AMD

Dženita Smailhodžić

STUDIES ON PATHOGENESIS, TREATMENT AND PREVENTION OF AGE-RELATED MACULAR DEGENERATION

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Studies on pathogenesis, treatment and prevention of age-related macular degeneration

ISBN: 978-94-91082-05-4

Coverdesign and layout: Ruth Visser, VisserVisuals Amsterdam, The Netherlands
Printed by: Gildeprint Drukkerijen BV, Enschede, The Netherlands

Financial contribution to the production of this thesis from Radboud University Nijmegen, Rotterdamse Stichting Blindenbelangen, Stichting Blindenhulp, Koninklijke Visio, Landelijke Stichting voor Blinden en Slechtzienden, Abbot B.V., Bayer Healthcare B.V., Merck Sharp and Dohme B.V., Ergra Low vision B.V., Haags Kunstogen Laboratorium, Alcon Nederland B.V., Bartimeus B.V., Synga Medical B.V., Bausch & Lomb B.V., Chipsoft B.V., Oculenti B.V., Low Vision Totaal B.V., Ursapharm Benelux B.V.,Théa Pharma B.V., Malosa Medical B.V., Sanmed B.V., Novartis Pharma B.V., Allergan B.V., Croma B.V., Zeiss B.V., Medical Workshop B.V., Abbott Medical Optics B.V., Rockmed B.V. and Carl Zeiss B.V. is gratefully acknowledged.

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STUDIES ON PATHOGENESIS, TREATMENT AND PREVENTION OF AGE-RELATED MACULAR DEGENERATION

Doctoral Thesis

to obtain the degree of doctor

from Radboud University Nijmegen

on the authority of the Rector Magnificus prof. dr. S.C.J.J. Kortmann,

according to the decision of the Council of Deans

to be defended in public

on Friday, December 20, 2013

at 10.30 hours

by

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Proefschrift

ter verkrijging van de graad van doctor

aan de Radboud Universiteit Nijmegen

op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,

volgens besluit van het college van decanen

in het openbaar te verdedigen

op vrijdag 20 december 2013

om 10.30 uur precies

door

Dženita Smailhodžić

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Voor mijn kinderen

Wees nooit bang om je hart te volgen

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1 (gene)
AMD	Age-related macular degeneration
AOPE	Apolipoprotein E
AP	Alternative complement pathway
ARMS2	Age-related maculopathy susceptibility 2 (gene)
BM	Bruch membrane
BMI	Body-mass index
CACD	Central areolar choroidal dystrophy
CARMS	Clinical Age-Related Maculopathy Staging
CFB	Complement factor B (protein)
CFD	Complement factor D (protein)
CFH	Complement factor H (protein)
CFI	Complement factor I (protein)
CFB	Complement factor B (gene)
CFH	Complement factor H (gene)
CFI	Complement factor I (gene)
CEPT	Cholesterylester transfer protein (gene)
CI	Confidence interval
CIRCL	Cologne Image Reading Center and Laboratory
CNV	Choroidal neovascularization
CP	Classical complement pathway
CRP	C-reactive protein
cSLO	Confocal scanning laser ophthalmoscope
C2	Complement component 2 (gene)
C3	Complement component 3 (gene)
C3	Complement component 3 (protein)
C3bBb	C3 convertase
C3d	Complement C3d
C5a	Complement C5a

DNA	Deoxyribonucleic acid
ELM	External limiting membrane
ETDRS	Early treatment diabetic retinopathy study
EUGENDA	European Genetic Database
LIPC	hepatic lipase (gene)
LogMAR	Logarithm of minimal angle of resolution
LP	Mannose-binding lection pathway
LPL	lipoprotein lipase (gene)
FA	Fluorescein angiography
FD-OCT	Fourier-Domain optical coherence tomography
GA	Geographic atrophy
GWAS	Genome-wide association study
HDL	high-density lipoprotein
HTRA1	Htra serine peptidase 1 (gene)
HTRA1	Htra serine peptidase 1 (protein)
OCT	Optical coherence tomography
ONL	Outer nuclear layer
OR	Odds ratio
PDT	Photo dynamic therapy
PED	Pigment epithelial detachment
PRPH2	Peripherin-2 (gene)
RPE	Retinal pigment epithelium
SE	Standard error
STGD1	Stargardt disease
VA	Visual acuity
VEGF-A	Vascular endothelial growth factor- A (gene)
VEGF-A	Vascular endothelial growth factor- A (protein)
TCC	Terminal complement complex

CHAPTER ONE

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

Affecting an estimated 30-50 million individuals, age-related macular degeneration (AMD) has reached epidemic proportions among the elderly and is a leading cause of blindness in Western society.[1] AMD is a complex, multifactorial disease that manifests clinically as a loss of central vision due to the loss of the central retina such that the patient can no longer read, recognize faces or discriminate colors.[2] Pathologically, AMD is characterized initially by changes in the retinal pigment epithelium (RPE), followed by RPE and photoreceptor cell loss and/or secondary angiogenesis.[2] In patients with the neovascular form of AMD, new choroidal blood vessels invade the subretinal and retinal spaces, causing exudation, hemorrhage and a rapidly progressive loss of vision. The majority of AMD patients with severe visual impairment have lost their central vision as a result of neovascular AMD.[3-6] Despite the benefits of intraocular injections of vascular endothelial growth factor A (VEGF-A) inhibitors, a large percentage of neovascular AMD patients continue to lose vision.[7,8] In patients with the advanced non-exudative form of AMD—more commonly known as geographic atrophy—vision declines at a slower rate, as atrophy of the RPE and photoreceptor cells progresses in the posterior pole. Unfortunately, no effective therapy for treating geographic atrophy has yet been developed.[9]

1. STAGES OF AMD

1.1 Early AMD

Visual acuity is relatively preserved in most patients with early stage AMD. The hallmark lesions of early AMD are drusen, focal extracellular deposits between the RPE and the inner layer of Bruch membrane (Figure 1; 2a-3b).[2] Early AMD is also characterized by a thickening, loss of normal architecture within Bruch's membrane and lipofuscin accumulation in the RPE.[10]

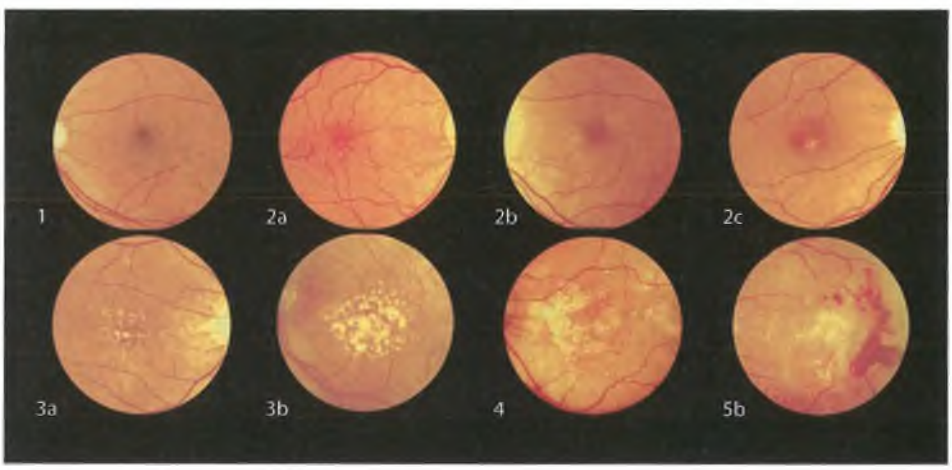
1.2 Late AMD

In time, early AMD can progress to late AMD. Late AMD can be classified into two clinical subtypes: geographic atrophy and neovascular atrophy. Geographic atrophy is characterized by hypo pigmented atrophic zone co-localized with RPE cell atrophy, degeneration of overlaying outer retinal layers which results in thinning of the retina (Figure 1; 4). Hyperpigmentation associated with compensatory RPE cell proliferation is commonly observed at the periphery of hypopigmented areas.[2] The defining characteristic of neovascular AMD is choroidal neovascularization (CNV), which is the formation of new blood vessels that arise primarily from the choroidal vessels that penetrate Bruch's membrane and infiltrate the subretinal and the retinal space. Those new blood vessels cause serous RPE detachment, hemorrhage and macular scarring (Figure 1; 5b).

1.3 Clinical Age-Related Maculopathy Staging (CARMS)

The increasing impact of AMD on society has led to a significant increase in both clinical and basic AMD research. The various stages of the disease must be defined clearly, and an accurate clinical diagnosis is essential in determining the prognosis and clinical management strategy. The classification of AMD for clinical studies has typically been based on the evaluation of stereophotographs at reading centers using standardized classification protocols.[11] The detailed AMD classification schemes involve the complex assessment of various parameters, including the number, area and size of drusen within multiple zones centered on the fovea. These schemes are difficult to apply in clinical practice, given the amount of time and the level of training required to use them properly. An alternative can be found in the Clinical Age-Related Maculopathy Staging (CARMS) system, which is a validated, reliable and easy-to-use grading scheme for both patient management and research purposes (Figure 1).[12]

FIGURE 1. Examples of age-related maculopathy grades based on the Clinical Age-Related Maculopathy Staging (CARMS) system.



1. No drusen.
2a. Several small drusen, but no retinal pigment epithelium (RPE) changes.
2b. RPE changes, but no drusen. 2c, Both small drusen and RPE changes.
3a. Several intermediate-size and large drusen.
3b. Drusenoid RPE detachment.
4. Geographic atrophy.
5b. Choroidal neovascular membrane with disciform scar.
Note: grade 5a (not shown) is serous RPE detachment.

2. IMAGING TECHNIQUES AS A DIAGNOSTIC TOOL OF AMD

AMD is best diagnosed using a combination of imaging techniques such as color fundus photography, fundus autofluorescence (FAF) and high-resolution spectral-domain optical coherence tomography (SD-OCT). These techniques are discussed in detail below.

2.1 Color fundus photography

The most commonly used imaging technique for diagnosing AMD is fundus photography. This technique simply consists of photographing the interior posterior surface of the eye—the retina, optic disc, macula and posterior pole—with the fundus photocamera.

2.2 Spectral-domain optical coherence tomography

High-resolution spectral-domain optical coherence tomography (SD-OCT) is a non-invasive technique that allows the clinician to acquire cross-sectional images of the retina within seconds (Figure 2). SD-OCT captures live images at near-microscopic resolution, thereby facilitating the identification of subtle morphological changes in the retina. Moreover, because this technique is non-invasive and does not use ionizing radiation, it is both easy to use and safe for the patient.

FIGURE 2. Image of a retina captured using SD-OCT.

SD-OCT scan through the fovea of a normal retina of a 65-year-old male. Different hyper-reflective bands can be defined that can be correlated with the anatomical layers of the outer retina. Outer nuclear layer (ONL); (1) external limiting membrane (ELM); (2) interface of the inner and outer segments of the photoreceptor layer (IPRL); (3) outer segment – RPE interdigitation; and (4) RPE/Bruch's membrane complex (RPE/BM).

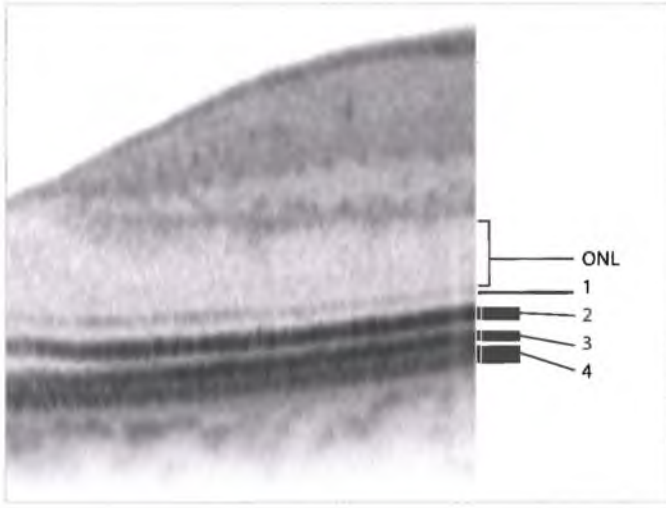
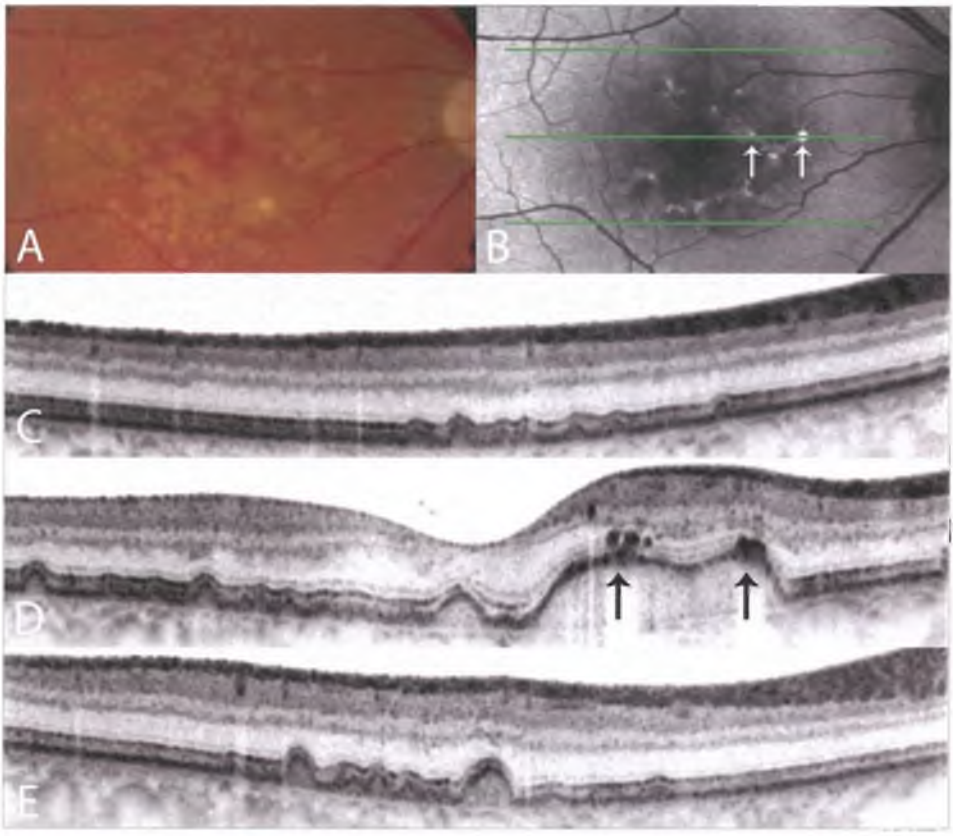


FIGURE 3: Subretinal pigment epithelium deposits in early AMD.
The dome-shaped elevations of the outer retinal layers in the SD-OCT scans (C–E) correspond to soft drusen visible by color fundus photography (A). Hyperpigmentation of the fundus corresponds to an increased FAF signal (B) and hyper-reflective structures that overlay the drusen (visible in the SD-OCT scan) (D). The green line indicates the position of the SD-OCT scans.



2.3 Fundus autofluorescence (FAF)

FAF imaging is a relatively new non-invasive imaging method that is used to create a topographic map of autofluorescent substances (e.g., lipofuscin) in the RPE (Figure 2B). Lipofuscin is a waste product that accumulates in the cytoplasm of RPE cells as a result of continuous phagocytosis of outer-segment disks that are shed by photoreceptor cells. The accumulation of lipofuscin in the RPE is a hallmark of aging, and the accumulation of excess lipofuscin granules in RPE cells contributes to the pathogenesis of various retinal diseases, including AMD. Increased autofluorescence is indicative of lipofuscin accumulation and RPE dysfunction, whereas an absence of autofluorescence reflects areas with a complete loss of the photoreceptor/RPE functional unit.

3. RISK FACTORS FOR THE DEVELOPMENT OF AMD

3.1. Advanced age is a high risk factor of AMD

AMD progresses over a long time frame, and the prevalence of AMD increases steeply over the age of 70. Nearly 30% of Americans over the age of 75 have signs of early AMD, and 7% have late-stage disease.[5, 13, 14] A sharp age-related increase in the prevalence of AMD was reported in data pooled from the Baltimore Eye Survey, the Blue Mountains Eye Study, the Beaver Dam Eye Study, the Rotterdam Study, the Melbourne Vision Impairment Project and the Salisbury Eye Evaluation Project. Among white females 50-54 years of age, 0.2% had late stage AMD, 1.5% for white females 70-74 years of age, and up to 16% of white women over the age of 80 had neovascular AMD and/or geographic atrophy. Because the average age of the general population is increasing rapidly, the prevalence of AMD is estimated to increase by 50% by the year 2020.[1]

3.2 Smoking and obesity are the strongest environmental risk factors for AMD

The relationship between smoking and AMD was investigated in numerous cross-sectional studies, cohort studies and case-control studies. The majority of these studies reported a statistically significant association between smoking and the AMD; in fact, smokers have a relative risk of developing late AMD that is 3.9-fold higher than the risk for non-smokers. Possible mechanisms by which smoking increases the risk of AMD include impaired antioxidant synthesis (e.g., impaired plasma vitamin C and carotenoid synthesis), induction of hypoxia, the generation of reactive oxygen species and an alteration in choroidal blood flow.

Both fat intake and obesity have also been associated with an increased risk of developing AMD.[15-17] Relative to the lowest-risk category, the relative risk of patients with a body-mass index (BMI) of ≥30 was 2.35, and relative risk was 2.32 for patients with a BMI of 25-29. The mechanism(s) by which obesity increases the risk of developing AMD may be related to increased oxidative stress,[18-20] increased inflammation,[21] and/or changes in lipoprotein profile.[22] These factors can also cause increased macular destruction and a decrease in the delivery of lutein and zeaxanthin to the macula of the eye.[22]

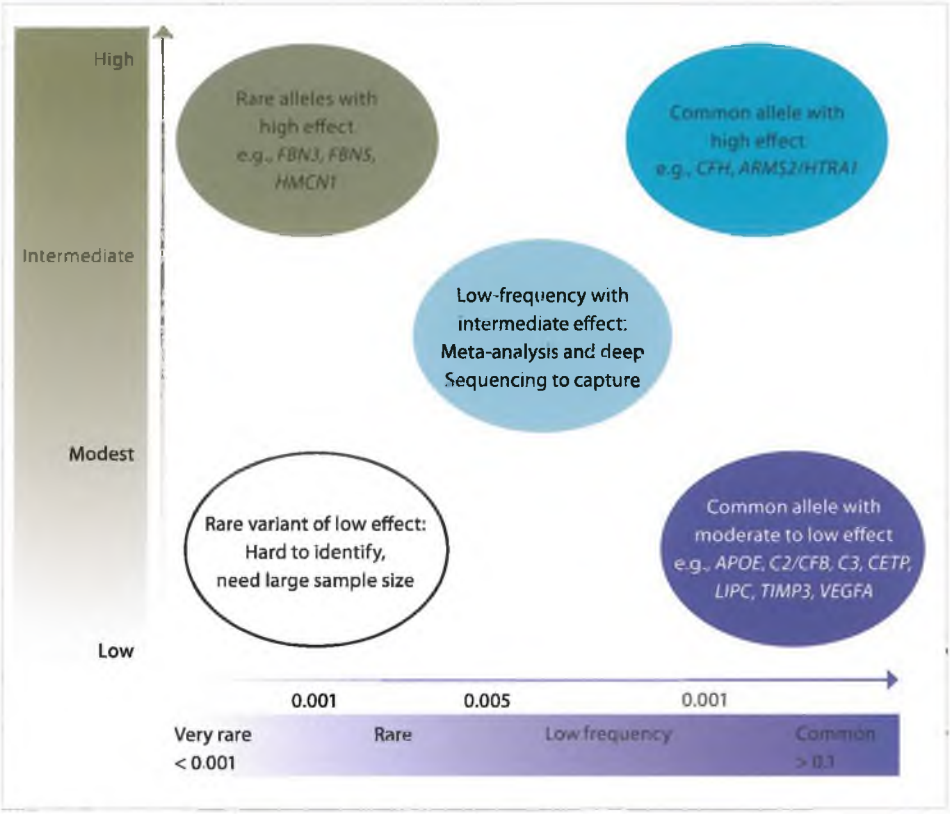
3.3 Genetic risk factors for AMD

Familial and twin-based studies have revealed that AMD pathogenesis has a strong genetic component. AMD may in fact be the best example of a highly prevalent disease in which genome-wide association studies have identified several common genetics variants that have relatively large effects.[23] A significant breakthrough in the AMD field was the identification of a strong association between AMD and a coding variant (Tyr402His) in the complement factor H (*CFH*) gene,[24-26] which encodes the main regulator of the alternative complement pathway.

A strong association was also found between AMD and a haplotype on chromosome 10q26, which contains the *ARMS2* and *HTRA1* genes.[27, 28]

Due to the strong effect of these variants, these associations were detected in relatively small sample sizes. Genome-wide association studies using larger patient cohorts and higher-resolution genotyping microarrays identified several associations with smaller effects between AMD and genetic variants in other complement factor genes (*C2/CFB, C3* and *CFI*)[29-32] and in genes encoding members of the high-density lipoprotein cholesterol pathway (*APOE, LIPC, CETP*)[33, 34] the extracellular collagen matrix pathway (*TIMP3, COL8A1, COL10A1*) and vascular endothelial growth factor (*VEFGA* and *TGFBR1*) (Figure 4).[35]

FIGURE 4. Genetic architecture of age-related macular degeneration.
From Priya RR, Chew EY, Swaroop A. Genetic Studies of Age-related Macular Degeneration : Lessons, Challenges, and Opportunities for Disease Management. Ophthalmology. 2012 Dec;119:2526-36.



4. PATHWAYS INVOLVED IN AMD PATHOGENESIS OF AMD

4.1 The role of the complement pathway in AMD

In the past ten years, significant progress has been made towards unraveling the pathogenic mechanisms that underlie AMD. Three separate lines of evidence have emerged to implicate chronic inflammation and complement activation as important factors driving the development of AMD and have formed the basis of a new paradigm in describing AMD pathogenesis. The first evidence dates from the mid 1990's and involves drusen, the hallmark lesions of developing AMD. Drusen are pathological deposits of extracellular material that form between the RPE and the stratified extracellular matrix known as Bruch's membrane.[17] At that time, proteins of the complement cascade were identified in drusen.[36] In the following years, it became clear that drusen contain alternative complement pathway proteins such as CFH and C3, the products of alternative complement activation and the terminal pathway proteins C5, C6, C7, C8, C9 either independently or in combination with the membrane attack complex.[37-41] This evidence indicates that local, complement-mediated inflammation occurs in the retina in AMD. The compositional profile of drusen has formed the basis of a hypothesis in which drusen are regarded as by-products of chronic local inflammatory events at the interface between the RPE and Bruch's membrane.[37, 40] Complement-mediated inflammation may be the first step in both drusen formation and the development of AMD.[42]

Numerous genetics studies have provided a second line of evidence to support the inflammation model of AMD pathogenesis. The strongest genetic association with AMD was found for genetic variants in the complement factor H (*CFH*) gene.[24-26] CFH is the primary inhibitor of the alternative complement pathway, which—due to the continuous activation of this pathway—plays a particularly important role in preventing the inappropriate overactivation of this pathway and subsequent damage to the body's own tissues.[31] Several minor AMD susceptibility loci were later found to encode complement component C3, factor B and factor I, thus further establishing a role for the alternative complement pathway in AMD pathogenesis.[29-32]

A third line of evidence supporting complement involvement in AMD came from studies that showed that AMD patients have higher levels of complement activation products in their blood.[43, 44] This increased concentration of activation products in circulating blood indicates that AMD-associated inflammation is not restricted to the retina, but is systemic.

4.1.1 How does the complement system lead to AMD?

The complement system is an ancient defense system and a major component of innate immunity that is unchanged throughout an individual's lifetime. The complement system can generate toxic activation products and plays crucial roles in the first line of defense to protect the

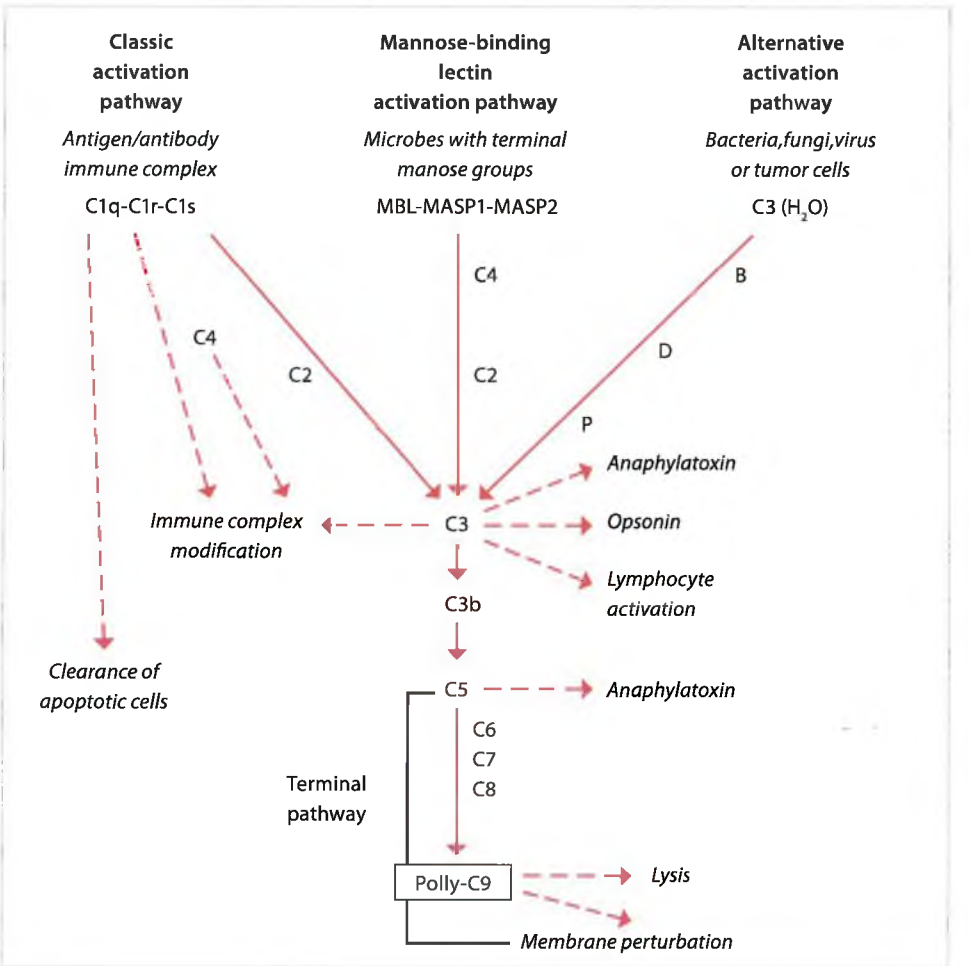
host from invading microorganisms, modulating the adaptive immune response and clearing apoptotic cells. The complement system consists of more than 30 proteins that circulate in the blood, usually as inactive pro-proteins. Activation converts an inactive pro-protein into an active protein; once activated, the complement cascade produces powerful anaphylatoxins such as C3a, C5a and the membrane attack complex (MAC), resulting in the phagocytosis of antigens (opsonization), the attraction of macrophages and neutrophils (chemotaxis), the rupture of the membranes of foreign cells (cell lysis) and the clumping of antigen-bearing agents (Table 1 and Figure 5).[9-11] These activation fragments are non-discriminatory and can therefore be delivered to the surface of any cell. Thus, the complement system can be extremely damaging to host tissues. Therefore, the toxic activity of the activated complement system must be focused at the surface of invading microorganisms while simultaneously sparing normal neighboring host cells. Complement activation is tightly regulated by several inhibitory proteins that are present in the plasma at considerably higher concentrations than the complement proteins themselves. The combination of integral cell membranes and the presence of regulatory proteins blocks complement effector molecules on the surface of host cells and protects these cells from the complement system.[8, 12, 13]

TABLE 1. The three main physiologic activities of the complement system

Activity	Complement protein responsible for activity
Host defense against infection	
Opsonization	Covalently bound fragments of C3 and C4
Chemotaxis and activation of leukocytes	Anaphylatoxins (C5a, C3a, and C4a); anaphylatoxin receptors on leukocytes
Lysis of bacteria and cells	Membrane-attack complex (C5b–C9)
Interface between innate and adaptive immunity	
Augmentation of antibody responses	C3b and C4b bound to immune complexes and to antigen, C3 receptors on B cells and antigen-presenting cells
Enhancement of immunologic memory	C3b and C4b bound to immune complexes and to antigen, C3 receptors on follicular dendritic cells
Disposal of waste	
Clearance of immune complexes from tissues	C1q, covalently bound fragments of C3 and C4
Clearance of apoptotic cells	C1q, covalently bound fragments of C3 and C4

FIGURE 5: The three complement activation pathways: the classical, mannose-binding lectin and alternative pathways.

Three pathways for complement activation have been identified—the classical pathway, the lectin pathway and the alternative pathway. Each of these pathways represents a unique method for activating the C3 molecule, initiating pro-inflammatory reactions and activating the terminal complement pathway. The basic steps of every complement pathway can be summarized as follows: initiation, formation of a C3 convertase, cleavage of C3, formation of a C5 convertase, cleavage of C5, and formation of the membrane attack complex. The classical and lectin pathways are activated by the binding of pathway-specific recognition molecules to their respective ligands. The classical pathway is activated by the binding of the C1complex to antibodies that are bound to an antigen on the surface of a bacterial cell. The recognition molecule of the classical pathway is C1q. The lectin pathway is activated by the binding of the mannose-binding lectin (MBL) on the surface of a bacterial cell. In contrast to the other two pathways, the alternative pathway is a spontaneous and constantly activated immune surveillance system without specific recognition molecules.



A single-nucleotide polymorphism (SNP) in a single complement gene can lead to deficient complement inhibition, thereby causing an uncontrolled inflammatory reaction and tissue-damaging effects, ultimately causing disease.[14-16] This may explain why individuals with diverse variants in complement system genes—as in the case of AMD—can respond to the same environmental triggers with differing levels of (harmful) inflammation. Changes in the retina occur during aging and can include alterations in the size and shape of RPE cells, thickening of Bruch's membrane, thickening of the internal limiting membrane and a decrease in retinal neuronal elements. These age-related changes in the retina may facilitate the development of AMD in elderly individuals with a genetic predisposition such as the coding variant Tyr402His in the *CFH* gene.

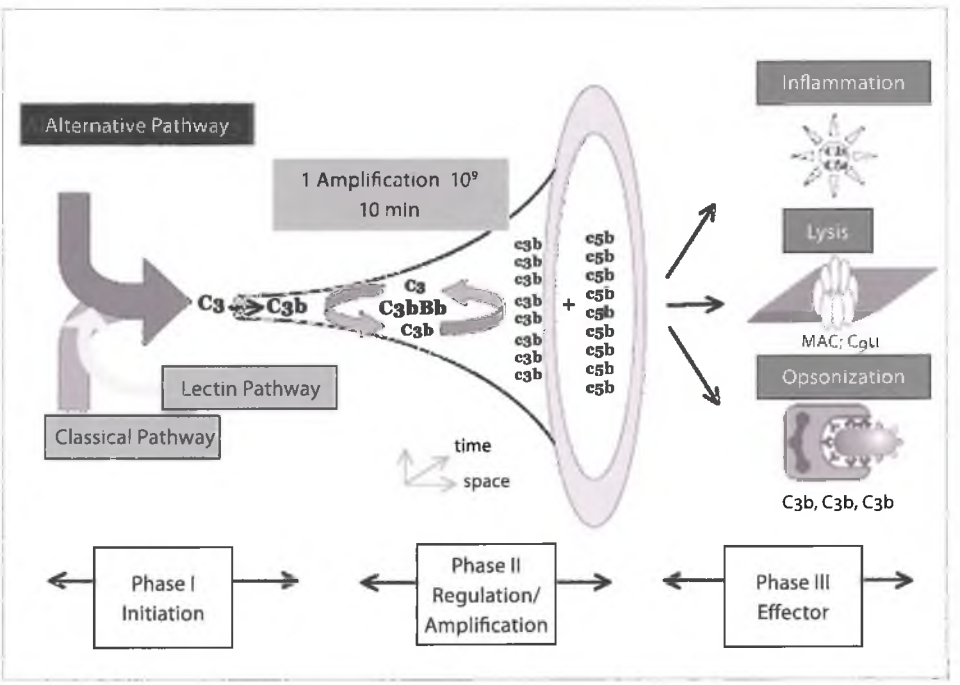
4.1.2 Activation and control of the alternative complement pathway

The alternative pathway is part of the innate immune defense system that provides one of the first—if not the first—level of protection against invading microbes; this pathway can be activated within one second after the host comes into contact with a microbe.In the initiation phase, C3—the "start" molecule of the alternative complement pathway—undergoes spontaneous hydrolysis into C3(H2O). This process is called the "tick-over" and occurs constitutively at a slow rate. Although not cleaved, the C3(H2O) molecule binds to Factor B, which is then cleaved—with the help of Factor D—into the Ba and Bb fragments (Figure 5). The newly generated C3(H20)Bb molecule is a C3 convertase that cleaves C3 into C3a and C3b. C3b can be deposited on any cell surface, where it can form a cluster (Figure 6). This initial step is non-discriminatory and can occur on both host cells and invading microbes. The second phase—the amplification/regulation loop—distinguishes between foreign and host cells and then determines whether complement activation must be inhibited or amplified (Figure 6). Strong amplification and unrestricted activation results from contact with an "unprotected" foreign surface. Unlike foreign microbes, the host's cells are equipped with membrane regulators such as DAF (CD55), MCP (CD46) and CR1 (CD35) and can also utilize fluid-phase regulators such as Factor H and Factor H-like protein 1 (FHL-1). Contact with a "protected" surface inhibits the cascade and terminates the reaction.

4.2 The chromosome 10q26 locus

The second major AMD susceptibility locus 10Q26 contains two genes, *age-related maculopathy susceptibility 2 (ARMS2)* and *Htra serine peptidase 1 (HTRA1)*.[27, 28, 45-47] Whether either of these genes plays a role in AMD is currently unclear.[27] A locus is the specific place on a chromosome where a gene is located. Several SNPs and an insertion-deletion (InDel) variant have been identified within a region 10q26 that has strong linkage disequilibrium, spans both genes and constitutes a high-risk haplotype.

FIGURE 6. Activation of the alternative complement pathway proceeds in two steps.
Phase I: **The Initiation Phase** is initiated by the spontaneous activation of C3. In phase II, **the amplification loop**, a spontaneously formed C3 convertase is stabilized by properdin, and high levels of C3b are generated and lead to the formation of the more efficient C3 convertase C3bBb. In this positive feedback loop, C3bBb activates more C3, generates C3a and C5a, which then initiate inflammation, producing more C3b and thus more C3bBb. Under certain circumstances, activation of the terminal pathway is a vital end result in cell lysis. However, in many situations, the release of the anaphylatoxins C3a, C4a and C5a or the formation of the opsonins C3b and iC3b may be more important. If activation proceeds to **the third phase**, the effector response is initiated, resulting in (1) the release of anaphylactic C3a and C5a, (2) composition of membrane attack complex (MAC), and (3) the opsonization of the foreign surface with C3b and phagocytosis.



The putative association between the *ARMS2* gene and AMD susceptibility has been supported by numerous independent studies.[28,48-51] Heterozygozity at the *ARMS2/LOC387715* (A69A/A69S) locus is associated with an odds ratio (OR) of 1.69–3.0 for advanced AMD, and homozygosity for the risk-conferring allele (A69S/A69S) is associated with an OR of 2.20–12.1 (after adjusting for demographic and behavioral risk factors).[49] However, the function of the ARMS2 protein and its subcellular localization are controversial.[49-52] One study found that the *ARMS2* gene encodes a mitochondrial protein, whereas another study suggested that ARMS2 is cytoplasmic, and a third study suggested that ARMS2 is secreted as an extracellular protein. Interestingly, the third

study identified that ARMS2 is a constituent of extracellular matrix providing a link between sporadic familial macular degeneration and age-related macular degeneration.[53]

The *HTRA1* gene is adjacent to the *ARMS2* gene in the 10q26 locus. Several studies have found that the rs11200638 SNP in the *HTRA1* promoter has also a strong association with AMD, with an OR of 1.60–2.61 and 6.56–10.0 in heterozygous and homozygous individuals, respectively.[47, 54-58] Elevated levels of *HTRA1* mRNA and/or HtrA1 protein in the drusen and choroidal neovascularization (CNV) components of AMD patients carrying the AA risk genotype have been reported in several studies.[47, 54, 59, 60] Moreover, evidence suggests that HtrA1 plays roles in both extracellular protein degradation and cellular growth or survival. Under chronic inflammatory conditions, the extracellular protease activity of HtrA1 may promote neovascularization by enhancing the degradation of extracellular matrix components through the increased expression of matrix metalloproteinases,[61] or by binding to transforming growth factor beta (TGF-β), an angiogenic factor whose expression has been linked to CNV.[62]

Fritsche et al. (2008) reported that the InDel polymorphism *372_815delins54 in the 3'-UTR of the *ARMS2* gene is also significantly associated with AMD.[52] Because the A69S and InDel polymorphisms are in perfect linkage disequilibrium and reside in the same haplotype, their effects are not independent. Although the precise biological functions and the potential roles of ARMS2 and Htra1 in AMD pathogenesis remain unknown, the strong effect of the 10q26 locus might be explained by a contribution of both genes. Consistent with this hypothesis, the InDel polymorphism can destabilize *ARMS2* mRNA while upregulating *HTRA1* gene transcription.[63]

4.3 High-density lipoprotein and Extracellular/collagen matrix pathway

Epidemiologic studies have indicated a link between cardiovascular risk factors and incidence of AMD. Cholesterol and lipids accumulate underneath the RPE with age and are present in the drusen.[64-67] Genetic variants that impact cholesterol levels in the macula and RPE, might impact drusen formation and thus modulate the risk of AMD. Alleles near *the cholesterylester transfer protein (CEPT), the lipoprotein lipase (LPL), the hepatic lipase (LIPC)* and *the ATP-binding cassette transporter A1 (ABCA1)* are associated with the change in high-density lipoprotein (HDL)-c levels in blood and are associated with increase but also with decreased the risk of AMD.[33, 34]

TIMP3 gene is also associated with increased the risk of AMD.[33] TIMP3 inhibits matrix metalloproteinases (MMP) and is involved in degradation of the extracelilular matrix.[53] It can also inhibit vascular endothelial factor (VEGF)-mediated angiogenesis independent of its MMP-inhibitory activity.[68] Mutations in this gene can cause Sorsby fundus dystrophy, an autosomal dominant macular dystrophy with clinical features similar to AMD and an early onset before 40 years.[69] This may indicate the important role of the extracellular matrix in the pathogenesis of AMD.

4.4 Angiogenesis signaling pathway

In the neovascular manifestation of AMD, pathological blood vessels arising from the choroid invade the retina and cause foveal hemorrhaging and scarring. Under physiological conditions, vascular endothelial growth factor (VEGF) signaling is usually the critical rate-limiting step in angiogenesis. The growth and maturation of new vessels are highly complex and coordinated processes that require the sequential activation of a series of receptors by numerous ligands.[70] VEGF-A is a key molecule in promoting angiogenesis and can cause vascular leakage and inflammation by triggering the increased permeability of capillary endothelial cells.[71, 72] VEGF-A and two receptors VEGFR-1 and VEGFR-2 regulates growth of blood vessel in physiological angiogenesis during embryogenesis,[73, 74] skeletal growth,[75, 76] and reproductive processes.[77-79] However, VEGF-A has also been implicated in pathological angiogenesis in malignant growths,[80] inflammation,[71] brain edema,[81, 82] and intraocular neovascularization. Intraocular neovascularization occurs as a consequence of diabetes mellitus,[83] occlusion of the central retinal vein,[84] retinopathy of prematurity,[85, 86] and AMD.[87-91] All of these conditions are associated with retinal ischemia. An increased expression of VEGF-A in the RPE and the outer nuclear layer has been reported in post-mortem maculae that were obtained from individuals with AMD.[88] The identification of VEGF-A as an important factor in the pathogenesis of AMD has led to the development of anti-VEGF-A therapies for neovascular AMD.[92]

5. TREATMENT OF NEOVASCULAR AMD

5.1 A sequence of improving therapies for treating neovascular AMD

Choroidal neovascularization (CNV) is the principal cause of severe AMD-associated vision loss. For many years, a diagnosis of CNV heralded an unstoppable decline in central vision in the majority of patients. Fortunately, however, in the past decade a series of improved therapies has emerged. The first breakthrough therapy was laser photocoagulation followed by photodynamic therapy (PDT) using benzoporphyrin (verteporfin, Visudyne; Novartis, Basel, Switzerland).[93] Although PDT therapy with verteporfin—which was approved for use in Europe in 2002—stems vision loss, it does not improve vision in the majority of patients.[94] The identification of VEGF-A as an important factor in the pathogenesis of neovascular AMD has led to the development of anti-VEGF-A therapies. The first anti-VEGF-A therapy, pegaptanib (Macugen; Pfizer, New York, NY) was approved in Europe in 2006 for the treatment of neovascular AMD.[93] Intravitreal pegaptanib slows vision loss but does not improve vision,[93] in contrast, ranibizumab was the first drug to reduce CNV and significantly improve central vision.[92, 95]

5.2 Current anti-VEGF-A therapies for treating neovascular AMD

Ranibizumab (Lucentis; Novartis, Basel, Switzerland; and Genentech Inc., South San Francisco, CA) is a humanized monoclonal antibody Fab fragment that inhibits all active forms of VEGF-A. In 2006, ranibizumab was approved by the US Food and Drug Administration for the treatment of AMD-associated choroidal neovascularization.[92,96] While awaiting approval for ranibizumab, ophthalmologists began treating patients with neovascular AMD using an off-label application of bevacizumab (Avestin, Genentech), a low-cost full-length anti–VEGF-A antibody with similar target specificity to ranibizumab.[97, 98] Bevacizumab is approved for treatment of metastatic colorectal cancer, advanced nonsquamous non–small cell lung cancer, metastatic kidney cancer and glioblastoma but not for AMD. Despite a lack of large-scale clinical trial data to support its use, bevacizumab become the most commonly used drug for treating neovascular AMD in the United States. In the Comparison of Age-Related Macular Degeneration Treatment Trials (CATT), 1208 neovascular AMD patients were randomly assigned to receive intravitreal injections of ranibizumab or bevacizumb either monthly or as needed with monthly evaluations.[8] After two years of treatment, bevacizumab and ranibizumab had similar effects on visual acuity when administered using the same schedule. An as-needed treatment regimen has been widely accepted in place of the monthly regimen that was tested in the trial. Thus, neovascular AMD patients are now treated initially with three monthly intravitreal anti-VEFG-A injections, followed by pro re nata (meaning "as needed") guided re-injections whenever signs of CNV activity are detected by ophthalmoscopy, SD-OCT or fluorescein angiography.

Anti-VEGF therapy is effective and is currently the standard treatment for neovascular AMD. The current treatments for neovascular AMD are designed to reduce the ongoing VEGF-stimulated new vessel growth; however, these treatments do not address the underlying pathology. Because of this, neovascular AMD patients are being re-injected with anti-VEGF on the regular basis, until they develop irreversible and profound retinal damage. Unfortunately, early AMD and geographic atrophy have no effective therapy.

6. PREVENTION OF AMD

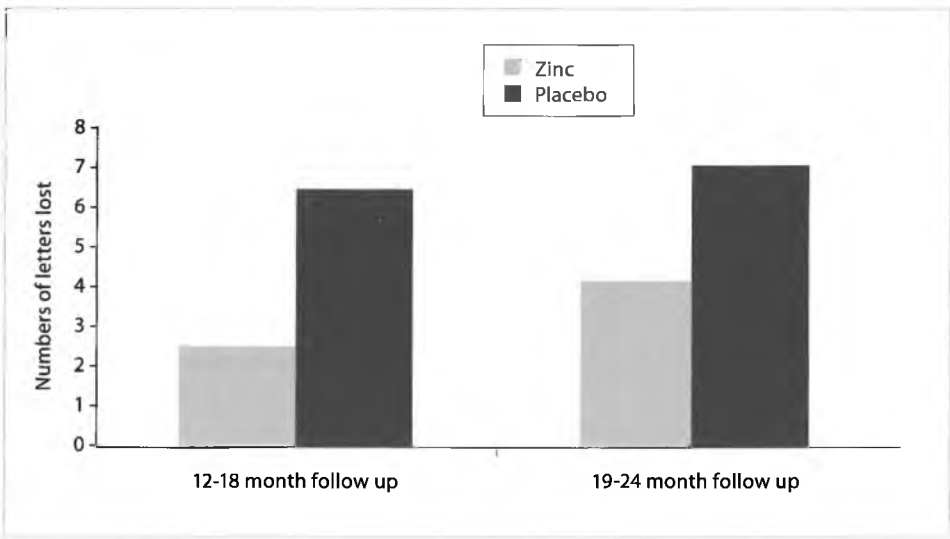
6.1 Dietary and supplement intake of antioxidants

Increased oxidative stress occurs during aging and has been linked to many chronic inflammatory diseases, including AMD. Like other chronic conditions such as hypertension and cardiovascular disease, diet can play a role in AMD. The current lack of effective treatment modalities—coupled with evidence supporting oxidative pathogenesis—has sparked interest in the potential preventive role of nutritional supplements that contain antioxidants such as zinc.

6.2 The first studies on zinc supplementation in AMD

Zinc is required for the catalytic, structural and regulatory functions of more than 300 enzymes.[99] In addition to its antioxidant effects, zinc is also an anti-inflammatory agent,[23] and zinc ions are essential for the normal development, differentiation and function of cells involved in both the innate and acquired immune systems.[25,26] This abundance of the functions of zinc has sparked the interest of researchers in many fields to investigate a beneficial effect of the supplementatio of zinc. In 1988, Newsome and co-workers published the results of a double-blind, randomized, placebo-controlled study of the effect of oral zinc sulphate on the visual acuity outcome in AMD.[100] Based on the results of this study, the investigators concluded that zinc slows the progression of AMD (Figure 7).[100] However, an analysis of the data revealed that this trial was unlikely to detect differences smaller than 72% between treatments and the results should therefore be viewed with skepticism.[101] In 1996, Stur et al. reported the effect of zinc supplements on the progression of exudative AMD in the second eye.[102] The subjects were randomly allocated into two groups; one group received 200 mg of zinc sulphate daily, and the other group received a placebo. The investigators concluded that oral zinc treatment had no short-term effect on the course of AMD in patients with an exudative form of the disease in one eye.[102] Despite a lack of convincing evidence, the marketing and use of antioxidants—and zinc in particular—in eye-targeted formulations has become common practice.

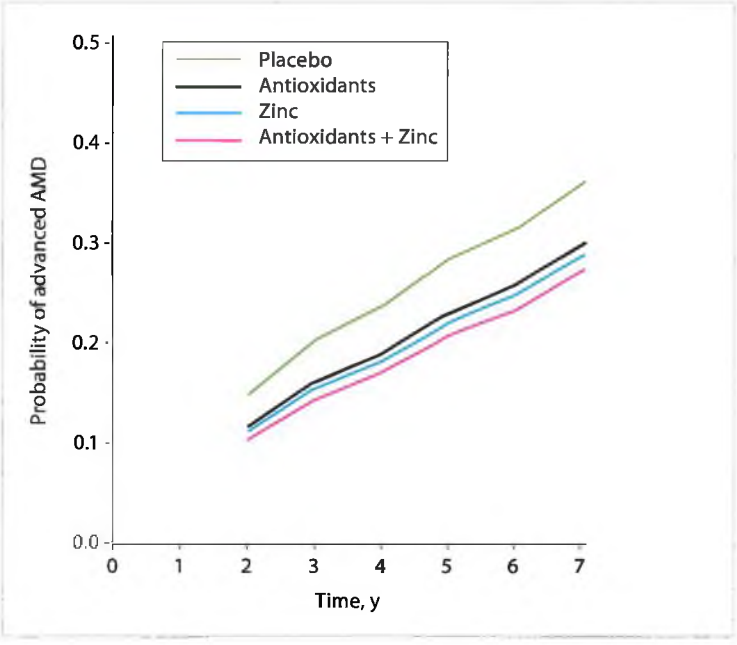
FIGURE 7: Visual acuity loss with time in the Newsome et al. (1988) study.
The effect of administering 200 mg of oral zinc sulphate, on the visual acuity outcome, in 151 subjects with AMD. After both 12 and 24 months of treatment, the zinc-treated patients had significantly less vision loss than the patients who received placebo.[100]



6.3 Zinc can reduce progression of AMD

Because of public health concerns regarding the widespread use of untested high-dose antioxidants and zinc supplements for treating AMD, the National Eye Institute at the National Institutes of Health in Bethesda, MD conducted the Age-Related Eye Disease Study (AREDS).[11] In this randomized, clinical trial, 3640 AMD patients were divided into four groups and received (1) antioxidants, (2) zinc, (3) antioxidants plus zinc, or (4) a placebo. In 2001, the data collected from AREDS revealed that patients who were treated with zinc—either alone or in combination with vitamins—had reduced progression from intermediate to advanced AMD (Figure 8).[11] The effect was not seen with antioxidants alone, or in subjects with earlier or later stages of disease. Based on these results, AREDS recommends that persons who are older than 55 years of age and who are at risk for developing advanced AMD should consider taking vitamin supplements plus zinc.[11]

FIGURE 8. Probability of advanced AMD by treatment arm in AREDS.
AMD patients were divided into four groups and received (1) antioxidants, (2) zinc, (3) antioxidants plus zinc, or (4) a placebo. The primary outcomes were progression to advanced AMD (an AMD event) and a 15-letter or more decrease in the visual acuity score (a VA event). An AMD event was defined as the development of neovascular AMD or the development of geographic atrophy that involved the central macula. Estimates of risk ratios derived from odds ratios suggest that risk is reduced by 17% and 21% for individuals taking either antioxidants or zinc, respectively. Individuals who took antioxidants together with zinc had a 25% reduction in risk.[11]



A population-based study published in 2008 by the Blue Mountains Eye Study confirmed the long-term beneficial effect of zinc in AMD patients.[103] More recently, the Rotterdam Study revealed that a diet rich in zinc, β-carotene, lutein/zeaxanthin and omega-3 fatty acids reduces the risk of developing early AMD.[9] In this population-based study, diets were assessed, and investigators assessed biological interactions with genetic variants by calculating the synergy index. The finding supported the hypothesis of biological interactions between the *CFH* gene Y402H variant and zinc, β-carotene, lutein/zeaxanthin and omega-3 fatty acids and between the *ARMS2* gene A69S variant and zinc and omega-3 fatty acids.[9] As a result of these findings, the Rotterdam Study recommended that clinicians give dietary advice to young individuals who are at risk in order to delay or even prevent AMD-related vision loss.[9] Despite the widespread use of zinc and antioxidants among AMD patients, the mechanism by which zinc exerts its beneficial effects in AMD patients has not yet been identified.

7. AIMS AND OUTLINE OF THESIS

Advanced age is a high risk factor for developing late AMD, and because the average age of our population is increasing, the prevalence of this widespread disease is on the rise. Therefore, in coming years, there will be an increasing need for novel preventive and therapeutic strategies. The design of optimal and appropriate therapies will require a comprehensive understanding of the factors that drive the pathogenesis of AMD. This thesis deals with three aspects of AMD: i) diagnosis of AMD, ii) systemic immunologic biomarkers and genetic variation that play a role in the development of AMD, iii) and whether these markers and genetic variation can be used to determine the effectiveness of current prevention and treatment options.

Chapter 1. In the past ten years, significant progress has been made towards unraveling the pathogenic mechanisms that underlie AMD. Chapter 1 provides the reader summary of the pathogenic mechanisms that underlie AMD. Next to this, current treatment and prevention options for AMD are highlighted. The background information in chapter 1 can be regarded as a starting point for this research project.

Chapter 2. Late-onset central areolar choroidal dystrophy (CACD) can easily be confused with geographic atrophy (GA) in AMD. Distinguishing AMD from AMD-mimicking late-onset macular dystrophies is essential both for determining a prognosis and clinical management strategy, and for research purposes. In this chapter we revealed identified several morphologically distinguishable features between CACD and AMD based on non-invasive novel imaging tools such as high-resolution spectral-domain optical coherence tomography (SD-OCT) and fundus autofluorescence (FAF).

Chapter 3A. Recently, two GWASs identified additional genetic markers, thus identifying novel AMD pathways. In Chapter 3, we analyzed the prevalence of new susceptibility loci in 1201 AMD patients and 562 controls. In addition, we measured the levels of apolipoprotein B, apolipoprotein A2, lipoprotein A, cholesterol, triglycerides, and HDL-cholesterol in serum samples. Our findings further highlight a role of the extracellular matrix and HDL metabolism in the pathogenesis of AMD.

Chapter 3B. Since CFH is a regulator of the alternative complement pathway and the CFH risk allele is strongly associated with AMD, it has been postulated that it is the deregulation of the alternative pathway that underlies the pathogenesis of AMD. In this chapter we describe serum complement activity in 197 confirmed AMD patients and 150 unaffected age-matched controls. Furthermore, we analyzed the roles of the risk alleles in genes that were previously identified as being associated with AMD. CFH and ARMS2/HTRA1 are the two major AMD susceptibility genes. However, the function of the ARMS2 protein is currently unknown, and its subcellular localization is unclear. The current findings suggest that CFH and ARMS2/HTRA1 share a common pathway in the pathogenesis of AMD.

Chapter 4. Currently, therapies that target VEGF-A are available for treating neovascular AMD. Several pharmacogenetic studies suggest that genetic variations may underlie the variable responses to treatment with anti-VEGF, although conflicting results have been reported. In Chapter 5, we described a large multi-center pharmacogenetic study to measure the impact of high-risk alleles on ranibizumab treatment and the age of onset for neovascular disease. We demonstrated that a cumulative effect of high-risk alleles leads to a younger age of disease onset in combination with poor response rates to ranibizumab treatment.

Chapter 5. The data collected from AREDS revealed that patients who were treated with zinc—either alone or in combination with vitamins—had reduced progression from intermediate to advanced AMD. Despite the wide spread use of zinc among AMD patients, how zinc actually exerts its positive effects has not yet been identified. In Chapter 6, we evaluated whether oral zinc supplementation affects complement catabolism in AMD patients. Our results show that this is indeed the case. Interestingly, we also found evidence that that suggest that systemic serum complement activation is further enhanced by active stages of AMD.

Chapter 6. In general discussion important findings are discussed and placed in current and future clinical prospective.

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Investigative Ophthalmology & Visual Science. 2011 Nov 21;52:8908-18.

CHAPTER TWO

IMAGING OF AGE-RELATED MACULAR DEGENERATION

Central areolar choroidal dystrophy and age-related macular degeneration: differentiating characteristics in multimodal imaging

1. SUMMARY

Late-onset central areolar choroidal dystrophy (CACD) may easily be confused with geographic atrophy (GA) in AMD. In order to detect discerning features, the morphological changes in CACD patients and in AMD patients were assessed with confocal scanning laser ophthalmoscopy (cSLO), fundus autofluorescence (FAF) and spectral domain optical coherence tomography (SD-OCT).

A total of 30 CACD patients with identified *PRPH2* gene mutations were analyzed and compared to 19 patients with early AMD and 13 patients with AMD-associated GA, respectively. The presence of drusen and pigment clumping was determined with color fundus photography. High-resolution in vivo imaging was performed with cSLO and SD-OCT (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany). FAF images and SD-OCT volume scans were analysed in each study eye.

Patients with CACD were significantly younger than patients with AMD, 53.2 years vs. 77.1 years, $p< 0.0001$. A positive family history for visual loss was significantly more common in CACD than in AMD (80% vs. 9.4%, $p < 0.0001$). On FAF, a "speckled" FAF pattern occurred significantly more often in CACD (85%) than in early AMD (5.6%), ($p<0.0001$). There was a significantly higher frequency of sub-RPE deposits in eyes with AMD compared to CACD (36.8% versus 2.1% of scans, $p=0.0019$). Reticular drusen could be visualized by SD-OCT and FAF imaging in 52.6% of the eyes with early AMD and in 100% of the eyes with GA, whereas this drusen phenotype did not manifest in eyes with CACD. Table 1 provides an overview of differences between CACD and AMD patients.

Although outer retinal atrophy is the clinical common feature in advanced CACD as well as GA, there are microstructural alterations on high-resolution SD-OCT and FAF imaging, which allow for the differentiation between CACD and AMD. The findings may help to identify patients where a diagnostic *PRPH2* screening is warranted.

2. INTRODUCTION

Central areolar choroidal dystrophy (CACD) is a hereditary retinal disorder which affects the macula, resulting in progressive and usually profound visual loss. The hallmark feature of the disorder represents a well-defined atrophy of the retinal pigment epithelium (RPE) and the choriocapillaris.[1] Four clinical stages of the diseases have been described.[2] In stage 1 CACD, subtle focal parafoveal pigmentary RPE changes can be observed on ophthalmoscopy. A typical stage 2 finding in the color image, is an oval to round, mildly atrophic hypopigmented area. This area, on a fundus autofluorescence (FAF) image, shows increased as well as decreased reflectivity resulting in a speckled FAF pattern. Stage 3 is characterized by one or more patches of well-demarcated RPE atrophy outside the fovea. In stage 4, the atrophic area involves the fovea, resulting in a markedly decreased visual acuity.[2,3]

Autosomal dominant CACD is most commonly caused by mutations in the *peripherin-2 (PRPH2)* gene (formerly known as *peripherin/RDS).*[4,5] More than 90 different *PRPH2* mutations associated with a wide spectrum of fundus alterations have been reported. To date, seven different mutations in the *PRPH2* gene have been identified to cause the CACD phenotype.[6-12] It may be challenging to diagnose CACD in the early stages of the disorder because of the relative nonspecific RPE abnormalities. Also, the late-onset variant may easily be confused with age-related macular degeneration (AMD) and, thus, be misdiagnosed.

AMD is a complex disease and as such the resultant of multiple factors including genetic and exogenous factors.[13] The early and intermediate stages of AMD are characterized by the presence of drusen and pigmentary changes. Choroidal neovascularization (CNV) is present in the exudative form while the advanced non-exudative form (geographic atrophy, GA) is characterized by atrophy of the retinal pigment epithelium (RPE), the photoreceptors, and the choriocapillaris. Approximately 20% of AMD-patients with severe visual impairment have lost central vision due to GA.[14-18] Major genetic risk factors for AMD are certain common variants in the *complement factor H (CFH)*[19-21] and the *age-related maculopathy susceptibility 2 (ARMS2/ LOC387715)* gene.[22-24] Subsequently, other complement factor genes, i.e., *C3, CFB/C2* and *CFI*, were found to be associated with AMD.[25-28] In contrast, a study involving 371 cases of AMD showed that the PRPH2 gene appears not to be involved in the pathogenesis of AMD.[29] Despite these apparent differences in the underlying pathophysiologal processes, CACD and AMD share many phenotypic characteristics. Up to a third of CACD patients develop visual loss at an older age, showing a considerable overlap with age of onset in AMD.[3] Besides the circumscribed atrophic lesions that characterizes the late stages in both disorders, drusen-like deposits have been described in limited numbers of patients with CACD.[30] In addition, the penetrance of CACD may be low (up to 21% non-penetrance), which may mask the autosomal dominant mode of inheritance in some families and may further impede the correct diagnosis.[3]

In the current study, we analyzed the morphologic findings of 30 genetically confirmed CACD patients and a representative cohort of patients with early and late stage non-exudative AMD, in order to detect discerning features on ophthalmoscopy, fundus autofluorescence (FAF), and spectral domain (SD)-optical coherence tomography (OCT).

3. PATIENTS AND METHODS

First, we evaluated 60 eyes of 30 genetically proven CACD patients in various stages of the disease. All patients with CACD were randomly chosen from a large CACD cohort and examined at the Departments of Ophthalmology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Twenty-nine CACD patients carried the p.Arg142Trp *PRPH2* mutation and one patient carried the p.Arg172Gln *PRPH2* mutation. Both mutations were previously associated with CACD.[6, 31] Subsequently, in order to detect discerning features between the CACD phonotype and AMD, we stratified CACD eyes into two groups: 1) early non-atrophic stages (CACD stage 1 and 2) and, 2) late atrophic stages (CACD stage 3 and 4). Only one eye of each patient was included into the statistic analysis, resulting in 20 CACD eyes in group 1 and 18 eyes in group 2.

In order to compare CACD phenotype with AMD, we evaluated 19 eyes (19 patients) with early AMD and 13 eyes (13 patients) with GA due to AMD. The diagnosis and grading of AMD was based on an international classification and grading system for age-related maculopathy and age-related macular degeneration, as described previously.[32] AMD patients were examined at the Department of Ophthalmology, University of Bonn, Germany. All patients with AMD were participants of the multicenter, prospective FAM (Fundus Autofluorescence in Age-related Maculadegeneration) study (ClinicalTrials.gov Identifier: NCT00393692). Eyes with hyperpigmentary changes and/or soft drusen without any sign of active or previous neovascular AMD or GA were included into the study. For comparison with late CACD stages, eyes with GA due to AMD were analyzed. These included eyes with similar FAF patterns to the "speckled" CACD FAF pattern in the perilesional zone of GA (i.e., the "diffuse fine granular", the "diffuse branching", and the "diffuse trickling" FAF phenotype; according to Holz et al.)[33] and without signs of active or previous neovascular AMD. Only one eye of each patient was included into the analysis but, in case of the bilateral early AMD of the bilateral GA only the right eye was included.

The 32 AMD patients were analyzed for the presence of the *PRPH2* p.Arg142Trp and the p.Arg172Gln mutations by direct Sanger sequencing. Genomic DNA was isolated from peripheral blood lymphocytes using standard extraction procedures. To inspect the c.424C>T (p.Arg142Trp) and c.515G>A (p.Arg172Gln) positions in exon 1 of the *PRPH2* gene, oligonucleotide

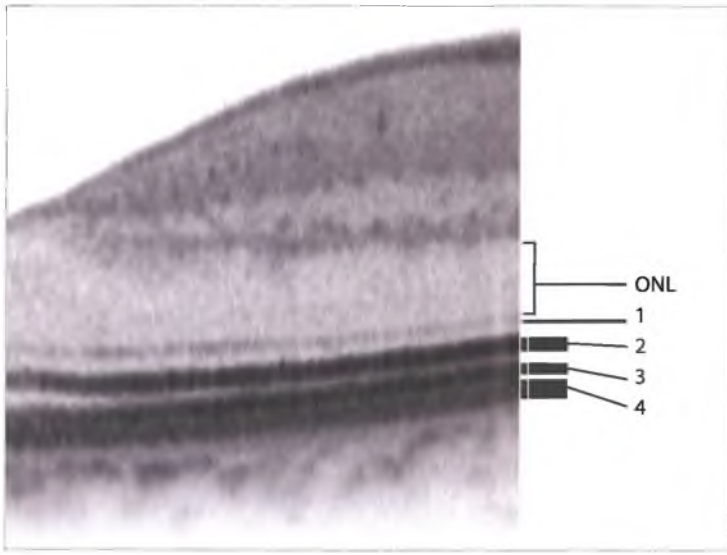
primers RDS.cEx01-F (5'-CTG CAC TTT TCC CAA GGC CCT AAG TC-3') and RDS.cEx01-R (5'- TGT CCC CAA TAT ATT CAT AGC TCT GAC CC-3') were used to PCR-amplify the exonic fragment from the patient DNAs. Direct sequencing was then achieved with primers RDS.cEx01-R, RDS-ex1Fa (5'-AGC CAA GTA TGC CAG ATG GA-3'), or RDS-ex1Ra (5'-AGC AGA AAG CAG CAG AGA GC-3') by using the Big Dye Terminator Cycle Sequencing Kit Version 1.1" (Applied Biosystems, Darmstadt, Germany). Reactions were analyzed with an ABI Prism Model 3130xl Sequencer (Applied Biosystems). The study was performed in accordance with the tenets of the Declaration of Helsinki (1983 revision) and in accordance with the Medical research Involving Human Subjects Act (WMO). The approval of the local ethics committee was obtained for both centers and a written informed consent to participate in this study was acquired from all subjects.

4. IMAGE ACQUISITION AND DATA ANALYSIS

Pupillary dilatation was achieved with topical 1.0% tropicamide and 2.5% phenylephrine prior to retinal imaging. Each participant underwent digital color fundus photographs (Imagenet; Topcon Corporation, Tokyo, Japan [Nijmegen], FF 450 Visupac ZK5, Carl Zeiss MediTec AG, Jena, Germany [Bonn]). High-resolution in vivo imaging was carried out with a combined instrument (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) that allows for simultaneous recording of SD-OCT and cSLO (confocal scanning laser ophthalmoscope) as described previously.[34] The cSLO images were obtained according to a standardized operation protocol as describe before.[34, 35] Nineteen horizontal SD-OCT scans through the macula area with simultaneous cSLO imaging were obtained for each study eye. Individual bands lying below the hyporeflective band of the outer nuclear layer (ONL) were identified based upon recent descriptions as depictured in figure 1.[36] Color fundus photography was used to identify the presence of drusen and pigmentary changes. FAF images were used to identify CACD stages as described previously.[3] In AMD patients, FAF images were analyzed according to the classification of FAF patterns in early AMD[37] and of abnormal FAF patterns in the junctional zone of GA, respectively.[33]

Microstructural changes were analyzed and categorized in consensus meetings of the authors. Subsequently, two graders (DS and MF) performed qualitative and quantitative analysis on fundus photography, FAF and SD-OCT images (19 single SD-OCT scans per study eye). Quantitative differences in the presence of retinal features were analyzed using Fisher's exact test and for the continuous variables we used the Mann-Whitney U test. In order to adjust the p-value levels for multiple testing Borforronni correction was preformed. Reported p-values are two-sided and considered statistically significant if lower than 0.002. Statistical analyses were performed using SPSS, version 17.0.

FIGURE 1. SD-OCT scan through the fovea of a normal retina of a 65-year old male
Different hyperreflective bands can be defined that appear to correlate with the anatomic layers of the outer retina.[36] ONL, outer nuclear layer; (1) external limiting membrane (ELM); (2) interface of the inner and outer segments of the photoreceptor layer (IPRL); (3) outer segment- the retinal pigment epithelium (RPE) interdigitation; and (4) RPE/Bruch membrane complex (RPE/BM).



5. RESULTS

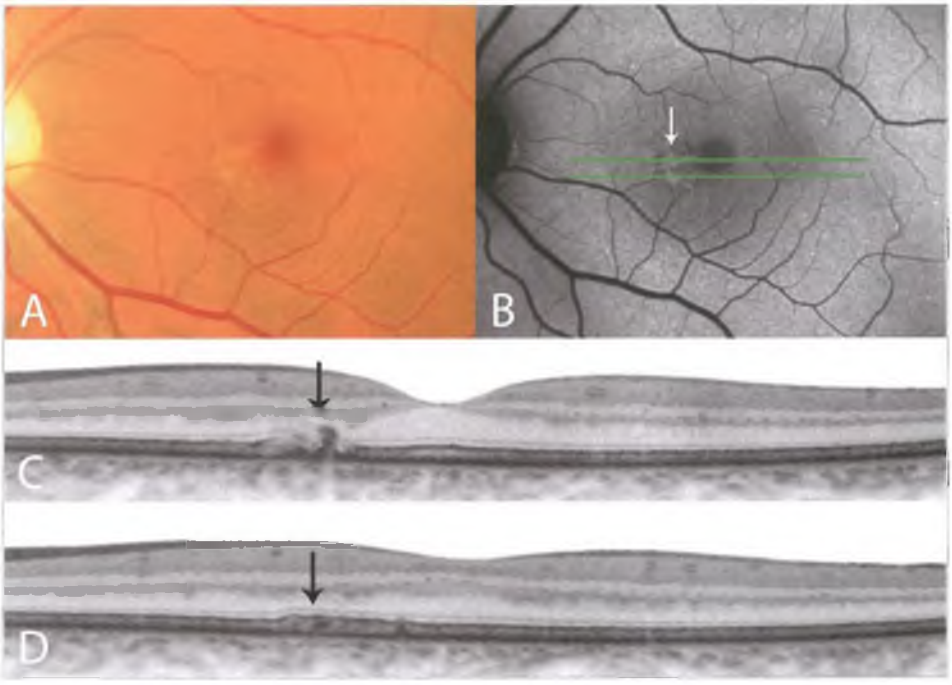
5.1 Description of CACD stages

5.1.1 Stage 1 CACD

Five eyes of 3 patients originating from two families had stage 1 CACD. The presence of focal parafoveal RPE-changes in a predominantly normal retina characterizes this early stage (Fig. 2). FAF imaging revealed lipofuscin accumulation indicated by an increased signal of these parafoveal lesions (Fig2B). Corresponding SD-OCT scans showed focal photoreceptor/RPE abnormalities, ranging from subtle disruption and elongation of the line representing the interface of the inner and outer segments of the photoreceptor layer (IPRL) to frank loss of the normal outer retinal anatomy (Fig. 2C, D).

FIGURE 2. Stage 1 CACD

The green line indicates the positions of the SD-OCT scans. Color fundus photography (A) shows a parafoveal increase of reflectivity with some pigment clumping, co-located with a discrete increase of fundus autofluorescence (FAF) (B). (C) Interruption of the normal anatomy of RPE and photoreceptor outer segments (arrow). (D) Subtle disruption, elongation and reflectivity loss of IPRL and the RPE/BM on SD-OCT (arrow).



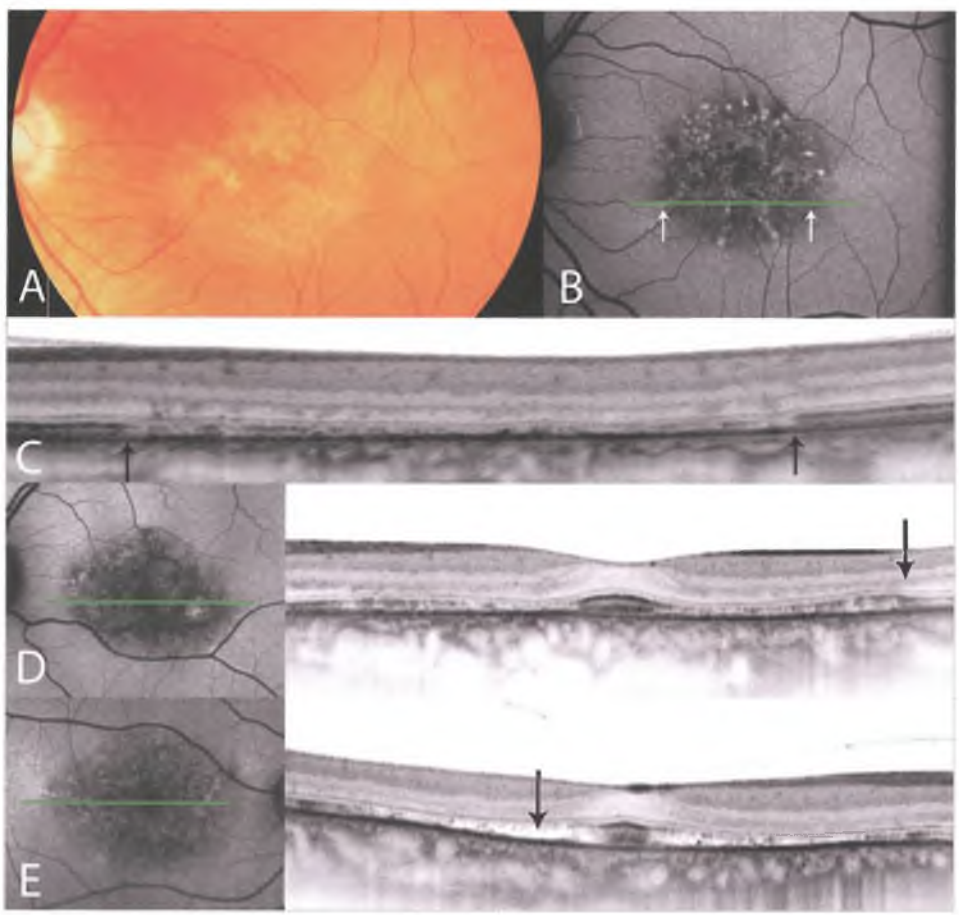
5.1.2 Stage 2 CACD

Twenty-seven eyes of 18 patients were diagnosed as stage 2 CACD. In this stage, a round and poorly demarcated area of hypopigmentation can be observed on color fundus photography (Fig. 3A). The most striking finding was a speckled FAF that covered the macula (Fig. 3B). SD-OCT scans of this area demonstrated a loss of reflectivity and interruption of the IPRL, and to a lesser extend of the external limiting membrane (ELM) (Fig. 3C). In addition, this area showed thinning and increased irregularity of the outer nuclear layer. At the central macula, relative preservation of the reflectivity of the outer photoreceptor segments was observed (Fig. 3 C, D). In 11 eyes (40.7%) focal elongation of the photoreceptor outer segments was present on the SD-OCT (Fig. 3D). In three eyes (two patients), slight detachment of the neurosensory retina was noted (Fig. 3E). Compared to stage 1, a high number of SD-OCT hyperreflective clumps

were noted, generally localized above the level of the RPE. Approximately 85% of the clumps correlated with markedly increased FAF signals (Fig. 4D-F).

FIGURE 3. Stage 2 CACD

The green line indicates the position of the SD-OCT scans. Color fundus photography (A) shows a hypopigmented area, co-located with a speckled fundus autofluorescence appearance at the macula (B). SD-OCT shows reflectivity changes and interruptions of the IPRL and thinning of the ONL (C). White arrows (B) indicate the area of changed SD-OCT between the black arrows in (C). In CACD stage 2 elongation of the IPRL/ RPE is visible in the transition zone (arrow). In late stage 2 CACD interruption or even loss of the IPRL together with relatively preserved sub-foveal reflectivity was notified, suggesting a partial detachment of the neurosensory retina (arrow).

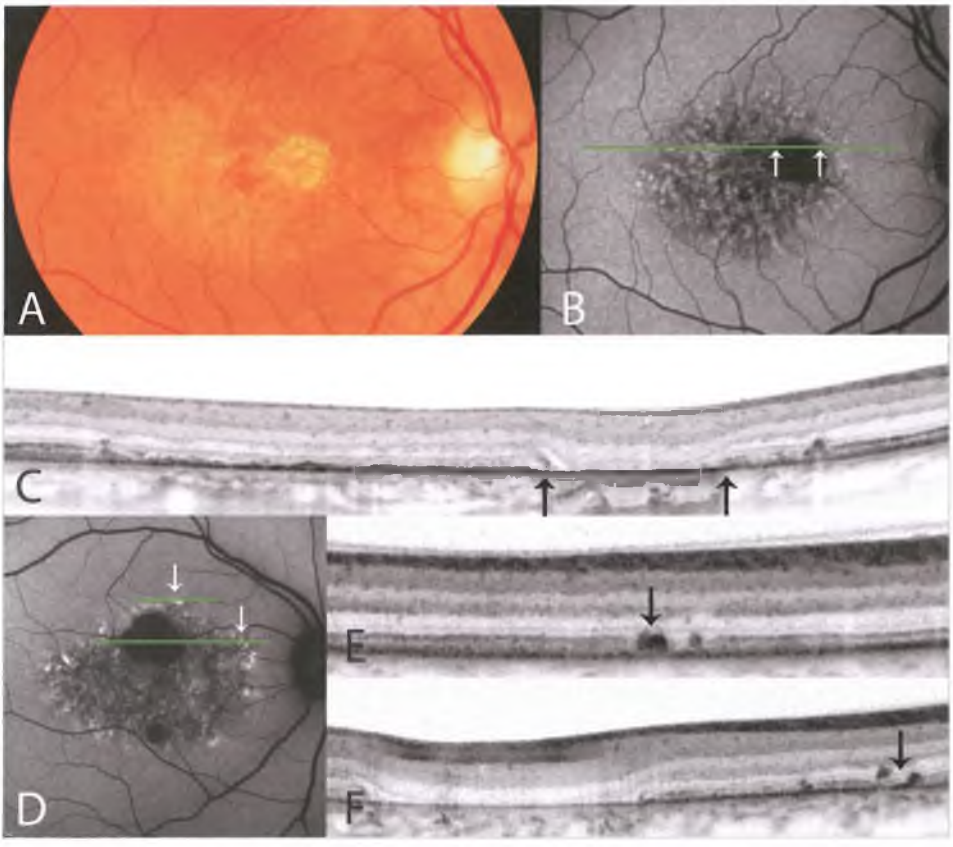


5.1.3 Stage 3 CACD

Fourteen eyes of 10 patients demonstrated well-defined atrophy not involving the macula and were thus classified with stage 3 CACD (Fig. 4). A typical speckled FAF pattern always surrounds the atrophic areas (Fig. 4B). As in stage 2, SD-OCT scans within speckled FAF revealed a reflectivity loss and interruption of IPRL (Fig. 4C). The atrophic borders were characterized by a relative sharp disruption of the RPE layer with ELM covering the borders (Fig. 4C, F). In 4 eyes (29%) rosette-like structures located at the photoreceptor layer were observed near the border of the atrophic area (Fig. 5D). Hyporeflective spaces, located mainly in the outer plexiform layer, were occasionally present (39% of stage 3 and 4).

FIGURE 4. Stage 3 CACD

The green line indicates the position of the SD-OCT scans. Color fundus photography (A) shows an atrophy zone co-located with an absent fundus autofluorescence (FAF) signal (B, arrows) embedded in a typical speckled FAF area. On SD-OCT (C) the atrophic area shows total absence of the outer retinal layers (black arrows). The outer retinal changes within the zone of speckled FAF appears comparable with stage 2 CACD on SD-OCT. As in stage 2 CACD, hyperfluorescent spots on FAF (D, white arrows) co-localise with hyperreflective clumping on SD-OCT (E, F, black arrow).

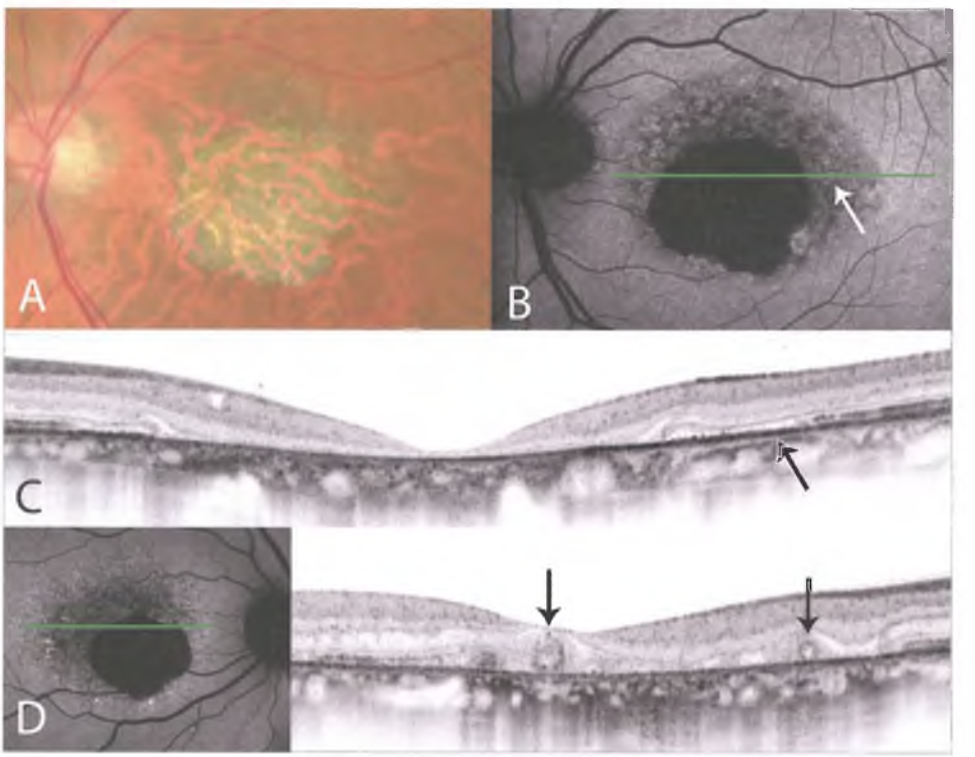


5.1.4 Stage 4 CACD

Fourteen eyes of nine patients demonstrated central atrophic lesions characteristic for CACD stage 4 (Fig. 5). In areas of atrophy, virtually all outer retinal layers were absent (Fig. 5C). Rosette-like structures originating from the photoreceptor layer were observed in 11 eyes (79%) near the border of the atrophic area (Fig. 5D). Within the atrophic zones 21% of eyes had clusters of hyperreflective material correlating with increased FAF.

FIGURE 5. Stage 4 CACD

The green line indicates the position of the SD-OCT scans. Stage 4 CACD is characterized by atrophy of the fovea (A). As in early stages, a typically speckled autofluorescence with outer retinal changes on SD-OCT can be observed (B, C, arrows). In tangential SD-OCT scans through the margin of chorioretinal atrophy, rosette-like structures could be detected in many cases (D, arrows).



5.2 CACD versus AMD

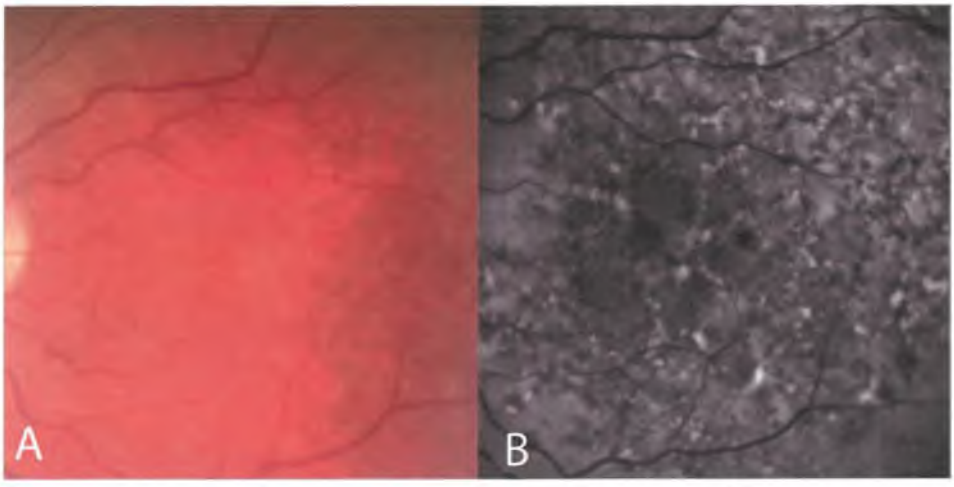
Patients with CACD were significantly younger than patients with AMD, 53.2 (SD 14.1) years vs. 77.1 (SD 7.93) years, $p< 0.0001$. A positive family history for visual loss was significantly more common in CACD than in AMD (80% vs. 9.4%, $p < 0.0001$). None of the AMD patients carried

the *PRPH2* p.Arg142Trp or the p.Arg172Gln gene mutation. Table 1 provides an overview of differences between CACD and AMD patients.

5.2.1 CACD stage 1 and 2 versus early AMD

On FAF, a speckled pattern of increased and decreased FAF signal as describe before[3] occurred significantly more often in CACD than in AMD (85% vs. 5.6%, p<0.0001). However, the "speckled" FAF pattern in early AMD as described by Bindewald and colleges (Fig. 6), was not as well demarcated and was more irregularly shaped than in CACD. In eyes with early AMD, the abnormal FAF pattern frequently extended beyond the macular and covered the entire posterior fundus.

FIGURE 6. The "speckled" fundus autofluorescence (FAF) pattern in early AMD
This FAF pattern (B) is characterized by the simultaneous presence of a variety of FAF abnormalities in a larger area of the FAF image. The changes may extend beyond the macular area and may cover the entire posterior fundus. Typically, these abnormalities include multiple small areas of irregularly increased and decreased FAF. The small areas of focal increased FAF may be punctuate or resemble linear structures. The corresponding abnormalities visible on color fundus photographs (A) include hyper- and hypopigmentation and multiple subconfluent and confluent drusen. From: Bindewald A, Bird AC, Dandekar SS, Dolar-Szczasny J, Dreyhaupt J, Fitzke FW, Einbock W, Holz FG, Jorzik JJ, Keilhauer C, Lois N, Mlynski J, Pauleikhoff D, Staurenghi G, Wolf S.Classification of fundus autofluorescence patterns in early age-related macular disease. Invest Ophthalmol Vis Sci. 2005 Sep;46(9):3309-14. Copyright: Association for Research in Vision and Ophthalmology



This contrasted with the sharply demarcated, regularly oval shaped "speckled" FAF pattern that was usually confined to the central macular in CACD (Fig. 3 versus Fig. 6). On SD-OCT imaging, in early AMD, focal accumulations of material under the RPE cell layer were significantly more

often present as compared to CACD ($p<0.0001$). The typical dome-shaped elevations of RPE layer corresponding with soft drusen were visible in 100% of eyes (42.6% of total scans) in early AMD (Fig. 7) and, elongated sub-RPE lesions were visible in 65% of eyes (13.4% of total scans) in early AMD (Fig. 8 C). None of the eyes with stage 1 and 2 CACD showed a reticular drusen whereas 50% of the eyes with early AMD eyes showed this characteristic finding ($p<0.0001$) (Fig. 9 D).

FIGURE 7. Sub-Retinal pigment epithelium deposits in early AMD

The dome-shaped elevations of the outer retinal layers in the SD-OCT scans (C-D) correspond to soft drusen on color fundus photography (A). Hyperpigmentations in the fundus image correspond to an increased fundus autofluorescence signal (B) and hyperreflective structures overlaying the drusen in the SD-OCT scan (D). The green line indicates the position of the SD-OCT scans.

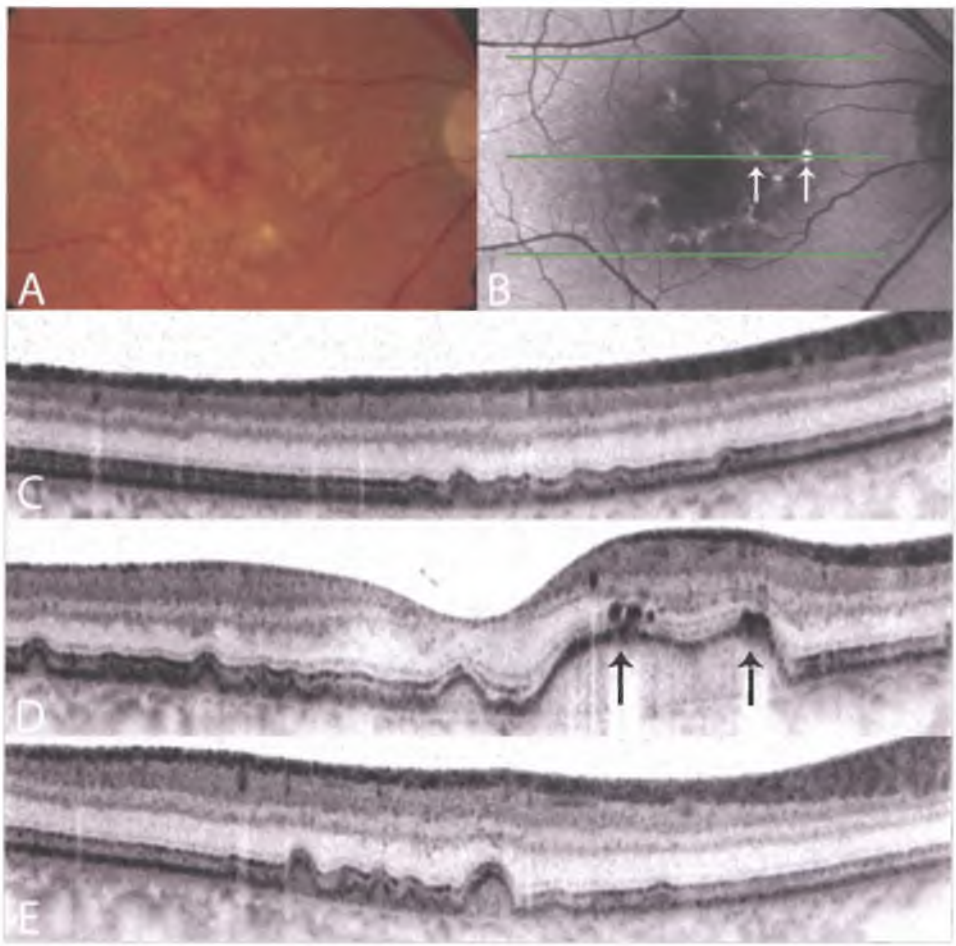
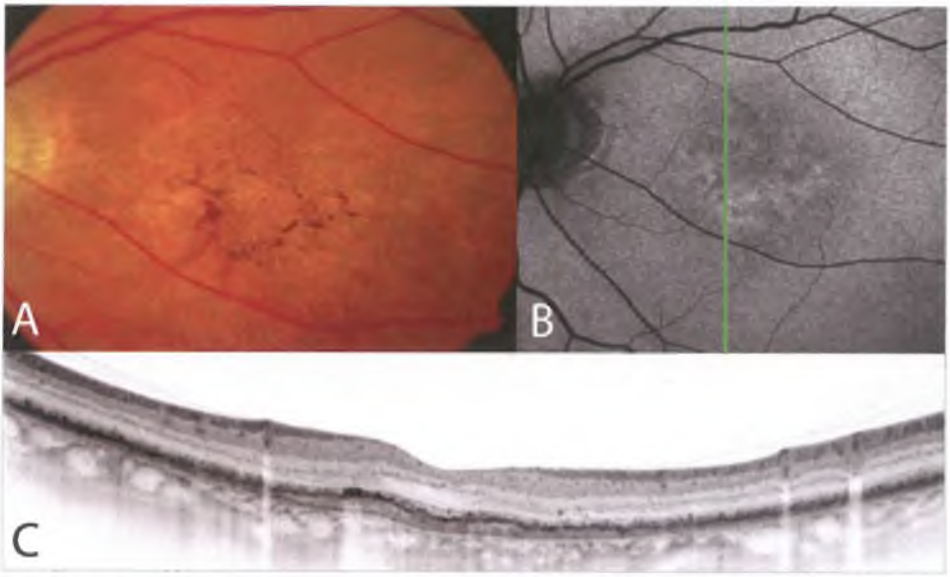


FIGURE 8. Sub-Retinal pigment epithelium deposits in early AMD

Sub-Retinal pigment epithelium deposits in early AMD: Elongated elevations of the outer retinal layers in the SD-OCT scan (C) may appear in areas with hyperpigmentation (A) and mildly increased fundus autofluorescence (B). Repeated fluorescein angiography showed no signs of CNV or scaring (data not shown). The green line indicates the position of the SD-OCT scans. In this eye, there is an extreme amount of deposit beneath the RPE. In most AMD eyes, this SD-OCT finding is more subtle.

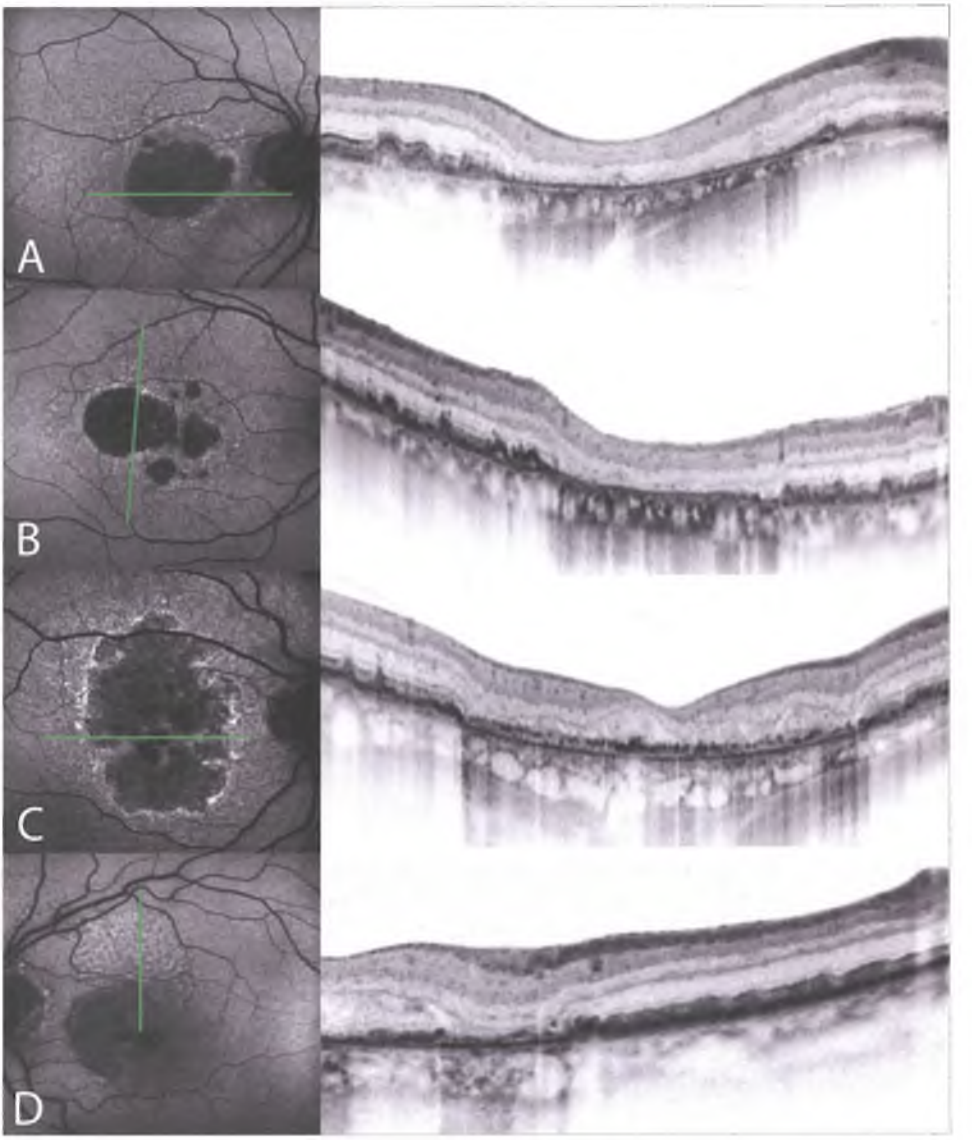


5.2.2 CACD stage 3 and 4 versus GA in AMD

On FAF imaging, among the eyes with GA due to AMD, 6 eyes showed the "diffuse branching" (Fig. 9A), 5 eyes the "diffuse fine granular" (Fig. 9B), and 2 eyes the "diffuse trickling" (Fig. 9C) FAF pattern in the perilesional zone of atrophy. Despite the phenotypic resemblance, the FAF changes in AMD that surround the atrophic lesion, were not as well demarcated from the surrounding retina and often extended beyond the macular area (Fig. 4B vs. 9 A-C). In contrast to AMD, atrophic lesions in CACD usually showed a sharply demarcated round to oval configuration (Fig. 4). Within SD-OCT scans, there was a significantly higher frequency of sub-RPE deposits in the peri-atrophic area in AMD compared to eyes with atrophic CACD ($p<0.0001$, Table 1). A reticular drusen pattern could be visualized by SD-OCT and FAF imaging in 92.3% of eyes with atrophic AMD (Fig. 9 D) while no eye with CACD showed this pattern. The atrophic lesions in AMD frequently showed irregular material remaining on the assumed Bruch's membrane, what gave a more irregular structure (Fig. 9C, D) compared to the blank appearance of remaining retinal layers in the atrophic lesion in eyes with CACD (Fig. 4 and 5).

FIGURE 9. Simultaneous fundus autofluorescence (FAF) and SD-OCT imaging in GA due to AMD

The green line indicates the position of the SD-OCT scans. (A) "diffuse branching" – (B) "diffuse fine granular" – (C, D) "diffuse trickling" – FAF pattern in the peri-atrophic zone (according to Holz et al. AJO 2007). SD-OCT imaging shows dome-shaped elevations (A, B) and elongated sub-RPE deposits (C) in der peri-atrophic area. (D) Reticular drusen pattern in FAF imaging with corresponding characteristic SD-OCT finding. Note the irregular structure of the remaining retinal layers within the atrophic lesion (C, D).



6. DISCUSSION

This study was conducted to systematically analyze and compare the morphologic changes in the various stages of AMD and CACD. in accordance with the ophthalmoscopical similarities of these retinal disorders, SD-OCT and FAF findings also show that CACD shares many morphologic characteristics with atrophic AMD. These include hyperpigmentations, abnormal FAF and occurrence of atrophy of the outer retinal SD-OCT layers in the advanced stages. Nevertheless, there are morphologic differences that may help in the differentiation between CACD and AMD (Table 1).

In all but the initial stage of CACD, a "speckled" FAF pattern that was sharply demarcated from the surrounding retina, regularly oval shaped and confined to the central macular was observed. In fact, this could be considered as the hallmark feature of CACD stage 2, 3 and 4 and, was the most prominent discriminating characteristic (Fig. 3). Although speckled FAF patterns previously have been described in AMD,[33, 38] the alterations were less regularly shaped, less well demarcated and often extended beyond the macular area. In the speckled FAF area in CACD, SD-OCT revealed a corresponding disorganization of the photoreceptors and a loss of photoreceptor outer segments, illustrated by a disruption of the inner-outer photoreceptor junction accompanied by a thinning in the outer nuclear layer. The predominant photoreceptor loss in CACD is a consequence of the underlying disease process. CACD is caused by a monogenetic defect in one of the two *PRPH2* genes and potential modifying environmental factors are thought to be of little relevance in this disorder.[3] The Peripherin-2 protein may function as an adhesion molecule involved in stabilization and compaction of the outer segment discs and/or in the maintenance of the curvature of the rim of the discs.[39-41] It has been demonstrated in the mice model that one functional *PRPH2* allele is unable to support the normal outer segments morphogenesis in cones and rods.[42] Cones with one functional *PRPH2* allele provide better sensitivity to light stimuli than those completely lacking *PRPH2*. These cones are, however, not as efficient in phototransduction as cones in wild-type mice.[42] In human CACD patients, there appears to be no detectable early cone dysfunction, but, like in mice, the disturbance of outer segment morphogenesis may results in an increased phagocytosis.[43-45] The resulting accelerated lipofuscin accumulation in the RPE may cause progressive impartiment of lysosomal funtions and,[43, 46] over time may contribute to RPE cell death.[47] Increased levels of RPE lipofuscin have been measured in a CACD patient carrying the p.Arg172Trp point mutation.[48, 49] It is plausible that the typical speckled FAF in the posterior pole of CACD stage 2-4 represents simultaneous occurrence of lipofuscin accumulation and focal atrophy of the photoreceptor/RPE functional unit. A reduction in cone density within areas of speckled FAF has also been described in 4 patients with macular dystrophies caused by the

maging technique		Features	Stage 1 and stage 2 CACD n=20 eyes	Early AMD n=20 eyes	P-value	Stage 3 and n=18 eyes
		Soft drusen (eyes)	2 (10%)	19 (95%)	< 0.0001	1 (5.6%)
unduscopy		Lobular configuration of atrophy (eyes)				2 (11.1%)
		Multifocal atrophy (eyes)				5 (27.8%)
		Abnormal FAF pattern (eyes)	17 (85%) speckled	1 (5%) speckled*	< 0.0001	25 (89%)
AF		Configuration / extend of abnormal FAF pattern (in the majority of eyes)	Oval shaped, sharply demarcated, confined to the central macular (2-3 disc areas)	Irregularly shaped, no sharp demarcation to surrounding retina, frequently 2-3 disc areas but also larger extend		Oval shaped, demarcated, to the centra (2-3 disc area
		Reticular drusen pattern (eyes)	0	10 (50%)	0.001	0
		Disruption of IPRL (eyes)	19 (95%)	14 (70%)	0.037	17 (94.4%)
		Disruption of IPRL (scans)	in 234 scans (61.6%)	in scans 34 (9%)	0.004	475 scans (89
		Dome-shaped sub-RPE deposits (eyes)	8 (25%)	27 (100%)	< 0.0001	6 (33.3%)
		Dome-shaped sub-RPE deposits (scans)	in 18 scans (4.7%)	in 162 (42.6%)	0.004	in 8 scans (2.
	Peri-atrophic	Elongated sub-RPE deposits (eyes)	0	13 (65%)	0.001	0
SD-OCT		Elongated sub-RPE deposits in scans (scans)	0	in scans 51 (13.4%)	0.004	0
		Reticular drusen pattern (no. of eyes)	0	10 (50.0%)	< 0.0001	0
		Sub-foveal changes/elongation (eyes)	11 (55%)	0	0.001	6 (33.3%)
	Within atrophy	Hyporeflective spaces (eyes)				7 (38.9%)
		Rosette-like structures (eyes)				9 (50%)

TABLE 1. Discriminating features between central areolar choroidal dystrophy (CACD) and age-related macular degeneration (AMD).

* according to Bindewald, et al. IOVS 2005
** according to Holz, et al. AJO 2007

The Bonferroni correction for multiple testing P > 0,002

SD-OCT = spectral domain optical coherence tomography
RPE = Retinal pigment epithelium
IPRL= Inner and outer segments of the photoreceptor layer

different *PRPH2* mutations.[50] Interestingly, in CACD stage 2 and 3 a sub-foveal reflectivity was often preserved, indicating that foveal cones may be less vulnerable to the effects of a *PRPH2* mutation. Highly heterogeneous FAF pattems in patients with GA in AMD have been reported previously.[50] In this study we selected and studied those FAF patterns of AMD-associated GA, that could be confused with speckled FAF pattern as observed in CACD.

Another observation in the CACD group constituted a rosette-like structures, mainly located at the border of the atrophic zone. These were not observed in the AMD group and appear to represent a morphologic feature associated with CACD. Rosette-like structures originating from photoreceptors were previously observed in retinas of mice with one functional *PRPH2* allele.[42] This histopathologic study indicated that the rosettes develop as a consequence of tensional forces exerted on cells by the ELM to overcome the excessive space and keep the photoreceptors close to each other. We hypothesize that our CACD population resembles this mouse model. However, this phenomenon is only observed in advanced CACD stages when the outer segments may lose their normal morphology and RPE apposition. Zweifel and colleges describe similar structural changes in the outer retinal layer, also in GA patients, and coined the term "outer retinal tabulations".[51] When we take this study into consideration, we must conclude that rosette-like structures are significantly more often present in CACD than in GA.

SD-OCT revealed sub-RPE deposits in eyes with AMD (Fig. 7 and 8), whereas these were extremely rare in CACD. We distinguished two types of sub-RPE deposits: "dome-shaped" and "elongated" sub-RPE deposits. The "dome-shaped" deposits on SD-OCT correlated with funduscopically typical soft drusen while the "elongated" deposits had no clear funduscopic correlation, but were frequently associated with hyperpigmentary changes and a mildly increased FAF signal (Fig. 8). Within the "elongated" deposits, in the most eyes a characteristic

SD-OCT alteration was subtle splitting of the IPRL but, in eyes with the "diffuse trickling" phenotype it may be markedly pronounced (Fig. 8).[52] The " elongated" deposits most likely correlate with basal laminar deposits (BLamD) histopathologically, which occur between the RPE basement membrane and the RPE plasma membrane.[53-55] BLamD have been identified in human donor eyes as hallmarks of AMD, particularly in late-stage AMD.[55-59] The higher prevalence of sub-RPE deposits in AMD patients was also associated with the late atrophic stage. The atrophic zones in GA were highly irregular and showed residual material on Bruch's membrane. In CACD, by contrast, the atrophic areas were rather smooth and homogeneous. Furthermore, reticular drusen frequently observed in AMD were never noted adjacent to or within the CACD lesions. The much higher occurrence of sub-RPE deposits, the reticular drusen and the irregular structure of retinal layers within the atrophic lesion in AMD patients, all indicate different pathogenetic mechanisms for CACD versus atrophic AMD although, the outer atrophy finally represents a common downstream pathogenetic pathway. CACD is a monogenetic disorder that appears to cause direct photoreceptor damage with subsequent RPE cell loss. AMD, on the other hand, is currently thought of as represents a complex, multifactorial disease characterized by initial (sub)-RPE alterations followed by either photoreceptor and RPE cell loss and/or secondary angiogenetic processes.

The prevalence of end-stage AMD has been estimated at 3% in people aged over 65 years, rising to 11% in those over 85 years,[60] therefore, AMD-mimicking diseases should be suspected in relative young patients with atrophic lesions. It seems reasonable to assume that late-onset macular dystrophies such as CACD are underdiagnosed and confused with AMD on a regular basis. The main reasons being the low prevalence of macular dystrophies and their phenotypic similarities with AMD, especially in the absence of a positive family history. Therapeutic options for both disorders are currently limited, however, this could change in the near future. Oxidative stress is involved in AMD pathogenesis and antioxidants, like zinc and certain vitamins, may slow the progression of the disease.[61] In the future, targeted manipulation of the alternative complement pathway, may provide a more powerful weapon in the battle against AMD-related vision loss.[62, 63] In CACD the development of gene therapy, appears currently the most promising approach. The proper differentiation between AMD and CACD becomes of further consequence when the autosomal dominant inheritance of the latter is taken into consideration, as well as the differences in visual prognosis.

This study identified several morphological distinguishing features between CACD and AMD based on non-invasive novel imaging tools, i.e., SD-OCT and FAF. These findings may aid the clinician in discerning these entities and may help in the identification of patients where genetic analysis for verification of a *PRPH2* mutation is warranted.

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Investigative Ophthalmology & Visual Science. 2011 Jul 25;52:5525-8.

CHAPTER THREE - A

PATHOGENESIS OF AGE-RELATED MACULAR DEGENERATION

Evaluation of serum lipid concentrations and genetic variants at high-density lipoprotein metabolism loci and TIMP3 in age-related macular degeneration

1. SUMMARY

Age-related macular degeneration (AMD) is a multifactorial disorder caused by genetic and environmental factors. Despite the late-onset of the disease, familial and twin-based studies have shown that AMD has a strong genetic component. Recently, two genome-wide association studies identified new genetic markers to be associated with AMD.[14, 15] Although these associations point towards a role of the high-density lipoprotein (HDL) metabolism in AMD, previous studies that have examined the association between serum HDL levels and AMD show conflicting results.[17-23] Some studies found no relationship,[21, 23] whereas others found that increased risk of AMD was associated with increased HDL levels,[17, 19] and yet others have shown an inverse relationship between HDL levels and AMD.[18, 20, 22] In present study we analyze the association between a new susceptibility locus near the *TIMP3* gene and genes of the high-density lipoprotein (HDL) metabolism and age-related macular degeneration (AMD), and evaluate serum lipid and lipoprotein levels in AMD patients compared with control individuals.

Single nucleotide polymorphisms in or near the *TIMP3*, *ABCA1*, *FADS1–3*, *CETP*, *LIPC*, and *LPL* genes were genotyped. Serum levels of apolipoprotein B (ApoB), apolipoprotein A2, lipoprotein a, cholesterol, triglycerides, and HDLcholesterol were determined.

Significant associations were found between AMD and variants in *ABCA1* and *FADS1–3*, and a nearly significant association in *TIMP3*. No significant associations were observed for variants in *LPL*, *LIPC*, and *CETP*. We also observed a significant elevation of ApoB levels in serum of AMD patients. Other lipids and lipoproteins were not significantly altered.

These results confirm associations of AMD with variants near the *TIMP3* gene and at loci involved in HDL metabolism. They further highlight a role of the extracellular matrix and the HDL metabolism in the pathogenesis of AMD. This study identified increased ApoB levels as a possible new serum biomarker for AMD.

2. INTRODUCTION

Age-related macular degeneration (AMD) is a multifactorial disorder caused by genetic and environmental factors. The most consistently identified environmental risk factor is smoking.[1] Despite the late-onset of the disease, familial and twin-based studies have shown that AMD has a strong genetic component .[2,3]

Single nucleotide polymorphisms (SNPs) in the *complement factor H* (*CFH*)[4-6] gene and in the *ARMS2/HTRA1*[7-9] gene are strongly associated with AMD. Genetic association studies identified variants in several other genes of the complement pathway such as *complement factor B* (*CFB*),[10] *component 2* (*C2*),[10] *component 3* (*C3*),[11] and *complement factor I* (*CFI*).[12] Together, these variants account for more than 50% of the disease risk.[13]

Recently, two genome-wide association studies identified additional genetic markers to be associated with AMD.[14,15] A new susceptibility locus was identified near the *TIMP3* gene.[15] TIMP3 is a metalloproteinase involved in degradation of the extracellular matrix. Mutations in *TIMP3* are also responsible for an early-onset autosomal dominant macular dystrophy.[16] In addition, loci involved in the high-density lipoprotein (HDL) cholesterol pathway were found to be associated with AMD. Associations were identified with variants in the hepatic lipase (*LIPC*) gene, the cholesterylester transfer protein (*CETP*) gene, the ATP-binding cassette transporter A1 (*ABCA1*) gene, the fatty acid desaturase gene cluster (*FADS1-3*), and the lipoprotein lipase (*LPL*) gene.[14,15]

Although these associations point towards a role of the HDL metabolism in AMD, previous studies that have examined the association between serum HDL levels and AMD show conflicting results.[17-23] Some studies found no relationship,[21,23] whereas others found that increased risk of AMD was associated with increased HDL levels[17,19] and yet others have shown an inverse relationship between HDL levels and AMD.[18,20,22] In this study we analyzed these new risk alleles in AMD patients and control individuals from a German/Dutch cohort, in order to evaluate their relevance in our population. In addition, we evaluated the concentration of lipids and lipoproteins in serum samples of AMD patients and control individuals.

3. PATIENTS AND METHODS

3.1 Study Population

The European Genetic Database (EUGENDA) is a German/Dutch project studying development and therapy of AMD. In the current study, 1201 AMD patients (827 Dutch and 374 German) and 562 controls (476 Dutch and 86 German) from EUGENDA were included. Patients of all AMD stages were included. AMD staging for the EUGENDA study was performed by the Cologne Image Reading Center and Laboratory (CIRCL). Color fundus photographs of both eyes of all cases were evaluated by two independent reading center graders according to the standard protocol of the CIRCL. AMD was defined as the presence of at least 10 small, hard drusen and pigmentary changes, or at least one intermediate size drusen. Control individuals exhibited no signs of AMD in either eye and showed no other macular pathology. AMD cases and control individuals of similar ages were collected, although the mean age of AMD patients (75.86 ± 8.16 years) was slightly higher than that of the control individuals (72.72 ± 6.63 years). All individuals were from the Nijmegen (Netherlands) and Cologne (Germany) area, respectively. The research protocols followed the tenets of the Declaration of Helsinki. All participants provided written informed consent. The protocols were reviewed and approved by the local institutional review boards.

3.2 Genotyping and lipid/lipoprotein measurements

Genomic DNA was extracted from peripheral blood samples using standard procedures. Genotyping of SNPs in the *TIMP3* (rs9621532), *LIPC* (rs10468017), *LPL* (rs12678919), *ABCA1* (rs1883025), *FADS1_3* (rs174547) and *CETP* (rs3764261) genes was carried out as previously described.[24] Serum levels of apolipoprotein B (ApoB), apolipoprotein A2 (ApoA2), lipoprotein a (Lpa), cholesterol, triglycerides and HDL-cholesterol (HDLC) were measured in a subset of patients and controls using standard procedures by a clinical chemistry laboratory (Architect analyzer, Abbott Diagnostics).

3.3 Statistical analysis

Differences between case and control subjects in baseline characteristics, mean serum lipid and lipoprotein levels and risk allele frequencies were tested using the Chi-square or Student's T-test, where appropriate. Linear regression was performed to determine whether genotypes in *LIPC*, *LPL*, *ABCA1*, *FADS1_3* and *CETP* are associated with ApoB and triglyceride levels. Reported p-values are two-sided and considered statistically significant if <0.05. Statistical analyses were performed using SPSS, version 16.0.

4. RESULTS

Baseline demographics of the Dutch-German cohort are shown in Table 1. The mean age of the AMD patients is slightly higher than in the control individuals. Female gender and current smoking status were significantly associated with AMD.

The risk allele distributions were analyzed in the Dutch-German cohort (Table 2). The risk allele frequency of rs1883025 in the *ABCA1* gene was significantly higher in AMD patients compared to control individuals (p=0.00027). The risk allele frequency of rs174547 in the *FADS1_3* gene was significantly elevated in AMD patients in the combined cohorts (p=0.015). A nearly significant association was observed for rs9621532 in the *TIMP3* gene (p=0.067). We did not find significant associations for SNPs in the *LPL, LIPC* and *CETP* genes.

TABLE 1. Baseline characteristics and mean lipid/lipoprotein levels in AMD cases and control individuals.

AMD risk factors	AMD n =1201	Control n= 562	P-value
Female	62.0%	56.2%	0.020
Age (mean)	75 86 ± 8 16	72.72 ± 6.63	<0.001
Smoking	n = 734	n = 472	
Never	42 9%	40 7%	
Past	45.1%	53.6%	0.068
Current	12 0%	5.7%	0 004
BMI (mean)	n = 717 25.96 ± 4.06	n = 452 25.92 ± 3.90	0.867
ApoB (mg/l)	n = 689 1012 ± 251	n = 398 979 ± 227	0 029
ApoA2 (mg/l)	n = 690 1615 ± 304	n = 398 1610 ± 313	0.802
Lpa (U/l)*	n = 691 168	n = 397 164	0 983
Cholesterol (mmol/l)	n = 792 5.93 ± 1.27	n = 521 5.88 ± 1.18	0.425
Triglycerides (mmol/l)	n = 780 1.90 ± 1 05	n = 521 1 92 ± 0 95	0 661
HDLC (mmol/l)	n = 805 1.46 ± 0.37	n = 521 1.44 ± 0.36	0.327

*** median values**

Mean (or median) serum levels of ApoB, ApoA2, Lpa, cholesterol, triglycerides, and HDLC in AMD patients and control individuals are presented in Table 1. Mean ApoB levels were significantly higher in AMD patients (1012 ± 521 mg/l) than in control individuals (979 ± 227 mg/l) in the Dutch-German cohort. No significant associations were found for the other lipids and lipoproteins.

ApoB levels were higher in individuals carrying the homozygous high-risk CC genotype (1007 ± 241 mg/l) compared to individuals carrying the homozygous low-risk TT genotype (940 ± 222 mg/l) (Table 3). The mean ApoB levels increased with the number of risk alleles in *ABCA1* (p = 0.041), but this finding did not remain significant after adjusting for age and gender (p = 0.061).

TABLE 2. Risk allele frequencies in AMD cases and control individuals.

Gene	SNP	Alleles (risk/nonrisk)	AMD	Controls	OR [95% CI]	P-value
TIMP3	rs9621532	A/C	0.966	0.953	1.41 [0.98-2.03]	0.067
LIPC	rs10468017	C/T	0.711	0.698	1.07 [0.91-1.25]	0.442
LPL	rs12678919	G/A	0.097	0.098	0.98 [0.77-1.26]	0.879
ABCA1	rs1883025	C/T	0.786	0.729	1.36 [1.15-1.61]	2.7 x 10-4
FADS1_3	rs174547	T/C	0.696	0.654	1.21 [1.04-1.41]	0.015
CETP	rs3764261	A/C	0.342	0.314	1.14 [0.97-1.33]	0.108

TABLE 3. Association between ApoB levels and number of risk alleles at HDL loci.

Gene	Risk allele	Mean ApoB in individuals carrying no risk alleles	Mean ApoB in individuals carrying 1 risk allele	Mean ApoB in individuals carrying 2 risk alleles	P-value	P*-value
LIPC	C	992 ± 241	998 ± 233	1002 ± 254	0.683	0.477
LPL	G	994 ± 243	1001 ± 231	1156 ± 390	0.126	0.208
ABCA1	C	940 ± 222	989 ± 245	1007 ± 241	0.041	0.061
FADS1_3	T	1005 ± 237	983 ± 241	1008 ± 240	0.364	0.364
CETP	A	995 ± 250	994 ± 232	1004 ± 248	0.817	0.762

* P-value adjusted for age and gender

5. DISCUSSION

In this study we confirmed the presence of associations between AMD and polymorphisms at loci of the HDL metabolism: *ABCA1* and *FADS1_3*, and observed a nearly significant association with a polymorphism near the *TIMP3* gene. No association was observed for SNPs in the *LIPC*, *LPL* and *CETP* genes. Lack of association has also been observed in other cohorts; e.g. the previously reported association with the variant in the *LIPC* gene was not identified in two of seven cohorts studied.[14] This suggests that these variants may be differentially distributed among different populations. Alternatively, our study may not have sufficient power to detect modest effects; in particular for the *LIPC* and *LPL* SNPs the effect in our cohort seems to be more modest than previously described in other cohorts.

We detected elevated serum ApoB levels in AMD patients compared to control individuals. The serum levels of other lipids and lipoproteins, including HDLC, did not differ significantly between AMD cases and control individuals.

These data add further support that several pathways contribute to the pathogenesis of AMD. Besides the well-established involvement of the complement system (CFH, C2, C3, CFB),[25] additional genes encoding components of the extracellular matrix (*ARMS2* and *TIMP3*),[15, 26] and genes of the HDL metabolism play a role in the pathogenesis of AMD. Although the genes involved in the HDL metabolism have a relatively small contribution to the development of AMD, they may reveal novel pharmacological targets to prevent AMD in individuals carrying high-risk alleles in these genes.

TIMP3 inhibits matrix metalloproteinases (MMP) and is involved in degradation of the extracellular matrix. It can also inhibit vascular endothelial factor (VEGF)-mediated angiogenesis independent of its MMP-inhibitory activity.[27] Mutations in this gene can cause Sorsby fundus dystrophy, an autosomal dominant macular dystrophy with clinical features similar to AMD and an early onset before 40 years. Both TIMP3 and ARMS2 seem to be involved in extracellular matrix function. It has been shown that ARMS2 interacts with several matrix proteins.[26] This highlights the important role of the extracellular matrix in the pathogenesis of AMD.

This study supports previous associations between AMD and HDL metabolism.[14, 15] It is unclear how polymorphisms in genes of the HDL metabolism can influence the development of AMD. Changes in HDL levels may lead to the accumulation of cholesterol and lipids in drusen. There has been some confusion since some alleles increase HDL levels and decrease the risk for AMD, while other alleles decrease HDL levels and increase the risk for AMD.[14, 15] Studies on the association between plasma HDL levels and AMD are also inconsistent. Studies have found no relationship,[21] a relationship between increased[17, 19] and decreased HDL levels[20, 22, 28] and AMD. In a recent study by Reynolds et al. elevated HDL levels in AMD were found to be associated

with the *LIPC* genotype.[28] In this study, we did not observe altered serum HDL levels in AMD, nor did we find an association with ApoA2, Lpa, cholesterol and triglyceride levels, despite that our study cohort was significantly larger and thus has more power than previous case-control studies.[20,21,28]

In this study we did find significantly elevated serum ApoB levels in AMD patients compared to controls, which may partially be explained by the *ABCA1* risk allele. A previous study also observed a marked increase of ApoB levels in AMD patients.[20] ApoB is a major low-density lipoprotein (LDL) transporting cholesterols to tissues. High levels of ApoB have been associated with atherosclerosis.[29] ABCA1 is known as the key transporter that facilitates this initial step in reverse cholesterol transport. In transgenic mice, ABCA1 overexpression raised plasma apoB levels by delayed ^{125}I-apoA-I catabolism without altering apoB secretion.[30] In AMD lipids accumulate in Bruch membrane, a process which may be mediated through ABCA1. Transgenic mice overexpressing apoB in the retinal pigment epithelium develop a phenotype similar to early human AMD.[31] Elevated levels of apoB lipoproteins are known to stimulate inflammation although the underlying etiology of chronic subclinical inflammation is not clear.[32] This may be another possible mode of action of lipoproteins in the pathogenesis of AMD.

In conclusion, these results confirm associations of AMD and the loci for *TIMP3* and genes of the HDL metabolism: *ABCA1* and *FADS1_3*. They further stress the role of the extracellular matrix and the HDL metabolism in the pathogenesis of AMD. Our study did not detect elevated HDL levels in AMD, but identified increased ApoB levels as a possible new serum biomarker for AMD.

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Ophthalmology. 2012 Feb;119:339-46.

CHAPTER THREE - B

PATHOGENESIS OF AGE-RELATED MACULAR DEGENERATION

Risk alleles in *CFH* and *ARMS2* are independently associated with systemic complement activation in age-related macular degeneration

1. SUMMARY

Systemic complement activation is associated with AMD and has mainly been attributed to a risk allele in the *complement factor H* (*CFH*) gene. The second major genetic risk factor for AMD is *Age-Related Maculopathy susceptibility 2* (*ARMS2/LOC387715*) SNP. The function of the *ARMS2* protein remains unknown, and controversy exists about its subcellular localization. Whether other important AMD genes also influence complement activation is unclear. In the present large case-control study complement activity and concentrations of complement components and their activation products are measured in AMD patients and in unaffected controls and correlated with genetic variants in the *CFH, ARMS2, C3, CFI* and *CFB* genes. In contrast to previous studies, we stratified the data by *CFH* and *ARMS2* genotypes to enable us to study the effects of these genotypes independently

Hemolytic complement assays (AP50, CP50, LP50), complement components (C3, CFB, CFI and CFH) and the activation products (C3d, C5a, SC5b-9) were analyzed in serum or plasma. Complement concentrations and their associations with SNPs in the *CFH, ARMS2, C3, CFB* and *CFI* genes were studied.

The AMD patients had increased activation of the alternative complement pathway ($p=0.0003$) and elevated levels of complement activation components C3d ($p<0.0001$) and C5a ($p<0.0001$), CFB ($p=0.002$), and an increased C3d/C3 ratio ($p<0.0001$) calculated as a measure of C3 activation. While the *CFH* risk genotype was significantly associated with the elevated C3d/C3 ratios obtained. In the absence of *CFH* risk alleles the *ARMS2* risk genotype also showed significantly increased levels of complement activation ($p=0.013$). Furthermore, the carriers of the *CFB* protective allele had lower CFB concentrations.

The current study found evidence showing that in AMD risk alleles in *CFH* and *ARMS2* are independently associated with complement activation. Especially the C3d/C3 ratio seems to be a strong marker for AMD. The findings suggest that *CFH* and *ARMS2* share a common pathway in the pathogenesis of AMD.

2. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible, severe visual loss in the developed world, with the prevalence of end-stage AMD being estimated at 3% in people aged over 65 years, rising to 11% in those over 85 years.[1] AMD is a genetically complex neurodegenerative retinal disease in whose development and progression both genetic and environmental factors are implicated. The most important environmental risk factors are smoking and obesity,[2] while the strongest genetic association has been found on chromosome 1q32 with common single nucleotide polymorphisms (SNPs) in the *complement factor H (CFH)* gene, the main regulator of the alternative complement pathway.[3-6] Subsequently, SNPs in other complement factor genes, i.e., *C3, CFB/C2* and *CFI*, were found to be associated with AMD as well.[7-10] Evidence that complement activation plays a role in the retina was provided by the presence of complement components C3 and C5, the membrane attack complex C5b-9, and CFH in drusen, the extracellular deposits under the retinal pigment epithelium (RPE), and pathological hallmarks of early disease pathogenesis.[4, 11, 12]

The second major genetic risk factor for AMD was identified at the chromosome 10q26 locus,[13-17] although controversy remains as to which gene at this locus is responsible for the high risk of AMD given that SNPs in the *Age-Related Maculopathy susceptibility 2* (*ARMS2/LOC387715*) gene and the adjacent *high-temperature requirement factor A1* (*HTRA1/PRSS11*) gene are in high linkage disequilibrium.[18-22] A recent study suggests that both *ARMS2* and *HTRA1* could be involved in the pathogenesis of AMD.[23] The function of the *ARMS2* protein remains unknown, and controversy exists about its subcellular localization. Although an interaction has been observed between *ARMS2, CFH,* and cigarette smoking, it is unclear whether there is a common causative pathway.[24, 25] Other recent studies demonstrated systemic complement activation in the serum of AMD patients,[26-28] which was mainly attributed to the risk allele in the *CFH* gene.

To add to this knowledge, we performed a large-scale, case-control study to evaluate systemic complement activation in AMD, while seeking to determine the role risk alleles in the complement genes (*CFH, C3, CFB, CFI*) and in the *ARMS2* gene play in complement activation. In contrast to previous studies, we stratified the data by *CFH* and *ARMS2* genotypes to enable us to study the effects of these genotypes independently.

3. PATIENTS AND METHODS

3.1 Study population

We evaluated 197 unrelated Caucasian patients with late-stage neovascular AMD and 150 unrelated Caucasian controls of similar age not showing any signs of AMD. The study participants were enrolled in EUGENDA, a multicenter database for the clinical and molecular analysis of AMD, between March 2006 and August 2008. All participants in the current study were examined at the Department of Ophthalmology of the Radboud University Nijmegen Medical Centre. Each participant underwent a general ophthalmologic examination, including best-corrected visual acuity using Early Treatment Diabetic Retinopathy Study (ETDRS) charts and digital color fundus photographs (Imagenet; Topcon Corporation, Tokyo, Japan). In addition, the AMD patients received fluorescein angiography on the same Topcon device. The diagnosis of AMD was based on a grading of the color fundus photographs and fluorescein angiograms as described previously.[29-31] The study was performed in accordance with the tenets of the Declaration of Helsinki (1983 revision) and the Medical Research Involving Human Subjects Act (WMO). Local ethics committee approval was obtained as well as written informed consent from all participants.

3.2 Complement measurements and genotyping

EDTA samples were placed on ice immediately after the blood draw and centrifuged (10 min at 550 g at 4 °C). Plasma samples were stored in aliquots at -80 °C within 1 hour after collection. Serum was prepared by coagulation at room temperature and after centrifugation the samples were stored at -80 °C within one hour after collection. Hemolytic complement activity assays for the classical pathway (CP50), the alternative pathway (AP50), and the mannose-binding lection pathway (LP50) were assessed in serum samples with the Wieslab kit (Wieslab, Lund Sweden) as described before.[32] Activation products C5a and the soluble end product of complement activation terminal complement complex (TCC, SC5b-9) were assessed in plasma samples as described before.[33, 34] In each case a standard with a known concentration of the assayed component was included. Complement component C3, CFB, CFH, CFI, and the activation fragment C3d were measured in serum samples as described before.[34, 35] The C3d/C3 ratio was calculated as a measure of C3 activation.

Genotyping of SNPs in the *CFI* (rs1003900), *CFB (*H9L; rs4151667), *C3 (*R102G; rs2230199) and *ARMS2 (*A69S; rs10490924) genes was carried out as described elsewhere.[36] The *CFH* variant Y402H (rs1061170) was analysed by direct sequencing of PCR products using forward primer TCATTGTTATGGTCCTTAGG and reverse primer AAAGACATGAACATGCTAGG.

3.3 Statistical analysis

First, we conducted a univariate logistic regression analysis to study associations between single risk factors and AMD. We subsequently performed multivariate logistic regressions to study the associations adjusted for covariates such as age, gender, BMI (Body Mass Index in kg/m^2 in three classes: <25, 25 -30 and >30), and smoking status (never, past or current), which are presented as odds ratios (ORs) with 95% confidence intervals (Cis). As reference categories we used the homozygous low-risk genotypes for all SNPs, the lowest categories for BMI and age, 'never smokers' for smoking status and 'males' for gender.

To correct for the amount of C3 in each individual, the C3d/C3 ratio was calculated as a measure for complement activation.[37] Differences in complement levels between case and control subjects were evaluated using the Mann-Whitney U test. The association between complement levels and *CFH* genotype was evaluated by stratification of individuals in homozygous low-risk (TT), heterozygous (CT) and homozygous high-risk genotype (CC) groups. To explore whether high-risk alleles in *CFH* (C allele; Y402H) and *ARMS2* (T allele; A69S) independently contribute to elevated serum complement levels we stratified the data for *CFH* as well as *ARMS2* risk alleles by creating four 'allele' groups: 1) participants without risk alleles in either gene, 2) those carrying the *ARMS2* risk allele, but not the *CFH* risk allele, 3) those with the *CFH* risk allele but not the *ARMS2* risk allele, and 4) participants carrying both the *CFH* and the *ARMS2* risk alleles. The association between the C3d/C3 ratios and AMD was assessed using general linear models with the C3d/C3 ratio defined as the dependent variable, which analysis was performed separately in the four allele groups. Relationships were adjusted for significant confounders only.

BMI and smoking history were unknown for 49 participants; we used the multiple imputation procedure in SPSS to impute missing data and based the logistic regression analysis on the imputed data. However, since none of the complement parameters measured showed a significant relationship with BMI and smoking status, we performed all analyses on complement levels using the original dataset.

Reported p-values are two-sided and considered statistically significant if lower than 0.05. Statistical analyses were performed using SPSS, version 17.0

4.RESULTS

4.1 Baseline demographic and genotype characteristics

Increasing age, BMI, current smoking, *CFH, ARMS2,* and *C3* genotypes were significantly associated with an increased risk of AMD. The *CFB* A-allele was significantly associated with a lower risk of AMD. In our study population the *CFI* polymorphism did not show an association with AMD (Table 1).

4.2 Elevated serum complement levels in AMD

The median serum concentrations of C3d, a marker of C3 activation, and C5a anaphylatoxin were significantly elevated in the patients ($p<0.001$) (Table 2). The C3d/C3 ratio was also significantly associated with AMD ($p<0.001$). Despite their higher median C3d and C5a serum concentrations, increased levels of the soluble terminal complement complex SC5b-9 were not observed in the patients. Increased activation of the alternative pathway appears to be the main cause of elevated serum complement levels ($p=0.003$), while activity assays did not demonstrate elevation of the classical and the mannose-binding lectin pathways. CFB was also significantly elevated ($p=0.002$) and CFI showed a trend ($p=0.068$) towards elevation in the patients. The median concentration of CFH was similar in the patients and the controls (Table 2).

Age, BMI and smoking history were not found to be associated with the C3d/C3 ratio, but the male gender was associated with significantly higher C3d/C3 levels ($p<0.001$) in the patients, although the C5a concentration did not differ significantly between the male and female patients.

4.3 Associations between *CFH, ARMS2* and *CFB* risk alleles, and complement activation

The level of complement activation, defined as the C3d/C3 ratio, was stratified by *CFH* genotype (Figure 1A) and by the presence of *CFH* and *ARMS2* risk alleles (Figure 1B). The patients carrying the *CFH* risk allele, either heterozygously or homozygously, had a significantly higher C3d/C3 ratio ($p=0.001$) than the controls with the *CFH* risk allele (Figure 1A). The patients carrying the *ARMS2* risk allele in the absence of *CFH* risk alleles had a significantly higher C3d/C3 ratio ($p=0.013$) than the controls with this profile (Figure 1B). This latter finding remained significant after correction for age, gender, smoking status, BMI, and *C3* and *CFB* genotypes. The patients and controls that did not carry the *CFH* or *ARMS2* risk alleles did not show a significant difference between C3d/C3 levels ($p=0.83$; Figure 1B).

After having stratified the serum complement levels for both study groups by *CFB* genotype, we found individuals carrying the protective *CFB*-A allele to have significantly lower CFB levels (Figure 1C). Polymorphisms in *C3* and *CFI* did not show any associations with altered serum concentrations.

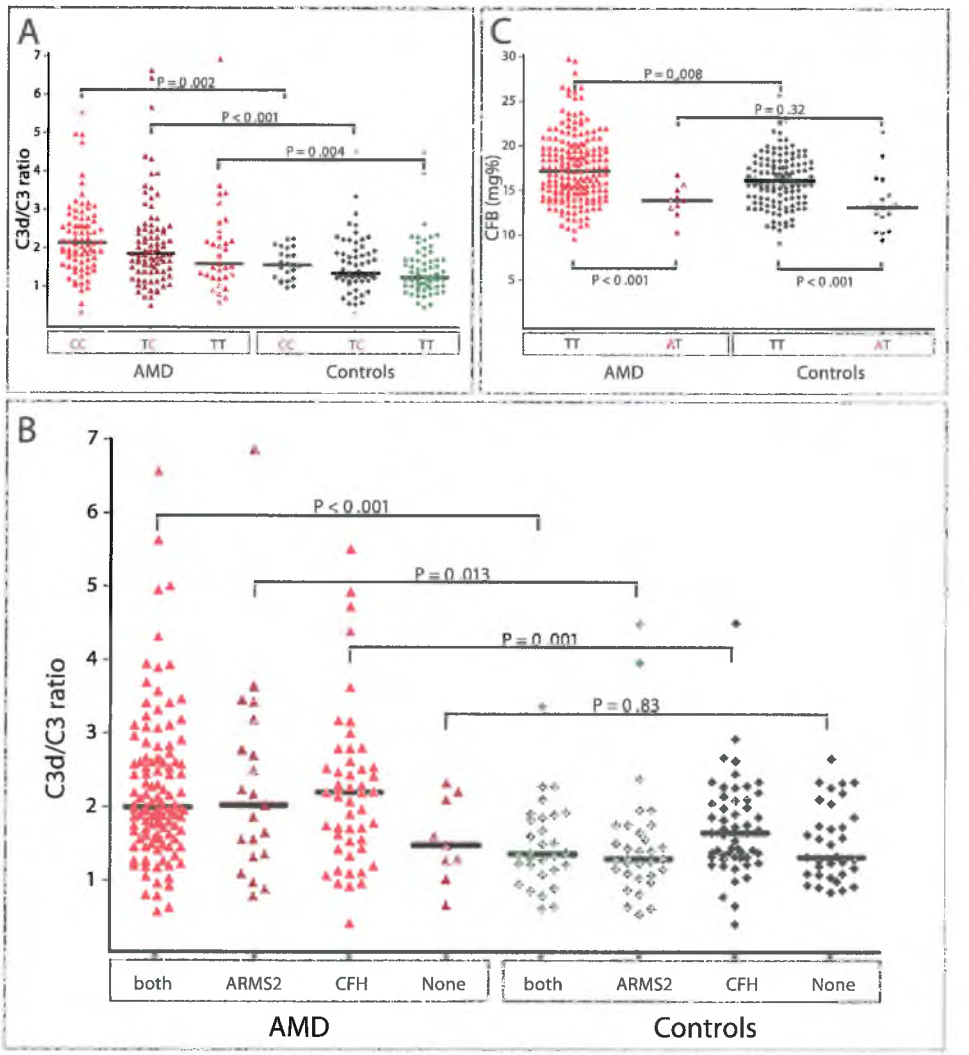
TABLE 1: Baseline demographics and genotype characteristics of the study population.

AMD risk factors		AMD n = 197	Controls n=150	OR (95% CI) *	P-value
Gender, female %		122 (61.9%)	83 (55.3%)	1.54 (0.90 - 2.63)	0.11
Age < 70		43 (21.8%)	62 (41.3%)	reference	
Age 70-80		92 (46.7%)	68 (45.3%)	1.92 (1.13 - 3.26)	0.016
Age > 80		62 (31.5%)	20 (13.3 %)	4.84 (2.45 - 9.60)	< 0.001
BMI < 25		75 (44 1 %)	71 (54 6 %)	reference	
BMI 25-29 9		67 (38 8 %)	49 (37 7 %)	1 53 (0 92 - 2 54)	0 15
BMI 30 or greater		29 (17.1 %)	10 (7.70 %)	3 07 (1 41 - 6 67)	0.001
Non smoker		66 (39.3%)	48 (36.9%)	reference	
Ex-smoker		71 (42.3%)	73 (56.2%)	0.87 (0.48 - 1.57)	0.71
Current smoker		31 (18.5 %)	9 (6.9%)	3.43 (1.03 - 11.5)	0.06
CFH Y402H	TT	30 (15 2%)	67 (44 7%)	reference	
	TC	87 (44 2%)	61 (40 7%)	3.80 (2.04 - 7 05)	< 0.001
	CC	80 (40 6%)	22 (14 7%)	9 21 (4.53 - 18.7)	< 0 001
ARMS2 A69S	GG	59 (29.9%)	87 (58.4%)	reference	
	GT	94 (47.7%)	58 (38.9%)	2.36 (1.42 - 3.94)	0,001
	TT	44 (22.3%)	4 (2.70%)	15.6 (5.10 - 47.8)	< 0.001
C3 R102G	CC	94 (49 5%)	80 (54 8%)	reference	
	CG	76 (40.0%)	58 (39 7%)	1 08 (0.66 - 1.76)	0.76
	GG	20 (10 5 %)	8 (5 50%)	2 98 (1 19 - 7 46)	0 02
CFB H9L	TT	180 (94.7%)	129 (87.8%)	reference	
	AT	10 (5.30%)	18 (12.2%)	0.38 (0.16 - 0.95)	0,042
CFI rs1003900	TT	52 (27.1%)	35 (24.3%)	reference	
	TC	92 (47 9%)	80 (55.6%)	0 76 (0.42 - 1 35)	0.35
	CC	48 (25.0%)	29 (23.0%)	1.08 (0.55 - 2.11)	0.83

*** ORs are based on a multivariable logistic regression model with gender, age, BMI and smoking status as covariates including imputations of missing values.**

AMD=Age-related macular degeneration
OR=Odds ratio
CI=Confidence interval
BMI=Body Mass Index
CFH=Complement factor H
ARMS2=Age-Related Maculopathy susceptibility 2
C3=Complement component C3
CFB=Complement factor B
CFI=Complement factor I

FIGURE 1: Associations between *complement factor H (CFH), Age-Related Maculopathy susceptibility 2 (ARMS2)* and *complement factor B (CFB)* risk alleles, and serum complement.



A. Complement activation was defined by the C3d/C3 ratio (y-axis) in the patients and the controls clustered by *CFH* genotype. Relative to the controls, complement activation is significantly elevated in the AMD group carrying all three CFH genotypes (CC, TC and TT).

B. C3d/C3 ratio (y-axis) clustered by *CFH* and ARMS2 allelic composition: 1) individuals carrying both the *CFH* and the *ARMS2* risk alleles, 2) individuals carrying the *ARMS2* risk allele, but not the *CFH* risk allele, 3) individuals carrying the *CFH* risk allele but not the *ARMS2* risk allele, and 4) individuals without risk alleles in either gene. Relative to the controls, complement activation was significantly elevated in the patients carrying either the *ARMS2* risk allele, the *CFH* risk allele, or risk alleles in both genes. Complement

activation is not elevated in the patients carrying neither allele. Note that, as shown in Figure 1B, after stratification for the ARMS2 risk allele the patients carrying the low-risk CFH genotype (TT) did not show elevated serum complement levels. The controls carrying risk alleles both in *ARMS2* and *CFH* do not have elevated complement activation.

C. CFB concentration (y-axis) clustered by *CFB* genotype in the patients and the controls. Individuals carrying the protective A allele in *CFB* have significantly lower serum CFB levels.

TABLE 2: Median complement activities and concentrations in the age-related macular degeneration (AMD) patients and the controls.

Complement (units)	AMD n = 197 Median (SD)	Controls n = 150 Median (SD)	P-value
Classical pathway activity (CP50) (%)*	110 (11.1)	108 (11.1)	0.643
Alternative pathway activity (AP50) (%)*	96.0 (9.00)	93.0 (12.4)	0.003
MBL pathway (LP50) (%)*	74.0 (43.2)	77.5 (40.5)	0.178
C3 (ug/ml)	7.79 (1.58)	7.75 (1.43)	0.903
C3d (ug/ml)	15.6 (6.90)	11.2 (5.20)	< 0.001
C5a (ug/ml)	0.22 (1.09)	0.15 (0.15)	< 0.001
TCC (SC5b-9) (AU/ml)	1.50 (23.1)	1.40 (19.3)	0.644
CFB (mg %)	16.9 (3.80)	15.9 (3.00)	< 0.001
CFH (mg %)	24.9 (5.10)	24.5 (5.00)	0.654
CFI (mg %)	69.1 (19.2)	64.9 (14.1)	0.068
C3d/C3 ratio	2.00 (1.09)	1.39 (0.65)	< 0.001

* Complement activity for each pathway was determined by ELISA32 and expressed as a percentage of the standard in the kit.

SD = Standard deviation

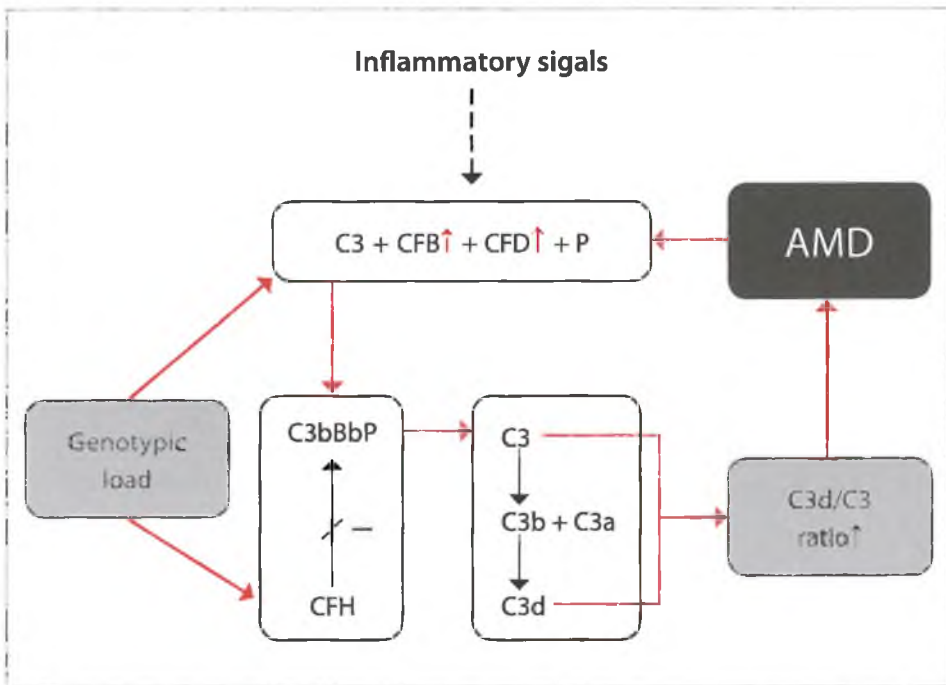
5. DISCUSSION

In our case-control study we examined systemic complement activation in AMD and analyzed the roles of the risk alleles in the *CFH*, ARMS2, *C3*, *CFI* and *CFB* genes earlier identified as being associated with AMD. Using complement activity assays in serum for all three complement pathways, we showed that the activity of the alternative complement pathway (AP50) was systemically increased in our AMD cohort, while the classical and mannose-binding lectin pathways were not altered relative to those observed in the unaffected controls. Since CFH is a regulator of the alternative complement pathway and the *CFH* risk allele is strongly associated with AMD, it has been postulated that it is the deregulation of the alternative pathway that underlies the pathogenesis of AMD.[3, 4] This is confirmed by our study. More recently, an association between AMD and a genetic variant in *SERPING1*, a regulator of the classical pathway, was suggested,[38] but this finding could not be replicated.[39, 40] Likewise our study does not support deregulation of the classical pathway in the pathogenesis of AMD.

We found the levels of complement activation fragments C3d and C5a in the AMD patients to be elevated relative to the levels obtained for the controls, which is in agreement with previous findings.[26-28] In line with other studies, we did not detect altered CFH levels in our AMD cohort,[26-28] which finding supports the notion that an altered function of CFH leads to a deregulation of the alternative complement pathway rather than an alteration in serum CFH levels. It is important to note that even in the presence of an increased C3 activation (i.e., an elevated C3d/C3 ratio) we found increased levels of AP50. Also, levels of CFB, an acute-phase protein, were significantly elevated in the patients.[41] Taking these observations together, we propose that the increased CFB contributes to increased C3 activation. In addition, less functional activity of CFH may potentially prolong the half-lives of the amplification converter C3bBb, further adding to the C3 activation. In addition to CFH, the acute-phase response of CFB and possibly other complement components involved in increased catabolism of C3 contribute to the pathogenesis of AMD. Scholl and co-workers demonstrated that in their AMD patients concentrations of a complement factor D was also higher.[28] The scheme depicted in Figure 2 summarizes our current understanding of C3 activation associated with AMD.

Conflicting results have been reported for terminal complement complex SC5b-9: elevated levels were observed in one study,[28] but not in another.[27] Despite increased activation of the complement cascade, we did not observe elevated levels of the soluble SC5b-9 assembly. Whether a systemic activation reflects local complement activation in the eye is still unclear. Although it has been demonstrated that drusen contain SC5b-9,[42] this complex may have been generated locally.

FIGURE 2. Inflammation model of C3 activation in age-related macular degeneration.
We propose that inflammation leads to enhanced levels of acute-phase response protein complement factor B (CFB), which, in the presence of high-risk alleles, increased complement factor D (CFD) levels and of properdin (P), contribute to increased alternative pathway activation and C3 catabolism and subsequently the C3d/C3 ratio. Increased C3 activation may directly contribute to the development and progression of AMD, making a self-perpetuating amplification loop of complement-mediated inflammation in AMD pathogenesis.



On a biochemical level, the high-risk CFH 402H variant has been shown to have a decreased interaction with C-reactive protein (CRP)[43] and sulfated GAGs at Bruch's membrane.[44,45] Since a binding of CFH to CRP and GAGs is essential to limit tissue complement attack,[46,47] it is plausible that at the level of Bruch's membrane impaired binding of the 402H variant results in even higher levels of deregulation and overactivation of the alternative complement pathway. Nevertheless, given the high variation of SC5b-9 concentrations we obtained, it is reasonable to conclude that our study lacks the power to detect a significant systemic SC5b-9 elevation. Since our study protocol was very strict, it is unlikely that the blood sampling procedure accounts for the high variation in complement concentrations. Thus, further investigation in this direction is required.

Furthermore, our study demonstrates that carriers of the *CFB* H9L protective allele have lower CFB concentrations, which may explain the protective nature of this polymorphism and supports our notion that CFB contributes to increased C3 activation. This finding corroborates the earlier hypothesis that the protective effect is caused by genetic variants in *CFB,* and not by those in *C2*, which are in high linkage disequilibrium.[8,26] A mouse model demonstrated that CFB deficiency was associated with a significant reduction in the size of choroidal neovascularization after laser injury.[48] Since inhibition of the complement system may slow or even arrest AMD progression, manipulation of the CFB concentration seems a promising therapeutic strategy for AMD. A major advantage of lowering the CFB concentration is that it may inhibit the chronic low-level alternative complement pathway overactivation, but will not affect the classical and mannose-binding lectin pathways required for host defense against infection. A limitation of our case-control study is that it does not provide information about the course of increased C3 catabolism in AMD patients over time. A longitudinal study approach is needed to document complement activity before and during early stages of the disease, which would be relevant to determine the timing of potential future therapy.

Consistent with previous findings, we found systemic complement activation to be associated with the *CFH* genotype .[26-28] After stratification for the *ARMS2* and *CFH* risk alleles, we additionally observed that the AMD patients who carry the *ARMS2* risk allele only (and not the *CFH* risk allele) had a significantly higher degree of complement activation than the controls. Two previous studies did not report such an association, but neither had stratified the data for these two risk alleles and thus could not delineate the independent effects of *ARMS2* and *CFH* on complement levels.[26, 27] Our findings suggest that ARMS2 itself is involved in the activation of the complement system. Interestingly, a genetic interaction between *CFH* and *ARMS2* risk alleles was reported earlier, already suggesting that the two alleles might be involved in a common disease pathway.[25]

The function of ARMS2 protein, however, remains controversial.[49-52] It has been proposed that the *ARMS2* locus encodes for a mitochondrial protein, implicating a role in the oxidative defense response.[49,50] Oxidative stress plays a fundamental role in AMD pathogenesis, and both in vitro and in vivo studies suggest that it leads to activation and deposition of complement on the endothelium.[53] Then again, Kortvely and co-workers state that ARMS2 is a secreted protein that interacts with extracellular matrix (ECM) proteins.[51] They found that ARMS2 directly binds to fibulins and EMILIN-2, proteins that participate in the assembly and stabilization of ECM structures and are important components of the elastic layer of Bruch's membrane.[51] The authors further hypothesize that *ARMS2* risk alleles may compromise the elastic fibers of Bruch's membrane.[51] It plausible to assume that matrix changes may result in a decreased interaction

between complement regulators and Bruch's membrane which can lead to increased catabolism of C3. As *ARMS2* and the adjacent gene *HTRA1* are in high linkage disequilibrium, it is possible that, rather than ARMS2, it is HTRA1 that is associated with AMD pathogenesis given that HTRA1 regulates the degradation of extracellular matrix proteoglycans.[54] Yet, a relationship between HTRA1 and the complement system regulators may still exist. Although our data suggest that ARMS2/HTRA1 and CFH are linked to AMD through a comparable pathological mechanism, further research is needed to shed more light on these various potential associations. Although our study indicates that *CFH* and *ARMS2* genotypes are related to complement activation, various factors mainly related to immune defense in case of an infection, may also lead to increased C3 activation.[55] However, it is unlikely that infection rates are differently distributed among the groups.

Interestingly, the serum of the AMD patients who did not carry the *CFH* and *ARMS2* risk alleles did not show elevated levels of complement activation (Figure 1B), indicating that other disease mechanisms are involved in this AMD subgroup. Another remarkable observation was that the controls carrying both the *CFH* and the *ARMS2* risk alleles did not show elevated complement activation (Figure 1B), suggesting that the presence of *CFH* and/or *ARMS2* risk alleles alone does not account for increased complement activation, but that a preceding and additional inflammatory signal is required (Figure 2). Multiple triggers may lead to such an initial inflammatory signal,[44] and they are likely derived from environmental exposures.

In conclusion, the findings of the present study demonstrate that systemic activation of the alternative complement pathway is associated with AMD, with the C3d/C3 ratio constituting a robust marker. In AMD acute-phase response protein CFB was elevated, which, in the presence of high risk alleles, increases alternative pathway activation and C3 catabolism. We found complement deregulation not only to be associated with *CFH* high-risk alleles, but also with *ARMS2* high-risk alleles. The current findings thus suggest that CFH and ARMS2 share a common pathway in the pathogenesis of AMD.

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Ophthalmology. 2012 Nov;119:2304-11.

CHAPTER FOUR

TREATMENT OF AGE-RELATED MACULAR DEGENERATION

Cumulative effect of risk alleles in *CFH, ARMS2* and *VEGFA* on the response to ranibizumab treatment in age-related macular degeneration

1. SUMMARY

Intravitreal ranibizumab injections are currently the standard treatment for neovascular AMD. However, a broad range of response rates have been observed. In the present pharmacogenetic study we evaluate the impact of high-risk alleles in *CFH*, *ARMS2*, vascular endothelial growth factor-A (*VEGFA)* on the response to ranibizumab treatment and on the age of treatment onset. As individual patients can carry a variable number of high-risk variants, we hypothesized that combinations of high-risk alleles may explain the heterogeneity in the age of neovascular disease manifestation and the variable responses to treatment with ranibizumab. Therefore, in contrast to previous studies, we stratified the data according to the number of high-risk alleles to enable us to study the combined effects of these genotypes in development and treatment of neovascular AMD.

We evaluated 420 eyes of 397 unrelated with active subfoveal choroidal neovascularization secondary to AMD. Genotyping of SNPs in the *CFH*, *ARMS2*, *VEGFA*, *KDR*, *LPR5* and *FZD4* genes was performed.

After ranibizumab treatment, AMD patients without risk alleles in the *CFH* and *ARMS2* genes (4.8%) demonstrated a mean VA improvement of 10 Early Treatment Diabetic Retinopathy Study (ETDRS) letters while no VA improvement was observed in AMD patients with four *CFH* and *ARMS2* risk alleles ($p=0.014$). Patients with four high-risk alleles in *CFH* and *ARMS2* were 5.2 years younger than patients with one or two risk alleles, respectively ($p<0.0001$). The mean age at which the first ranibizumab treatment was carried out among AMD patients with all 6 risk alleles in *CFH*, *ARMS2* and *VEGFA* was 65.9 years versus 75.3 years in patients with none or one high-risk allele ($p=0.001$). After ranibizumab treatment, patients with 6 high-risk alleles demonstrated a mean VA loss of 10 ETDRS letters ($p<0.0001$).

In this study we evaluated the largest pharmacogenetic AMD cohort reported to date. A cumulative effect of high-risk alleles in *CFH*, *ARMS2* and *VEGFA* appears associated with a younger age of onset in combination with poor response rates to ranibizumab treatment.

2. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in Western society,[1] with the neovascular form responsible for 80% of severe vision loss.[2, 3] Neovascular AMD is characterized by choroidal neovascularization (CNV), an outgrowth of blood vessels that invade the sub-retinal and intra-retinal space causing exudation, hemorrhage and subsequently rapid visual decline. Important triggers for angiogenesis and vascular permeability are vascular endothelial growth factor A (VEGF-A), and its receptor VEGFR-2 (also known as KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase).[4] The identification of VEGF as important factor in the pathogenesis of neovascular AMD,[5-7] has led to the development of anti-VEGF therapy aimed to neutralize VEGF-A activity. Previously available treatments for neovascular AMD, such as laser photocoagulation and photodynamic therapy, generally did not result in visual improvement in the majority of patients.[8]

Ranibizumab (Lucentis; Novartis, Basel, Switzerland; and Genentech Inc., South San Francisco, CA), a humanized monoclonal antibody Fab fragment that inhibits all active forms of VEGF-A, was approved in 2006 by the Food and Drug Administration for the treatment of AMD-associated choroidal neovascularization.[9] Anti-VEGF therapy is the first treatment to significantly improve visual acuity in a large number of patients, representing an unparalleled advance in the treatment of neovascular AMD.[8] However, a broad range in response rates to anti-VEGF therapy has been observed, as some patients showed a better outcome compared to the average, while others showed VA deterioration instead of improvement. The reasons for different responses to anti-VEGF treatment are poorly understood.[10] Because of the invasive and time-consuming nature of anti-VEGF therapy, it is of importance to understand which factors influence treatment response. Genetic variation has been recognized as an important determinant of individual variability of drug response, and the identification of such variants has enhanced patient care – particularly in oncology and cardiology.[11, 12] Knowledge of genetic variants that influence anti-VEGF therapy may facilitate a personalized treatment approach for neovascular AMD patients.

In the past decade, the identification of several genetic risk factors involved in AMD has provided important insights into the pathogenesis of the disease. The strongest genetic association has been found with a common variant in the *complement factor H* (*CFH*) gene, the main regulator of the alternative complement pathway. [13, 14] The second major genetic risk factor for AMD was identified at the chromosome 10q26 locus,[15] encompassing the *Age-Related Maculopathy susceptibility 2* (*ARMS2/LOC387715*) gene and the adjacent *high-temperature requirement factor A1* (*HTRA1/PRSS11*) gene. Several recent studies suggest that genetic background may play a role in

the varying response to treatment with ranibizumab.[16-19] Conflicting results have been reported for the role of the *CFH* gene in response to ranibizumab treatment. Homozygous carriers of the 402H high-risk allele in CFH (CC genotype) had a lower VA outcome in one study,[16] but better in another.[18] Yet another study concluded that there was no association between the *CFH* Y402H genotype and VA after ranibizumab treatment.[17] For *ARMS2/HTRA1*, one study reported that heterozygous carriers of the high-risk *HTRA1* allele were associated with better VA outcomes,[18] while another study presented lower VA outcomes associated with the homozygous high-risk *ARMS2/HTRA1* genotype.[19]

To clarify and elaborate on the impact of the high-risk alleles in *CFH* and *ARMS2* on the treatment with ranibizumab, we conducted a multicenter study, which allowed a thorough analysis in the largest series of neovascular AMD patients studied to date. Furthermore, we analyzed the role of single nucleotide polymorphisms (SNPs) in VEGF-A, in its receptor VEGFR-2 (KDR) and also in genes involved in angiogenesis and vascularisation (*LPR5* and *FZD4).*[16] As individual patients can carry a variable number of high-risk variants, we hypothesized that combinations of high-risk alleles may explain the heterogeneity in the age of neovascular disease manifestation and the variable responses to treatment with ranibizumab. Therefore, in contrast to previous studies, we stratified the data according to the number of high-risk alleles to enable us to study the combined effects of these genotypes in development and treatment of neovascular AMD.

3. PATIENTS AND METHODS

3.1 Study population

We evaluated 420 eyes of 397 unrelated Caucasian patients at an age of 50 years or more with active subfoveal choroidal neovascularization secondary to AMD. All study participants were enrolled in EUGENDA, a multicenter database for the clinical and molecular analysis of AMD, and venous blood for genotyping was drawn before onset of treatment. One hundred seventy-two eyes (41%) were examined and treated at the Department of Ophthalmology of the Radboud University Nijmegen Medical Center, the Netherlands; 193 eyes (46%) were examined and treated at the Department of Ophthalmology, University of Cologne, Germany; and 55 eyes (13%) were examined and treated at the Department of Ophthalmology, McGill University Health Center, Montreal, Canada. All participants were enrolled between June 2008 and June 2010. The study was performed in accordance with the tenets of the Declaration of Helsinki (1983 revision) and the Medical Research Involving Human Subjects Act (WMO). The approval of the local ethics committee was obtained for all three centers and written informed consent was acquired from all participants.

The diagnosis and grading of AMD was based on an international classification and grading scheme for age-related maculopathy and AMD, as described previously in another study.[2] The diagnosis "active subfoveal choroidal neovascularization secondary to AMD" was established by retinal specialists based on ophthalmic examination, fluorescein angiography (FA) and spectral domain (SD)-optical coherence tomography (OCT).[16-18] Prior to retinal imaging, pupillary dilatation was achieved with topical 1.0% tropicamide and 2.5% phenylephrine. Digital color fundus photographs was carried out with; Imagenet, Topcon Corporation, Tokyo, Japan, [Nijmegen], Zeiss, FF450 IR [Montreal] and Canon CF-60 DSi, digital fundus camera, Canon, Haag-Streit Deutschland GmbH, Wedel, Germany [Cologne]. Each participant underwent fluorescein angiography with; a combined instrument Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany [Nijmegen, Montreal and Cologne] or with Imagenet, Topcon Corporation, Tokyo, Japan, [Nijmegen]. The lesion type of the choroidal neovascularization was determined by FA, and was classified into 4 categories: occult with no classic lesion, minimally classic lesion, predominantly classic lesion and retinal angiomatous proliferation. [9, 20]

3.2 Study design

According to the standard protocol for anti-VEGF therapy, all patients were treated with 3 initial monthly intravitreal 0.5 mg ranibizumab injections, followed by on demand re-injections whenever signs for CNV activity were detected on funduscopy, SD-OCT or angiography.[21] Therefore, the follow-up protocol and the total number of injections varied among patients. Since VA change is maximal after 3 monthly injections,[22] and in order to achieve a comparable and standardized evaluation of the response to ranibizumab treatment, we used data only after 3 consecutive ranibizumab injections. Each participant underwent best-corrected visual acuity (BCVA) assessments prior to and after treatment with three ranibizumab injections. For 347 (81.6 %) patients the Snellen VA were collected retrospectively at a later time point after treatment, while 78 (18.4%) patients were followed prospectively during treatment using ETDRS VA. Venous blood samples for the genotyping were collected before onset of the treatment and VA were retrospectively collected. Patients were excluded from this study if they had received other prior treatments for active subfoveal CNV secondary to AMD. If the second eye became eligible for ranibizumab treatment during the course of the study, then both eyes were included in the study. Age at the first ranibizumab injection was collected, as well as the duration of visual complaints prior to the treatment, but this was not available for all patients (n=218, 52%). Genotyping of SNPs in the *CFH* (Y402H; rs1061170), *ARMS2* (A69S; rs10490924), *VEGFA* (rs699947 and rs833069), *KDR* (rs2071559 and rs7671745), *LPR5* (rs3736228), *FZD4* (rs10898563) was performed with TaqMan probes and primers, using assays developed by Applied Biosystems, and an ABI 7900HT system (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands).

3.3 Statistical analysis

Snellen VA was converted to the logarithm of minimal angle of resolution (logMAR) VA for the purpose of statistical analysis. Change in VA was calculated as the difference between VA at baseline and VA at follow-up. Levene's test for equality of variances was used to test variability of VA changes between Snellen and ETDRS techniques. The association between genotype and visual response after three ranibizumab injections was assessed with linear mixed models using the delta VA as the dependent variable. Association between single genotypes and the age of neovascular disease onset, defined as the age when the first ranibizumab injection was administered, was assessed with linear mixed models using the age of onset as the dependent variable. Relationships were adjusted for significant confounders.

To explore whether a genetic interaction exists between high-risk alleles in *CFH* (C allele; Y402H) and *ARMS2* (T allele; A69S) we stratified the data (the age of onset and delta VA) according to the number of high–risk alleles by creating five 'allele' groups: (0) patients without high-risk alleles in either gene, (1) patients carrying one high-risk allele, (2) patients carrying two high-risk alleles, (3) patients carrying three high-risk alleles, and (4) patients carrying all four high-risk alleles. Adding other risk alleles to the additive model only showed significant results for the VEGFA rs699947 polymorphism. We therefore stratified the data (age of onset and delta VA) according to the number of high-risk alleles in *CFH*, *ARMS2* and *VEGFA* by creating 5 'allele' groups: (0) patients without or with one high-risk allele, (1) patients carrying two and tree high-risk alleles, (2) AMD patients carrying four high-risk alleles (3) AMD patients carrying five high-risk alleles (4) patients carrying all six high-risk alleles. Relationships were assessed using linear mixed models, and were adjusted for significant confounders. Reported p-values are two-sided and considered statistically significant if lower than 0.05. Statistical analyses were performed using SPSS, version 18.0 (SPSS, Inc., Chicago, IL).

4. RESULTS

In this study we evaluated various factors that may influence treatment response to intravitreal ranibizumab injections in 420 eyes of 397 unrelated Caucasian neovascular AMD patients. After 3 ranibizumab injections, the study population on average showed visual improvement, with a median VA prior to and after treatment of 0.54 and 0.44 logMAR, respectively. An increased age at the onset of treatment was significantly associated with loss in VA after three ranibizumab injections (0.02 LogMAR or 1 ETDRS letter loss per 4 years, $p=0.003$), while a poor baseline VA was significantly associated with a greater VA improvement after ranibizumab treatment ($p<0.001$). Individuals carrying the *CFH* low-risk genotype (TT, n=78, 18.6%) performed significantly better

TABLE 1. The effect of genotype on the visual acuity (VA) response after three ranibizumab injections and on the age when the first ranibizumab injection was administered.

Genotype		Mean VA change* (SE), logMAR	P-value	Mean difference in age§ (SE), years	P-value
CFH rs1061170 (Y402H), n= 420					
TT	n= 78 (18.6)	0.105 (0.039)	0.009	+ 1.70 (1.1)	0.11
TC	n= 198 (47.1)	0.031 (0.031)	0.31	+ 1.13 (0.8)	0.18
CC	n= 144 (34.4)	0	reference	0	reference
ARMS2 rs10490924 (A69S), n= 420					
GG	n= 141 (33.6)	0.033 (0.037)	0.37	+ 0.65 (1.0)	0.50
GT	n= 175 (41.7)	0.061 (0.035)	0.08	+ 2.06 (0.9)	0.026
TT	n= 104 (24.8)	0	reference	0	reference
VEGFA rs699947, n= 400					
AA	n= 117 (29.3)	0.048 (0.040)	0.23	+ 0.64 (1.1)	0.55
CA	n= 194 (48.5)	0.035 (0.033)	0.29	+ 1.43 (0.9)	0.11
CC	n= 89 (22.3)	0	reference	0	reference
VEGFA rs833069, n= 393					
TT	n= 168 (42.7)	- 0.020 (0.044)	0.66	- 0.55 (1.2)	0.64
TC	n= 173 (44.0)	- 0.014 (0.044)	0.74	- 0.74 (1.2)	0.54
CC	n= 52 (13.2)	0	reference	0	reference
KDR rs2071559, n= 393					
AA	n= 91 (23.2)	0.007 (0.040)	0.86	+ 0.12 (1.1)	0.91
GA	n= 197 (50.1)	- 0.064 (0.034)	0.06	+ 1.84 (0.9)	0.043
GG	n= 105 (26.7)	0	reference	0	reference
KDR rs7671745, n= 388					
GG	n= 129 (33.2)	- 0.029 (0.049)	0.55	+ 1.81 (1.3)	0.16
AG	n= 214 (55.2)	- 0.051 (0.045)	0.27	+ 0.80 (1.2)	0.52
AA	n= 45 (11.6)	0	reference	0	reference
FZD4 rs10898563, n=397					
AA	n= 135 (34.0)	- 0.018 (0.042)	0.67	+ 1.56 (1.1)	0.17
AG	n= 193 (48.6)	- 0.040 (0.040)	0.33	+ 1.06 (1.0)	0.32
GG	n= 69 (17.4)	0	reference	0	reference
LRP5 rs3736228, n= 388					
TT	n= 230 (59.3)	- 0.011 (0.041)	0.80	0.01 (1.1)	0.99
CT	n= 100 (25.8)	- 0.002 (0.046)	0.96	0.27 (1.3)	0.83
CC	n= 58 (14.9)	0	reference	0	reference

* Mean visual acuity improvement in LogMAR (0.02 LogMAR=1 ETDRS letter).

§ Positive age difference indicate older age, and negative age difference indicate younger age when the first ranibizumab injection was given.

VA= visual acuity, SE = Standard error, LogMAR = The logarithm of minimal angle of resolution, ETDRS = Early Treatment Diabetic Retinopathy Study

than those carrying the high-risk genotype (CC, n=144, 34.4%) (5.3 ETDRS letters better, p=0.009, Table 1). Furthermore, male patients (n=240, 57.1 %) were affected with neovascular leakage due to AMD 2.1 years earlier than female patients (p=0.004). Patients carrying the heterozygous *ARMS2* (GT) and *KDR* (GA) genotype were significantly older at the first injection compared to patients carrying the homozygous high-risk genotypes (p=0.026 and p=0.043, Table 1). We did not observe significant associations with *FZD4* and *LRP5* genotypes. In our study population the CNV type did not show an association with change in VA after 3 consecutive ranibizumab injections (p=0.14). The duration of visual complaints prior to treatment was not significantly different between the genotype groups. We did not encounter a difference in variability between the different between the change in VA techniques, as the standard deviation of the change in VA was very similar for Snellen and ETDRS (0.29 and 0.25 LogMAR, p=0.26).

4.1 Combined effect of *CFH* and *ARMS2* high-risk alleles

AMD patients who did not carry high-risk alleles in *CFH* and *ARMS2* demonstrated significantly more improvement in VA after ranibizumab treatment compared to carriers of all four high-risk alleles in these two genes (p=0.009, Table 2). A mean VA improvement of 10 ETDRS letters was observed in patients without any high risk alleles (n=20, 4.8%), 5 ETDRS letters in patients with one or two risk alleles (n=267, 63.5%) and 2.5 ETDRS letters in carriers of three risk alleles in *CFH* and *ARMS2* (n=104, 24.8%), while no mean improvement was observed in patients carrying all four high-risk alleles (n=29, 6.9%), (Figure 1A). A significant association between the number of high-risk alleles in *CFH* and *ARMS2* and the age at the first ranibizumab treatment was observed (p=0.002, Figure 1B). Carriers of 4 risk alleles in the *CFH* and *ARMS2* genes were 4.4 years younger at treatment onset than the carriers of 3 high-risk alleles (p=0.006) and 5.2 years younger than the AMD carriers of 1 or 2 risk alleles, respectively (p<0.0001, Table 2).

4.2 Combined effect of *CFH, ARMS2* and *VEGFA* high-risk alleles

Adding the *VEGFA* rs699947 SNP to the stratification model demonstrated a significant decrease in VA after ranibizumab treatment in the group carrying all 6 high-risk alleles in *CFH, ARMS2* and *VEGFA* compared to the remaining AMD patients. Carriers of all 6 risk alleles demonstrated a mean loss of 10 ETDRS letters after treatment (n=8, 2%), while all other allele groups demonstrated an improvement in VA after treatment (Figure 2A). In addition, a significant decrease of the age of neovascular onset was observed. The mean age at which the first ranibizumab treatment was carried out among the carriers of all 6 high-risk alleles in *CFH, ARMS2* and *VEGFA* was 65.9 years versus 75.3 years in the AMD carriers of none or one high-risk alleles (n=35, 8.8%, p=0.001, Table 2 and Figure 2B).

TABLE 2. The effect of high-risk alleles in *complement factor H (CFH), Age-Related Maculopathy susceptibility 2 (ARMS2)* and *vascular endothelial growth factor A (VEGFA)* on visual acuity (VA) response after three ranibizumab injections and on the age in years when the first ranibizumab injection was administered.

Number of high-risk alleles in *CFH* and *ARMS2*		**Mean VA change* (SE), logMAR**	**P-value**	**Mean difference in age§ (SE), years**	**P-value**
0	n= 20 (4.8%)	0.20 (0.08)	0.014	+ 2.8 (2.1)	0.19
1 and 2	n= 267 (63.5%)	0.10 (0.06)	0.07	+ 5.2 (1.5)	<0.0001
3	n= 104 (24.8%)	0.05 (0.06)	0.45	+ 4.4 (1.6)	0.006
4	n= 29 (6.9%)	0.00	reference	0.0	reference
Number of high-risk alleles in *CFH, ARMS2* and *VEGFA*		**Mean VA change* (SE), logMAR**	**P-value**	**Mean difference in age§ (SE), years**	**P-value**
0 and 1	n= 35 (8.8%)	0.40 (0.11)	< 0.0001	+ 9.4 (2.9)	0.001
2 and 3	n= 208 (52%)	0.32 (0.10)	0.001	+ 7.8 (2.7)	0.003
4	n= 116 (29%)	0.31 (0.10)	0.002	+ 7.2 (2.7)	0.008
5	n= 33 (8.3%)	0.29 (0.11)	0.008	+ 6.9 (2.9)	0.018
6	n= 8 (2%)	0.00	reference	0.0	reference

* Mean visual acuity improvement in LogMAR (0.02 LogMAR=1 ETDRS letter).
§ Positive age difference indicate older age when the first ranibizumab injection was given.

VA= visual acuity
SE = Standard error
LogMAR = The logarithm of minimal angle of resolution
CFH = Complement factor gene
ARMS2 = Age-Related Maculopathy susceptibility 2 gene
VEGFA = Vascular endothelial growth factor A gene
ETDRS = Early Treatment Diabetic Retinopathy Study

FIGURE 1. Cumulative effect of *complement factor H (CFH)* and *Age-Related Maculopathy susceptibility 2 (ARMS2)* risk alleles on the change in visual acuity (VA) after ranibizumab treatment (A) and on the age of neovascular onset (B).

A. VA change after 3 ranibizumab injections from baseline (y-axis, 0.20 corresponds to 10 Early Treatment Diabetic Retinopathy Study letters, a positive change indicates VA gain and a negative change indicates VA) clustered by a *CFH* and *ARMS2* allelic composition (x-axis): (4) participants carrying all four high-risk alleles (n=29, 6.9%), (3) patients with three high-risk alleles (n=104, 24.8%), (2-1) patients carrying one or two risk alleles (n=267, 63.5%), and (0) participants without high-risk alleles in either genes (n=20, 4.8%). The carriers of all four *CFH* and *ARMS2* high-risk alleles demonstrated a significantly lower mean VA than the AMD patients carrying two, one or no high-risk *CFH* and *ARMS2* alleles.

B. The age when first ranibizumab injection has been administrated (y-axis) clustered by *CFH* and *ARMS2* allelic composition as mentioned above. The carriers of all four alleles in *CFH* and *ARMS2* received their first ranibizumab injection at a significantly younger age, indicating that those high-risk genes lead to an earlier onset of neovascular AMD.

FIGURE 1.

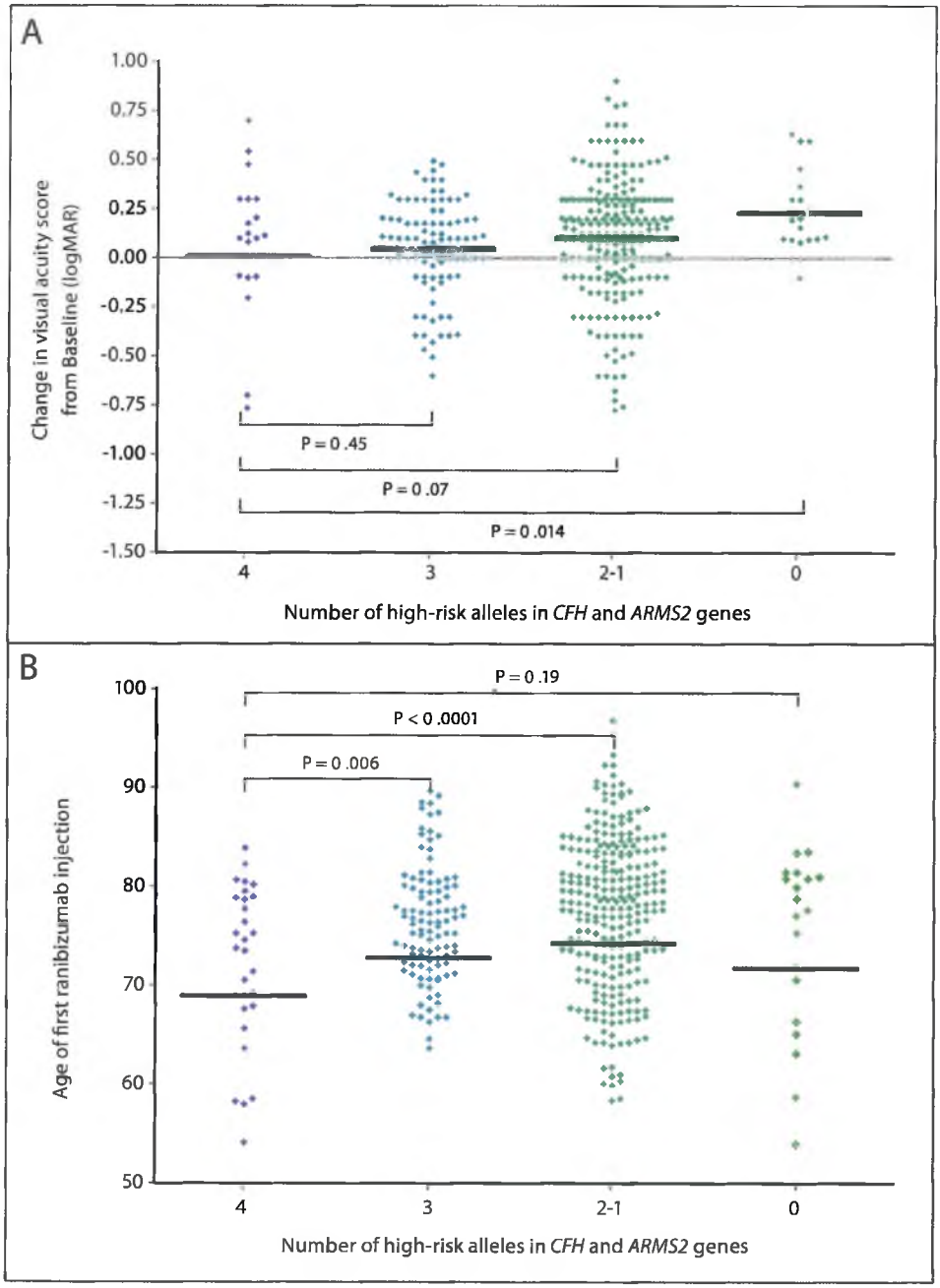
A
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
-1.25
-1.50
Change in visual acuity score from Baseline (logMAR)
P = 0.45
P = 0.07
P = 0.014
4
3
2-1
0
Number of high-risk alleles in CFH and ARMS2 genes
B
100
90
80
70
60
50
Age of first ranibizumab injection
P = 0.19
P < 0.0001
P = 0.006
4
3
2-1
0
Number of high-risk alleles in CFH and ARMS2 genes

FIGURE 2.

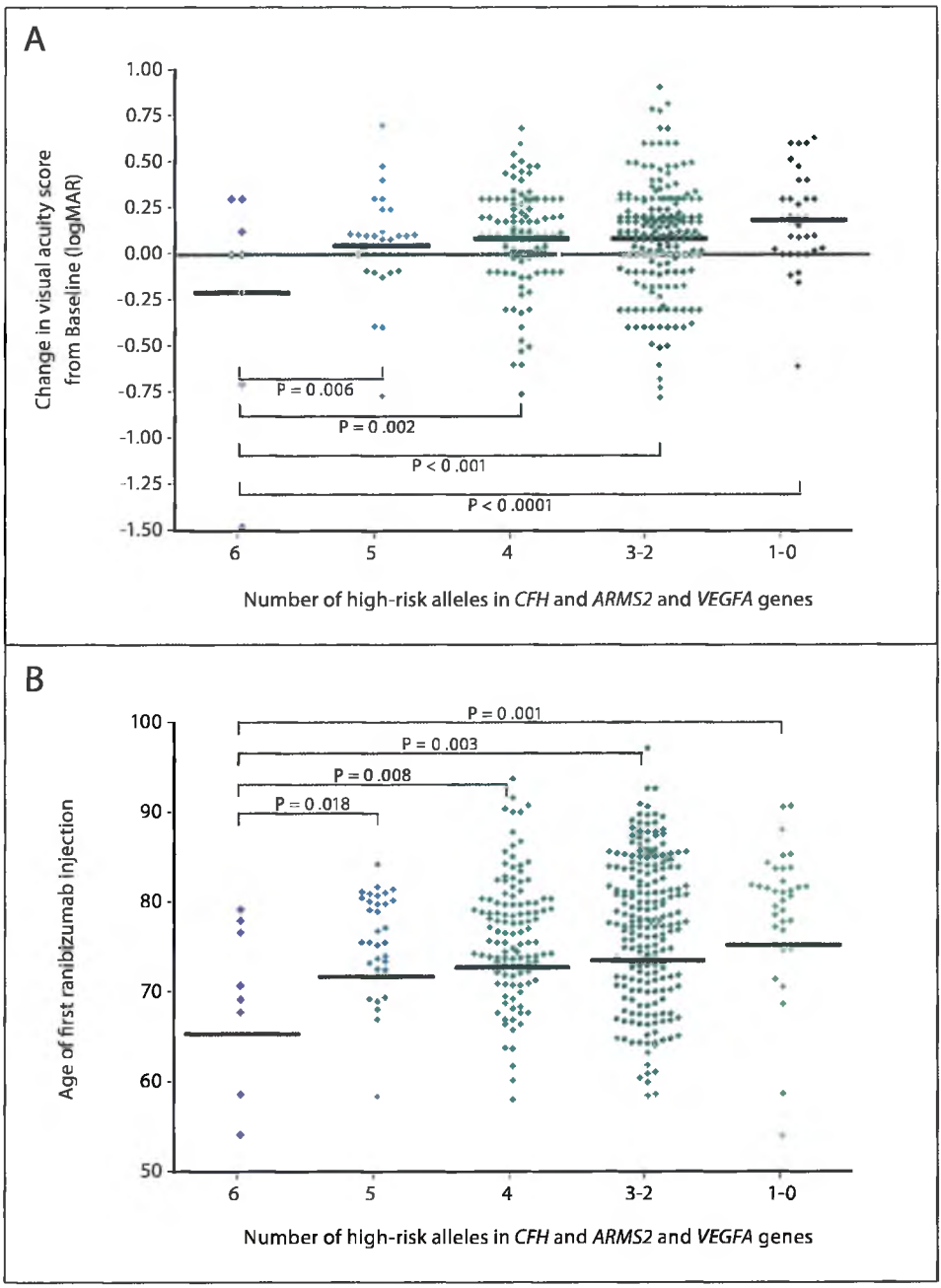
A
Change in visual acuity score from Baseline (logMAR)
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
-1.25
-1.50
P = 0.006
P = 0.002
P < 0.001
P < 0.0001
6
5
4
3-2
1-0
Number of high-risk alleles in CFH and ARMS2 and VEGFA genes
B
Age of first ranibizumab injection
100
90
80
70
60
50
P = 0.001
P = 0.003
P = 0.008
P = 0.018
6
5
4
3-2
1-0
Number of high-risk alleles in CFH and ARMS2 and VEGFA genes

FIGURE 2. Cumulative effect of *complement factor H (CFH), Age-Related Maculopathy susceptibility 2 (ARMS2),* and *vascular endothelial growth factor A (VEGFA)* high-risk alleles on the change in visual acuity (VA) after ranibizumab treatment (A) and on the age of neovascular onset (B).

A. VA change after 3 ranibizumab injections from baseline (y-axis, 0.20 corresponds to 10 Early Treatment Diabetic Retinopathy Study letters, a positive change indicates VA gain and a negative change indicates VA loss) clustered by *CFH, ARMS2* and *VEGFA* allelic composition (x-axis): (6) patients carrying all six high-risk alleles (n=8, 2%), (5) AMD patients carrying 5 risk alleles (n=33, 8.3%), (4) participants with four risk alleles (n=116, 29%), (3-2) patients carrying three or two risk alleles (n=208, 52%), and (1-0) patients carrying one or no high-risk alleles in *CFH, ARMS2* and *VEGFA* genes (n=35, 8.8%). Compared the remaining AMD patients, the carriers of all 6 high-risk alleles demonstrated a significant worsening of VA.

B. The age when the first ranibizumab injection was administrated (y-axis) clustered by *CFH, ARMS2* and *VEGFA* allelic composition as mentioned above. Adding the *VEGFA* high-risk allele to the stratification model demonstrated a younger age at which the first ranibizumab treatment was carried out compared to the stratification model based on *CFH* and *ARMS2* risk alleles. The mean age among the carriers of all 6 high-risk alleles in *CFH, ARMS2* and *VEGFA* were 65.9 years versus 75.3 years in the AMD carriers of no or one high-risk alleles (p=0.001).

5. DISCUSSION

Pharmacogenetics is the field of study that examines the impact of genetic variation on the response to drugs. As more eye diseases are linked to their underlying genetic defects, pharmacogenetics and genomics will play an increasingly important role in clinical trial design and eventually in everyday ophthalmology practice. Genetic factors are known to play a major role in the pathogenesis of AMD, and it has been suggested that genetic factors may also influence response to anti-VEGF treatment in neovascular AMD.[16-19] In this large series of patients, we evaluated the role of the high-risk alleles in the *CFH, ARMS2, VEGFA, KDR, FZD4* and *LPR5* genes on VA outcome after treatment with ranibizumab injections. Consistent with a previously conducted study, we demonstrated that carriers of the high-risk *CFH* genotype show less improvement in VA after treatment.[16]

In order to test the possibility of an additive genetic effect on the response to ranibizumab treatment, we stratified the data according to the number of the high-risk alleles in *CFH, ARMS2* and/or *VEGFA*. After stratification for the number of risk alleles in *CFH* and *ARMS2*, we observed that the carriers of 4 risk alleles demonstrated no mean VA improvement after 3 injections and received ranibizumab treatment 5 years earlier. In AMD patients who in addition also carried two *VEGFA* risk alleles, ranibizumab treatment was needed almost 10 years earlier. The *VEGFA*

SNP also had an additional negative effect on the VA response rate of ranibizumab. A previous study demonstrated a potential association between the *VEGFA* rs699947 risk genotype and neovascular lesions that remained exudative after several photodynamic therapies.[23] Taking those observations together, we conclude that a cumulative effect of high-risk alleles in *CFH*, *ARMS2* and *VEGFA* may lead to a younger age of onset in combination with worse response rates to ranibizumab treatment. The carriers of 4 high-risk alleles in *CFH, ARMS2* or *VEGFA* demonstrate a better VA improvement than the AMD carriers of 4 high-risk alleles in *CFH* and *ARMS2*, indicating that *CFH* and *ARMS2* genes have greater consequence on the response to ranibizumab treatment than the *VEGFA* gene.

Although our study indicates that an additive effect of *CFH, ARMS2* and *VEGFA* genotypes is partially responsible for a decreased response rate to the ranibizumab treatment, the mechanism by which these genotypes interact with anti-VEGF therapy is unknown. Because anti-VEGF drug is locally injected, it is unlikely that inherited pharmacokinetic effects are responsible for the variation in the concentration of the drug reaching its target. AMD is strongly associated with *CFH* and *ARMS2* polymorphisms, and both genes are independently related to the progression of AMD from early to advanced stages.[24] CFH is the main regulator of the alternative complement pathway and the *CFH* 402H variant is associated with an altered function of the CFH protein, leading to a deregulation and an over-activation of the alternative complement pathway.[25] In a recent study we demonstrated that increased complement activation in serum is associated with the *ARMS2* high-risk allele, suggesting that this locus is also involved in complement activation.[26]

At the biochemical level, bioactive complement components C3a and C5a induce VEGF expression in RPE cells, and cause a reduction in CNV when neutralized.[27, 28] Another important finding is that the CC genotype of the *VEGFA* rs699947 SNP, localized in the promoter region of the *VEGFA* gene, is associated with a higher VEGF production.[29] Taking observations together, we propose that *CFH* and *ARMS2/HTRA1* are both associated with an over-activation of the alternative complement pathway and, in combination with the *VEGFA* polymorphism may lead to potentially higher levels of (local) VEGF production. As a result of increased VEGF production choroidal neovascularization may develop sooner, consequently leading to CNV formation at a younger age, as demonstrated in our study.

It is not clear whether AMD patients with a high-risk genetic background develop more devastating type of AMD or less anti-VEGF responsiveness. It is plausible that high VEGF expression levels may cause specific phenotypic CNV characteristics or submacular fibrosis, which may lead to a decreased response to anti-VEGF therapy. It is unclear whether such phenotypic characteristics correlate with genetic background. One study suggested that type

of neovascular membranes may be influenced by the *CFH* and *HTRA1* genetic variations.[45] Yet another study showed that it is not the type of neovascularisation that is associated with worse visual outcome after ranibizumab treatment, but the size of the retinal pigment epithelium area that is affected.[30] Consistent with previous findings, the CNV type did not show an association with response to anti-VEGF treatment in our study population.[30] Conflicting results have been reported concerning association between *CFH* and *ARMS2/HTRA1* genotypes and size of the CNV lesion.[31-34] Nevertheless, further investigation in the mechanism by which these genotypes interact with anti-VEGF therapy is required.

High-risk genotypes tested in our study are predictive for progression of AMD and response to anti-VEGF treatment, which underlines the potential of genetic screening in predicting the development of end-stage AMD. Identifying high-risk individuals during the early stage of the disease could lead to an awareness, and a more targeted education about the adoption of a healthy lifestyle and the beneficial use of antioxidants and zinc supplementation in slowing down the progression of AMD.[35] Identifying high-risk individuals during advanced stages of the disease could lead to a more frequent clinical surveillance in such individuals, and could be used for identifying patients for future clinical trials designed to evaluate new treatments. Therapeutic options for late stage AMD are currently limited, however, this could change in the near future. Ongoing clinical trials are currently testing therapeutic effects of complement inhibition in neovascular and non-exudative AMD.[36] One clinical trial is using a combination of intravitreal therapy of anti-C5-aptamer (ARC1905) and ranibizumab.[36] AMD patients with high-risk genotypes and decreased response rates to ranibizumab treatment may have a greater benefit of a combination therapy with complement inhibition and anti-VEGF. A limitation of the study is that the conclusions are based on limited number of AMD patients with 4 or 6 high-risk alleles in *CFH, ARMS2* and *VEGFA* genes. Therefore, we recommend these results to be validated in other patient cohorts. The vast majority of patients are in the middle of the distribution and a wide range in response to ranibizumab treatment was observed among allele groups, indicating that also other factors contribute to the response rate. Additional studies on the effect of phenotypic and environmental parameters, additional genetic variants and possibly proteomic profiling, may lead to a more accurate prediction model, which would provide the basis towards personalized medicine for neovascular AMD.

In conclusion, our findings demonstrate a cumulative effect of high-risk alleles, leading to a younger age of neovascular AMD onset in combination with poor response rates to intravitreal ranibizumab treatment. Because genetic variation partially explains the wide range of response to ranibizumab treatment, genetic screening has the potential to identify high-risk individuals and may in future help clinicians to tailor medical care to individual needs.

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CHAPTER FIVE

PREVENTION OF AGE-RELATED MACULAR DEGENERATION

Zinc supplementation inhibits complement activation in age-related macular degeneration

1. SUMMARY

Complement-mediated inflammation plays a pivotal role in the pathogenesis of age-related macular degeneration (AMD), the leading cause of blindness in the Western world. Oral zinc supplementation can reduce the progression of AMD but the precise mechanism of this protective effect is as yet unclear. We investigated whether zinc supplementation directly affects the degree of complement activation in AMD.

In an open-label clinical study, randomly selected 72 AMD patients in various stage of AMD received a daily supplement of 50 mg zinc sulphate and 1 mg cupric sulphate for three months. Serum complement catabolism— defined as the C3d/C3 ratio—was measured at baseline, throughout the three-months of supplementation and after discontinuation of zinc administration. Secondly, we investigated the effect of zinc on complement activation *in vitro*. *In vitro* zinc sulphate directly inhibits complement catabolism in hemolytic assays and membrane attack complex (MAC) deposition on RPE cells.

AMD patients with high levels of complement catabolism at baseline exhibited a steep decline in serum complement activation ($p<0.0001$) during the 3-month zinc supplementation period. Individuals who were homozygous for the *CFH* Y402H high-risk genotype had a significantly faster decline in their C3d/C3 ratio ($p=0.016$) in after the first month of zinc administration. There was no significant association between C3d/C3 ratio and *ARMS2* genotype. Patients with late-stage AMD in both eyes but no evidence of disease activity had significantly lower levels of serum complement catabolism than AMD patients with large drusen and/or drusenoid retinal pigment epithelial (RPE) detachment ($p=0.005$).

The level of serum complement catabolism correlates with the AMD stage, indicating that the level of serum complement catabolism may be a sign of disease activity. Daily administration of 50 mg zinc sulphate inhibits complement catabolism in AMD patients. However, this effect occurs only in patients with a high baseline level of complement catabolism. This could be the mechanism by which zinc slows AMD progression and it also explains why this effect occurs in some but not all patients.

2. INTRODUCTION

Worldwide, age-related macular degeneration (AMD) affects 30-50 million people and is the leading cause of blindness in the Western world.[1-4] The hallmark lesions of early- and intermediate-stage AMD are drusen, which are pathological deposits of extracellular material that form between the retinal pigment epithelium and Bruch membrane.[5] The late stages of AMD can be separated into geographic atrophy and neovascular AMD.[5] Geographic atrophy is characterized by the development of central retinal atrophy.[5] In neovascular AMD, choroidal blood vessels invade the central retina and subretinal space.[5] Although the neovascular AMD accounts for 10% of all AMD patients, it is responsible for the majority of AMD-related severe visual impairment.[6-9] Despite the beneficial effects of intraocular injections of vascular endothelial growth factor A (VEGF-A) inhibitors,[10,11] a large percentage of neovascular AMD patients continue to lose vision.[12,13] For geographpic atrophy no effective therapy is currently available.

The complement system is a major component of innate immunity with crucial roles in the first line defense against invading microorganisms, clearance of the apoptotic cells and modulation of the adaptive immune response.14 There are three pathways of complement activation, i.e., the classical, the lectin and the alternative pathway.14 The discovery that drusen contain proteins of the alternative complement pathway led to the hypothesis that drusen could be involved in local complement-mediated inflammation.15,16 Moreover, the discovery of a strong association between AMD and genetic variants in the complement factor H gene *(CFH)*, a major inhibitor of the alternative pathway, provided a second line of evidence in support of the inflammation model.17-20 In addition to *CFH,* several other AMD risk variants have been found in genes underlying the alternative pathway.21-24 Several studies reported systemic activation of the alternative pathway, supporting that complement inflammation in AMD is not restricted to the retina.25-28 The discovery of complement as a major contributing factor to AMD pathogenesis has sparked considerable interest in this system as a potential therapeutic target, and several complement inhibitors are currently being tested in clinical trials.29 However, it is likely to be several years before any of the complement inhibiting drugs will be approved for routine use in clinical practice, assuming they are eventually found to be safe and effective

The Age-Related Eye Disease Study (AREDS) found that the risk of progressing from intermediate to advanced AMD can be reduced by supplementation with zinc either given alone or in combination with antioxidants.[30] A report published by the Blue Mountains Eye Study, a population-based study, confirmed the beneficial effect of zinc in AMD patients.[31] In addition, the large population-based Rotterdam Study found that a high dietary intake of zinc

reduces the increased risk for AMD that is associated with the Y402H *CFH* variant, suggesting a link between complement and zinc.[32] While many studies have focused on understanding the anti-oxidative mechanism of zinc,[33,34] to date, it is unknown whether there is an effect on the complement catabolism. The goal of the present study was to investigate whether zinc affects the activity of the alternative complement pathway in vitro as well as in patients with AMD.

3. PATIENTS AND METHODS

3.1 Study population of the clinical study

This study was performed in accordance with the Declaration of Helsinki and the Dutch Medical Research Involving Human Subjects Act. Prior to the study, we obtained approval from the local ethics committee as well as written informed consent from all participants. This clinical study is registered with the Netherlands Trial Register, number NTR2605. Patients with various stages of AMD were selected at random from the EUGENDA database. We excluded individuals who had a core body temperature above 38°C and/or received antibiotics at baseline. In addition, we excluded patients who were receiving intraocular anti-angiogenic treatment, individuals with atypical hemolytic uremic syndrome or membranoproliferative glomerulonephritis type 2 and patients who received local or systemic steroid therapy within the three months prior to the trial. A total of 72 AMD patients were included in this study.

3.2 Study design

For a period of three months, 72 AMD patients received a daily oral supplement containing 50 mg zinc sulphate and 1 mg cupric sulphate in capsule form. The primary endpoint of the study was a change in serum complement catabolism. AMD patients have increased serum levels of C3 and the metabolic byproduct C3d, most prominent marker of chronic activation of the alternative complement pathway.[28] To correct for individual variations in the level of C3, complement activation was defined as the C3d/C3 ratio as described.[28] The second endpoint of the study was the correlation of serum complement catabolism during zinc administration and genotypes of AMD risk variants in *CFH* or *ARMS2*. During the course of the study, six venous blood samples were collected. One sample was collected prior to zinc supplementation and served as the baseline sample. Three samples were collected at the end of months 1, 2 and 3 of the three-month period of zinc supplementation. We collected a fifth blood sample two months after ending the zinc administration (i.e., at the end of month 5) to check for any reversible effects on complement activation. A final blood sample was collected in months 14-22. From one month prior to zinc supplementation through the end of month 5, the patients

were prohibited to take any type of nutritional supplement; from month 5 onwards, the patients were free to take supplements at their own discretion.

To identify clinical manifestations associated with intermittent infections, at every visit, we performed a general physical examination, measured the serum C-reactive protein (CRP) levels and administered a questionnaire that was aimed at identifying clinical manifestations associated with intermittent infections. At every visit, we also assessed the best-corrected visual acuity using Early-Treatment Diabetic Retinopathy Study (ETDRS) charts. In addition, we imaged the retinas using high-resolution spectral-domain optical coherence tomography (SD-OCT) to detect neovasular manifestation of AMD. We performed color fundus photography at baseline to assist in AMD grading based on the 5-grade Clinical Age-Related Maculopathy Staging (CARMS) classification scale.[35] In a recent study we measured C3d/C3 levels in 119 unaffected age-matched controls,[28] and we used those C3d/C3 ratios as normal values.

3.3 Complement measurements and genotyping in AMD patients

Serum was prepared by coagulation at room temperature and after centrifugation the samples were stored at -80 °C within one hour after collection. C3 and C3d were measured in serum samples as described.36,37 All complement measurements in this study were performed in a single experiment, except for the final sample in months 14-22. The CFH (Y402H; rs1061170) and ARMS2 (A69S; rs10490924) SNPs were genotyped as described.38 Serum zinc concentration was measured by atomic absorption spectroscopy with the spectrophotometer 1100 B from Perkin Elmer. CRP levels were measured by Abbott Architect C16000 system. The immunoturbidimetric test for CRP was provided by Abbott Diagnostics (Abbott Diagnostics).

3.4 Statistical analysis

Data for the hemolytic assay and the RPE cell assay were analyzed using one-way ANOVA with Dunnett's multiple comparison test. Changes in serum complement level were analyzed using a linear mixed-effects model with C3d/C3 ratio as the dependent variable. A fixed effects model was used, including the interaction between time and baseline C3d/C3 ratio. The associations between the C3d/C3 ratio throughout the study and *CFH* and *ARMS2* genotype, age, gender, CRP level and zinc level were also studied using a linear mixed-effects model. In the final model, only significant predictors were used ($p < 0.05$). Changes in serum zinc concentration were analyzed using a linear mixed-effects model with zinc concentration as the dependent variable.

We further analyzed baseline characteristics of AMD patients with a relatively high baseline complement catabolism. In an exploratory analysis, baseline characteristics were compared between patients with near normal vs. elevated C3d/C3 ratio at baseline. As a cut-off point, a C3d/C3 ratio of 2.1 was used, corresponding with the mean plus one standard deviation level of C3d/C3 ratio among the n=150 age-matched healthy controls.[28] The Mann-Whitney *U*

test and the Student t-test were used to compare baseline visual acuity and age between the two groups, respectively. Visual acuity changes during the course of the study were assessed by generalized estimated equations (GEE). The GEE model estimated the probability of low vision (LogMAR < 0.5) versus high vision (LogMAR > 0.5), with time and baseline C3d/C3 ratio as predictors. The correlation between baseline visual acuity and CARMS was tested using the Spearman's rank correlation coefficient. Reported p-values are two-sided, and differences were considered to be statistically significant if lower than 0.05.

3.5 Stratification of systemic complement catabolism according to CARMS

The association between systemic complement catabolism and baseline CARMS classification was tested using general linear models with a post-hoc Bonferroni correction. Because patients often display different stages of AMD in each eye, we created five groups for both eyes. These groups were based on the CARMS classification as follows: (CARMS grade 2:2), several small drusen and/or RPE changes in both eyes; (CARMS grade 3:3), several intermediate-size and large drusen and/or drusenoid RPE detachment in both eyes; (CARMS grade 2: 4-5), small drusen and/or RPE changes in one eye and geographic atrophy or choroidal neovascularisation in the other eye; (CARMS grade 3:4-5), large drusen and/or drusenoid RPE detachment in one eye and geographic atrophy or choroidal neovascularisation in the other eye; and (CARMS grade 4-5:4-5), geographic atrophy or choroidal neovascularisation in both eyes. The baseline C3d/C3 ratio was used as the dependent variable, and relationships were adjusted for the *CFH* and *ARMS2* genotypes. In order to adjust the p-value levels for multiple testing as in CARMS, Borforronni correction was preformed. Reported p-values are two-sided, and differences were considered to be statistically significant if lower than 0.01. All statistical analyses were performed using SPSS, version 18.0.

3.6 *In vitro* hemolytic assays and membrane attack complex (MAC) deposition on the retinal pigment epithelium (RPE) cells

Human serum was prepared from blood of several healthy volunteers after written informed consent had been obtained with the specific permit (418/2008) from the ethics committee of Lund University. Rabbit erythrocytes were washed in 2.5 mM veronal buffer pH 7.3, supplemented with 70 mM NaCl, 140 mM glucose, 0.1% porcine gelatin and 7 mM $MgCl_2$. Different concentrations (0-64 μM) of zinc sulphate (Merck) were pre-incubated with 2% serum in the same buffer for 1.5 h at 37°C, followed by 1 h incubation at 37°C together with the erythrocytes. The amount of lysed erythrocytes was determined from the amount of released hemoglobin at 405 nm using Cary 50 MPR microplate reader (Varian).

To study the effect of zinc on membrane MAC deposition on human RPE cells subjected to oxidative stress, RPE cells (ARPE-19, ATCC) were cultured in DMEM/F12 media (HyClone), supplemented with 10% FCS (Gibco) and antibiotics (HyClone). After detachment using trypsin,

the cells were incubated in medium containing 10 mM H_2O_2 for 2 h at 37°C, to induce oxidative stress. After washing with PBS, the cells were incubated with 5% human serum, together with 0-250 µM zinc sulphate, in the veronal buffer defined above , for 1 h at 37°C. The amount of MAC deposited on the RPE cells was detected using a monoclonal C9 neoepitope antibody (aE11, Hycult), which only recognizes C9 in the C5b-9 complex, followed by a FITC-conjugated secondary antibody and flow cytometric analysis (Partec).

4. RESULTS

4.1 Effect of zinc supplementation on complement catabolism in AMD patients

To evaluate the effect of receiving zinc supplements on systemic complement catabolism, AMD patients received oral zinc sulphate. The baseline characteristics of the study population are presented in Table 1. Serum zinc concentration increased significantly during the supplementation period ($p<0.0001$) and returned to baseline levels two months after the

TABLE 1. Summary of the baseline characteristics of the study group and age-matched control group.

Baseline charecteristics	AMD group, n=72	Control group, n=119
Mean age — yrs. ± SD	73.9 ± 8.3	71.4 ± 5.6
Sex, male — No. (%)	29 (40.3)	50 (42.0)
CFH (Y402H, rs1061170) and ARMS2 (A69S; rs10490924) genotypes — No. (%)		
CFH low-risk TT genotype	1 (1.4)	54 (45.4)
CFH high-risk CT genotype	36 (50.0)	51 (42.9)
CFH high-risk CC genotype	34 (47.2)	14 (11.8)
ARMS2 low-risk GG genotype	19 (26.4)	69 (58.0)
ARMS2 high-risk TG genotype	30 (41.7)	44 (37.0)
ARMS2 high-risk TT genotype	22 (30.6)	5 (4.2)
Serum C-reactieve protein — No. (%)		
< 5 — mg/l	52 (72.2)	93 (78.2)
5 - 15 — mg/l	18 (25.0)	23 (19.3)
16 - 45 — mg/l	2 (2.8)	3 (2.5)

SD= Standard deviation

zinc supplements were discontinued (Fig. 1). The mean complement activation level, defined as the C3d/C3 ratio, in the 72 patients showed tendency to decline (albeit not significantly; p=0.15) during the three months of zinc supplementation. The decline in C3d/C3 ratio was strongly correlated with the baseline C3d/C3 ratio (p<0.0001). The AMD patients with relatively high baseline levels of serum complement catabolism exhibited a steep decline in their C3d/C3 ratio during the administration of zinc sulphate (p<0.0001), after which the C3d/C3 ratio remained at this lower level for the following two months, even in the absence of zinc supplementation. Measurements performed at least nine months later (in months 14-22) showed that complement activation had returned to baseline levels in this group. The AMD patients who already had a relatively low C3d/C3 ratio at baseline showed no decline in C3d/C3 ratio throughout the treatment period. The Figure 2 shows raw data of different C3d/C3 ratios during the study period, the statistic method is not based on cut-off point as used in the figure 2. There was no significant association between C3d/C3 ratio and age or gender throughout the course of the study.

FIGURE 1. Serum zinc concentration and C3d/C3 ratio throughout the study period.
During the three months daily zinc supplementation, serum zinc concentration increased significantly (p<0.0001). After zinc supplementation was discontinued, the serum zinc levels returned to baseline levels within 2 months. The C3d/C3 ratio showed non-significant decline during zinc supplementation (p=0.15).

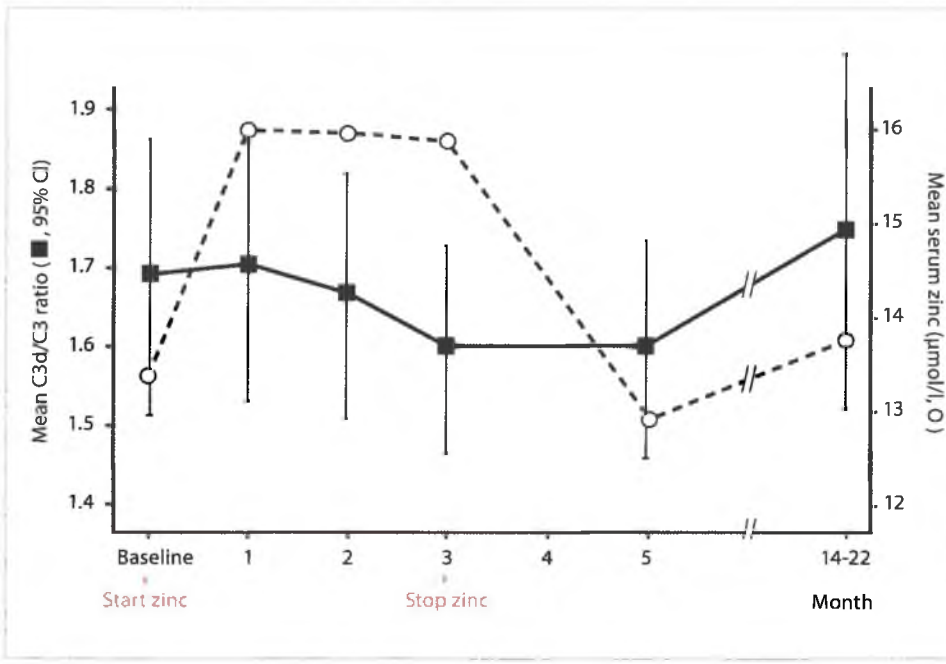
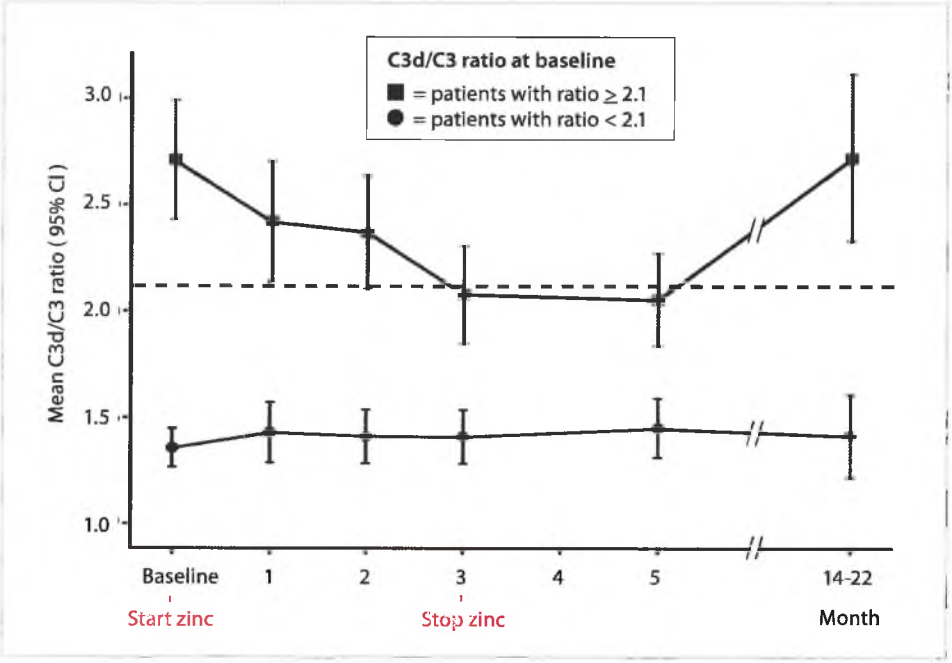


FIGURE 2. The effect of zinc supplementation on patients with different level of complement catabolism at baseline.

The patients with high serum complement catabolism had a steep decline in C3d/C3 ratio during the administration of zinc sulphate ($p<0.0001$). The dashed line shows the mean C3d/C3 ratio in the control group plus one standard deviation from the mean.[28] Please note, the cut-off point is only used for illustration of different C3d/C3 ratios during the study period and is not used in the statistic analysis.



4.2 Correlation between the serum complement catabolism and the genotype

The baseline C3d/C3 ratios did not differ significantly between high and low risk *CFH* genotypes ($p=0.95$). The decrease in C3d/C3 ratio, however, was more pronounced in patients homozygous for the *CFH* Y402H risk variant at 1 month compared with patients heterozygous or homozygous for low-risk *CFH* genotypes ($p=0.016$). There was no significant association between C3d/C3 ratio and *ARMS2* genotype.

4.3 Intermittent infections and the C3d/C3 ratio

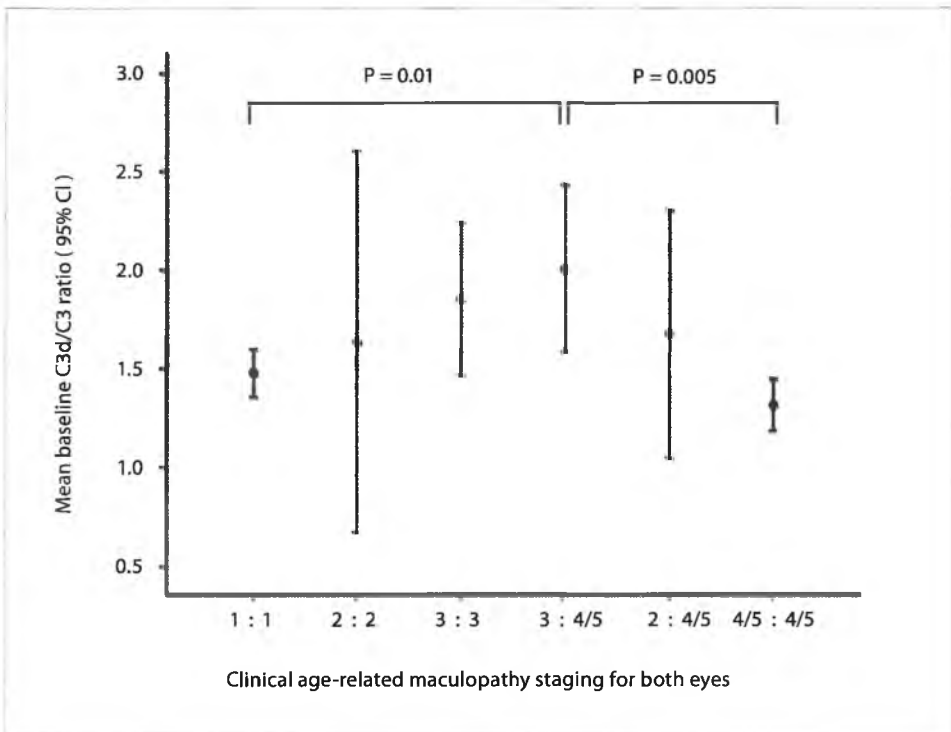
Serum CRP levels were measured at every visit and were not significantly associated with the C3d/C3 ratio ($p=0.17$). Questionnaires demonstrated that antibiotics were prescribed in 10 patients during the study period. Use of antibiotics was not related to increased CRP levels, increased body temperature or elevated C3d/C3 ratio (data not shown) in these individuals.

4.4 Association between the stage of AMD and serum complement catabolism

We further analyzed the clinical characteristics of AMD patients with a relatively high baseline complement catabolism. The group with relatively high C3d/C3 ratio had significantly better visual acuity ($p < 0.003$) and were significantly younger ($p<0.0001$, Table 2). Baseline visual acuity was associated strongly with the CARMS classification ($r=0.71$, $p<0.0001$). We tested whether the level of serum complement catabolism was associated with the stage of AMD. The baseline C3d/C3 ratio appeared to be significantly associated with the baseline CARMS classification ($p=0.006$) and Figure 3.

FIGURE 3. The correlation between AMD stage and C3d/C3 ratio.

AMD patients with several intermediate-size and large drusen and/or drusenoid RPE detachment (CARMS grade 3) in one eye and geographic atrophy or neovascular (CARMS stage 4 or 5) had significantly the highest C3d/C3 ratio. The patients who had geographic atrophy or neovascular AMD in both eyes (CARMS 4-5:4-5) had significantly lower C3d/C3 levels.



CARMS grade 1; No drusen. CARMS grade 2; several small drusen and/or RPE changes. CARMS grade 3; several intermediate-size and large drusen and/or drusenoid RPE detachment. CARMS grade 4; geographic atrophy. CARMS grade 5; choroidal neovascularisation.

TABLE 2. Summary of the baseline characteristics of the AMD patients with relatively high and normal baseline C3d/C3 ratio.

Baseline characteristics of AMD patients	Baseline C3d/C3 < 2.1 n =56	Baseline C3d/C3 ≥ 2.1 n=16
Mean age — yrs. ± SD	76.1 (7.04)	65.9 (7.57)
Sex, male — No. (%)	23 (41.1)	6 (37.5)
Mean serum zinc — µmol/l ± SD	13.4 (2.90)	13.3 (2.67)
Median visual acuity OD— logMAR	1.00	0.15
Median visual acuity OS— logMAR	0.59	0.14
Median serum C-reactief protein — mg/l	5	5
CFH (Y402H; rs1061170) and ARMS2 (A69S, rs10490924) genotypes — No. (%)		
CFH low-risk TT genotype	0	1 (6.3)
CFH high-risk CT genotype	28 (50.9)	8 (50.0)
CFH high-risk CC genotype	27 (49.1)	7 (43.7)
ARMS2 low-risk GG genotype	15 (27.3)	4 (25.0)
ARMS2 high-risk TG genotype	23 (41.8)	7 (43.8)
ARMS2 high-risk TT genotype	17 (30.9)	5 (31.2)

SD= Standard deviation. OD=Oculus dexter. OS=Oculus sinister. logMAR= The logarithm of minimal angle of resolution.

TABLE 3. Summary of the baseline clinical age-related maculopathy staging (CARMS) of the study group and age-matched control group for both eyes.

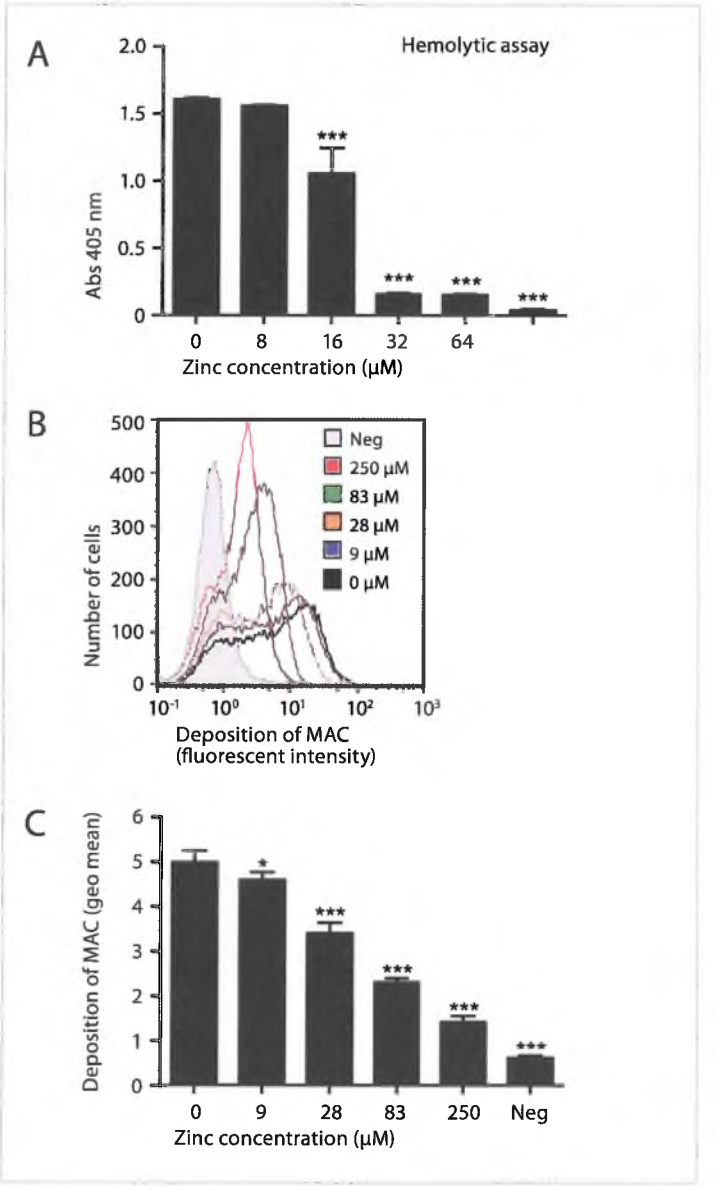
Clinical Age-Related Maculopathy Staging (CARMS) for both eyes		Median Visual Acuity (LogMAR)		No. (%)
1 : 1	Grade 1 in both eyes (healthy controls)	0.00	0.00	119 (62.6)
2 : 2	Grade 2 in both eyes	0.02	0.02	4 (3.7)
2 : 4-5	Grade 2 in the first eye and grade 4 or 5 in the second eye	0.02	1.06	9 (4.7)
3 : 3	Grade 3 in both eyes	0.14	0.14	10 (5.3)
3 : 4-5	Grade 3 in the first and stages 4 or 5 in the second eye	0.14	1.06	19 (10.0)
4-5 : 4-5	Grades 4 or 5 in both eyes	1.06	1.06	29 (15.3)

LogMAR= The logarithm of minimal angle of resolution. CARMS grade 1; No drusen. CARMS grade 2; several small drusen and/or RPE changes. CARMS grade 3; several intermediate-size and large drusen and/or drusenoid RPE detachment. CARMS grade 4; geographic atrophy. CARMS grade 5; choroidal neovascularisation.

FIGURE 4. The effect of zinc on the hemolytic activity of human serum and on the membrane attack complex (MAC) deposition on retinal pigment epithelial (RPE) cells.

A. The alternative complement pathway hemolytic activity of human serum was tested after pre-incubation of human serum with 0-64 μM zinc sulphate. Zinc sulphate inhibits the lysis of rabbit erythrocytes in a dose-dependent manner.

B-C. the amount of MAC deposited on RPE cells exposed to oxidative stress can be reduced in a dose dependent manner by zinc. *p<0.05 and ***p<0.001.



4.5 Effect of zinc on complement catabolism *in vitro*

To demonstrate the *in vitro* effect of zinc on the complement activity of human serum, we performed an alternative pathway hemolytic assay. Results showed that zinc sulphate inhibits the lysis of rabbit erythrocytes in a dose-dependent manner (Fig. 4A). Interestingly, the effect of zinc was more pronounced when serum was pre-incubated with zinc before performing the hemolytic assay (data not shown).

Retina is exposed to high levels of oxidative stress from light exposure and metabolic processes.[39] We tested *in vitro* whether zinc could also protect the RPE from a oxidative stress related damage from the complement system. The test results show that the amount of MAC deposited on RPE cells exposed to oxidative stress can be reduced in a dose dependent manner by zinc sulphate (Fig. 4B-C). In the negative controls, zinc and serum were omitted.

5. DISCUSSION

In the past decade, it has become increasingly clear that complement-mediated inflammation plays a fundamental role in the etiology of AMD.[16,40] Our results show that zinc inhibits complement activation in AMD patients who have a high level of complement catabolism. The AREDS1 study concluded that the five-year progression from intermediate to advanced AMD can be reduced by 25% with zinc plus antioxidants and by 21% with only zinc supplementation.[30] Based on these findings, we surmise that the mechanism by which zinc supplementation delays AMD progression is by reducing the level of complement activation, but only in those AMD patients with high complement catabolism. This could also imply that treatment with complement inhibitors should be targeted at patients with elevated complement activity, defined by the the serum C3d/C3 ratio. It is unlikely that the study results were influenced by the statistical phenomenon of 'regression to the mean' because the C3d/C3 ratio returned to baseline levels for both groups after discontinuation of zinc administration. Because serum CRP levels were not significantly associated with C3d/C3 ratio, it is unlikely that the observed change in complement catabolism can be ascribed to an intermittent infection.

In support of the hypothesis that zinc administration affects complement catabolism, we demonstrated *in vitro* that zinc sulphate directly inhibits complement activation in human serum in a dose-dependent manner. The effect of zinc was more pronounced when serum was pre-incubated with zinc before performing the hemolytic assay, suggesting that zinc may inhibit the spontaneous activation of the alternative pathway of complement. In addition to the direct inhibitory effect of zinc on the complement cascade, we also demonstrated that during oxidative challenge the presence of zinc sulphate diminishes MAC deposition on RPE

cells, thereby preventing complement-mediated cytolysis and apoptosis. This implies that zinc not only has the ability to reduce systemic activation of the alternative complement pathway, but may also diminish complement activation locally on RPE cells. Zinc concentrations are in physiological levels,[39,41] and therefore have biomedical significance. Interestingly, a recent biochemical study demonstrated that factor H-C3b complexes are precipitated by zinc which also inhibits complement activation.[42]

Although several studies have reported systemic complement activation in AMD patients,[25-28] the extent to which systemic inflammation can manifest in the eye is unclear. Characterization of AMD patients with a high baseline level of complement activation revealed that the majority of these patients had large drusen and/or drusenoid RPE detachment rather than geographic atrophy and neovascular AMD. Large drusen and/or drusenoid RPE detachment The intermediate stage of the disease is characterized by a large region of drusen under the retina, relative intact retinal anatomy and relatively preserved visual acuity. However, 42% of patients with drusenoid RPE detachment progress to end-stage AMD and develop profound and irreversible visual loss within five years.[43] The level of serum complement catabolism correlates with the AMD stage, indicating that the level of serum complement catabolism may be a sign of disease activity.

In addition to its direct inhibitory effect on complement cascades, zinc may also reduce the effects of oxidative stress, which can indirectly lead to reduced activation of the alternative complement pathway. Patients who were homozygous for the CFH Y402H high-risk variant had a more rapid and profound decline in C3d/C3 ratio. The Rotterdam study showed that high dietary zinc intake reduces the risk of AMD associated with the CFH 402H variant, suggesting a relationship between zinc and this genotype.[32] The CFH binds the malondialdehyde (MDA) epitopes generated during oxidative stress and prevents MDA-mediated proinflammatory signals in the retinal pigment epithelium and macrophages, and this protective effect is reduced in individuals carrying the CFH Y402H risk variant.[44] As with CFH, zinc can also inhibit MDA-mediated signals on the surface of damaged cells,[45] which may explain why zinc supplementation can be beneficial in individuals carrying the CFH 402H high-risk genotype. Further investigation in the mechanism by which these genotypes interact with zinc supplementation is required.

This study has some limitations that should be addressed. A relatively small number of subjects were included, and zinc was administered for a relatively brief period of time. Because of the slow natural progression of AMD, we were unable to detect a direct protective effect of zinc on visual acuity. Larger patient cohorts and a longer period of zinc supplementation should be

studied to corroborate and extend our findings, especially to determine the duration and the maximum effect of zinc on complement acitvity.

Our findings demonstrate that increased levels of complement catabolism in AMD patients can be normalized by the daily oral administration of 50 mg zinc sulphate. Molecular targeting of the complement system may represent an effective approach to prevent and treat AMD. Zinc supplementation represents a safe and cost-effective approach to lower complement catabolism in AMD.

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CHAPTER SIX

GENERAL DISCUSSION

1. DISTINGUISHING AMD FROM AMD-MIMICKING LATE-ONSET MACULAR DYSTROPHIES

1.1 Late-onset central areolar choroidal dystrophy and AMD

Late-onset central areolar choroidal dystrophy (CACD) can easily be confused with geographic atrophy (GA) in AMD.[1,2] CACD is most commonly caused by mutations in the *peripherin-2* (*PRPH2*) gene (formerly known as *peripherin/RDS*).[3,4] Correctly diagnosing the condition is essential both for determining a prognosis and clinical management strategy, and for research purposes. In Chapter 2, we describe the morphological findings of patients with genetically confirmed CACD and a representative cohort of patients with early AMD and geographic atrophy due to AMD in order to detect discerning features using ophthalmoscopy, fundus autofluorescence (FAF), and spectral domain-optical coherence tomography (SD-OCT). Compared to patients with AMD, the patients with CACD were significantly younger and had a significantly higher positive family history for vision loss. Our study further identified several morphologically distinguishable features between CACD and AMD based on non-invasive novel imaging tools such as SD-OCT and FAF. The results of our study will likely help clinicians discern CACD from GA due to AMD and may facilitate the identification of patients for whom a genetic analysis to verify a putative *PRPH2* mutation is warranted. Table 1 provides a quick check-list of features that can be used to alert the clinician to the possibility of a rare inherited macular disorder, such as CACD, as opposed to the ubiquitous geographic AMD.

TABLE 1. Combination of features that can be used to identify diseases that mimic geographic atrophy in AMD

Relatively young patients with atrophic retinal lesions
A positive family history of vision loss
Presence of typical FAF patterns of dystrophy
Absence of a low prevalence of sub-RPE deposits on SD-OCT
The predominant photoreceptor loss on SD-OCT
Absence of a low prevalence of reticular drusen pattern

1.2 Late-onset Stargardt disease and AMD

It seems reasonable to assume that late-onset macular dystrophies such as CACD are underdiagnosed and confused with AMD on a regular basis. The primary reasons are the low prevalence of macular dystrophies and their phenotypic similarities with AMD, particularly in patients who do not have a positive family history. Interesting, it is recently demonstrated that Stargardt disease (STGD1), caused by mutation in ABCA4 gene, also occurs above 50 years of age or older,[5] indicating that late-onset STGD1 can also be confused with AMD. Distinguishing between late-onset STGD1 and AMD is important because of the different consequences of dietary supplementation but also because of correct selection for the future treatment options. AREDS advises supplementation with the vitamin A derivative beta-carotene for AMD patients to prevent the disease from progressing to an advanced stage.[6] Beta-carotene supplementation may increase lipofuscin accumulation,[7] and thus may possibly accelerate retinal disease pathogenesis in STGD1 patients. On the other hand, the discovery of the complement system as a major contributing factor to AMD pathogenesis has sparked considerable interest in this system as a potential therapeutic target, and several complement inhibitors are currently being tested in phase 1 and 2 clinical trials.[8, 9] In future, misdiagnosis can lead to an unnecessary inhibition of the immune system in patients with late-onset dystrophy. Developing effective gene-therapies is currently the most promising approach for treating STGD1.

1.3 Genes of retinal dystrophies and AMD

Several reports describe a classification system based on FAF patterns in AMD patients.[10-13] FAF patterns that were characterized as "fine granular" and "speckled" or "fine granular with punctuated spots" are highly reminiscent of the FAF findings in CACD and late-onset STGD1 patients.[10-13] We suggest that at least some of the putative "AMD" patients who exhibit these FAF patterns may in fact be misdiagnosed CACD or STGD1 patients. Recently, same research group indeed demonstrated that the fine granular pattern with punctate spots (GPS[+]) phenotype is accounted for by monoallelic variants in *ABCA4* and unlikely by the well-established AMD risk-increasing alleles at *CFH*, *ARMS2*, and *C3*.[14] The carrier frequencies of severe and moderate *ABCA4* mutations in the general population are ~0.008 and ~0.01, respectively.[15] Although variants in the *ABCA4* gene have been associated with AMD, this association is controversial,[16, 17] and it is conceivable that misdiagnosis of late-onset STGD1 may account for the associations that have been reported in some studies.[18, 19] Various genes that are linked to other macular dystrophies such as Sorsby fundus dystrophy (*STGD3*), Best disease (*VMD*, *VMD2*), Doyne honeycomb retinal dystrophy (*EFEMP1*), Stargardt-like macular dystrophy (*STGD3*, *ELVOL4*), cone dystrophy (*CORDX1*, *RPGR*), malattia leventinese (*MLVT*, *EFEMP1*), and adult vitelliform dystrophy (*AVMD*, *RDS*) have not been associated with AMD susceptibility.[20, 21] This absence of genetic associations indicates that different genetic pathogenic mechanisms are likely involved in the development of retinal dystrophies and AMD.

2. PATHWAYS INVOLVED IN AMD PATHOGENESIS

2.1 High-density lipoprotein and extracellular/collagen matrix pathways

Disease-associated variants located near *CFH* (1q31) and in a cluster of genes near *ARMS2* (10q26) were identified through genome-wide association studies (GWASs). Recently, two GWASs identified additional genetic markers, thus identifying novel pathways.[22, 23] A new susceptibility locus was identified near the *TIMP3* gene, which encodes a metalloproteinase that is involved in degrading the extracellular matrix.[22] Mutations in *TIMP3* have previously been linked to an early-onset autosomal dominant macular dystrophy.[24] In addition, loci in the high-density lipoprotein (HDL) cholesterol pathway were found to be associated with AMD. Specifically, associations were identified for variants in the hepatic lipase (*LIPC*) gene, the cholesterylester transfer protein (*CETP*) gene, the ATP-binding cassette transporter A1 (*ABCA1*) gene, the fatty acid desaturase gene cluster (*FADS1-3*), and the lipoprotein lipase (*LPL*) gene.[23]

An early hallmark of AMD is the appearance of drusen, which are extracellular deposits of proteins and lipids under the RPE. Although these genetic associations suggest a role of HDL metabolism in AMD, previous studies that examined the relationship between serum HDL levels and AMD yielded conflicting results.[25-31] Some studies found no relationship[25, 26], whereas other studies found that an increased risk of AMD was associated with increased HDL levels;[27, 30] finally, some studies found an inverse correlation between HDL levels and AMD.[28, 29, 31] In Chapter 4, we analyzed the prevalence of these new risk alleles in 1201 AMD patients and 562 controls. In addition, we measured the levels of apolipoprotein B (ApoB), apolipoprotein A2, lipoprotein A, cholesterol, triglycerides, and HDL-cholesterol in serum samples obtained from AMD patients and controls. Our results confirm associations between AMD and variants near the *TIMP3* gene and at loci involved in HDL metabolism. Our findings further highlight a role of the extracellular matrix and HDL metabolism in the pathogenesis of AMD.

2.2 Complement pathway

Since CFH is a regulator of the alternative complement pathway and the *CFH* risk allele is strongly associated with AMD, it has been postulated that it is the deregulation of the alternative pathway that underlies the pathogenesis of AMD.[32, 33] For our case-control study described in Chapter 3, a cohort of 197 confirmed AMD patients and 150 unaffected age-matched controls were prospectively recruited. We performed hemolytic complement assays for all three complement cascades, the complement components C3, CFB, CFI, and CFH and the activation products C3d, C5a, and SC5b-9 in the serum and plasma of AMD patients and age-matched controls. Furthermore, we analyzed the roles of the risk alleles in the *CFH*, *CFB*, *C3*, *CFI*, and *ARMS2* genes that were previously identified as being associated with AMD. In contrast

to previous studies, we stratified the data by *CFH* and *ARMS2* genotypes to enable us to study the effects of these genotypes independently.

We found that AMD patients have a systemically increased activation of their alternative complement pathway, indicating that activation of the alternative complement pathway is not limited to the retina but is increased systemically. Recently, an association between AMD and a genetic variant in *SERPING1*, a regulator of the classical pathway, was suggested,[34] but this finding could not be replicated.[35, 36] Likewise our study does not support deregulation of the classical pathway in the pathogenesis of AMD. It is important to note that even in the presence of increased C3 activation (i.e., an elevated C3d/C3 ratio), we found increased activity of the alternative complement pathway. In addition, the levels of the acute-phase protein CFB were significantly elevated in these patients. Based on all of these observations, we propose that increased CFB levels also contribute to increased C3 activation.

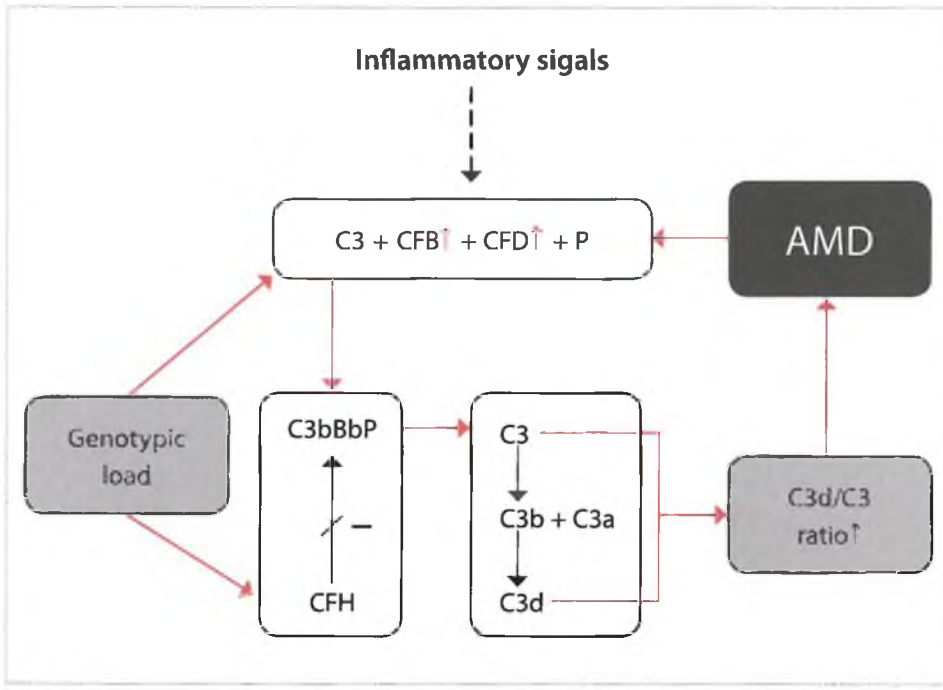
2.3 Genetic control of the complement pathway

complement activation is associated with the rs1061170 (Y402H) variant in the *CFH* gene. In agreement with other studies, we did not detect altered CFH levels, supporting the idea that altered CFH function leads to a deregulation in the alternative pathway rather than a change in serum CFH levels. CFB is part of the alternative pathway C3 and C5 convertase enzymes, which are required for complement activation and amplification. We demonstrated that carriers of the *CFB* H9L allele have lower CFB concentrations, which may explain the protective nature of this variant.[40] Furthermore, this finding supports the previous hypothesis that the protective effect arises from genetic variants in the *CFB* gene, not by variants in the *C2* gene, which are in high linkage disequilibrium.[40, 41] To date, no functional study has investigated the effect of the *CFB* H9L polymorphism. However, the minor allele of the *CFB* polymorphism, rs641153 (R32Q), is also associated with protection from AMD.[40] A functional study demonstrated that CFB_{32Q} is less efficient at forming AP convertase.[42] It is likely that the lower CFB concentration associated with CFB_{9L} also leads to decreased AP convertase production.

Most interestingly, we found that complement deregulation in AMD was not only associated with *CFH* high-risk alleles, but it was also associated with the *ARMS2* A69S high-risk allele. *CFH* and *ARMS2* are the two major AMD susceptibility genes.[43-47] The function of the ARMS2 protein is currently unknown, and its subcellular localization is unclear.[48-51] Both *ARMS2* and the adjacent *HTRA1* gene are in high linkage disequilibrium, and genetic studies have failed to pinpoint the susceptibility causal gene.[52-56] Findings from our study for the first time suggest that ARMS2/HTRA1 may be involved in activating the complement system and that the *CFH* and *ARMS2/HTRA1* genes may play a common role in the pathogenesis of AMD. Another remarkable

observation was that control subjects who carry both the *CFH* and *ARMS2* risk alleles did not have elevated complement activation, suggesting that the presence of the *CFH* and/or *ARMS2* risk alleles is not sufficient to account for increased complement activation; thus, a preceding and additional inflammatory signal is likely required. Multiple factors might trigger such an initial inflammatory signal,[57] and these factors are likely derived from environmental exposures. The scheme depicted in Figure 2 summarizes our current understanding of how C3 activation is associated with AMD.

FIGURE 1. Inflammation model of C3 activation in age-related macular degeneration.
We hypothesize that inflammation leads to increased levels of the acute-phase response protein complement factor B (CFB), which in the presence of high-risk alleles and increased levels of complement factor D (CFD) and properdin (P) contributes to increased activation of the alternative pathway and increased C3 catabolism, ultimately increasing the C3d/C3 ratio. Increased C3 activation may contribute directly to the development and progression of AMD, creating a self-perpetuating amplification loop of complement-mediated inflammation in AMD pathogenesis.



2.4 Complement activation is central to AMD pathogenesis

Both *ARMS2* and the adjacent *HTRA1* gene are in high linkage disequilibrium.[52-56] Recently, the protease HTRA1 protease was reported to activate the alternative complement pathway through factor D.(ARVO abstract C. Stanton et al 2011) Data from this recent study revealed that HTRA1 cleaves the activation peptide of profactor D to generate factor D. Factor D catalyzes the cleavage of factor B in the C3bB complex, which is a rate-limiting step in complement activation. Another study found that AMD patients indeed have elevated levels of factor D in their serum,[65] adding further support to our finding that the ARMS2/HTRA1 haplotype is involved in complement activation; this study also supports our hypothesis that in addition to factor H, the acute-phase proteins such as complement factors B and D also contribute to the increased C3 activation that occurs in AMD pathogenesis. Thus, in addition to genetic predisposition based on common variants in complement genes, other pathological pathways converge at the site of inflammation and also contribute to complement activation. Therefore, complement-mediated inflammation is a key component in the network of cellular signaling pathways underlying AMD pathogenesis.

2.5 Beyond the *CFH Y402H* polymorphism

CFH interacts with the level of complement activation in a complex manner, and the CFHR1 and CFHR3 deletion in combination with the CFH/FHL1 haplotype may be more important than the *CFH* Y402H variant itself. Therefore, it will be interesting if further studies will look beyond the *CFH* Y402H polymorphism. Notably, the gene that encodes CFH also encodes an alternatively transcribed and truncated protein called factor H-like 1 (FHL1), which shares the first seven functional domains with CFH and therefore has some of the same enzymatic functions as CFH.[58, 59] A deletion in the *CFHR3* and *CFHR1* genes is a third polymorphism in this region that is closely related to *CFH* . This deletion is associated with a decreased risk of developing AMD.[60] *CFHR3* and *CFHR1* encode proteins that function independent of the effect of CFH.[61, 62] The CFHR1 protein inhibits the production of C5 convertase (C3bBb3b),[62] and CFHR3 acts together with CFI to degrade C3b in the absence of CFH.[61] Like CFHR1, CFHR3 also inhibits C5 convertase.[61] Both CFHR1 and CFHR3 compete with CFH for binding to C3b, and the presence of either CFHR1 or CFHR3 inhibits CFH binding.[61] Deletion of the genes—and therefore the reduction (in heterozygosity) or absence (in homozygosity) of the CFHR3 and CFHR1 proteins—translates to a deficiency of these anti-inflammatory molecules and an increase in the activity of CFH, thereby resulting in a lower inflammatory state (at least in the retina in the case of AMD).[61] The deletion of both alleles of *CFHR3* and *CFHR1* protects against AMD but is an important risk factor for atypical hemolytic uremic syndrome.[63] A recent study found that patients with AMD had fewer auto-antibodies against fragments of the CFH protein than age-matched controls.[64] This finding reflects the much lower frequency of the CFHR3 and CFHR1 deletion among AMD cases relative to controls and suggests a protective effect of these proteins.

3. ENVIROMENTAL EXPOSURES AND COMPLEMENT CATABOLISM

Tobacco smoking and obesity are the two most important environmental and behavioral risk factors for AMD, and they exhibit their influence—at least partially—by affecting the complement cascade. Smoke-induced damage in AMD is mediated through direct oxidation, depletion of antioxidant protection, immune system activation and atherosclerotic vascular changes.[66] Cigarette smoke activates C3 *in vitro* and renders C3 less susceptible to inactivation by CFH and CFI;[67] *in vivo*, tobacco activates the classical pathway.[68] Halkes et al. found that C3 levels increase in humans after consuming fat.[69] Activation of the alternative complement pathway and increased C3, CFB, CFH and CFI levels have been reported in obese patients. Adipose tissue is a major source of CFD and other complement regulatory proteins.[70-72] HDL, which has a protective effect against AMD, inhibits binding of the terminal complement complex to the cell surface, thus interfering with either the insertion of C9 into the membrane or its polymerization.[73] In addition, the intake of certain dietary compounds in AMD patients may alter complement activation. AREDS reported that high dietary intake of omega-3 long-chain polyunsaturated fatty acids was associated with a decreased risk of developing neovascular AMD.[74] The Blue Mountains Study supports these findings and also found that weekly consumption of fish and nuts decreases the risk of AMD, where as high LDL cholesterol increases this risk.[75] According to the Beaver Dam Study[76] and a study of a cohort containing 33,000 subjects,[77] exercise can decrease the risk of developing AMD. Interestingly, moderate exercise exerts an anti-inflammatory effect via the complement system—CFB is decreased, whereas protective CFH is increased.[78]

4. TARGETING COMPLEMENT ACTIVATION IN AMD

4.1. Current drugs that inhibit the complement system

The current therapies for treating neovascular AMD are designed to reduce the ongoing VEGF stimulus—and hence inhibit the growth of new vessels—but do not address the underlying pathology. Moreover, no effective therapy has been developed for treating early AMD or GA. Inhibitors of the complement system suppress complement activation by preventing the formation of key elements within the proteolytic cascade. Molecular targeting of the complement system may represent a novel approach to preventing and/or treating AMD.

Both CFH and C3 are central to the complement cascade, and successfully enhancing CFH function and/or inhibiting C3 activation will inhibit downstream events. CFH is the principal inhibitor of the complement system, and boosting its production or activity may represent a promising therapeutic approach. For example, a research group in Germany successfully

produced a culture from which biologically active recombinant human CFH can be extracted. The complement components C3a and C5a promote choroidal neovascularization,[79] and C5a increases VEGF secretion from human RPE cells.[80] Inhibition of these complement components could therefore potentially avoid choroidal neovascularization. The compstatin derivative POT-4 is the first complement inhibitor to be evaluated as a treatment for neovascular AMD. POT-4 is a peptide that can be administered by intra-vitreal injection; it then binds to C3 and inhibits C3 binding to C3b in C3 convertase. The injected compound forms a gel that slowly releases the molecule over many months, therefore allowing for relatively long intervals between treatments. This drug has now passed phase I testing.(Kaushal S at el. Complement C3 inhibitor POT-4: clinical safety of intravitreal administration. 2009. ARVO abstract – 5010 – A611) A small study performed using a primate model of AMD found an improvement in drusen in four test subjects.[81] With respect to testing in patients, a large randomized phase II trial is scheduled and will further test the efficacy, safety, and pharmacodynamic profile of the compound.

Therapies designed to disrupt the complement cascade at the level of C5 are also under development and may be useful for treating AMD. A second intravitreal complement inhibitor, ARC1905, is currently being tested in combination with ranibizumab in phase I trials for treating both dry and neovascular AMD. ARC1905 is an aptamer-based C5 inhibitor that blocks the cleavage of C5 into the fragments C5a and C5b.[82] Eculizumab (Soliris) is currently the only licensed monoclonal antibody-based complement inhibitor that has received FDA approval (in 2007).[83] Eculizumab is now in phase II trials for treating GA and early AMD.[82] This drug is administered intravenously over 6 months in weekly doses during the initial induction period followed by twice-weekly maintenance doses. Like ARC1905, eculizumab binds the complement component C5 and prevents C5 cleavage, its downstream activation, and the formation of the membrane attack complex (MAC).[83] However, a recent study found that C3 and C5a receptor (C5aR) deficiencies lead to an inhibition of VEGF-A isoforms and results in profoundly impaired epithelial ovarian cancer growth and reduced tumor vascularization.[84] Therefore, eculizumab may be more suitable for treating neovascular AMD. Two peptidomimetic C5aR antagonists,JSM-7717 and JPE-1375, are currently in preclinical assessment for treating AMD.[82] Other therapeutic antibodies that are currently being evaluated for treating (dry) AMD include anti-factor D (FCFD4514S) and TA106.[82] Although it is still in preclinical development, the CFB inhibitor TA106 is being investigated primarily as an inhaled formulation for treating severe chronic asthma that is resistant to current therapies.[82]

4.2 Zinc inhibits complement catabolism in AMD

Over the past decade, it has become increasingly evident that complement-mediated inflammation plays a fundamental role in the etiology of AMD.[85] The AREDS study concluded

that the typical five-year progression from intermediate to advanced AMD can be reduced up to 25% by treatment with zinc and antioxidants.[6] Despite the wide spread use of zinc among AMD patients, how zinc actually exerts its positive effects has not yet been resolved. in Chapter 6, we evaluated whether oral zinc supplementation affects complement catabolism in AMD patients. Our results show that this is indeed the case, although the effect was limited to patients with a high baseline level of complement catabolism. Interestingly, we also found that enhanced complement catabolism was apparently specific to patients with intermediate-stage AMD. Between the findings of the AREDS study and our own study, we can conclude that restoring functional zinc homeostasis in patients with intermediate AMD can normalize the hyperactive alternative complement pathway, perhaps explaining the protective role that zinc can play in AMD patients.

Furthermore, in our study, the patients who were homozygous for the *CFH* Y402H high-risk variant had a faster and more profound decline in their C3d/C3 ratio. We also found that the patients with advanced bilateral AMD, profound retinal damage, and no evidence of active disease had complement levels that were comparable to control subjects. Although the relatively small sample size in this study does not allow for us to draw generalized conclusions, these findings suggest that systemic serum complement activation is further enhanced by active stages of AMD.

4.3 Zinc as a complement inhibitor

The mechanism by which zinc interacts with the complement system may lie within its ability to bind to complement fragments. The most critical step in the activation of the alternative complement pathway is the formation of the unstable C3 convertase C3bBb, which cleaves C3 to generate the active fragment C3b.[86] Deposition of C3b on the target surface activates the effector molecules C3a, C5a, and the membrane attack complex (MAC), causing inflammation and cell lysis.[86] Zinc alone can potently inhibit the activity of C3 convertases in both the classical and the alternative complement pathways.[87] When both CFH and zinc are present *in vitro*, they cause a cumulative inhibition of the alternative pathway.[87] Moreover, zinc can bind to the MAC and prevent complement-mediated cytolysis.[88]

4.4 Zinc as an antioxidant and anti-inflammatory agent

In addition to its direct inhibitory effect on the complement cascades, zinc can also reduce oxidative stress, thereby decreasing activation of the alternative complement pathway. Increased oxidative stress occurs with aging,[89-91] and has been linked to many chronic inflammatory diseases, including AMD.[92] Oxidative damage is closely associated with free radicals such as superoxide O_2^- and the reactive oxygen species H_2O_2 and ·OH, which are continuously produced *in vivo* under aerobic conditions.[93] Free radicals are known as reactive

oxygen species (ROS), and zinc decreases ROS via several mechanisms.[93] First, zinc is an inhibitor of NADPH oxidases, a group of plasma membrane enzymes that catalyze the synthesis of the free radical superoxide O_2^- from O_2.[93] Furthermore, zinc activates intracellular defense systems to counter oxidative stress, including the enzyme copper-zinc superoxide dismutase. Zinc also induces metallothioneins, which are highly effective ·OH scavengers.[93] Moreover, zinc deficiency is known to induce oxidative stress.[94] In addition to its antioxidant effects, zinc acts as an anti-inflammatory agent,[95] and zinc ions are essential for the normal development, differentiation, and function of cells in both the innate and acquired immune systems.[96, 97] Zinc supplementation has beneficial effects in treating acute lower respiratory infections, chronic hepatitis C, diarrhea, shigellosis, leprosy, tuberculosis, and acute cutaneous leishmaniasis.[98] Zinc may exert its anti-inflammatory effect by inhibition of NF-κB, there by decreasing the production of the inflammatory cytokines IL-1β, IL-6, IL-8, tumor necrosis factor- α (TNF-α), and MCP-1 via up-regulation of the zinc-finger protein A20.[93]

Recently, a link was discovered between oxidative stress defense, the high-risk *CFH* Y402H polymorphism, and complement activation.[99] Malondialdehyde (MDA) and its condensation products are reliable markers for oxidative stress and have been associated with many disorders, including AMD.[92, 100] These MDA-modified cellular proteins can trigger an inflammatory response and are recognized by the innate immune system.[101-103] CFH can block MDA-mediated proinflammatory effects in RPE cells and macrophages, the homozygous *CFH* 402H high-risk variant have a reduced capacity to generate anti-inflammatory complement iC3b fragments.[99] Zinc can also inhibit MDA on the surface of damaged cells.[104] Thus, in addition to its ability to directly inhibit complement cascades, zinc can also reduce oxidative stress, which can indirectly reduce the activation of the alternative complement pathway.

4.5 Zinc homeostasis in aging

Under normal conditions, zinc is abundant in the human retina and the RPE-choroid complex; however, in donor eyes obtained from patients with AMD, the levels of zinc were reduced by 23%.[105] It is not clear whether this decrease in local zinc levels in AMD patients is in response to the ever-increasing oxidative stress in the aging outer retina, thereby preventing activation of the complement system. Progressively increasing levels of the enzyme copper-zinc superoxide dismutase are associated with the progressive clinical stages of AMD, suggesting that the RPE responds to progressive oxidative stress by up-regulating the expression of antioxidant enzymes.[106] Moreover, a rise in metallothione in levels has been widely documented in various aging organs and tissues due to a chronic inflammatory state mediated by proinflammatory cytokines. However, reduced plasma zinc ion bioavailability has been measured in elderly subjects.[107] Moreover, proteins that are involved in zinc homeostasis may be altered during

aging, thus leading to peripheral zinc deficiency and an accumulation of extracellular zinc.[108] Therefore, aging has been suggested to be a condition associated with zinc dyshomeostasis rather than zinc deficiency per se.[108] Although acquired zinc deficiency is relatively uncommon, some Western diets are low in zinc.[109] The reduced levels of zinc in the RPE-choroid complex of AMD patients may therefore be a consequence of aging, low dietary zinc, and/or larger demands for zinc due to increasing oxidative stress and chronic inflammation.

4.6 Systemic versus local complement inflammation

Although several studies have reported systemic complement activation in AMD patients, the extent to which systemic complement activation reflects pathological events in the eye—and the extent to which systemic inflammation can manifest in the eye—remains unclear. In chapter 6, we linked the degree of serum complement activation to the clinical stages of AMD and found that that the level of serum complement activation is correlated with highly localized disease processes in the retina. This finding suggests that the increased complement activation found in the serum of AMD patients is not a downstream product of (massive) local complement activation in the eye. However, additional research is needed to appreciate the clinical relevance of measuring complement activation in the serum of AMD patients.

5. TOWARDS PERSONALIZED MANAGEMENT OF AMD PATIENTS

5.1 Pharmacogenetics

Standards of medical care have traditionally been based on the results of epidemiological studies using large cohorts. However, large cohort studies cannot account for the genetic variability of individuals within a population. Pharmacogenetics is a field of study that examines the impact of genetic variation on the response to specific medications and therapies. Currently, therapies that target VEGF-A are available for treating neovascular AMD.[110] Several pharmacogenetic studies suggest that genetic variations in the *CFH* and *ARMS2/HTRA1* genes may underlie the variable responses to treatment with anti-VEGF, although conflicting results have been reported.[111-114] Table 2 provides an overview of pharmacogenetic studies that studied the effect genotypes on the outcome of anti-VEGF treatment. Although SNPs in the *VEGFA* gene may not be strongly associated with AMD, they might contribute to the effectiveness of anti-VEGF treatment.[111] In Chapter 5, we described a large multi-center pharmacogenetic study to measure the impact of high-risk alleles in *CFH*, *ARMS2*, and *VEGFA* on ranibizumab treatment and the age of onset for neovascular disease. Unlike previous studies, we stratified our data according to the number of high-risk alleles, thus allowing us to study the combined effects of these genotypes. From this study, we conclude that a cumulative effect of high-risk alleles in

TABLE 2. An overview of pharmacogenetic studies that studied the effect genotypes on the outcome of anti-VEGF treatment.

Study	Number of patients	Treatment	Conclusions
Tian 2012	144	Avastin	*CFH* high-risk CC and *ARMS2* high-risk TT genotype have a worse response
Yamashiro 2012	105	Lucentis	No association with *CFH* or *ARMS2* genotype
Nischler 2011	197	Avastin	*CFH* high-risk CC genotype has a worse response
Kloeckner-Gruissem 2011	215	Lucentis	*CFH* high-risk CC genotype has a worse response
McKibbin 2011	104	Lucentis	*CFH* low-risk TT and *HTRA1* low-risk *AA* genotype may lead to a better response
Teper 2010	90	Lucentis	*ARMS2* low-risk GG genotype has a worse response
Lee 2009	156	Lucentis	*CFH* high-risk CC genotype requires more treatments
Brantley 2007	86	Avastin	*CFH* high-risk CC genotype has a worse response

CFH, *ARMS2*, and *VEGFA* appears to be associated with a younger age of onset in combination with a weak response to ranibizumab treatment. Thus, the high-risk genotypes that were tested in our study can predict AMD progression and the response to anti-VEGF treatment, which underscores the value of genetic screening in predicting the treatment outcome. In our study, we observed a wide variety in the response to ranibizumab treatment among the various allele groups, indicating that additional factors likely contribute to the efficacy of ranibizumab treatment. For example, other genes and other non-genetic factors may help determine the treatment response. Other factors that can potentially influence the effect of anti-VEGF treatment include gender as well as age, comorbidity and the duration of CNV leakage prior to the onset of treatment. Other factors such as the neovascular involvement of both eyes and the education level and mobility of the patient can affect patient compliance and can therefore indirectly influence the response to anti-VEGF treatment. Future studies should be performed using even larger patient cohorts in order to confirm and extend our findings and investigate whether these putative factors affect the treatment outcome.

5.2 Prediction models for AMD

The discovery of multiple AMD-associated loci has given researchers the opportunity to explore the value of various risk prediction models for clinical and public health. One's genotype is independent of external factors and does not change throughout an individual's lifetime. The early detection of genetic risk factors may translate into more efficacious preventive regimens and interventions. This is particularly relevant for late-onset diseases such as AMD, in which the signs of the disease may not appear until the fifth or sixth decade of life. Combined, the *CFH* and *ARMS2/HTRA1* genes contribute to approximately 50% of the genetic risk associated with AMD. The remaining heritability may be attributed to additional loci with smaller effects that are often missed in GWASs due to the sample size and/or adjustments for multiple testing. A recent meta-analysis of AMD-GWASs from 18 centers identified or confirmed 19 AMD loci with modest effect, including seven novel loci. (M.C. Schu, *et al.* Meta-analysis of genome-wide association studies identifies 19 loci associated with AMD risk. Invest Ophthalmol Visual Sci, 53 (2012) (ARVO E-Abstract 2259)

Genetic tests for AMD are currently commercially available; however, the predictive value of common genetic variants for AMD and other complex disorders has seen limited success because the value of rare variants has not yet been appreciated.[115] Next-generation sequencing provides the opportunity for researchers to identify rare or even patient-specific variants with large effect.[116] However, it will be necessary to functionally characterize the effect of such variants on the function of the affected proteins to confirm their potential pathogenicity. Although the risks associated with some genetic variants are high, our pharmacogenetic data support the hypothesis that the cumulative risk of combining risk variants is likely more complex than a simple sum of probabilities would suggest. Building combined haplotype- and rare variant-based predictive model(s) for AMD using next-generation sequencing will likely provide a more accurate estimate of the total risk. With respect to AMD, non-genetic risk factors contribute considerably to the disease's development. An optimum prediction model should therefore include a more comprehensive set of rare and common susceptibility variants together with environmental factors, aging, and diet.

5.3 Antioxidants versus other complement inhibitors

Pivotal studies performed during the past decade have changed our understanding of the molecular mechanisms underlying AMD. These findings have paved the way for the exploration of a new therapeutic paradigm for managing AMD, namely the targeting of specific molecular components in the complement pathway. Nevertheless, it will take at least several years before any of the aforementioned drugs will be approved for routine use in clinical practice, assuming they are eventually found to be safe and effective. It is interesting to question whether zinc alone—or in combination with other antioxidants—can inhibit the complement system at

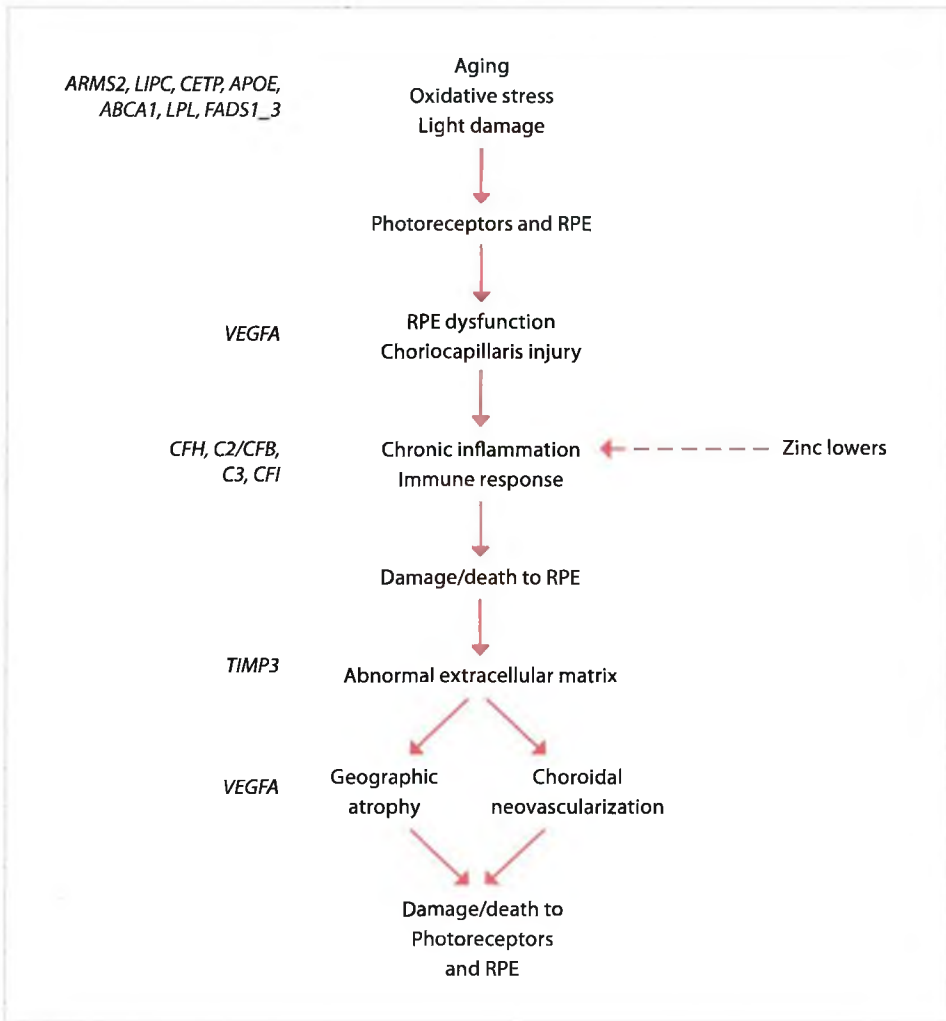
levels comparable to the drugs that are currently being tested for treating AMD. Oxidative stress is associated with inflammation and the pathogenesis of AMD. Zinc is both an antioxidant and an anti-inflammatory agent and we found that zinc can decrease complement catabolism in AMD. Oxidative stress following stroke is associated with an activation of the inflammatory system. Recently, the inhibition of the complement component C3 by the antioxidant N-tert-butyl-α-phenylnitrone was found to have a neuro protective effect following cerebral ischemia and reperfusion in mice.117 A second study found that lutein supplementation can also decrease complement catabolism in AMD patients.(T. Berendschot, personal communication) The major advantage of this approach is that antioxidants are already being used in clinical trials for treating AMD patients and appear to be safe, so these drugs will likely reach clinical practice faster than other drugs that inhibit the complement cascade. Another advantage is that antioxidants can inhibit chronic low-level alternative complement pathway overactivation but will not affect block complement catabolism which also protect the host from infection. In our opinion, antioxidants should be considered a legitimate treatment for AMD.

5.4 The personalized use of antioxidants

Another question that must be addressed is whether all AMD patients—irrespective of their disease stage—should be treated with antioxidants or future (as yet undiscovered) complement inhibitors. My answer to this question is both "yes" and "no"—all AMD patients should be treated with antioxidants, but every AMD patient does not require complement inhibition therapy. Our results show that zinc affects complement catabolism only in AMD patients who have a high level of complement catabolism. Based on the findings of the AREDS study and our own results, we conclude that restoring functional zinc homeostasis in patients with intermediate AMD may normalize the overactivity of the alternative complement pathway, possibly explaining the protective role that zinc plays in AMD patients. On the other hand, patients with a relatively low baseline level of complement activation had no change in activation during the study period. It is possible that zinc supplementation inhibits complement activation only in patients who have increased C3 catabolism. However, the beneficial effect of zinc in treating AMD was confirmed by a large population-based study that found that a high dietary intake of zinc reduces the risk of developing early AMD.[118] In the early stages of the disease, zinc supplementation may reduce the risk of developing AMD by preventing complement overactivation from occurring. Therefore, zinc supplementation might have the highest benefit in early AMD, but because of the slow progression of the disease, the effects of the treatment could be very subtle and therefore underappreciated. Late-stage AMD patients with profound retinal damage and low complement catabolism may not experience visual improvement and thus might not benefit from complement inhibition therapy; nevertheless, AREDS reported that antioxidants decrease the mortality rate of late-stage AMD patients.[119] Other late-stage patients may have increased

complement catabolism and may therefore experience an improvement in visual acuity from antioxidant therapy. Thus, for practical reasons, we recommend treating all AMD patients with antioxidants. Future studies may be needed to develop the optimum combination and dosage of antioxidants to treat patients in specific stages of AMD.

5.5 Overview of pathways involved in pathogenesis of AMD studied in this thesis.
Different genetic risk factors contribute to the pathogenesis of AMD by different pathological mechanisms. Our results show that zinc inhibits complement activation in AMD patients. This could be the mechanism by which zinc slows AMD progression.



6. VISION OF THE FUTURE OF AMD TREATMANT

Despite considerable effort by numerous research groups to understand the pathogenesis of AMD, much work remains to be done. For example, we must identify the initial event that triggers the immune response in AMD. Potential candidates include microbial infection, photo-oxidative stress, smoking, and many other genetic and environmental factors. In stark contrast to conventional therapies, this new paradigm in treating and managing AMD focuses specifically on treating the disease in a much earlier stage, before vision loss has occurred.

In addition to gene–gene interactions and gene copy number variations, epigenetic modifications provide another level of gene regulation. For example, environmental exposure can affect the epigenetic regulation of many genes, meaning that the DNA sequence itself is normal but that the gene's expression has been adversely affected. Interestingly, a recent study of twins with AMD found that the regulatory regions of the *IL17RC* gene, was hypomethylated, which led to increased gene expression. Based on these results, the authors of this study proposed that a chronic increased level of the IL17RC protein in the retina likely promotes inflammation and the recruitment of immune cells that then damage the retina. Moreover, the Rotterdam Study found that eating a diet rich in zinc, β-carotene, lutein/zeaxanthin, and omega-3 fatty acids can counteract one's increased genetic risk for developing AMD,[118] which also supports a gene-environment interaction hypothesis. This suggests that the notion of "genetic" risk remaining constant and independent of external factors throughout an individual's lifetime must be abandoned. In abandoning this outdated notion, we may provide patients with a powerful incentive to urge personal responsibility and adopt long-term changes in their behavior, as this may be the easiest way to decrease one's genetic predisposition for developing AMD. Therefore, although estimating one's personal overall AMD risk may be important, estimating one's risk reduction by adopting a healthy lifestyle and diet is essential. More importantly, understanding one's overall risk for AMD without knowing how to reduce that risk is useless.

To facilitate more personalized management of AMD, future studies must focus on the effects of environmental factors and diet, as well as strategies to identify high-risk individuals. Next-generation sequencing may be able to provide detailed and accurate molecular profiling for individual patients. Targeting key steps in AMD pathogenesis using specific drugs will be the most efficient approach to restoring balance to the system, thereby treating the disease. Moreover, a therapeutic approach such as switching protective genes "on" and switching high-risk genes "off" represents an entirely different level of personalized medicine in the future. Twenty years from now (or perhaps even sooner), I would like to be able to say to my patient;

"I'm not just treating AMD; I'm treating your AMD."

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CHAPTER SEVEN

SUMMARY
SAMENVATTING
LIST OF PUBLICATIONS
DANKWOORD
CURRICULUM VITAE

SUMMARY

Affecting an estimated 30-50 million individuals, age-related macular degeneration (AMD) has reached epidemic proportions among the elderly and is a leading cause of blindness in Western society. AMD manifests clinically as a loss of central vision due to the loss of the central retina such that the patient can no longer read, recognize faces or discriminate colors. The late stages of AMD can be separated into geographic atrophy and neovascular AMD. Geographic atrophy is characterized by the development of central retinal atrophy. Other retinal diseases, such as late-onset central areolar choroidal dystrophy (CACD), may easily be confused with geographic atrophy (GA) in AMD. Correctly diagnosing the condition is essential for determining the prognosis, a clinical management strategy, and for research purposes. In order to detect discerning features, the morphological changes in CACD patients and in AMD patients were assessed with confocal scanning laser ophthalmoscopy (cSLO), fundus autofluorescence (FAF) and spectral domain optical coherence tomography (SD-OCT). Although outer retinal atrophy is a common clinical feature in advanced CACD as well as GA, there are microstructural alterations on high-resolution SD-OCT and FAF imaging, which allow for the differentiation between CACD and AMD. These findings may aid the identification of patients with CACD caused by mutations in the *PRPH2* gene, which can further be confirmed with a genetic screening (Chapter 2).

AMD is a multifactorial disorder caused by genetic and environmental factors. Despite the late onset of the disease, familial and twin-based studies have shown that AMD has a strong genetic component. Genome-wide association studies identified several genetic markers to be associated with AMD. Some of these associations point towards a role of the high-density lipoprotein (HDL) metabolism in AMD, but previous studies that have examined the association between serum HDL levels and AMD show conflicting results. Some studies found no relationship, whereas others found that increased risk of AMD was associated with increased HDL levels, and yet others have shown an inverse relationship between HDL levels and AMD. In Chapter 3 we analyzed the association between a susceptibility locus near the *TIMP3* gene and genes of the HDL metabolism and AMD, and evaluated serum lipid and lipoprotein levels in AMD patients compared to control individuals. Our results confirm associations of AMD with variants near the *TIMP3* gene and at loci involved in HDL metabolism. They further highlight a role of the extracellular matrix and the HDL metabolism in the pathogenesis of AMD. This study identified increased ApoB levels as a possible new serum biomarker for AMD (Chapter 3A).

The hallmark lesions of early- and intermediate-stage AMD are drusen, which are pathological deposits of extracellular material that form between the retinal pigment epithelium and Bruch

membrane. The discovery that drusen contain proteins from the alternative complement pathway led to the hypothesis that drusen could be considered the byproduct of local complement-mediated inflammation. Systemic complement activation is associated with AMD and has mainly been attributed to a risk allele in the *complement factor H* (*CFH*) gene. The second major genetic risk factor for AMD is *Age-Related Maculopathy susceptibility 2* (*ARMS2/LOC387715*) SNP. The function of the ARMS2 protein remains unknown, and controversy exists about its subcellular localization. Whether other important AMD genes also influence complement activation is unclear. In Chapter 4, we measured complement activation in plasma/serum samples of AMD patients and control individuals, and stratified the data by *CFH* and *ARMS2* genotypes. In contrast to previous studies, this enabled us to study the effects of these genotypes independently. The findings of our study demonstrate that systemic activation of the alternative complement pathway is associated with AMD, with the C3d/C3 ratio constituting a robust marker. In AMD, the acute-phase response protein CFB was elevated, which, in the presence of high-risk alleles, increases alternative pathway activation and C3 catabolism. Furthermore, our study demonstrates that carriers of the *CFB* H9L protective allele have lower CFB concentrations, which may explain the protective nature of this polymorphism. We found complement deregulation not only to be associated with *CFH* high-risk alleles, but also with *ARMS2* high-risk alleles. Our findings thus suggest that CFH and ARMS2 share a common pathway in the pathogenesis of AMD (Chapter 3B).

In neovascular AMD, choroidal blood vessels invade the central retina and subretinal space; although this form of AMD accounts for 10% of all patients with AMD, it is responsible for the majority of AMD-related severe visual impairment. Intravitreal ranibizumab injections are currently the standard treatment for neovascular AMD. However, a broad range of response rates have been observed, for which the reasons are poorly understood. In Chapter 5 we evaluated the impact of high-risk alleles in *CFH*, *ARMS2*, and vascular endothelial growth factor-A (*VEGFA*) on the response to ranibizumab treatment and on the age of treatment onset. As individual patients can carry a variable number of high-risk variants, we hypothesized that combinations of high-risk alleles may explain the heterogeneity in the age of neovascular disease manifestation and the variable responses to treatment with ranibizumab. Therefore, in contrast to previous studies, we stratified the data according to the number of high-risk alleles to enable us to study the combined effects of these genotypes in development and treatment of neovascular AMD. Our findings demonstrate a cumulative effect of high-risk alleles, leading to a younger age of neovascular AMD onset in combination with poor response rates to intravitreal ranibizumab treatment. Because genetic variation partially explains the wide range of response to ranibizumab treatment, genetic screening has the potential to identify high-risk individuals and may in future help clinicians to tailor medical care to individual needs (Chapter 4).

The complement system is a major component of our immune system that plays a crucial role in the first line defense against invading micro-organisms and in the modulation of adaptive immune response. Complement-mediated inflammation plays a pivotal role in the pathogenesis of AMD, and AMD patients have increased levels of serum complement catabolism. Large population-based studies have demonstrated that oral zinc supplementation can reduce the progression of AMD, although the precise mechanism of this protective effect is as yet unclear. The discovery of the complement system as a major contributing factor to AMD pathogenesis has sparked considerable interest in this system as a potential therapeutic target, and several complement inhibitors are currently being tested in phase 1 and 2 clinical trials. In chapter 6, we investigated whether zinc supplementation directly affects the degree of complement activation in AMD. In this open-label clinical study, a cohort of 72 patients in various stage of AMD received a daily oral supplement containing 50 mg zinc sulphate. Serum complement catabolism— defined as the C3d/C3 ratio—was measured at baseline, throughout the three-month period of zinc supplementation and after discontinuation of zinc administration. The high-risk genetic variants in *CFH* (Y402H) and *ARMS2* (A69S) were determined, and the stage of the disease was assessed in accordance with clinical age-related maculopathy staging (CARMS). The level of serum complement catabolism correlates with the AMD stage, indicating that the level of serum complement catabolism may be a sign of disease activity. Our study demonstrates that increased levels of complement catabolism in AMD patients can be lowered towards normal values by daily administration of 50 mg zinc sulphate. Patients who were homozygous for the *CFH* Y402H high-risk variant had a more rapid and profound decline in C3d/C3 ratio. Because the results of the present study show that zinc can inhibit complement catabolism, giving zinc supplementation to AMD patients may be considerably safer and more cost-effective for preventing and treating AMD (Chapter 5).

In addition to gene–gene interactions and gene copy number variations, epigenetic modifications provide another level of gene regulation. For example, environmental exposure can affect the epigenetic regulation of many genes, meaning that the DNA sequence itself is normal but that the gene's expression has been adversely affected. This suggests that the notion of "genetic" risk remaining constant and independent of external factors throughout an individual's lifetime must be abandoned. In abandoning this outdated notion, we may provide patients with a powerful incentive to urge personal responsibility and adopt long-term changes in their behavior, as this may be the easiest way to decrease one's genetic predisposition for developing AMD (Chapter 6). Therefore, although estimating one's personal overall AMD risk may be important, estimating one's risk reduction by adopting a healthy lifestyle and diet is essential. More importantly, understanding one's overall risk for AMD without knowing how to reduce that risk is useless. To facilitate more personalized

management of AMD, future studies must focus on the effects of environmental factors and diet, as well as strategies to identify high-risk individuals. Next-generation sequencing may be able to provide detailed and accurate molecular profiling for individual patients. Targeting key steps in AMD pathogenesis using specific drugs will be the most efficient approach to restoring balance to the system, thereby treating the disease. Moreover, a therapeutic approach such as switching protective genes "on" and switching high-risk genes "off" represents an entirely different level of personalized medicine in the future (Chapter 6). Twenty years from now (or perhaps even sooner), I would like to be able to say to my patient;

"I'm not just treating AMD; I'm treating your AMD."

SAMENVATTING

Met een geschatte 30-50 miljoen mensen die aan leeftijdsgebonden maculadegeneratie (LMD) lijden heeft deze aandoening epidemische vormen aangenomen en is in de westerse wereld de hoofdoorzaak van blindheid bij ouderen. Klinisch manifesteert LMD zich als verlies van de gezichtsscherpte als gevolg van 'slijtage' van het centrale gedeelte van het netvlies (de gele vlek of macula lutea) waardoor de patiënt niet langer kan lezen, geen gezichten meer kan herkennen en geen kleuren kan onderscheiden. LMD kent twee eindstadia: geografische atrofie (droge) en neovasculaire (natte of exsudatieve) LMD. Geografische atrofie wordt gekenmerkt door veranderingen en uiteindelijk verlies (atrofie) van weefsel in de macula. Sommige netvliesaandoeningen, zoals late centrale areolaire choroïdale dystrofie (CACD), worden gemakkelijk verward met geografische atrofie bij LMD. In het gevorderde stadium van deze twee aandoeningen wordt atrofie van de buitenste retinalagen gevonden. Een juist onderscheid tussen deze aandoeningen is cruciaal, zowel voor een adequate prognose en behandelstrategie, als voor onderzoeksdoeleinden. Om onderscheidende kenmerken te identificeren zijn de morfologische veranderingen bij patiënten met CACD en LMD in kaart gebracht met drie beeldvormende onderzoeken (confocale laserscan oftalmoscopie (cSLO), fundus autofluorescentie (FAF) en spectraal-domein optische coherentietomografie (SD-OCT)). Dit proefschrift laat zien dat met de zeer gedetailleerde beeldvorming van de SD-OCT en FAF microstructurele veranderingen zichtbaar worden die onderscheid tussen CACD en LMD mogelijk maken. Dankzij deze bevindingen kunnen vermoedelijke CACD patiënten gemakkelijker en sneller worden opgespoord, waarna met genetisch onderzoek naar de oorzakelijk mutaties in het PRPH2-gen de diagnose gesteld kan worden (Hoofdstuk 2)

LMD is een multifactoriële aandoening die zowel door genetische als door omgevingsfactoren wordt veroorzaakt. Ondanks de manifestatie op latere leeftijd heeft familie- en tweelingonderzoek uitgewezen dat er bij LMD sprake is van een sterke genetische component. Genoombrede associatiestudies hebben verschillende genetische markers gevonden die met LMD zijn geassocieerd. Een aantal van deze associaties lijken aan te geven dat high-density lipoproteïnemetabolisme (HDL-metabolisme) een rol speelt bij LMD, al lieten eerdere studies naar de relatie tussen HDL-concentraties in het serum en LMD tegenstrijdige resultaten zien. Sommige van deze studies vonden geen relatie, terwijl andere een verhoogd risico op LMD relateerden aan een verhoogd HDL-gehalte en weer andere studies juist een omgekeerde relatie tussen HDL en LMD beschreven. In Hoofdstuk 3 onderzochten we de relatie tussen een gevoeligheidslocus in de buurt van het*TIMP3*-gen en genen van het HDL-metabolisme en

LMD en hebben we de concentraties van serumlipiden en -lipoproteïnen van LMD-patiënten en controlepersonen vergeleken. De resultaten bevestigden dat LMD was geassocieerd met varianten in de buurt van het*TIMP3*-gen en de genloci die betrokken zijn bij het HDL-metabolisme. Ook onderstrepen onze bevindingen de rol van de extracellulaire matrix en het HDL-metabolisme in de pathogenese van LMD. Onze studie wijst een verhoogde ApoB-concentratie aan als potentiële biomarker voor LMD (Hoofdstuk 3A).

Drusen zijn afwijkingen die typisch zijn voor vroege – en intermediaire stadia van LMD. Drusen ontstaan door pathologische afzettingen van extracellulair materiaal tussen het retinale pigmentepitheel en het membraan van Bruch. De ontdekking dat drusen complement eiwitten bevatten, gaf aanleiding tot de hypothese dat drusen een nevenproduct zijn van een locale ontstekingsreactie. Het complementsysteem is een belangrijk onderdeel van ons immuunsysteem dat een essentiële rol speelt in de verdediging tegen binnendringende micro-organismen en bij de regulatie van immuunreacties. Systemische complementactivatie is geassocieerd met LMD en wordt voornamelijk toegeschreven aan een risico-allel in *complementfactor H ofwel het CFH-gen.* De tweede belangrijke risicofactor voor LMD is de genetische gevoeligheid voor *leeftijdsgebonden maculopathie 2* (*ARMS2/LOC387715*) SNP. De functie van het *ARMS2*-gen is onbekend en men is het niet eens over de subcellulaire locatie van het eiwit. Het is niet duidelijk of bij LMD nog andere genen een rol spelen bij complementactivatie. In Hoofdstuk 3B hebben wij complementactivatie gemeten in het plasma/serum van LMD-patiënten en vergeleken met een controlegroep. De data werden vervolgens gestratificeerd op basis van de *CFH* en *ARMS2* genotypen. Anders dan in eerdere studies waren wij hierdoor in staat de effecten van deze genotypen onafhankelijk van elkaar te onderzoeken. Wij vonden dat systemische activatie van de alternatieve route van het complementsysteem (bepaald door de C3d/C3-ratio) inderdaad is geassocieerd met LMD. Bij LMD patiënten vonden we verhoogde concentraties van CFB, een zogenaamd 'acute fase-eiwit'. Samen met de genetisch risicofactoren leidt dit tot een versterkte activatie van de alternatieve complementroute. Verder liet deze studie zien dat CFB-concentraties lager waren bij dragers van het beschermende *CFB* H9L-allel, wat een mogelijke verklaring biedt voor het beschermingsmechanisme van dit polymorfisme. Ook constateerden we dat verstoorde complementregulatie niet alleen geassocieerd is met de *CFH* risico-allelen, maar ook met de *ARMS2* risico-allelen. Onze bevindingen wijzen derhalve op een mogelijk gemeenschappelijk pathogenetisch mechanisme van CFH en ARMS2 bij LMD (Hoofdstuk 3B).

Het kenmerk van neovasculaire LMD is de ingroei van choroïdale bloedvaten in en onder de macula. Hoewel slechts 10% van alle patiënten met LMD aan deze vorm lijden, komt ernstige slechtziendheid vooral voor bij patienten met de neovasculaire vorm van LMD. De huidige

behandeling bestaat uit intravitreale injecties met ranibizumab, maar het effect van de behandeling verschilt aanzienlijk per patiënt. De oorzaak van deze verschillen wordt niet goed begrepen. In hoofdstuk 5 onderzochten wij daarom de invloed van risico-allelen in het *CFH en ARMS2-gen*, en de vasculaire endotheliale groeifactor A (*VEGF-A)* in relatie tot de respons op ranibizumab en de leeftijd bij aanvang van de behandeling. Onze hypothese was dat de verschillen in leeftijd waarop LMD zich manifesteert en de respons op de behandeling met ranibizumab te maken hadden met het type en het aantal van de bovenstaande genetische risicofactoren. De data werden – in tegenstelling tot eerdere studies – daarom gestratificeerd op het aantal aanwezig risico-allelen. We vonden een cumulatief effect van risico-allelen, zowel op het ontwikkelen van LMD op jongere leeftijd als een verminderde respons op ranibizumab. Door genetische screening op de risico-allelen bij LMD kunnen we mensen met een verhoogd risico vroegtijdig opsporen, daarnaast kan de clinicus het behandelplan beter afstemmen op de individuele patiënt. (Hoofdstuk 4).

Zoals hierboven geschetst, spelen complementgemedieerde ontstekingen een centrale rol in de pathogenese van LMD. Bij LMD patiënten wordt bijvoorbeeld vaker een verhoogde activatie van het complement systeem gevonden. Grootschalige populatiestudies hebben laten zien dat orale suppletie van zink het beloop van LMD kan vertragen. Het is nog niet duidelijk hoe de beschermende werking van zink ontstaat. In Hoofdstuk 6 hebben wij gekeken of zinksuppletie een direct effect had op de mate van complementactivatie. In deze open-label studie ontving een cohort van 72 patiënten met verschillende stadia van LMD gedurende 3 maanden dagelijks een orale dosis van 50 mg zinksulfaat. De mate van complementactivatie - gedefinieerd als de C3d/C3-ratio - werden gemeten voor, tijdens en na de studie. De aanwezigheid van de genetische risicovarianten *CFH* (Y402H) en *ARMS2* (A69S) werd vastgesteld en het stadium van de LMD werd bepaald met CARMS (een klinisch classificatiesysteem voor LMD). Bij analyse van de data bleek de mate van complementactivatie te correleren met het ziektestadium; mogelijk geeft de mate van activatie een indicatie voor de ziekteactiviteit. Onze studie liet verder zien dat een verhoogde complementactivatie bij LMD-patiënten tot normale niveaus kan worden teruggebracht door dagelijkse toediening van 50 mg zinksulfaat. Bij patiënten met het homozygote *CFH* Y402H-risicovariant daalde de C3d/C3 ratio sneller en sterker dan in de patiënten zonder deze genetische risicofactor. Remming van het complementsysteem staat sterk in de belangstelling bij onderzoek naar preventie en behandeling van LMD. Klinische studies naar verschillende complementremmers zijn reeds gestart en bevinden zich in fase-1 en -2. De resultaten van de studie in dit proefschrift laat zien dat verlaging van de complementactivatie ook met zink tabletten kan worden verkregen. Uit oogpunt van veiligheid en kosteneffectiviteit zou onderzocht moeten worden of zinksuppletie de voorkeur verdient boven behandelingen met complementremmers. (Hoofdstuk 5).

Naast interacties tussen genen en variaties in het aantal kopieën van genen, spelen ook epigenetische modificaties een rol in de regulatie van genen. Dit betekent dat omgevingsfactoren de regulatie van veel genen kan beïnvloeden. Hierbij verandert de expressie van genen, terwijl de DNA-structuur zelf onveranderd blijft. De idee dat het "genetisch" risico gedurende het hele leven constant is en onafhankelijk is van externe factoren, moeten we dus loslaten. Dit inzicht betekent dat het genetisch risico wel degelijk kan worden beïnvloed door levenstijl, bijvoorbeeld roken, en dat LMD patiënten hierop moeten worden aangesproken. In individuele gevallen kan het belangrijk kan zijn om het persoonlijke risicoprofiel voor LMD te bepalen (Chapter 6). Echter, kennis van een risicoprofiel is zinloos als we niet weten hoe we dit risico kunnen verminderen. Om de opsporing en behandeling van LMD beter af te stemmen op de individuele patiënt, zou toekomstig onderzoek zich kunnen richten op het ontwikkelen van methoden om personen met een hoog risico vroegtijdig op de sporen alsmede op de invloed die omgevingsfactoren en voeding hebben op de ontwikkeling van LMD. Met 'next-generation sequencing' of NSG-technieken waarmee al het erfelijk materiaal van een persoon kan worden bekeken, kan een veel gedetailleerder en accurater moleculair profiel worden verkregen. De meest efficiënte benadering zou kunnen bestaat uit het ingrijpen in de specifieke fasen van de pathogenese van LMD met de daarvoor toegesneden medicijnen, in een poging de balans in het systeem te herstellen en het beloop van de ziekte (beter) te beheersen. Daarnaast zal het in de toekomst mogelijk worden beschermende genen 'aan' en risicogenen 'af' te zetten, waarmee er sprake zal zijn van een geheel nieuwe dimensie in patiëntspecifieke behandeling (Chapter 6). Over twintig jaar (of misschien wel eerder) hoop ik tegen mijn patiënt te kunnen zeggen:

"Ik behandel niet de LMD; ik behandel *uw* LMD."

LIST OF PUBLICATIONS

D. Smailhodzic, A.I. den Hollander, van de Ven JP, R.A.C. van Huet, J.M.M. Groenewoud, Y.T.E. Lechanteur, S. Fauser, C. de Bruijn, M.R. Daha, G.J. van der Wilt, C.B. Hoyng and B.J. Klevering. Zinc supplementation lowers complement overactivation in patients with age-related macular degeneration. Submitted

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Manuel M. Hermann, Freekje van Asten, Philipp S. Muether, **D. Smailhodzic**, Peter Lichtner, Carel B. Hoyng, Bernd Kirchhof, Christian Grefkes, Anneke I. den Hollander, Sascha Fauser. Polymorphisms in the vascular endothelial growth factor receptor 2 (VEGFR2/KDR) gene are associated with better response rates to ranibizumab treatment in age-related macular degeneration. *Submitted*

Chloe M. Stanton, Elod Kortvely, Caroline Hayward, John R. Yates, **D. Smailhodzic**, Kevin J. Chalmers, Ulrich Luhmann, Malcolm G. Dunlop, Robin Ali, Osamu Onodera, Anneke den Hollander, Marius Ueffing, Alan F. Wright. The HTRA1 protease activates the alternative complement pathway by cleavage of factor D, influencing systemic complement activation in age-related macular degeneration. *Submitted*

DANKWOORD

Veel mensen denken dat onderzoek doen saai is. Hoe kan dit van toepassing zijn op de ontdekkingsreis die onderzoek doen eigenlijk is?
Onderzoek kent geen begin en geen eind. Daar komt nog bij dat het belangrijkste instrument – je verstand – niet altijd te vertrouwen is. Immers, dat wat je zoekt ga je ook vinden. In deze wereld van 'to catch the uncatchable, to see the unvisable' is veel creativiteit, moed en toewijding nodig. Omdat beelden sterker spreken dan duizend woorden, spreek ik hieronder mijn dank uit in beelden.

Beste prof. dr. Hoyng, beste Carel. Misschien geïnspireerd door de middeleeuwse stad Korčula, geboorteplek van ontdekkingsreiziger Marco Polo, zie ik jou als een luid lachende, wijndrinkende schipper, die 's werelds wildste zeeën aandurft en als een ware ontdekkingsreiziger, onderzoeksschip OOGHEELKUNDE naar onbekende wateren koerst. Zo heb jij de contouren van mijn onderzoek vormgegeven en mij geholpen mijn koudwatervrees te overwinnen. Dank je voor je onbevreesdheid, je onuitputtelijke energie en je lust 'to discover and rule them all'.

Beste dr. Den Hollander, beste Anneke. Eigenlijk past hier de titel prof., maar die komt vast nog. Rots in de branding en mijn steun en toeverlaat. Als ik aan jou denk zie ik het Vrijheidsbeeld. Jij staat er en houdt hoog in de lucht trots de fakkel vast, een hoop- en oriëntatiepunt voor verloren schepen in de donkere nacht. Dank je voor je intelligentie, voor je rust en dat jij er altijd voor mij bent.

Beste dr. Klevering, beste Jeroen. Als ik aan jou denk dan zie ik een heldere denker die in staat is om elk probleem op te lossen. Ik zie je vliegen en waken over ons allemaal. En zo heb jij ook over mij gewaakt toen het minder goed ging totdat 'de trein weer begon te rijden'. Dank je voor de academische discussies en je onberispelijk gevoel voor taal. Dank je voor die keren dat jij mij een spiegel voorhield. Dank je voor je wijze en goedbedoelde adviezen binnen en buiten de OK.

Beste prof. Daha, beste Mo. Elke keer wanneer ik met jou in dezelfde kamer was dacht ik: 'Knoop goed in je oren wat deze man te zeggen heeft'. Als een wijze shaman kun jij adviezen geven die veel verder gaan dan de wereld van de moleculen. Dank je voor je laagdrempeligheid, je 'zoom out'-vermogen en je aanstekelijke, jeugdige energie.

Beste prof. dr. Klaver, beste Caroline. Of kan ik beter prof. dr. Clever zeggen? Met verwondering keek ik naar hoe jij moeiteloos 'data-blokken' van rechts naar links verplaatste en hoe gemakkelijk je er mee kon spelen. Ongelofelijk hoeveel ik in de drie uur die ik met jou doorbracht, geleerd heb! Nadien ben ik zelf eindeloos met blokken gaan stapelen om te kijken wat er te zien valt, tot de dag voorbij was en ik bij mezelf dacht: 'Weer een dag mogen spelen en ik krijg hier nog voor betaald ook'. Dank je voor je inspiratie!

Beste drs. Groenwoud, beste Hans. Als ik een vogel was in het land van de statistiek was jij mijn vleugels. Ik zal nooit vergeten hoe wij een overleg voerden toen ik hoogzwanger was en me niet zo goed voelde, terwijl ik plat op de grond lag. En het gezicht van je collega die binnenkwam en tot de volgende conclusie kwam: 'Hans, er ligt een vrouw op de vloer!'

Beste prof. dr. Keunen, beste Jan. Als ik aan jou denk dan zie ik een vaderfiguur. Beschermer van de assistenten. Dank je voor je toewijding en betrokkenheid bij de mensen om je heen.

Beste dr. Boon, beste Camiel. Je bent inmiddels oogarts maar het beeld dat ik van jou heb stamt nog uit de tijd dat wij elkaar pas hadden ontmoet: een gedreven jonge promovendus, rond brilletje op en een lok a la Lucky Luke. Dank je voor je betrouwbaarheid, je enthousiasme en je wil om van elke dag 'de beste dag' te maken.

Beste John, als ik aan jou denkt dan zie ik een soldaat. Strak gekleed, de haren kort en op hun plek, geen kleur bekennen en liefst geen emoties. Want, echte mannen hebben geen emoties, toch? Dank je voor je vriendschap, je snelheid en dat ik altijd op je kan rekenen.

Beste Ramon, ik zie jou rondrennen en zorgen dat iedereen zich comfortabel voelt. 'Hai, ga jij maar lekker zitten; ik haal zo een lekker kopje verse muntthee met appeltaart'. Dank je voor je vriendschap, je zorgzaamheid en je digitale kennis.

Beste Yara, als ik aan jou denk, denk ik aan de zee. Diep en oppervlakkig, warm en koud, vol met leven en toch transparant, bewegingloos en in staat tot grote kracht. Dank je voor je vriendschap, je stabiliteit en je aanwezigheid.

Beste Freekje, als ik aan jou denk dan zie ik 'Singing in the rain' van Gene Kelly. Met dansschoenen aan, luchtig tapdansend door het leven. Dank je voor je Nederlandse nuchterheid en je ongecompliceerdheid a la 'Freekje'.

Beste Asha, Frederique, Bert, Ngaisah, Anne, Anja, Bjorn, Tiny, Maria en Agnes. Als ik aan jullie denk, denk ik aan magische wezens die in de nacht komen helpen. En wanneer ik 's ochtends wakker word, is er heel veel werk verzet. Veel dank voor jullie hulp!

Beste Nicole, ik fietste op mijn zware mamafiets tegen de wind in, met muziek in de oren en verzonken in gedachten. Toen fietste jij voorbij, gebogen om geen wind te vangen, doelgericht en snel. Een vrouw die weet wat ze wil en geen energie verspilt. Ik dacht toen: 'Dzenita, maak je hoofd leeg en fiets gewoon naar je werk. En koop een lichte racefiets'! Dank je dat jij zo anders bent dan ik.

Beste Paul Kalkbrenner, La-33, Black Eyed Peas, Kylie Minogue, Underworld, Moby en honderden andere muzikanten. Dankzij jullie muziek vond mijn beweeglijke brein de rust om uren achtereen achter computers te blijven zitten en na te denken. Dank aan alle muzikanten, jullie maken mijn brein minder grijs!

Dragi moji roditeljiji Ajša I Mirsade, hvala vam za vašu ljubav. Hvala vam za slobudu koju ste mi dali da postanem čovjek koji jesam i koji želim da budem.

Lieve Lyra en Enna, jullie geboorte viel samen met die van dit proefschrift. Dat maakte het promotietraject nog uitdagender! Zo ook het voltooien van het dankwoord, want Lyra kruipt steeds tussen mij en het toetsenbord. Ik dank jullie voor jullie verrijking van mijn leven en ziel.

Lieve Maarten, als ik aan jou denk, begint het met een ontbijt op zondagochtend: de banaan-pannenkoeken, de vanillesuiker en Frank Sinatra. 's Middags trekken wij onze wandelschoenen aan en trekken we een jungle in, op zoek naar een avontuur. 's Avonds zeg je: 'Schat, trek je glitterjurk aan, er is een leuk feest'. Dank je dat je zo veelzijdig bent. Dank je dat jij mijn thuis bent.

Beste allen, nogmaals dank voor het geven van een deel van jullie wezen en leven, dat ik uiteindelijk heb mogen benutten om dit proefschrift te schrijven. Daarom is dit niet mijn proefschrift. Het is van ons.

Onderzoeksgroep afdeling Oogheelkunde RadboudUMC

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 4 juli 1976 te Sarajevo, Bosnië en Herzegovina, waar ze haar jeugd en middelbare school tijd doorbracht.

Na het behalen van het diploma Nederlands in 1995, het HAVO diploma in 1996 en het VWO diploma in 1997, begon ze aan de studie Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Daarnaast werkte ze als opticien. In dit werk werd ze verliefd op de perfectie en helderheid van het menselijk oog en ontdekte dat werken met mensen beter bij haar paste dan alleen wetenschappelijk onderzoek.

In september 2001 begon ze dan ook aan de studie Geneeskunde aan Radboud Universiteit Nijmegen, welke werd afgerond in juni 2008. Tijdens haar wetenschappelijke stage bij prof. dr. Carel B. Hoyng konden haar interesse in het menselijk oog gecombineerd worden met patiënt gerelateerd wetenschappelijk onderzoek.

Aansluitend, in juli 2008, begon ze aan haar promotie onderzoek onder begeleiding van prof. dr. Carel B. Hoyng, dr. Anneke I. den Hollander, dr. B. Jeroen Klevering en prof. dr. M Daha bij de afdeling Oogheelkunde van het Radboud UMC. Sinds mei 2012 is ze werkzaam als arts-assistent oogheelkunde in opleiding tot oogarts.

STELLINGEN

AMD

STUDIES ON PATHOGENESIS, TREATMENT AND PREVENTION OF AGE-RELATED MACULAR DEGENERATION

1. Complement inflammatie speelt een centrale rol in de pathogenese van AMD.
2. De twee belangrijkste risico factoren voor *AMD, CFH* en *ARMS2*, delen een gezamenlijke route in het ontstaan van AMD.
3. Het immuunsysteem breekt een eenmaal beschadigde macula verder af, en draagt daarmee bij aan het ontstaan van slechtziendheid door AMD.
4. Om complement activatie te voorkomen is het beter om zink te suppleren in de vroege fase van AMD.
5. Om complement remmers te doen slagen in de strijd tegen AMD is het noodzakelijk om de juiste patiënten te selecteren en lang te vervolgen.
6. Grote studies waarin anti-VEGF therapie bij AMD getest werd, informeren ons over de effecten van deze behandeling onder optimale condities. Helaas, is deze therapie minder effectief in de weerbarstige klinische praktijk.
7. Genen kun je veranderen, maar het is essentieel om ze eerst te begrijpen.
8. Genetische screening bij AMD patiënten verdient een plaats in de kliniek.
9. Wie geen prikkelende stellingen poneert, kan beter het onderzoeksveld verlaten.
10. Nuanceren kan ook handig zijn.
11. Als je geblinddoekt de staart van de olifant vastpakt ga je denken dat het een touw is. Zo is het ook met de wetenschap. Conclusies zijn sterk afhankelijk van de data in je hand, terwijl de werkelijkheid er heel anders uitziet.
12. Multitasken is veel dingen tegelijk slecht tot matig doen.
13. Alleen mijn grootste vijand wens ik toe dat waar hij bang voor is, hem nooit overkomt.

Dženita Smailhodžić
20 december 2013

AMD

Dženita Smailhodžić