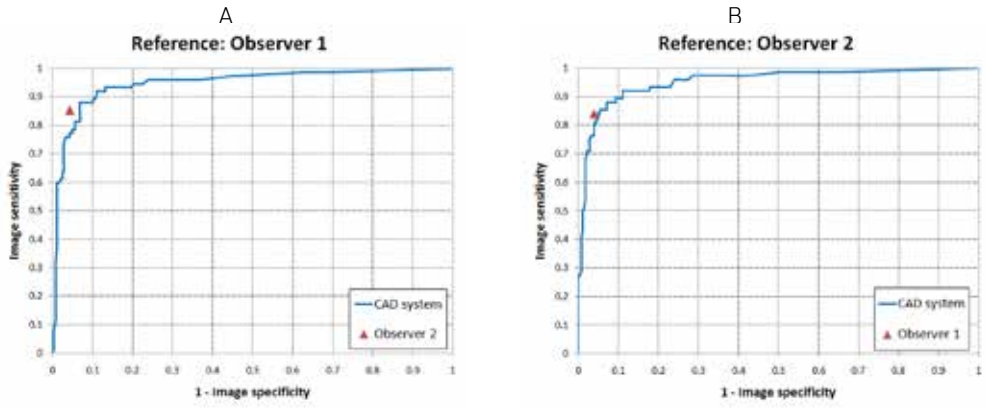
Figures 5A and 5B show ROC curves using Observer 1 and Observer 2 as reference standards, obtaining Az values of 0.948 and 0.954, respectively. Observer 2 reached an image sensitivity of 0.85 and an image specificity of 0.96, as shown in Figure 4, whereas Observer 1 obtained an image sensitivity of 0.84 and image specificity of 0.96 (Figure 5). Table 5 shows the contingency table and κ agreement between the observers and between the CAD system and the observers for AMD risk assessment. The threshold for the CAD system was set at the cutoff point that maximizes sensitivity + specificity.

FIGURE 4. ICC values for drusen area and maximum druse diameter between the CAD system and the observers



ICC values for (A) drusen area and (B) maximum druse diameter. ICC values are calculated for the values obtained inside and outside the ETDRS grid, as well as for the total image.
ICC, intraclass correlation coefficient; CAD, computer aided diagnosis; ETDRS, Early Treatment Diabetic Retinopathy Study.

FIGURE 5. ROC curves for the CAD system



A, Observer 1 and B, Observer 2 as reference standard. The corresponding observer performance compared to the reference standard is also plotted as a point in the graph.
ROC, receiver operating characteristic; CAD, computer aided diagnosis.

TABLE 5. Contingency table for AMD risk assessment between Observer 1 and Observer 2

Assessment	Low Risk	High Risk	κ (95% CI)
Observer 1 vs. Observer 2			
Low risk	268	11	0.807 (0.731-0.833)
High risk	12	64	
CAD system vs. Observer 1			
Low risk	261	10	0.765 (0.684-0.846)
High risk	19	65	
CAD system vs. Observer 2			
Low risk	259	10	0.760 (0.679-0.841)
High risk	20	66	

AMD, age-related macular degeneration; CAD, computer aided diagnosis; κ, kappa; CI, confidence interval.

DISCUSSION

In this study, a supervised machine learning algorithm for automated AMD classification based on drusen identification and quantification was developed. Our system was able to perform equally as experienced human graders with respect to AMD risk assessment, drusen localization and determination of mean drusen area with a dataset considerably larger than those used in previous publications.[25-27,29,31,41-46]

Detecting drusen on color fundus images is a challenging task, as shown by the differences in observer annotations in Figure 2. These differences illustrate the need for a robust and accurate system for drusen detection. This would help in eliminating intra- and interobserver variability and the subjective character of manual drusen detection. For this reason, automated drusen detection on color fundus photographs has been a field of interest for the last couple of decades. However, many systems still require human adjustments or close supervision by experts and are therefore still amenable to subjective input.[26,31,32,44-46] Unsupervised automatic detection systems have been developed, but most have failed to achieve acceptable performances compared to human graders,[41-43] or are only able to give categorized outcome values.[25,47]

In addition to detection, accurate localization and segmentation of drusen are very important to adequate quantification of drusen load in an image. In contrast to other methods,[27,29] where the performance analysis was carried out by pixel-to-pixel comparison, we performed FROC analysis,[38,48] stressing the importance of a correct localization and segmentation of individual lesions and providing higher statistical power than conventional ROC analysis for this task.[48]

With respect to quantification of the total drusen area, there is high agreement between the observers and the proposed CAD system (Figure 4). The CAD system also showed similar agreement with the observers independently of the camera used for the acquisition. Howev-

er, a more exhaustive analysis should be made in order to evaluate the effect of the image quality of manual and automatic drusen quantification. In a previously proposed drusen quantification method, a slightly higher ICC value was reported (ICC = 0.92) than the ground truth based on the average grading of eight experts.[27] However, in that study, images with the highest variability among observers were excluded from the study. This was the case for five images, resulting in the exclusion of more than 20% of the total dataset, which is likely to influence the outcome.

For the estimation of maximum druse diameter, agreement between the CAD system and the observers was lower with a maximum ICC value of 0.69. However, the interobserver agreement on this measurement also decreased. These lower values might be explained by the fact that a correct druse diameter depends on accurate druse delineation, which may be hampered by several factors. For human observers the main problem lies in the analysis and classification of complex morphological patterns that characterize drusen,[29] whereas the CAD system is impeded mostly by low image quality, poor contrast, or neighboring artifacts.

In this study, we also examined AMD risk assessment, and we showed that the CAD system performs as well as the human observers (Figure 4, Table 4). Images incorrectly classified by the system in the low-risk stage corresponded mainly to cases of disagreement between the observers (40% of misclassified images) or low quality images where the system was unable to localize low contrast drusen. Other studies have tried to identify AMD with automatic methods.[49,50] However, these were aimed primarily at identifying the presence or absence of disease instead of trying to separate high-risk from low-risk AMD patients, which is clinically more relevant. Zheng et al developed an algorithm for identification of AMD with a sensitivity of 99.4% and a specificity of 100%.[50] However, the authors compared their CAD system only to a single human observer, which can lead to false high performance.

In contrast to previously published CAD systems,[25-27,29,31,32,42-46] our software performs drusen quantification independently of a fixed region of interest. With full image drusen detection, more information from the image is extracted which can be beneficial if our method would be deployed in clinical studies of AMD. For example, in studies of the cuticular drusen subtype of AMD diagnosis is based on a typical pattern of innumerable small drusen on fluorescein angiography (FA), not only in the macular region, but also in the peripheral retina.[34,51] It would be very valuable to evaluate this drusen pattern to see if regions identified on FA are also detected by the CAD system on color images.

In our system, misclassification of candidates as true drusen often occurred due to reflections of the internal limiting membrane or because of the presence of non-AMD-related abnormalities. Adding better features to characterize these regions or including them as samples in the learning process of the CAD system might solve this problem in the future. Depending on their number and size, false-positive drusen detection might lead to incorrect AMD risk assessment. However, in our study, this did not occur very often. It is possible that these false-positive drusen have a relatively low probability to be a true druse, which is accounted for during computation of AMD risk. In addition, we did not consider pigmentary changes for automatic AMD risk assessment. This could be unfortunate if we wanted to separate patients with early AMD from healthy controls. For detection of patients at high risk of developping late-stage AMD, this distinction is not relevant because no AMD and early AMD were both considered low risk. However, if we wanted to use the system for classifying groups of patients in different AMD stages in new studies, the need for a well-defined control group is high. We will investigate automatic detection of pigmentary changes in upcoming studies. We are not aware of any implementation of AMD screening programs, but there have been studies evaluating cost-effectiveness of such programs.[52,53] However, the proposed programs are based on self-testing, whereas screening based on evaluation of color fundus images would be preferable. Deployment of human graders in such a broad setting would be costly and time consuming, and implementation of an automatic detection system would circumvent these problems. The CAD system could, for example, be installed in opticians' offices and be implemented in routine evaluation of elderly people. High-risk individuals would be selected on site and referred for further ophthalmologic evaluation.

CONCLUSIONS

We have developed and evaluated a machine learning system for identification of high-risk AMD patients. Our system allows for accurate detection and quantification of drusen location, area, and size, with a performance equal to human observers under stringent testing conditions. Implementation of our system allows for quick and reliable diagnosis of AMD in screening as well as in research programs. Additionally, there is a need for detailed phenotyping of large datasets in order to gain more insight into risk factors and disease mechanisms involved in AMD.[34] With the use of an automatic detection system, identification of homogeneous AMD subgroups and genotype-phenotype correlations should be achievable in a broader context.[23]

REFERENCES

1. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. *N Engl J Med* 2008;358:2606-2617.
2. Seddon JM, Reynolds R, Yu Y, Daly MJ, Rosner B. Risk models for progression to advanced age-related macular degeneration using demographic, environmental, genetic, and ocular factors. *Ophthalmology* 2011;118:2203-2211.
3. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* 2001;119:1417-1436.
4. Evans JR. Antioxidant vitamin and mineral supplements for slowing the progression of age-related macular degeneration. *Cochrane Database Syst Rev* 2006;CD000254.
5. Krishnadev N, Meleth AD, Chew EY. Nutritional supplements for age-related macular degeneration. *Curr Opin Ophthalmol* 2010;21:184-189.
6. Mares JA, Voland RP, Sondel SA, et al. Healthy lifestyles related to subsequent prevalence of age-related macular degeneration. *Arch Ophthalmol* 2011;129:470-480.
7. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res* 2001;20:705-732.
8. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol* 1995;39:367-374.
9. Bressler NM, Bressler SB, Seddon JM, Gragoudas ES, Jacobson LP. Drusen characteristics in patients with exudative versus non-exudative age-related macular degeneration. *Retina* 1988;8:109-114.
10. Bressler SB, Maguire MG, Bressler NM, Fine SL. Relationship of drusen and abnormalities of the retinal pigment epithelium to the prognosis of neovascular macular degeneration. The Macular Photocoagulation Study Group. *Arch Ophthalmol* 1990;108:1442-1447.
11. Holz FG, Wolfensberger TJ, Piguet B, et al. Bilateral macular drusen in age-related macular degeneration. Prognosis and risk factors. *Ophthalmology* 1994;101:1522-1528.
12. Klein R, Davis MD, Magli YL, Segal P, Klein BE, Hubbard L. The Wisconsin age-related maculopathy grading system. *Ophthalmology* 1991;98:1128-1134.
13. Klein R, Klein BE, Jensen SC, Meuer SM. The five-year incidence and progression of age-related maculopathy: the Beaver Dam Eye Study. *Ophthalmology* 1997;104:7-21.
14. Smiddy WE, Fine SL. Prognosis of patients with bilateral macular drusen. *Ophthalmology* 1984;91:271-277.
15. Bressler NM, Silva JC, Bressler SB, Fine SL, Green WR. Clinicopathologic correlation of drusen and retinal pigment epithelial abnormalities in age-related macular degeneration. *Retina* 1994;14:130-142.
16. Age-Related Eye Disease Study Research Group. The Age-Related Eye Disease Study system for classifying age-related macular degeneration from stereoscopic color fundus photographs: the Age-Related Eye Disease Study Report Number 6. *Am J Ophthalmol* 2001;132:668-681.
17. Klaver CC, Assink JJ, van Leeuwen R, et al. Incidence and progression rates of age-related maculopathy: the Rotterdam Study. *Invest Ophthalmol Vis Sci* 2001;42:2237-2241.
18. Seddon JM, Sharma S, Adelman RA. Evaluation of the clinical age-related maculopathy staging system. *Ophthalmology* 2006;113:260-266.
19. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. *Ophthalmology* 2001;108:697-704.
20. Yehoshua Z, Wang F, Rosenfeld PJ, Penha FM, Feuer WJ, Gregori G. Natural history of drusen morphology in age-related macular degeneration using spectral domain optical coherence tomography. *Ophthalmology* 2011;118:2434-2441.
21. Chiu SJ, Izatt JA, O'Connell RV, Winter KP, Toth CA, Farsiu S. Validated automatic segmentation of AMD pathology including drusen and geographic atrophy in SD-OCT images. *Invest Ophthalmol Vis Sci* 2012;53:53-61.
22. Scholl HP, Peto T, Dandekar S, et al. Inter- and intra-observer variability in grading lesions of age-related maculopathy and macular degeneration. *Graefes Arch Clin Exp Ophthalmol* 2003;241:39-47.
23. Quellec G, Russell SR, Seddon JM, et al. Automated discovery and quantification of image-based complex phenotypes: a twin study of drusen phenotypes in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:9195-9206.
24. Niemeijer M, van Ginneken B, Russell SR, Suttorp-Schulten MS, Abramoff MD. Automated detection and differentiation of drusen, exudates, and cotton-wool spots in digital color fundus photographs for diabetic retinopathy diagnosis. *Invest Ophthalmol Vis Sci* 2007;48:2260-2267.
25. Liang Z, Wong DWK, Liu J, Chan KL, Wong TY. Towards automatic detection of age-related macular degeneration in retinal fundus images. *Conf Proc IEEE Eng Med Biol Soc. 2010;* 2010;4100-4103. doi:10.1109/IEMBS.2010.5627289
26. Jain N, Farsiu S, Khanifar AA, et al. Quantitative comparison of drusen segmented on SD-OCT versus drusen delineated on color fundus photographs. *Invest Ophthalmol Vis Sci* 2010;51:4875-4883.
27. Mora AD, Vieira PM, Manivannan A, Fonseca JM. Automated drusen detection in retinal images using analytical modelling algorithms. *Biomed Eng Online* 2011;10:59.
28. Ong BB, Lee N, Lee WP, et al. Optimisation of an automated drusen-quantifying software for the analysis of drusen distribution in patients with age-related macular degeneration. *Eye (Lond)* 2013.
29. Rapantzikos K, Zervakis M, Balas K. Detection and segmentation of drusen deposits on human retina: potential in the diagnosis of age-related macular degeneration. *Med Image Anal* 2003;7:95-108.
30. Sanchez CI, Niemeijer M, Isgum I, et al. Contextual computer-aided detection: improving bright lesion detection in retinal images and coronary calcification identification in CT scans. *Med Image Anal* 2012;16:50-62.
31. Smith RT, Chan JK, Nagasaki T, Sparrow JR, Barbazetto I. A method of drusen measurement based on reconstruction of fundus background reflectance. *Br J Ophthalmol* 2005;89:87-91.
32. Smith RT, Sohrab MA, Pumariega NM, et al. Drusen analysis in a human-machine synergistic framework. *Arch Ophthalmol* 2011;129:40-47.
33. Fauser S, Smailhodzic D, Caramoy A, et al. Evaluation of serum lipid concentrations and genetic variants at high-density lipoprotein metabolism loci and TIMP3 in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:5525-5528.
34. van de Ven JP, Smailhodzic D, Boon CJ, et al. Association analysis of genetic and environmental risk factors in the cuticular drusen subtype of age-related macular degeneration. *Mol Vis* 2012;18:2271-2278.
35. Jain AK, Duin RPW, Jianchang M. Statistical pattern recognition: a review. *IEEE T Pattern Anal* 2000;22:4-37.
36. Ho TK. The random subspace method for constructing decision forests. *IEEE T Pattern Anal* 1998;20:832-844.
37. Arlot S, Celisse A. A survey of cross-validation procedures for model selection. *Stat Surv* 2010;4:40-79.
38. He X, Frey E. ROC, LROC, FROC, AFROC: an alphabet soup. *J Am Coll Radiol* 2009;6:652-655.
39. Koch GG. Intraclass correlation coefficient. In: Kotz S, Johnson NL, eds, *Encyclopedia of Statistical Sciences*. New York: John Wiley & Sons; 1982:213-217.
40. Grading diabetic retinopathy from stereoscopic color fundus photographs--an extension of the modified Airlie House classification. ETDRS report number 10. Early Treatment Diabetic Retinopathy Study Research Group. *Ophthalmology* 1991;98:786-806.
41. Goldbaum MH, Katz NP, Nelson MR, Haff LR. The discrimination of similarly colored objects in computer images of the ocular fundus. *Invest Ophthalmol Vis Sci* 1990;31:617-623.
42. Kirkpatrick JN, Spencer T, Manivannan A, Sharp PF, Forrester JV. Quantitative image analysis of macular drusen from fundus photographs and scanning laser ophthalmoscope images. *Eye (Lond)* 1995;9(pt 1):48-55.
43. Morgan WH, Cooper RL, Constable IJ, Eikelboom RH. Automated extraction and quantification of macular drusen from fundal photographs. *Aust N Z J Ophthalmol* 1994;22:7-12.
44. Peli E, Lahav M. Drusen measurement from fundus photographs using computer image analysis. *Ophthalmology* 1986;93:1575-1580.
45. Sebag M, Peli E, Lahav M. Image analysis of changes in drusen area. *Acta Ophthalmol* 1991;69:603-610.
46. Shin DS, Javornik NB, Berger JW. Computer-assisted, interactive fundus image processing for macular drusen quantitation. *Ophthalmology* 1999;106:1119-1125.
47. Brandon L, Hoover A. Drusen Detection in a Retinal Image Using Multi-level Analysis. In: Ellis RE, Peters TM (eds), *Medical Image Computing and Computer-Assisted Intervention - MICCAI 2003*. Berlin: Springer; 2003:618-625.
48. Metz CE. Receiver operating characteristic analysis: a tool for the quantitative evaluation of observer performance and imaging systems. *J Am Coll Radiol* 2006;3:413-422.
49. Agurto C, Barriga ES, Murray V, et al. Automatic detection of diabetic retinopathy and age-related macular degeneration in digital fundus images. *Invest Ophthalmol Vis Sci* 2011;52:5862-5871.
50. Zheng Y, Hijazi MHA, Coenen F. Automated "Disease/No Disease" Grading of Age-Related Macular Degeneration by an Image Mining Approach. *Invest Ophthalmol Vis Sci* 2012;53:8310-8318.

51. van de Ven JP, Boon CJ, Smailhodzic D, et al. Short-term changes of Basal laminar drusen on spectral-domain optical coherence tomography. *Am J Ophthalmol* 2012;154:560-567.
52. Karnon J, Czoski-Murray C, Smith K, et al. A preliminary model-based assessment of the cost-utility of a screening programme for early age-related macular degeneration. *Health Technol Assess* 2008;12:iii-iv, ix-124.
53. Karnon J, Czoski-Murray C, Smith KJ, Brand C. A hybrid cohort individual sampling natural history model of age-related macular degeneration: assessing the cost-effectiveness of screening using probabilistic calibration. *Med Decis Making* 2009;29:304-316.
54. Bellman R. On the Theory of Dynamic Programming. *Proc Natl Acad Sci U S A* 1952;38:716-719.

APPENDIX A

Druse Candidate Extraction

In this step, pixels that are potentially bright lesion pixels are extracted by convolving the green channel of the color fundus image with a group of Gaussian filters. These filters are based on Gaussian derivatives up to second order at different scales of information.[30] A kNN classifier is then trained to classify every pixel in the image on the basis of the filter responses.[35] No preprocessing of the image is needed previous to the druse candidate extraction step, such as suppression of luteal pigmentation.[26,31,32] After classification, a pixel probability map is obtained that indicates the probability of each pixel to be part of a bright lesion. Neighboring pixels with similar probability were grouped into druse candidates. Algorithms that perform optic disc segmentation and vessel segmentation were also applied in order to remove candidates that overlapped with these anatomical landmarks and to use in further processing.[30]

Druse Candidate Segmentation

In order to find the border of the drusen candidates, dynamic programming is applied around the local maxima of the calculated pixel probability map.[54] During this process, the gradient magnitude of the Gaussian derivatives is used as cost function to guide the algorithm to the candidate borders.

Druse Candidate Classification

In order to determine whether a druse candidate is a true druse or not, a classification step using a LDA is performed.[35] For each druse candidate, a total of 109 features based on color, intensity, contextual information and shape are extracted (Table 6).[30] These features exploit the different characteristics that the drusen show in color fundus images.

TABLE 6. Features for druse candidate classification

Feature	Number	Criteria
Shape	1-5	Area, perimeter, compactness, length and width of the candidate.
Context	6,7	Average and standard deviation of vessel pixel probability at the candidate border.
	8	Distance to the closest candidate.
	9,10	Number and average pixel probability of neighboring candidates in a radius of 50 pixels.
Intensity	11-33	Features measuring the contrast of the candidate in the RGB channels.
	33-81	Mean and standard deviation of Gaussian filter bank outputs.
Color	82-105	Average and standard deviation inside and outside the candidate using the planes of the Luv color space and HSI color space.
Misc.	106-109	Average, standard deviation, maximum and median pixel probability inside the candidate.

RGB, red-green-blue; Luv, luminescence-saturation-hue angle color space adopted by the International Commission on Illumination; HSI, hue-saturation-intensity.

AMD Risk Assessment

To separate high-risk from low-risk patients, a weighted histogram of the calculated drusen probabilities in the image is created to encode drusen extension and size. The value h_n of the histogram bin n is defined as:

$$h_n = \sum_{i \in L_n} p_i$$

where p_i is the posterior probability of druse candidate i, and L_n is the group of candidates whose size is $\tau n \leq d_i < \tau(n+1)$, in which d_i the size (μm) of candidate i. Terms τ and n control the bin size and the histogram resolution, respectively, and values were chosen as $n = 0, \ldots, 36$ and $\tau = 10$ μm. The last bin ($n = 36$) takes all the candidates with sizes d_i larger than 360 μm into account. A random forest classifier is then trained by using the histogram bins as features to distinguish high-risk patients.[36]

COMPLEMENT ACTIVATION IN AGE-RELATED MACULAR DEGENERATION

CHAPTER 8

COMPLEMENT ACTIVATION LEVELS ARE RELATED TO DISEASE STAGE IN AGE-RELATED MACULAR DEGENERATION

Yara T.E. Lechanteur,
Tina Schick,
Mohammed R. Daha,
Lebriz Altay,
Sandra Liakopoulos,
Dzenita Smailhodzic,
Anneke I. den Hollander,
Carel B. Hoyng,
B. Jeroen Klevering.

Submitted

IMPORTANCE Overactivation of the complement system plays an important role in the development of age-related macular degeneration (AMD), but not much is known yet about complement activity during disease progression.

OBJECTIVE To study the levels of complement activation in different stages of the disease and how this may be influenced by genes and treatment.

DESIGN, SETTING, AND PARTICIPANTS Case-control study with AMD patients and controls from the European Genetic Database collected between 2007 and 2011. Patients were individuals who visited the outpatient ophthalmology clinics of tertiary referral centers or volunteers collected through news advertisements. Controls were healthy volunteers or spouses of participating patients. We included 797 patients and 945 control individuals. Patients were classified as having early AMD, intermediate AMD, geographic atrophy, active choroidal neovascularization (CNV) or inactive CNV. We determined systemic levels of complement component 3 (C3) and its activation product C3d to calculate the C3d/C3 ratio as a measure of complement activation.

MAIN OUTCOMES AND MEASURES General linear models were used to evaluate the differences in complement activation between AMD stages. In a similar way we evaluated the effect of anti-vascular endothelial growth factor (VEGF) treatment and eight genetic polymorphisms in complement genes.

RESULTS Significant differences in complement activation were observed between AMD stages. The C3d/C3 ratio was lowest in controls ($P < .05$ compared to all AMD groups except for inactive CNV) and highest in patients with GA and intermediate AMD. Four polymorphisms in *CFH*, *CFB* and *C3* were significantly associated with systemic complement activation. The association between AMD stage and complement activation was only present in patients with ≥ 6 risk alleles. Patients who received anti-VEGF treatment in the past three months demonstrated lower C3d/C3 levels ($P = .005$).

CONCLUSIONS AND RELEVANCE We hypothesize that complement activation in AMD is a dynamic rather than a static process in individuals with a genetic predisposition. Local disease activity may elicit a systemic inflammatory signal leading to systemic activation and amplification of the complement pathway. These results shed new light on the relation between complement activation and AMD, and could have implications for patient selection and the window of opportunity for treatment with complement inhibitors.

Age-related macular degeneration (AMD) is a chorioretinal disease of the posterior pole of the retina characterized by the presence of drusen and pigmentary changes in the early stages of the disease, and geographic atrophy (GA) and choroidal neovascularization (CNV) in the advanced stages. The etiology of AMD is multifactorial and includes both environmental and genetic risk factors.[1] A major role in the pathophysiology of AMD is attributed to the alternative pathway of the complement system. The evidence for this relationship comes from several lines of research. First, already two decades ago immunohistochemical studies demonstrated the presence of complement components in drusen.[2-4] Next, several genetic variants in genes encoding components and regulators of the complement system were associated with an increased risk of AMD.[5-14] In addition, serum and plasma components of the alternative pathway were shown to be elevated in AMD.[15-20]

The complement system is part of our innate immunity and is important in the defense against pathogens and host homeostasis. The alternative pathway is continuously activated at a low level and this activation is tightly regulated by several inhibitory proteins.[21] It is thought that disturbances of these regulating mechanisms lead to an increase in complement activity and subsequently an increased inflammatory reaction, ultimately resulting in tissue damage and AMD.[22] Systemic levels of complement components have been associated with polymorphic variants in complement genes (*C3*, *CFH* and *CFB*), indicating that at least part of the complement activation is influenced by genetics.[15,17,18,23]

Modulation of the complement system by targeting the regulating components is now the focus for the development of new treatment modalities for AMD. Several clinical trials have been initiated to study the effect of complement inhibition on dry AMD. Promising results were derived from the phase II study on lampalizumab, an antigen-binding fragment against factor D, and phase III studies are ongoing.[24,25] In another clinical trial, we showed that daily administration of zinc sulphate can lower complement activity in AMD patients with a high baseline level of complement activation, suggesting a direct influence of zinc on the complement system. Subanalyses from this study showed that the level of complement activation varies between different stages of AMD.[26]

The aim of the current study was to evaluate the association between systemic complement activation and different stages of AMD in a larger cohort, and to define the role of genetic polymorphisms and treatment with anti-vascular endothelial growth factor (VEGF) injections on this relationship.

METHODS

Study Participants

All participants were recruited from the European Genetic Database (EUGENDA, www.eugenda.org), a multicenter database for clinical and molecular analysis of AMD. This study adhered to the tenets of the declaration of Helsinki and was approved by the local ethics committees of the Radboud university medical center and the University of Cologne. All study subjects provided written informed consent prior to participation.

We selected all unrelated AMD patients (≥ 50 years of age) and unrelated control individuals (≥ 65 years of age) with gradable fundus photographs and optical coherence tomograms of both eyes, for whom the level of complement activity had been analyzed. Participants where the ophthalmic examination and date of phlebotomy were separated by more than one month were not eligible for inclusion. Also, inclusion of AMD patients with choroidal neovascularisation (CNV) was limited to those individuals for whom blood collection took place on the same day as ophthalmic examination, to ensure that active CNV could be distinguished from inactive CNV. We excluded all participants with other pathology possibly affecting AMD staging (such as high myopia) and patients in whom retinal surgery was performed. Further, individuals with CRP levels > 10 mg/L and patients with a difference of more than one AMD stage between the left and right eye (e.g. "no AMD" and "intermediate AMD"; "no AMD" or "early AMD" and "advanced AMD") were excluded. We documented if and when participants had received intravitreal injections with anti- VEGF.

AMD Staging

AMD staging was performed using stereo fundus photographs and spectral domain optical coherence tomograms (SD-OCT). In patients with suspicion for neovascular AMD, fluorescein angiography was performed additionally. We performed grading according to the protocol of the Cologne Image Reading Center and Laboratory (CIRCL) by certified graders (TS, SL, LA). Study participants were classified as controls (no AMD) or patients with early, intermediate or advanced AMD (Table 1). The latter group was further specified into geographic atrophy, active CNV and inactive CNV.

TABLE 1. Classification of eyes with AMD according to the criteria defined by the CIRCL grading protocol

AMD stage	Criteria
Control	no drusen or only small drusen (< 63 µm diameter) or only pigmentary abnormalites or < 10 small drusen + pigmentary abnormalities
Early AMD	≥ 10 small drusen + pigmentary abnormalities or 1-14 intermediate drusen (≥ 63 and < 125 µm)
Intermediate AMD	≥ 1 large drusen (≥ 125 µm diameter) or ≥ 15 intermediate drusen or geographic atrophy (RPE atrophy ≥ 175 µm) not in the central circle of the ETDRS grid
Advanced AMD	
GA	GA (RPE atrophy ≥ 175 µm) secondary to AMD involving the central subfield of the ETDRS grid
Active CNV	CNV within the ETDRS grid secondary to AMD with signs for CNV activity (hemorrhage on FP and/or leakage on FA and/or subretinal and/or intraretinal fluid on SD-OCT)
Inactive CNV	CNV within the ETDRS grid secondary to AMD without signs for CNV activity

AMD, age-related macular degeneration; CIRCL, Cologne Image and Reading Center Laboratory; RPE, retinal pigment epithelium; ETDRS, Early Treatment Diabetic Retinopathy Study; GA, geographic atrophy; CNV, choroidal neovascularization; FP, color fundus photography; FA, fluorescein angioigraphy; SD-OCT, spectral domain optical coherence tomography.

Since this study focused on the relationship between AMD stage and complement activity, we had to attribute AMD stage not only to eyes, but also to individuals. To take a difference in AMD stage between both eyes into account, we classified patients based on the worst eye. Patients with GA in at least one eye no signs of CNV in the fellow eye were staged as *advanced AMD with GA*. The term *advanced AMD with active CNV* was used when at least on eye had active CNV. We used *advanced AMD with inactive CNV* for patients inactive CNV in at least one eye but without active CNV. Patients with GA in one eye and CNV in the other eye were classified as *advanced AMD with active* or *inactive CNV*, depending on CNV activity. We deemed reliable classification impossible when the AMD stage between the left and right eye differed by more than one stage: these individuals were excluded from analysis.

Complement and CRP Measurements

C3d and C3 measurements were determined from serum samples. Serum was prepared by coagulation at room temperature. After centrifugation, samples were stored at -80°C within one hour after collection. Complement component C3 and its activation product C3d were measured in serum samples as described previously.[27,28] All measurements were performed in a single assay. C-reactive protein (CRP) levels were measured with the Abbott Architect C16000 system (Abbott Diagnostics). We excluded patients with CRP values above 10 mg/L, because these values are suggestive for viral or bacterial inflammation or disease activity in chronic inflammatory conditions such as rheumatoid arthritis and will lead to a rise in complement activation.[29]

Genetic Analysis

DNA was extracted from venous blood samples. The DNA was analyzed for eight SNPs in four genes encoding components or regulators of the complement pathway and previously associated with AMD: *CFH* (rs1061170; rs12144939; rs800292), *CFB* (rs4151667; rs641153), *C3* (rs433594; rs2230199) and *CFI* (rs10033900) (Table 3). The SNPs were genotyped using competitive allele-specific polymerase chain reaction assays (KASPar SNP Genotyping System, KBiosciences).

Statistical Analysis

The activation fragment C3d is the most prominent marker for chronic complement activation because it reflects the complement turnover. The C3d level depends on the concentration of its parent molecule C3, therefore the C3d/C3 ratio was calculated as a value of complement activation that is independent on individual variations in the level of C3.[30] We used the natural logarithm of the C3d/C3 ratio for the analyses because of the skewness of the data. To analyze the association between complement activation and AMD stage, genetic polymorphisms in the complement genes and recent treatment with anti-VEGF injections we used univariate general linear models. All analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY). *P* values < .05 were considered to be significant.

RESULTS

A total of 2116 participants of the EUGENDA database had gradable images and measurements of complement activity. We excluded 374 participants for the following reasons: a difference of more than 1 AMD stage between the left and right eye (n = 159); CRP levels > 10 mg/L (n = 148); other retinal pathology that could interfere with the diagnosis of AMD, such as myopic degeneration (n = 59), or previous retinal surgery (n = 8). This left us with 797 AMD patients and 945 control individuals. Table 2 provides an overview of the different disease stages of the selected individuals, along with basic descriptive information of the control group and the five patient groups.

TABLE 2. Overview of AMD stages and information on age, sex and C3d/C3 ratio

	Control (n = 945)	Early AMD (n = 270)	Intermediate AMD (n = 144)	GA (n = 62)	Active CNV (n = 270)	Inactive CNV (n = 51)
Mean Age (SD)	72.3 (5.9)	73.1 (7.5)	73.8 (8.1)	78.0 (8.4)	78.4 (7.3)	78.8 (8.3)
Female sex (%)	529 (56.0)	157 (58.1)	103 (71.5)	36 (58.1)	173 (64.1)	30 (58.8)
Median C3d/C3 ratio (IQR)	3.99 (3.06-5.31)	4.21 (3.34-5.57)	4.89 (3.57-6.09)	4.75 (3.86-5.75)	4.41 (3.43-5.83)	4.10 (3.31-5.76)

AMD, age-related macular degeneration; GA, geographic atrophy; CNV, choroidal neovascularization; SD, standard deviation; IQR, interquartile range.

Significant differences in complement activation (C3d/C3 ratio) were observed between AMD stages (Figure 1A). Control individuals had a significantly lower level of complement activation as compared to all other AMD groups (*P* < .05), except for the group with inactive CNV (*P* > .05). Complement activation levels were significantly higher in intermediate AMD compared to early AMD and active CNV (*P* < .05). Patients with GA also showed high levels of complement activation, but this was not significantly different from other AMD stages. This may be due to the relatively low number of patients in the GA group.

We next evaluated the influence of eight SNPs in four complement genes on the level of complement activation. Four SNPs were significantly associated with altered levels of complement activation: rs2230199 in the *C3* gene, rs4151667 in the *CFB* gene, and rs12144939 and rs800292 in the *CFH* gene (Table 3). For a more detailed analysis of the relationship between these genetic polymorphisms and complement activation in AMD, we divided the patients and controls in a high and a low genetic risk group based on the numbers of risk alleles (defined as the complement raising alleles) in these three SNPs. Individuals with 6 or more risk alleles were classified in the high genetic risk group, those with 5 or less risk alleles in

the low genetic risk group. The association between AMD stage and complement activation was more pronounced in the high genetic risk group as compared to the whole population (Figure 1B). In the low genetic risk group an association between AMD stage and complement activation was no longer discernible.

In the AMD groups with active or inactive CNV, we evaluated whether recent treatment with anti-VEGF medication had an effect on complement activity. We compared patients who received intraocular injections with anti-VEGF medication within three months prior to participation with patients who did not receive treatment in this time period, and observed that the treated group had lower levels of complement activation (Figure 2; $P = .005$). We corrected for this effect by removing recently treated patients from the active CNV group. In the entire dataset, the level of complement activity in the untreated active CNV patients was no longer significantly different from the group with intermediate AMD ($P > .05$). In the subgroup with high risk levels the complement activity was still significantly different from the patients with intermediate AMD but no longer from the group with GA. The level of complement activity of the treated patients with active CNV was more comparable to that of the inactive CNV group and the control group ($P > .05$) and significantly lower than the complement activity in the groups with intermediate AMD and GA in the entire dataset. These observations were even stronger in the high genetic risk group. Because only 10 patients with inactive CNV had recently received anti-VEGF treatment, we did not perform sub-analysis on this group.

TABLE 3. Association between complement activation and AMD-associated SNPs in genes involved in the complement system

SNP	Median C3d/C3 (IQR)	*P* value*
CFH_rs12144939		.001
GG (n = 1137)	4.3156 (3.2765-5.7061)	
GT (n = 479)	3.8836 (3.0089-5.1291)	
TT (n = 53)	3.7163 (2.8681-4.6098)	
CFH_rs800292		.017
GG (n = 1019)	4.2193 (3.2558-5.6201)	
GA (n = 567)	4.0281 (3.0924-5.3429)	
AA (n = 79)	4.2253 (3.1941-5.5930)	
CFB_rs4151667		< .001
TT (n = 1537)	4.2252 (3.2393-5.6387)	
TA/AA (n = 128)	3.3738 (2.7701-4.3484)	
C3_rs2230199		.026
CC (n = 863)	4.0127 (3.1256-5.3761)	
CG (n = 467)	4.2755 (3.2522-5.7709)	
GG (n = 71)	4.2608 (3.2368-5.8480)	

AMD, age-related macular degeneration; SNP, single nucleotide polymorphism; IQR, interquartile range; CFH, complement factor H; CFB, complement factor B; C3, complement component 3.
** Corrected for AMD status (yes / no), age, sex, and body mass index.*

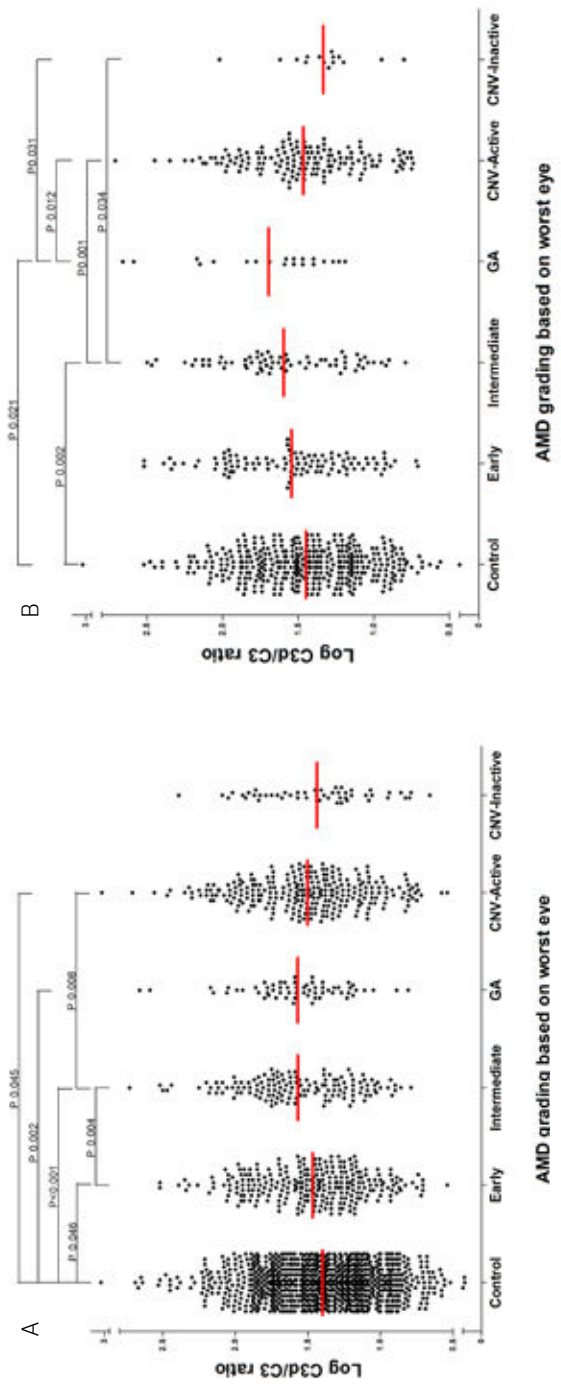


FIGURE 1. Association between serum complement and AMD staging in the total population (A) and the high genetic risk group (B)

A. Control individuals had the lowest C3d/C3 ratios, significantly lower than all patient groups except the patients with inactive CNV. Patients with intermediate AMD and GA had the highest complement activation levels. B. Subgroup analyses showing only patients and controls with a high genetic risk profile (6 or more risk alleles in SNPs in C3 (rs2230199), CFB (rs4151667) and CFH (rs12144939, rs800292)). Patients with intermediate AMD and GA had significantly higher C3d/C3 ratios compared to controls and patients with active and inactive CNV.
Red lines represent mean levels of log C3d.C3 ratio per subgroup.
P values are corrected for age, sex and body mass index.
AMD, age-related macular degeneration; GA, geographic atrophy; CNV, choroidal neovascularization.

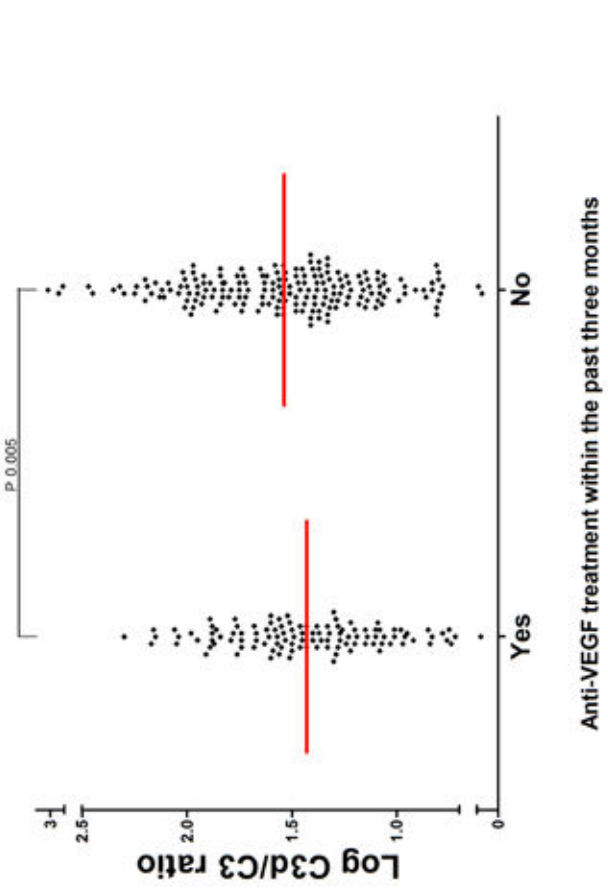


FIGURE 2. Association between the level of serum complement activation and presence or absence of recent anti-VEGF treatment in patients with active or inactive CNV

Comparison of patients who received intraocular injections with anti-VEGF medication within three months prior to participation with patients who did not receive treatment in this time period. The treated group had significantly lower levels of complement activation. Red lines represent mean levels of log C3d/C3 ratio per subgroup.
VEGF, vascular endothelial growth factor; CNV, choroidal neovascularization.

DISCUSSION

In this study we demonstrated that the level of complement activation differs among AMD patients, depending on their disease stage. All AMD stages, except the inactive CNV group, showed significantly higher C3d/C3 ratios compared to the controls. Patients with intermediate AMD and GA had the highest C3d/C3 ratios. These significant differences were only observed in participants with 6 or more risk alleles in SNPs in *C3* (rs2230199), *CFB* (rs4151667) and *CFH* (rs12144939, rs800292), and not in the subgroup of patients with a low genetic risk, suggesting that in this last group the complement pathway is not the driving force behind AMD development. In addition, the level of complement activation in CNV patients recently treated with anti-VEGF injections was significantly lower than in untreated CNV patients. Patients with active CNV showed lower levels of complement activation as compared to patients with GA and intermediate AMD. Sub analyses showed that this effect could largely be attributed to an effect of anti-VEGF treatment.

Although we did not measure the levels of complement activation prospectively during the entire disease process, comparing C3d/C3 levels in patient groups with identical AMD stages implies that complement activation is a dynamic process in genetically predisposed individuals. Complement activation in AMD appears to increase with local disease activity. This is counterintuitive: a higher level of complement activity is thought to be a contributing factor and not the result of the local disease process in AMD.[31] The differences between AMD stages with low complement activity (controls, early AMD and inactive CNV) and stages with higher activity (intermediate AMD, GA and active CNV) are difficult to explain. We are not aware of any studies that have evaluated the association between complement activation and AMD stages over time. Such a prospective study would be needed to confirm our findings. In particular, the lower level of complement activity in patients with active CNV treated with anti-VEGF suggests a direct influence of the AMD disease process on complement activity levels. It has been demonstrated that small amounts of intravitreally administered anti-VEGF drugs enter the systemic circulation and, although this may affect systemic VEGF levels, an effect on systemic complement activity has never been reported.[32] Rather, it seems that these patients are transferring from the active CNV group to the group with inactive disease with respect to the significantly lower C3d/C3 levels found in these patients.

We can merely speculate on the mechanisms underlying the observed association between AMD stage and complement activity. Intuitively, it seems unlikely that a localized macular disease as AMD has an effect on systemic complement activation. This is supported by this and previous studies demonstrating that polymorphisms in complement genes increase the chance of AMD development and progression, and these genetic risk factors drive systemic complement activation.[15,17,18,23] This supports the hypothesis of a chronic low-grade systemic complement activation, ultimately leading to changes in the eye, with the macula being the main site of disease activity because of its high metabolic properties.[22] One could thus argue that a rise in this low-grade complement activation may trigger AMD progression. This

does, however, not explain the decrease in complement activity in the fibrotic stage with or without treatment, which rather points toward an effect of local disease activity on systemic complement activation. An immunohistochemical study showed that SNPs in complement genes were related to complement activation in the vitreous of AMD patients but not with complement activation in the Bruch membrane – choroid interface, further suggesting that local disease activity is important.[33]

The variation in complement activity was most explicit in the subgroup with a high genetic risk based on four SNPs in three complement genes. In fact, after exclusion of these high-risk participants, the effect was no longer discernible in the remaining group. One other study reported an interesting observation on the association between complement levels and AMD with respect to genotype. Reynolds at al demonstrated that the odds ratios for association of complement levels with disease increased with adjustment for genetic variants,[16] suggesting that other factors than merely genetic factors influence complement activation. It seems plausible that in the setting of an increased activity of the complement system, a small, local stimulus is sufficient for further amplification of the complement response. We hypothesize that in these high genetic risk patients the active macular disease process elicits a systemic inflammatory signal that results in amplification and activation of complement as measured by elevated C3d/C3 levels.

Our results may have implications for the rationale and timing of complement inhibiting treatment in AMD. The first results from a clinical trial with eculizumab, a C5 complement inhibitor, were not convincing.[34] More recently, promising results were derived from the phase II study on lampalizumab, an antigen-binding fragment against factor D.[24,25] It is very important to select the right group of patients for a clinical trial. We showed that the relationship between complement activation levels and AMD stage is limited to a subgroup of individuals carrying multiple risk alleles in *CFH*, *CFB* and *C3*. These patients carrying high risk genetic variants may thus respond better to complement inhibiting treatments. In addition, trials tend to select patients with advanced AMD, while our current study suggests that patients with intermediate AMD have higher levels of complement activation. Further research should focus on the best timing for complement inhibition for AMD and the identification of suitable subgroups of patients for this treatment. Besides the presence of high risk variants, high baseline levels of complement activity could help in identifying these subgroups. This study shows that patients in the intermediate stage display high levels of complement activity and might also be considered for complement inhibition. The potential gain of the therapy would be much higher if initiated in an earlier disease stage to avoid sustained tissue damage by complement activation products. The downside is that only a minority of patients with intermediate AMD eventually progress toward late AMD.[35,36]

The robust dataset from the EUGENDA database is a major strength of this study. We have a large set of control individuals and AMD patients that allow us to study the relation between complement activation and disease stage in detail. A downside is that we currently do not have follow up of patients over time. We cannot exclude the possibility that our results merely reflect a selection process in which only individuals with higher C3d/C3 ratios progress toward the next disease stage. However, this would not explain why patients with inactive CNV display very low complement levels. Another limitation is the fact that especially for GA and inactive CNV our numbers are relatively small due to the fact that we collected our patients primarily in a tertiary referral center. Especially in the low genetic risk group, these subgroups are underrepresented. Despite statistical significance, our results should be interpreted with caution. We believe that our study contributes to the discussion on the pathogenesis of AMD and opens avenues to explore these relationships further in a prospective setting.

CONCLUSIONS

The level of complement activation appears to vary with the clinical AMD stage, in particular in individuals with high risk variants in complement genes. This leads us to hypothesize that complement activation in AMD at least in patients with high risk variants, is a dynamic rather than a static process, and that the active AMD process elicits a systemic inflammatory signal that results in amplification and activation of the complement pathway.

REFERENCES

1. de Jong PT. Age-related macular degeneration. *N Engl J Med* 2006;355:1474-1485.
2. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* 2002;134:411-431.
3. Johnson LV, Ozaki S, Staples MK, Erickson PA, Anderson DH. A potential role for immune complex pathogenesis in drusen formation. *Exp Eye Res* 2000;70:441-449.
4. van der Schaft TL, Mooy CM, de Bruijn WC, de Jong PT. Early stages of age-related macular degeneration: an immunofluorescence and electron microscopy study. *Br J Ophthalmol* 1993;77:657-661.
5. Fagerness JA, Maller JB, Neale BM, Reynolds RC, Daly MJ, Seddon JM. Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* 2009;17:100-104.
6. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
7. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
8. Li M, Atmaca-Sonmez P, Othman M, et al. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat Genet* 2006;38:1049-1054.
9. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
10. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet* 2011;43:1232-1236.
11. Helgason H, Sulem P, Duvvari MR, et al. A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nat Genet* 2013;45:1371-1374.
12. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet* 2013;45:813-817.
13. Seddon JM, Yu Y, Miller EC, et al. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat Genet* 2013;45:1366-1370.
14. van de Ven JP, Boon CJ, Fauser S, et al. Clinical Evaluation of 3 Families With Basal Laminar Drusen Caused by Novel Mutations in the Complement Factor H Gene. *Arch Ophthalmol* 2012;130:1038-1047.
15. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet* 2010;19:209-215.
16. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci* 2009;50:5818-5827.
17. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One* 2008;3:e2593.
18. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
19. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. *Arch Ophthalmol* 2007;125:515-519.
20. Stanton CM, Yates JR, den Hollander AI, et al. Complement factor D in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2011;52:8828-8834.
21. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* 2015;6:262.
22. Bora NS, Matta B, Lyzogubov VV, Bora PS. Relationship between the complement system, risk factors and prediction models in age-related macular degeneration. *Mol Immunol* 2015;63:176-183.
23. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One* 2014;9:e93459.
24. Hoffmann-La Roche. A Study Investigating the Efficacy and Safety of Lampalizumab Intravitreal Injections in Patients With Geographic Atrophy Secondary to Age-Related Macular Degeneration (CHROMA). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 Nov 29]. Available from: https://clinicaltrials.gov/show/NCT02247479 NLM Identifier: NCT02247479.
25. Hoffmann-La Roche. A Study Investigating the Safety and Efficacy of Lampalizumab Intravitreal Injections in Patients With Geographic Atrophy Secondary to Age-Related Macular Degeneration (SPECTRI). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2015 Nov 29]. Available from: https://clinicaltrials.gov/show/NCT02247531 NLM Identifier: NCT02247531.
26. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One* 2014;9:e112682.
27. Reddingius RE, Schroder CH, Daha MR, Monnens LA. The serum complement system in children on continuous ambulatory peritoneal dialysis. *Perit Dial Int* 1993;13:214-218.
28. Siezenga MA, Chandie Shaw PK, van der Geest RN, et al. Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. *Clin Exp Immunol* 2009;157:98-103.
29. Clyne B, Olshaker JS. The C-reactive protein. *J Emerg Med* 1999;17:1019-1025.
30. Rother E, Lang B, Coldewey R, Hartung K, Peter HH. Complement split product C3d as an indicator of disease activity in systemic lupus erythematosus. *Clin Rheumatol* 1993;12:31-35.
31. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res* 2001;20:705-732.
32. Avery RL, Castellarin AA, Steinle NC, et al. Systemic pharmacokinetics following intravitreal injections of ranibizumab, bevacizumab or aflibercept in patients with neovascular AMD. *Br J Ophthalmol* 2014;98:1636-1641.
33. Loyet KM, Deforge LE, Katschke KJ, Jr., et al. Activation of the alternative complement pathway in vitreous is controlled by genetics in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2012;53:6628-6637.
34. Yehoshua Z, de Amorim Garcia Filho CA, Nunes RP, et al. Systemic complement inhibition with eculizumab for geographic atrophy in age-related macular degeneration: the COMPLETE study. *Ophthalmology* 2014;121:693-701.
35. Joachim N, Mitchell P, Burlutsky G, Kifley A, Wang JJ. The Incidence and Progression of Age-Related Macular Degeneration over 15 Years: The Blue Mountains Eye Study. *Ophthalmology* 2015;122:2482-2489.
36. Klein R, Klein BE, Knudtson MD, Meuer SM, Swift M, Gangnon RE. Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. *Ophthalmology* 2007;114:253-262.

CHAPTER 9

A NOVEL COMPLOTYPE COMBINATION ASSOCIATES WITH AGE-RELATED MACULAR DEGENERATION AND HIGH COMPLEMENT ACTIVATION LEVELS *IN VIVO*

Constantin C. Paun,
Yara T.E Lechanteur,
Joannes M. M. Groenewoud,
Lebriz Ersoy,
Tina Schick,
Mohamed R. Daha,
Sascha Fauser,
Carel B. Hoyng,
Anneke I. den Hollander,
Eiko K. de Jong.

Sci Rep **2016;6:26568**

The complement system is the first line of defense against foreign intruders, and deregulation of this system has been described in multiple diseases. In age-related macular degeneration (AMD), patients have higher complement activation levels compared to controls. Recently, a combination of three single nucleotide polymorphisms (SNPs) in genes of the complement system, referred to as a complotype, has been described to increase complement activation *in vitro*. Here we describe a novel complotype composed of *CFB* (rs4151667) – *CFB* (rs641153) – *CFH* (rs800292), which is strongly associated with both AMD disease status (*P* $5.84*10^{-13}$) and complement activation levels *in vivo* (*P* $8.31*10^{-9}$). The most frequent genotype combination of this complotype was associated with the highest complement activation levels in both patients and controls. These findings are relevant in the context of complement-lowering treatments for AMD that are currently under development. Patients with a genetic predisposition to higher complement activation levels will potentially benefit the most of such treatments.

The complement system is part of our innate immunity where it acts as a first line of defense against foreign intruders, and as a surveillance system to discriminate between healthy host tissue, cellular debris and apoptotic cells.[1,2] The complement system can be triggered through one of its three pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). All three pathways converge at the level of complement component 3 (C3), which is cleaved into C3a – a potent proinflammatory molecule – and C3b – an opsonin.[1]

After C3 cleavage, a subsequent cascade of enzymatic reactions lead to the formation of the membrane-attack-complex, which is responsible for disrupting the target cell membrane by forming transmembrane pores.[3] Unlike the CP and the LP, which need certain triggers to become activated, the AP is always at a low level of activation via a process called "tick-over", a spontaneous conversion of C3 to a bioactive form C3(H_2O).[4,5] This conversion leads to a conformational change that allows for the binding of complement factor B (FB), similar to C3b and, through a series of subsequent steps, forms the initial C3 convertase C3(H_2O)Bb.[1,5] This convertase cleaves C3 molecules into C3a and C3b.[5,6] In plasma, AP amplification is controlled by complement factor H (FH), which inactivates the C3 convertase and catalyses complement factor I (FI) degradation of C3b.[7] Dysregulation of the AP is associated with a number of diseases,[8] a strong example being age-related macular degeneration (AMD).[9-12]

AMD is a progressive retinal disease that leads to vision loss in the elderly population.[13] It is a multifactorial disease caused by both genetic and environmental factors. Several lines of evidence support the involvement of the complement system in the pathology of AMD. Some of the highest genetic risk for AMD is conferred by single nucleotide polymorphisms (SNPs) in or near genes of the complement system.[14] Additionally, complement activation levels in plasma / serum are significantly higher in patients compared to controls,[9-12] and complement components have been described in the composition of retinal deposits called drusen, which are a hallmark of the disease.[15]

Currently, AMD therapies that aim to inhibit or lower complement activation are being developed,[16,17] but it has been suggested that one of these inhibitors, lampalizumab, is effective only in a subset of patients that carry a specific genotype.[18] Therefore, it is important to understand the genetic risk factors that influence complement activation in order to identify those individuals that would benefit the most from such treatments.

Several studies have evaluated the effect of SNPs on complement activity, and only moderate effects have been observed.[19-21] *In vitro* studies show that complement activity can increase six-fold when multiple SNPs in the complement system interact together.[20] Such combinations of SNPs in the complement system are called complotypes. Harris et al. defined the complotype as any inherited pattern of genetic variants in complement genes which alters risk for both inflammatory disorders and infectious diseases involving the complement system.[22] Until now, the best studied complotype *in vitro* is composed of three functional variants from the AP: *C3* (rs2230199; R102G), *CFB* (rs641153; R32Q) and *CFH* (rs800292; V62I). All three SNPs are individually associated with AMD.[23-25] Although the presence of all three SNPs led to markedly higher complement activity *in vitro*, the effect of the complotype has so far neither been investigated in human plasma samples, representative of the *in vivo* situation, nor been associated with any disease.

In a recent study, we have found another functional SNP in *CFB* (rs4151667) to be more strongly associated with complement activation than the individual SNPs in the most studied complotype (*C3* [rs2230199], *CFB* [rs641153], and *CFH* [rs800292]).[9] The aims of this study, therefore, are: 1) to expand the complotype with the *CFB* variant (rs4151667) we found to be highly associated with complement activity; 2) to evaluate the relation of the complotype with complement activation in human plasma samples, representative of the *in vivo* situation; and 3) to investigate the association between the complotype and AMD.

METHODS

Study Population

In this study, 3042 participants from the European Genetic Database (EUGENDA, www.eugenda.org), over the age of 50 years, were included. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO) and was approved by the local ethics committee of the University Hospitals in Cologne and Nijmegen. Written informed consent was obtained from all participants. AMD and control status were assigned by multimodal image grading that included stereo fundus photographs, fluorescein angiograms and spectral domain optical coherence tomograms. The grading was performed according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (TR, LE) as previously described.[26]

Age, sex, and body mass index (BMI) were obtained by standardized interviewer-assisted questionnaires.

Complement Measurements and Genetic Analysis

Complement component C3 and the activation fragment C3d were measured in serum samples as previously described,[9] and the C3d/C3 ratio was calculated as a measure of complement activation. The complement activation data were skewed and had several outliers at the high end of the value range. In order to reduce the risk of outlier effects distorting the data, five percent of the highest values from the entire dataset were excluded from our analysis. After the exclusion of the outliers, the remaining skewness of the C3d/C3 data was normalized by Log10 transformation.

Genomic DNA was extracted from peripheral blood samples using standard procedures. Four SNPs, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153), and *C3* (rs2230199) were genotyped using the KASPar SNP Genotyping System by LGC Genomics.

Statistical Analysis

All associations were calculated using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA). Associations with complement activation were analyzed using General Linear Models with C3d/C3 as the dependent variable. The models were corrected for age, sex, BMI, and disease status.

The associations between AMD phenotype and the individual SNPs or the complotype were evaluated using logistic regression. To determine if the SNPs were independently associated with the disease, all four SNPs were included in the logistic regression model at once.

To avoid being relevant only to our sample set (overfitting), the most informative complotype combination was determined by calculating the variable importance in a random forest analysis using the R package (RandomForest version 4.6-10). In the first analysis, C3d/C3 was included as the dependent variable for the regression type random forest test. In the second analysis, the disease status was defined as the classifier for a classification type of random forest. For both analyses, the number of predictors sampled for splitting at each node was set to two. All other options were left at default setting.

RESULTS

The study was performed in three consecutive steps. First, the individual associations of *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153), and *C3* (rs2230199) with AMD and with complement activation were verified. Next, we determined the most informative complotype for complement activation. Finally, we analyzed the association of the resulting complotype with the disease and with complement activation.

Individual Association of CFH (rs800292), CFB (rs4151667), CFB (rs641153) and C3 (rs2230199) with AMD and Complement Activation

In a previous study, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153), and *C3* (rs2230199) were tested for their association with AMD in 2655 individuals.[9] For the purpose of this study, 387 additional individuals were genotyped, amounting to a total of 3042 subjects (1615 AMD and 1427 controls). The mean age was 75 years for AMD and 70 years for controls. The sex distribution was: 41% males to 59% females. All four SNPs were significantly associated with AMD (Supplementary Table 1). SNPs *CFH* (rs800292; minor allele A), *CFB* (rs4151667; minor allele A) and *CFB* (rs641153; minor allele A) are protective, whereas the *C3* (rs2230199; minor allele C) infers increased risk of AMD.

To determine the association of these SNPs with complement activation levels, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153), and *C3* (rs2230199) were included in a single general linear model, corrected for age, sex, BMI, and disease status. The model revealed significant independent associations with complement activation levels for all four SNPs. Figure 1 illustrates *P* values, mean log-transformed complement activation levels, and genotype distribution for the four tested SNPs.

When we looked at the difference in mean complement activation level between the genotypes for each SNP, the high-risk *C3* (rs2230199) genotype (GG) showed higher complement activation levels than the heterozygous (CG) and ancestral (CC) genotype. The protective *CFH* (rs800292) genotype (AA) showed lower complement activation levels than the other genotypes (Figure 1). However, a statistically significant difference in mean complement levels was only observed between the heterozygous genotype (GA) and the major genotype (GG) (P = .002), presumably due to the limited number of individuals carrying the AA genotype. The protective *CFB* (rs641153) genotype (AA) and the heterozygous (GA) genotype displayed lower mean complement activation levels than the ancestral (GG) genotype. For *CFB* (rs4151667), the homozygous protective genotype for AMD (AA) could not be statistically compared to the homozygous ancestral genotype (TT), due to low number of individuals in this genotype group. The observed effects are driven by the difference in mean complement activation levels between the heterozygous (TA) genotype and the ancestral (TT) genotype (Figure 1).

The Most Informative SNP Combination in Determining Complement Activation or AMD Status

As all four SNPs were individually and independently associated with both complement activation and AMD status, the next step aimed to assess which combination of SNPs best predicted these associations. It was impossible to introduce genotype combinations of all 4 SNPs into the model because of the very low samples number of individuals in each of the resulting groups. For this reason, only combinations of 3 SNPs were considered.

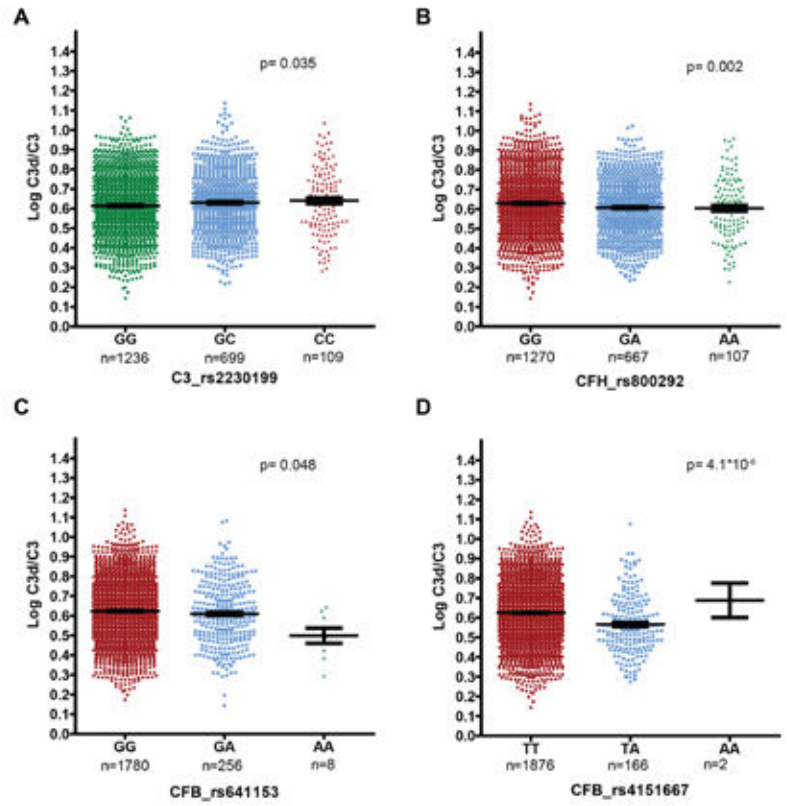
In order to determine which combination of SNPs could best explain complement activation and disease status, two random forest analyses were performed. In the first analysis, the ratio of C3d/C3 as a measure of complement activation was used as the dependent variable, whereas the second analysis was classified on AMD disease status. Variable importance analyses in both tests revealed that the SNP combination composed of *CFB* (rs4151667) – *CFB* (rs641153) – *CFH* (rs800292) was the strongest predictor for both complement activation and AMD status (Table 1). For the purpose of clarity, this combination of SNPs will be referred to as the novel complotype in the remainder of the manuscript.

Association of the Novel Complotype with AMD

Mathematically, there are 27 possible genotype combinations for a complotype composed of three SNPs. To ensure a meaningful interpretation of the statistical analyses, we included only those genotype combinations that were represented by at least ten individuals in both the patient and control groups. In our cohort, we observed seven genotype combinations that met these criteria. The distribution of all genotype combinations in our cohort is shown in Supplementary Table 2.

To determine the association of the novel complotype with AMD, a logistic regression anal-

FIGURE 1. Plasma complement activation levels (log-transformed C3d/C3 ratio) for C3, CFH, and CFB genotype groups



Each genotype per single nucleotide polymorphism (SNP) is plotted on the X axis in an individual dot plot. The homozygous genotypes conferring increased risk for age-related macular degeneration (AMD) are indicated in red; the homozygous genotypes that are protective for AMD are indicated in green. The number of individuals carrying a specific genotype is indicated below each genotype. The Y axis represents the log-transformed C3d/C3 ratio level as a measure of complement activation. The P values represent the overall significance for each SNP included in the model.

TABLE 1. Variable importance scores of C3, CFB and CFH genotypes and genotype combinations on complement activation levels and AMD status

Variables	%IncMSE	IncNode Purity	Mean Decrease Accuracy	Mean Decrease Gini
C3 (rs2230199)	5.33	0.06	6.59	1.85
CFB (rs4151667)	13.24	0.20	2.51	0.92
CFB (rs641153)	5.77	0.11	6.08	2.41
CFH (rs800292)	6.07	0.15	13.24	6.57
C3 (rs2230199) – *CFB* (rs4151667) – *CFB* (rs641153)	8.83	0.34	8.71	5.07
C3 (rs2230199) – *CFB* (rs4151667) – *CFH* (rs800292)	9.08	0.35	13.12	9.66
C3 (rs2230199) – *CFB* (rs641153) – *CFH* (rs800292)	10.88	0.33	13.84	11.85
CFB (rs4151667) – *CFB* (rs641153) – *CFH* (rs800292)	18.58	0.62	18.47	17.28
C3 (rs2230199) – *CFB* (rs4151667) – *CFB* (rs641153) – *CFH* (rs800292)	10.25	0.49	14.07	15.36

AMD, age-related macular degeneration.
Mean decrease accuracy and mean decrease Gini measure variable importance in predicting disease status.
%IncMSE and IncNode Purity are measures for variable importance in predicting complement activation.
For all variables, the highest values represent the best predictors.

TABLE 2. Association between the novel complotype and AMD

Genotype combination for the novel complotype	Controls	AMD	OR (95% CI)*	*P* value*
TT – GG – GG	607	916	1	$5.84*10^{-13}$
TA – GG – GA	47	23	0.30 (0.17-0.51)	$1.01*10^{-5}$
TA – GG – GG	55	65	0.74 (0.49-1.10)	.131
TT – GA – GA	74	47	0.36 (0.24-0.54)	$6.65*10^{-7}$
TT – GA – GG	112	106	0.57 (0.42-0.78)	$3.2*10^{-4}$
TT – GG – AA	59	48	0.56 (0.37-0.86)	.007
TT – GG – GA	406	370	0.58 (0.48-0.70)	$7.32*10^{-9}$

AMD, age-related macular degeneration; OR, odds ratio; CI, confidence interval.
Bonferroni corrected threshold for statistical significance is P < .008.
** Corrected for age and sex.*

ysis was performed. A strong overall association of the novel complotype with AMD ($P = 5.84*10^{-13}$) was observed. In our analysis of the genotype combinations within the novel complotype, the most frequent genotype combination found in controls (TT – GG – GG) was set as reference. The logistic regression analyses corrected for age and sex revealed that, in comparison to TT – GG – GG, the other six genotype combinations were protective for AMD (Table 2).

Association of the Novel Complotype with Complement Activation

Finally, to determine the association of the novel complotype with complement activation, a general linear model was built, corrected for age, sex, BMI, and disease status. This model showed that the novel complotype was highly associated with complement activation levels ($P = 8.31*10^{-9}$). When we compared the different genotype combinations with one another, the TT – GG – GG combination was associated with the highest mean complement activation levels (Figure 2). The difference in mean complement activation levels between all genotype combinations, tested in a post-hoc Bonferroni corrected manner, are presented in Supplementary Table 3. When comparing complement activation levels between AMD patients and controls, we only observed a significant difference for genotype combination TT – GG – GA (Figure 2).

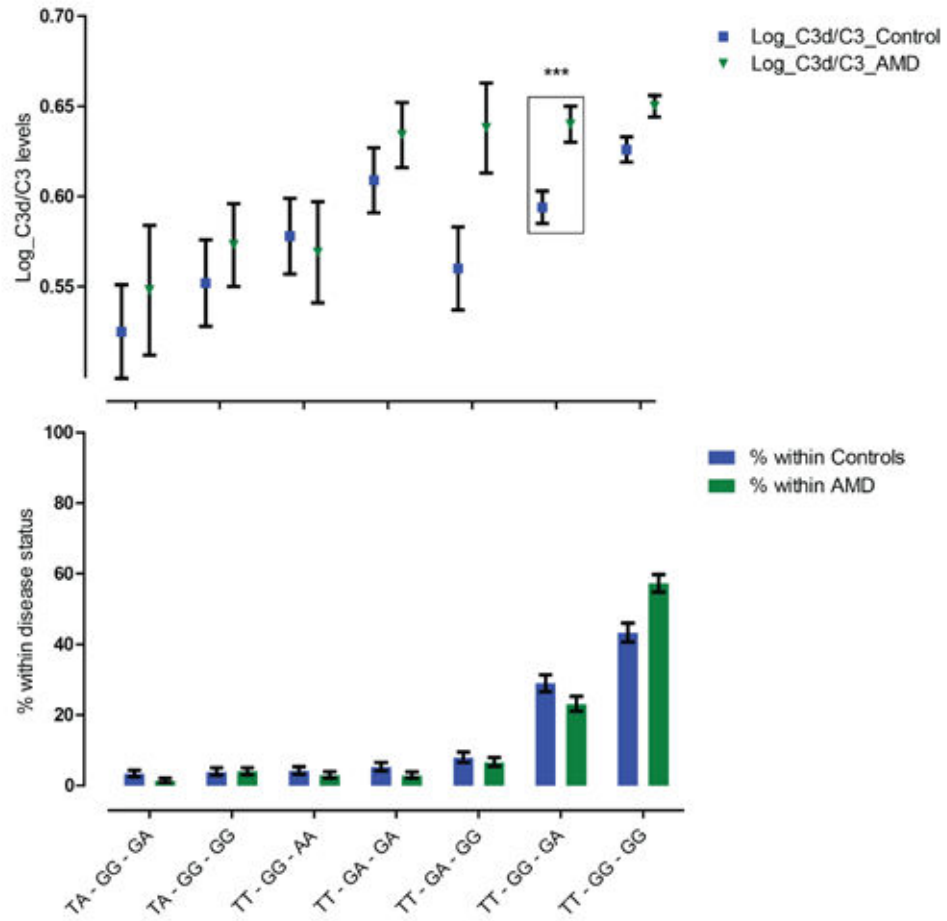
DISCUSSION

In a large case-control study, we show that carrying multiple AMD protective genotypes for *CFB* (rs4151667), *CFB* (rs641153), and *CFH* (rs800292) leads to lower levels of complement activation in plasma compared to the most frequent genotype combination of these SNPs in control individuals. This novel complotype was identified as the most predictive SNP combination for determining both complement activation levels and AMD status. This combination of SNPs, drawn from an *in vivo* setting, is different from what has previously been suggested on the basis of *in vitro* data.[20]

It is well established that SNPs in complement components *C3*, *CFB*, and *CFH* influence the risk for AMD.[24,25,27] In this study, we confirmed that four common functional SNPs, *CFH* (rs800292), *CFB* (rs4151667), *CFB* (rs641153), and *C3* (rs2230199), are associated with AMD. The minor alleles of the *CFH* and the *CFB* SNPs are protective,[23,24] whereas the minor allele of the *C3* SNP confers increased risk of AMD.[25] The well-known AMD SNP *CFH* (rs1061170; Y402H) was not included in this study because it was not associated with complement activation in our previous study.[9] This SNP was not described to alter AP convertase regulation, but rather it displays differential binding to C-reactive protein and malondialdehyde.[28,29]

Higher levels of systemic complement activation in patients compared to controls have been described in multiple studies.[9,10,12,30] As the proteins encoded by *CFH*, *CFB* and *C3* are key components of the AP of the complement system,[31] the contribution of these SNPs to disease

FIGURE 2. Mean C3d/C3 level and frequency of genotype combinations in AMD patients and controls



The blue and green bars represent the percentage of individuals carrying a specific genotype combination within their own disease status. The green triangles and blue squares represent mean C3d/C3 values for the corresponding genotype combination.
The only genotype combination showing a significant difference in complement levels between AMD patients and controls was observed for TT – GG – GA with a P value of $3*10^{-4}$ (after Bonferroni correction statistical significance is achieved at $P < .007$).
AMD, age-related macular degeneration.

susceptibility possibly comes from their impact on AP activation.

FH is a major regulator of the AP.[32] One of the ways in which it down-regulates complement activity is to bind C3b as a cofactor for its inactivation.[33] The A allele (p.62I) of the *CFH* (rs800292; V62I) SNP is a gain of function variant. *In vitro* experiments showed that the resulting protein binds more efficiently to C3b than the protein resulting from the G allele (p.V62) of this SNP, thus leading to more complement inhibition.[19] This is in line with our results, demonstrating that the *CFH* (rs800292) GG genotype was associated with decreased risk for AMD and lower levels of complement activation than the AA genotype.

FB binds hydrolyzed C3(H_2O) or C3b, which is then cleaved by complement factor D to form the AP C3 convertase that cleaves C3 to C3a and C3b, thus fueling the AP amplification loop.[22] The A (p.32Q) allele of rs641153 (p.R32Q) leads to a FB protein with decreased potential to form the C3 convertase and amplify complement activation.[34] The second *CFB* SNP (rs4151667; L9H) resides in the signal peptide, and it has been proposed that it could alter CFB secretion.[24] In this study, the A alleles of both *CFB* SNPs were found to be protective for AMD and to lead to lower complement activation levels, even in heterozygous state, than the major homozygous genotype. The homozygous protective genotypes for *CFB* (rs4151667) were too rare for any reliable conclusions to be drawn.

C3 plays a central role in the complement system.[35] The G (p.102G) allele of *C3* (rs2230199; R102G) decreases the efficiency of regulation of C3b by FH, thus leading to an increase in complement activation. These observations are in accordance with the results in the present study, where the GG genotype is associated with risk for AMD and displays higher levels of complement activation than the CC genotype (Figure 1A). Even though it plays such an important role, it was not part of the most predictive complotype in the present study.

Several *in vitro* studies have shown that having multiple SNPs in complement genes would lead to higher complement activation.[20,36] In the present study, the novel complotype composed of *CFB* (rs4151667) – *CFB* (rs641153) – *CFH* (rs800292) had a larger effect on complement activation than the initially studied complotype *C3* (rs2230199) – *CFB* (rs641153) – *CFH* (rs800292) (Table 1).[20] The higher predictive value of the newly described complotype with respect to AMD might be related to the fact that it is composed of protective SNPs only rather than of a combination of polymorphisms with opposing effects on AMD susceptibility. When comparing the strongest effect (odds ratio [OR] 0.3) of this new complotype on the risk of AMD with the odds ratios of the 38 individual loci described in the newest AMD GWAS, we notice that the effect size is close to both the *CFH* (OR 0.38) and the *ARMS2* (OR 2.81), albeit reverse, locus.[37] It is worth to mention that the OR of 0.3 for the complotype is seen when comparing TA – GG – GA to TT – GG – GG, which has only two alleles difference out of the six.

This study is the first to analyze this specific complotype combination for its association with AMD and complement activity. Although it would have been interesting to study the simultaneous presence of all four genotyped SNPs, cohorts even larger than ours are needed to avoid the problem of small genotype combination groups that cannot be reliably compared.

Intriguingly, the homozygous genotypes associated with the highest complement activation levels in all three SNPs (TT – GG – GG) in the novel complotype are found most frequently in both AMD patients and controls. This is in contrast to what was proposed in a theoretical model, where the extreme genotype combinations were expected to be at the lower end of the carrier frequency spectrum.[22] With fewer than ten individuals for patients or controls, the combination of all heterozygous genotypes was rare. The combination where all SNPs had the homozygous protective genotypes (AA – AA – AA) was not present in our cohort at all. In our study, therefore, the frequency distribution is skewed toward complement-raising genotypes. This could potentially be explained by the fact that our cohort has a mean age of 73 years and might therefore be enriched for alleles that promote survival. In this case, the alleles that give higher complement activation could offer better lifetime protection against infection. However, these same genetic variants would potentially induce low-grade inflammation, and its effect would only be observed later in life, as is the case for AMD, a disease that is prevalent in the elderly population. In support of this hypothesis, immune genes have been described to have the highest rate of positive selection.[38] Upon examination of the amino acid conservation of the SNPs in the present study, in humans three complement-raising variants are the reference amino acid, compared to only one in primates (Supplementary Table 4).

A significant difference in complement levels was observed between AMD patients and controls carrying the TT – GG – GA genotype combination. Although the highest mean difference was observed between the groups carrying the TT – GA – GG combination, this difference was not significant due to the high standard error.

The four most prevalent genotype combinations are all associated with high levels of complement activation in AMD patients with only minor differences between the groups. The three genotype combinations that are least prevalent are associated with lower complement activity. If we look at the specific genotype combinations, some interesting observations can be made. First of all, the TT – GG – GG genotype combination is associated with the highest complement activation levels and is more prevalent in AMD patients (57.3%) than in controls (43.3%). The TA – GG – GG genotype, which is only different with respect to 1 risk allele in *CFB* (rs4151667), is at the lower end of complement activation. The only other genotype combination with TA instead of TT for *CFB* (rs4151667) is also associated with lower complement activation. This suggest that this SNP might be the most important of the three SNPs in the novel complotype, and is the driving force behind the influence on complement activation. This is also evident in the results from the random forest analyses, where *CFB* (rs4151667) is the strongest predictor for complement activation compared to the other individual SNPs. Another interesting observation from Figure 1 is the difference in complement activation between genotype combinations TT – GG – AA and TT – GA – GA. Both combinations include four risk alleles and two protective alleles, but the difference in complement activation is striking, especially in the AMD group. Perhaps the presence of two protective alleles in one SNP, as in the TT – GG – AA genotype combination, has a stronger influence on complement

activity than the combination of two heterozygous SNPs (TT – GA – GA). Observations like ours may help to clarify this and warrant further research, preferentially in an even larger dataset.

One of the major strengths of this study is the use of the large EUGENDA dataset. To the best of our knowledge, this is one of the largest datasets of complement activation to date. For the evaluation of mean differences in complement activation at a population level, as we have done in this study, a single measurement of C3 and C3d in each individual is sufficient. However, if complement activation would be used on an individual basis, such as for the selection of patients for clinical trials, multiple measurements over time would be preferred to correct for individual variations in complement activation.

CONCLUSIONS

The current study has demonstrated that a novel complotype composed of *CFB* (rs4151667) in combination with *CFB* (rs641153) and *CFH* (rs800292) is strongly associated with complement activation and AMD status. These findings are relevant in the context of future complement-lowering treatments for AMD. In the era of personalized medicine, we are moving toward a more individualized approach to the treatment of diseases. To evaluate new treatment strategies, we need detailed information to determine how subgroups of patients with a higher treatment response potential should be defined. In this case, genotype-based patient stratification may identify those individuals that are genetically predisposed to having the highest complement levels, potentially making them the best candidates for complement-inhibiting therapies in AMD.

REFERENCES

1. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* 2015;6:262.
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010;11:785-797.
3. Peitsch MC, Tschopp J. Assembly of macromolecular pores by immune defense systems. *Curr Opin Cell Biol* 1991;3:710-716.
4. Lachmann PJ, Halbwachs L. The influence of C3b inactivator (KAF) concentration on the ability of serum to support complement activation. *Clin Exp Immunol* 1975;21:109-114.
5. Pangburn MK, Schreiber RD, Muller-Eberhard HJ. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med* 1981;154:856-867.
6. Isenman DE, Kells DI, Cooper NR, Muller-Eberhard HJ, Pangburn MK. Nucleophilic modification of human complement protein C3: correlation of conformational changes with acquisition of C3b-like functional properties. *Biochemistry* 1981;20:4458-4467.
7. Morgan BP, Meri S. Membrane proteins that protect against complement lysis. *Springer Semin Immunopathol* 1994;15:369-396.
8. Holers VM. The spectrum of complement alternative pathway-mediated diseases. *Immunol Rev* 2008;223:300-316.
9. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One* 2014;9:e93459.
10. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet* 2010;19:209-215.
11. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci* 2009;50:5818-5827.
12. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
13. de Jong PT. Age-related macular degeneration. *N Engl J Med* 2006;355:1474-1485.
14. Schramm EC, Clark SJ, Triebwasser MP, Raychaudhuri S, Seddon JM, Atkinson JP. Genetic variants in the complement system predisposing to age-related macular degeneration: a review. *Mol Immunol* 2014;61:118-125.
15. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* 2002;134:411-431.
16. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One* 2014;9:e112682.
17. Yehoshua Z, de Amorim Garcia Filho CA, Nunes RP, et al. Systemic complement inhibition with eculizumab for geographic atrophy in age-related macular degeneration: the COMPLETE study. *Ophthalmology* 2014;121:693-701.
18. Volz C, Pauly D. Antibody therapies and their challenges in the treatment of age-related macular degeneration. *Eur J Pharm Biopharm* 2015;95:158-172.
19. Tortajada A, Montes T, Martinez-Barricarte R, Morgan BP, Harris CL, de Cordoba SR. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. *Hum Mol Genet* 2009;18:3452-3461.
20. Heurich M, Martinez-Barricarte R, Francis NJ, et al. Common polymorphisms in C3, factor B, and factor H collaborate to determine systemic complement activity and disease risk. *Proc Natl Acad Sci U S A* 2011;108:8761-8766.
21. Pechtl IC, Kavanagh D, McIntosh N, Harris CL, Barlow PN. Disease-associated N-terminal complement factor H mutations perturb cofactor and decay-accelerating activities. *J Biol Chem* 2011;286:11082-11090.
22. Harris CL, Heurich M, Rodriguez de Cordoba S, Morgan BP. The complotype: dictating risk for inflammation and infection. *Trends Immunol* 2012;33:513-521.
23. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* 2005;102:7227-7232.

24. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.
25. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 2007;357:553-561.
26. Ristau T, Ersoy L, Lechanteur Y, et al. Allergy is a protective factor against age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:210-214.
27. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
28. Sjoberg AP, Trouw LA, Clark SJ, et al. The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. *J Biol Chem* 2007;282:10894-10900.
29. Weismann D, Hartvigsen K, Lauer N, et al. Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. *Nat New Biol* 2011;478:76-81.
30. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One* 2008;3:e2593.
31. Lachmann PJ. The amplification loop of the complement pathways. *Adv Immunol* 2009;104:115-149.
32. McHarg S, Clark SJ, Day AJ, Bishop PN. Age-related macular degeneration and the role of the complement system. *Mol Immunol* 2015;67:43-50.
33. Pangburn MK. Host recognition and target differentiation by factor H, a regulator of the alternative pathway of complement. *Immunopharmacology* 2000;49:149-157.
34. Montes T, Tortajada A, Morgan BP, Rodriguez de Cordoba S, Harris CL. Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. *Proc Natl Acad Sci U S A* 2009;106:4366-4371.
35. Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev* 2001;180:35-48.
36. Lay E, Nutland S, Smith JE, et al. Complotype affects the extent of down-regulation by Factor I of the C3b feedback cycle in vitro. *Clin Exp Immunol* 2015;181:314-322.
37. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet* 2016;48:134-143.
38. Nielsen R, Bustamante C, Clark AG, et al. A scan for positively selected genes in the genomes of humans and chimpanzees. *PLoS Biol* 2005;3:e170.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1. Association between AMD and SNP genotypes in the *CFH*, *CFB* and *C3* genes

SNP	cDNA change	AMD	Control	OR (95% CI)	*P* value
***CFH* V62I rs800292**	c.134G>A				
GG		1101	801		$7.4*10^{-11}$
GA		445	539	0.63 (0.435-0.913)	.015
AA*		62	77	0.564 (0.477-0.667)	$2.4*10^{-11}$
***CFB* L9H rs4151667**	c.26T>A				
TT*		1511	1291		.02
TA		99	127	0.828 (0.038-17.888)	.904
AA		1	1	0.654 (0.486-0.881)	.005
***CFB* R32 Q rs641153**	c.95G>A				
GG*		1436	1195		$3.0*10^{-4}$
GA		171	217	0.545 (0.138-2.152)	0.387
AA		4	7	0.623 (0.492-0.788)	$7.9*10^{-5}$
***C3* R102G rs2230199**	c.304G>C				
CC*		911	901		$2.4*10^{-6}$
CG		570	471	1.183 (1.003-1.396)	.046
GG		117	47	2.59 (1.775-3.777)	$7.8*10^{-7}$

Analyses were performed by logistic regression analysis.
*The genotypes marked with * are 4 the ancestral variants.*
Variables entered in the model: CFH rs800292, CFB rs4151667, CFB 5 rs641153, C3 rs2230199, age and sex.
Bonferroni corrected threshold for statistical significance is P < .004.

SUPPLEMENTARY TABLE 2. Genotype combination frequency for the novel complotype

CFB (rs4151667) - *CFB* (rs641153) - *CFH* (rs800292)	Control n (%)	AMD n (%)	Total
AA - GG - GG	1 (0.1)	1 (0.1)	2
TA - GA - AA	0 (0.0)	2 (0.1)	2
TT - AA - GA	1 (0.1)	1 (0.1)	2
TA - GA - GA	5 (0.4)	1 (0.1)	6
TT - AA - GG	6 (0.4)	3 (0.2)	9
TA - GG - AA	7 (0.5)	3 (0.2)	10
TA - GA - GG	10 (0.7)	5 (0.3)	15
TT - GA - AA	11 (0.8)	9 (0.6)	20
TA - GG - GA	47 (3.4)	23 (1.4)	70
TA - GG - GG	55 (3.9)	65 (4.1)	120
TT - GG - AA	59 (4.2)	48 (3.0)	107
TT - GA - GA	74 (5.3)	47 (2.9)	121
TT - GA - GG	112 (8.0)	106 (6.6)	218
TT - GG - GA	406 (29.0)	370 (23.1)	776
TT - GG - GG	607 (43.3)	916 (57.3)	1523
Total	1401 (100)	1600 (100)	3001

SUPPLEMENTARY TABLE 3. Differences in mean complement activation levels between genotype combinations

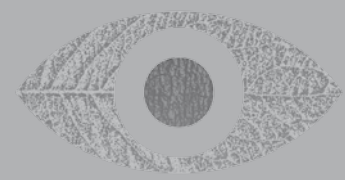
CFB (rs4151667) - *CFB* (rs641153) - *CFH* (rs800292)		Mean Difference (I-J)	Std. Error	*P* value
TA - GG - GA	TA - GG - GG	-0.024	0.026	1
	TT - GA - GA	-0.041	0.027	1
	TT - GA - GG	-0.085*	0.024	.011
	TT - GG - AA	-0.06	0.027	.524
	TT - GG - GA	-0.079*	0.022	.006
	TT - GG - GG	-0.100*	0.021	$6.1*10^{-5}$
TA - GG - GG	TA - GG - GA	0.024	0.026	1
	TT - GA - GA	-0.017	0.024	1
	TT - GA - GG	-0.061	0.021	.074
	TT - GG - AA	-0.036	0.023	1
	TT - GG - GA	-0.055*	0.018	.043
	TT - GG - GG	-0.076*	0.017	$1.9*10^{-4}$
TT - GA - GA	TA - GG - GA	0.041	0.027	1
	TA - GG - GG	0.017	0.024	1
	TT - GA - GG	-0.044	0.021	.843
	TT - GG - AA	-0.019	0.024	1
	TT - GG - GA	-0.038	0.018	.826
	TT - GG - GG	-0.059*	0.018	.019
TT - GA - GG	TA - GG - GA	0.085*	0.024	.011
	TA - GG - GG	0.061	0.021	.074
	TT - GA - GA	0.044	0.021	.843
	TT - GG - AA	0.025	0.021	1
	TT - GG - GA	0.006	0.014	1
	TT - GG - GG	-0.015	0.014	1
TT - GG - AA	TA - GG - GA	0.06	0.027	.524
	TA - GG - GG	0.036	0.023	1
	TT - GA - GA	0.019	0.024	1
	TT - GA - GG	-0.025	0.021	1
	TT - GG - GA	-0.019	0.018	1
	TT - GG - GG	-0.04	0.018	.464
TT - GG - GA	TA - GG - GA	0.079*	0.022	.006
	TA - GG - GG	0.055*	0.018	.043
	TT - GA - GA	0.038	0.018	.826
	TT - GA - GG	-0.006	0.014	1
	TT - GG - AA	0.019	0.018	1
	TT - GG - GG	-0.021	0.008	.215
TT - GG - GG	TA - GG - GA	0.100*	0.021	$6.1*10^{-5}$
	TA - GG - GG	0.076*	0.017	$1.9*10^{-4}$
	TT - GA - GA	0.059*	0.018	.0189
	TT - GA - GG	0.015	0.014	1
	TT - GG - AA	0.04	0.018	.464
	TT - GG - GA	0.021	0.008	.215

**The mean difference is significant at the .05 level. All P values were adjusted for multiple comparisons: Bonferroni.*
The general linear model was corrected for age, sex, BMI and disease status.

SUPPLEMENTARY TABLE 4. Amino acid conservation for *CFH* (rs800292, p.V62I) - *CFB* (rs4151667, p.L9H) - *CFB* (rs641153, p.R32Q) - *C3* (rs2230199, p.R102G)

Species	*CFH* p.V62I	*CFB* p.L9H	*CFB* p.R32Q	*C3* p.R102G
Human	V	L	R	R
Chimp	I	L	Q	R
Mouse	I	L	R	G
Dog	I	L	A	G
Cat		L	G	G
Cow		L	G	G

GENERAL DISCUSSION



CHAPTER 10

GENERAL DISCUSSION

1. PRIMARY FINDINGS OF THIS THESIS AND CLINICAL IMPLICATIONS

1.1 DEVELOPMENT OF AMD

AMD is a multifactorial disease and a broad range of genetic, environmental and sociodemographic risk factors are associated with disease development. Numerous studies have identified risk factors for AMD, and in this next section the most important findings are highlighted.

Risk factors for AMD development

Environmental / Demographic risk factors

The most consistently established risk factors for AMD are age and smoking.[1-8] A higher body mass index is also thought to be associated with AMD,[2,9-14] although some studies were unable to replicate this association.[1,15,16] For other potential risk factors that have been studied, the results are even less unambiguous. Significant associations with AMD have been reported for low physical activity,[17] sunlight exposure,[18,19] hypertension,[20-22] and female sex.[23,24]

Genetic risk factors

Several genes in the complement pathway have been associated with AMD. The strongest associations have been described for variants in the complement factor H (*CFH*) and the age-related maculopathy susceptibility 2 (*ARMS2*) genes. A more detailed discussion on the complement system and AMD is provided in section 3. Other associations with AMD have been found for genes involved in several AMD-related pathways. These include the high-density lipoprotein cholesterol pathway and the extracellular matrix pathway.[25,26] In recent years, large genome-wide association (GWAS) study have uncovered more than 30 susceptibility loci that harbor common and rare risk variants for AMD.[27]

GWAS studies have made a significant contribution to identify the genetic causes of AMD. However, since we know that gene-gene and/or gene-environment interactions may also be involved,[10,13,28-30] the need for other methods to identify AMD-associated loci remains. In **Chapter 4** we evaluate the interaction between glutathione S-transferase (GST) polymorphisms in a large AMD case control dataset. Even though no GST polymorphisms have ever surfaced in large GWAS studies, one study group reported an association between specific combinations of GST polymorphisms and AMD.[31] In our dataset, which was much larger than the datasets of the original study, we were unable to replicate these findings. With our study we want to highlight the importance of replication of new findings and the need for large datasets to identify gene-gene interactions in AMD.

Age at onset of neovascular AMD

The onset of neovascularization in AMD varies greatly, in some patients occurring as early as age 50, whereas others are well past the age of 90. Research that has focused on identifying risk factors for an earlier age at onset of AMD is limited. In **Chapter 2** we demonstrate that

major risk factors for the development of AMD, the *CFH* Y402H and the *ARMS2* A69S risk alleles and smoking, are also important players in the age at onset of neovascular AMD. Accumulation of these factors may accelerate neovascularization substantially by one or two decades. This finding has a high clinical value, as it will aid the timely identification of high-risk individuals, who might benefit from more frequent ophthalmic examination to screen for the first signs of neovascular disease. In addition, it will enable the initiation of possible countermeasures at an early stage, where they may be more effective. Such measures do not only consist of cessation of smoking, but a healthy diet can also reduce genetic risks. Data from the Rotterdam study showed that individuals with a high genetic risk of AMD (defined as risk alleles in *CFH* and *ARMS2*) can reduce this genetic risk of AMD by consuming a diet rich in zinc, ω-3 fatty acids, β-carotene and lutein/zeaxanthin.[32,33] Regular consumption of fish also decreased the risk of late AMD in individuals with a high genetic risk.[33,34] These reports underline the need for general health education on proper food patterns and special attention, meaning an early identification, to high risk individuals.

Familial AMD

AMD is known to cluster in families and the first studies to reveal a role for genetics in AMD were familial and twin based studies.[35-38] Several studies have also suggested a different distribution of common environmental, genetic and/or phenotypic characteristics in familial versus sporadic AMD. We describe the results from our cohort in **Chapter 5**. The frequencies of common risk alleles in *CFH* and *C3* are higher in familial cases and controls compared to sporadic cases and controls. In line with this, the frequencies of protective alleles in *CFH* and *CFB* are lower (**Chapter 5**). This shows that in general the genetic burden in AMD families is higher and points toward an important role of the complement system in familial AMD. However, this also indicates that there must be other factors in these families that determine why one individual actually develops AMD and his/her sibling with the identical risk factors in complement genes does not. For the majority of families these factors are not different from the common genetic and environmental risk factors for AMD, and the prevalence of AMD can be explained by clustering of risk factors in these families. However, for a subset of families this observation does not hold, and unexplained variables are responsible for the high frequency of AMD (**Chapter 6**). Already in 1998 Klaver and colleagues concluded that clustering of common genetic risk factors in affected families does not explain the number of affected family members in large AMD families.[39] We have now shown that addition of known common environmental factors is still not enough to explain the familial clustering of AMD. Several rare variants with a high impact on disease risk have been identified in genes involved in the complement pathway,[40-48] and it is thought that the clustering of AMD in families may in part be explained by these rare variants. Screening for known rare variants in our AMD families did not yield any results, and these families are thus of particular interest for additional genetic testing, such as whole exome sequencing to search for rare variants that have not yet been identified. Even though the occurrence of some rare variants may

be limited to specific populations, families or geographic regions, their identification can be very important for the families that carry these variants. This means that family members without clinical signs of AMD can be tested for carriership of the variant that is found in their family, and this will provide these individuals with a better insight in their disease risk. Carriers of the rare variants can be counseled and provided with the tools to decrease their risk of developing AMD.

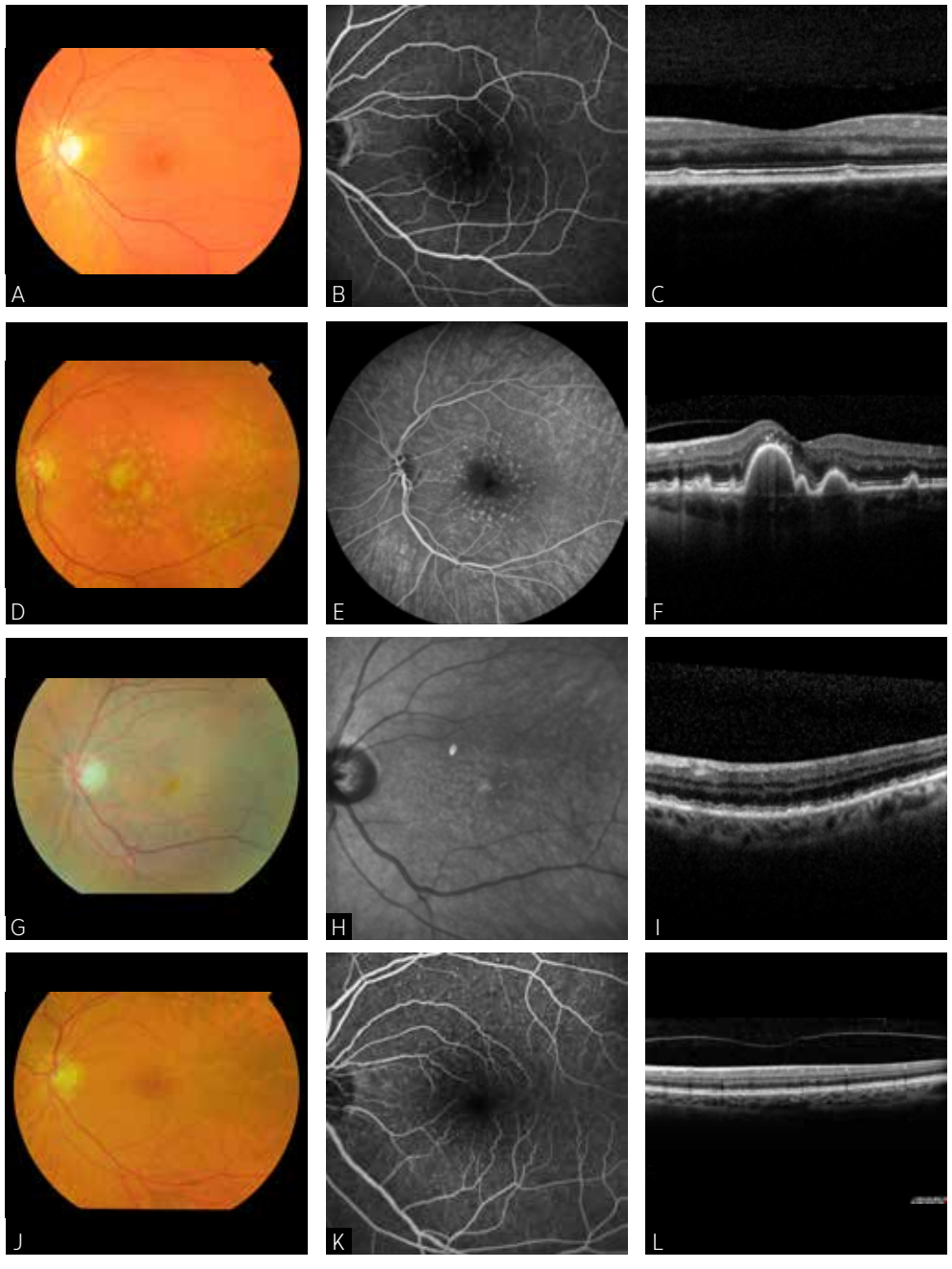
Phenotypic variations

With upcoming new treatment modalities for AMD, mostly aimed at slowing or preventing the progression of dry AMD, more and more interest is directed toward phenotyping of AMD patients. A detailed phenotyping can aid in the identification of certain subgroups of patients within the AMD spectrum who share clinical characteristics that may distinguish them from the classical AMD phenotype. An example of this is the cuticular drusen phenotype, characterized by innumerable, small , subretinal drusen with a typical "stars-in-the-sky" appearance on fluorescein angiography.[49] It is thought that this phenotype accounts for approximately 10% of the AMD spectrum, and it has been shown that although there is a clear overlap in risk factors for cuticular drusen and AMD, there are also differences.[45,50] Another example are reticular (pseudo-)drusen, which are associated with progression toward geographic atrophy (GA).[51,52] Figure 1 gives an overview of different drusen phenotypes that can be observed in AMD. The ever expanding spectrum of diagnostic tools may aid in the identification of new subgroups. Automatic grading systems, like the one described in **Chapter 7**, are able to objectively categorize and quantify clinical characteristics such as drusen on color fundus images. This will allow us to study different patterns of drusen distribution, for example inside or outside the central macular grid, and determine whether correlate to known risk factors (genotype-phenotype correlations) or aspects related to AMD progression. In the future, the automatic quantification and evaluation of phenotypes combining information from multimodal imaging techniques will further contribute to disease classification.

Neovascular versus geographic AMD

Even though GA and CNV are considered to be two distinct phenotypes, they share a similar risk profile with respect to environmental, clinical and genetic factors.[1,13,53-57] This suggests that both disease entities share a common pathophysiological pathway, and this seems logical since both stages are preceded by early and intermediate AMD stages, characterized by drusen and RPE alterations. In addition, both GA and CNV can manifest in the same patient and even within the same eye, and this so-called mixed phenotype shares the same genetic, epidemiological and clinical features as GA and CNV separately.[58] On the other hand, there are also some differences. For example, the *ARMS2/HTRA1* locus is associated with both types of advanced AMD, but the risk for CNV is much higher compared to that for GA,[59,60] and recently other genetic risk variants in the loci *CETP*, *MMP9* and *SYN3/TIMP3* were also found to be significantly different between disease subtypes.[27] It has also been suggested that

FIGURE 1. Overview of different drusen phenotypes in age-related macular degeneration



A-C, Hard drusen are visible as small yellowish points on FP (A). FA shows corresponding hyperfluorescent spots (B). Small depositions under the RPE are identified on OCT (C). D-F, Soft drusen are typically larger than hard drusen and their borders are less well-defined. FP, FA and OCT images of soft drusen are seen on images D, E, and F respectively. G-I, Reticular drusen are visible on FP (G) as grayish deposits in a network-like pattern. They are better seen on infra red images (H) and OCT imaging shows an irregular pattern of elevations internal to the RPE (I). J-L, The cuticular drusen phenotype is characterized by numerous small, hard drusen (J). They are even better visible on FA, where the typical "stars-in-the-sky" appearance is shown (K). On OCT, multiple hard drusen are visible (L).
FP, color fundus photography; FA, fluorescein angiography; RPE, retinal pigment epithelium; OCT, optical coherence tomography

some environmental risk factors such as cigarette smoking and hypertension confer different risks for GA and CNV.[3,61,62] In **Chapter 9** we also show that levels of complement activation differ between GA and both active and inactive CNV. Based on these findings we propose that even though there is a common starting point for GA and CNV, subtle influences of genetic and non-genetic factors determine the development toward either endpoint. In this view, GA may be regarded as the natural endpoint for AMD, except when something acute goes wrong and CNV develops. It is very important to separate these groups when performing analyses for AMD, especially for AMD risk prediction.[63]

1.2 PROGRESSION OF AMD

Several factors that are involved in the development of AMD, also play a role in AMD progression. For example, genetic risk variants in the major complement genes (*CFH*, complement components 2 and 3 [*C2* and *C3*] and complement factor B [*CFB*]) and ARMS2 are independently associated with progression.[64-66] Of the non-genetic factors, age, smoking, and BMI have consistently been linked to AMD progression.[65-67]

Second eye progression

Patients who have developed end-stage AMD in one eye are at high risk of progressing toward end-stage AMD in the second eye. Cumulative incidence rates have been reported to be 10% to 14%, 28% to 31%, and 36% to 37% after 1, 3, and 4 years of follow-up, respectively.[68,69] In **Chapter 3** we show that both genetic and environmental risk factors determine the risk of second eye progression. We were the first to report that the most common AMD risk alleles in *CFH*, *C3*, and *ARMS2* do not seem to play a role. These findings were later corroborated by the Comparison of AMD Treatments Trials (CATT) Research Group, also supporting the notion that the range of risk is restricted because most of these patients are high risk individuals since they had already progressed toward end-stage AMD in one eye.[70] We also describe other risk alleles in complement factor I (*CFI*) and lipoprotein lipase (*LPL*) that influence second eye progression, suggesting that in this stage other genetic risk profiles are important for a faster progression toward second eye involvement. In addition, whereas

genetic influences seem to be different from those that cause a risk for AMD development and progression, it is noteworthy that, in our study, the common environmental risk factors, smoking and BMI, are also of importance in this stage of disease progression. In the CATT study smoking and age were not associated with second eye progression, but these results may be limited by the fact that they only had two years of follow up. They did show an increased risk for females for second eye progression, in line with our findings. Where the role of sex is controversial in AMD development, these results may indicate that sex differences may exert an effect on progression.
In the eye clinics, the patients at risk for second eye progression are an important subgroup. They are the ones who will ask the ophthalmologist how long they will be able to see well with their 'good' eye and what they can do to prevent themselves from becoming blind in the near future. Of course, these patients need regular eye examinations and they should receive an amsler chart for everyday self-control. In addition, it is the job of the ophthalmologist to inform them about the risks of smoking and overweight, and they should be stimulated to persue a healthy lifestile. Next to this, we know from the AREDS trials, that these patients will benefit from the use of nutritional supplements. However, since these supplements are not covered by the health insurance companies in the Netherlands, the effects of a healthy diet rich in lutein and zeaxanthin should also be emphasized. In the future, this patient group may benefit from preventive treatment in the form of anti-VEGF eye drops. Even though recent clinical trials did not show a beneficial effect of anti-VEGF eye drops in the setting of CNV,[71-73] it would be worthwhile to find out if they can prevent or delay the onset of CNV formation.

1.3 THE COMPLEMENT SYSTEM AND ITS ROLE IN AMD DEVELOPMENT AND PROGRESSION

Already in the early nineties, the Dutch ophthalmologist van der Schaft identified components of the complement system in drusen, and this led to a series of other publications that supported these findings and formed the basis for the hypothesis that inflammation and, more specifically, the complement system play an important role in AMD pathogenesis.[74-76] Genetic variants in the *CFH* gene are strongly associated with AMD risk, and this discovery in 2005 formed the second line of evidence that implicated chronic local inflammation and activation of the complement system in AMD.[77-79] In the following years other single nucleotide polymorphisms (SNPs) in genes involved in the complement system have been associated with AMD.[80,81] The second major AMD susceptibility locus is located on chromosome 10q26 and contains two genes in high linkage disequilibrium: *ARMS2* and Htra serine peptidase 1 (*HTRA1*).[82-85] Even though there is no doubt about the association with AMD, there is no agreement on the causal variant at this locus. The underlying pathophysiological mechanisms remain unclear as well.[82,86-89] Several recent publications have linked *ARMS2/HTRA1* to inflammation, complement components and complement activity, suggesting a direct or indirect influence on the complement pathway.[90-93] The third line of evidence that the

complement system is strongly associated with AMD came from the finding that the mean level of complement activation in plasma or serum is higher in patients with AMD compared to controls.[16,91,94-96] Several of the SNPs in complement genes that are associated with AMD exert a direct effect on systemic complement activation.[91,94,95,97]
In **Chapter 8** of this thesis we evaluate the relationship between complement activation and different AMD stages in detail. We demonstrate that complement activation is highest in patients with intermediate AMD and geographic atrophy (GA) and lowest in control individuals and patients with inactive choroidal neovascularization (CNV), suggesting that complement activation in AMD is a dynamic process. These differences seem most prominent in cases with a high genetic predisposition based on four SNPs in *CFH*, *CFB* and *C3*. In **Chapter 9** we combine risk alleles in *CFB* and *CFH* to form a novel combination of complement genotypes (also referred to as a complotype), that is strongly associated with both AMD disease status as well as complement activation. Different genotype combinations of this complotype lead to differences in complement activation in both control individuals and patients.
In this era where we are moving more and more toward a personalized therapeutic approach it is very important to identify subgroups of patients who share a common pathophysiological mechanism of disease and who might benefit most from therapeutic strategies that are currently being developed. Several lines of research should be further explored to come toward this goal. First of all, genetics can play a major role in this and although we already know a great deal through GWAS studies, the next step should be to focus in more detail on clusters of genotypes or complotypes, because risk alleles that are associated with AMD seem to enhance each other when they are present in different combinations. Secondly, by evaluating several components (genetic and/or biochemical) of one pathway at the same time, we might be able to get a more general idea of the functionality of the pathway and gain insight in the disease from a broader perspective. Thirdly, the finding that complement activation differs among the AMD stages underlines the need for detailed phenotyping. This may aid in the identification of subtypes of AMD, and raises the question at what stage of the disease a new treatment should be started in order to be most successful.
Also in the design of future clinical trials possible subgroups of patients should be identified and evaluated separately. For example, in the current trial on lampalizumab, an anti factor D antibody, it would be very interesting to see if patients with a high risk genetic profile – based on the number of risk alleles in complement genes – show a better response to the treatment compared to patients with a low risk genetic profile.

2. TOWARD PERSONALIZED MEDICINE IN AMD

2.1 RISK FACTORS: FROM POPULATION TO THE INDIVIDUAL PATIENT

Risk factors are identified in large datasets and odds ratios or disease risks are determined based on the average distribution of risk factors among patients and controls. How can we

translate these population-based risks toward disease prediction for the individual patient? Several direct-to-consumer genome tests have been made publically available and allow consumers to determine their personal disease risk. For AMD, these tests are based on a small number of SNPs associated with AMD, and a lifetime risk score is calculated based on these SNPs and a lifetime population risk. A recent report from Buitendijk et al. showed that while these tests were accurate with respect to genotyping, they were unreliable when it came to disease prediction and yielded contradictory results for the same individual most likely because of a limited set of genetic markers and differences in methodology and population used as reference.[98]

Different problems arise when it comes to accurate risk prediction. First of all, it is very important to determine what you want to predict and for whom. As becomes clear from **Chapters 2-4** there are different risk factors and/or different effect sizes depending on whether you want to predict disease development or (second eye) progression. In addition, in the case of familial clustering of AMD we are not always able to rely on the common risk factors for disease prediction, and a much more individual approach is needed in those families where there is a substantial difference between the expected and actual number of affected family members as described in **Chapter 6**. Rare genetic variants are thought to explain these differences, but so far rare variants have been identified only in a minority of families with AMD. Therefore, in these families, exome sequencing or whole genome sequencing should be the next step.

Another problem of accurate risk prediction lies in the complex interplay of genetic and non-genetic risk factors. Each individual carries a different subset of risk factors and protective variants and together they determine the time of disease onset and the course of the disease. Due to the existence of interaction between factors, many of which are still unknown to us, the same risk factor can have a very different effect in one individual compared to another.

2.2 PREDICTION MODELS FOR AMD

Several study groups have developed a prediction model or risk algorithm for AMD, which are summarized in Table 1. When we take a closer look into these models, several observations can be made. First of all, there is not one model that includes exactly the same variables as any other model. This is very understandable since most study groups use predefined datasets and can only include variables that are actually measured in their datasets, but this makes direct comparison of model performance already very difficult. Some of the infrequently used variables seem very promising and warrant further research. Reynolds et al include several complement components in their model and show that the area under the curve (AUC) increases significantly.[16] The only other model that evaluates complement components is that from Sharma et al, but they only included factor H, and their dataset had a very small sample size.[99] Evaluation of complement components in study subjects

is a rather costly analysis and requires a strict protocol of sample handling, which may be the main reason why this has not been explored by other study groups. Another aspect is the inclusion of rare variants in the risk algorithm as was done by Seddon et al.[64] Since we know that rare variants can explain part of the missing heritability in AMD, it is important to incorporate known rare variants in our disease models. However, this also comes with some limitations, since the occurrence of rare variants may be limited to certain populations or geographic areas.

The second aspect that differs among prediction models is the study design. Many models are based on case-control datasets, which leads to an overestimation of disease risk and hampers the translation to the population as a whole because the extreme ends of the disease spectrum – controls and advanced AMD – are over-represented compared to the patients with early and intermediate disease stages. In addition, most of the models built on prospective data are derived from the Age-Related Eye Disease Study (AREDS), a randomized controlled trial on the use of antioxidants and zinc supplementation, and one may question the generalizability of these results. The same is true for the model from the Complications of Age-related Macular Degeneration Prevention Trial (CAPT) Research Group, where all the so-called non-cases had large drusen in at least both eyes and no 'true' AMD-free controls were included. The model from the Three Continent AMD Consortium (3CC), that was build on the data from the Rotterdam Study and validated in the Beaver Dam and Blue Mountain Eye Study Groups, is the only other model that uses prospective data. They are faced with all the advantages of a prospective dataset, but the consequence is the relatively low number of incident advanced cases, which limits variable inclusion and subdivision in AMD subtypes. Thirdly, the start and endpoints used in the different prediction models are not the same. Most models predict end-stage disease, i.e. GA or CNV. Given the notion that there are some differences in risk factors for GA and CNV as described in section 1.1, this will lead to a less accurate prediction. Several studies have therefore developed separate models for GA and CNV, sometimes even including different risk factors in each model. It is not only important to take the endpoint of the model into account, but also the baseline AMD stage is very important. In many studies, the non-cases are not pure controls (i.e. free of any AMD), but a mixed group of controls and early AMD. However, in most of the models based on the AREDS data, the non-cases include patients with unilateral advanced AMD at baseline, who are at risk of developing end-stage AMD in the other eye. As we know from the literature and **Chapter 3** of this thesis, the genetic risk factors for second eye progression are different from the factors that determine disease onset or progression, and this will also influence the model performance.

These examples underline the previous statement that it is extremely important to determine what you want to predict and for whom you want to predict. If the goal is to predict lifetime risk of AMD, than a model is needed based on data with a long follow-up. However, if a model is to be used for the identification of eligible patients for a randomized controlled trial evaluating a possible treatment for AMD, than a model is needed that can identify

TABLE 1. Overview of published prediction models for AMD

					Prediction Model Variables						
Author	Year	Design	NonCases / Cases	Follow up	Demographic / Environmental	Genetic	Ocular	AUC	Sens	Spec	Validation
Gold[100]	2006	Case-Control	275 / 548 (GA / CNV only)	No	-	*CFH, C2/CFB*	-	-	0.77	0.58	Yes
Hughes[101]	2007	Case-Control	266 / 401 (CNV only)	No	Smoking	*CFH and ARMS2 haplotypes*	-	-	-	-	No
Jakobsdottir[102]	2008	Case-Control / family cohort	168 / 798 (all AMD stages)	No	Age, sex, smoking	*CFH, ARMS2, C2/CFB*	-	-	0.70	0.74	No
Jakobsdottir[103]	2009	Case-Control	142 / 640 (all AMD stages)	No	-	*CFH, ARMS2, C2/CFB*	-	0.79	-	-	No
Reynolds[16]	2009	Case-Control	60 / 120 (GA / CNV only)	No	Age, sex, smoking, BMI, complement fragments	*CFH, ARMS2, C3, C2/CFB, CFI*	-	0.94	-	-	No
Seddon[65]	2009	RCT (AREDS)	1167* / 279 (GA / CNV only)	6.3yrs	Age, sex, smoking, BMI, education, antioxidants/zinc	*CFH, ARMS2, C3, C2/CFB*	Baseline grade	0.82	0.83	0.68	No
Gibson[104]	2010	Case-Control	470 / 470 (all AMD stages)	No	Age, sex, smoking	*CFH, ARMS2, C3, SERPING1*	-	0.83	0.76	0.76	No
McKay[105]	2010	Case-Control	436 / 437 (GA / CNV only)	No	Age, smoking	*CFH haplotypes, ARMS2, C3, C2/CFB*	-	0.86	-	-	No
Zanke[106]	2010	Risks from literature	-	No	Smoking	*CFH haplotypes, ARMS2, C3, mtA4917G*	-	-	-	-	No
Chen[10]	2011	Case-Control / AREDS	509 / 1335 (GA / CNV only)	No	Age, sex, smoking, BMI	*CFH, ARMS2, C3, C2/CFB*	-	0.82	0.76	0.75	No
Hageman[107]	2011	Case-Control	467 / 482 (CNV only)	No	Age, sex	*CFH, ARMS2, C3, C2/CFB, CFHR4, CFHR5, F13B*	-	0.82	0.82	0.63	Yes
Klein[108]	2011	RCT (AREDS)	2034* / 688 (GA / CNV only)	9.3yrs	Age, smoking, family history	*CFH, ARMS2*	Baseline grade, drusen size	0.87†	-	-	Yes
Seddon[66]	2011	RCT (AREDS)	2118* / 819 (GA / CNV only)	9.2yrs	Age, sex, smoking, BMI, education, antioxidants	*CHF, ARMS2, C3, C2/CFB*	Baseline grade, drusen size	0.90† 0.92‡	-	-	Yes (internal)
Spencer[109]	2011	Case-Control	216 / 349 (all AMD stages)	No	Age, smoking	*CFH, ARMS2, C3, C2/CFB*	-	0.84	0.85	0.65	Yes
		(Nested) case-Control	148 /85 (all AMD stages)	No	Age, smoking	*CFH, ARMS2, C3, C2/CFB*	-	-	0.80	0.71	Yes
Ying[110]	2011	RCT (CAPT)	878§ / 64 (endpoint GA#)	5yrs	Age, smoking, hypertension	-	Baseline grade, night vision score	0.76	-	-	No
Grassmann[111]	2012	Case-Control	796 / 986 (GA / CNV only)	No	-	*CFH, ARMS2, C3, C2/CFB, APOE, LIPC, PLA2G12A, TIMP3*	-	0.82	-	-	Yes (internal)
McCarthy[112]	2012	RCT (AREDS)	1897* / 114 (CNV only)	3yrs	Age, sex, smoking, antioxidants/zinc	*CFH, ARMS2*	Baseline grade, fellow eye	0.88	-	-	Yes (internal)
			1897* / 59 (GA only)	3yrs	Age, sex smoking, antioxidants/zinc	*CFH*	Baseline grade	0.89	-	-	Yes (internal)
Yu[113]	2012	RCT (AREDS)	1982 / 578 (GA / CNV only)	10.3yrs	Age, sex, smoking, BMI, education, antioxidants	*CFH, ARMS2, C3, C2/CFB, CFI, APOE, LIPC, TIMP3, CETP, ABCA1, COL8A1*	Fellow eye status	0.88† 0.90‡	-	-	No
Buitendijk[114]	2013	Cohort Study (Rotterdam)	4171 / 121 (GA / CNV only)	10.7yrs	Age, sex, smoking, BMI	*CFH, ARMS2, C3, C2/CFB, CFI, LIPC, TIMP3, CETP, ABCA1, COL8A1, TGBR1, SKIV2L, IER3/DDR1, ADAMTS9, LPL, SLC16A8, VEGFA, MYRIP, RAD51B, TNFRSF10A, B3GALTL*	Baseline grade	0.88	-	-	Yes

Perlee[63]	2013	RCT (AREDS)	1433* / 603 (CNV only)	10yrs	Age, sex, smoking, BMI, education, antioxidants/zinc	*CFH, ARMS2, C3, C2/CFB, CFHR4, CFHR5, F13B*	Baseline grade	0.96	-	-	Yes (internal)
			1433* / 379 (GA only)	10yrs	Age, sex, smoking, BMI, education, antioxidants/zinc	*CFH, ARMS2, C3, C2/CFB, CFHR4, CFHR5, F13B*	Baseline grade	0.96	-	-	Yes (internal)
Seddon[67]	2013	RCT (AREDS)	2105* / 809 (GA / CNV only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB*	Baseline grade	0.86† 0.88‡	-	-	Yes
			2105* / 454 (CNV only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB*	Baseline grade	0.83† 0.88‡	-	-	Yes
			2105* / 355 (GA only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB*	Baseline grade	0.86† 0.88‡	-	-	Yes
Sharma[99]	2013	Case-Control	33 / 73 (AMD stage NR)	No	Stress, comorbidity, serum markers (CFH, CCL2, SOD1)	*CCL2*	-	0.91	0.97	0.88	No
Chiu[115]	2014	RCT (AREDS)	6992* / 1185 eyes (GA / CNV only)	6.3yrs	Age, sex, smoking, education, race	-	Drusen size and type, pigment abnormality	0.88	0.88	0.74	Yes
Ristau[116]	2014	Case-Control	1014 / 445 (CNV only)	No	Age, education, sunlight exposure, allergy, fish consumption, physical exercise, alcohol	*CFH, ARMS2*	-	0.92	-	-	Yes (internal)
Seddon[64]	2015	RCT (AREDS)	2117* / 834 (GA / CNV only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB, COL8A1, RAD51B*	Baseline grade	0.91‡	-	-	Yes (internal)
			2117* / NR (CNV only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB, COL8A1, RAD51B*	Baseline grade	0.90‡	-	-	Yes (internal)
			2117* / NR (GA only)	8.8yrs	Age, sex, smoking, BMI, education	*CFH, ARMS2, C3, C2/CFB, COL8A1, RAD51B*	Baseline grade	0.92‡	-	-	Yes (internal)

AMD, age-related macular degeneration; AUC, area under the curve; GA, geographic atrophy; CNV, choroidal neovascularization; BMI, body mass index; RCT, randomized controlled trial; NR, not reported
** NonCases include patients with GA / CNV in one eye at baseline*
† Results based on 5 yrs
‡ Results based on 10 yrs
§ All of the NonCases had large drusen in both eyes
 # CAPT endpoint GA: development of a total of > 1 disc area of new, additional atrophy when all areas of GA (> 250 m in diameter) within 3000 µm of the foveal center were combined

patients who are at high risk of progressing toward end-stage disease in a relatively short time-period. In this situation, a model like that from McCarthy et al could be of use since it predicts AMD progression within 3 years. In addition, a model can be used to identify subgroups of patients, such as the families that we describe in **Chapter 6** where the clustering of AMD within the families could not be explained by the common genetic and environmental risk factors.

In conclusion, we are still a long way from the optimal predicton model to allow for individual risk prediction. Models can serve several purposes; they can be used for the classification of study populations into high and low risk groups, which can be useful for further studies, either on treatment or on further unraveling disease mechanisms. For now, the models are not refined enough for individual disease prediction and application in the general population. In addition, there are currently no available treatment options for the early AMD stages. Even if we could provide people with an individual risk profile, we currently cannot offer them other interventions than the preventive measures that are already known to most individuals: a balanced diet, the use of nutritional supplements, regular physical activity and to stop smoking.

An accurate prediction model would have to include interaction terms, haplotypes or complotypes, rare genetic variants, epigenetic effects and biomarkers (such as complement components), all of which are not - or very poorly - represented in the models that are currently available. Exclusion of these factors, for practical, statistical or financial reasons, will hamper adequate risk prediction. Another factor that should definitely be included in a predictive model for AMD is family history. Since still 40% of the heritability of AMD is unexplained, family history may serve as a marker for unknown genetic variants and may therefore aid in a more accurate risk prediction.

2.3 WHAT IS NEEDED TO MOVE FORWARD TOWARD PERSONALIZED MEDICINE IN AMD?

In order to provide individuals with a personal disease risk or a personal treatment strategy, we need to be able to divide individuals in subgroups. We need predictive tests to identify those individuals who are at high risk to develop end-stage AMD, predictive tests to identify individuals at high risk for progression of AMD, and predictive tests to estimate treatment response for the individual patient. To cover all these facets of AMD, we need extensive knowledge on risk factors for development, progression, and treatment. But it is not enough to focus on genetics and environmental factors. We have to move forward and explore new upcoming fields of proteomics and metabolomics and focus more on gene-gene and gene-environment interactions. Large datasets are needed to evaluate these areas and we highlight the importance of collaborations between different studygroups. Only then, we can obtain enough power to stratify study subjects into subgroups. These subgroups are of the most importance when it comes to selecting appropriate treatment strategies for AMD. Not every patient will respond in the same way to specific treatments, this is inherent to the multifactorial etiology of the disease. Before more progression can be made in the field of AMD treatment, we have to learn how to select patients that share a more common etiology in order to create more homogeneous subgroups based on disease pathology. For example, patients with a more active complement system may be more sensitive to treatment with complement inhibitors. On the other hand, patients with a lower activity of the complement system may be more susceptible for treatments that target other pathways involved in AMD. If we don't know how to differentiate these subgroups, than we may face disappointing results from current clinical trials focusing on new treatment modalities. There is still a lot to discover about the role of different biomarkers in disease development and progression, and more knowledge is essential for classification and selection of possible treatment markers en treatment strategies.

3. FUTURE PERSPECTIVES

3.1 FINDING THE UNEXPLAINED VARIANCE IN AMD

The heritability of AMD is estimated at about 47%.[117] A recent study of over 12 million genetic variants analyzed in 16,144 patients and 17,832 controls, identified 52 common and rare variants on 34 loci, explaining 27.2% of disease variability and thus almost 60% of the total heritability.[27] This still leaves 40% of the heritability of AMD unexplained. In part, this may be explained by the discovery of additional variants not studied so far. It seems unlikely that many other common variants with a significant impact on disease risk still play a role, as they would have been picked up in one of the many GWAS studies performed so far. More likely, the answer may be found in the identification of rare variants. However, this will not explain all of the missing 40%. Gene-gene and gene-environment interactions as well as epigenetics may also play a role, and further research is needed to shed light on this. In addition, non-genetic studies, such as metabolomics, may play a role in the identification of new pathways associated with AMD, and subsequent dissection of these pathways may contribute to the discovery of new genetic variants in AMD.

3.2 SCREENING FOR AMD

In **Chapter 4** we describe a computer aided diagnosis system that is able to detect and quantify the presence of drusen in color fundus photographs with an accuracy that is equal to that of human graders. In addition, a classification in low risk and high risk individuals can be made. Development of automatic grading software opens new possibilities for screening for AMD in the elderly population. For diabetic retinopathy (DR), screening systems have been proved to be cost-effective and they are implemented on a large scale.[118] Most DR screening programs are based on human evaluation of color fundus photographs, but with the growing diabetic population these manual screening methods will not be able to meet the screening needs.[119]

For AMD, currently no screening programs are available. Some cost-utility models have been developed, but the proposed programs were based on self-testing,[120,121] or dialated fundus examinations.[122,123] Recently, Chan et al evaluated the cost-effectiveness of AMD screening performed simultaneously with DR screening for diabetic patients in Hong Kong. They reported that the total cost per quality-adjusted life year gained was $12 712, which is highly cost effective.[124] In the proposed screening system, mydriatic fundus photographs are manually graded by trained ophthalmologists and optometrists. An automatic grading system would further increase the cost-effectiveness and with the increased growth of the elderly population, this option should be seriously considered.

3.3 PREVENTION OF AMD DEVELOPMENT AND PROGRESSION

Vitamin supplements

Supplementation with the AREDS formulation (antioxidant vitamins C and E, lutein, zeaxanthin and zinc) has been shown to slow down progression to end-stage AMD by 25% in high-risk patients.[125] In 2014, we showed that oral zinc supplementation decreases complement activation in AMD patients, but only in the presence of a high baseline level of complement catabolism.[126] In addition to its role as a complement inhibitor, zinc also has antioxidant properties that further dampen the activation of the alternative complement pathway. Based on what we know today, zinc might be a realistic candidate drug to slow down AMD progression through complement inhibition and should be evaluated in a large prospective clinical trial.
Who should be advised to take vitamin supplements? In current clinical practice patients with unilateral advanced AMD and patients with bilateral intermediate stage disease are advised to consider the use of vitamin supplements in order to slow down disease progression.[125] However, it is not unlikely that there are other patient groups who might benefit from nutritional supplements. For example, individuals from families with a high prevalence of AMD are at increased risk of developing end-stage AMD and the complement system seems to play a more important role in the development of familial AMD (**Chapter 5**). However, these individuals may report to an ophthalmologist at a relatively young age, because of an increased awareness of their disease risk and they may not show any signs of AMD yet. It seems counterintuitive not to advise the use of nutritional supplements in these patients but further research is warranted to prove the beneficial effect in selected subgroups of individuals known to be at high risk of development of end-stage AMD.

Complement inhibitors

Modulation of the complement system by targeting the regulating components is now the focus for the development of new treatment modalities for AMD and especially GA. Several clinical trials have been initiated to study the effect of complement inhibition on dry AMD. Table 2 gives an overview of the complement inhibiting therapies for AMD and their stage of development. Until recently, results were not very promising, if any results were published at

all. Events took another turn when the first results from the phase II study on lampalizumab were published, suggesting that intravitreally administered lampalizumab, an antigen-binding fragment against factor D, could successfully slow down the progression of GA. At the moment, several phase III studies for lampalizumab are ongoing.
The progression rate of GA is highly variable among different studies and ranges from 1.2 to 2.8 mm^2 per year.[127] A clinical trial designed to detect a decline in the progression rate needs to be well powered. A possible explanation for the disappointing results so far may be due to underpowered study designs.
In addition, we need to determine the optimal timeframe to initiate complement inhibition. As shown in table 2, almost all trials included only patients with end-stage AMD, and evaluated the effect on the progression rate of GA. However, we do not know whether complement activation has an influence on GA progression. Maybe the role of complement (over) activation in the pathogenesis of dry AMD involves the earlier stages of the disease. We observe in our study in **Chapter 8** that patients with intermediate AMD have levels of complement activation that are as high as the levels in patients with GA. We therefore propose that clinical trials should also include patients in nonadvanced AMD stages in order to determine if complement inhibitors can prevent or slow down development of GA. This requires an accurate identification of those patients at the highest risk of progressing toward end-stage AMD, because the majority of intermediate AMD patients never develops GA or CNV.
We further highlight the importance of genetics. As shown in this thesis, the relationship between AMD stage and complement activation was only observed in individuals with a high genetic risk (and thus a higher baseline complement activity). This is in line with our previous finding in our study on zinc supplementation: only in patients with a high level of complement activation at baseline was zinc supplementation successful in lowering complement activity.[126] It is therefore likely that there is a subgroup of patients who would benefit most from complement inhibition, and they might be identified through genetic testing.
Besides the presence of high risk variants, high baseline levels of complement activity could help in identifying these subgroups. We should not only evaluate genetic polymorphisms of complement genes, but should also bear in mind that patients with low baseline levels of complement activity might benefit less from complement inhibition. Since systemic complement measurements are greatly influenced by viral or bacterial infections and disease activity of other inflammatory conditions, alternative approaches may be needed to determine an individual's level of complement activity when using such a measure to select individual patients for treatment. For example, complement activation levels may be measured at multiple timepoints, or selection of patients for treatment could be based on a prediction rule which should not only be based on a single measurement of the C3d/C3 ratio, but should also include genetic variants, disease stage and other factors influencing complement activity.
It is important that future clinical trials evaluating complement inhibitors are well powered and include a sample size that is large enough for these kind of subanalyses.

TABLE 2. Overview of clinical trials on anti-complement therapies for AMD

Drug	Administration	Study group characteristics	Current stage	Preliminary / Final results
Lampalizumab (Humanized mouse antibody – targets factor D)	Intravitreal	Patients with GA without CNV in both eyes; follow up 48 weeks – 2 years; Estimated inclusion: 936	Phase III studies (Chroma and Spectri) ongoing	Phase II: 20% reduction in GA growth rate at 18 months[128]
Eculizumab (Humanized mouse antibody – targets C5 convertase)	Intravenous	30 patients with GA; follow up 26 weeks	Phase II study completed	No effect upon GA size or growth rate at 6 or 12 months[129]
LFG316 (fully-human IgG antibody – targets C5)	Intravitreal	158 patients with GA in at least one eye; follow up 18 months	Phase II completed	No results reported
CLG561 with or without LFG316 (anti C5)	Intravitreal	Patients with GA in both eyes; follow up 12 months; Estimated inclusion: 114	Phase II study recruiting	No results reported
POT-4 (blocks cleavage of C3 to its active products)	Intravitreal	27 patients with neovascular AMD	Phase I completed	No results reported
ARC1905 (aptamer – prevents cleavage of C5)	Intravitreal	50 patients with drusen and/or GA in both eyes	Phase I completed	No results reported
TT30 (CFH-recombinant fusion protein)	-	-	Preclinical	-
TA106 (antibody – targets factor B)	-	-	Preclinical	-
JPE-1375 / JSM-7717 (receptor antagonist for C5a)	-	-	Preclinical	-

AMD, age-related macular degeneration; GA, geographic atrophy; CNV, choroidal neovascularization. From: https://clinicaltrials.gov/

REFERENCES

1. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. *Ophthalmology* 2001;108:697-704.
2. Age-Related Eye Disease Study Research Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology* 2000;107:2224-2232.
3. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. *JAMA* 1996;276:1141-1146.
4. Christen WG, Glynn RJ, Manson JE, Ajani UA, Buring JE. A prospective study of cigarette smoking and risk of age-related macular degeneration in men. *JAMA* 1996;276:1147-1151.
5. Hyman LG, Lilienfeld AM, Ferris FL, 3rd, Fine SL. Senile macular degeneration: a case-control study. *Am J Epidemiol* 1983;118:213-227.
6. Klein R, Klein BE, Linton KL, DeMets DL. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. *Am J Epidemiol* 1993;137:190-200.
7. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. The Blue Mountains Eye Study. *Arch Ophthal* 1996;114:1518-1523.
8. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. *Arch Ophthalmol* 1996;114:1193-1196.
9. Adams MK, Simpson JA, Aung KZ, et al. Abdominal obesity and age-related macular degeneration. *Am J Epidemiol* 2011;173:1246-1255.
10. Chen Y, Zeng J, Zhao C, et al. Assessing susceptibility to age-related macular degeneration with genetic markers and environmental factors. *Arch Ophthalmol* 2011;129:344-351.
11. Hogg RE, Woodside JV, Gilchrist SE, et al. Cardiovascular disease and hypertension are strong risk factors for choroidal neovascularization. *Ophthalmology* 2008;115:1046-1052 e1042.
12. Klein R, Deng Y, Klein BE, et al. Cardiovascular disease, its risk factors and treatment, and age-related macular degeneration: Women's Health Initiative Sight Exam ancillary study. *Am J Ophthalmol* 2007;143:473-483.
13. Seddon JM, George S, Rosner B, Klein ML. CFH gene variant, Y402H, and smoking, body mass index, environmental associations with advanced age-related macular degeneration. *Hum Hered* 2006;61:157-165.
14. Smith W, Mitchell P, Leeder SR, Wang JJ. Plasma fibrinogen levels, other cardiovascular risk factors, and age-related maculopathy: the Blue Mountains Eye Study. *Arch Ophthalmol* 1998;116:583-587.
15. Buch H, Vinding T, la Cour M, Jensen GB, Prause JU, Nielsen NV. Risk factors for age-related maculopathy in a 14-year follow-up study: the Copenhagen City Eye Study. *Acta Ophthalmol Scand* 2005;83:409-418.
16. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. *Invest Ophthalmol Vis Sci* 2009;50:5818-5827.
17. Knudtson MD, Klein R, Klein BE. Physical activity and the 15-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. *Br J Ophthalmol* 2006;90:1461-1463.
18. Delcourt C, Carriere I, Ponton-Sanchez A, Fourrey S, Lacroux A, Papoz L. Light exposure and the risk of age-related macular degeneration: the Pathologies Oculaires Liees a l'Age (POLA) study. *Arch Ophthalmol* 2001;119:1463-1468.
19. Cruickshanks KJ, Klein R, Klein BE, Nondahl DM. Sunlight and the 5-year incidence of early age-related maculopathy: the beaver dam eye study. *Arch Ophthalmol* 2001;119:246-250.
20. Hogg RE, Woodside JV, Gilchrist SE, et al. Cardiovascular disease and hypertension are strong risk factors for choroidal neovascularization. *Ophthalmology* 2008;115:1046-1052.e1042.
21. Klein R, Klein BE, Tomany SC, Cruickshanks KJ. The association of cardiovascular disease with the long-term incidence of age-related maculopathy: the Beaver Dam Eye Study. *Ophthalmology* 2003;110:1273-1280.
22. Fraser-Bell S, Wu J, Klein R, et al. Cardiovascular risk factors and age-related macular degeneration: the Los Angeles Latino Eye Study. *Am J Ophthalmol* 2008;145:308-316.
23. Evans JR. Risk factors for age-related macular degeneration. *Prog Retin Eye Res* 2001;20:227-253.
24. Smith W, Mitchell P, Wang JJ. Gender, oestrogen, hormone replacement and age-related macular degeneration: results from the Blue Mountains Eye Study. *Aust N Z J Ophthalmol* 1997;25 Suppl 1:S13-15.
25. Johnson LV, Anderson DH. Age-related macular degeneration and the extracellular matrix. *N Engl J Med* 2004;351:320-322.

26. Curcio CA, Presley JB, Malek G, Medeiros NE, Avery DV, Kruth HS. Esterified and unesterified cholesterol in drusen and basal deposits of eyes with age-related maculopathy. *Exp Eye Res* 2005;81:731-741.
27. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet* 2016;48:134-143.
28. Schmidt S, Hauser MA, Scott WK, et al. Cigarette smoking strongly modifies the association of LOC387715 and age-related macular degeneration. *Am J Hum Genet* 2006;78:852-864.
29. Seitsonen SP, Onkamo P, Peng G, et al. Multifactor effects and evidence of potential interaction between complement factor H Y402H and LOC387715 A69S in age-related macular degeneration. *PLoS One* 2008;3:e3833.
30. Naj AC, Scott WK, Courtenay MD, et al. Genetic factors in nonsmokers with age-related macular degeneration revealed through genome-wide gene-environment interaction analysis. *Ann Hum Genet* 2013;77:215-231.
31. Oz O, Aras Ates N, Tamer L, Yildirim O, Adiguzel U. Glutathione S-transferase M1, T1, and P1 gene polymorphism in exudative age-related macular degeneration: a preliminary report. *Eur J Ophthalmol* 2006;16:105-110.
32. Ho L, van Leeuwen R, Witteman JC, et al. Reducing the genetic risk of age-related macular degeneration with dietary antioxidants, zinc, and omega-3 fatty acids: the Rotterdam study. *Arch Ophthalmol* 2011;129:758-766.
33. Wang JJ, Buitendijk GH, Rochtchina E, et al. Genetic susceptibility, dietary antioxidants, and long-term incidence of age-related macular degeneration in two populations. *Ophthalmology* 2014;121:667-675.
34. Wang JJ, Rochtchina E, Smith W, et al. Combined effects of complement factor H genotypes, fish consumption, and inflammatory markers on long-term risk for age-related macular degeneration in a cohort. *Am J Epidemiol* 2009;169:633-641.
35. Klein ML, Mauldin WM, Stoumbos VD. Heredity and age-related macular degeneration. Observations in monozygotic twins. *Arch Ophthalmol* 1994;112:932-937.
36. Heiba IM, Elston RC, Klein BE, Klein R. Sibling correlations and segregation analysis of age-related maculopathy: the Beaver Dam Eye Study. *Genet Epidemiol* 1994;11:51-67.
37. Meyers SM, Greene T, Gutman FA. A twin study of age-related macular degeneration. *Am J Ophthalmol* 1995;120:757-766.
38. Hammond CJ, Webster AR, Snieder H, Bird AC, Gilbert CE, Spector TD. Genetic influence on early age-related maculopathy: a twin study. *Ophthalmology* 2002;109:730-736.
39. Klaver CC, Wolfs RC, Assink JJ, van Duijn CM, Hofman A, de Jong PT. Genetic risk of age-related maculopathy. Population-based familial aggregation study. *Arch Ophthalmol* 1998;116:1646-1651.
40. Boon CJ, Klevering BJ, Hoyng CB, et al. Basal laminar drusen caused by compound heterozygous variants in the CFH gene. *Am J Hum Genet* 2008;82:516-523.
41. Duvvari MR, Paun CC, Buitendijk GH, et al. Analysis of rare variants in the C3 gene in patients with age-related macular degeneration. *PLoS One* 2014;9:e94165.
42. Helgason H, Sulem P, Duvvari MR, et al. A rare nonsynonymous sequence variant in C3 is associated with high risk of age-related macular degeneration. *Nat Genet* 2013;45:1371-1374.
43. Raychaudhuri S, Iartchouk O, Chin K, et al. A rare penetrant mutation in CFH confers high risk of age-related macular degeneration. *Nat Genet* 2011;43:1232-1236.
44. Seddon JM, Yu Y, Miller EC, et al. Rare variants in CFI, C3 and C9 are associated with high risk of advanced age-related macular degeneration. *Nat Genet* 2013;45:1366-1370.
45. van de Ven JP, Boon CJ, Fauser S, et al. Clinical evaluation of 3 families with basal laminar drusen caused by novel mutations in the complement factor H gene. *Arch Ophthalmol* 2012;130:1038-1047.
46. van de Ven JP, Nilsson SC, Tan PL, et al. A functional variant in the CFI gene confers a high risk of age-related macular degeneration. *Nat Genet* 2013;45:813-817.
47. Yu Y, Triebwasser MP, Wong EK, et al. Whole-exome sequencing identifies rare, functional CFH variants in families with macular degeneration. *Hum Mol Genet* 2014;23:5283-5293.
48. Zhan X, Larson DE, Wang C, et al. Identification of a rare coding variant in complement 3 associated with age-related macular degeneration. *Nat Genet* 2013;45:1375-1379.
49. Grassi MA, Folk JC, Scheetz TE, Taylor CM, Sheffield VC, Stone EM. Complement factor H polymorphism p.Tyr402His and cuticular Drusen. *Arch Ophthalmol* 2007;125:93-97.
50. van de Ven JP, Smailhodzic D, Boon CJ, et al. Association analysis of genetic and environmental risk factors in the cuticular drusen subtype of age-related macular degeneration. *Mol Vis* 2012;18:2271-2278.
51. Finger RP, Wu Z, Luu CD, et al. Reticular pseudodrusen: a risk factor for geographic atrophy in fellow eyes of individuals with unilateral choroidal neovascularization. *Ophthalmology* 2014;121:1252-1256.

52. Marsiglia M, Boddu S, Bearelly S, et al. Association between geographic atrophy progression and reticular pseudodrusen in eyes with dry age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2013;54:7362-7369.
53. Francis PJ, George S, Schultz DW, et al. The LOC387715 gene, smoking, body mass index, environmental associations with advanced age-related macular degeneration. *Hum Hered* 2007;63:212-218.
54. Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat Genet* 2006;38:1055-1059.
55. Seddon JM, Francis PJ, George S, Schultz DW, Rosner B, Klein ML. Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. *JAMA* 2007;297:1793-1800.
56. Magnusson KP, Duan S, Sigurdsson H, et al. CFH Y402H confers similar risk of soft drusen and both forms of advanced AMD. *PLoS Med* 2006;3:e5.
57. Sepp T, Khan JC, Thurlby DA, et al. Complement factor H variant Y402H is a major risk determinant for geographic atrophy and choroidal neovascularization in smokers and nonsmokers. *Invest Ophthalmol Vis Sci* 2006;47:536-540.
58. Saade C, Ganti B, Marmor M, Freund KB, Smith RT. Risk characteristics of the combined geographic atrophy and choroidal neovascularisation phenotype in age-related macular degeneration. *Br J Ophthalmol* 2014;98:1729-1732.
59. Sobrin L, Reynolds R, Yu Y, et al. ARMS2/HTRA1 locus can confer differential susceptibility to the advanced subtypes of age-related macular degeneration. *Am J Ophthalmol* 2011;151:345-352.e343.
60. Sobrin L, Ripke S, Yu Y, et al. Heritability and genome-wide association study to assess genetic differences between advanced age-related macular degeneration subtypes. *Ophthalmology* 2012;119:1874-1885.
61. Complications of Age-related Macular Degeneration Prevention Trial (CAPT) Research Group. Risk factors for choroidal neovascularization and geographic atrophy in the complications of age-related macular degeneration prevention trial. *Ophthalmology* 2008;115:1474-1479.
62. Hyman L, Schachat AP, He Q, Leske MC. Hypertension, cardiovascular disease, and age-related macular degeneration. Age-Related Macular Degeneration Risk Factors Study Group. *Arch Ophthalmol* 2000;118:351-358.
63. Perlee LT, Bansal AT, Gehrs K, et al. Inclusion of genotype with fundus phenotype improves accuracy of predicting choroidal neovascularization and geographic atrophy. *Ophthalmology* 2013;120:1880-1892.
64. Seddon JM, Silver RE, Kwong M, Rosner B. Risk Prediction for Progression of Macular Degeneration: 10 Common and Rare Genetic Variants, Demographic, Environmental, and Macular Covariates. *Invest Ophthalmol Vis Sci* 2015;56:2192-2202.
65. Seddon JM, Reynolds R, Maller J, Fagerness JA, Daly MJ, Rosner B. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. *Invest Ophthalmol Vis Sci* 2009;50:2044-2053.
66. Seddon JM, Reynolds R, Yu Y, Daly MJ, Rosner B. Risk models for progression to advanced age-related macular degeneration using demographic, environmental, genetic, and ocular factors. *Ophthalmology* 2011;118:2203-2211.
67. Seddon JM, Reynolds R, Yu Y, Rosner B. Validation of a prediction algorithm for progression to advanced macular degeneration subtypes. *JAMA Ophthalmol* 2013;131:448-455.
68. Macular Photocoagulation Study Group. Risk factors for choroidal neovascularization in the second eye of patients with juxtafoveal or subfoveal choroidal neovascularization secondary to age-related macular degeneration. *Arch Ophthalmol* 1997;115:741-747.
69. Solomon SD, Jefferys JL, Hawkins BS, Bressler NM. Incident choroidal neovascularization in fellow eyes of patients with unilateral subfoveal choroidal neovascularization secondary to age-related macular degeneration: SST report No. 20 from the Submacular Surgery Trials Research Group. *Arch Ophthalmol* 2007;125:1323-1330.
70. Maguire MG, Daniel E, Shah AR, et al. Incidence of choroidal neovascularization in the fellow eye in the comparison of age-related macular degeneration treatments trials. *Ophthalmology* 2013;120:2035-2041.
71. Csaky KG, Dugel PU, Pierce AJ, et al. Clinical evaluation of pazopanib eye drops versus ranibizumab intravitreal injections in subjects with neovascular age-related macular degeneration. *Ophthalmology* 2015;122:579-588.
72. Singh R, Wurzelmann JI, Ye L, et al. Clinical evaluation of pazopanib eye drops in healthy subjects and in subjects with neovascular age-related macular degeneration. *Retina* 2014;34:1787-1795.
73. Danis R, McLaughlin MM, Tolentino M, et al. Pazopanib eye drops: a randomised trial in neovascular age-related macular degeneration. *Br J Ophthalmol* 2014;98:172-178.
74. van der Schaft TL, Mooy CM, de Bruijn WC, de Jong PT. Early stages of age-related macular degeneration: an immunofluorescence and electron microscopy study. *Br J Ophthalmol* 1993;77:657-661.

75. Johnson LV, Ozaki S, Staples MK, Erickson PA, Anderson DH. A potential role for immune complex pathogenesis in drusen formation. *Exp Eye Res* 2000;70:441-449.
76. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* 2002;134:411-431.
77. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science* 2005;308:421-424.
78. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 2005;308:419-421.
79. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science* 2005;308:385-389.
80. Ennis S, Gibson J, Cree AJ, Collins A, Lotery AJ. Support for the involvement of complement factor I in age-related macular degeneration. *Eur J Hum Genet* 2010;18:15-16.
81. Francis PJ, Hamon SC, Ott J, Weleber RG, Klein ML. Polymorphisms in C2, CFB and C3 are associated with progression to advanced age related macular degeneration associated with visual loss. *J Med Genet* 2009;46:300-307.
82. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* 2008;40:892-896.
83. Lee SJ, Kim NR, Chin HS. LOC387715/HTRA1 polymorphisms , smoking, and combined effects on exudative age-related macular degeneration in a Korean population. *Clin Experiment Ophthalmol* 2010;38:698-704.
84. Rivera A, Fisher SA, Fritsche LG, et al. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum Mol Genet* 2005;14:3227-3236.
85. Yang Z, Tong Z, Chen Y, et al. Genetic and functional dissection of HTRA1 and LOC387715 in age-related macular degeneration. *PLoS Genet* 2010;6:e1000836.
86. Kanda A, Chen W, Othman M, et al. A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. *Proc Natl Acad Sci U S A* 2007;104:16227-16232.
87. Kortvely E, Hauck SM, Duetsch G, et al. ARMS2 is a constituent of the extracellular matrix providing a link between familial and sporadic age-related macular degenerations. *Invest Ophthalmol Vis Sci* 2010;51:79-88.
88. Wang G, Spencer KL, Court BL, et al. Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria. *Invest Ophthalmol Vis Sci* 2009;50:3084-3090.
89. Xu YT, Wang Y, Chen P, Xu HF. Age-related maculopathy susceptibility 2 participates in the phagocytosis functions of the retinal pigment epithelium. *Int J Ophthalmol* 2012;5:125-132.
90. Yasuma TR, Nakamura M, Nishiguchi KM, et al. Elevated C-reactive protein levels and ARMS2/HTRA1 gene variants in subjects without age-related macular degeneration. *Mol Vis* 2010;16:2923-2930.
91. Smailhodzic D, Klaver CC, Klevering BJ, et al. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. *Ophthalmology* 2012;119:339-346.
92. Zeng F, Zhang M, Xu Y, Xu H. ARMS2 interference leads to decrease of proinflammatory mediators. *Graefes Arch Clin Exp Ophthalmol* 2013;251:2539-2544.
93. Cascella R, Ragazzo M, Strafella C, et al. Age-related macular degeneration: insights into inflammatory genes. *J Ophthalmol* 2014;2014:582842.
94. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. *PLoS One* 2008;3:e2593.
95. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Hum Mol Genet* 2010;19:209-215.
96. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. *Arch Ophthalmol* 2007;125:515-519.
97. Ristau T, Paun C, Ersoy L, et al. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. *PLoS One* 2014;9:e93459.
98. Buitendijk GH, Amin N, Hofman A, van Duijn CM, Vingerling JR, Klaver CC. Direct-to-consumer personal genome testing for age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:6167-6174.
99. Sharma NK, Sharma SK, Gupta A, Prabhakar S, Singh R, Anand A. Predictive model for earlier diagnosis of suspected age-related macular degeneration patients. *DNA Cell Biol* 2013;32:549-555.
100. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 2006;38:458-462.

101. Hughes AE, Orr N, Patterson C, et al. Neovascular age-related macular degeneration risk based on CFH, LOC387715/HTRA1, and smoking. *PLoS Med* 2007;4:e355.
102. Jakobsdottir J, Conley YP, Weeks DE, Ferrell RE, Gorin MB. C2 and CFB genes in age-related maculopathy and joint action with CFH and LOC387715 genes. *PLoS One* 2008;3:e2199.
103. Jakobsdottir J, Gorin MB, Conley YP, Ferrell RE, Weeks DE. Interpretation of genetic association studies: markers with replicated highly significant odds ratios may be poor classifiers. *PLoS Genet* 2009;5:e1000337.
104. Gibson J, Cree A, Collins A, Lotery A, Ennis S. Determination of a gene and environment risk model for age-related macular degeneration. *Br J Ophthalmol* 2010;94:1382-1387.
105. McKay GJ, Dasari S, Patterson CC, Chakravarthy U, Silvestri G. Complement component 3: an assessment of association with AMD and analysis of gene-gene and gene-environment interactions in a Northern Irish cohort. *Mol Vis* 2010;16:194-199.
106. Zanke B, Hawken S, Carter R, Chow D. A genetic approach to stratification of risk for age-related macular degeneration. *Can J Ophthalmol* 2010;45:22-27.
107. Hageman GS, Gehrs K, Lejnine S, et al. Clinical validation of a genetic model to estimate the risk of developing choroidal neovascular age-related macular degeneration. *Hum Genomics* 2011;5:420-440.
108. Klein ML, Francis PJ, Ferris FL 3rd, Hamon SC, Clemons TE. Risk assessment model for development of advanced age-related macular degeneration. *Arch Ophthalmol* 2011;129:1543-1550.
109. Spencer KL, Olson LM, Schnetz-Boutaud N, et al. Using genetic variation and environmental risk factor data to identify individuals at high risk for age-related macular degeneration. *PLoS One* 2011;6:e17784.
110. Ying GS, Maguire MG. Development of a risk score for geographic atrophy in complications of the age-related macular degeneration prevention trial. *Ophthalmology* 2011;118:332-338.
111. Grassmann F, Fritsche LG, Keilhauer CN, Heid IM, Weber BH. Modelling the genetic risk in age-related macular degeneration. *PLoS One* 2012;7:e37979.
112. McCarthy LC, Newcombe PJ, Whittaker JC, et al. Predictive models of choroidal neovascularization and geographic atrophy incidence applied to clinical trial design. *Am J Ophthalmol* 2012;154:568-578.e512.
113. Yu Y, Reynolds R, Rosner B, Daly MJ, Seddon JM. Prospective assessment of genetic effects on progression to different stages of age-related macular degeneration using multistate Markov models. *Invest Ophthalmol Vis Sci* 2012;53:1548-1556.
114. Buitendijk GH, Rochtchina E, Myers C, et al. Prediction of age-related macular degeneration in the general population: the Three Continent AMD Consortium. *Ophthalmology* 2013;120:2644-2655.
115. Chiu CJ, Mitchell P, Klein R, et al. A risk score for the prediction of advanced age-related macular degeneration: development and validation in 2 prospective cohorts. *Ophthalmology* 2014;121:1421-1427.
116. Ristau T, Ersoy L, Hahn M, et al. Nongenetic risk factors for neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2014;55:5228-5232.
117. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. *Am J Hum Genet* 2011;88:76-82.
118. Javitt JC, Aiello LP. Cost-effectiveness of detecting and treating diabetic retinopathy. *Ann Intern Med* 1996;124:164-169.
119. Bhaskaranand M, Ramachandra C, Bhat S, et al. Automated Diabetic Retinopathy Screening and Monitoring Using Retinal Fundus Image Analysis. *J Diabetes Sci Technol* 2016.
120. Karnon J, Czoski-Murray C, Smith K, et al. A preliminary model-based assessment of the cost-utility of a screening programme for early age-related macular degeneration. *Health Technol Assess* 2008;12:iii-iv, ix-124.
121. Karnon J, Czoski-Murray C, Smith KJ, Brand C. A hybrid cohort individual sampling natural history model of age-related macular degeneration: assessing the cost-effectiveness of screening using probabilistic calibration. *Med Decis Making* 2009;29:304-316.
122. Hopley C, Salkeld G, Wang JJ, Mitchell P. Cost utility of screening and treatment for early age related macular degeneration with zinc and antioxidants. *Br J Ophthalmol* 2004;88:450-454.
123. Rein DB, Saaddine JB, Wittenborn JS, et al. Cost-effectiveness of vitamin therapy for age-related macular degeneration. *Ophthalmology* 2007;114:1319-1326.
124. Chan CK, Gangwani RA, McGhee SM, Lian J, Wong DS. Cost-Effectiveness of Screening for Intermediate Age-Related Macular Degeneration during Diabetic Retinopathy Screening. *Ophthalmology* 2015;122:2278-2285.
125. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* 2001;119:1417-1436.

126. Smailhodzic D, van Asten F, Blom AM, et al. Zinc supplementation inhibits complement activation in age-related macular degeneration. *PLoS One* 2014;9:e112682.
127. Schmitz-Valckenberg S, Sahel JA, Danis R, et al. Natural History of Geographic Atrophy Progression Secondary to Age-Related Macular Degeneration (Geographic Atrophy Progression Study). *Ophthalmology* 2015.
128. Holz F, Schmitz-Valckenberg S, Yaspan B, Li Z, Henry E, Strauss E. The MAHALO phase II results- lampalizumab (anti-factor D) in patients with geographic atrophy. Paper presented at the annual meeting of EURETINA, London, UK. Abstract retrieved from http://www.euretina.org/london2014/programme/free-papers-details.asp?id=3439&day=0. 2014.
129. Yehoshua Z, de Amorim Garcia Filho CA, Nunes RP, et al. Systemic complement inhibition with eculizumab for geographic atrophy in age-related macular degeneration: the COMPLETE study. *Ophthalmology* 2014;121:693-701.

CHAPTER 11

SUMMARY
SAMENVATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE
DANKWOORD

SUMMARY

Age-related macular degeneration (AMD) is a disease affecting the central retina and the most common cause of blindness in developed countries. AMD is a multifactorial disease and both environmental and genetic risk factors are involved in the pathogenesis. The aim of this thesis is to gain more insight in risk factors for disease development and progression and to characterize different subgroups of patients, such as individuals from AMD families. In addition, the role of the complement system in the course of the disease is evaluated.

Chapter 1 serves as an introduction to the clinical and pathophysiological characteristics of AMD. It also provides the reader with basic knowledge on risk factors and available treatment and prevention strategies.

Chapters 2-4 focus on the identification of risk factors for AMD.
In **Chapter 2** we report our findings on risk factors for an earlier age at onset of neovascular AMD. We evaluate a retrospective cohort of 275 patients with an average age at onset of 75 years of age and show that in individuals homozygous for *CFH* and *ARMS2* risk alleles and who smoke cigarettes, the onset of neovascular AMD may be accelerated by as much as one or two decades. These patients should be identified at an early age, in order to benefit from possible countermeasures as early as possible.
Patients who have already developed end-stage AMD in one eye are at increased risk of developing choroidal neovascularization (CNV) or geographic atrophy (GA) in the second eye. In **Chapter 3** we determine risk factors for this second eye progression. Female sex, a higher age, a body mass index (BMI) above 30kg/m^2 and smoking are all associated with a faster progression toward end-stage AMD in the fellow eye. In addition, genetic polymorphisms in the *CFI* and *LPL* genes also increase the risk of second eye progression. No increased risk is observed for polymorphisms in the *CFH* and *ARMS2* genes, the two major genes for AMD development and progression in one eye.
The presumed association of polymorphisms in the glutathione-S transferase (GST) genes and AMD is discussed in **Chapter 4**. A few studies, all characterized by small study populations, identified a relationship between GST polymorphisms and AMD. In a larger sample size of 477 AMD patients and 359 controls, we cannot confirm these associations.

Patients with a positive family history for AMD are at increased risk to develop AMD. We focus in more detail on familial AMD in **Chapters 5 and 6**.
We know that certain lifestyle factors are significantly associated with AMD in non-familial cases, but not in familial cases. In **Chapter 5** we investigate whether the contribution of common genetic variants and complement activation levels differs between familial and sporadic cases with AMD. The A69S polymorphism in the *ARMS2* gene is more strongly associated with sporadic AMD compared with familial AMD. In contrast, systemic complement activation is more associated with familial AMD, especially in patients with a densely affected family.

Our results point toward a more important role of the complement system in familial AMD. Several groups have developed a prediction model for AMD. In clinical practice, individuals with a positive family history for AMD are particularly interested in their disease risk. In **Chapter 6** we evaluate if these prediction models – that are mostly based on common environmental and genetic risk factors – can be applied to individuals from families affected with AMD. For most families, clustering of common risk factors explains the high frequency of disease. However, in a subset of densely affected families, we observed low predicted risk scores for all affected family members, suggesting that other risk factors, such as rare genetic variants, explain the aggregation of AMD in these families.

In **Chapter 7** we present an automatic computer system that allows for drusen detection and quantification with a performance equal to that of human observers. Automatic detection systems can be of additional value in research programs and screening settings.

The alternative pathway of the complement system is thought to be the most important pathway involved in AMD. AMD patients have higher levels of complement activation compared to controls and several polymorphisms in complement genes are associated with AMD. **Chapters 8 and 9** discuss the role of the complement pathway in AMD.
Chapter 8 describes the levels of complement activation in different stages of AMD. Complement activation in AMD appears to increase with local disease activity, with the lowest levels in controls and patients with inactive CNV and higher levels in patients with intermediate AMD, GA and active CNV. These results are only observed in a subgroup of patients with a high genetic risk, based on variants in several complement genes. Our results have implications for patient selection and time of initiation of treatment with complement inhibitors and should be evaluated in a prospective setting.
In **Chapter 9** we report our finding of a novel complotype – a combination of three single nucleotide polymorphisms (SNPs) in genes of the complement system – that is strongly associated with AMD and complement activation. The complotype is composed of 2 SNPs in *CFB* and one SNP in *CFH*. It is important to identify these complotypes because they can aid in the stratification of patients in subgroups that might be more eligible for complement-inhibiting therapies in AMD.

Chapter 10 discusses the findings of this thesis in a broader context. Research focused on the identification of risk factors for AMD is very important, since it will allow us to identify high risk individuals for disease development or progression. These individuals should be identified at an early age and provided with the proper tools and education to limit their risk of losing vision from advanced AMD. An overview of the currently available prediction models for AMD is presented. Because of the multifactorial etiology of the disease, it is likely that not all patients will benefit from the same treatment. Stratification of patients in subgroups based on their genetic risk profile or complement activity is important for the proper selection of eligible candidates for treatment with complement inhibitors.

SAMENVATTING

Leeftijdsgebonden maculadegeneratie (LMD) is een ziekte van het centrale deel van de retina en de meest voorkomende oorzaak van blindheid in ontwikkelde landen. LMD is een multifactoriële aandoening en zowel omgevingsfactoren als genetische risico factoren spelen een rol in the pathogenese. Het doel van dit proefschrift is meer te weten te komen over risico factoren voor LMD ontwikkeling en progressie en om verschillende subgroepen van patiënten te karakteriseren, zoals individuen uit LMD families. Daarnaast wordt ook de rol van het complement systeem in het beloop van de ziekte bekeken.

Hoofdstuk 1 dient als introductie in de klinische en pathofysiologische karakteristieken van LMD. Het verschaft de lezer de basiskennis over risico factoren en beschikbare behandelingen en preventieve maatregelen.

Hoofdstuk 2-4 gaan dieper in op het identificeren van risico factoren voor LMD.
In **Hoofdstuk 2** rapporteren we onze bevindingen over risico factoren voor een jongere leeftijd van ontstaan van neovasculaire LMD. We evalueren een retrospectief cohort van 275 patiënten met een gemiddelde leeftijd van 75 jaar bij het ontstaan van neovasculaire LMD en we laten zien dat in individuen die homozygoot zijn voor risico allelen in *CFH* en *ARMS2* en ook roken, de beginleeftijd tot wel een of twee decaden vervroegd wordt. Deze patiënten zouden op jonge leeftijd geïdentificeerd moeten worden, om in een zo vroeg mogelijk stadium te kunnen profiteren van mogelijke tegenmaatregelen.
Patiënten die reeds in een oog eindstadium LMD hebben ontwikkeld, hebben verhoogd risico op het ontwikkelen van een choroidale neovascularizatie (CNV) of geografische atrofie (GA) in het tweede oog. In **Hoofdstuk 3** bepalen we risico factoren voor deze progressie in het tweede oog. Vrouwelijk geslacht, een hogere leeftijd, een body mass index (BMI) boven de 30 kg/m^2 en roken zijn allemaal geassocieerd met een snellere progressie naar eindstadium LMD in het tweede oog. Daarnaast verhogen ook genetische polymorfismen in de *CFI* en *LPL* genen het risico op progressie in het tweede oog. Er wordt geen verhoogd risico gezien voor polymorfismen in de *CFH* en *ARMS2* genen, de twee belangrijkste genen voor LMD ontwikkeling en progressie in het eerste oog.
De veronderstelde associatie tussen polymorfismen in de glutathion-S transferase (GST) genen en LMD wordt bediscussieerd in **Hoofdstuk 4**. Een aantal studies, allen gekenmerkt door kleine studie populaties, beschreven een relatie tussen GST polymorfismen en LMD. In een grotere studie groep van 477 LMD patiënten en 359 controles, kunnen wij deze associaties niet bevestigen.

Patiënten met een positieve familie voorgeschiedenis voor LMD hebben verhoogd risico om LMD te ontwikkelen. We gaan nader in op familaire LMD in **Hoofdstuk 5 en 6**.
We weten dat bepaalde leefstijl factoren significant geassocieerd zijn met LMD in niet-fami-

liaire patiënten, maar niet in familiaire patiënten. In **Hoofdstuk 5** onderzoeken we of de bijdrage van veelvoorkomende genetische varianten en complement activatie levels verschillen tussen familaire en sporadische patiënten met LMD. Het A69S polymorfisme in het *ARMS2* gen is sterker geassocieerd met sporadische LMD dan met familiaire LMD. Tegengesteld hieraan lijkt systemische complement activatie meer geassocieerd met familiaire LMD, met name in patiënten met een sterk belaste familie. Onze resultaten wijzen op een belangrijkere rol van het complement systeem in familiaire LMD.
Verschillende groepen hebben een predictiemodel voor LMD ontwikkeld. In de klinische praktijk, zijn het individuen met een positieve familie voorgeschiedenis voor LMD die vooral geïnteresseerd zijn in hun risico op ziekte. In **Hoofdstuk 6** evalueren we of deze predictiemodellen – die meestal gebaseerd zijn op veelvoorkomende omgevingsfactoren en genetische risico factoren – toegepast kunnen worden op individuen van LMD families. In de meeste families kan clustering van veelvoorkomende risico factoren het veelvuldig voorkomen van LMD verklaren. Echter, in een subset van sterk belaste families, zien we lage voorspelde risico scores voor alle aangedane familieleden, wat suggereert dat andere risico factoren, zoals zeldzame genetische varianten, het voorkomen van LMD in deze families verklaren.

In **Hoofdstuk 7** presenteren we een automatisch computer system dat in staat is om drusen te detecteren en kwantificeren op een niveau gelijk aan dat van menselijke graders. Automatische detectie systemen kunnen van toegevoegde waarde zijn in onderzoeksprogramma's en screenings programma's.

De alternatieve route van het complement systeem wordt geduid als de meest belangrijkste 'pathway' die betrokken is bij LMD. LMD patiënten hebben hogere complement activatie levels dan controles en verschillende polymorfismen in complement genen zijn geassocieerd met LMD. **Hoofdstuk 8 en 9** bediscussiëren de rol van het complement systeem in LMD.
Hoofdstuk 8 beschrijft complement activatie levels in verschillende stadia van LMD. Complement activatie lijkt toe te nemen met de lokale ziekte activiteit bij LMD, waarbij de laagste levels gevonden worden in controles en patiënten met inactieve CNV en de hoogste levels in patiënten met intermediaire LMD, GA en actieve CNV. Deze resultaten worden alleen gezien in een subgroep van patiënten met een hoog genetisch risico, gebaseerd op varianten in verschillende complement genen. Onze resultaten hebben implicaties voor de selectie van patiënten voor behandeling met complement inhibitoren en voor de timing van de start van behandeling, en zouden in een prospectieve setting moeten worden geëvalueerd.
In **Hoofdstuk 9** rapporteren we onze bevindingen over een nieuw complotype – een combinatie van drie 'single nucleotide polymorphisms' (SNPs) in genen van het complement systeem – die sterk geassocieerd is met LMD en complement activatie. Dit complotype bestaat uit 2 SNPs in *CFB* en een SNP in *CFH*. Het is belangrijk om deze complotypes te identificeren omdat zij kunnen helpen bij de stratificatie van patiënten in subgroepen die mogelijk meer in aanmerking komen voor complement-inhiberende behandelingen voor LMD.

Hoofdstuk 10 plaatst de bevindingen uit dit proefschrift in een bredere context. Onderzoek dat zich richt op de identificatie van risico factoren voor LMD is erg belangrijk, aangezien het ons in staat stelt individuen te identificeren met een hoog risico voor ziekte ontwikkeling of progressie. Deze individuen zouden we op jonge leeftijd moeten identificeren en moeten voorzien van de juiste handvatten en informatie om hun risico op verlies van gezichtsvermogen door LMD te beperken. Een overzicht van de huidige predictiemodellen voor LMD wordt gepresenteerd.
Vanwege de multifactoriële aard van de ziekte, is het aannemelijk dat niet alle patiënten baat zullen hebben bij dezelfde behandeling. Stratificatie van patiënten in subgroepen gebaseerd op hun genetisch risico profiel of op basis van complement activiteit is belangrijk voor de juiste selectie van geschikte kandidaten voor behandeling met complement remmers.

LIST OF PUBLICATIONS

Genotypes in glutathione S-transferase are not associated with age-related macular degeneration in a caucasian population.
Lechanteur YT, Peters WH, te Morsche RH, Hoyng CB, Klevering BJ, den Hollander AI.
Submitted

Prediction in families with age-related macular degeneration: Clustering of risk factors or rare genetic variants?
Kersten E, Lechanteur YT, Saksens NT, Geerlings MJ, Schick T, Fauser S, Boon CF, den Hollander AI, Hoyng CB.
Submitted

Complement activation levels are related to disease stage in age-related macular degeneration.
Lechanteur YT, Schick T, Daha MR, Altay L, Liakopoulos S, Smailhodzic D, den Hollander AI, Hoyng CB, Klevering BJ.
Submitted

Rare variants in *CFH* and *CFI* result in decreased C3b degradation in patients with age-related macular degeneration
Geerlings MJ, Kremlitzka M, Bakker B, Nilsson SC, Saksens NT, Lechanteur YT, Pauper M, Corominas J, Fauser S, Hoyng CB, Blom AM, de Jong EK, den Hollander AI.
Submitted

Analysis of risk alleles and complement activation levels in familial and non-familial age-related macular degeneration.
Saksens NT, Lechanteur YT, Verbakel SK, Groenewoud JM, Daha MR, Schick T, Fauser S, Boon CJ, Hoyng CB, den Hollander AI.
PLoS One 2016;11:e0144367

A novel complotype combination associates with age-related macular degeneration and high complement activation levels in vivo.
Paun CC, Lechanteur YT, Groenewoud JM, Altay L, Schick T, Daha MR, Fauser S, Hoyng CB, den Hollander AI, de Jong EK.
Sci Rep 2016;6:26568

A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants.
Fritsche LG, Igl W, Bailey JN, Grassmann F, Sengupta S, Bragg-Gresham JL, Burdon KP, Hebbring SJ, Wen C, Gorski M, Kim IK, Cho D, Zack D, Souied E, Scholl HP, Bala E, Lee KE, Hunter DJ, Sardell RJ, Mitchell P, Merriam JE, Cipriani V, Hoffman JD, Schick T, Lechanteur YT, Guymer RH, Johnson MP, Jiang Y, Stanton CM, Buitendijk GH, Zhan X, Kwong AM, Boleda A, Brooks M, Gieser L, Ratnapriya R, Branham KE, Foerster JR, Heckenlively JR, Othman MI, Vote BJ, Liang HH, Souzeau E, McAllister IL, Isaacs T, Hall J, Lake S, Mackey DA, Constable IJ, Craig JE, Kitchner TE, Yang Z, Su Z, Luo H, Chen D, Ouyang H, Flagg K, Lin D, Mao G, Ferreyra H, Stark K, von Strachwitz CN, Wolf A, Brandl C, Rudolph G, Olden M, Morrison MA, Morgan DJ, Schu M, Ahn J, Silvestri G, Tsironi EE, Park KH, Farrer LA, Orlin A, Brucker A, Li M, Curcio CA, Mohand-Saïd S, Sahel JA, Audo I, Benchaboune M, Cree AJ, Rennie CA, Goverdhan SV, Grunin M, Hagbi-Levi S, Campochiaro P, Katsanis N, Holz FG, Blond F, Blanché H, Deleuze JF, Igo RP Jr, Truitt B, Peachey NS, Meuer SM, Myers CE, Moore EL, Klein R, Hauser MA, Postel EA, Courtenay MD, Schwartz SG, Kovach JL, Scott WK, Liew G, Tan AG, Gopinath B, Merriam JC, Smith RT, Khan JC, Shahid H, Moore AT, McGrath JA, Laux R, Brantley MA Jr, Agarwal A, Ersoy L, Caramoy A, Langmann T, Saksens NT, de Jong EK, Hoyng CB, Cain MS, Richardson AJ, Martin TM, Blangero J, Weeks DE, Dhillon B, van Duijn CM, Doheny KF, Romm J, Klaver CC, Hayward C, Gorin MB, Klein ML, Baird PN, den Hollander AI, Fauser S, Yates JR, Allikmets R, Wang JJ, Schaumberg DA, Klein BE, Hagstrom SA, Chowers I, Lotery AJ, Léveillard T, Zhang K, Brilliant MH, Hewitt AW, Swaroop A, Chew EY, Pericak-Vance MA, DeAngelis M, Stambolian D, Haines JL, Iyengar SK, Weber BH, Abecasis GR, Heid IM.
Nat Genet 2016;48:134-43

Genetic variants and systemic complement activation levels are associated with serum lipoprotein levels in age-related macular degeneration.
Paun CC, Ersoy L, Schick T, Groenewoud JM, Lechanteur YT, Fauser S, Hoyng CB, de Jong EK, den Hollander AI.
Invest Ophthalmol Vis Sci 2015;56:7766-73

History of sunlight exposure is a risk factor for age-related macular degeneration.
Schick T, Ersoy L, Lechanteur YT, Saksens NT, Hoyng CB, den Hollander AI, Kirchhof B, Fauser S.
Retina 2016;36:787-90

Association of smoking and *CFH* and *ARMS2* risk variants with younger age at onset of neovascular age-related macular degeneration.
Lechanteur YT, van de Camp PL, Smailhodzic D, van de Ven JP, Buitendijk GH, Klaver CC, Groenewoud JM, den Hollander AI, Hoyng CB, Klevering BJ.
JAMA Ophthalmol 2015;133:533-41

Zinc supplementation inhibits complement activation in age-related macular degeneration.
Smailhodzic D, van Asten F, Blom AM, Mohlin FC, den Hollander AI, van de Ven JP, van Huet RA, Groenewoud JM, Tian Y, Berendschot TT, Lechanteur YT, Fauser S, de Bruijn C, Daha MR, van der Wilt GJ, Hoyng CB, Klevering BJ.
PLoS One 2014;9:e112682

Predicting non-response to ranibizumab in patients with neovascular age-related macular degeneration.
van Asten F, Rovers MM, Lechanteur YT, Smailhodzic D, Muether PS, Chen J, den Hollander AI, Fauser S, Hoyng CB, van der Wilt GJ, Klevering BJ.
Ophthalmic Epidemiol 2014;21:347-55

Nutritional risk factors for age-related macular degeneration.
Ersoy L, Ristau T, Lechanteur YT, Hahn M, Hoyng CB, Kirchhof B, den Hollander AI, Fauser S.
Biomed Res Int 2014;2014:413150

Environmental and genetic risk factors for retinal angiomatous proliferation.
Caramoy A, Ristau T, Lechanteur YT, Ersoy L, Müller S, Gelisken F, Hoyng CB, Kirchhof B, den Hollander AI, Fauser S.
Acta Ophthalmol 2014;92:745-8

Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels.
Ristau T, Paun C, Ersoy L, Hahn M, Lechanteur YT, Hoyng C, de Jong EK, Daha MR, Kirchhof B, den Hollander AI, Fauser S.
PLoS One 2014;9:e93459

Allergy is a protective factor against age-related macular degeneration.
Ristau T, Ersoy L, Lechanteur YT, den Hollander AI, Daha MR, Hahn M, Hoyng CB, Fauser S.
Invest Ophthalmol Vis Sci 2014;55:210-4

Automatic drusen quantification and risk assessment of age-related macular degeneration on color fundus images.
van Grinsven MJ, Lechanteur YT, van de Ven JP, van Ginneken B, Hoyng CB, Theelen T, Sánchez CI.
Invest Ophthalmol Vis Sci 2013;54:3019-27

Genetic, behavioral, and sociodemographic risk factors for second eye progression in age-related macular degeneration.
Lechanteur YT, van de Ven JP, Smailhodzic D, Boon CJ, Klevering BJ, Fauser S, Groenewoud JM, van der Wilt GJ, den Hollander AI, Hoyng CB.
Invest Ophthalmol Vis Sci 2012;53:5846-52

Short-term changes of Basal laminar drusen on spectral-domain optical coherence tomography.
van de Ven JP, Boon CJ, Smailhodzic D, Lechanteur YT, den Hollander AI, Hoyng CB, Theelen T.
Am J Ophthalmol 2012;154:560-7

CURRICULUM VITAE

Yara Terefech Esther Lechanteur was born on november 28th, 1986 in Addis Abeba, Ethiopia. She was adopted by Dutch parents in 1988. In 2004 she graduated from secondary school at "Liemers College" in Zevenaar, and started her medical studies in the same year at the Radboud University Nijmegen. It was during her internship in ophthalmology, that her particular interest in ophthalmology emerged. This was further reinforced during her scientific internship under the supervision of prof. dr. C.B. Hoyng at the department of ophthalmology at the Radboud university medical center. After graduation in 2010, she became a member of the scientific research group of prof. dr. C.B. Hoyng when she started her Ph.D. project on age-related macular degeneration. Her research results are presented in this thesis.
In January 2015, she started a residency in ophthalmology at the same institute.

DANKWOORD

Tijdens mijn promotietraject heb ik veel geschreven. Artikelen, abstracts, verhalen bij presentaties, en ga zo maar door. Wat voelt het nu vreemd om hier op dit punt aan te komen: het schrijven van mijn dankwoord. Het meest gelezen, en misschien ook wel meest gewaardeerde hoofdstuk van dit proefschrift. En dat is terecht, zonder de toewijding, steun en support van velen was het niet tot zo'n mooi resultaat gekomen. Een aantal mensen wil ik graag persoonlijk toespreken.

Allereerst het team van mijn promotoren en co-promotoren.
Prof. dr. Hoyng, beste Carel, mijn eerste herinnering aan jou was tijdens mijn co-schap oogheelkunde. Ik liep met jouw spreekuur mee en jij sprak vol passie en enthousiasme over je vak. Daarna wist ik het zeker: ik wordt oogarts! Toen jij me enkele jaren later vroeg of ik na mijn wetenschappelijke stage wilde blijven als promovenda twijfelde ik dan ook geen seconde. Ik heb me door jou altijd gesteund en gewaardeerd gevoeld. Jij was degene die zicht had op mijn promotie en die van bovenaf goed in de gaten hield welke kant we op gingen. Bedankt voor het geloof en vertrouwen dat je vanaf het eerste moment in mij hebt gehad.
Prof. dr. Klevering, beste Jeroen, na het eerste jaar raakte jij bij mijn onderzoek betrokken en ben jij degene geweest die geholpen heeft alle zaken weer op een rij te krijgen. Je hield mijn belangen in de gaten en hielp mijn projecten gestalte te geven. En op de momenten dat ik het even niet meer zag zitten kon ik bij jou aankloppen. Als ik na een overleg jouw kamer verliet dan zag ik het weer positief in en kon ik met frisse moed aan de slag. Ook nu ik in opleiding ben staat jouw deur voor mij open en dat voelt goed. Bedankt voor je betrokkenheid, je geduld en de koffie momenten bij Valdin.
Prof. dr. den Hollander, beste Anneke, vanaf het eerste moment was jij betrokken bij mijn onderzoek. Een bron van kennis op het gebied van genetica en moleculaire wetenschappen. Ook bij de projecten die een minder genetische insteek hadden bleef je betrokken en je wist met jouw commentaar op mijn artikelen menig project tot een hoger niveau te tillen. Jouw kwaliteiten zijn onmisbaar voor onze afdeling en het was dan ook met veel trots dat ik heb geluisterd naar jouw voordracht toen je in Seattle de Cogan award in ontvangst mocht nemen. Bedankt voor je inzet, het laagdrempelige contact en je bijdrage aan mijn projecten.
Prof. dr. van der Wilt, beste Gert Jan, het was prettig om er iemand bij te hebben die de zaken vanuit een andere invalshoek bekeek. Je wist altijd de juiste vragen te stellen waardoor ik scherp kon blijven. Helaas hebben we het grote project waar we aan gewerkt hebben niet kunnen voltooien, maar ik heb veel geleerd van het proces. Bedankt voor je kritische blik.

Daarnaast een woord van dank voor degenen die bij specifieke projecten een rol hebben gespeeld.
Dr. Theelen, beste Thomas, bedankt voor de begeleiding en betrokkenheid bij de drusenteller. And the same goes for Dr. Sánchez, dear Clarissa, thank you for your guidance in this project.

Prof. dr. Klaver, beste Caroline, bedankt voor alle keren dat ik een beroep heb mogen doen op jouw kennis en expertise. Met jouw heldere blik wist je altijd een ander licht op het probleem te schijnen.
Prof. dr. Daha, dank voor de inspirerende overlegmomenten over het complement artikel.
A big thank you to our collaborators from cologne, especially Sascha Fauser, Sandra Liakopoulos, Tina Schik, and Lebriz Altay. The EUGENDA database would not be what it is today, without our fruitful collaboration.
Dr. de Jong, beste Eiko, ook met jou heb ik mogen samenwerken aan een artikel. Je duidelijke visie en planning, en gevoel voor rechtvaardigheid zijn me bijgebleven. Daarnaast kon ik ook altijd bij jou terecht met mijn persoonlijke perikelen. Je bood een luisterend oor en wist mij ook te overtuigen bij mezelf te blijven en de juiste keuzes te maken. Dank daarvoor.
Drs. Groenewoud, beste Hans, wat een geluk dat we zo vaak op jou konden terugvallen met alle vragen over statistiek. De uren die we samen achter SPSS hebben doorgebracht zijn er nogal wat, jou was geen moeite te veel. Dank voor je geduld, je enthousiasme en onuitputtelijke drive om door te gaan tot we een antwoord hadden.

Prof. dr. Keunen, beste Jan, sinds mijn start van de opleiding val ik onder uw vleugels. Het eerste jaar is niet altijd makkelijk voor mij geweest, er was niet altijd een evenwichtige balans tussen de opleiding, de promotie en de privé-situatie. Ik heb de steun en het begrip dat ik van u heb ontvangen zeer gewaardeerd. Hetzelfde geldt voor mijn mentor. Dr. Eggink, beste Cathrien, ook jouw deur stond voor mij open en het is fijn te weten dat dat nog steeds zo is.

Dan mijn directe collega's op de werkvloer.
John, Dzenita, Mahesh, Ramon, Shazia, Nicole, Freekje, Constantin, Michel, Myrte, Nathalie, Laura, Maartje, Stanley, Roos, en Eveline. Onderzoek doen doe je samen, dat hebben jullie wel bewezen. Bedankt voor alle afleiding en gezelligheid. Ik heb enorm veel lol gehad en van jullie genoten. Van ARVO trips tot pubquiz tot swap-avondjes, bedankt! Ook nu ik in opleiding ben, maak ik deel uit van een hele leuke groep. Milan, Irene, Samuël, Linda, Carla, Paulien, Ann-Laure, John, Dzenita, Anita, Stefan, Jelina, Ellen, Ramon, Frank, Nicole, Artin, Myrte, en Anna. Bedankt voor leuke etentjes en gezellige spellenavonden, en ook op de poli kan ik op jullie rekenen. Bert en Tiny, bedankt voor alle energie die jullie in het verwerken van de samples hebben gestopt. Ik heb veel waardering voor de grenzeloze inzet en passie die jullie altijd hebben laten zien. Frederieke en Bjorn, bedankt voor de enorme inspanningen om de koppeling tussen EUGENDA en MCCD te maken en voor alle genetische bepalingen die jullie hebben gedaan. Dames (en de enkele heer) van het secretariaat (Anja, Berna, Karlijn, Charlotte, Bart en Francis) en dames van het trialcentrum (Anne, Maria, en Asha). Veel dank voor de gezellige momenten en afleiding! Ook nu ik beneden ben vind ik afleiding op de verpleegpost en de administratie, bedankt allemaal!

Een speciaal woord van dank aan mijn pafanimfen, ik had er bijna 3!
Dzenita, wat ben ik blij met jou als paranimf aan mijn zijde. Ik heb veel respect voor de keuzes die je maakt, keuzes die misschien niet altijd door iedereen gewaardeerd worden, maar waar jij wel achter staat. Dat maakt jou tot de sterke vrouw die jij bent. Ik bewonder je. Bedankt voor wat je in mij ziet en voor de vriendschap die we delen. Ramon, onze vriendschap begon in 2004 toen we in dezelfde werkgroep startten aan de opleiding geneeskunde. Wat hebben we veel meegemaakt en het is toch wel bijzonder om uiteindelijk allebei hetzelfde pad af te leggen. Ik kan altijd op je rekenen, jouw hulp lijkt soms bijna een vanzelfsprekendheid. Daarom op deze plek, zwart op wit, mijn dank voor alles wat je voor me hebt gedaan! Met jou naast me als paranimf, kan het niet meer mis gaan. Freekje, ook jij mag in dit rijtje niet ontbreken, jij bent mijn onderzoeks-maatje. De juiste persoon om even mee te sparren over de inhoud van mijn artikelen en om raad te vragen bij de statistiek en epidemiologie. En ook buiten het werk vonden we elkaar, bij Multivox! Jij maakt je dromen waar in de verenigde staten, daar heb ik veel respect voor. Helaas kan je daardoor niet mijn paranimf zijn, maar een speciaal plekje in mijn dankwoord verdien je zeker.

En ook buiten de oogheelkunde heb ik veel steun ervaren van mijn vrienden.
Lianne, Nicky, Nienke, Monique, Maaike, Wienke, Lyvonne, Joep en Ramon. Bedankt voor alle gezellige spellenavonden, weekendjes weg en etentjes. Wat fijn dat we na al die tijd nog zo'n leuke club vormen! Seline en Manja, bedankt voor de altijd gezellige avondjes samen! En zo leuk dat de club alleen maar groter wordt met de kindjes erbij! Letty, bedankt voor alle gezellige theetjes en etentjes. Wat leuk dat we nu weer meer contact hebben nadat je naar Rotterdam verhuisd bent! Sandra en Marieke, ook jullie hebben met enige regelmaat voor de nodige afleiding gezorgd. Dat er nog maar veel "spelletjes voor 9 + 1/2 + 1/2" mogen volgen! Lieve Jos en Jikke, ook jullie zijn een heel groot deel van mijn promotie heel betrokken geweest. Ik kijk met veel plezier (en een beetje heimwee) terug op een mooie tijd. Dat de corfu app nog steeds bestaat zegt volgens mij wel genoeg! Lieve Young Ones en sopranen 1 van Multivox, bedankt voor alle afleiding en gezelligheid op de maandagavonden en daarbuiten. Wat zijn we een leuke club!

Het laatste plek in het dankwoord wordt bewaard voor de meest belangrijke mensen: familie!
Lieve papa en mama. Bedankt voor jullie onvoorwaardelijke steun en liefde. Jullie staan altijd voor me klaar, ook als het jullie zelf even niet mee zit. Ik hoop dat mijn kinderen later net zo trots op mij zijn en van mij houden zoals ik van jullie.
Lieve Judith, Lotte en Jesse, ook jullie bedankt voor alle gezelligheid en fijne momenten samen. Tamirat, Lowie en Maaike mogen natuurlijk ook niet ontbreken!
Lieve oma, wat fijn dat jij er nog bent en ook dit bijzondere moment mee kan maken.
Lieve Anne, Wim en Wietze, bedankt dat jullie mij in jullie armen gesloten hebben. Nu ik meer tijd heb, kom ik ook wat vaker mee naar de boot!

Lieve Geert, jij bent er altijd voor mij geweest. Wat ben ik blij dat wij ooit samen zijn gaan 'hardlopen'. Bij jou kan ik 100% mezelf zijn en dat voelt goed! Bedankt voor al je begrip en geduld als ik weer eens hele vrijdagen en weekenden op het werk zat. Zonder jou had ik dit nooit kunnen doen.

Lieve Hanna, jij bent het beste wat mij overkomen is. Ik hou van je vrolijkheid, je knuffels, je lach. En wat een leuk vooruitzicht dat je er binnenkort een broertje of zusje bij krijgt. Je wordt vast een geweldige grote zus!

TOWARD PERSONALIZED MEDICINE IN AGE-RELATED MACULAR DEGENERATION