# Genetic biomarkers for precision medicine in age-related macular degeneration

LAURA LORÉS DE MOTTA

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**Doctoral Thesis**

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by

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*A mis abuelos*

# Contents

# List of abbreviations

### Genes and proteins

| | |
|---|---|
| *ABCA1* | ATP-binding cassette subfamily A member 1 |
| *ACAD10* | Acyl-CoA dehydrogenase family member 10 |
| *ACADSB* | Acyl-coenzyme A dehydrogenase, short/branched chain |
| *ADAMTS9-AS2* | ADAM metallopeptidase with thrombospondin type 1 motif *9* antisense RNA 2 |
| *APOE* | Apolipoprotein E |
| *ARHGAP21* | Rho GTPase activating protein 21 |
| *ARMS2* | Age-related maculopathy susceptibility 2 |
| *B3GALTL* | Beta 3-glucosyltransferase |
| *C10orf88* | Chromosome 10 open reading frame 88 |
| *C2* | Complement component 2 |
| *C20orf85* | Chromosome 20 open reading frame 85 |
| *C3* | Complement component 3 |
| C5 | Complement component 5 |
| *C9* | Complement component *9* |
| *CCT3* | Chaperonin containing TCP1 subunit 3 |
| *CD63* | CD63 molecule |
| *CETP* | Cholesterylester transfer protein |
| *CFB* | Complement factor B |
| *CFD* | Complement factor D |
| *CFH* | Complement factor H |
| *CFHL-1* | Complement factor H-like protein 1 |
| *CFHR4* | Complement factor H related 4 |
| *CFI* | Complement factor I |
| *CNN2* | Calponin 2 |
| *COL10A1* | Collagen type X alpha 1 chain |
| *COL4A3* | Collagen type IV alpha 3 chain |
| *COL8A1* | Collagen type VIII alpha 1 chain |
| *CTRB1* | Chymotrypsinogen B1 |
| *CTRB2* | Chymotrypsinogen B2 |
| *CXCL8* | C-X-C motif chemokine ligand 8 |
| *FBN2* | Fibrillin 2 |
| *HIF1A* | Hypoxia inducible factor 1 alpha subunit |
| *HTRA1* | HtrA serine peptidase 1 |
| IL-6 | Interleukin 6 |
| *KCNT2* | Potassium sodium-activated channel subfamily T member 2 |
| *KMT2E* | Lysine methyltransferase 2E |
| *LIPC* | Lipase C, hepatic type |
| MAC-IP | Membrane attack complex inhibitory protein |
| *MIR6130* | MicroRNA 6130 |
| *MMP9* | Matrix metalloproteinase *9* |

| | |
|---|---|
| *NPLOC4* | NPL4 homolog, ubiquitin recognition factor |
| *NRP1* | Neuropilin 1 |
| *PEDF* | Pigment epithelium-derived factor |
| *PGF* | Placental growth factor |
| *PILRA* | Paired immunoglobin like type 2 receptor alpha |
| *PILRB* | Paired immunoglobin like type 2 receptor beta |
| *PLA2G12A* | Phospholipase A2 group XIIA |
| *PRLR* | Prolactin receptor |
| *RAD51B* | RAD51 paralog B |
| *RDH5* | Retinol dehydrogenase 5 |
| *RORB* | RAR related orphan receptor B |
| *SERPINF1* | Serpin family F member 1 |
| *SKIV2L* | Ski2 like RNA helicase |
| *SLC16A8* | Solute carrier family 16 member 8 |
| *SPEF2* | Sperm flagellar 2 |
| *SRPK2* | SRSF protein kinase 2 |
| *SYN3* | Synapsin III |
| *TGFBR1* | Transforming growth factor beta receptor 1 |
| *TIMP3* | Tissue inhibitor of metallopeptidases 3 |
| *TMEM97* | Transmembrane protein 97 |
| *TNFRSF10A* | TNF receptor superfamily member 10a |
| *TRPM3* | Transient receptor potential cation channel subfamily M member 3 |
| *TSPAN10* | Tetraspanin 10 |
| *UNC93B1* | Unc-93 homolog B1 |
| *VEGF(A)* | Vascular endothelial growth factor (A) |
| VEGFR1/*FLT1* | Vascular endothelial growth factor receptor 1 / fms related tyrosine kinase 1 |
| VEGFR2/*KDR* | Vascular endothelial growth factor receptor 2 / kinase insert domain receptor |
| *VTN* | Vitronectin |

**Other abbreviations**

| | |
|---|---|
| aHUS | Atypical hemolytic uremic syndrome |
| AMD | Age-related macular degeneration |
| AP | Alternative pathway |
| AREDS | Age-Related Eye Disease Study |
| BMI | Body mass index |
| BMSC | Bone marrow-derived stem cells |
| Bp | Base pair |
| BrM | Bruch's membrane |
| BRAMD | Comparing the Effectiveness of Bevacizumab to Ranibizumab in Patients with Exudative Age-Related Macular Degeneration |
| BVZ | Bevacizumab |
| CATT | Comparison of AMD Treatments Trials |
| CDD | Conserved Domain Database |

| | |
|---|---|
| Chr | Chromosome |
| CI | Confidence interval |
| CIDR | Center for Inherited Disease Research |
| CIRCL | Cologne Image Reading Center and Laboratory |
| CMT | Central macular thickness |
| CNV | Choroidal neovascularization |
| CP | Classical pathway |
| CRT | Central retinal thickness |
| CS | Contrast sensitivity |
| CSMT | Central subfield macular thickness |
| DA | Disk area |
| ECM | Extracellular matrix |
| ERC | European Research Council |
| ETDRS | Early Treatment Diabetic Retinopathy Study |
| ETR | Manchester Eye Tissue Repository |
| EUGENDA | European Genetic Database |
| FA | Fluorescein angiography |
| FDA | Food and Drug Administration |
| FFA | Fundus fluorescein angiography |
| Fw | Forward |
| GA | Geographic atrophy |
| GWAS | Genome-wide association study |
| hCNSSC | Human central nervous system stem cells |
| HDL | High-density lipoprotein |
| hESC | Human embryonic stem cell |
| HLA | Human leukocyte antigen |
| IAMDGC | International AMD Genomics Consortium |
| iPSC | Induced pluripotent stem cells |
| IVAN | Alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation |
| LD | Linkage disequilibrium |
| LDL | Low-density lipoprotein |
| logMAR | Logarithm of minimal angle of resolution |
| LP | Lectin pathway |
| MA | Minor allele |
| MAC | Membrane attack complex |
| MAF | Minor allele frequency |
| N | Number |
| NA | Not applicable |
| nAMD | Neovascular age-related macular degeneration |
| NF | Not found |
| NI | Not indicated |
| nvAMD | Neovascular age-related macular degeneration |
| OCT | Optical coherence tomography |
| OR | Odds ratio |

| | |
|---|---|
| PBST | Phosphate buffered saline with Tween-20 |
| PCA | Principal components analysis |
| PLIER | Probe Logarithmic Intensity Error |
| PRN | Pro re nata |
| QC | Quality control |
| RAC | Rare allele count |
| RAP | Retinal angiomatous proliferation |
| RPE | Retinal pigment epithelium |
| Rv | Reverse |
| SAB | Standard assay buffer |
| SD | Standard deviation |
| SE | Standard error |
| SKAT-O | Optimal unified sequence kernel association test |
| SNP | Single nucleotide polymorphism |
| SPR | Surface plasmon resonance |
| TE | Treat-and-extend |
| TFT | Total foveal thickness |
| TLR | Toll-like receptor |
| VA | Visual acuity |

# 1

# Introduction

Adapted from "Exploring the use of molecular biomarkers for precision medicine in age-related macular degeneration"

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*"All men naturally desire knowledge. An indication of this is our esteem for the senses; for apart from their use we esteem them for their own sake, and most of all the sense of sight. Not only with a view to action, but even when no action is contemplated, we prefer sight, generally speaking, to all the other senses. The reason of this is that of all the senses sight best helps us to know things, and reveals many distinctions."*

Metaphysics 1st book, Aristotle, 350 B.C.E

# 1 Age-related macular degeneration (AMD)

## 1.1 AMD leads to vision loss in the elderly

In the process of vision, reflected light enters the eye through the cornea and is focused onto the retina, located in the posterior section of the eye (Figure 1A). The retina is the light-sensitive tissue that transforms light photons into electrical signals, which are transmitted to the brain and allow visual perception. It is composed of a layer of supporting cells, the retinal pigment epithelium (RPE) and the neurosensory retina, which consists of several layers of neural cells including the photoreceptors. Nutrients, oxygen, biomolecules, fluids and metabolic waste products are exchanged between the RPE and the bloodstream via the vessel network of the choroid (the choriocapillaris), passing through its innermost extracellular matrix layer; the Bruch's membrane (Figure 1A).

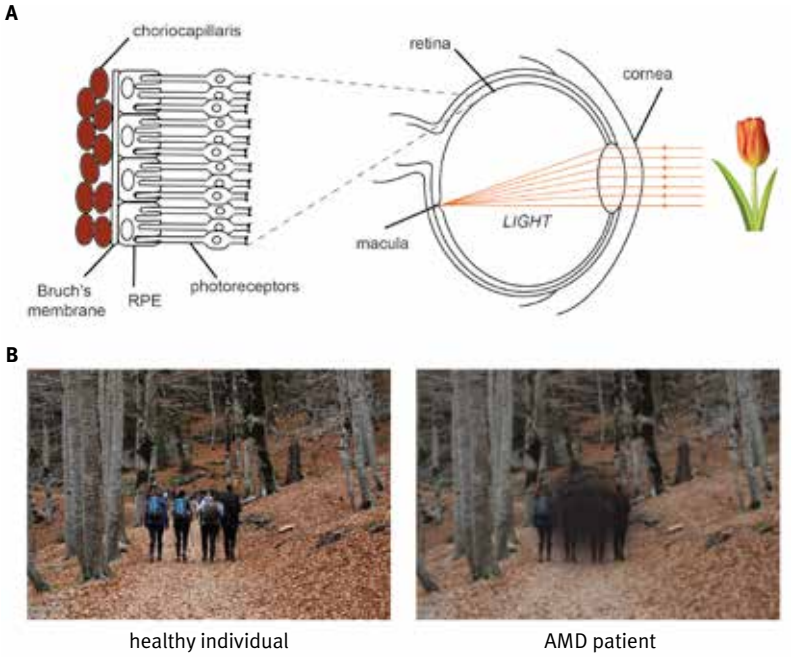


FIGURE 1 **Vision process and AMD**

**A)** Schematic representation of the eye, the retina and the choriocapillaris. Light enters the eye and is projected onto the retina. The macula is located in the center of the retina. In the retina, the photoreceptors are located in the outer section, adjacent to the retinal pigment epithelium (RPE). The Bruch's membrane is a collagen- and elastin-rich layer located between the RPE and the choriocapillaris. **B)** Depiction of vision loss in AMD patients. Vision of a healthy individual on the left and distortion of vision in an AMD patient on the right.

Age-related macular degeneration (AMD) is a progressive disease that leads to the degeneration of the macula, which is located in the center of the retina (Figure 1A). The macula is

responsible for central vision, sharp vision and color vision. Consequently, AMD patients lose their central vision field, have blurred vision and have less ability to discriminate colors (Figure 1B).

The prevalence of AMD increases exponentially with age (Figure 2). While around 1% of people between 65 and 69 years old are affected by AMD, in the 80-84 year range 7% are affected, and in the 85-89 year range the prevalence reaches 14%. Approximately half of these AMD patients are visually impaired.[1]

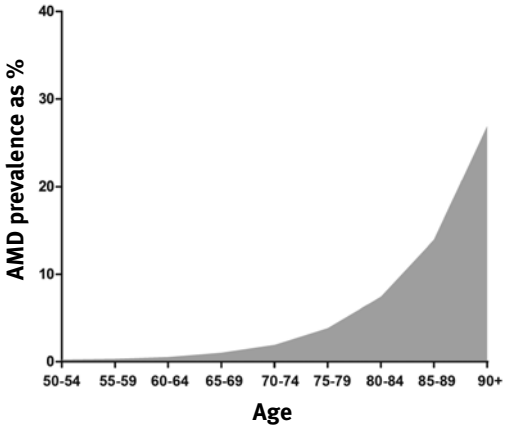


FIGURE 2 **Estimated prevalence of advanced AMD per age-category in Europe (adapted from Colijn JM et al., 2017)**

## 1.2 Age-related macular degeneration is a highly prevalent disease and impacts quality of life

AMD is the leading cause of blindness in the elderly in the Western world, and the third most common cause of severe visual impairment worldwide.[2,3] The prevalence is higher in Europeans compared to Asians and Africans.[4] Due to an increased aging of the population, the number of people affected by the disease in Europe is expected to rise up to 4.8 million by 2040. Worldwide, that number is expected to reach 26.2 million.[4,5] Considering that half of these patients are expected to be visually impaired, health care efforts need to be intensified in order to reduce the number of AMD patients who suffer from vision loss in the coming years.[5]

AMD impacts quality of life mainly due to visual acuity loss; it can impair face recognition and daily life activities such as reading, watching TV, cooking, travelling, cleaning, self-care, shopping and driving. AMD patients also have a higher risk of falling, and fear of falling results in limitation of activities. As a consequence, AMD patients require up to eight times more assistance. Moreover, AMD patients experience poorer life satisfaction, greater stress, and higher rates of depression compared to healthy individuals.[6]

## 1.3 Clinical characteristics of AMD

The hallmark of AMD is the presence of drusen. Drusen are deposits of extracellular debris that localize under the RPE, recognizable as yellowish spots on photographs of the fundus of the eye (Figure 3). Drusen are a possible consequence of RPE disruption, and contain lipids, lipoproteins and inflammatory factors, suggesting a local chronic inflammatory state.[7-9] Aging can lead to drusen formation, however, the number of drusen around the macular zone in AMD is notably higher compared to what would be expected in a healthy retina. In early stages, AMD is characterized by the appearance of more than 10 small drusen or 1-14 intermediary-sized drusen, and pigmentary changes can also be observed (Figure 3A).[10] When drusen increase in number and size, AMD is classified as intermediate AMD (Figure 3B). While in these stages visual acuity is well preserved,[11] AMD patients can progress to an advanced stage in which vision loss occurs.[12] Advanced AMD can be classified in two types: geographic atrophy (GA, Figure 3C) and choroidal neovascularization (CNV, Figure 3D). CNV, also referred to as neovascular AMD (nvAMD), involves the abnormal growth of blood vessels from the choriocapillaris invading the retina, with subsequent leakage and bleeding, and provokes a vision-threatening scar in the macula. GA is characterized by atrophy of the retina, resulting from gradual loss of photoreceptors, RPE cells and the choriocapillaris.[13,14] The prevalence of both advanced types is similar,[4] however, nvAMD accounts for most of the visual acuity loss caused by AMD.[15]

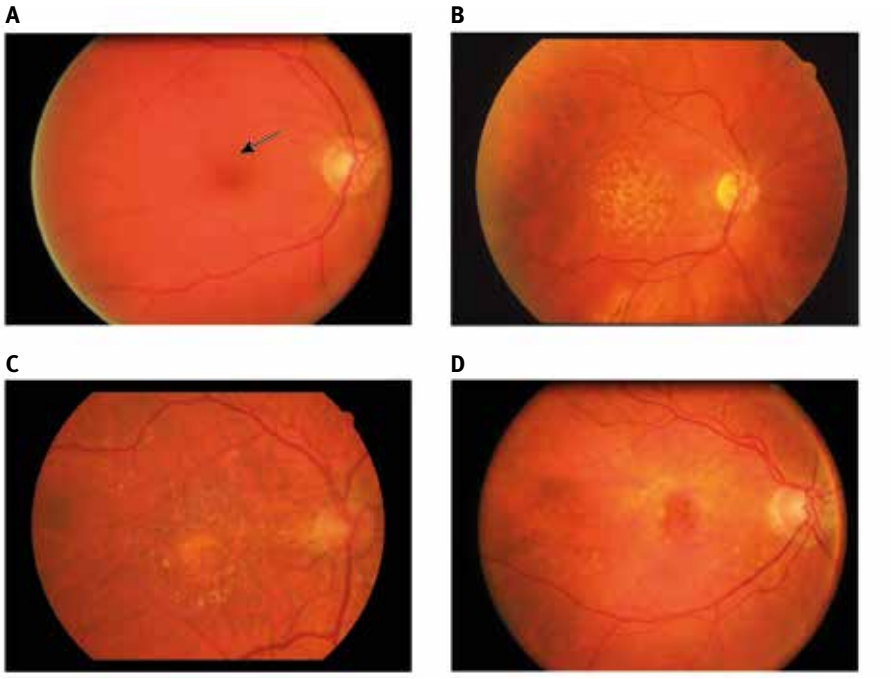


FIGURE 3 **Fundus photographs of AMD patients**

**A)** Early AMD; the arrow indicates the drusen in the macular zone. **B)** Intermediate AMD. **C)** Geographic atrophy. **D)** Choroidal neovascularization.
The patients depicted in this figure were included in the studies presented in this thesis.

## 1.4 Risk factors for AMD development – Genetic factors play a major role

AMD is a multifactorial disease; both environmental and genetic factors influence disease risk. Increasing age is the strongest risk factor for AMD development. Other factors that have consistently been associated with AMD risk are smoking, previous cataract surgery and family history of AMD. Smokers have approximately two times more chance of developing AMD compared to non-smokers (odds ratio [OR]≈2), making smoking the strongest modifiable risk factor for AMD. Patients who have undergone cataract surgery have approximately a three-fold increased chance of developing AMD (OR≈3), and family members of an AMD patient have a six-fold increased chance (OR≈6). Other reported environmental factors that potentially influence disease risk are higher body mass index, history of cardiovascular disease and hypertension.[16]

Genetic factors play a major role in the disease etiology, explaining up to 71% of the disease variation.[17] The first single nucleotide polymorphisms (SNPs) found to be associated with AMD were rs1061170 or p.Y402H in the complement factor H (*CFH*) gene and rs10490924 or p.A69S in the age-related maculopathy susceptibility 2 (*ARMS2*) gene, both conferring a nearly three-fold increase in the risk of developing AMD.[18-21] The largest case-control association study performed to date for AMD included 16,144 patients and 17,832 controls, and identified 52 independent genetic variants across 34 loci (Table 1). Additionally, this study assessed whether genetic variants could explain the phenotypic differences observed between GA and CNV, the advanced types of AMD. SNP rs142450006, located in the *MMP9* gene, was found to be associated exclusively with CNV. Moreover, besides the 52 independently associated genetic variants, four genes showed a rare variant burden associated with AMD: *CFH*, *CFI*, *TIMP3* and *SLC16A8*.[22]

## 1.5 Pathways involved in AMD etiology

Although many of the underlying mechanisms of AMD still need to be elucidated, several pathways are known to be involved in the disease pathogenesis. These pathways include angiogenesis, complement system, lipid metabolism and extracellular matrix remodeling.

### 1.5.1 Angiogenesis

Angiogenesis is the process of formation of new vessels, sprouting from pre-existing ones. In AMD, angiogenesis occurs in the neovascular form, in which new leaky vessels are formed in the choroidal microvascular bed.[14] Under normal conditions, vessels in the choroid are quiescent. However, hypoxia, inflammation and oxidative stress can lead to the production of angiogenic factors, which in turn induce the breakdown of the endothelial cell basal membrane, the proliferation and migration of endothelial cells, and the degradation of the surrounding extracellular matrix, eventually leading to new vessel formation.[23,24]

TABLE 1 34 loci influence the risk of AMD development (adapted from Fritsche et al., 2016)

| Chr. | Locus name | Number of signals | Lead variant | Major/ minor allele | OR | *P*-value |
|---|---|---|---|---|---|---|
| 1 | *CFH* | 8 | rs10922109 | C/A | 0.38 | $9.6 \times 10^{-618}$ |
| 2 | *COL4A3* | 1 | rs11884770 | C/T | 0.9 | $2.9 \times 10^{-8}$ |
| 3 | *ADAMTS9-AS2* | 1 | rs62247658 | T/C | 1.14 | $1.8 \times 10^{-14}$ |
| 3 | *COL8A1* | 2 | rs140647181 | T/C | 1.59 | $1.4 \times 10^{-11}$ |
| 4 | *CFI* | 2 | rs10033900 | C/T | 1.15 | $5.4 \times 10^{-17}$ |
| 5 | *C9* | 1 | rs62358361 | G/T | 1.8 | $1.3 \times 10^{-14}$ |
| 5 | *PRLR-SPEF2* | 1 | rs114092250 | G/A | 0.7 | $2.1 \times 10^{-8}$ |
| 6 | *C2-CFB-SKIV2L* | 4 | rs116503776 | G/A | 0.57 | $1.2 \times 10^{-103}$ |
| 6 | *VEGFA* | 1 | rs943080 | T/C | 0.88 | $1.1 \times 10^{-14}$ |
| 7 | *PILRB-PILRA* | 1 | rs7803454 | C/T | 1.13 | $4.8 \times 10^{-9}$ |
| 7 | *KMT2E-SRPK2* | 1 | rs1142 | C/T | 1.11 | $1.4 \times 10^{-9}$ |
| 8 | *TNFRSF10A* | 1 | rs79037040 | T/G | 0.9 | $4.5 \times 10^{-11}$ |
| 9 | *TGFBR1* | 1 | rs1626340 | G/A | 0.88 | $3.8 \times 10^{-10}$ |
| 9 | *TRPM3* | 1 | rs71507014 | GC/G | 1.1 | $3.0 \times 10^{-8}$ |
| 9 | *MIR6130-RORB* | 1 | rs10781182 | G/T | 1.11 | $2.6 \times 10^{-9}$ |
| 9 | *ABCA1* | 1 | rs2740488 | A/C | 0.9 | $1.2 \times 10^{-8}$ |
| 10 | *ARMS2-HTRA1* | 1 | rs3750846 | T/C | 2.81 | $6.5 \times 10^{-735}$ |
| 10 | *ARHGAP21* | 1 | rs12357257 | G/A | 1.11 | $4.4 \times 10^{-8}$ |
| 12 | *RDH5-CD63* | 1 | rs3138141 | C/A | 1.16 | $4.3 \times 10^{-9}$ |
| 12 | *ACAD10* | 1 | rs61941274 | G/A | 1.51 | $1.1 \times 10^{-9}$ |
| 13 | *B3GALTL* | 1 | rs9564692 | C/T | 0.89 | $3.3 \times 10^{-10}$ |
| 14 | *RAD51B* | 2 | rs61985136 | T/C | 0.9 | $1.6 \times 10^{-10}$ |
| 15 | *LIPC* | 2 | rs2043085 | T/C | 0.87 | $4.3 \times 10^{-15}$ |
| 16 | *CETP* | 2 | rs5817082 | C/CA | 0.84 | $3.6 \times 10^{-19}$ |
| 16 | *CTRB2-CTRB1* | 1 | rs72802342 | C/A | 0.79 | $5.0 \times 10^{-12}$ |
| 17 | *TMEM97-VTN* | 1 | rs11080055 | C/A | 0.91 | $1.0 \times 10^{-8}$ |
| 17 | *NPLOC4-TSPAN10* | 1 | rs6565597 | C/T | 1.13 | $1.5 \times 10^{-11}$ |
| 19 | *C3* | 3 | rs2230199 | C/G | 1.43 | $3.8 \times 10^{-69}$ |
| 19 | *APOE* | 2 | rs429358 | T/C | 0.7 | $2.4 \times 10^{-42}$ |
| 19 | *CNN2* | 1 | rs67538026 | C/T | 0.9 | $2.6 \times 10^{-8}$ |
| 20 | *MMP9* | 1 | rs142450006 | TTTTC/T | 0.85 | $2.4 \times 10^{-10}$ |
| 20 | *C20orf85* | 1 | rs201459901 | T/TA | 0.76 | $3.1 \times 10^{-16}$ |
| 22 | *SYN3-TIMP3* | 1 | rs5754227 | T/C | 0.77 | $1.1 \times 10^{-24}$ |
| 22 | *SLC16A8* | 1 | rs8135665 | C/T | 1.14 | $5.5 \times 10^{-11}$ |

Vascular endothelial growth factor A (VEGFA, also referred to as VEGF) has been established as the key mediator of pathological neovascularization in the eye.[25] In AMD, elevated VEGF levels were found in post-mortem retinas and choroidal neovascular membranes of eyes of AMD patients, as well as in animal models with induced choroidal neovascularization.[26-29] Inhibition of VEGF in mice was also found to inhibit choroidal neovascularization.[30] In addition, more recent genetic studies showed that genetic variation in the *VEGFA* gene is associated with AMD risk.[31]

*VEGFA* is expressed by a variety of cells, including the RPE and immune cells. Transcription of the *VEGFA* gene leads to several alternatively spliced isoforms, with $VEGFA_{165}$ being the most highly expressed in the RPE.[32,33] The tyrosine kinase receptor VEGFR-2 is the key receptor for angiogenesis and is the predominantly expressed receptor on vascular endothelial cells.[34] Neuropilin 1 (NRP-1) is a co-receptor for VEGFR-2 that upon VEGF binding enhances the transduction signal up to 6-fold (Figure 4A).[35,36] One of the key regulators of VEGF expression is hypoxia-inducible factor 1α (HIF-1α), which in hypoxic conditions will induce VEGF expression.[37] Inflammatory cytokines such as IFN-γ, TNF-α and IL-1β can also promote VEGF expression.[38] Besides the pathological effect of elevated VEGF levels, further studies have observed that VEGF represents a survival factor required for neuronal cells and blood vessel homeostasis, and is constitutively produced at low levels by several cell types of the retina.[39,40]

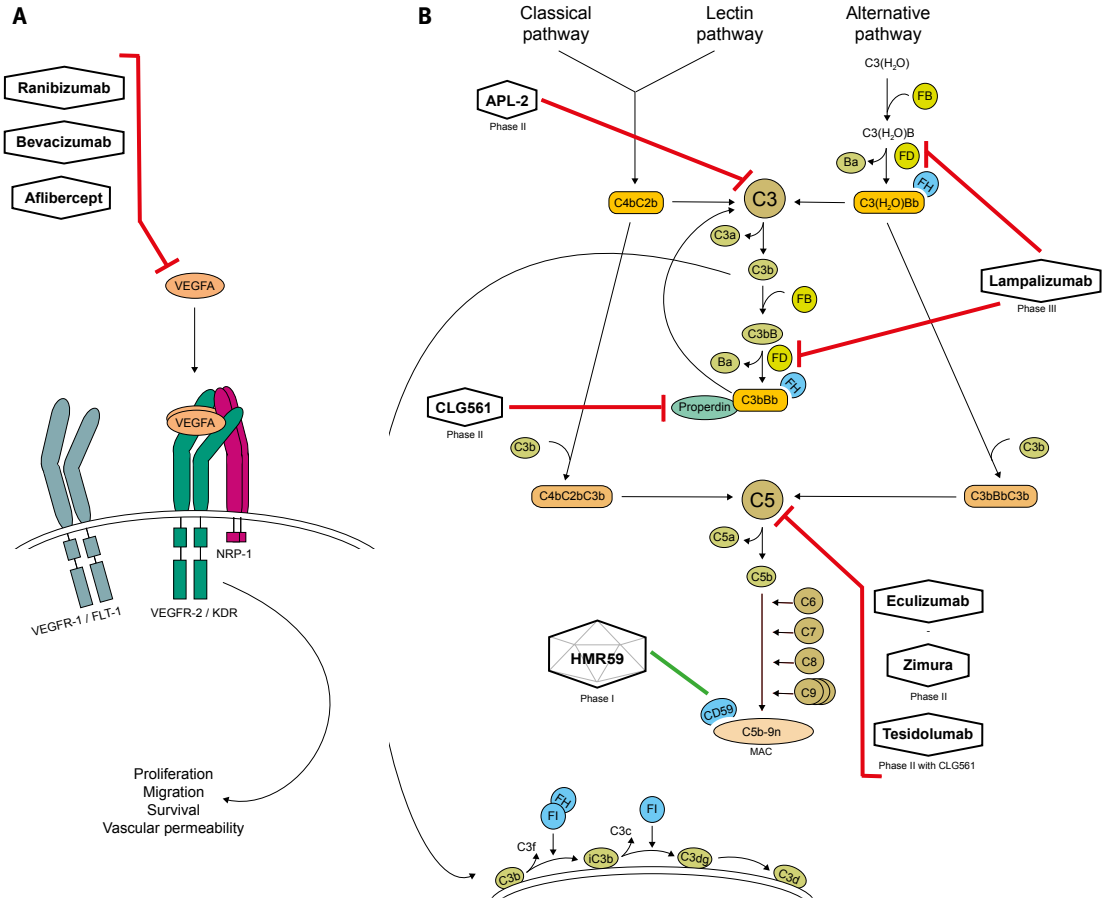


**FIGURE 4 The VEGFA and complement pathways are targets for therapy for AMD**

VEGF = Vascular endothelial growth factor, VEGFR = Vascular endothelial factor receptor, FLT-1 = Fms related tyrosine kinase 1, KDR = Kinase insert domain receptor, FB = Complement factor B, FD = Complement factor D, FH = Complement factor H, C3 to 9 = Complement component 3 to 9, MAC = Membrane attack complex, Complement factor I = FI.

C4bC2b, C3($H_2O$)Bb and C3bBb are C3 convertases, C4bC2bC3b and C3bBbC3b are C5 convertases. A red line towards the target indicates inhibition, whereas a green line indicates augmentation. **A)** Schematic representation of the VEGFA pathway in which therapies used for the treatment of neovascular AMD are depicted. VEGFA can bind either to VEGF receptor 1 (VEGFR-1 or FLT-1) or to VEGF receptor 2 (VEGFR-2 or KDR). Binding to VEGFR-1 can positively or negatively regulate VEGFR-2 activity. Upon VEGF binding to VEGFR-2, the receptor is phosphorylated, which in turn leads to the activation of signaling pathways for proliferation, migration and survival of endothelial cells, as well as to increased vascular permeability. **B)** Schematic representation of the complement cascade in which complement inhibiting therapies in clinical trials are depicted. The complement system can be initiated by three different pathways: the classical pathway , activated by antibody-antigen complexes, the lectin pathway, activated by lectin or ficolin binding to carbohydrates, and the alternative pathway (AP), which is constitutively activated at a low level due to the spontaneous hydrolysis of small amounts of C3 into $C3_{H2O}$. All three pathways lead to the formation of C3 convertases (C4bC2b for the CP and LP, and C3(H2O)Bb for the AP) that catalyze a proteolytic cleavage of the central component C3 into the potent anaphylatoxin C3a and the opsonization molecule C3b, which can be further cleaved into C3d on the cell surface. At the cell surface, factor D (FD) cleaves C3b-bound factor B (FB) to Ba and Bb, the latter forming the AP C3 convertase (C3bBb). This convertase will then cleave more C3, initiating an amplification loop. The alternative pathway can also be activated through properdin. Properdin recognizes pathogens or apoptosis markers and attracts C3b to the targeted cell surface generating and stabilizing new convertases. This amplification leads to a high amount of deposited C3b that, downstream, will bind the C3 convertases forming the C5 convertases (C4bC2bC3b for the CP and LP, and C3bBbC3b for the AP). The C5 convertases will then cleave complement C5 into C5a and C5b initiating a common terminal pathway. C5a is another potent anaphylatoxin and C5b can bind complement C6 and C7. This complex binds to the cell membrane and recruits C8 and several molecules of C9 to form the membrane-attack complex (MAC), a pore capable of osmotic cytolysis. In order to regulate the rapid activation and exponential effects of the complement cascade, soluble and membrane-bound proteins act as inhibitors. Factor H (FH) is the main regulator of the AP, inhibiting the system at different levels. It disassembles C3Bb convertases by competing with Bb. C3b can also be degraded by Factor I (FI), of which FH is a cofactor. FH recognizes the host cells by surface pattern binding, having an essential role in preventing self-attack. Another inhibitor of the system acting on the terminal pathway is MAC-inhibitory protein (MAC-IP, also known as CD59), which also recognizes host cells, and inhibits the formation of the MAC.

### 1.5.2 The complement system

The complement system is an integral part of our innate immunity and connects the innate to the adaptive immune response. It consists of a complex network of plasma and membrane-associated proteins that, in order to maintain homeostasis and protect against foreign intruders, are capable of orchestrating a rapid and efficient response via inflammation, opsonization and cytolysis (Figure 4B).[41]

The first evidence for the involvement of the complement system in AMD came from histological and molecular analysis of drusen components, which revealed components of the complement cascade such as C3a and vitronectin.[42-45] The first genetic studies corroborated this finding when the genetic variant p.Y402H in the *CFH* gene was found to be strongly associated with AMD.[18-20] Further genetic studies revealed AMD-associated variants in the *CFI*, *C2*/*CFB*, *C3*, *C9* and *VTN* complement genes (Table 1).[22] Finally, systemic measurements of complement activation fragments revealed elevated levels of Ba, Bb,

C3a, C3d, and C5a in AMD patients compared to controls, suggesting a chronic over-activation of the complement system.[46-52] A life-long aberrant activation of the complement system determined by genetic variants, together with an environment with high oxidative stress, may lead to a chronic level of inflammation in the aging retina, resulting in the degeneration of the macular structures.[53]

### 1.5.3 Lipid metabolism

The lipid metabolism is also known to be involved in AMD pathogenesis. Genetic variants in the hepatic lipase C (*LIPC*), cholesterylester transfer protein (*CETP*), apolipoprotein E (*APOE*), ATP-binding cassette subfamily A member 1 (*ABCA1*) lipid-related genes have been seen to modulate AMD disease risk (Table 1).[22,54] Moreover, variants in these genes and in the *VEGFA* gene have also been associated with high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol and/or triglyceride levels.[55] Other evidence supporting the relevance of the lipid metabolism in AMD is the accumulation of lipids in drusen, which represent around the 40% of their content.[8,56] Systemic levels of lipids, lipoproteins and fatty acids have been evaluated and, although no clear associations have been found for the majority of the measurements, several studies suggest that elevated HDL cholesterol levels might be associated with a higher risk for AMD development.[57]

### 1.5.4 Extracellular matrix remodeling

Extracellular matrix remodeling is regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metallopeptidases (TIMPs), and is known to be altered in AMD.[58] Aging leads to a thickening of the Bruch's membrane, altering its permeability and limiting the exchange of metabolites between the choroid and the RPE. This, in turn, contributes to drusen formation.[59] Moreover, MMP degradation of components of the capillary basement membrane for new vessel outgrowth takes place in the neovascularization process.[60] Further evidence of an altered extracellular matrix integrity in AMD comes from genetic studies, in which genetic variants in the tissue inhibitor of metalloproteinases 3 gene (*TIMP3*) and the metalloproteinase 9 (*MMP9*) have been associated with AMD. Additionally, genetic variants in fibrillin 2 (*FBN2*, involved in elastic fiber assembly), transforming growth factor beta receptor 1 (*TGFBR1*, involved in extracellular matrix production), collagen type VIII alpha 1 chain (*COL8A1*) and collagen type X alpha 1 chain (*COL10A1*) have been described to influence AMD disease risk (Table 1).[22,61-63]

## 1.6 Therapeutic options for AMD

### 1.6.1 Supplements

Dietary supplementation with vitamins and zinc is proven to reduce the risk of progression to advanced AMD. These supplements act against oxidative stress, which is thought to be

one of the drivers of AMD pathogenesis.[64,65] Oxidative stress refers to a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses. The retina is highly susceptible to oxidative stress due to the sunlight exposure, high oxygen consumption and high concentration of polyunsaturated fatty acids. Moreover, oxidative stress increases with age and is associated with smoking, another AMD risk factor.[65] The notion that oxidative stress may play an important role in AMD development and progression led to development of the Age-Related Eye Disease Study (AREDS) clinical trial that evaluated the effect of high doses of vitamin C, vitamin E, beta-carotene and zinc on AMD progression. In 2001, the AREDS trial concluded that patients with intermediate AMD in at least one eye receiving this formulation reduced their risk of progression to advanced AMD by 25% at 5 years. Moreover, in those patients, a 19% reduction in moderate vision loss was additionally observed.[66] An AREDS2 supplementation trial followed in 2013, describing an improved formula with lutein and zeaxanthin substituting beta-carotene. This formula showed the same effects, but is preferred as beta-carotene conferred risk for lung cancer in former smokers.[67] Clinicians have rapidly adopted the AREDS recommendations and the oral use of antioxidants combined with zinc is currently prescribed for intermediate or unilateral advanced AMD.

Supplements have been proven to reduce AMD progression in the overall patient population. However, the effect of these supplements is variable and the protective effects on progression to advanced AMD range from an OR of 0.47 to an OR of 0.91.[66] The identification of factors involved in this variability could lead to an improvement in the treatment strategy and therefore on its effects. Indeed, the patient's genetic background may be one of the factors underlying these differences, as may influence the effects of the individual components of the formula. Therefore, it has been hypothesized that the effectiveness of the treatment with dietary supplements in reducing disease progression may improve tailoring the prescription to each individual genetic makeup.

### 1.6.2 Anti-VEGF antibodies for neovascular AMD

The discovery of VEGF as the main driver of pathological neovascularization, lead to the development of anti-VEGF therapies for cancer, and soon also for the CNV form of AMD.[68] Nowadays, the gold-standard treatment for CNV consists of intravitreal injections of anti-VEGF antibodies. Anti-VEGF antibodies for CNV treatment include ranibizumab (Lucentis; Novartis, Basel, Switzerland, and Genentech Inc., South San Francisco, USA), bevacizumab (Avastin, Genentech, South San Francisco, USA), and aflibercept (EYLEA, Regeneron Pharmaceutical Inc, Tarrytown, USA, Figure 4A).

Ranibizumab is a recombinant monoclonal antibody fragment against all VEGFA isoforms, which was approved by the United States Food and Drug Administration (FDA) for the treatment of CNV in 2006 after showing efficacy in the MARINA and ANCHOR trials.[69,70] Bevacizumab is a full-length monoclonal antibody, also against all VEGFA isoforms. This drug has been approved by the FDA for the treatment of several cancer types, however, it is administered off-label for the treatment of CNV. The Comparison of AMD Treatments

Trials (CATT) and the Inhibition of VEGF in Age-related choroidal Neovascularisation (IVAN) clinical trials demonstrated similar outcomes after bevacizumab treatment compared to ranibizumab.[71-74] The anti-VEGF agents ranibizumab and bevacizumab are administred as a loading dose of three monthly injections and the follow-up treatment differs among clinics. The most used strategies are pro re nata (PRN) and treat-and-extend (TAE). In the PRN, patients are followed monthly but only injected when needed, whereas in the TAE protocol, patients receive an injection every visit, but the time between visits is extended or shortened depending on the disease progression.[75,76] Finally, aflibercept consists of key domains of VEGFR1 and VEGFR2 fused with a portion of human antibody. Unlike ranibizumab and bevacizumab, aflibercept binds VEGFA, VEGFB and placental growth factor (PGF), acting as a VEGF decoy-receptor. Aflibercept was granted the FDA approval in 2011, and has similar efficacy outcomes as ranibizumab, however, a less frequent dosing is needed, once every two months instead of monthly (Figure 4A).[77,78]

The use of anti-VEGF drugs to treat CNV has significantly changed the prognosis of the disease and has led to significant improvements in visual acuity. Nevertheless, a more detailed analysis of individual patient outcomes shows that not all patients benefit equally from this therapy. Vision remains stable or improves in approximately 80% of the patients, but 20% of treated patients continue to lose vision despite treatment.[69,70] Along the same line, anatomical changes in the retina after treatment, reflecting fluid clearance, are also variable among patients.[71]

Understanding the reasons underlying this variability in treatment outcome after anti-VEGF injections can help improve treatment strategies, but also would allow early identification of poor responders, and would enable individual treatment optimization. Clinical and epidemiological factors that have repeatedly been associated with worse treatment outcome include baseline parameters such as older age, larger neovascular lesion, larger retinal tissue thickness and lower visual acuity.[79] These factors are highly correlated and indicative of longer disease duration, highlighting the importance of initiating treatment in an early phase. Nevertheless, these factors cannot fully explain the wide range in treatment outcomes.[80] Due to the highly heritable nature of AMD, it has been hypothesized that genetic factors may influence treatment outcome. Genetic markers are independent of disease duration and therefore may explain treatment outcome variability.

### 1.6.3 Under development: Complement inhibiting therapies for geographic atrophy in AMD

Anti-VEGF treatment is only applicable to CNV, which affects only half of the advanced AMD patients. For the other half, who suffer from GA, no treatment is available yet. A prime candidate target in AMD is the complement system, and several therapies aiming to inhibit complement activity are being developed (Figure 4B).[81] These therapies aim to slow down disease progression and to prevent the development of GA, but may also be useful for CNV patients in combination with anti-VEGF drugs.

Complement-inhibiting therapies that have gone through clinical trials include APL-2, Lampalizumab, Eculizumab, Tesidolumab, CLG561, Zimura and AAVCAGsCD59 (Figure 4B). These drugs inhibit the complement system at different levels of the proteolytic cascade.

APL-2 (Apellis Pharmaceuticals, Crestwood, USA), a reformulated version of POT-4, is a cyclic peptide inhibitor of complement component 3 (C3). This drug is currently being tested in a phase II clinical trial (https://clinicaltrials.gov, NCT02503332). In a press release, Apellis Pharmaceuticals communicated that this clinical trial has resulted in a significant reduction in the rate of geographic lesion growth over 12 months.[82] Lampalizumab (Genentech Inc., South San Francisco, CA) is an antigen-binding fragment of a humanized monoclonal antibody that targets complement factor D (FD). The phase II clinical trial for Lampalizumab (MAHALO) has been completed, and yielded promising results with a 20% reduction in atrophy area progression at month 18 for the monthly treated group compared to placebo.[83] Lampalizumab is currently being evaluated in two phase III clinical trials (SPECTRI and CHROMA, NCT02247531 and NCT02247479 respectively). Recently, Genentech revealed in a press release that SPECTRI did not meet its primary endpoint of reducing mean change in GA lesion area, and that they are expecting the results of CHROMA to be evaluated in November 2017.[84] Eculizumab (Soliris, Alexion Pharmaceuticals, New Haven, USA) is a humanized monoclonal antibody targeting complement 5 (C5). Eculizumab has been approved for the treatment of paroxysmal nocturnal hemoglobinuria. In a phase II clinical trial in AMD (COMPLETE, NCT00935883), systemically administered eculizumab was well-tolerated, however, it did not decrease the growth rate of geographic atrophy significantly.[85] Another drug targeting C5 is Zimura (Ophtotech, USA), a chemically synthesized aptamer. This drug is currently in phase II/III trial (NCT02686658). Tesidolumab (LFG316, Novartis, Basel, Switzerland/MorphoSys, Planegg, Germany) is a human monoclonal antibody also targeting C5. The phase II clinical trial has been completed (NCT015275000); however, the results have not yet been published. Currently, another phase II trial is ongoing which analyzes CLG561 (Novartis, Basel, Switzerland), a human antibody Fab that neutralizes properdin, as monotherapy or in combination with tesidolumab (NCT02515942). Finally, the first gene therapy tested for GA treatment is HMR59 (AAVCAGsCD59, Hemera Biosciences Inc., Newton, USA), and its safety is currently being evaluated in a phase I clinical trial (NCT03144999). This therapy consists of a single injection of an adeno-associated virus that transfects the retinal cells, leading to expression of a soluble form of MAC-inhibitory protein (MAC-IP), also named CD59 (Figure 4B).

These therapies may not work equally in all AMD patients, and may be more effective in AMD patients in which the complement system is most over-activated. Identification of genetic variants as biomarkers for complement activation in AMD may be useful for selecting patients for complement inhibiting therapies.

# 2 Genetic association studies for understanding complex traits

Genetic association studies are used to study multifactorial traits and to assess whether one or more genetic variants co-occur with a trait more often than would be expected by chance.[86] Genetic association studies allow the identification of risk factors and can give insights into the biological mechanisms underlying a trait.[18,87] An association between a genetic variant and a trait indicates a direct or indirect relationship. In the latter case, the associated variant is a marker for the causal variant as they are, to some degree, co-inherited. The indirect associations are usually weaker than the direct associations, however, can be useful to identify causal genes.[88]

There are two different strategies for performing association studies: the (targeted) candidate gene approach, and the (untargeted) genome-wide approach. In the candidate gene approach, genetic variants within a pre-specified gene or group of genes are analyzed. The variants chosen for analysis can be also pre-specified. This type of analysis is based on a hypothesis, and therefore, based on *a priori* knowledge of the gene's function and the investigated trait.[89] In contrast, genome-wide association studies (GWAS) explore variants distributed genome-wide and therefore entail a hypothesis-free approach.[90] GWAS typically analyze common variants, however, analysis of single rare variants in the same manner has little statistical power due to the low allele frequencies. In order to overcome this limitation, rare variants can be grouped by gene, and the burden of rare genetic variation in each gene can be evaluated, increasing in this way the statistical power.[91] One of the most important considerations in genetic association analysis is to avoid confounding due to population stratification; differences in allele frequencies across populations and differences in phenotype prevalence can lead to spurious results.[90]

# 3 Pharmacogenetics and precision medicine for AMD

## 3.1 Potential impact of pharmacogenetics

Precision medicine aims to improve healthcare through individualized selection of treatment options, taking into account each patient's characteristics and individual needs. The field of precision medicine has moved forward rapidly in the last decades thanks to association studies, which have identified genetic markers that predict response to treatment in many different diseases.[92] Genetic screening prior to treatment is now increasingly being implemented in the healthcare system.[93-95] A prime example is the oncology field, where, for instance, genetic variants in the *DPYD* gene are highly recommended to be screened to

avoid toxicity from fluoropyrimidine drugs.[96] Other examples include the anti-coagulant warfarin, for which a GWAS successfully identified genetic variants with an effect on the maintenance dose,[97] and genotype-guided prescription has been established to improve safety and effectiveness, and to reduce healthcare costs.[98-100]

## 3.2 First steps in the use of genetic biomarkers for precision medicine for AMD: where are we now and what is needed?

The use of dietary supplements has been proven to be effective in reducing the progression to late AMD and several groups have investigated whether the response is variable depending on the *CFH* rs1061170 and *ARMS2* rs10490924 genotypes. At the beginning of this thesis, there was a discrepancy in the results of such studies and therefore not a clear biomarker.[101-103] More comprehensive genetic studies are needed but it has not been the purpose of this thesis, however, the studies on this topic have been analyzed and compared in the discussion section (chapter 4, section 1.1).

The only available therapy to treat AMD symptoms is anti-VEGF injections for the CNV form, however, as mentioned in section 1.6.2, treatment response is highly variable among CNV patients. Identification of pharmacogenetic associations for AMD is highly relevant as they may help understand the causes underlying this variability, can be used for anti-VEGF therapy planning and can help selecting patients for new therapy options. Before the start of this thesis work, several genetic variants such as rs1061170 in *CFH*, rs10490924 in *ARMS2*, rs699947 in *VEGFA* and rs4576072 in *KDR* had been identified in candidate gene studies, which suggested a role for genetic variation in treatment response, however, a systematic analysis of the 52 AMD-associated variants had not been performed yet.[104-127] One GWAS had been carried out which did not identify any statistically significant association, however, this study was very limited in sample size (n=65). Additionally, the role of rare genetic variants, which may have stronger effects on treatment response, had not been explored yet.[128] As a consequence, more comprehensive association analyses were needed in order to find genetic biomarkers for treatment response to anti-VEGF therapy in CNV.

For the treatment of advanced GA, complement inhibiting therapies are currently under development, but these will most probably not be equally effective in all AMD patients. Genetic variants that reflect complement activation in AMD patients could be used as biomarkers for patient selection in complement inhibiting therapies. At the start of this thesis work, three studies had analyzed the association of AMD variants with systemic complement activation measurements.[48,50,52] Those studies had successfully identify variants in the *CFH*, *CFB*/*C2*, *C3* and *ARMS2* genes associated with systemic measurements of complement activation such as C3a, C5a, Ba and the C3d/C3 ratio. These results suggested that genetic variants could be useful biomarkers of complement activation in AMD. However, a comprehensive analysis of the genetic influence on complement activation had not yet been performed, nor had the 52 AMD-associated variants been comprehensively analyzed yet.

# 4 Aims and outline of this thesis

Taking first steps towards a personalized treatment for AMD patients, we sought to identify genetic factors associated with response to anti-VEGF therapy, and to identify genetic variants associated with complement activation in AMD.

**1 Genetic factors associated with response to anti-VEGF therapy in AMD**

Chapter 2 describes the different approaches we have taken for the identification of genetic variants associated with treatment response to anti-VEGF therapy in CNV. Chapter 2.1 describes a candidate gene study of the *NRP1* gene, which identified rs2070296 as a new genetic variant associated with treatment response. Taking a hypothesis-free approach as a next step, we performed a GWAS study using pooled DNA in chapter 2.2. This study was performed in collaboration with the group of Prof. Baird from the Centre for Eye Research Australia, and lead to the identification of rs4910623 in the *OR52B4* gene as a new SNP associated with treatment response. In chapter 2.3, leading a large collaborative study, we performed a large GWAS for treatment response. In this study we also evaluated previously reported associations and the 52 AMD-associated variants, which were not found to influence treatment outcome in our study. Additionally, we performed genome-wide gene-based tests of rare variants and identified that rare variation in the *C10ORF88* and *UNC93B1* genes seems to have a profound impact on treatment outcome.

**2 Genetic factors associated with complement activation in AMD**

Chapter 3 describes studies on the complement system, which can be considered a first step in order to identify the most suitable patients for therapies targeting the complement system. In chapter 3.1, we performed a GWAS on systemic complement activation levels in order to identify genetic variants that associate with complement activation levels. We identified two independent signals at the *CFH*/*CFHR* locus. The top SNPs for these loci were rs3753396 located in *CFH* and rs6685931 located in the complement factor H related 4 (*CFHR4*) gene. The results of this study lead to further investigation of the role of FHR-4 in AMD, which is described in chapter 3.2. The biochemical studies together with genetic analyses presented in this chapter support that FHR-4 is a new complement component involved in AMD, and represents a promising new target for treatment.

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# 2

# Genetic biomarkers for anti-VEGF therapy

# 2.1

# *A genetic variant in NRP1 is associated with worse response to ranibizumab treatment in neovascular age-related macular degeneration*

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# Abstract

**Objective**: The aim of the study was to investigate the role of single nucleotide polymorphisms (SNPs) located in the neuropilin-1 (*NRP1*) gene in treatment response to antivascular endothelial growth factor (VEGF) therapy for neovascular age-related macular degeneration (nvAMD).

**Methods**: Four SNPs in the *NRP1* gene (rs2229935, rs2247383, rs2070296 and rs2804495) were genotyped in a study cohort of 377 nvAMD patients who received the loading dose of three monthly ranibizumab injections. Treatment response was assessed as the change in visual acuity after three monthly loading injections compared with baseline.

**Results**: SNP rs2070296 was associated with change in visual acuity after 3 months of treatment. Patients carrying the GA or AA genotypes performed significantly worse than individuals carrying the GG genotype ($P$=0.01). A cumulative effect of rs2070296 in the NRP1 gene and rs4576072 located in the VEGF receptor 2 (*VEGFR2* or *KDR*) gene, previously associated with treatment response, was observed. Patients carrying two risk alleles performed significantly worse than patients carrying zero or one risk allele ($P$=0.03) and patients with more than two risk alleles responded even worse to the therapy ($P$=3x10$^{-3}$). The combined effect of these two SNPs on the response was also seen after 6 and 12 months of treatment.

**Conclusion**: This study suggests that genetic variation in *NRP1*, a key molecule in VEGFA-driven neovascularization, influences treatment response to ranibizumab in nvAMD patients. The results of this study may be used to generate prediction models for treatment response, which in the future may help tailor medical care to individual needs.

# Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the western world[1]. The neovascular, or wet, form of AMD (nvAMD) is the most aggressive, being responsible for around 90% of the vision loss caused by the disease[2].

The first choice therapy for nvAMD consists of intravitreal injections of antivascular endothelial growth factor (VEGF) drugs. Although this treatment has dramatically changed the prognosis of the disease with a significant mean improvement in visual acuity (VA)[3], a high variability in response rates has been described. Approximately 10% of the treated patients do not respond to anti-VEGF therapy and still lose more than 15 Early Treatment Diabetic Retinopathy Study (ETDRS) letters 2 years after the start of treatment[3,4], which is comparable to the natural course of the disease[5].

To date, several studies have suggested that genetic variants can influence this variability in treatment response[6-16]. These studies have mainly focused on single nucleotide polymorphisms (SNPs) located in AMD-associated loci, but common variants in VEGF family members, cytokines, and proteins involved in development and maintenance of the retinal vasculature have also been explored. Not all studies showed consistent results[9,16], however, due to a high variability in study designs it is difficult to reliably compare the outcomes of these studies. Therefore, the relevance and basis of the genetic component of this diverse response to treatment still needs to be elucidated.

Recently, two SNPs in the VEGF receptor 2 (*VEGFR2* or *KDR*) gene, which encodes the main receptor of VEGFA on vascular endothelial cells[17], have been associated with better anti-VEGF response rates[12]. Consequently, other molecules involved in this pathway are also potential candidates to influence treatment response. Neuropilin-1 (NRP1) is a coreceptor of VEGFA that binds to the predominant isoform, $VEGFA_{165}$[18], and forms a complex with VEGFR2, which enhances the transduction of downstream signaling[19-22]. Recent studies have implicated NRP1 signaling pathways in pathological neovascularization of the retina[23] and NRP1 has been described to be involved in VEGFA-mediated vascular leakage[19]. Indeed, NRP1 has been shown to affect the evolution of the choroidal neovascularization in AMD[24] and has been proposed as a new target molecule for AMD treatment[25]. Moreover, NRP1 seems to play a role in the cancer prognosis when treated with anti-VEGF compounds[26], which makes this molecule a compelling candidate for being involved in response variation.

This study aimed to determine whether genetic variants in the *NRP1* gene influence treatment response to anti-VEGF therapy in patients with nvAMD.

# Patients and methods

## Study population

The study cohort comprised 377 eyes of 377 treatment-naive patients aged 50 years or older with active choroidal neovascularization secondary to AMD. A total of 145 patients were treated at the Department of Ophthalmology of the Radboud University Medical Center, Nijmegen, the Netherlands, 182 at the University of Cologne, Germany; and the remaining 50 patients at the McGill University Health Center, Montreal, Canada. The patients from the German and Dutch clinics were enrolled between 2008 and 2010 in the European Genetic Database (EUGENDA), a multicenter database for the clinical and molecular analysis of AMD.

The study was performed in accordance with the tenets of the Declaration of Helsinki (7$^{th}$ revision). Approval of the local ethics committee was obtained for all three centers and written informed consent was acquired from all participants.

The diagnosis of active nvAMD was determined by retinal specialists based on ophthalmic examination, spectral-domain optical coherence tomography (OCT) (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) or fluorescein angiography (FA) (Spectralis HRA+OCT, Heidelberg Engineering; or Imagenet, Topcon Corporation, Tokyo, Japan). Exclusion criteria included any previous ophthalmic surgery, except for cataract removal, and retinal disorders other than AMD. If both eyes received treatment, the first eye to receive treatment was chosen as the study eye. If treatment started simultaneously, the study eye was chosen randomly.

All patients were treated between 2007 and 2009 with three consecutive monthly intravitreal injections of 0.5 mg ranibizumab (Lucentis; Novartis Pharmaceuticals UK Limited, Surrey, UK). VA was assessed in all cases before treatment (baseline) and after the three loading monthly injections. After the loading dose, patients were followed up on a monthly basis and treated on a pro re nata regimen at the clinics of Nijmegen and Cologne. At the clinic of Montreal, the patients were further managed through a treat-and-extend regimen. OCT, best-corrected VA, fundus examination and FA were used alone or in combination to evaluate the effectiveness of the treatment. Recurrence or persistence of the choroidal neovascularization was defined as fluid seen by OCT, loss of VA of five ETDRS letters or more, leakage seen on FA, or new macular hemorrhage or fluid. In case of persistence or recurrence of the choroidal neovascularization, patients received three consecutive monthly ranibizumab injections. If available, VA was collected after 6 and 12 months of treatment. For 304 patients, Snellen VA measurements were collected retrospectively and 73 patients were followed up prospectively using ETDRS VA. Treatment response was defined as the change in VA after the three first months of treatment compared with baseline. Longterm treatment response was defined as the change in VA after 6 and 12 months of treatment. Age at first ranibizumab injection, sex and other baseline variables were collected using questionnaires or retrieved from the patient files.

## Genotyping

The SNPs rs2229935, rs2247383, rs2070296 and rs2804495 were selected from the major haploblocks of the *NRP1* gene for genotyping (see Table, Supplemental digital content 1, http://links.lww.com/FPC/A912, which details the chromosomal location of the SNPs). Two SNPs, rs2070296 (p.Ala179=) and rs2229935 (p.Tyr422=), were located in the coding region of *NRP1*.

Genotyping of the SNPs was performed using competitive allele-specific KASP genotyping chemistry (LGC, Hoddesdon, UK). Primers and probes were developed by LGC, (see Table, Supplemental digital content 1, http://links.lww.com/FPC/A912, which describes the probes used). Quality control of the genotyping assays was assessed using duplicate DNA samples in each run, achieving a concordance of 100% of the results.

Sanger sequencing of exon 4 of the *NRP1* gene (*NM_003873.5*) was performed in 11 patients for which genotyping by KASPar of SNP rs2070296 was not successful. Primers were designed using Primer3 software[27] (see Table, Supplemental digital content 1, http://links.lww.com/FPC/A912, which describes the primers used). PCR was performed, and the amplicons were sequenced using an automated sequencer (BigDye Terminator, version 3, 3730 DNA analyzer; Applied Biosystems; Waltham, Massachusetts, USA). Sequences were assembled and analyzed using ContigExpress (Vector NTI Advance, version 11.0, Life Technologies).

## Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, New York, USA). ETDRS and Snellen VA records were converted to the logarithm of minimal angle of resolution (logMAR) for the purpose of statistical analysis. Change in VA after 3, 6 and 12 months was calculated as the difference between VA at baseline and VA at the different time points.

Deviation of the genotype frequencies from those expected under Hardy-Weinberg equilibrium was assessed by means of a $\chi^2$ test. To determine the influence of the baseline variables on the change in VA after 3 months Spearman's correlation was used for the continuous variables, and Kruskal-Wallis or Mann-Whitney U tests were performed for the categorical variables.

The association of the different SNPs with the change in VA after 3, 6 and 12 months was assessed using Mann-Whitney U tests. Bonferroni's procedure was applied to correct for four tests ($P \leq 0.01$ were considered statistically significant).

To analyze the combined effect of *NRP1* rs2070296 and *KDR* rs4576072 on the change in VA after 3, 6 and 12 months, patients were combined into three groups of approximately

equal size (carriers of less than two risk alleles, of two risk alleles, or of more than two risk alleles), and a Mann-Whitney U test was performed. Only the patients who were successfully genotyped for rs4576072 in a previous study[12] were included in the analysis (n=353). The rs4576072 major allele (T) has been reported to lead to a worse response to therapy[12], therefore, this allele was considered the risk allele.

TABLE 1 **Characteristics of the study cohort.**

| | | |
|---|---|---|
| **Demographics** | | |
| | Age at first injection (years), mean (SD) | 77.11 (7.46) |
| | Female gender, n (%) | 215 (57.0) |
| **Disease history** | | |
| | Hypertension (n=259)[a], n (%)[b] | 154 (59.5) |
| | Diabetes mellitus (n=259)[a], n (%)[b] | 47 (18.1) |
| **Other environmental factors** | | |
| | BMI (kg/m$^2$) (n=258)[a], median (quartiles) | 25.39 (23.52 - 28.49) |
| **Ophthalmological details** | | |
| | Baseline VA (logMAR), median (quartiles) | 0.543 (0.398 - 1.000) |
| | Equivalent baseline VA (ETDRS letters)[c], median (quartiles) | 57.9 (35.0 - 65.1) |
| | Change in VA after 3 months (logMAR)[d], median (quartiles) | 0.097 (0.000 - 0.259) |
| | Equivalent change in VA after 3 months (ETDRS letters)[c,d], median (quartiles) | 4.8 (0.0 - 12.2) |
| | Change in VA after 6 months (logMAR)[d], median (quartiles) (n=262) | 0.090 (-0.097 - 0.223) |
| | Equivalent change in VA after 6 months (ETDRS letters)[c,d], median (quartiles) | 4.5 (-4.9 - 11.2) |
| | Change in VA after 12 months (logMAR)[d], median (quartiles) (n=240) | 0.040 (-0.192 - 0.204) |
| | Equivalent change in VA after 12 months (ETDRS letters)[c,d], median (quartiles) | 2 (-9.6 - 10.2) |
| | Type of CNV (n=335)[a] | |
| | Occult with no classic, n (%)[b] | 199 (59.4) |
| | RAP, n (%)[b] | 21 (6.3) |
| | Minimally classic, n (%)[b] | 42 (12.5) |
| | Predominantly classic, n (%)[b] | 73 (21.8) |
| | Lesion size (DA) (n=285)[a] | |
| | <2, n (%)[b] | 92 (32.3) |
| | 2-4, n (%)[b] | 91 (31.9) |
| | 4-6, n (%)[b] | 43 (15.1) |
| | >6, n (%)[b] | 59 (20.7) |

SD, standard deviation; n, number of patients; VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution; ETDRS, Early Treatment Diabetic Retinopathy Study; CNV, choroidal neovascularization; RAP, retinal angiomatous proliferation; DA, disk areas.

[a] For the remaining patients no data were available.

[b] Valid percentage.

[c] ETDRS letters equivalents were calculated in the following manner: ETDRS letters = 85 – logMAR/0.02 for logMAR values.

[d] Change in VA after 3, 6 and 12 months was calculated in the following manner: VA prior to treatment - VA after 3, 6 or 12 months of treatment.

TABLE 2 **Association of genotypes in *NRP1* with response to ranibizumab treatment.**

| SNP | N (%) | ΔVA after 3 months (logMAR), median (quartiles)[a] | *P*-value[b] |
|---|---|---|---|
| rs2229935 | | | |
| **CC** | 203 (57.2) | 0.100 (0.000 - 0.301) | Reference |
| **CT** | 132 (37.2) | 0.079 (0.000 - 0.198) | 0.12 |
| **TT** | 20 (5.6) | 0.085 (-0.075 - 0.273) | 0.50 |
| **CT or TT** | 152 (42.8) | 0.079 (0.000 - 0.198) | 0.11 |
| rs2247383 | | | |
| **CC** | 123 (35.2) | 0.097 (-0.077 - 0.273) | Reference |
| **CT** | 169 (48.4) | 0.097 (0.000 - 0.242) | 0.94 |
| **TT** | 57 (16.3) | 0.090 (-0.064 - 0.238) | 0.69 |
| **CT or TT** | 226 (64.8) | 0.097 (0.000 - 0.242) | 0.84 |
| rs2070296 | | | |
| **GG** | 270 (71.6) | 0.100 (0.000 - 0.287) | Reference |
| **GA** | 98 (26.0) | 0.079 (-0.097 - 0.195) | 0.04 |
| **AA** | 9 (2.4) | 0.000 (-0.097 - 0.040) | 0.04 |
| **GA or AA** | 107 (28.4) | 0.040 (-0.097 - 0.184) | 0.01 |
| rs2804495 | | | |
| **TT** | 167 (49.1) | 0.098 (0.000 - 0.240) | Reference |
| **TG** | 147 (43.2) | 0.097 (0.000 - 0.273) | 0.74 |
| **GG** | 26 (7.6) | 0.138 (-0.088 - 0.300) | 0.84 |
| **TG or GG** | 173 (50.9) | 0.097 (0.000 - 0.279) | 0.72 |

SNP, single nucleotide polymorphism; N, number; VA, visual acuity; logMAR=logarithm of the Minimum Angle of Resolution.

[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment.

[b] *P*-values were calculated using Mann-Whitney U tests.

# Results

Demographics and ophthalmological details of the patients are described in Table 1. Older age at first injection ($P$=0.01), having a better baseline VA ($P<10^{-3}$) and having diabetes mellitus ($P$=0.02) were associated with worse response after 3 months of treatment (see Table, Supplemental digital content 2, http://links.lww.com/FPC/A913, which describes the results of the association tests). The type of choroidal neovascularization showed a trend towards statistical significance ($P$=0.06). These baseline variables were not associated with the SNPs of interest ($P>0.05$, lowest $P$=0.22) (see Table, Supplemental digital content 3, http://links.lww.com/FPC/A914, which describes the results of the association tests).

Over 90% of patients were successfully genotyped for SNPs rs2229935, rs2247383, rs2070296 and rs2804495 (Table 2). None of the SNPs showed deviations from Hardy-

Weinberg equilibrium in the study cohort (*P*=0.81, 0.93, 0.98 and 0.41 respectively). The GA or AA genotypes of SNP rs2070296 were found to be associated with a significantly reduced improvement in VA after 3 months (*P*=0.01) compared with the GG genotype, showing a linear trend for the three genotype groups (Fig. 1a). The SNPs rs2229935, rs2248383 and rs2804495 were not found to be associated with treatment response (Table 2).

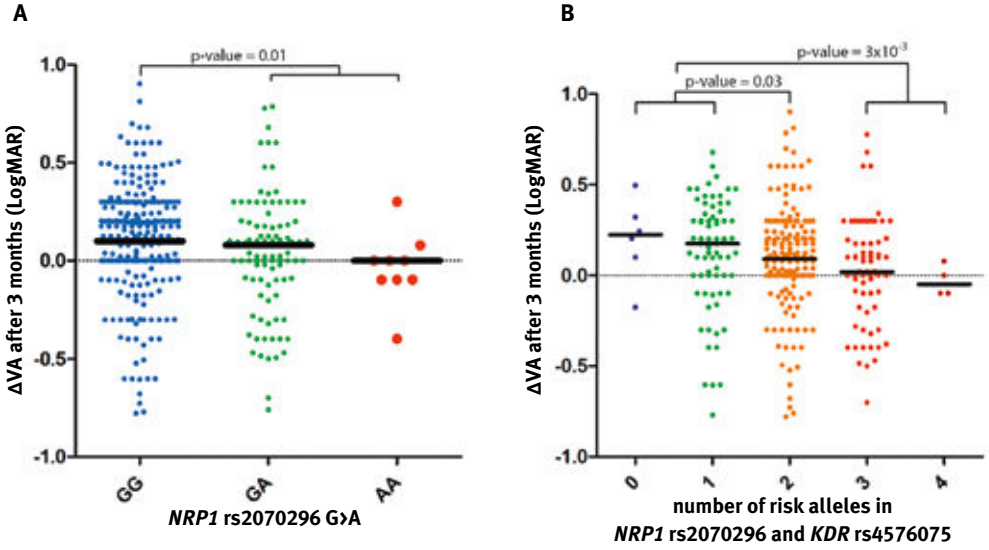


FIGURE 1 **Effect of genetic variants in *NRP1* and *KDR* on response to ranibizumab treatment in nvAMD.**

Change in visual acuity after 3 months of ranibizumab treatment stratified by *NRP1* rs2070296 genotype. **(A)** Change in visual acuity after 3 months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 **(B)** and *KDR* rs4576075 (T).
The median change in visual acuity for each group is depicted in both figures.
logMAR, logarithm of minimal angle of resolution; nvAMD, neovascular age-related macular degeneration; VA, visual acuity.

A combined analysis of *NRP1* rs2070296 and the previously associated SNP rs4576072 in *KDR*[12] revealed a decrease in the change in VA after 3 months depending on the number of risk alleles (Fig. 1b). Patients who carried two risk alleles responded significantly worse to therapy than did carriers of one or zero allele (median of 0.090 logMAR or 4.5 ETDRS letters gained vs. 0.196 logMAR or 10 ETDRS letters gained, *P*=0.03), and carriers of more than 2 alleles had even worse response rates (median of 0.020 logMAR or 1 ETDRS letter gained, $P=3x10^{-3}$) (Fig. 1B and Table 3).

Besides the variability in treatment regimens after the first loading injections, we evaluated whether the effect of rs2072096 in *NRP1* remained significant after 6 and 12 months of treatment. This SNP was not associated with the change in VA after 6 and 12 months

(Table 4). However, the combined effect of this SNP in *NRP1* and rs4576072 in the *KDR* gene did influence long term response (Fig. 2 and Table 5).

TABLE 3 **Combined effect of the risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576072 (T) on response to ranibizumab treatment.**

| Number of risk alleles | N (%) | ΔVA after 3 months (logMAR), median (quartiles)[a] | *P*-value[b] |
|---|---|---|---|
| ‹2 | 79 (22.4) | 0.196 (0.000 – 0.321) | Reference |
| 2 | 201 (56.9) | 0.090 (0.000 – 0.204) | 0.03 |
| ›2 | 73 (20.7) | 0.020 (-0.097 – 0.180) | $3x10^{-3}$ |

N, number of patients; VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution.
[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment.
[b] *P*-values were calculated using Mann-Whitney U tests.

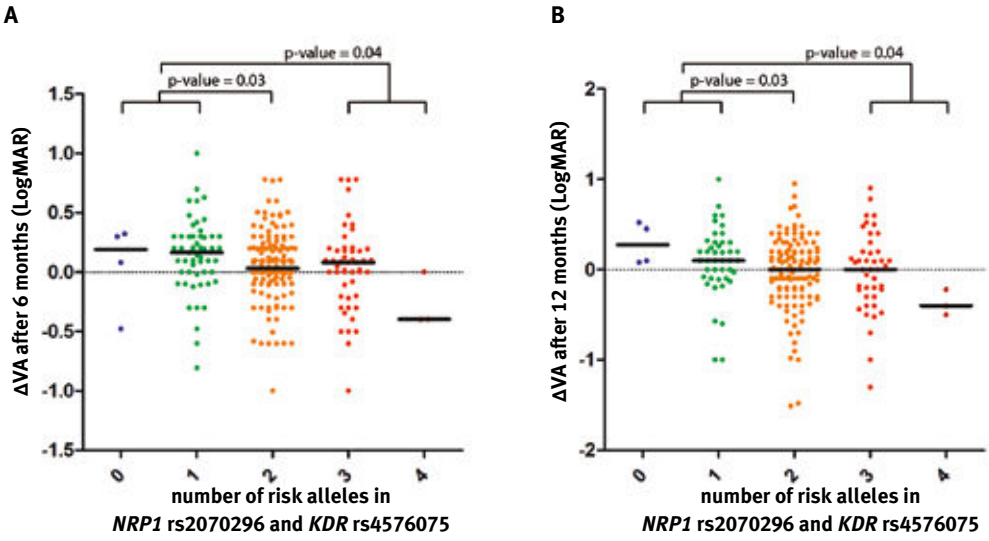


FIGURE 2 **Effect of genetic variants in *NRP1* and *KDR* on long term response to ranibizumab treatment in nvAMD.**

**(A)** Change in visual acuity after 6 months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T). **(B)** Change in visual acuity after 12 months of ranibizumab treatment stratified by the number of risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576075 (T).
The median change in visual acuity for each group is depicted in both figures.
logMAR, logarithm of minimal angle of resolution; nvAMD, neovascular age-related macular degeneration; VA, visual acuity.

TABLE 4 **Association of genotypes in rs2070296 *NRP1* with long term response to ranibizumab treatment.**

| | ΔVA after 6 months (logMAR) | | | ΔVA after 12 months (logMAR) | | |
|---|---|---|---|---|---|---|
| **SNP** | **N (%)** | **Median (quartiles)[a]** | ***P*-value[b]** | **N (%)** | **Median (quartiles)[a]** | ***P*-value[b]** |
| **rs2070296** | | | | | | |
| **GG** | 188 (71.8) | 0.097 (-0.092 - 0.257) | Reference | 180 (75.0) | 0.078 (-0.120 - 0.218) | Reference |
| **GA** | 67 (25.6) | 0.040 (-0.080 - 0.194) | 0.38 | 53 (22.1) | 0.020 (-0.222 - 0.252) | 0.64 |
| **AA** | 7 (2.7) | 0.000 (-0.398 - 0.090) | 0.21 | 7 (2.9) | -0.097 (-0.400 - 0.100) | 0.33 |
| **GA or AA** | 74 (28.2) | 0.020 (-0.098 - 0.194) | 0.25 | 60 (25.0) | 0.010 (-0.222 - 0.203) | 0.46 |

VA, visual acuity; logMAR = logarithm of the Minimum Angle of Resolution; SNP, single nucleotide polymorphism; N, number.

[a] Change in VA after 6 or 12 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 6 or 12 months of treatment.

[b] *P*-values were calculated using Mann-Whitney U tests.

TABLE 5 **Combined effect of the risk alleles in *NRP1* rs2070296 (A) and *KDR* rs4576072 (T) on long term response to ranibizumab treatment.**

| Number of risk alleles | N (%) | ΔVA after 6 months (logMAR), median (quartiles)[a] | ***P*-value[b]** | N (%) | ΔVA after 12 months (logMAR), median (quartiles)[a] | ***P*-value[b]** |
|---|---|---|---|---|---|---|
| <2 | 57 (22.7) | 0.164 (-0.010 - 0.301) | Reference | 47 (20.4) | 0.100 (-0.079 - 0.301) | Reference |
| 2 | 140 (55.8) | 0.031 (-0.097 – 0.203) | 0.03 | 137 (59.7) | 0.000 (-0.199 - 0.201) | 0.03 |
| >2 | 54 (21.5) | 0.034 (-0.209 – 0.184) | 0.04 | 46 (20.0) | 0.000 (-0.325 - 0.200) | 0.04 |

N, number of patients; VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution.

[a] Change in VA after 6 and 12 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 6 or 12 months of treatment.

[b] *P*-values were calculated using Mann-Whitney U tests.

# Discussion

We evaluated the role of four SNPs located in *NRP1* (rs2229935, rs2247383, rs2070296 and rs2804495) in response to anti-VEGF treatment. The SNP rs2070296 was found to be significantly associated with a fewer gain in letters. Depending on the genotype, patients showed a different response following an additive model in which the minor allele (A) leads to worse response to treatment. In median, the nine patients that carried the homozygous AA genotype didn't improve their VA and performed five ETDRS letters (one line) worse than the homozygous GG group. As a recent study showed that most patients perceive one line of the ETDRS chart as an improvement[28], this difference could be clinically relevant. This effect was not seen after 6 and 12 months of treatment. Nevertheless, the dilution of the effect seen in the change in VA after the loading dose of three ranibizumab injections,

could be due to variability in the treatment regime and progression of the disease, which makes the comparison of the long term response difficult.

We defined treatment response as change in VA after three consecutive loading injections compared with baseline. VA is an important functional outcome measure, which is most relevant for patients, and therefore, it has been extensively used to evaluate treatment response in nvAMD[7,12,29-36]. Most patients achieve the largest change in VA after the three first monthly injections[3] and this time interval can be predictive of long-term response[37]. Therefore, this finding not only expands the knowledge of the mechanisms that underlie the variability in the response, but also could be implemented in future prediction models. Despite that, we encourage the evaluation of the effect of this SNP using also anatomic features defined by OCT. The patients in our study cohort were treated between 2007 and 2009, and at that time, OCT scans were not implemented routinely during treatment regimes.

Although our study detected a significant association of rs2070296 with anti-VEGF treatment response, further studies are required to confirm our findings and to determine whether this SNP or other genetic variants in *NRP1* are driving the effect. A more extensive analysis of additional genetic variants in *NRP1* could reveal other SNPs associated with variability in the response. Furthermore, examination of low frequency and rare variants could reveal variants with a higher impact on the trait and major clinical relevance.

The *NRP1* gene has also been implicated in treatment response to anti-VEGF therapy in cancer. A SNP in the 3'UTR of *NRP1* has been associated with better progression-free survival in recurrent ovarian cancer treated with bevacizumab (Avastin; Genentech Inc., San Francisco, California, USA)[38], an anti-VEGF drug also used off-label for the treatment of nvAMD. *NRP1* is expressed in endothelial cells and upregulated in numerous tumor cell types[39-48], which has been associated with poorer outcomes in several cancers such as breast cancer[42], osteosarcoma[46] and nasopharyngeal carcinoma[48]. Therefore, the interest in developing new therapies targeting NRP1 in cancer is increasing[49]. Moreover, an improved effect of an anti-VEGF drug combined with anti-NRP1 antibodies has been described in tumor treatment.[50] In addition, NRP1 has been proposed as a potential biomarker for treatment response in advanced gastric cancer treated with bevacizumab[26]. In a recent study, Raimondi and colleagues described that NRP1 promotes angiogenesis in a VEGFR2/VEGFA independent manner. In this novel mechanism, NRP1 forms a complex with ABL1 that leads to the activation of paxillin in a fibronectin dependent manner which enhanced motility *in vitro* and angiogenesis *in vivo*. Moreover, in a mouse model of oxygen-induced retinopathy, treatment with imatinib (an ABL1 inhibitor used for the treatment of leukemia) reduced angiogenesis. Consequently, imatinib was proposed as a new therapy for nvAMD targeting NRP1[25].

The wide range of response to anti-VEGF therapy observed in nvAMD patients has drawn much attention in the pharmacogenetic research field. The findings described in this study, together with the findings of Hermann *et al.*[12] and Lotery *et al.*[9], suggest that variants in

components of the neovascularization pathways play an important role in treatment response to anti-VEGF therapy in AMD. The study by Hermann et al. showed that rs4576072 in *KDR* is associated with response after 12 months of treatment[12]. In the current study we demonstrated a significant cumulative effect of this SNP and SNP rs2070296 in *NRP1* in the response to ranibizumab treatment after the three loading injections, and also after 6 and 12 months of treatment. This finding is specifically interesting for the development of prediction models based on relevant clinical parameters, environmental and genetic factors, which would allow patients to be grouped for different regimen doses or therapies.

In summary, our findings suggest that genotyping of SNPs in *NRP1*, in combination with SNPs in other genes as *KDR*, could be used as a rapid preclinical tool for selection of the optimal treatment for individual patients, which besides anti-VEGF treatment could also involve targeting of NRP1. In the future, genetic testing of such variants may help to predict outcome of nvAMD treatment, and to tailor medical care to individual needs.

# Supplemental content

SUPPLEMENTAL DIGITAL CONTENT TABLE 1 **Chromosomal location of the *NRP1* SNPs and primers used for genotyping.**

| SNP | Chromosomal location (chr, bp)[a] | Primers KASPar sequencing | Primers Sanger sequencing |
|---|---|---|---|
| **rs2229935** | 10, 33510663 | Primer Allele FAM (C): AGCTTACCTGT-TATCTTGCAACCG<br>Primer Allele HEX (T): CAGCTTACCTGT-TATCTTGCAACCA<br>Primer Common: GGGAAACTGG-CATATCTATGAGATTTGAA | NA |
| **rs2247383** | 10, 33489052 | Primer Allele FAM (C): CAGAATTGGAG-GGAGGCCAGG<br>Primer Allele HEX (T): CAGAATTGGAG-GGAGGCCAGA<br>Primer Common: CCACTGGGAA-CAGAACGCTAATGTA | NA |
| **rs2070296** | 10, 33552695 | Primer Allele FAM (A): CCAGGATA-ATCTCTGACATCTTTGGT<br>Primer Allele HEX (G): CAGGATA-ATCTCTGACATCTTTGGC<br>Primer Common: ATCCCAACAGCCTT-GAATGCACTTATATT | Fw: CTGAACTTGACTTTCCATACCC<br>Rv: TGCTTTGTTTTCCAGTGTCC |
| **rs2804495** | 10, 33612500 | Primer Allele FAM (G): ACTACTAACTGC-CCTAGATACCAG<br>Primer Allele HEX (T): CACTACTAACTG-CCCTAGATACCAT<br>Primer Common: CTCTTCTCTGGTT-GATTGGCCTGTA | NA |

SNP, single nucleotide polymorphism; chr, chromosome; bp, base pair; NA = not applicable; Fw, forward; Rv, reverse.

[a] The chromosomal location is based on the assembly of February 2009 (GRCh37/hg19).[51]

SUPPLEMENTAL DIGITAL CONTENT TABLE 2 **Influence of the baseline variables on response to ranibizumab treatment.**

| | | ΔVA after 3 months (logMAR)[a] | |
|---|---|---|---|
| | | ***P*-value[b]** | **Correlation coefficient/ median (quartiles)** |
| **Demographics** | | | |
| | Age at first injection | 0.01 | -0.130 |
| | Gender | 0.38 | Female: 0.079 (0.000 - 0.222) |
| | | | Male: 0.100 (-0.020 - 0.300) |
| **Disease history** | | | |
| | Hypertension (n=259)[c] | 0.91 | Yes: 0.104 (0.000 - 0.250) |
| | | | No: 0.079 (0.000 - 0.301) |
| | Diabetes mellitus (n=259)[c] | 0.02 | Yes: 0.000 (-0.204 - 0.176) |
| | | | No: 0.107 (0.000 - 0.301) |
| **Other environmental factors** | | | |
| | BMI (kg/m2) (n=258)[c] | 0.22 | -0.077 |
| **Ophthalmological details** | | | |
| | Baseline VA (logMAR) | $<10^{-3}$ | 0.195 |
| | Change in VA after 3 months (logMAR) | NA | NA |
| | Type of CNV (n=335)[c] | 0.06 | Occult with no classic: 0.097 (0.000 - 0.204) |
| | | | RAP: 0.107 (-0.048 - 0.301) |
| | | | Minimally classic: 0.000 (-0.099 - 0.176) |
| | | | Predominantly classic: 0.100 (0.045 – 0.311) |
| | Lesion size (DA) (n=285)[c] | 0.23 | <2: 0.100 (-0.015 – 0.296) |
| | | | 2-4: 0.090 (0.000 – 0.222) |
| | | | 4-6: 0.100 (0.000 – 0.300) |
| | | | >6: 0.000 (-0.097 – 0.176) |

VA, visual acuity; logMAR = logarithm of the Minimum Angle of Resolution; NA, not applicable; n, number of patients; CNV, choroidal neovascularization; RAP, retinal angiomatous proliferation; DA, disk areas.

[a] Change in VA after 3 months (logMAR) was calculated in the following manner: VA prior to treatment - VA after 3 months of treatment.

[b] *P*-values were calculated using Spearman correlations for the independent continuous variables and Kruskal-Wallis tests or Mann-Whitney U tests for the categorical variables.

[c] For the remaining patients no data were available.

SUPPLEMENTAL DIGITAL CONTENT TABLE 3 Influence of *NRP1* SNPs on potential confounding factors.

| | Potential confounding factors of ΔVA after 3 months (logMAR) | | | |
|---|---|---|---|---|
| | Age at first injection (years) | Baseline VA (logMAR) | Diabetes mellitus | Type of CNV |
| | *P*-value[a] | *P*-value[a] | *P*-value[a] | *P*-value[a] |
| rs2229935 | 0.23 | 0.23 | 0.38 | 0.22 |
| rs2247383 | 0.89 | 0.30 | 0.37 | 0.93 |
| rs2070296 | 0.50 | 0.34 | 0.30 | 0.30 |
| rs2804495 | 0.85 | 0.47 | 0.79 | 0.39 |

VA, visual acuity; logMAR, logarithm of the Minimum Angle of Resolution; CNV, choroidal neovascularization.

[a] *P*-values were calculated using Kruskal-Wallis tests for continuous outcome variables and test for categorical outcome variables.

# Additional information

## Acknowledgments

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## Conflicts of Interest

None declared.

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# 2.2

# *GWAS study using DNA pooling strategy identifies association of variant rs4910623 in OR52B4 gene with anti-VEGF treatment response in age-related macular degeneration*

Riaz M*, Lorés-Motta L*, Richardson AJ, Lu Y, Montgomery G, Omar A, Koenekoop RK, Chen J, Muether P, Altay L, Schick T, Fauser S, Smailhodzic D, van Asten F, de Jong EK, Hoyng CB, Burdon KP, MacGregor S, Guymer RH, den Hollander AI, Baird PN

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# Abstract

Pooled DNA based GWAS to determine genetic association of SNPs with visual acuity (VA) outcome in anti-vascular endothelial growth factor (anti-VEGF) treated neovascular age-related macular degeneration (nAMD) patients. We performed pooled DNA based GWAS on 285 anti-VEGF treated nAMD patients using high density Illumina 4.3M array. Primary outcome was change in VA in Early Treatment Diabetic Retinopathy Study (ETDRS) letters after 6 months of anti-VEGF treatment (patients who lost ≥5 ETDRS letters classified as non-responders and all remaining classified as responders). GWAS analysis identified 44 SNPs of interest: 37 with strong evidence of association ($p<9x10^{-8}$), 2 in drug resistance genes ($p<5x10^{-6}$) and 5 nonsynonymous changes ($p<1x10^{-4}$). In the validation phase, individual genotyping of 44 variants showed three SNPs (rs4910623 $p=5.6x10^{-5}$, rs323085 $p=6.5x10^{-4}$ and rs10198937 $p=1.30x10^{-3}$) remained associated with VA response at 6 months. SNP rs4910623 also associated with treatment response at 3 months ($p=1.5x10^{-3}$). Replication of these three SNPs in 376 patients revealed association of rs4910623 with poor VA response after 3 and 6 months of treatment ($p=2.4x10^{-3}$ and $p=3.5x10^{-2}$, respectively). Meta-analysis of both cohorts (673 samples) confirmed association of rs4910623 with poor VA response after 3 months ($p=1.2x10^{-5}$) and 6 months ($p=9.3x10^{-6}$) of treatment in nAMD patients.

# Background

Age-related macular degeneration (AMD) is a common complex progressive neurodegenerative disease in the elderly, which can lead to irreversible severe vision loss[1]. Vision is threatened when AMD advances to its late sequelae of either geographic atrophy (GA) or choroidal neovascularization (CNV) also known as nAMD[2]. One of the most important regulators of the neovascularization process is vascular endothelial growth factor A (VEGF-A)[3]. Currently, the most effective treatment for nAMD is inhibition of VEGF through the use of recombinant, humanised anti-VEGF monoclonal antibodies such as ranibizumab (Lucentis), aflibercept (Eylea) or off-label bevacizumab (Avastin). These drugs have been shown in multiple studies[4-8] to be efficacious in improving vision, but a varying response to anti-VEGF treatment has been observed. Approximately 10% of patients showed no improvement in visual acuity (VA) (loss of › 15 ETDRS letters), and exhibited a continuous decline in VA over two years of treatment similar to that previously reported for both ANCHOR and MARINA trials[5,6]. This range of variable VA response may in part be explained by genetic predisposition.

It is well established that genetic factors are associated with risk of developing AMD[9]. Several of the genes associated with AMD are also genes encoding components of the neovascularization pathway and have previously been investigated in variation to anti-VEGF treatment response studies[8,10-12]. However, conflicting findings have been reported thus far.

The current study aimed to investigate associations with genetic variants in a hypothesis-free manner using a genome-wide association study (GWAS). We investigated whether genetic factors influencing ranibizumab treatment outcomes in nAMD patients could be identified through the initial use of a GWAS pooling strategy followed by a technical validation and subsequent replication in an independent cohort.

# Results

The objective of this study was to investigate whether genetic variants could be identified that might influence the treatment outcome after anti-VEGF treatment in nAMD patients. The demographic characteristics of patients of the Melbourne discovery cohort and the replication cohort are shown in Table 1. A total of 297 patients from the Melbourne discovery cohort (285 individuals used initially in the pooled GWAS, plus an additional 12 ranibizumab-treated AMD patients were included, giving a total of 297 individuals) and 376 patients from the replication cohort met the study inclusion criteria with a mean age of 79.2 years and 77.1 years, respectively. A mean baseline VA (at the time of first injection) of 51 ETDRS letters was observed in the Melbourne discovery cohort and 52.6 ETDRS letters in the replication cohort (Table 1). In the Melbourne discovery cohort, sex, smoking, type of lesion, size of CNV and number of injections up to 6 months of treatment,

showed no significant association with change in VA at 3 and 6 months of treatment (p>0.05). Baseline VA showed a consistent negative association with change in VA at 3 and 6 months (p<0.001) in both the Melbourne and replication cohort. In addition, age at the time of nAMD treatment was associated with change in VA at 3 months (p<0.001) but not at 6 months in the replication cohort (p>0.05) (Supplementary Table S1). Both the discovery and replication cohort have similar demographic and clinical characteristics except for the size of the CNV.

TABLE 1 **Patient demographics and clinical characteristics of the Melbourne discovery cohort and the replication cohorts**

| | Discovery Cohort (Melbourne) (n=297) | Replication Cohort (Nijmegen/Cologne/Montreal) (n=376) |
|---|---|---|
| **Sex** | | |
| **Female** | 181 (60.9%) | 214 (56.9%) |
| **Male** | 116 (39.1%) | 162 (43.1%) |
| **Age (years), mean±SD** | 79.2±7.1 | 77.1±7.4 |
| **Range** | (53-102) | (53-97) |
| **Baseline Visual Acuity (ETDRS letters), mean ±SD** | 51.0±17.5 | 52.6±18.0[∞] |
| **Range** | (2-88) | (1-85) |
| **Type of lesion** | | |
| **Predominantly Classic** | 23% | 19.4% |
| **Non-Predominantly Classic** | 77% | 69.7% |
| **Missing Data** | 11% | 10.9% |
| ***[+]Size of CNV** | | |
| **‹ 2 optic-disc area** | 44% | 24.5% |
| **› 2 Optic-disc area** | 29% | 51.3% |
| **Missing Data** | 27% | 24.2% |
| **Number of Injections at 6 months, mean ±SD** | 4.7 ± 1.3 | NA |
| **^Number of Responders/Non-responders** | | |
| **3 Month** | 84%/16% | 82%/18% |
| **6 Month** | 79%/21% | 76%/24% |

SD = Standard Deviation, ETDRS = Early Treatment Diabetic Retinopathy Study, *In the discovery cohort, one optic-disc area is equal to 2.54 $mm^2$, based on an optic disc diameter of 1.8mm, [+] In the replication cohort optic disc measurement is based on each patient optic disc area, NA = Not available, ^Non-responders classified as those who showed loss of ≥5 EDTRS letters VA from baseline; all remaining patients were classified as responders. [∞] 303 Snellen visual acuity values were first converted into approximate ETDRS letters using a chart that has all three measurement to read the equivalent number of ETDRS letters. It is based on formula ETDRS letters = 85-((-(logSnellen))/0.02)[40].

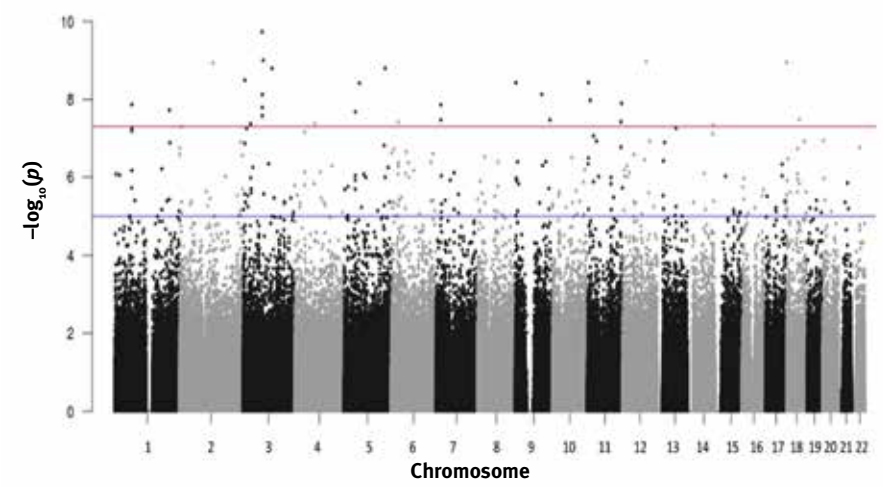


FIGURE 1 **Manhattan plot of SNPs tested in a pooled GWAS for response to anti-VEGF treatment.**

The X-axis indicates the chromosomal position of the SNPs and the Y-axis shows their corresponding *P*-value (-$\log_{10}$). The red line indicates the threshold for genome-wide significance ($p<5x10^{-8}$) and the blue line indicates the threshold for suggestive association ($p<1x10^{-5}$).

## First Phase – Genome wide association Study

First phase results from the pooled GWAS are shown in a Manhattan plot in Figure 1. A total of 44 SNPs from the pooled GWAS were selected for technical validation through independent individual genotyping on the basis of a genome-wide significant *p*-value (37 SNPs, $p<9x10^{-8}$), SNPs in genes involved in drug resistance response ('pharmagenes') (2 SNPs, $p<5x10^{-6}$) and SNPs leading to a missense change in a coding region of a gene (5 SNPs, $p<1x10^{-4}$) (Table 2).

## Second Phase – Validation

In the second phase, all 44 SNPs from the first phase were genotyped in 297 patients (the same cohort as in phase 1 but with the addition of 12 extra patient samples who became available between the time of phase1 and phase2). This individual genotyping confirmed the 3 SNPs (rs4910623, rs323085, rs10158937) to be significantly ($p<0.05$) associated with response at 6 months of ranibizumab treatment after Bonferroni correction for 44 independent tests. SNP rs4910623 showed the highest significance ($p=5.7x10^{-5}$), in which the 'G' risk allele led to a worse response (OR=2.58 [95% CI=1.63-4.10]). While the C allele of both rs323085 and rs10158937 ($p=6.5x10^{-4}$ and $p=1.30x10^{-3}$, respectively) appeared protective (OR=0.16 [95% CI= 0.06-0.46] and OR=0.32 [95% CI=0.17-0.65], respectively) (Table 3).

TABLE 2 **Selected SNPs from the pooled GWAS comparing responders and non-responders at 6 months ranibuzimab treatment and validation results using an independent genotyping technique in the Melbourne discovery cohort**

| SNP | Chr. | Position+ | Gene | Effect Allele | Pooled GWAS | | Technical Replication | |
|---|---|---|---|---|---|---|---|---|
| **Pooled GWAS SNPs reaching P ≤ 9x10$^{-8}$** | | | | | ***P-Value*** | **OR** | ***P-Value*** | **OR(95% CI)** |
| **rs4910623** | **11** | **4389639** | ***OR52B4*** | **G** | **3.69x10$^{-9}$** | **3.14** | **5.65x10$^{-5}$** | **2.58 (1.63-4.10)** |
| **rs323085** | **18** | **49290621** | ***LOC100287225*** | **C** | **3.29x10$^{-8}$** | **0.22** | **6.53x10$^{-4}$** | **0.16 (0.06-0.46)** |
| **rs10158937** | **1** | **66144876** | ***LEPR*** | **C** | **5.36x10$^{-8}$** | **0.28** | **1.30x10$^{-3}$** | **0.32 (0.17-0.65)** |
| rs2475779 | 5 | 157541895 | *CLINT1* | T | 1.59x10$^{-9}$ | 0.29 | 1.36x10$^{-6}$ | 0.40 (0.23-0.70) |
| rs59741976 | 3 | 74593662 | *CNTN3* | T | 2.65x10$^{-8}$ | 0.32 | 1.84x10$^{-3}$ | 0.31 (0.15-0.65) |
| rs4655583 | 1 | 66155407 | *LEPR* | A | 1.37x10$^{-8}$ | 0.28 | 2.38x10$^{-3}$ | 0.34 (0.17-0.69) |
| rs7857431 | 9 | 132529504 | *PTGES* | T | 3.36x10$^{-8}$ | 0.25 | 4.58x10$^{-6}$ | 0.12 (0.03-0.53) |
| rs794009 | 4 | 40139719 | *N4BP2* | A | 6.91x10$^{-8}$ | 0.35 | 5.47x10$^{-3}$ | 0.40 (0.22-0.77) |
| rs10234065 | 7 | 19547138 | *TWISTNB* | T | 1.37x10$^{-8}$ | 0.31 | 5.61x10$^{-3}$ | 0.13 (0.03-0.56) |
| rs1447830 | 3 | 74613171 | *CNTN3* | T | 7.56x10$^{-9}$ | 0.30 | 5.63x10$^{-3}$ | 0.35 (0.17-0.74) |
| rs2110470 | 7 | 19509870 | *TWISTNB* | A | 3.35x10$^{-8}$ | 0.35 | 5.65x10$^{-6}$ | 0.40 (0.21-0.77) |
| rs1892535 | 1 | 66097181 | *LEPR* | T | 6.50x10$^{-8}$ | 0.31 | 6.50x10$^{-3}$ | 0.38 (0.19-0.77) |
| rs1573317 | 18 | 382268 | *COLEC12* | T | 1.11x10$^{-9}$ | 0.27 | 7.60x10$^{-3}$ | 0.36 (0.18-0.77) |
| rs13154178 | 5 | 42828101 | *SEPP1* | A | 2.09x10$^{-8}$ | 0.36 | 8.20x10$^{-3}$ | 0.49 (0.30-0.83) |
| rs4909963 | 11 | 11119228 | *GALNTL4* | T | 9.85x10$^{-9}$ | 0.24 | 8.32x10$^{-3}$ | 0.20 (0.06-0.67) |
| rs79966776 | 3 | 74582701 | *CNTN3* | A | 1.61x10$^{-8}$ | 0.32 | 9.72x10$^{-3}$ | 0.42 (0.22-0.81) |
| rs510549 | 3 | 111700305 | *ABHD10* | T | 1.60x10$^{-9}$ | 0.34 | 1.07x10$^{-2}$ | 0.53 (0.33-0.87) |
| rs6917419 | 6 | 27243480 | *PRSS16* | T | 3.80x10$^{-8}$ | 0.33 | 1.41x10$^{-2}$ | 0.32 (0.13-0.80) |
| rs12117294 | 1 | 209814879 | *LAMB3* | T | 1.89x10$^{-8}$ | 0.34 | 1.84x10$^{-2}$ | 0.46 (0.25-0.88) |
| rs10767060 | 11 | 23468443 | *LOC100500938* | T | 8.72x10$^{-8}$ | 0.38 | 2.23x10$^{-2}$ | 0.58 (0.37-093) |
| rs17770298 | 9 | 101208288 | *GABBR2* | A | 7.44x10$^{-9}$ | 0.18 | 2.40x10$^{-2}$ | 0.19 (0.05-081) |
| rs11131078 | 3 | 7548067 | *GRM7* | T | 3.23x10$^{-9}$ | 0.13 | 2.63x10$^{-2}$ | 0.58 (0.37-0.94) |
| rs292998 | 5 | 58032485 | *RAB3C* | G | 3.86x10$^{-9}$ | 0.38 | 2.94x10$^{-2}$ | 0.61 (0.39-0.95) |
| rs772433 | 2 | 7838257 | *LOC339788* | A | 5.06x10$^{-8}$ | 0.36 | 4.24x10$^{-2}$ | 0.62 (0.40-0.98) |
| rs3806586 | 2 | 128433897 | *LIMS2* | T | 1.16x10$^{-9}$ | 0.26 | 4.96x10$^{-2}$ | 0.52 (0.28-1.00) |
| rs9644866 | 9 | 2290590 | *SMARCA2* | T | 3.72x10$^{-9}$ | 0.17 | 5.60x10$^{-2}$ | 0.63 (0.40-1.01) |
| rs10050214 | 4 | 78733015 | *CNOT6L* | T | 4.22x10$^{-8}$ | 0.38 | 6.35x10$^{-2}$ | 0.40 (0.28-1.04) |
| rs12638297 | 3 | 29748169 | *RBMS3* | T | 4.29x10$^{-8}$ | 0.31 | 6.40x10$^{-2}$ | 0.50 (0.25-1.04) |
| rs4449299 | 3 | 14598965 | *GRIP2* | A | 5.50x10$^{-8}$ | 0.39 | 8.79x10$^{-2}$ | 0.64 (0.39-1.07) |
| rs1353892 | 12 | 90716019 | *C12orf37* | T | 1.06x10$^{-9}$ | 0.34 | 9.71x10$^{-2}$ | 0.67 (0.42-1.07) |
| rs659910 | 11 | 131769454 | *NTM* | T | 1.27x10$^{-8}$ | 0.39 | 1.67x10$^{-1}$ | 0.71 (1.00-1.03) |
| rs10141328 | 14 | 96752555 | *ATG2B* | C | 7.78x10$^{-8}$ | 0.16 | 1.90x10$^{-1}$ | 0.73 (0.47-1.16) |
| rs9323992 | 14 | 98649816 | *C14orf64* | T | 4.59x10$^{-8}$ | 0.38 | 5.14x10$^{-1}$ | 0.84 (0.50-1.41) |
| rs7432690 | 3 | 77450363 | *ROBO2* | T | 9.91x10$^{-10}$ | 0.14 | 6.81x10$^{-1}$ | 1.09 (0.72-1.67) |
| rs141659302 | 11 | 128802177 | *TP53AIP1* | T | 3.79x10$^{-8}$ | 0.41 | 9.99x10$^{-1}$ | 0.74 (0.00-0.00) |
| rs7320683 | 13 | 71787948 | *DACH1* | A | 5.38x10$^{-8}$ | 2.86 | 1.00 | 1 (-) |
| rs291477 | 3 | 73807858 | *PDZRN3* | G | 1.85x10$^{-10}$ | 2.91 | 1.00 | 1 (-) |
| **Pooled GWAS SNPs in known pharmagenes showing suggestive significance** | | | | | | | | |
| rs3804938 | 3 | 7550294 | *GRM7* | C | 1.35x10$^{-7}$ | 0.13 | 1.01x10$^{-2}$ | 0.54 (0.34-0.87) |
| rs4148732 | 7 | 87234049 | *ABCB1* | G | 2.72x10$^{-6}$ | 0.22 | 1.00x10$^{-3}$ | 0.08 (0.02-0.38) |

| Pooled GWAS SNPs leading to a missense change in gene | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| rs3877899 | 5 | 42801268 | *SEPP1* | A | $9.02x10^{-7}$ | 0.36 | $1.15x10^{-3}$ | 0.34 (0.18-0.66) |
| rs34677 | 5 | 33998768 | *AMACR* | T | $3.22x10^{-5}$ | 0.35 | $3.0x10^{-3}$ | 0.26 (0.11-064) |
| rs3784588 | 15 | 31294654 | *TRPM1* | T | $4.73x10^{-5}$ | 0.27 | $9.96x10^{-1}$ | 0.04 (1.00-1.03) |
| rs17659179 | 18 | 47511113 | *MYO5B* | A | $8.20x10^{-5}$ | 0.28 | $4.75x10^{-2}$ | 0.29 (0.09-0.99) |
| rs17673268 | 9 | 368128 | *DOCK8* | T | $1.54x10^{-5}$ | 0.41 | $2.44x10^{-1}$ | 0.63 (1.00-1.03) |

For technical replication *P* values were calculated using logistic regression adjusted for baseline visual acuity, OR = odds ratio, SNP: single nucleotide polymorphism, Chr: chromosome, BP: base pair position on chromosome. SNPs that remained significantly associated after Bonferroni correction for multiple testing are highlighted in bold.+ Representing position from phase 3 1000G CEU reference panel.

TABLE 3 **Genotypic distribution and allele frequency of rs4910623, rs323085 and rs10158937 and association with VA response at six and three months of anti-VEGF treatment in the Melbourne discovery cohort**

| 6 Month | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **SNP** | **A1** | **A2** | **Genotype frequency *** | | **Allele frequency +** | | ***P-Value*** | **OR 95% CI** |
| | | | **Responders** | **Non-responders** | **Responders** | **Non-Responders** | | |
| rs4910623 | **G** | A | 0.23/0.52/0.25 | 0.54/0.36/0.10 | 0.49/0.51 | 0.72/0.28 | **$5.7x10^{-5}$** | 2.58 (1.63-4.10) |
| rs323085 | **C** | T | 0.30/0.28/0.69 | 0/0.70/0.93 | 0.17/0.83 | 0.03/0.97 | $6.5x10^{-4}$ | 0.16 (0.06-0.46) |
| rs10158937 | **C** | A | 0.06/0.33/0.61 | 0/0.17/0.83 | 0.23/0.77 | 0.08/0.92 | $1.3x10^{-3}$ | 0.32 (0.17-0.65) |
| **3 Month** | | | | | | | | |
| rs4910623 | **G** | A | 0.25/0.51/0.24 | 0.52/0.37/0.11 | 0.51/0.49 | 0.71/0.29 | **$1.5x10^{-3}$** | 2.23 (1.36-3.66) |
| rs323085 | **C** | T | 0.02/0.25/0.73 | 0.02/0.22/0.76 | 0.14/0.86 | 0.13/0.87 | 0.81 | 0.92 (0.47-1.81) |
| rs10158937 | **C** | A | 0.06/0.33/0.62 | 0.02/0.13/0.85 | 0.22/0.78 | 0.09/0.91 | $9.0x10^{-3}$ | 0.36 (0.17-0.77) |

Definition of response was the same as in the pooled GWAS: Non-responders are patients who lost ≥ 5 ETDRS letters and the remainder were classified as responders *P*-Value: Calculated using logistic regression adjusted for baseline visual acuity, OR = odd ratio, representing trend per copy effect of risk or minor allele on VA outcome, highlighted in bold for each SNP, CI: Confidence Interval,
* Genotype frequency A1A1/A1A2/A2A2, +Allele frequency A1/A2.

Since a loading dose of three monthly injections is prescribed to all patients, we determined if the effect of these three selected SNPs in treatment outcome could be seen at this time point. The G allele of rs4910623 showed an association with worse outcome at three months of treatment ($p=1.5x10^{-3}$, OR=2.23 [95% CI=1.36-3.66]).

SNP rs10158937 also showed an association with the C allele leading to a better response after 3 months ($p=9.0x10^{-3}$, OR=0.36 [95% CI=0.17-0.77]), consistent with the six months result. However rs323085 did not show significant association at three months (Table 3).

## Third Phase – Replication

In the third phase, we analysed the association of the three SNPs with treatment outcome in a replication cohort of European descent, consisting of 215 ranibizumab-treated AMD patients following pro re nata treatment protocol. The effect of rs4910623 replicated at six months of treatment ($p=3.5x10^{-2}$, OR=1.71 [95% CI=1.04-2.80]). At the 3 month treatment time point all replication patients were included in the analysis (n = 376) as they were all treated in a similar manner to the discovery cohort. The effect of this SNP on treatment response was also similar at this time point ($p=2.4x10^{-3}$, OR=1.8 [95% CI=1.24-2.71]). SNPs rs323085 and rs10158937 were not associated with treatment response in the replication cohort (Table 4).

TABLE 4 **Genotypic distribution and allele frequency of rs4910623, rs323085 and rs10158937 and association with VA response at six and three months of anti-VEGF treatment in the replication cohort**

| **6 Month** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **SNP** | **A1** | **A2** | **Genotype frequency *** | | **Allele frequency +** | | ***P-Value*** | **OR 95% CI** |
| | | | **Responders** | **Non-responders** | **Responders** | **Non-Responders** | | |
| rs4910623 | G | A | 0.24/0.48/0.28 | 0.34/0.54/0.12 | 0.48/0.52 | 0.62/0.38 | $3.5x10^{-2}$ | 1.71(1.04-2.80) |
| rs323085 | C | T | 0.02/0.25/0.74 | 0.00/0.21/0.79 | 0.14/0.86 | 0.10/0.90 | 0.46 | 0.74(0.34-1.63) |
| rs10158937 | C | A | 0.05/0.32/0.63 | 0.02/0.30/0.67 | 0.21/0.79 | 0.17/0.83 | 0.58 | 0.84(0.45-1.57) |
| **3 Month** | | | | | | | | |
| rs4910623 | G | A | 0.22/0.50/0.28 | 0.39/0.46/0.15 | 0.47/0.53 | 0.62/0.38 | $2.4x10^{-3}$ | 1.83 (1.24–2.71) |
| rs323085 | C | T | 0.03/0.24/0.73 | 0/0.23/0.77 | 0.15/0.85 | 0.11/0.89 | 0.34 | 0.74 (0.50–1.33) |
| rs10158937 | C | A | 0.04/0.32/0.64 | 0.06/0.35/0.59 | 0.20/0.80 | 0.23/0.77 | 0.25 | 1.31 (0.83-2.06) |

Definition of response was the same as in the pooled GWAS: Non-responders are patients who have lost ≥ 5 ETDRS letters and the remainder were classified as responders *P*-Value: Calculated using logistic regression adjusted for baseline visual acuity and age, OR = odd ratio, representing trend per copy effect of risk or minor allele on VA outcome, highlighted in bold for each SNP, CI: Confidence Interval, * Genotype frequency A1A1/A1A2/A2A2, + Allele frequency A1/A2.++ In replication cohort pr re nata treated patients included n = 215.

The frequency of rs4910623 risk allele (G) in the Melbourne discovery cohort was 71% and 72% for the non-responder group (3 and 6 months respectively) in comparison to 51% and 49% in the responders group (3 and 6 months, respectively) (Table 3). A similar change in allele frequency was also seen in the replication cohort with the frequency of the risk allele G being 62% and 38% in the non-responder group compared to 48% and 52% in the responders group (3 and 6 months (pro re nata patients), respectively) (Table 4). The overall distribution of change in VA corresponding to each genotype of rs4910623 in both cohorts is shown in Figure 2.

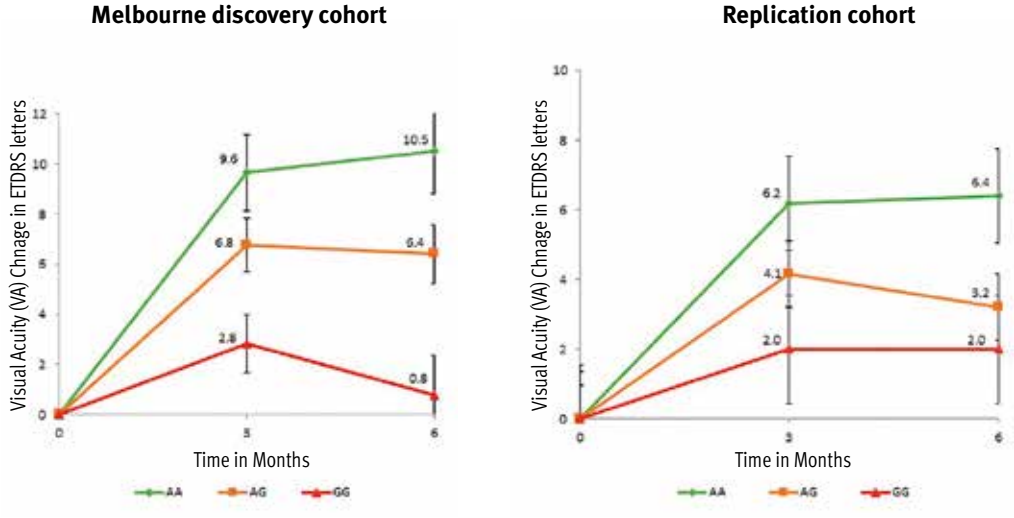


FIGURE 2 **Mean change in visual acuity (VA) after three and also the six month time period (pro re nata treated only) of treatment stratified by rs4910623 genotype for the Melbourne discovery and replication cohorts.**

Vertical lines represent the standard error (SE) of the mean change in VA.

## Meta-Analysis

A meta-analysis of SNP rs4910623 in both the Melbourne discovery cohort and the replication cohort showed differences in allele distribution between responders and non-responders at 3 months ($p=1.21x10^{-5}$) and 6 months ($p=9.3x10^{-6}$) of treatment (only pro re nata patients from the replication cohort were included) indicating the influence of rs4910623 on outcome of ranibizumab treatment in AMD patients (Table 5).

TABLE 5 **Meta-analysis of the Melbourne discovery cohort and the replication cohort for SNP rs4910623 at 3 and 6 months of ranibizumab treatment in AMD patients**

| **Meta-Analysis** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **3 Month** | | | | **6 Month*** | | | |
| **SNP** | **Gene** | **A1** | **A2** | ***P-Value*** | **N** | **Direction** | **Z score** | ***P-Value*** | **N** | **Direction** | **Z score** |
| rs4910623 | *OR52B4* | G | A | $1.2x10^{-5}$ | 673 | ++ | 4.37 | $9.3x10^{-6}$ | 512 | ++ | 4.43 |

Meta-analysis was performed using METAL.* At 6 month only pro re nata treated patient were included from replication cohort.

# Discussion

We sought to identify genetic variants that influence the ranibizumab treatment response in AMD. Using a pooled DNA GWAS approach, we identified SNP rs4910623 in the promoter region of the *OR52B4* gene as being associated with a worse response to ranibizumab treatment. The association was validated in the same cohort through individual genotyping and confirmed in an independent replication cohort of AMD patients of European descent. Individuals carrying the risk allele G did not respond as well to ranibizumab treatment in either cohort after 3 or 6 months of treatment compared to those with the A allele. In the replication cohort we observed significant association of rs4910623 with change in VA in patients on pro re nata treatment regimen at 6 months. However, 47 patients from Montreal were not included in this analysis as they followed a treat-and-extend regimen. We undertook an additional sensitivity analysis where these 47 patients were included back into the 6 month analysis time point to provide a total of 262 patients. A similar trend for association of the G allele of rs4910623 was seen ($p=4.6x10^{-2}$, OR=1.53 [95% CI=1.00-2.30]). As this group consisted of only 47 patients it was too small to draw any conclusive conclusions about whether this treatment protocol significantly affected the association of the G allele of rs4910623 with change in VA. In the meta-analysis we found significant association of rs4910623 patients with change in VA at both 3 and 6 months of treatment.

Two other SNPs, rs323085 and rs10158937, located *202 kb 3'* of *LOC100287225* and 42 kb at 3' of *LEPR* gene respectively, did not remain associated with change in VA after replication. The inconsistency in the results for these two SNPs could indicate that they are false positive findings.

The baseline characteristics of both the Melbourne discovery and replication cohort did not reveal significant association with change in VA except for the baseline VA in both the discovery and replication cohort at 3 and 6 months of treatment. Baseline VA is negatively associated with change in VA, thus lower baseline VA will lead to a greater VA gain after treatment and vice versa. Therefore our findings are in agreement with the ANCHOR and MARINA trials where higher VA at baseline was associated with a smaller gain in VA at 12 and 24 months of treatment, respectively[13]. This in-turn reflects a floor and ceiling effect of baseline VA[14]. Based on definition of response in our study it may have floor effect of baseline VA where individuals with higher baseline VA will have limited chance to gain vision after the treatment and vice versa. On the other hand, the baseline VA is also reported as one of the predictors of treatment outcome for ranibizumab[15]. To overcome these potential issues we incorporated baseline VA as a covariate into our analysis. However, there was a difference in baseline CNV size in the discovery and replication cohort. This is probably due to differences in measurements between the cohorts with the Melbourne discovery cohort being based on one optic-disc area is equal to 2.54 $mm^2$, using an optic disc diameter of 1.8 mm whereas the replication cohort was based on each patient's optic disc area.

Using the HaploReg database[16], SNP rs4910623 is predicted to alter the GATA and TCF4 regulatory motifs 22 bp upstream of the *OR52B4* gene. We also analysed the surrounding variants within its linkage disequilibrium (LD) block from the 1000 Genomes Project ($r^2$ = 0.8, 250 Kb). A number of the identified SNPs in this LD block are predicted to alter transcriptional binding sites (Supplementary Table S3). Thus, this region may play an important role in transcriptional regulation of this gene.

The OR52B4 protein (Q8NGK2, UniprotKB accession number) consists of 314 amino acids (aa) and is a member of the G-coupled protein receptor (GPCR) family, which is located in the plasma membrane of the cell. Interestingly we find the conserved domain of Q8NGK2[17] is a 7-transmembrane domain (7tm) region which is highly conserved with the 7tm_4 super family domain of all the olfactory receptors (33-321 aa) and also the 7tm_1 domain of the 7 transmembrane receptor (rhodopsin family) (43-294 aa) as indicated using the NCBI Conserved Domain Database (CCD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?seqinput=NP_001005161.2). Rhodopsin is also member of GPCR family and it functions to capture photons of light and trigger the intra-cellular photo-transduction cascade, forming the molecular basis of vision[18]. GPCRs are highly selective and expressed in specific tissues and cells, making them particularly important as potential drug targets as ›50% of therapeutic agents in the market target these receptors[19].

To assess expression of the *OR52B4* gene in human eye tissue, we used online resource: "The Ocular Tissue Database"[20]. Expression of the *OR52B4* gene was present in the retina, optic nerve and choroid (Supplementary Table S4). The identification of olfactory gene expression in the eye is relatively new, but not surprising, as recently published expression profiling of a number of olfactory receptors (olfr) in mouse cornea, retina and choroid[21]. Their analysis of eye tissue expression confirmed the presence of many olfr, with one of them, *olfr547* representing a homolog of the human *OR52B4* gene (85% identity). Interestingly, olfr547 is a close relative of olfr78, which has recently been shown to function in regulation of blood pressure in smooth muscle cells[22,23]. Expression of genes in the olfr pathway in mouse ocular tissues is mediated by the olfaction specific Gα protein, localised in the nuclear layer of the retina and previously thought to be only expressed in the olfactory epithelium[21]. Moreover, olfactory genes have been shown to be associated with cancer and other blood diseases, suggesting expression and function of olfactory proteins in a number of different pathways[23-25]. Interestingly, another olfactory receptor (*OR10J5*) was recently reported by *Kim and colleagues*[26] to control cell migration and phosphorylation of signaling during angiogenesis in mice. However the functional role of *OR52B4* gene in angiogenesis needs to be elucidated and it has so far not previously been reported to be associated with pharmacogenetic response to AMD.

Previous studies investigating genetic factors in anti-VEGF treatment outcome have assessed known AMD risk-associated genes and genes encoding components of the neovascularization pathway[8,10-12]. However, most of these studies have produced variable results; for example, the Y402H variant in the *CFH* gene has been associated with poor VA outcome in some studies but in other studies has been reported to show no association

with VA. Similarly, inconsistent results of association were reported for *ARMS2/HTRA1*[11,27]. Our pooled GWAS findings did not identify genome-wide significance for SNPs in the previously associated known AMD risk genes *CFH*, *VEGF-A*, *HTRA1*/*ARMS2* (Supplementary Table S5). These findings are in agreement with our previous study[28] and the CATT study, the largest current cohort in which major AMD risk alleles have been investigated for treatment outcome following treatment with bevacizumab, where no association with the *CFH*, *VEGF*, *VEGFR2* and *HTRA1*/*ARMS2* genes was identified with anti-VEGF treatment response[11,12]. To date, only one prior GWAS has been undertaken to identify genetic risk factors for treatment response to AMD. That study was conducted on 65 patients and reported the SNP rs7607942 near the *ERBB4* gene to be associated with poor VA outcome after 6 months of treatment, although this SNP did not reach genome-wide significance (uncorrected $p=6.692x10^{-6}$)[29]. Major drawbacks of that study were the small sample size and lack of replication of the reported association in an independent ranibizumab-treated AMD cohort. We did not find any association of this SNP in the current study ($p>0.05$).

We chose to use a pooled DNA GWAS strategy to investigate response to anti-VEGF treatment in AMD as this provided a cost-effective and efficient approach to identify genetic associations[23,30-33]. We were able to technically validate ~70% of the 44 SNPs that were identified in the pooling strategy at a nominal significance level. Whilst most SNPs replicated, the level of significance of the SNPs diminished following technical validation. Possible explanations for the diminished significance may be due to experimental error during pool construction, array-specific errors resulting from the use of a limited number of arrays per pool, variation in allele frequency due to small sample size in some of the pools, allele-specific bias skewing the results for some SNPs, and/or small differences in the set of samples used in the validation stage[34,35].

For the replication cohort, the number of injections at 6 month of treatment was not available, however it is interesting to note that a variation in treatment regime through change in number of injections between 3 and 6 months does not appear to ameliorate the genetic effect seen in the current study. However, after individual genotyping, replication and meta-analysis, SNP rs4910623 remained associated with worse functional outcome (change in VA) after the anti-VEGF treatment in AMD. Currently there is much interest in the role of anatomical features such as fluid clearance as a measure of treatment response. However, it was not considered in this analysis but this would be useful to examine in the future as to whether this SNP also affects fluid clearance and central macular thickness (CMT) on OCT following anti-VEGF treatment. To our knowledge, SNP rs4910623 and the 2 other SNPs associated with good response in the discovery phase have not previously been reported as associated with anti-VEGF treatment outcome in any type of cancer, thus it will be interesting to investigate the role of these SNPs in cancer following anti-VEGF treatment. Furthermore, much work needs to be undertaken to examine the biological basis of this phenomenon as well as the functional role of *OR52B4* gene in models of angiogenesis, such studies could lead to the development of other therapies for AMD patients that can be used as an adjunct to existing treatment. This, in turn, offers the prospect of personalising treatment, based on genotype and opens up the route for exploring other drug treatment opportunities. In

conclusion, we report for the first time a pooled DNA based GWAS on pharmacogenetic response to ranibizumab treatment in AMD that identifies association of variant rs4910623 in the *OR52B4* gene. This finding was replicated in an independent replication cohort, suggesting that this gene may be involved in the response to anti-VEGF treatments in AMD patients of European decent. Finally, our data suggests that the SNP rs4910623 could be used as a diagnostic genetic marker before anti VEGF treatments for AMD.

# Methods

## Patient Recruitment and Data Collection

### Melbourne Discovery Cohort

The study was approved by the Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital (RVEEH) and performed in accordance to the tenets of the Declaration of Helsinki (7th revision). Written informed consent was obtained for all study participants before participation. Patients were included in the study when they were ›50 years of age, presented with an active sub-foveal CNV secondary to AMD, and received intravitreal anti-VEGF injections. Neovascularization was confirmed by fundus photography (Canon CR6-45NM; Canon Saitama, Japan), fundus fluorescein angiography (FFA) using IMAGEnet 2000 (Topcon Corporation, Tokyo, Japan), and optical coherence tomography (OCT) with Stratus OCT version 5.0.1 (Carl Zeiss Meditec, Dublin, CA) or Cirrus HD-OCT version 6.0.0.599 (Carl Zeiss Meditec). Exclusion criteria were nAMD associated with non-AMD conditions such as degenerative myopia, angioid streaks and hereditary retinal disorders. In addition bilateral nAMD patients who had different VA response between both eyes and treatment with either laser photocoagulation or photodynamic therapy before anti-VEGF treatment as previously reported were also excluded[36]. Initially a total of 315 nAMD patients were retrospectively recruited from the medical retinal clinic of the RVEEH. A total of 297 patients meet the eligibility criteria consisting of 277 patients with unilateral nAMD and 20 patients with bilateral nAMD who had the same (either gain or loss) VA response in both eyes. A total of 18 bilateral patients did not meet the eligibility criteria because they had differing VA response in both eyes.

VA was measured using an Early Treatment Diabetic Retinopathy Study (ETDRS) chart at 4-metre distance at each visit. The majority of nAMD patients were treated with monthly ranibizumab injections (Lucentis; Novartis Pharma AG, Basel, Switzerland) for 3 months of initial dosing except for 27 (9.0%) patients who first received a single injection of bevacizumab (Avastin; Roche, Basel, Switzerland) who then went on to receive normal dosing using ranibizumab. After 3 monthly injections, pro re nata treatment regimen were adopted for follow-up injections, that was based on evaluation by a retinal specialist, who considered loss of VA ≥ 5 EDTRS letters, the presence of fluid on an OCT scan or presence

of chronic or new retinal haemorrhage as criteria for treatment. Data on demographics and clinical history (including VA and treatment regimens) were collected for all participating patients.

## Replication Cohort

Ethical approval for the replication cohort was obtained from the local ethic committees of the Radboud university medical center, University of Cologne, and McGill University Health Centre. Written informed consent was acquired from all participants.

The replication cohort consisted of 376 treatment naïve patients of European descent with choroidal neovascularization secondary to AMD. Patients were diagnosed by retinal specialists based on ophthalmic examination, spectral-domain OCT (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) or fluorescein angiography (FA) (Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany; or Imagenet, Topcon Corporation, Tokyo, Japan). All included patients were over the age of 50 years, had not undergone any previous ophthalmic surgery, except for cataract removal, and did not have other retinal disorders besides AMD.

A total of 144 patients were treated at the Department of Ophthalmology of the Radboud university medical centre, Nijmegen, the Netherlands and 182 at the University of Cologne, Germany. These patients were included in the European Genetic Database (EUGENDA), a multicenter database for the clinical and molecular analysis of AMD. The remaining 50 patients were treated at the McGill University Health Center, Montreal, Canada. All patients received the loading dose of three consecutive monthly intravitreal injections of 0.5 mg ranibizumab (Lucentis; Novartis Pharma AG, Basel, Switzerland). Afterwards, they were followed up monthly and treated on a pro re nata regimen at the clinics of Nijmegen and Cologne and on a treat-and-extend regimen at the clinic of Montreal. In Nijmegen and Cologne (pro re nata regimen), in cases of persistence or recurrence of choroidal neovascularization, which was defined as loss of VA of ≥ 5 ETDRS letters, leakage seen on FA, fluid seen by OCT, or new macular hemorrhage or fluid, three consecutive monthly ranibizumab injections were administered. VA was assessed before treatment and after the three loading injections for all patients. VA was also recorded after 6 months of treatment for 262 patients. For 303 patients, Snellen VA measurements were recorded retrospectively and 73 patients were followed up prospectively using ETDRS VA. Baseline variables were collected using questionnaires or retrieved from the patient files. One eye was selected per patient; if both eyes received treatment, the first eye treated was selected and if treatment started simultaneously, the study eye was chosen randomly.

## First phase – Pooled DNA Based GWAS in the Melbourne Discovery Cohort

In the first phase of the study, a GWAS using a DNA pooling approach was conducted (Macgregor et al.[37]). A peripheral blood sample was collected from each patient and DNA was extracted (Qiagen, Victoria, Australia). Genomic DNA was quantified by Nanodrop

Specrophotometer (Thermo Scientific) for a DNA concentration ≥175ng/µl. A total of 285 samples were selected for the pooling cohort (12 samples were excluded due to a low DNA concentration required for pooling). Each pool was given a unique ID number, based on presenting VA. Non-responders from each pool were classified as those who showed loss of ≥5 EDTRS letters VA following 6 months of ranibizumab treatment and all remaining patients were classified as responders. Six equimolar DNA pools were generated with a pool size varying from 9 to 133 samples (Supplementary Table S2). An equal amount of DNA from each sample (varying from 0.2µg to 0.6µg) was added to the appropriate pool, with a minimum volume of 5µl per sample to reduce pipetting errors at low volume. Pooled genomic DNAs were assayed using an Illumina Human Omni5-Quad Bead Chip that captured 4.3 million SNP markers. We randomised the pooled samples on the genotyping arrays to avoid spurious associations that may arise from array position. Approximately 4,600 indels or copy number variants were removed from the analyses. We applied stringent quality control (QC) as previously described (Lu et al.[38]). Briefly, we excluded SNPs with minor allele frequency (MAF) ‹1% from the reference panel of CEU (Utah residents with ancestry from northern and western Europe) samples in the 1000 Genome Project. Thus, the number of SNPs was reduced to 2.5 million. Further QC criteria for excluding SNPs from the analysis were: 1) SNPs with more than 10% negative probe scores; 2) a sum of the mean raw red and green intensity values less than 1200 for each array (to ensure calibration); 3) SNPs with a small number of expected probes in pools (SNPs with MAF between 1-5% required at least half of the expected number of probes, and SNPs with MAF › 5% required at least a third; the expected number of probes was calculated as the average number of probes per pool per SNP multiplied by the number of samples in each pool); 4) non-autosomal SNPs; 5) SNPs where the variance of the estimated allele frequency was significantly different between all pools. After applying these QC filters, a total of 1,940,408 SNPs were retained for further analysis. A GWAS was carried out between responders and non-responders from three different baseline VA categories and then results were combined in a meta-analysis, which included 225 responders and 60 non-responders.

## Second Phase – Technical Replication in the Melbourne Discovery Cohort

In the second phase, the most interesting associations found in the pooled DNA GWAS were validated by undertaking individual genotyping of the original 285 samples, plus the inclusion of the additional 12 patient samples that subsequently became available whose initial concentration was not enough for the pooling GWAS. These samples were genotyped individually for 44 SNPs using the MassArray platform (Agena Bioscience, San Diego, CA) and performed as previously described[36].

## Third Phase – Replication in an Independent Cohort of European Descent

In the third phase of the study, SNPs that showed association ($p<0.05$ after correction for multiple testing) in the second phase of the study were analysed in an independent ranibizumab-treated AMD cohort of European descent. DNA was extracted and quantified in Nijmegen, the Netherlands. Subsequently, DNA samples of a total of 376 AMD patients

were genotyped in Melbourne using the MassArray platform (Agena Bioscience, San Diego, CA) as previously described[36].

## Statistical analysis

Linear regression was undertaken to assess the role of non-genetic factors (potential cofounders), including age, sex, smoking status, CNV size, lesion type, number of injections and baseline VA, on the dependent variable "change in VA" at 3 and 6 months respectively.

The GWAS of pooled DNA was undertaken at the Queensland Institute of Medical Research (QIMR) as previously described[37]. A customised pipeline developed at QIMR was used for data analysis[37,38]. Briefly, a GWAS was run within each pool comparison, i.e. a linear model based approach was used for association between SNP and allele frequency difference in responder compared to non-responder pools accounting for pooling errors. A meta-analysis was performed summarising the GWAS results from the multiple pool comparisons, weighted by inverse variances. Finally, we applied post-analysis checks to the meta-analysis results in order to reduce the chance of false positive findings, filtering any SNP that had results from only one of the multiple pool comparisons and those with large discrepancies in association results of the SNP itself and its proxies.

For individual genotyping data, logistic regression was used to assess differences in allele frequency between the responder and non-responder groups (defined in the same manner as for the GWAS) and adjusted for baseline VA in the discovery cohort and baseline VA and age at first injection in the replication cohort. In this replication cohort, different treatment regimens were administered after the 3 initial monthly injections. Therefore, we stratified the analysis for the replication cohort into two groups; pro re nata and treat-and-extend. These groups consisted of 215 pro re nata (Nijmegen and Cologne) and 47 treat-and-extend (Montreal) patients respectively. Results are reported as odds ratios (OR), 95% confidence intervals (95% CI) and p value with statistical significance being defined as $p < 0.05$ following Bonferroni correction for multiple testing. For the analyses, the statistical software Plink V1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) and SPSS IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, New York, USA) were used. To prevent any possible confounding, the analyses performed in the both the Melbourne discovery and the replication cohort were corrected for baseline VA.

Meta-analysis of the discovery and replication cohorts was performed using Metal software[39]. For 3 month meta-analysis all the patients from Melbourne discovery and the replication cohort were included because they all were treated with 3 initial doses of anti-VEGF injections while for the 6 months analysis we considered the fact that the Melbourne discovery cohort was treated using a pro re nata strategy. Therefore, we included only the 215 pro re nata treated patients from Nijmegen and Cologne and excluded the 47 patients from Montreal who were treated with a treat-and-extend regimen.

# Supplementary material

TABLE S1 **Analysis of the influence of demographic and clinical variables on the change in VA after 3 and 6 month of anti-VEGF treatment in the Melbourne discovery cohort and the replication Cohort**

| | Melbourne Discovery Cohort | | | | Replication Cohort | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 Months | | 6 Months | | 3 Months | | 6 Months | |
| **Characteristics** | ***P*-Value** | **β (CI 95%) SE** | ***P*-Value** | **β (CI 95%) SE** | ***P*-Value** | **β (CI 95%) SE** | ***P*-Value** | **β (CI 95%) SE** |
| Gender (Male vs Female) | 0.86 | -0.26 (-2.7-2.1) 0.93 | 0.84 | -0.311(-3.4-2.84)1.58 | 0.89 | 0.33 (-1.13 - 4.78) 2.26 | 0.33 | 2.78 (-2.88 – 8.45) 2.86 |
| Age | 0.27 | -1.10 (-0.2-0.05) 0.10 | 0.45 | -0.07(-0.2-0.13)0.10 | **<0.01** | -0.42 (-0.70 - -0.13) 0.14 | 0.06 | -0.37 (-0.75 – 0.11) 0.19 |
| Baseline VA | **<0.001** | -0.24 (-0.3 -0.17) 0.03 | **<0.001** | -0.32 (-0.32 to -0.15)0.04 | **0.001** | -0.21 (-0.33 - -0.09) 0.06 | **0.04** | -0.18 (-0.35 – - 0.007) 0.09 |
| Smoking | 0.71 | 0.53 (-1.9-2.9) 1.48 | 0.42 | 1.27(-1.84 4.38) 1.58 | 0.33 | 2.21 (-2.29 - 6.71) 2.28 | 0.37 | 2.61 (-3.14 – 8.36) 2.90 |
| Type of lesion | 0.30 | 2.1 (-1.2-5.5) 2.0 | 0.45 | -1.74(-6.2 to 2.78)2.2 | 0.63 | 1.23 (-3.87 – 6.33) 2.58 | 0.50 | -2.21 (-8.68 – 4.27) 3.27 |
| Size of CNV | 0.91 | 0.18 (-2.8-3.2) 1.83 | 0.53 | -1.27(-5.2 to 2.73)2.0 | 0.12 | -0.48 (-1.08 – 0.133) 0.31 | 0.08 | -0.71 (-1.51 - 0.09) 0.41 |
| Number of injections | - | - | 0.25 | -3.4(-5.9 to -1.9)0.71 | - | - | - | - |

*P*-value calculated using a linear regression test for change in VA and non-genetic covariates, β: coefficient of the linear regression, CI: confidence Interval, SE: Standard Error, VA: Visual Acuity, CNV: Choroidal Neovascularization.

TABLE S2 **Design of DNA pools, pool comparisons and meta-analysis in pooled GWAS in the Melbourne discovery cohort (1st phase of study)**

| Pool ID (responder) | Presenting VA ETDRS Letters | Pool size | Male/Female n (%) | Mean age ± SD (years) | | Pool ID (non-responders) | Presenting VA ETDRS Letters | Pool size | Male/Female N (%) | Mean age ± SD (years) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | = 36 -69 | 133 | 48(17.0)/85(29.9) | 78.9±6.3 | VS | 2 | =36 -69 | 44 | 21(7.4)/23(8.0) | 79.0±8.6 |
| 3 | ≤ 35 | 46 | 23(8.0)/23(8.0) | 79.9±7.0 | | 4 | ≤ 35 | 7 | 1(0.3)/6(2.1) | 80.8±7.6 |
| 5 | ≥ 70 | 46 | 19(6.6)/27(9.5) | 79.8±7.7 | | 6 | ≥ 70 | 9 | 3(1.1)/6(2.1) | 80.7±8.0 |

| **Meta-analysis of pool comparisons** | **Responder/Non-responders** |
|---|---|
| 1, 3,5 vs. 2,4,6 | (225 individuals)/ (60 individuals) |

Different pool sizes lead to differences in pooling variance; thus pools were combined to have similar sizes (responders checked for association against non-responders). Mean age (at time of first treatment with anti-VEGF). SD=standard deviation. vs.=versus.

TABLE S3 HaploReg database showing variants located within predicted regulatory regions and in LD ($r^2$ › 0.8) with rs4910623 (+/- 250Kb)

| Chr | pos (hg19) | LD($r^2$) | LD(D') | Variant | Ref | Alt | EUR (freq) | Motifs Changed | RefSeq Genes | Functional Annotation |
|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 4372783 | 0.87 | 0.96 | rs4910613 | A | C | 0.49 | - | 16kb 3' of *OR52B4* | - |
| 11 | 4377981 | 0.94 | 0.98 | rs10835954 | C | T | 0.5 | Pou5f1 | 11kb 3' of *OR52B4* | - |
| 11 | 4387760 | 0.99 | 1 | rs12293167 | G | T | 0.5 | 5 altered motifs | 731bp 3' of *OR52B4* | - |
| 11 | 4389587 | 1 | 1 | rs7929171 | G | C | 0.51 | DMRT1,DMRT4,LUN-1 | *OR52B4* | 5'-UTR |
| **11** | **4389639** | **1** | **1** | **rs4910623** | **G** | **A** | **0.51** | **GATA,TCF4** | **22bp 5' of *OR52B4*** | **-** |
| 11 | 4395551 | 0.91 | -0.98 | rs1895914 | C | T | 0.51 | Nkx2,Sox | 5.9kb 5' of *OR52B4* | - |
| 11 | 4396029 | 0.92 | -0.99 | rs12575038 | T | C | 0.51 | TR4 | 6.4kb 5' of *OR52B4* | - |
| 11 | 4399936 | 0.88 | -0.99 | rs7124081 | A | C | 0.52 | CEBPB | 6.2kb 3' of *TRIM21* | |

HaploReg http://www.broadinstitute.org/mammals/haploreg/haploreg.php

TABLE S4 The Ocular Tissue Database shows expression of the *OR52B4* gene in adult human eye tissues

| Seq name | start | stop | strand | Probe Id | GENE | [1]PLEIR Score in different tissues of Eye | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Choroid & RPE | Optic nerve | Optic nerve head | Retina |
| chr11 | 4388614 | 4391330 | - | 3360136 | *OR52B4* | 17.3781 | 22.4866 | 22.9475 | 25.5724 |

[1] Expression of genes in the Ocular Tissue Database (https://genome.uiowa.edu/otdb/) is represented as an Affymetrix Probe Logarithmic Intensity Error (PLIER) number. RPE: retinal pigment epithelium

TABLE S5 Pooled GWAS association of previously associated known AMD risk genes with change in VA at 6 month of anti-VEGF treatment in Melbourne discovery cohort

| GENE | SNP | *P*-value |
|---|---|---|
| *VEGF-A* | | |
| | rs699946 | 0.24 |
| | rs3025000 | 0.04 |
| *ARMS2* | | |
| | rs10490924 | 0.85 |
| *HTRA1* | | |
| | rs2284665[+] | 0.09 |
| | rs932275[+] | 0.06 |
| *CFH* | | |
| | rs3753394 | 0.54 |
| | rs800292 | 0.33 |
| | rs1065489 | 0.23 |
| | rs7529589* | 0.55 |

[+]SNPs in high LD with rs11200638 ($r^2$›0.8). *SNP in high LD with rs1061170 ($r^2$=1)

# Additional information

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## Authors' contributions

PNB, RHG : designed the study, MR, LLM, AJR, SM, LY, GM, and KB performed experiments and data analysis, RHG, AO, RKK, JC, PM, LA, TS, SF, DS, FVA, EKdJ, CBH Sample collection, MR, LLM, AJR, EKdJ, SM, LY, RHG, AIdH, PNB manuscript preparation and proof reading.

## Competing Financial Interest

No competing financial interest exists for any author.

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# 2.3

# *Rare variants in C10ORF88 and UNC93B1 are associated with response to anti-VEGF therapy in age-related macular degeneration*

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# Abstract

**Importance**: Visual acuity (VA) outcome differs considerably among neovascular age-related macular degeneration (nAMD) patients treated with anti-VEGF drugs.

**Objective**: The purpose of this study was to identify genetic factors associated with variability in the response to anti-VEGF therapy for nAMD. Identification of pharmacogenetic associations may help to understand the mechanisms underlying this variability, as well as to pave the way for personalized treatment in nAMD.

**Design**: Cohort study.

**Setting**: Multicenter.

**Participants**: 2,058 nAMD patients (678 were included in the discovery phase, and 1,380 in the replication phase).

**Main Outcome(s) and Measure(s):** VA change after the loading dose of three monthly anti-VEGF injections.

**Results**: The mean age at baseline for all patients included in the study was 78 years (SD=7.4), and 58.8% of the patients were of female sex. The mean baseline VA was 51.3 Early Treatment Diabetic Retinopathy Study (ETDRS) letters (SD=20.3), and the mean change in VA after the loading dose of 3 monthly injections was a gain of 5.1 ETDRS letters (SD=13.9). Genome-wide single variant analyses of common variants revealed five independent loci that reached a *P*-value$<10^{-5}$. After replication and meta-analysis of the lead variants, rs12138564 located in the *CCT3* gene remained nominally associated with a better treatment outcome ($P=1.38\times10^{-5}$, $\beta=0.034$, SE($\beta$)=0.008). Genome-wide gene-based analyses of rare variants showed genome-wide significant associations for the C10orf88 and *UNC93B1* genes ($P=4.22\times10^{-7}$ and $P=6.09\times10^{-7}$, respectively), in both cases leading to a worse treatment outcome. Patients carrying rare variants in the *C10orf88* gene lost a mean of 30.6 ETDRS letters after treatment, and patients carrying rare variants in *UNC93B1* lost a mean 26.5 ETDRS letters after treatment.

**Conclusions and relevance:** Our results suggest that rare protein-altering variants in the *C10orf88* and *UNC93B1* genes are associated with worse response to anti-VEGF therapy for nAMD. There was a limited contribution of common genetic variants to variability in nAMD treatment response with a nominal association of rs12138564 in *CCT3* with better treatment outcome. This study for the first time identified a role for rare genetic variation in treatment response of nAMD, which may be used to adapt treatment strategies to individual needs.

# Introduction

Advanced age-related macular degeneration (AMD) is a leading cause of blindness in the elderly[1,2]. The most vision-impairing type of advanced AMD is the neovascular form (nAMD), which is responsible for the majority of the visual acuity (VA) loss caused by this disease[3]. In nAMD, the proliferation of aberrant new blood vessels leads to severe scarring of the central retina[4]. Currently, the most effective treatment for nAMD is intravitreal injections of anti-vascular endothelial growth factor (VEGF) antibodies[5,6]. Although this treatment has resulted in dramatic improvements in VA for many nAMD patients, a high degree of variability in response to these drugs has been observed. In fact, around 10% of the nAMD patients show a decline in VA of at least 15 letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) letter chart despite treatment[5-7].

Early identification of patients with poor treatment response is a critical step in optimizing AMD treatment. Patients classified as non-responders based on an absence of VA improvement after anti-VEGF injections, may indeed have better outcomes with higher frequency of dosing along with regular monitoring[8]. Also, alternative therapies with the potential for longer action are currently being developed for nAMD[9], and it is possible that other therapeutic options will become available for patients who do not achieve improvements in vision with current strategies. Therefore, establishing which factors are involved in treatment response variability could aid in the stratification of patients for the best treatment regime or therapeutic option[10].

Several studies have shown that clinical and epidemiological factors associated with disease severity before treatment can influence the anti-VEGF treatment outcome[11]. Genetic factors have also been indicated to affect treatment outcome in nAMD patients, although contradictory results have been reported[12]. Anti-VEGF treatment seemed to be less effective in nAMD patients carrying the AMD risk variant rs1061170 (p.Y402H) in the *CFH* gene in a meta-analysis of 13 different studies[13]. In addition, other studies have reported pharmacogenetic associations with other AMD-associated variants, variants involved in the VEGF neovascularization pathway and variants in other genes[12,14-31]. However, pharmacogenetic analysis in randomized controlled trials such as the CATT and IVAN studies did not reveal any associations with genetic variants[32-36].

The pharmacogenetic studies reported so far focus on a limited amount of variants or are limited in sample size and therefore statistical power[12,14-33]. Moreover, these studies have been restricted to the analysis of common variants. Rare variants, which may have stronger effects and can pinpoint underlying mechanisms by identifying the gene involved, have not yet been explored[37,38]. Consequently, larger and more comprehensive genetic studies are needed, targeting both common and rare variation in the genome.

Employing the resources of the International AMD Genomics Consortium (IAMDGC) and additional nAMD cohorts treated with anti-VEGF, we performed a multicenter genome-wide association study in order to: 1) evaluate in a hypothesis-free approach the association

of common genetic variants with VA treatment response to anti-VEGF therapy for nAMD, and 2) evaluate for the first time the cumulative effect of rare protein-altering variants on VA treatment response to anti-VEGF therapy for nAMD. Identification of pharmacogenetic associations is of great relevance in the AMD field, as they can help to identify underlying causal genes and thus mechanisms, suggest potential new drug targets, and can be used as robust biomarkers for precision medicine.

# Methods

## Study cohorts

Patients for both the discovery and replication cohorts of this study were recruited from multiple clinics, as detailed in Table 1. The discovery cohort included nAMD patients who were evaluated as part of the International AMD Genomics Consortium (IAMDGC) project[39]. All groups collected data according to Declaration of Helsinki principles. Study participants provided informed consent, and protocols were reviewed and approved by local ethics committees.

Inclusion criteria for all study center patients were defined as neovascularization secondary to AMD confirmed by fluorescein angiogram or optical coherence tomography, age greater or equal to 50 years, and a loading dose of three consecutive injections of bevacizumab (Hoffmann-La Roche, Basel, Switzerland) or ranzibizumab (Hoffmann-La Roche, Basel, Switzerland / Novartis Basel, Switzerland) therapy at monthly intervals (± two weeks). We excluded eyes with other retinal morbidities such as myopia greater than 8 diopters, ocular surgery during follow-up or in the two months prior to treatment, previous treatment for neovascular disease, macular hole, staphyloma, or a VA lower than 2.3 logMAR (0 ETDRS letters). Information about gender, age at first injection, as well as baseline VA before anti-VEGF treatment and after three monthly injections (± two weeks) was collected. VA was collected in ETDRS letters or Snellen and was transformed into logMAR for analysis. Influence of the variables age and baseline VA on the change in VA after 3 months was assessed including these variables in a general linear model.

## Exome array genotyping and quality control

DNA samples from patients included in the discovery cohort were uniformly genotyped with a custom-modified HumanCoreExome array (Illumina) by the IAMDGC, at the Center for Inherited Disease Research (CIDR). Genotype quality control and imputation was performed by the IAMDGC, as has been previously detailed[39]. Imputation provided information on 28,930,739 variants. Principal components analysis (PCA) was performed and only European-descent individuals, based on the PCA, were included in the analysis.

Informative PCA eigenvalues were additionally used as covariates to adjust for population stratification. Identity-by-descent analysis was performed and samples with a relatedness score (PI-HAT)›0.25 were excluded.

## Treatment outcome measurement

The outcome measure was a functional response defined as the change in visual acuity after treatment (ΔVA), which was defined as the initial VA before treatment subtracted from the final VA after three anti-VEGF injections. The logMAR VA values for the initial and final visual acuity distributions were evaluated for normal distribution amongst each study cohort, and outliers (defined as mean+3*standard deviation) were adapted to mean±2*SD.

## Genome-wide analyses of common variant

Variants with imputation quality score ($R^2$) less than 0.6 and minor allele frequency (MAF)‹0.05 were excluded from the common variant analysis. Genome-wide single variant association analyses using the ΔVA as the testing variable were performed including the first two ancestry principal components (PC1, PC2), baseline VA and age at first injection as a covariates. Common variants (MAF≥0.05) were tested via a quantitative linear regression model (linear-Wald testing) using the q.lm package in the EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS). In order to account for inter-cohort heterogeneity, the analyses were performed separately in the five discovery cohorts and covariate-adjusted estimates of the genetic effects were subsequently combined in a meta-analysis, which comprised a total of 678 unrelated European descent nAMD patients. Meta-analysis was performed with METAL[40] based on effect size estimates, and standard errors per cohort. Genomic control was applied and if lambda was greater than 1, adjustments were made for the inflation factor per cohort. A threshold for suggestively associated variants from the discovery cohort was set at $P=10^{-5}$. Final results were visualized using Manhattan and Q-Q plots generated with the R package qqman (https://cran.r-project.org/web/packages/qqman/index.html). Locus Zoom was used to visualize the LD structure block in each associated locus (http://locuszoom.org/)[41].

## Genotyping and association analyses in replication cohorts

The lead variants of the loci that reached the threshold for suggestive significance ($P$-value‹$10^{-5}$) were selected for genotyping in independent replication cohorts. These were rs241692 (chr3:60,410,187), rs12138564 (chr1:156,291,600), rs13002976 (chr2:10,678,538), rs242939 (chr17:43,895,579), and rs2237435 (chr7:41,731,053).

Genotyping in the replication phase was performed using KASP technology (LGC Group, Middlesex, United Kingdom) and the MassARRAYiPlex technology (Agena Bioscience, San Diego, CA, USA. All variants fell within Hardy-Weinberg equilibrium measures.

TABLE 1 Demographics and clinic characteristics of the discovery and replication cohorts

| | Discovery phase (n=678) | | | | | Replication phase (n=1,380) | | | | | | Total (n=2,058) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cologne | Jerusalem | Nijmegen | Melbourne | Sydney | Jerusalem | Melbourne | Leeds | BRAMD | IVAN | EUGENDA | |
| **N** | 155 | 113 | 121 | 119 | 170 | 146 | 88 | 220 | 215 | 542 | 169 | |
| **Age at first injection (years) [mean (SD)]** | 76.9 (7.2) | 77.4 (7.5) | 77.5 (7.2) | 79.1 (7.2) | 77.4 (7.9) | 79.3 (8.8) | 79.7 (6.9) | 80.2 (5.9) | 77.6 (7.0) | 77.6 (7.4) | 76.7 (7.5) | 78.0 (7.4) |
| **Sex (female) [%]** | 58.7 | 60.2 | 49.6 | 57.1 | 62.9 | 61.0 | 46.6 | 62.7 | 54.4 | 58.5 | 68.0 | 58.8 |
| **Baseline VA (logMAR) [mean (SD)]** | 0.657 (0.367) | 0.757 (0.616) | 0.682 (0.373) | 0.715 (0.350) | 1.039 (0.407) | 0.598 (0.562) | 0.762 (0.504) | 0.659 (0.289) | 0.509 (0.265) | 0.654 (0.333) | 0.547 (0.362) | 0.674 (0.406) |
| **Equivalent baseline VA (ETDRS letters) [mean (SD)]** | 52.2 (18.4) | 47.2 (30.8) | 50.9 (18.7) | 49.3 (17.5) | 33.1 (20.4) | 55.1 (28.1) | 46.9 (25.2) | 52.1 (14.5) | 59.6 (13.3) | 52.3 (16.7) | 57.7 (18.1) | 51.3 (20.3) |
| **Change in VA after 3 months (logMAR) [mean (SD)]** | 0.051 (0.274) | 0.065 (0.515) | 0.124 (0.284) | 0.150 (0.231) | 0.107 (0.310) | 0.106 (0.384) | 0.161 (0.301) | 0.116 (0.203) | 0.085 (0.176) | 0.094 (0.237) | 0.106 (0.207) | 0.101 (0.277) |
| **Equivalent change in VA after 3 months (ETDRS letters) [mean (SD)]** | 2.6 (13.7) | 3.3 (25.8) | 6.2 (14.2) | 7.5 (11.6) | 5.4 (15.5) | 5.3 (19.2) | 8.1 (15.1) | 5.8 (10.2) | 4.3 (8.8) | 4.7 (11.9) | 5.3 (10.4) | 5.1 (13.9) |

Cologne = University Hospital of Cologne, Cologne; Jerusalem = Hadassah-Hebrew University Medical Center, Jerusalem; Nijmegen = Radboud university medical center, Nijmegen; Melbourne = Centre for Eye Research Australia, Melbourne; Sydney = Centre for Vision Research, Sydney; Leeds = St. James's University Hospital, Leeds; BRAMD = BRAMD trial cohort, IVAN = alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN) trial cohort, EUGENDA = European Genetic Database study cohort, N = number of patients, SD = standard deviation, VA = visual acuity, logMAR = logarithm of the minimum angle of resolution, ETDRS = Early Treatment Diabetic Retinopathy Study. ETDRS letters equivalents were calculated in the following manner: ETDRS letters = 85 – logMAR/0.02. Change in VA after 3 months of treatment was calculated in the following manner: $VA_{\text{before treatment}} - VA_{\text{after 3 months of treatment}}$.

TABLE 2 Single-variant association analyses of five lead variants in the discovery GWAS and replication phase

| | | | | | Discovery phase (n=678) | | Replication phase (n=1,380) | | Discovery & replication (n=2,058) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Chr.** | **Position[a]** | **Lead variant** | **MA** | **Gene[b]** | **β (SE)** | ***P-value*** | **β (SE)** | ***P-value*** | **β (SE)** | ***P-value*** |
| 1 | 156,291,600 | rs12138564 | T | *CCT3* | 0.079 (0.016) | $5.74\times10^{-7}$ | 0.019 (0.009) | 0.029 | 0.034 (0.008) | $1.38\times10^{-5}$ |
| 2 | 10,678,538 | rs13002976 | G | *NOL10* | -0.080 (0.016) | $1.21\times10^{-6}$ | 0.009 (0.008) | 0.265 | -0.009 (0.008) | 0.219 |
| 3 | 60,410,187 | rs241692 | G | *FHIT* | 0.213 (0.042) | $4.46\times10^{-7}$ | -0.023 (0.017) | 0.187 | 0.011 (0.016) | 0.493 |
| 7 | 41,731,053 | rs2237435 | A | *INHBA* | -0.081 (0.018) | $6.58\times10^{-6}$ | 0.015 (0.009) | 0.109 | -0.005 (0.008) | 0.526 |
| 17 | 43,895,579 | rs242939 | C | *CRHR1* | 0.128 (0.027) | $2.25\times10^{-6}$ | -0.021 (0.017) | 0.214 | 0.020 (0.014) | 0.162 |

Chr = Chromosome, Pos = Position, MA = Minor allele, SE = Standard error.

[a]Chromosomal position according to the NCBI RefSeq hg19 human genome reference assembly. [b]Closest gene to the lead variant.

The association analysis in the replication cohort was performed with EPACTS in the same manner as the discovery cohort, except for the inclusion of PCA components as covariates. The results of all cohorts were combined in a meta analysis based on effect size estimates and standard errors. Subsequently, an overall meta-analysis of the discovery and replication phase was performed in the same manner (11 cohorts, n=2,058). Meta-analyses were performed using the software METAL[40].

## Gene-based analysis of rare variants

We performed gene based analyses using the optimal unified sequence kernel association test, SKAT-O[42], as implemented in EPACTS(http://genome.sph.umich.edu/wiki/EPACTS). When the majority of variants in a gene are associated with a trait and the effects are in the same direction, the burden test is most powerful, in contrast, SKAT is most powerful when most of the variants are not associated and the effects are in different directions. We used the SKAT-O test as it maximizes power compared to the traditional burden test and SKAT maintaining the power in both scenarios[42]. Rare and low frequency (MAF<0.05) protein-altering variants (missense, nonsense or affecting canonical splice sites) were included in the analysis. Imputed variants were only included if imputation quality ($R^2$) was ≥0.8. As in the single variant analysis for common variants, only non-related individuals of European descent were included in the analysis. Association tests were adjusted for age, baseline VA and the first two ancestry principal components, and a sensitivity analysis adjusting for ten ancestry principal components was additionally performed. The summary statistics for each single variant were extracted from a comparable single variant analysis using EPACTS (http://genome.sph.umich.edu/wiki/EPACTS).

# Results

## Characteristics of the study cohorts

We collected demographic and VA treatment response information for 2,058 nAMD patients who received anti-VEGF therapy. In the discovery phase, 678 patients of five different cohorts were genotyped with exome arrays by the IAMDGC[39] and used for genome-wide association analyses on common and rare variants. In the replication phase, 1,380 individuals from six different cohorts were genotyped for common variants identified in the discovery study.

Demographics and clinical parameters of the study cohorts are described in Table 1. The mean change in VA after the loading dose of 3 monthly injections for all patients included in the study was 0.101 logMAR (logarithm of the minimum angle of resolution), which corresponded to a gain of 5.1 ETDRS letters, and varied per cohort (Table 1). Age and visual

acuity at baseline have been the factors most consistently described as influencing change in VA after anti-VEGF treatment[43-46]. This was also true for the total patient population and in the majority of the individual cohorts (eTable 1). Therefore, these variables were included as covariates in all our subsequent analyses.

## Genome-wide association analyses of common variants identify a nominal association of rs12138564 in the *CCT3* gene with better response to anti-VEGF therapy in nAMD

In the discovery phase, we performed genome-wide single-variant association analyses on the change in VA after 3 monthly anti-VEGF injections. Linear regression models adjusted for age, baseline VA and the first two ancestry principal components were conducted on 6,089,769 quality-controlled common variants (minor allele frequency, MAF≥0.05).

We identified a total of 111 variants that reached a suggestive significance level (*P*-value<$10^{-5}$) (Figure 1A). The overall genomic inflation factor was very low ($\lambda = 1.01$), implying no confounding effect of the population's genetic structure on the association analyses (eFigure 1). Consecutive conditional analysis revealed that these variants were distributed across five different loci, for which the lead variants were rs12138564, rs13002976, rs241692, rs2237435 and rs242939. The results for these variants are described in Table 2, and the details of the associations per cohort are presented in eTable 2.

In the replication phase, the lead variants of the five associated loci were analyzed in six independent anti-VEGF treated nAMD patient cohorts, which comprised a total of 1,380 patients. SNP rs12138564 on chromosome 1 was nominally associated with functional treatment response in the replication phase, showing the same direction of effect as in the discovery phase ($P_{replication}$=0.029, β=0.019, SE(β)=0.009) (Table 2). This association was mainly driven by the cohort of St. James's University Hospital, Leeds ($P_{Leeds}$=0.028, β=0.044, SE(β)=0.020) as no association was observed for this variant in any of the other five replication cohorts (eTable 2). The other four lead variants did not show an association in the replication phase.

The results of the discovery and replication phase were combined in an overall meta-analysis of eleven cohorts, including 2,058 nAMD patients (Table 2, eTable 2). The association of SNP rs12138564 with functional treatment response remained nominally significant, showing a positive effect of the minor allele on treatment outcome ($P_{meta}$=1.38x$10^{-5}$, β=0.034, SE(β)=0.008) (Table 2, eTable 2). SNP rs12138564 is located in intron 8 of the Chaperonin Containing TCP1 Subunit 3 (*CCT3*) gene (Figure 1B). The other four lead variants did not replicate and their association was lost after the meta-analysis of the discovery and replication cohorts (Table 2, eTable 2).

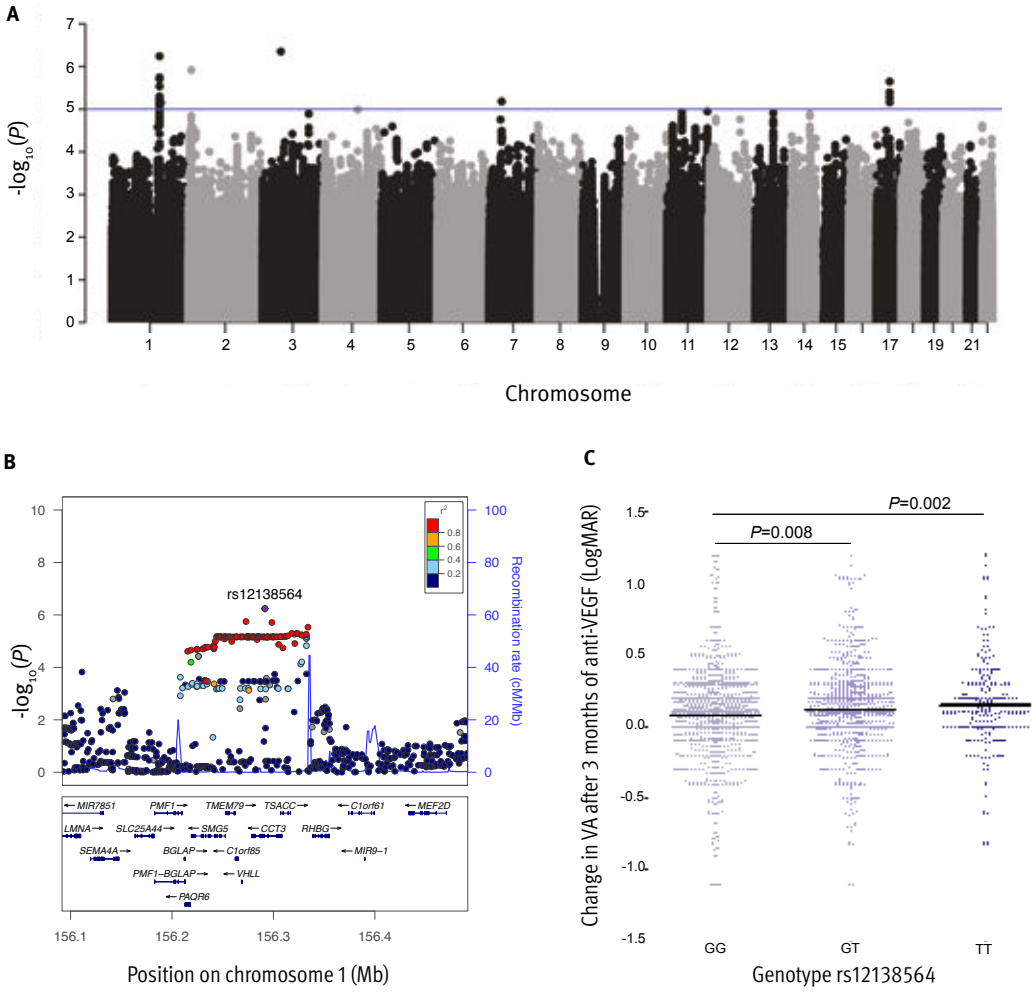



**FIGURE 1 Single-variant association analyses of common variants on response to anti-VEGF therapy in neovascular AMD**

**A)** Genome-wide single-variant association analysis for common variants in a discovery cohort of 678 nAMD patients identified five loci reaching suggestive significance. The Manhattan plot illustrates the -log(*P*-values) of each individual SNP tested for association. The horizontal blue line indicates the threshold considered for suggestive significance (*P*-value<$10^{-5}$). **B)** Locus track plot showing a detailed view of the chromosome 1 locus with rs12138564 in the *CCT3* gene as the lead SNP shown with a purple rhombus. The linkage disequilibrium (LD) structure of the area was constructed with this SNP as reference. **C)** Change in VA after 3 months of anti-VEGF treatment stratified by rs12138564 genotype in the discovery and replication cohorts. The homozygous genotype for the reference allele (GG) showed a mean improvement in VA of 0.079 logMAR or approximately 4 ETDRS letters, whereas the heterozygous GT group showed a mean improvement in VA of 0.118 LogMAR or approximately 6 ETDRS letters (*P*=$8x10^{-3}$), and the homozygous TT group an improvement 0.150 logMAR or approximately 7.5 ETDRS letters (*P*=$2x10^{-3}$).
VA = visual acuity, logMAR = logarithm of the minimum angle of resolution.

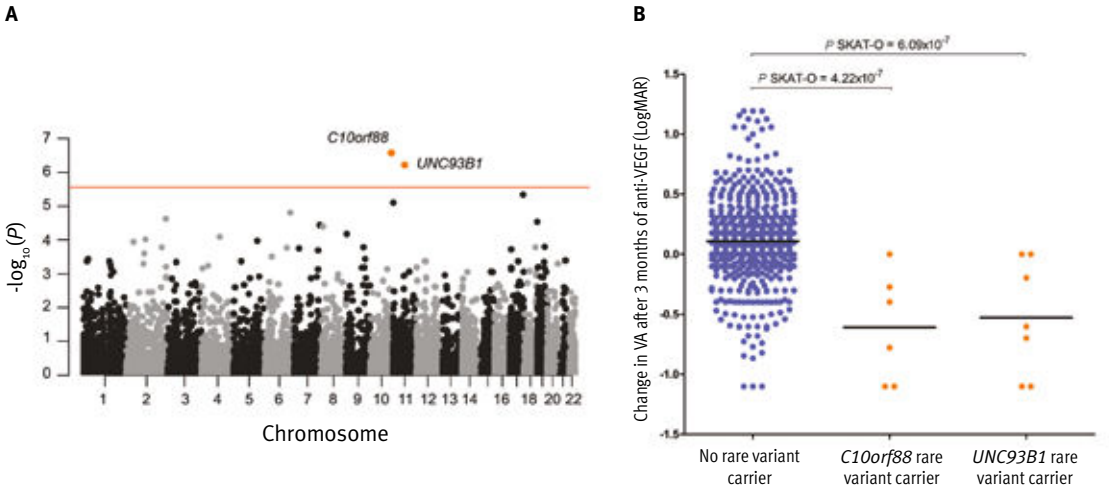



FIGURE 2 **Gene-based analysis of rare variants on response to anti-VEGF therapy in neovascular AMD**

**A)** Manhattan plot illustrating the -log(*P*-values) of each gene analyzed in the SKAT-O gene-based test of rare variants. The red line indicates the genome-wide significance level ($P<3.38x10^{-6}$=0.05/14,788). A genome-wide significant burden of rare protein-altering variants in the *C10orf88* and *UNC93B1* genes was associated with worse response to anti-VEGF therapy.
**B)** Change in VA after 3 months of anti-VEGF treatment stratified by non-carriers of rare variants and carriers of rare variants in *C10orf88* and *UNC93B1*. Horizontal lines indicate the mean change in VA after 3 months of anti-VEGF treatment per group. Carriers of rare variants in *C10orf88* who did not carry rare variants in *UNC93B1* were included in the SKAT-O test of *UNC93B1* as non-carriers and vice versa. Non-carriers gained on average 0.109 logMAR or 5.5 ETDRS letters of VA after treatment, while carriers of rare variants in *C10orf88* lost 0.609 logMAR or 30.6 ETDRS letters and carriers of rare variants in *UNC93B1* lost 0.529 logMAR or 26.5 ETDRS letters on average.
VA = visual acuity, logMAR = logarithm of the minimum angle of resolution.

Next, we analyzed the genotypic effects of SNP rs12138564 on change in VA in the discovery and replication cohorts combined. The heterozygous GT genotype group showed an increased improvement in VA after anti-VEGF treatment compared to the reference GG genotype group ($P=8x10^{-3}$), and the homozygous TT group showed the largest improvement ($P=2x10^{-3}$). The homozygous genotype for the reference allele (GG) showed a mean improvement in VA of 0.079 logMAR or approximately 4 ETDRS letters, whereas the heterozygous GT group showed a mean improvement in VA of 0.118 LogMAR or approximately 6 ETDRS letters, and the homozygous TT group an improvement 0.150 logMAR or approximately 7.5 ETDRS letters (Figure 1C).

Additionally, variants shown to be associated with treatment response in previous studies[12,14-31] were extracted from the GWAS results. No variants reached genome-wide significance, nor were they suggestively associated (*P*-value<0.05/18=0.003) with functional treatment response in this study (eTable 3). We also determined whether the 52 AMD-associated variants reported by the largest AMD case-control GWAS study performed so far[39] were associated with functional treatment outcome. Out of the 52

AMD-associated variants, 47 were present in one or more of our study cohorts but none of these were found to be associated with VA response at either suggestive significance (*P*-value<0.05/47=0.001) nor at the genome-wide significance level (eTable 4).

## Gene-based analysis identifies a burden of rare variants in the *C10Orf88* and *UNC93B1* genes associated with worse response to anti-VEGF therapy in nAMD

Using the rare variation content of the IAMDGC exome array, we analyzed the cumulative effect of rare protein-altering variants on nAMD functional treatment response. The analysis was performed on the 678 patients of European descent genotyped with exome arrays. We performed SKAT-O[42] gene-based tests of quality controlled variants with a MAF<0.05. A total of 58,414 protein-altering variants classified as missense (leading to an amino acid change), nonsense (introducing a stop codon) or affecting canonical splice sites were included in the analysis. These variants were distributed in a total of 14,788 genes. The association analyses were adjusted for age, baseline VA and the first two ancestry principal components.

We identified two genes associated with VA treatment response at a genome-wide significance level (*P*-value<$3.38 \times 10^{-6}$=0.05/14,788): chromosome 10 open reading frame 88 (*C10orf88*, *P*=$4.22 \times 10^{-7}$) and unc-93 homolog B1 (*UNC93B1*, *P*=$6.09 \times 10^{-7}$) (Table 3, Figure 2A). Sensitivity analysis was carried out adjusting for eight additional ancestry principal components, and yielded comparable results (*C10orf88*, *P*=$2.24 \times 10^{-7}$; *UNC93B1*, *P*=$1.65 \times 10^{-7}$). Patients who did not carry a rare variant in either *C10orf88* or *UNC93B1* gained on average 0.109 logMAR or 5.5 ETDRS letters of VA after treatment. In contrast, the mean change in VA after treatment for the carriers of rare variants in *C10orf88* was a loss of 0.609 logMAR or 30.6 ETDRS letters. The carriers of rare variants in *UNC93B1* showed a substantial decrease in VA after treatment as well, losing 0.529 logMAR or 26.5 ETDRS letters on average (Figure 2B).

TABLE 3 **Gene-based analysis of rare variants on response to anti-VEGF therapy in neovascular AMD**

| Gene | Chromosome | Chromosomal position[a] | N rare variants | RAC | *P*-value |
|---|---|---|---|---|---|
| *C10orf88* | 10 | 124,692,082 – 124,712,511 | 3 | 7 | $4.22 \times 10^{-7}$ |
| *UNC93B1* | 11 | 67,765,163 – 67,770,499 | 2 | 14 | $6.09 \times 10^{-7}$ |

N = number, RAC = rare allele count

[a]Chromosome and chromosomal position according to the NCBI RefSeq hg19 human genome reference assembly.

All variants included in the burden tests for these two genes had a high imputation quality score ($R^2$) of 1. For *C10orf88*, three rare protein-altering variants were included in the test; two of them leading to an amino acid change (c.412G>A; p.Glu138Lys and c.827T>C;

p.Ile276Thr) and one variant introducing a stop codon (c.1258C>T; p.Gln420*). The p.Glu138Lys and p.Ile276Thr variants individually showed a nominal association with worse response to treatment ($P$=4.96x10$^{-4}$ and $P$=2.33x10$^{-5}$, respectively). The variant p.Gln420* was present in only one individual and did not show an association at the single variant level ($P$=0.120). The variants p.Glu138Lys and p.Gln420* had a CADD score >20, which indicates that they are among the 1% most deleterious substitutions in the genome (eTable 5).

Two variants contributed to the burden of the *UNC93B1* gene, both leading to an amino acid change (c.385C>A; p.Leu129Ile and c.626C>T; p.Pro209Leu). Both variants individually showed a nominal association with worse response to treatment ($P$=3.33x10$^{-7}$ for p.Leu129Ile and $P$=4.21x10$^{-7}$ for p.Pro209Leu), and had an assigned CADD score >20 (eTable 5). These two variants are in high linkage disequilibrium (r2$\simeq$1), and were simultaneously present in 7 individuals, all belonging to the Hadassah-Hebrew University Medical Center cohort from Jerusalem.

Finally, we extracted the results of the genes in which a burden of rare variation has been shown in AMD (*CFH*, *CFI*, *TIMP3* and *SLC16A8*) from the gene-based analysis[39]. A suggestive association ($P$-value<0.05/4=0.013) was found for *SLC16A8* ($P$=0.007) (eTable 6); while none of the genes reached the genome-wide significance level.

# Discussion

We have undertaken the largest multicenter pharmacogenetic cohort study of nAMD patients reported so far, and performed for the first time both a genome-wide single variant analysis for common variants and a gene-based analysis for the cumulative effect of rare variants.

We evaluated the role of genetic variation on primary functional response: change in VA after the loading dose of three monthly injections. We aimed to understand the genetic component of the variability in functional response because VA is the most relevant outcome measure for patients as it directly influences their quality of life[47]. Whether our findings on the functional response can be related to an anatomical response, commonly defined as the change in central macular thickness or retinal fluid clearance as measured by optical coherence tomography, remains to be further investigated[48]. We chose the time interval of 3 months, as three loading injections are administered widely in contrast to the follow-up treatment, which is variable per clinic and will impact on VA outcomes[49]. The majority of patients show the most improvement in their VA after the three first monthly injections[5], and this time interval can thus be predictive of a longer-term response[15]. Moreover, long-term treatment response, is likely to be affected by non-adherence to treatment protocols. As there is no consensus in the definition of a functional non-response, we analyzed the trait in a continuous manner. This type of exploratory analysis represents the full spectrum of the patient population and may facilitate comparison and interpretation of study results.

We investigated the treatment response to bevacizumab or ranzibizumab therapy in a combined analysis. The CATT[50,51] and IVAN[52,53] clinical trials have demonstrated similar outcomes after bevacizumab treatment compared to those after ranibizumab treatment, and therefore the factors underlying the treatment response variability may be similar. In order to increase our statistical power, we combined results from both treatments in a unique analysis.

The heritability of treatment response to anti-VEGF in nAMD is unknown and difficult to estimate with our sample size. Nevertheless, our study had 80% power to detect common variants of moderate effects, explaining at least 6.2% of the variance in treatment response[54]. However, no single-variant associations were found at genome-wide significance level, suggesting a limited effect of individual common variants on treatment outcome.

Our genome-wide single variant association analysis of common variants identified the variant rs12138564, located in intron 8 of the *CCT3* gene, to be nominally associated with treatment response after replication analysis, and therefore, this variant may merit further investigation in alternative cohorts.

We did not find any of the previously reported variants[12,14-31] to be associated with functional response at a genome-wide level nor at suggestive significance level (*P*-value<0.003). This may be due to differences between the studies in response definition and patient population in terms of disease stage and other environmental factors, or due to spurious findings. However, our results support the notion that none of the previously identified genetic markers, individually, are strong determinants of overall functional treatment response. Furthermore, we also did not find a genome-wide nor a suggestive association (*P*-value <0.001) for any of the 52 AMD-associated variants reported in the largest AMD GWAS performed so far[39].

The rare variant burden test revealed two genes associated with primary functional response at a genome-wide level: *C10orf88* and *UNC93B1*. The function of C10orf88 is still uncharacterized and therefore the biological link to treatment response in nAMD is unclear. It has been suggested that common variants in *C10orf88* are associated with vitamin D levels, although it cannot be excluded that the effect might be driven by the neighbouring gene *ACADSB* (acyl-Coenzyme A dehydrogenase)[55]. Additionally, *C10orf88* is expressed at low levels in the retina and RPE/choroid[56]. UNC93B1 is involved in the innate and adaptive immune response by regulating toll-like receptor (TLR) signaling[57,58]. Regulation of the TLR and their subsequent signaling pathways has been found to be associated with AMD in apoptotic response[59], as well as with activation of microglia leading to retinal inflammation[60]. TLRs are found on retinal and choroidal vasculature[61], and interact with CD46, known to be associated with regulation of the complement immune system. Dysregulation in TLR signaling and the complement system may contribute to the pathogenesis of AMD[62-64]. Therefore, the identification of rare genetic variation in *UNC93B* in our study may point towards an immune component in treatment response in AMD. Of

note, the *UNC93B* variants driving the effect in the SKAT-O test were only found in patients belonging to the Jerusalem cohort. This is in line with the higher allele frequency found for the Jewish population as compared to the non-Finish European population in the Ashkenazi Genome Consortium and ExAC databases[65,66], and therefore, this finding may be related to the genetic nature of that specific population.

Carriers of rare variants in the *C10orf88* and *UNC93B* genes lost on average 6 and 5 lines of vision in the ETDRS letter chart respectively. The effect of rare variants in VA outcome after treatment was thus very large, making these findings potentially relevant for the clinical practice[67]. Interestingly, when analyzing the genes with a burden of rare variants associated with AMD[39], we found a suggestive association (*P*-value<0.013) with functional treatment outcome for the gene *SLC16A8*.

# Conclusions

Our multicenter GWAS suggests that the variability in primary functional treatment outcome in nAMD is probably not explained by large effects of individual common variants. We identified a common variant in the *CCT3* gene nominally associated with functional primary response to anti-VEGF therapy for nAMD. However, rare genetic variants seem to have large effects on treatment outcome, as a burden of rare protein-altering variants in the *C10orf88* and *UNC93B1* genes was associated with worse VA treatment response. This suggests that rare genetic variants might be used as biomarkers for VA response in nAMD, and may allow patients to be stratified for different regimen doses or therapies.

ETABLE 1 **Association of baseline variables with response to anti-VEGF for neovascular AMD**

| | Discovery phase (n=678) | | | | | Replication phase (n=1,380) | | | | | | Total (n=2,058) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cologne | Jerusalem | Nijmegen | Melbourne | Sydney | Jerusalem | Melbourne | Leeds | BRAMD | IVAN | EUGENDA | |
| **N** | 155 | 113 | 121 | 119 | 170 | 146 | 88 | 220 | 215 | 542 | 169 | |
| **Sex (*P*-value)** | 0.558 | 0.035 | 0.989 | 0.656 | 0.686 | 0.616 | 0.364 | 0.057 | 0.021 | 0.556 | 0.316 | 0.087 |
| **Baseline VA (*P*-value)** | 0.001 | $2.03x10^{-8}$ | 0.004 | $7.17x10^{-7}$ | $7.00x10^{-6}$ | $7.33x10^{-12}$ | $4.04x10^{-7}$ | $3.36x10^{-8}$ | $1.22x10^{-4}$ | $5.53x10^{-18}$ | $1.38x10^{-4}$ | $3.41x10^{-71}$ |
| **Age (*P*-value)** | 0.016 | 0.275 | 0.041 | 0.140 | 0.077 | 0.002 | 0.286 | 0.138 | 0.610 | 0.579 | 0.061 | $8.55x10^{-7}$ |

Cologne = University Hospital of Cologne, Cologne; Jerusalem = Hadassah-Hebrew University Medical Center, Jerusalem; Nijmegen = Radboud university medical center, Nijmegen; Melbourne = Centre for Eye Research Australia, Melbourne; Sydney = Centre for Vision Research, Sydney; Leeds = St. James's University Hospital, Leeds; BRAMD = BRAMD trial cohort, IVAN = alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN) trial cohort, EUGENDA = European Genetic Database study cohort; N = number; VA = visual acuity.

ETABLE 2 **Single-variant association analyses of lead variants in the discovery GWAS and replication phase per cohort**

| | Cohort | chr1 rs12138564 | | | chr2 rs13002976 | | | chr3 rs241692 | | | chr7 rs2237435 | | | chr17 rs242939 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MAF | β (SE) | *P*-value | MAF | β (SE) | *P*-value | MAF | β (SE) | *P*-value | MAF | β (SE) | *P*-value | MAF | β (SE) | *P*-value |
| Discovery | Cologne | 0.312 | 0.052 (0.031) | 0.093 | 0.409 | -0.025 (0.031) | 0.436 | 0.067 | 0.188 (0.069) | 0.007 | 0.287 | -0.084 (0.035) | 0.018 | 0.074 | 0.041 (0.058) | 0.485 |
| | Jerusalem | 0.275 | 0.006 (0.072) | 0.935 | 0.384 | -0.132 (0.073) | 0.075 | 0.092 | 0.331 (0.114) | 0.004 | 0.276 | -0.054 (0.086) | 0.528 | 0.088 | 0.288 (0.120) | 0.018 |
| | Nijmegen | 0.309 | 0.088 (0.040) | 0.031 | 0.410 | -0.123 (0.036) | 0.001 | 0.051 | 0.233 (0.098) | 0.019 | 0.275 | -0.13 (0.041) | 0.002 | 0.094 | 0.178 (0.064) | 0.007 |
| | Melbourne | 0.251 | 0.113 (0.028) | $1.04x10^{-4}$ | 0.387 | -0.081 (0.030) | 0.009 | NA* | NA* | NA* | 0.291 | -0.072 (0.033) | 0.032 | 0.079 | 0.151 (0.051) | 0.004 |
| | Sydney | 0.315 | 0.073 (0.035) | 0.036 | 0.358 | -0.096 (0.038) | 0.012 | 0.068 | 0.178 (0.077) | 0.022 | 0.304 | -0.048 (0.039) | 0.217 | 0.104 | 0.111 (0.051) | 0.030 |
| Replication | Jerusalem | 0.459 | 0.427 (1.141) | 0.709 | 0.398 | 1.69 (1.331) | 0.207 | NA^ | NA^ | NA^ | 0.337 | 0.443 (1.183) | 0.709 | 0.092 | 0.503 (1.654) | 0.761 |
| | Melbourne | 0.268 | 0.025 (0.045) | 0.583 | NA^ | NA^ | NA^ | 0.080 | -0.113 (0.068) | 0.099 | 0.288 | -0.024 (0.04) | 0.554 | 0.080 | -0.007 (0.075) | 0.921 |
| | Leeds | 0.306 | 0.044 (0.020) | 0.028 | 0.420 | 0.014 (0.018) | 0.460 | 0.053 | -0.025 (0.042) | 0.561 | 0.276 | 0.029 (0.02) | 0.162 | 0.063 | 0.047 (0.038) | 0.214 |
| | BRAMD | 0.285 | 0.016 (0.019) | 0.379 | 0.404 | 0.007 (0.017) | 0.704 | 0.051 | -0.041 (0.038) | 0.287 | 0.273 | 0.028 (0.019) | 0.138 | 0.079 | -0.052 (0.031) | 0.094 |
| | IVAN | 0.311 | 0.009 (0.014) | 0.515 | 0.428 | 0.002 (0.014) | 0.892 | 0.067 | 0.021 (0.027) | 0.442 | 0.282 | 0.009 (0.015) | 0.556 | 0.070 | -0.04 (0.027) | 0.131 |
| | EUGENDA | 0.270 | 0.017 (0.025) | 0.498 | 0.363 | 0.044 (0.023) | 0.061 | 0.067 | -0.075 (0.044) | 0.089 | 0.249 | 0.001 (0.024) | 0.968 | 0.051 | 0.009 (0.052) | 0.855 |

Cologne = University Hospital of Cologne, Cologne; Jerusalem = Hadassah-Hebrew University Medical Center, Jerusalem; Nijmegen = Radboud university medical center, Nijmegen; Melbourne = Centre for Eye Research Australia, Melbourne; Sydney = Centre for Vision Research, Sydney; Leeds = St. James's University Hospital, Leeds; BRAMD = BRAMD trial cohort, IVAN = alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN) trial cohort, EUGENDA = European Genetic Database study cohort; chr = chromosome; MAF = Minor allele frequency; SE = Standard error; NA = Not applicable

*Not included due to MAF <0.05, ^ Not included due to call rate <0.9

ETABLE 3 **Single-variant association analysis of variants previously associated with response to anti-VEGF therapy in neovascular AMD**

| Study | Chr. | Position[a] | SNP | Gene[b] | Allele | *P*-value Cologne | *P*-value Nijmegen | *P*-value Jerusalem | *P*-value Melbourne | *P*-value Sydney | β (SE) Meta-analysis | *P*-value Meta-analysis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wickremasinghe SS et al., 2011; Bakbak B et al., 2015* | 19 | 45,412,079 | rs7412 | *APOE* | T | 0.935 | 0.715 | 0.453 | 0.185 | 0.178 | -0.003 (0.027) | 0.926 |
| Wickremasinghe SS et al., 2011; Bakbak B et al., 2015* | 19 | 45411941 | rs429358 | *APOE* | C | 0.111 | 0.696 | 0.996 | 0.203 | 0.434 | 0.055 (0.027) | 0.043 |
| Brantley MA et al., 2007; Imai D et al., 2010*; Kloeckener-Gruissem B et al., 2011; Nischler C et al., 2011; McKibbin M et al., 2012; Smailhodzic D et al., 2012; Menghini M et al., 2012; Tian J et al., 2012*; Hautamaki A et al., 2014; Matsumiya W et al., 2014*; Veloso CE et al., 2014; Piermarocchi S et al., 2015; Medina F et al., 2015*; Shah AR at ., 2016. | 1 | 196,659,237 | rs1061170 | *CFH* | T | 0.074 | 0.446 | 0.984 | 0.397 | 0.546 | 0.028 (0.015) | 0.059 |
| Teper SJ et al., 2010; McKibbin M et al., 2012; Kang HK et al., 2012*; Tian J et al., 2012*; Abedi F et al., 2013; Kitchens JW et al., 2013; Valverde-Megías A et al., 2017 | 10 | 124,220,544 | rs11200638[#] | *HTRA1* | A | 0.141 | 0.729 | 0.892 | 0.790 | 0.864 | 0.011 (0.014) | 0.416 |
| Hautamaki A et al., 2013; Hautamaki A et al., 2014; Lazzeri S et al., 2015 | 4 | 74,606,024 | rs4073 | *CXCL8* | T | 0.403 | 0.814 | 0.532 | 0.325 | 0.921 | 0.014 (0.016) | 0.390 |
| Imai D et al., 2010* | 17 | 1,673,276 | rs1136287 | *SERPINF1* | T | 0.615 | 0.330 | 0.152 | 0.625 | 0.154 | 0.019 (0.016) | 0.226 |
| Wang VM et al., 2012 | 4 | 110,638,810 | rs2285714 | *PLA2G12A* | T | 0.849 | 0.852 | 0.708 | 0.805 | 0.109 | -0.014 (0.015) | 0.368 |
| Nakata I et al., 2011* | 6 | 43,732,669 | rs699946 | *VEGFA* | G | 0.377 | 0.141 | 0.614 | 0.819 | 0.297 | -0.007 (0.019) | 0.687 |
| Imai D et al., 2010*; Lazzeri S et al., 2013; Cruz-Gonzalez F et al., 2014; Hautamaki A et al., 2014 | 6 | 43,736,389 | rs699947 | *VEGFA* | C | 0.479 | 0.087 | 0.613 | 0.838 | 0.972 | -0.008 (0.015) | 0.581 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Abedi F et al., 2013; Chang W et al, 2013* | 6 | 43,742,579 | rs833069~ | *VEGFA* | C | 0.933 | 0.539 | 0.784 | 0.460 | 0.435 | 0.009 (0.015) | 0.559 |
| Zhao L et al., 2013 | 6 | 43,826,627 | rs943080 | *VEGFA* | T | 0.452 | 0.571 | 0.467 | 0.558 | 0.918 | -0.010 (0.015) | 0.492 |
| Hermann MM et al., 2014 | 4 | 55,986,238 | rs4576072+ | *KDR* | C | NA | NA | NA | NA | NA | NA | NA |
| Hermann MM et al., 2014 | 4 | 55,966,801 | rs6828477 | *KDR* | T | 0.337 | 0.554 | 0.950 | 0.696 | 0.773 | 0.012 (0.016) | 0.433 |
| Lazzeri S et al., 2015 | 4 | 55,992,366 | rs2071559 | *KDR* | G | 0.975 | 0.306 | 0.435 | 0.723 | 0.005 | 0.009 (0.015) | 0.555 |
| Lorés-Motta L et al., 2016 | 10 | 33,552,695 | rs2070296 | *NRP1* | T | 0.847 | 0.204 | 0.561 | 0.509 | 0.018 | -0.031 (0.021) | 0.136 |
| Riaz M and Lorés-Motta L et al., 2016 | 11 | 4,389,639 | rs4910623 | *OR52B4* | A | 0.185 | 0.161 | 0.756 | 0.158 | 0.126 | 0.022 (0.015) | 0.155 |

SNP = Single nucleotide polymorphism, Cologne = University Hospital of Cologne, Cologne; Jerusalem = Hadassah-Hebrew University Medical Center, Jerusalem; Nijmegen = Radboud university medical center, Nijmegen; Melbourne = Centre for Eye Research Australia, Melbourne; Sydney = Centre for Vision Research, Sydney; Leeds = St. James's University Hospital, Leeds; BRAMD = BRAMD trial cohort, IVAN = alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN) trial cohort; EUGENDA = European Genetic Database study cohort; SE = Standard error; NA = Not applicable

[a] Chromosomal position according to the NCBI RefSeq hg19 human genome reference assembly. [b] Closest gene.* Association identified in a non-European descent population.

+ Variant did not pass imputation quality control. # In linkage disequilibrium ($r^2$>0.9) with rs10490924. ^In linkage disequilibrium ($r^2$>0.9) with rs833061 and rs1413711. ~ In linkage disequilibrium ($r^2$>0.9) with rs3025000.

ETABLE 4 **Single-variant association analysis of variants previously associated with AMD**

| Locus name | Variant[a] | Allele | *P*-value Cologne | *P*-value Nijmegen | *P*-value Jerusalem | *P*-value Melbourne | *P*-value Sydney | β (SE) Meta-analysis | *P*-value Meta-analysis |
|---|---|---|---|---|---|---|---|---|---|
| *CFH* | rs10922109 | C | 0.536 | 0.575 | 0.702 | 0.585 | 0.355 | -0.013 (0.018) | 0.454 |
| *CFH* | rs570618 | G | 0.074 | 0.435 | 1.000 | 0.414 | 0.546 | 0.028 (0.015) | 0.060 |
| *CFH* | rs121913059 | NF | NF | NF | NF | NF | NF | NF | NF |
| *CFH* | rs148553336 | NF | NF | NF | NF | NF | NF | NF | NF |
| *CFH* | rs187328863 | C | 0.017 | 0.686 | 0.109 | 0.186 | 0.472 | 0.068 (0.039) | 0.077 |
| *CFH (CFHR3/CFHR1)* | rs61818925 | G | 0.297 | 0.549 | 0.594 | 0.150 | 0.737 | -0.019 (0.017) | 0.275 |
| *CFH* | rs35292876 | C | 0.013 | 0.379 | 0.736 | 0.105 | 0.707 | 0.029 (0.042) | 0.487 |
| *CFH* | rs191281603 | NF | NF | NF | NF | NF | NF | NF | NF |
| *COL4A3* | rs11884770 | C | 0.719 | 0.329 | 0.962 | 0.627 | 0.477 | 0.007 (0.018) | 0.689 |
| *ADAMTS9-AS2* | rs62247658 | C | 0.716 | 0.552 | 0.866 | 0.058 | 0.452 | 0.028 (0.015) | 0.056 |
| *COL8A1* | rs140647181 | C | 0.327 | 0.061 | 0.389 | 0.089 | 0.774 | -0.112 (0.049) | 0.022 |
| *COL8A1* | rs55975637 | G | 0.951 | 0.541 | 0.607 | 0.088 | 0.388 | -0.022 (0.021) | 0.310 |
| *CFI* | rs10033900 | C | 0.532 | 0.955 | 0.571 | 0.543 | 0.390 | -0.002 (0.016) | 0.925 |
| *CFI* | rs141853578 | NF | NF | NF | NF | NF | NF | NF | NF |
| *C9* | rs62358361 | G | NF | 0.007 | 0.307 | 0.812 | 0.151 | -0.115 (0.076) | 0.130 |
| *PRLR/SPEF2* | rs114092250 | G | 0.372 | 0.799 | NF | 0.272 | 0.614 | -0.029 (0.05) | 0.565 |
| *C2/CFB/SKIV2L* | rs116503776 | G | 0.086 | 0.806 | 0.448 | 0.632 | 0.146 | -0.001 (0.027) | 0.958 |
| *C2/CFB/SKIV2L* | rs144629244 | G | 0.500 | NF | 0.280 | NF | 0.523 | -0.05 (0.085) | 0.558 |
| *C2/CFB/SKIV2L (PBX2)* | rs114254831 | G | 0.416 | 0.811 | 0.148 | 0.014 | 0.271 | 0.036 (0.017) | 0.033 |
| *C2/CFB/SKIV2L* | rs181705462 | G | 0.402 | NF | 0.927 | 0.482 | 0.209 | -0.046 (0.057) | 0.422 |
| *VEGFA* | rs943080 | NF | NF | NF | NF | NF | NF | NF | NF |
| *KMT2E/SRPK2* | rs1142 | C | 0.941 | 0.406 | 0.955 | 0.668 | 0.396 | -0.005 (0.016) | 0.751 |
| *PILRB/PILRA* | rs7803454 | C | 0.461 | 0.240 | 0.020 | 0.956 | 0.292 | 0.037 (0.019) | 0.050 |
| *TNFRSF10A* | rs79037040 | G | 0.094 | 0.952 | 0.268 | 0.280 | 0.041 | 0.015 (0.016) | 0.325 |
| *MIR6130/RORB* | rs10781182 | G | 0.498 | 0.646 | 0.220 | 0.209 | 0.777 | 0.005 (0.016) | 0.729 |
| *TRPM3* | rs71507014 | GC | 0.696 | 0.074 | 0.401 | 0.780 | 0.753 | -0.017 (0.014) | 0.236 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *TGFBR1* | rs1626340 | G | 0.673 | 0.094 | 0.260 | 0.393 | 0.076 | 0.013 (0.02) | 0.530 |
| *ABCA1* | rs2740488 | C | 0.305 | 0.999 | 0.019 | 0.774 | 0.177 | 0.009 (0.018) | 0.631 |
| *ARHGAP21* | rs12357257 | G | 0.308 | 0.710 | 0.718 | 0.077 | 0.249 | -0.016 (0.017) | 0.372 |
| *ARMS2/HTRA1* | rs3750846 | C | 0.153 | 0.673 | 0.892 | 0.790 | 0.753 | 0.012 (0.014) | 0.409 |
| *RDH5/CD63* | rs3138141 | C | 0.620 | 0.567 | 0.630 | 0.877 | 0.267 | 0.004 (0.023) | 0.856 |
| *ACAD10* | rs61941274 | G | 0.203 | 0.699 | NF | 0.367 | 0.017 | -0.014 (0.064) | 0.831 |
| *B3GALTL* | rs9564692 | C | 0.999 | 0.544 | 0.384 | 0.452 | 0.907 | -0.002 (0.017) | 0.913 |
| *RAD51B* | rs61985136 | C | 0.142 | 0.360 | 0.268 | 0.628 | 0.243 | -0.007 (0.016) | 0.672 |
| *RAD51B* | rs2842339 | G | 0.368 | 0.651 | 0.255 | 0.511 | 0.527 | -0.006 (0.024) | 0.820 |
| *LIPC* | rs2043085 | C | 0.224 | 0.418 | 0.956 | 0.809 | 0.656 | -0.009 (0.015) | 0.536 |
| *LIPC* | rs2070895 | G | 0.242 | 0.274 | 0.158 | 0.463 | 0.914 | 0.004 (0.018) | 0.844 |
| *CETP* | rs5817082 | C | 0.114 | 0.052 | 0.105 | 0.393 | 0.425 | 0.033 (0.018) | 0.072 |
| *CETP* | rs17231506 | C | 0.384 | 0.041 | 0.932 | 0.492 | 0.037 | -0.017 (0.016) | 0.286 |
| *CTRB2/CTRB1* | rs72802342 | C | 0.750 | 0.497 | 0.522 | 0.794 | 0.837 | -0.001 (0.035) | 0.973 |
| *TMEM97/VTN* | rs11080055 | C | 0.108 | 0.862 | 0.837 | 0.349 | 0.483 | 0.002 (0.015) | 0.891 |
| *NPLOC4/TSPAN10* | rs6565597 | C | 0.188 | 0.977 | 0.036 | 0.047 | 0.885 | -0.021 (0.017) | 0.215 |
| *C3* | rs2230199 | G | 0.554 | 0.671 | 0.886 | 0.725 | 0.291 | 0.002 (0.019) | 0.897 |
| *C3* | rs147859257 | G | NF | NF | NF | 0.062 | NF | -0.226 (0.12) | 0.062 |
| *C3 (NRTN/FUT6)* | rs12019136 | G | 0.219 | 0.572 | 0.435 | 0.953 | 0.023 | 0.02 (0.042) | 0.633 |
| *CNN2* | rs67538026 | C | 0.983 | 0.221 | 0.893 | 0.903 | 0.197 | 0 (0.017) | 0.999 |
| *APOE* | rs429358 | C | 0.111 | 0.696 | 0.996 | 0.203 | 0.434 | 0.055 (0.027) | 0.043 |
| *APOE(EXOC3L2/ MARK4)* | rs73036519 | G | 0.193 | 0.820 | 0.430 | 0.192 | 0.464 | -0.005 (0.018) | 0.787 |
| *MMP9* | rs142450006 | TTTTC | 0.582 | 0.146 | 0.715 | 0.494 | 0.153 | 0.002 (0.024) | 0.944 |
| *C20ORF85* | rs201459901 | TA | 0.565 | 0.220 | 0.838 | 0.048 | 0.738 | -0.012 (0.034) | 0.723 |
| *SYN3/TIMP3* | rs5754227 | C | 0.390 | 0.313 | 0.010 | 0.689 | 0.706 | 0.015 (0.025) | 0.553 |
| *SLC16A8* | rs8135665 | C | 0.405 | 0.602 | 0.354 | 0.731 | 0.898 | -0.003 (0.018) | 0.855 |

NF = Not found, SE = Standard error. [a] Chromosomal location of the SNP as in Fritsche et al., 2016

ETABLE 5 **Rare protein-altering variants in *C10orf88* and *UNC93B1* included in the gene-based analysis**

| Gene | Variant[a] | Protein change | Imputation quality ($R^2$) | CADD score[b] | RAC | Single variant P-value | β (SE) |
|---|---|---|---|---|---|---|---|
| *C10orf88* | c.412G>A | p.Glu138Lys | 1 | 24.6 | 3 | $4.96x10^{-4}$ | -0.618 (0.177) |
| | c.827T>C | p.Ile276Thr | 1 | 0.038 | 3 | $2.33x10^{-5}$ | -0.749 (0.176) |
| | c.1258C>T | p.Gln420* | 1 | 38 | 1 | 0.120 | <0 |
| *UNC93B1* | c.385C>A | p.Leu129Ile | 1 | 24.9 | 7 | $3.33x10^{-7}$ | -0.606 (0.118) |
| | c.626C>T | p.Pro209Leu | 1 | 25.6 | 7 | $4.21x10^{-7}$ | -0.596 (0.117) |

RAC = Rare allele count, SE = Standard error

[a] Positions according to the NCBI RefSeq hg19 human genome reference assembly.

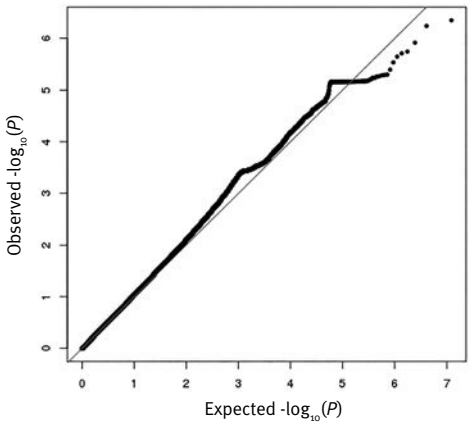
[b] The CADD score refers to the PHRED-like scaled C-score for which ≥20 indicates that the variant is predicted to be in the 1% most deleterious substitutions in the human genome.

ETABLE 6 **Gene-based analysis of rare variants in genes previously associated with AMD**

| Gene | Chromosome | Chromosomal position[a] | N rare variants | RAC | *P*-value |
|---|---|---|---|---|---|
| *CFH* | 1 | 196,621,252-196,716,375 | 14 | 40 | 0.541 |
| *CFI* | 4 | 110,662,068-110,723,117 | 10 | 26 | 0.026 |
| *TIMP3* | 22 | 33,198,100-33,255,356 | 2 | 4 | 0.163 |
| *SLC16A8* | 22 | 38,474,406-38,478,804 | 5 | 21 | 0.007 |

N = number, RAC = rare allele count

[a] Chromosome and chromosomal position according to the NCBI RefSeq hg19 human




EFIGURE 1 **Q-Q plot of the single-variant association analysis of response to anti-VEGF therapy in neovascular AMD**

Shown as black dots are the observed P-values ($-\log_{10}(p)$) compared to those expected under the null hypothesis.

In the meta-analysis, adjustment for the inflation factor of the different cohorts was conducted when λ>1 (University Hospital of Cologne cohort λ=1.020, Hadassah-Hebrew UMC cohort λ=0.99, Radboud umc cohort λ=1.001, Centre for Eye Research Australia cohort λ=1.005 and Centre for Vision Research cohort λ=1.005).

# Additional information

## Author contributions

AIdH, PNB, IC designed the study, and were involved in data acquisition and results interpretation. LLM, MR and MG performed all the statistical analysis in parallel, interpreted the collected data and results, and drafted the manuscript. JC and PM developed pipelines for data analysis and were involved in the results interpretation. ML carried out the genotyping of the EUGENDA, Leeds and BRAMD replication cohorts. AJR performed the DNA extraction and genotyping of the Melbourne replication cohort. MG performed the genotyping of the Jerusalem replication cohort. FvA, PM, AJC, HLG, CP, MCB, MAM-S, MA, UC, RG, MMcK, CFI, ROS, AO, JC, RKK, SF, RHG, CBH, AJL, PM were involved in the clinical data acquisition. IMH, LGF were involved in the exome array data acquisition. EKdJ was involved in the results interpretation. All authors revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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The sponsor or funding organizations had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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# 3

# Genetic biomarkers for complement inhibiting therapies

# 3.1

# *GWAS reveals genetic variants in CFH and CFHR4 associated with systemic complement activation levels: implications in age-related macular degeneration*

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# Abstract

**Purpose**: To identify genetic variants associated with complement activation, which could help to select age-related macular degeneration (AMD) patients for complement inhibiting therapies.

**Design**: Genome-wide association study (GWAS) followed by replication and meta-analysis.

**Participants**: 2,245 AMD patients and controls.

**Methods**: A GWAS on serum C3d/C3 levels was performed in 1,548 AMD patients and controls. After genotype imputation and quality control, 9,972,920 variants were included in the analysis. For replication and meta-analysis, 697 additional individuals were genotyped for the lead SNPs in the associated signals. A model for complement activation including the identified genetic and non-genetic factors was built, and the variance explained was estimated. Haplotype analysis was performed for eight SNPs across the *CFH*/*CFHR* locus. Association with AMD was performed for the variants and haplotypes found to influence complement activation.

**Main Outcome Measure**: Normalized C3d/C3 levels as a measure of systemic complement activation.

**Results**: Associations with complement activation were identified at the *CFH*/*CFHR* locus. Complement activation was independently associated with rs3753396 located in *CFH* ($P_{discovery}$=1.09x10$^{-15}$, $P_{meta}$=3.66x10$^{-21}$, β=0.141, SE=0.015) and rs6685931 located in *CFHR4* ($P_{discovery}$=8.18x10$^{-7}$, $P_{meta}$=6.32x10$^{-8}$, β=0.054, SE=0.010). A model including age, AMD disease status, body mass index, triglycerides, rs3753396, rs6685931, and previously identified SNPs, explained 18.7% of the variability in complement activation. Haplotype analysis revealed three haplotypes (H1-2 and H6 containing rs6685931, and H3 containing rs3753396) associated with complement activation. Haplotypes H3 and H6 conferred stronger effects on complement activation compared to the single variants ($P$=2.53x10$^{-14}$, β=0.183, SE=0.024 and $P$=4.28x10$^{-4}$, β=0.144, SE=0.041 respectively). Association analyses with AMD revealed that SNP rs6685931 and haplotype H1-2 containing rs6685931 associated with a risk for AMD development, while SNP rs3753396 and haplotypes H3 and H6 were not associated with AMD.

**Conclusions**: SNP rs3753396 in *CFH* and SNP rs6685931 in *CFHR4* are associated with systemic complement activation levels. The stronger effects of haplotypes H3 and H6 on complement activation suggest that other variants at the *CFH*/*CFHR* locus also influence this trait. SNP rs6685931 in *CFHR4*, and its linked haplotype H1-2, conferred also a risk for AMD development, and therefore could be used to identify AMD patients that would benefit most from complement inhibiting therapies.

# Background

The complement system is an integral part of our innate immunity. Its best known physiological functions are host defense against foreign intruders and homeostasis maintenance.[1] It consists of more than 30 plasma proteins and cellular components that interact in proteolytic cascades for an efficient and rapid activation leading to inflammation, opsonization and targeted cytolysis.[2]

The complement system can be activated by three different pathways: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). The CP is activated by antibody-antigen complexes and the LP by lectin or ficolin binding to carbohydrates, both on the surfaces of pathogens. In contrast, the AP is constitutively activated at a low level in a process known as *tick-over*.[3]

All three pathways lead to the formation of C3 convertases that catalyze a proteolytic cleavage of complement component 3 (C3) into the potent anaphylatoxin C3a, and C3b, an opsonization molecule that can be further cleaved into C3d. C3b can also bind the cleaved form of factor B (FB, Bb) to form the AP C3 convertase (C3bBb) that will cleave more C3, initiating an amplification loop. Downstream in the cascade C5 convertases are formed, initiating the terminal pathway with the subsequent formation of additional activation products as well as the membrane-attack complex (MAC) that is responsible for cytolysis.[4]

The complement system can be rapidly amplified and therefore several inhibitory proteins such as complement factor H (FH) and complement factor I (FI) are in place regulating complement activity.[4]

Deregulation and deficiencies of the complement system have been reported to be associated with numerous inflammatory, autoimmune, neurodegenerative and infectious disorders.[5] A prime example of a multifactorial disease associated with a deregulation of the complement system is age-related macular degeneration (AMD). AMD is characterized by a progressive degeneration of the central retina, and is responsible for the majority of vision loss in the elderly with a pooled prevalence of 8.9 %.[6,7] AMD entails a major health problem as in 2020, the number of people affected by a form of this disease is projected at 196 million, raising to 288 million in 2040.[8] Several lines of evidence point towards an over-activation of the complement system in AMD, mainly through a dysregulation of the AP. Multiple genetic variants in or near complement genes (*CFH*, *C3*, *CFI*, *C2*/*CFB* locus and *C9*) have been strongly associated with AMD.[9,10] Moreover, complement components have been described in drusen, the hallmark of the disease,[11-14] and complement activation fragments in plasma/serum such as Ba, C3a, C3d and C5a have been found to be significantly elevated in AMD patients compared to controls.[15-21] Currently, there is no treatment available for the majority of AMD cases, nor is there an effective means to halt AMD progression. Therefore, therapies for AMD, as well as for other diseases involving complement deregulation, are being developed aiming to inhibit or lower complement activation.[22-24]

Systemic complement activation levels demonstrate considerable variation among individuals.[16-20] As a consequence, patients who have higher levels of complement activation may benefit more than others from the upcoming therapies. A better understanding of the factors that influence complement activation would facilitate the selection of the most suitable patients for complement inhibiting therapies. Genetic markers are robust biomarkers that could be included in prediction models for complement activation. Several studies have previously evaluated the effect of genetic variation on complement activity; however, these studies were restricted to a limited number of single nucleotide polymorphisms (SNPs).[16-19,21,25]

The aim of this study was to perform the first genome-wide association study (GWAS) on systemic complement activation levels. Identification of genetic variants explaining complement activation levels will contribute to a better understanding of the molecular mechanisms of complement related diseases, will pinpoint potential drug targets, and will facilitate the selection of patients for complement inhibiting therapies.

# Results

## Characteristics of the study cohorts

We evaluated the association of genetic variants with systemic complement activation levels through a GWAS in a discovery cohort of 1,548 individuals, followed by replication in an independent cohort of 697 individuals. For both cohorts, demographics and information about AMD disease status, body mass index (BMI), triglycerides and high-density lipoprotein (HDL)-cholesterol was collected (Table 1).

TABLE 1 **Demographics and other characteristics of the discovery and replication cohorts**

| | Discovery cohort (n=1,548) | Replication cohort (n=697) |
|---|---|---|
| **Complement activation ln(c3d/c3), mean (SD)** | 1.459 (0.407) | 1.464 (0.398) |
| **Age, mean (SD)** | 73.2 (7.8) | 73.3 (7.7) |
| **Sex (female), %** | 60 | 58.8 |
| **AMD disease status (control), (%)** | 53.7 | 37.4 |
| **BMI kg/m2, median (quartiles)** | 25 (23 – 28) | 25 (23 – 28) |
| **Triglycerides mmol/l, median (quartiles)** | 1.620 (1.170 - 2.220) | 1.620 (1.165 - 2.210) |
| **HDL cholesterol mmol/l, mean (SD)** | 1.489 (0.377) | 1.478 (0.403) |
| **Clinic (Radboud university medical center), %** | 53.5 | 63 |

BMI=Body mass index, HDL=High-density lipoprotein, SD=standard deviation.

TABLE 2 **General linear model for systemic complement activation levels including environmental factors**

| | β | SE (β) | *P*-value | $R^2$ |
|---|---|---|---|---|
| **Age (years)** | 0.003 | 0.001 | 0.002 | 0.010 |
| **Sex (female)** | -0.025 | 0.018 | 0.155 | 0 |
| **Disease status (AMD)** | 0.074 | 0.017 | $6.700x10^{-5}$ | 0.013 |
| **BMI (kg/m²)** | -0.012 | 0.002 | $8.635x10^{-8}$ | 0.034 |
| **Triglycerides (mmol/l)** | -0.130 | 0.010 | $5.723x10^{-39}$ | 0.101 |
| **HDL cholesterol (mmol/l)** | 0.015 | 0.026 | 0.545 | 0.024 |
| **Clinic (University Hospital of Cologne)** | -0.064 | 0.016 | $6.800x10^{-5}$ | 0.005 |

BMI=Body mass index, HDL=High-density lipoprotein, SE=standard error.
$R^2$=0.141 (adjusted $R^2$=0.138).

Higher complement activation levels were associated independently with older age, AMD disease status, lower BMI and lower triglycerides levels as previously described.[21,26] Differences were also observed between the sample collection clinics (Table 2, available at www.aaojournal.org). Therefore, these factors were included as covariates in all consecutive analyses.

## GWAS identifies two independent signals at the *CFH*/*CFHR* locus to be associated with systemic complement activation

We carried out a GWAS of normalized C3d/C3 levels as a measure of systemic complement activation. Following quality control, a total of 1,548 individuals and 9,972,920 variants were included in the analysis. The study had ›80% of power to detect common variants (minor allele frequency ≥5%) explaining ≥2.6% of variance in complement activation levels.

A total of 280 variants reached genome-wide significance (Manhattan plot Fig. 1a, QQplot Fig. 2 (available at www.aaojournal.org), $\lambda_{GC}$=0.999). All variants, except for one, were located on chromosome 1 at the *CFH*/*CFHR* locus (chr1:196.643.724-197.061.086). The only variant outside of this locus was located on chromosome 6 near the *PSORS1C1* gene, but could not be verified by Sanger sequencing. SNP rs3753396 (c.2016A›G, p.Gln672Gln) located in exon 14 of the complement factor H (*CFH*) gene showed the strongest association with complement activation levels (*P*=$1.09x10^{-15}$, β=0.145, SE=0.018, Table 3, locus zoom depicted in Fig. 1b).

Conditional analysis on the lead SNP revealed a second independent signal with a *P* value close to genome-wide significance for which the strongest associated variant was rs6685931. This SNP was also located at the *CFH*/*CFHR* locus, specifically in intron 1 (c.59-4315T›C) of the complement factor H related 4 (*CFHR4*) gene (*P*=$8.18x10^{-7}$, β=0.068, SE=0.014, Table 3, locus zoom depicted in Fig. 1c).

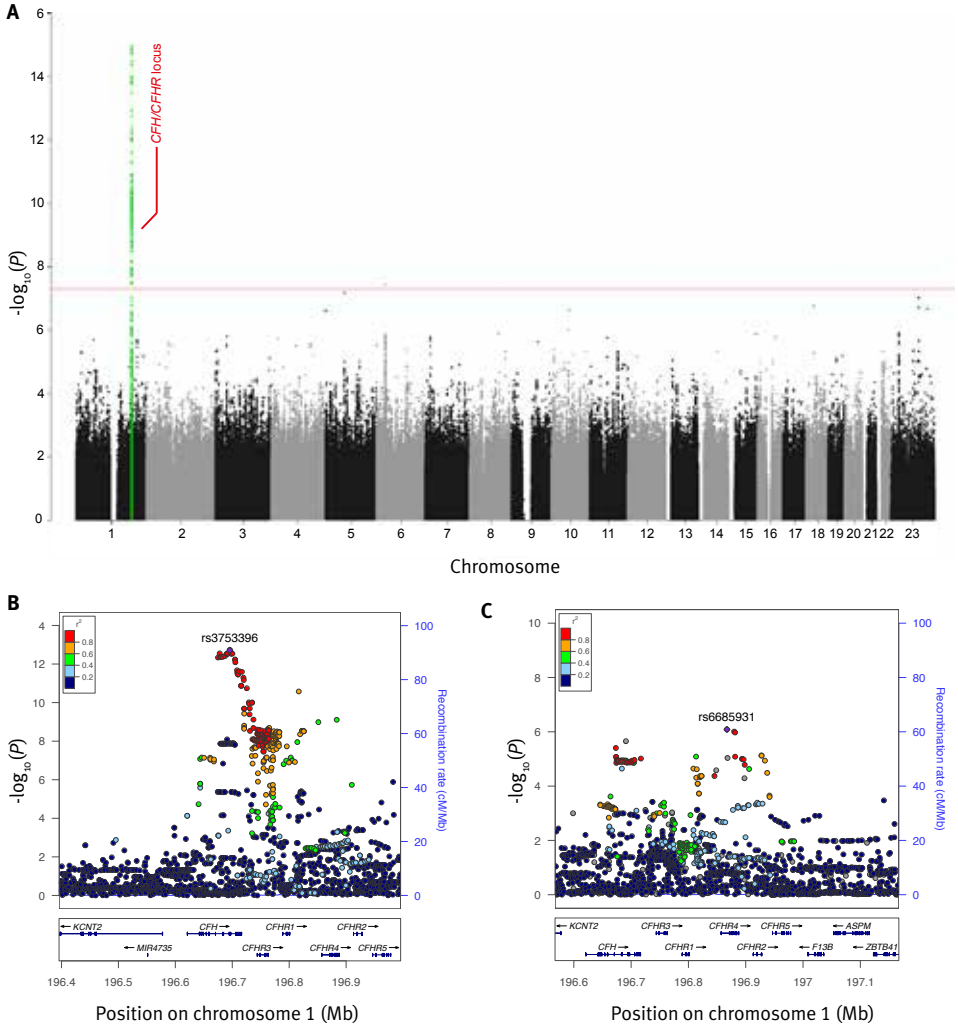


**FIGURE 1 GWAS identifies two independent signals at the *CFH*/*CFHR* locus associated with systemic complement activation levels**

**(A)** Manhattan plot illustrating the *P*-values of each individual SNP tested for association with systemic complement activation. The red horizontal line indicates the threshold considered for genome-wide significance ($P=5x10^{-8}$). **(B)** Locus zoom plot showing a detailed view of the chromosome 1 signal. The lead SNP rs3753396, is located in the *CFH* gene. SNPs are colored based on their LD estimate ($r^2$) to the lead SNP. **(C)** Locus zoom plot showing a detailed view of the signal on chromosome 1 after conditioning the association analysis for rs3753396. Here, the lead SNP rs6685931, is located in the *CFHR4* gene. SNPs are colored based on their LD estimate ($r^2$) to the lead SNP.

Variants shown to be associated with complement activation fragments in previous studies were extracted from the GWAS results.[17,18] SNP rs800292 in *CFH*, and the two SNPs in linkage disequilibrium rs4151667 in *CFB* and rs9332739 in *C2* were nominally associated with systemic complement activation levels in the current study, showing the same direction of the effect. SNP rs2230199 in *C3* and SNP rs10490924 in *ARMS2* could not be replicated (Table 4, available at www.aaojournal.org).

TABLE 3 **Meta-analysis of discovery and replication cohorts identifies two signals at the CFH/CFHR locus associated with systemic complement activation levels**

| | | | | Discovery cohort (n=1,548) | | | Replication cohort (n=697)[§] | | | Meta-analysis (n=2,245)[¶] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lead variant (MA)** | **Imputation quality (Rsq)*** | **Chr.: Position[†]** | **Gene[‡]** | **MAF** | **β (SE)** | ***P*-value** | **MAF** | **β (SE)** | ***P*-value** | **β (SE)** | ***P*-value** |
| rs3753396 (G) | - | 1:196,695,742 | *CFH* | 0.168 | 0.145 (0.018) | $1.091x10^{-15}$ | 0.147 | 0.131 (0.027) | $1.390x10^{-6}$ | 0.141 (0.015) | $3.664x10^{-21}$ |
| rs6685931 (C) | 0.99 | 1:196,867,233 | *CFHR4* | 0.439 | 0.068 (0.014) | $8.184x10^{-7}$ | 0.493 | 0.038 (0.014) | $8.620x10^{-3}$ | 0.054 (0.010) | $6.320x10^{-8}$ |

MA=Minor allele, Chr=chromosome, MAF=Minor allele frequency, SE=Standard error.
*Not applicable for genotyped variants. [†]Chromosome and chromosomal positions described according to the NCBI RefSeq hg19 human genome. [‡]Closest gene to the lead variant. [§]Replication cohort for rs6685931 consisted of 686 individuals. [¶]Meta-analysis for rs6685931 was performed in a total of 2234 individuals.

TABLE 4 **Association of variants identified in previous studies**

| **Gene** | **Genetic variant** | **Study** | **Allele** | **Complement activation measurement** | **Direction of the effect** | ***P*-value** | **This study** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | **Allele** | **AF** | ***P*-value** | **β** | **SE (β)** | **Replicated / Not replicated** |
| *CFH* | rs800292 | Hecker et al., 2009 | G | Ba | + | $7.06x10^{-6}$ | G | 0.79 | $2.545x10^{-4}$ | 0.058 | 0.016 | Replicated |
| *CFH* | rs800292 | Hecker et al., 2009 | G | C3d | + | 0.0013 | G | 0.79 | $2.545x10^{-4}$ | 0.058 | 0.016 | Replicated |
| *CFB* | rs4151667 | Hecker et al., 2009 | T | Ba | + | $3.86x10^{-6}$ | T | 0.95 | $3.48x10^{-6}$ | 0.151 | 0.032 | Replicated |
| *C2* | rs9332739 | Hecker et al., 2009 | G | Ba | + | $1.98x10^{-6}$ | G | 0.96 | $1.63x10^{-6}$ | 0.157 | 0.033 | Replicated |
| *C3* | rs2230199 | Reynolds et al., 2009 | G | C5a | + | 0.04 | G | 0.22 | 0.044 | -0.034 | 0.017 | Not replicated |
| *ARMS2* | rs10490924 | Reynolds et al., 2009 | T | C5a | + | 0.02 | T | 0.30 | 0.417 | 0.012 | 0.014 | Not replicated |

AF=Allele frequency, SE=Standard error.

TABLE 5 **Meta-analysis of discovery and replication cohorts identifies two signals at the *CFH*/*CFHR* locus associated with systemic complement activation levels: analysis adjusted for AMD status**

| | | | Discovery cohort (n=1,548) | | Replication cohort (n=697)[§] | | Meta-analysis (n=2,245)[¶] | |
|---|---|---|---|---|---|---|---|---|
| **Lead variant (MA)** | **Chr.: Position[†]** | **Gene[‡]** | **β (SE)** | ***P*-value** | **β (SE)** | ***P*-value** | **β (SE)** | ***P*-value** |
| rs3753396 (G) | 1:196695742 | *CFH* | 0.145 (0.018) | $1.316x10^{-15}$ | 0.128 (0.027) | $1.717x10^{-6}$ | 0.1396 (0.015) | $6.741x10^{-21}$ |
| rs6685931 (C) | 1:196867233 | *CFHR4* | 0.061 (0.014) | $9.561x10^{-6}$ | 0.030 (0.015) | 0.043 | 0.046 (0.010) | $3.928x10^{-6}$ |

MA=Minor allele, Chr=Chromosome, MAF=Minor allele frequency, SE=Standard error.
*Chromosome and chromosomal position according to the NCBI RefSeq hg19 human genome. [†]Closest gene to the lead variant. [§]Replication cohort for rs6685931 consisted of 686 individuals. [¶]Meta-analysis for rs6685931 was performed in a total of 2234 individuals.

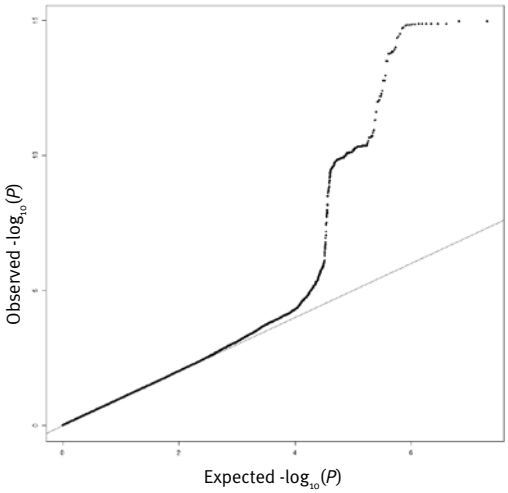


FIGURE 2 **Q-Q plot of the GWAS on systemic complement activation levels**

Shown as black dots are the observed *P*-values ($-\log_{10}(P)$). Lack of population stratification was confirmed by a genomic inflation factor ($\lambda$) for the trend of 0.*999*.

## Replication in an independent cohort confirms the effect of rs3753396 in *CFH* and rs6685931 in *CFHR4* on systemic complement activation

Replication analysis of rs3753396 in *CFH* and rs6685931 in *CFHR4* in an independent cohort of 697 study participants confirmed both variants to be significantly associated with systemic complement activation levels (rs3753396: $P=1.39\text{x}10^{-6}$, $\beta=0.131$, SE=0.027, rs6685931: $P=8.62\text{x}10^{-3}$, $\beta=0.038$, SE=0.014, Table 3). Subsequent meta-analysis showed associations for both rs3753396 ($P=3.66\text{x}10^{-21}$, $\beta=0.141$, SE=0.015) and rs6685931 ($P=6.32\text{x}10^{-8}$, $\beta=0.054$, SE=0.010), confirming that two independent signals at the *CFH*/*CFHR* locus are associated with higher complement activation levels (Table 3). Sensitivity analyses adjusting for AMD disease status showed comparable results (Table 5, available at www.aaojournal.org), and neither an interaction between clinic and the identified SNPs ($P_{\text{rs3753396 x clinic}}=0.436$, $P_{\text{rs6685931 x clinic}}=0.676$), nor an interaction between AMD status and the identified SNPs ($P_{\text{rs3753396 x AMD status}}=0.557$, $P_{\text{rs6685931 x AMD status}}=0.658$) was detected.

Next, mean complement activation levels in the genotype groups of rs373396 and rs6685931 were analyzed. For rs3753396 in *CFH*, the heterozygous AG genotype group showed higher complement activation levels compared to the reference AA genotype group ($P=6.23\text{x}10^{-18}$, $\beta=0.152$, SE=0.018), and for the homozygous GG group these levels were even higher ($P=2.39\text{x}10^{-7}$, $\beta=0.267$, SE=0.052, Fig. 3a). In the case of rs6685931 in *CFHR4*, a similar effect was observed: the heterozygous TC genotype group had higher complement activation levels than the reference TT genotype ($P=10^{-3}$, $\beta=0.063$, SE=0.019) and for the homozygous CC group the levels were even higher ($P=3.62\text{x}10^{-7}$, $\beta=0.118$, SE=0.023, Fig. 3b). Analysis of the cumulative effect of both SNPs showed that the main effect on systemic complement activation levels is driven by rs3753396 in *CFH*, and rs6685931 in *CFHR4* introduces additional variation to the rs3753396 genotypes (Fig. 3c).

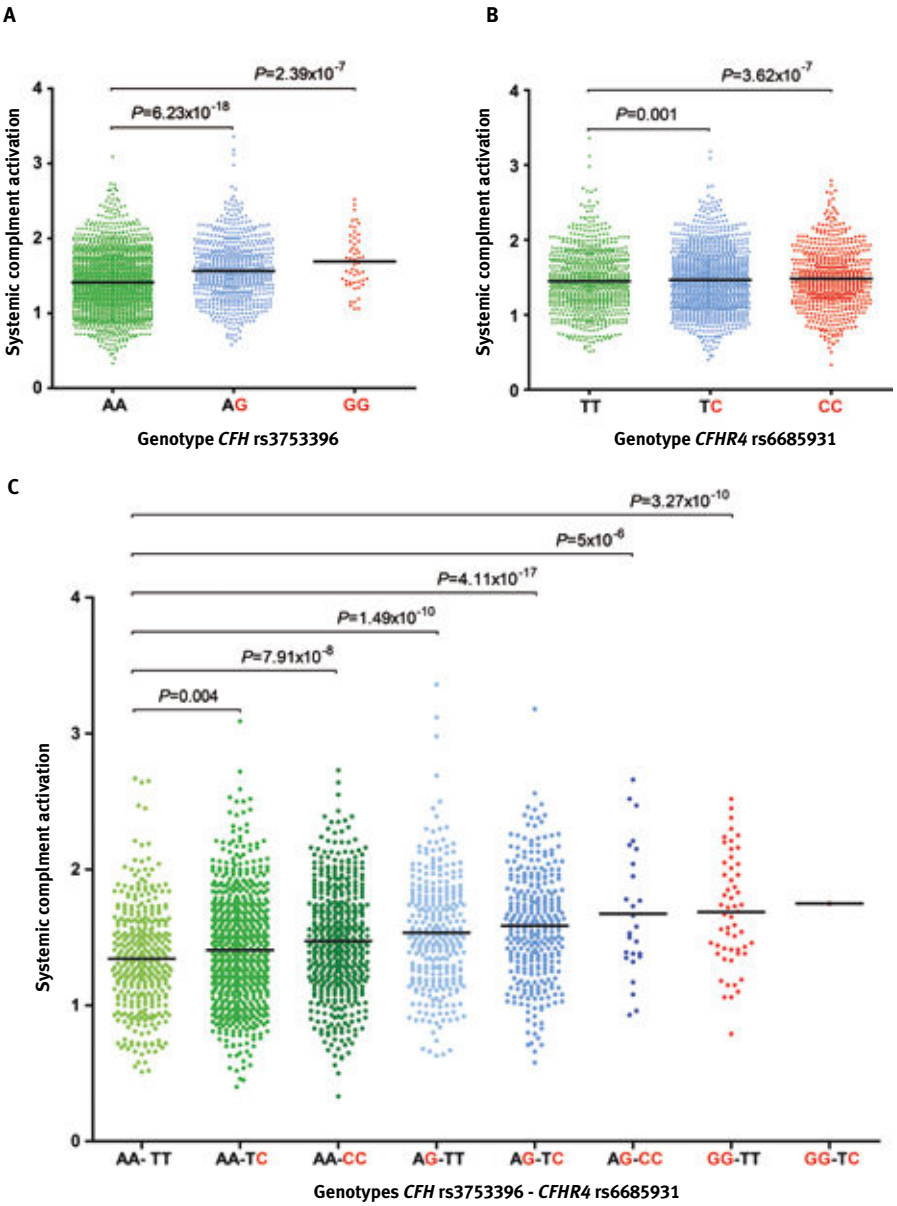


FIGURE 3 **Systemic complement activation levels stratified by rs3753396 and rs6685931 genotypes: rs6685931 introduces additional variation on the main effect of rs3753396**

The Y axes represent the ln-transformed C3d/C3 as a measure of systemic complement activation. Horizontal bars indicate the mean values for each genotype group. The complement-raising alleles for both SNPs are indicated in red. Association analyses included the 2,245 individuals from the discovery and the replication cohorts. **(A)** Distribution of complement activation levels for each genotype of rs3753396 in *CFH*. **(B)** Distribution of complement activation levels for each genotype of rs6685931 in *CFHR4*. *P*-values were calculated adjusting the model for rs3753396. **(C)** Distribution of complement activation levels over the genotype combinations of rs3753396 in *CFH* and rs6685931 in *CFHR4*.

## A model of genetic and non-genetic variables explains 18.7% of the variability in complement activation

General linear models were built in order to determine how much of the variation could be explained by factors found to be associated with systemic complement activation.

A model including only non-genetic factors (age, AMD disease status, BMI and triglycerides) explained 12.6% of the variability in systemic complement activation. With the addition of SNP rs3753396 to the model, 16.3% of the variability could be explained, and by including SNP rs6685931, a total of 17.3% was explained. We additionally incorporated SNPs associated with complement activation fragments in a previous study that replicated in our GWAS: rs800292 in *CFH* and rs9332739 in *C2*.[18] Only rs9332739 remained independently associated with systemic complement activation levels, and the variance explained by the model rose to 18.7% (adjusted $R^2$, Table 6).

TABLE 6 **A model of genetic and non-genetic variables explains 18.7% of the variability in systemic complement activation**

| | | β | SE (β) | *P*-value |
|---|---|---|---|---|
| ***CFH* rs3753396** | **AG** | 0.196 | 0.023 | $8.772x10^{-17}$ |
| | **GG** | 0.330 | 0.066 | $6.461x10^{-7}$ |
| ***CFHR4* rs6685931** | **TC** | 0.070 | 0.024 | 0.003 |
| | **CC** | 0.125 | 0.033 | $1.620x10^{-4}$ |
| ***CFH* rs800292** | **GA** | -0.011 | 0.023 | 0.639 |
| | **AA** | 0.027 | 0.046 | 0.555 |
| ***C2* rs9332739** | **GC** | -0.185 | 0.034 | $4.674x10^{-8}$ |
| | **CC** | 0.168 | 0.213 | 0.431 |
| **Age (years)** | | 0.004 | 0.001 | 0.005 |
| **Disease status (AMD)** | | 0.035 | 0.020 | 0.089 |
| **BMI (kg/m²)** | | -0.012 | 0.003 | $2x10^{-6}$ |
| **Triglycerides (mmol/l)** | | -0.131 | 0.011 | $1.177x10^{-33}$ |

BMI=Body mass index, SE= Standard error.

$R^2$=0.193 (adjusted $R^2$=0.187). The model included the 1,548 individuals of the discovery phase.

## Haplotypes across the *CFH*/*CFHR* locus have stronger effects on systemic complement activation levels compared to individual variants

In order to assess whether more variants at the *CFH*/*CFHR* locus influence systemic complement activation, and to determine the cumulative effect of several variants on the same haplotype, we evaluated the effect of distinct haplotypes across the *CFH*/*CFHR* locus on systemic complement activation.

TABLE 7 Association of haplotypes across the *CFH*/*CFHR* locus with systemic complement activation levels

| Haplotype | Alleles *CFH* rs3753396 – *CFHR4* rs6685931 | HF | β | SE (β) | *P*-value |
|---|---|---|---|---|---|
| H2 | A-T | 0.18 | Reference | Reference | Reference |
| H1-2 | A-C | 0.36 | 0.062 | 0.019 | $1.148x10^{-3}$ |
| H3 | G-T | 0.14 | 0.183 | 0.024 | $2.531x10^{-14}$ |
| H4 | A-T | 0.10 | 0.013 | 0.026 | 0.607 |
| H5 | A-T | 0.04 | -0.058 | 0.038 | 0.128 |
| H1-1 | A-T | 0.03 | -0.053 | 0.043 | 0.218 |
| H6 | A-C | 0.03 | 0.144 | 0.041 | $4.823x10^{-4}$ |
| H7 | A-C | 0.03 | 0.060 | 0.046 | 0.192 |
| H8 | A-T | 0.03 | -0.007 | 0.048 | 0.890 |

HF=Haplotype frequency, SE=Standard error.
Haplotype association analyses with AMD were performed on the 1,548 individuals of the discovery cohort. Haplotypes are coded as in Hageman et al., 2005. If two different sub-haplotypes based on the extra allele in SNP rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2. Alleles associated with higher complement levels are underlined. The reference haplotype was set to the most common haplotype not carrying any complement-raising allele for rs3753396 or rs6685931.

TABLE 8 Predicted haplotypes across the *CFH*/*CFHR* locus

| Haplotype | rs3753394 | rs529825* | rs800292* | rs3766404 | rs1061170 | rs203674 | rs3753396† | rs1065489† | rs6685931 |
|---|---|---|---|---|---|---|---|---|---|
| H1-2 (rs6685931-C) | C | G | G | T | C | G | A | G | C |
| H2 | C | A | A | T | T | T | A | G | T |
| H3 | T | G | G | T | T | T | G | T | T |
| H4 | C | G | G | C | T | T | A | G | T |
| H5 | T | G | G | T | T | T | A | G | T |
| H1-1 (rs6685931-T) | C | G | G | T | C | G | A | G | T |
| H6 | T | G | G | T | T | G | A | G | C |
| H7 | T | G | G | T | C | G | A | G | C |
| H8 | T | G | G | C | T | T | A | G | T |

*SNPs in linkage disequilibrium ($r^2=1$). †SNPs in linkage disequilibrium ($r^2=1$).

Haplotypes are coded as in Hageman et al., 2005. If two different sub-haplotypes based on the extra allele in SNP rs6685931 were found, the Hageman haplotypes were recoded as 1 or 2.

TABLE 9 **Association of complement-raising SNPs and haplotypes with AMD: SNP rs6685931 and haplotype H1-2 confer a risk for AMD**

| | OR | CI | *P*-value |
|---|---|---|---|
| **SNP rs3753396** | 1.031 | 0.839 - 1.223 | 0.756 |
| **SNP rs6685931** | 1.631 | 1.489 - 1.772 | $5.889 \times 10^{-12}$ |
| **Haplotype H3** | 1.015 | 0.911 - 1.130 | 0.795 |
| **Haplotype H6** | 0.828 | 0.637 - 1.075 | 0.135 |
| **Haplotype H1-2** | 1.318 | 1.223 - 1.420 | $1.382 \times 10^{-12}$ |

OR=Odds ratio, CI=Confidence interval.
Single variant and haplotype association analyses with AMD were performed on the 1,548 individuals of the discovery cohort. Haplotype analyses were based on $X^2$ tests that compared the frequency of the analyzed haplotypes in cases vs. controls.

Haplotypes previously described for AMD already included rs3753396, the lead variant associated in the GWAS,[27] and were expanded by adding rs668593, the second independent signal. In total, seven SNPs across the *CFH*/*CFHR* locus yielded nine different haplotypes with a predicted population frequency higher than 1% (Table 7, Table 8 available at www.aaojournal.org).

Association with systemic complement activation levels revealed haplotypes with stronger effects on complement activation compared to the single SNPs identified in the GWAS. Haplotypes H1-2, H3 and H6 were associated with higher systemic complement activation levels. Haplotype H3 carrying the complement-raising allele of rs3753396 (G) had a stronger effect on complement activation levels ($P=2.53 \times 10^{-14}$, $\beta=0.183$, SE=0.024) compared to the complement-raising allele of rs3753396 in the single variant analysis ($\beta=0.141$, SE=0.015). Haplotypes H1-2 and H6 both carried the complement-raising allele for rs6685931 (C). Haplotype H6 showed a stronger effect on complement activation levels ($P=4.82 \times 10^{-4}$, $\beta=0.144$ SE=0.041) compared to the single variant analysis for rs6685931 ($\beta=0.054$, SE=0.010) (Table 7, Table 8 available at www.aaojournal.org).

## SNP rs6685931 in *CFHR4* and haplotype H1-2 confer a risk for AMD

In order to identify genetic biomarkers that are relevant in the context of disease, we explored whether the SNPs and haplotypes associated with systemic complement activation levels associate also with AMD.

SNP rs3753396 in *CFH* was not associated with AMD ($P=0.76$). In contrast, the complement-raising allele of rs6685931 in *CFHR4* (C) was associated with an increased risk for AMD ($P=5.89 \times 10^{-12}$, OR=1.631 CI=1.489-1.772) (Table 9). These results are in concordance with the largest GWAS on AMD reported to date (rs3753396: $P=3 \times 10^{-3}$; rs6685931: $P=1.02 \times 10^{-495}$, OR>1).[9]

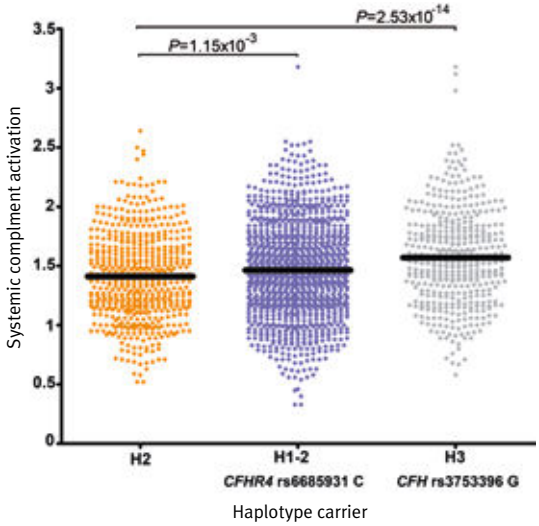


FIGURE 4 **Complement activation levels stratified by common haplotypes across the *CFH*/*CFHR4* locus: the AMD risk haplotype H1-2 shows high complement activation levels, and the non-AMD-associated H3-1 haplotype shows the highest**

Horizontal bars indicate the mean values for each haplotype carrier group. Haplotype carriers included in the graph had a posterior probability higher than 0.75. The haplotype group colors indicate the association with AMD: orange=protective, blue=risk conferring, grey=not associated. Association analyses were carried out on the 1,548 patients genotyped with exome-array.

TABLE 10 **Association of the 52 AMD variants with systemic complement activation levels**

| Variant* | AMD risk-increasing allele | Locus name | AF† | β † | SE (β) † | *P*-value† | *P*-value†‡ |
|---|---|---|---|---|---|---|---|
| rs10922109 | C | *CFH* | 0.629 | 0.089 | 0.013 | $5.389x10^{-11}$ | $1.406x10^{-9}$ |
| rs570618 | T | *CFH* | 0.421 | -0.002 | 0.013 | 0.876 | 0.629 |
| rs121913059§ | T | *CFH* | - | - | - | - | - |
| rs148553336 | T | *CFH* | 0.995 | -0.128 | 0.097 | 0.186 | 0.219 |
| rs187328863 | T | *CFH* | 0.042 | 0.028 | 0.036 | 0.445 | 0.668 |
| rs61818925 | G | *CFH(CFHR3/CFHR1)* | 0.637 | 0.015 | 0.015 | 0.306 | 0.187 |
| rs35292876 | T | *CFH* | 0.016 | 0.031 | 0.052 | 0.550 | 0.648 |
| rs191281603 | G | *CFH* | 0.01 | 0.041 | 0.088 | 0.639 | 0.578 |
| rs11884770 | C | *COL4A3* | 0.738 | -0.022 | 0.015 | 0.146 | 0.176 |
| rs62247658 | C | *ADAMTS9-AS2* | 0.417 | 0.007 | 0.013 | 0.616 | 0.475 |
| rs140647181 | C | *COL8A1* | 0.018 | -0.092 | 0.057 | 0.109 | 0.106 |
| rs55975637 | A | *COL8A1* | 0.129 | -0.015 | 0.020 | 0.473 | 0.427 |
| rs10033900 | T | *CFI* | 0.495 | 0.004 | 0.014 | 0.762 | 0.664 |
| rs141853578 | T | *CFI* | 0.003 | 0.292 | 0.133 | 0.028 | 0.042 |
| rs62358361 | T | *C9* | 0.014 | 0.025 | 0.057 | 0.668 | 0.742 |
| rs114092250 | G | *PRLR/SPEF2* | 0.975 | -0.046 | 0.045 | 0.300 | 0.297 |
| rs116503776 | G | *C2/CFB/SKIV2L* | 0.872 | 0.059 | 0.020 | 0.003 | 0.005 |
| rs144629244 | A | *C2/CFB/SKIV2L* | 0.010 | 0.067 | 0.065 | 0.300 | 0.360 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| rs114254831 | G | *C2/CFB/SKIV2L* | 0.242 | -0.01 | 0.015 | 0.508 | 0.426 |
| rs181705462 | T | *C2/CFB/SKIV2L* | 0.008 | -0.008 | 0.075 | 0.921 | 0.877 |
| rs943080 | T | *VEGFA* | 0.510 | -0.026 | 0.013 | 0.056 | 0.075 |
| rs1142 | T | *KMT2E/SRPK2* | 0.371 | 0.002 | 0.014 | 0.883 | 0.948 |
| rs7803454 | T | *PILRB/PILRA* | 0.184 | -0.01 | 0.017 | 0.574 | 0.484 |
| rs79037040 | T | *TNFRSF10A* | 0.518 | 0.011 | 0.013 | 0.411 | 0.338 |
| rs10781182 | T | *MIR6130/RORB* | 0.301 | -0.008 | 0.015 | 0.594 | 0.701 |
| rs71507014 | G | *TRPM3* | 0.421 | -0.005 | 0.014 | 0.732 | 0.724 |
| rs1626340 | G | *TGFBR1* | 0.793 | -0.009 | 0.017 | 0.598 | 0.468 |
| rs2740488 | A | *ABCA1* | 0.740 | -0.009 | 0.015 | 0.555 | 0.403 |
| rs12357257 | A | *ARHGAP21* | 0.238 | -0.001 | 0.016 | 0.927 | 0.915 |
| rs3750846 | C | *ARMS2/HTRA1* | 0.297 | 0.01 | 0.014 | 0.468 | 0.866 |
| rs3138141 | A | *RDH5/CD63* | 0.210 | 0.046 | 0.021 | 0.030 | 0.045 |
| rs61941274 | A | *ACAD10* | 0.013 | 0.003 | 0.072 | 0.971 | 0.980 |
| rs9564692 | C | *B3GALTL* | 0.727 | -0.008 | 0.015 | 0.618 | 0.506 |
| rs61985136 | T | *RAD51B* | 0.619 | 0.007 | 0.014 | 0.620 | 0.541 |
| rs2842339 | G | *RAD51B* | 0.095 | 0 | 0.023 | 0.994 | 0.975 |
| rs2043085 | T | *LIPC* | 0.616 | 0.029 | 0.014 | 0.036 | 0.039 |
| rs2070895 | G | *LIPC* | 0.800 | -0.03 | 0.017 | 0.071 | 0.062 |
| rs5817082 | C | *CETP* | 0.745 | 0.017 | 0.016 | 0.284 | 0.402 |
| rs17231506 | T | *CETP* | 0.326 | -0.001 | 0.014 | 0.924 | 0.723 |
| rs72802342 | C | *CTRB2/CTRB1* | 0.929 | 0.003 | 0.028 | 0.914 | 0.932 |
| rs11080055 | C | *TMEM97/VTN* | 0.503 | 0.015 | 0.013 | 0.246 | 0.206 |
| rs6565597 | T | *NPLOC4/TSPAN10* | 0.391 | 0.003 | 0.015 | 0.824 | 0.862 |
| rs2230199 | G | *C3* | 0.224 | -0.034 | 0.017 | 0.044 | 0.060 |
| rs147859257 | G | *C3* | 0.006 | 0.098 | 0.089 | 0.270 | 0.385 |
| rs12019136 | G | *C3(NRTN/FUT6)* | 0.958 | -0.012 | 0.035 | 0.740 | 0.689 |
| rs67538026 | C | *CNN2* | 0.556 | 0.001 | 0.015 | 0.960 | 0.900 |
| rs429358 | T | *APOE* | 0.883 | 0.022 | 0.021 | 0.311 | 0.294 |
| rs73036519 | G | *APOE(EXOC3L2/MARK4)* | 0.697 | 0.012 | 0.015 | 0.453 | 0.477 |
| rs142450006 | TTTTC | *MMP9* | 0.858 | 0.009 | 0.020 | 0.667 | 0.705 |
| rs201459901 | T | *C20orf85* | 0.948 | -0.024 | 0.030 | 0.425 | 0.444 |
| rs5754227 | T | *SYN3/TIMP3* | 0.871 | -0.003 | 0.020 | 0.895 | 0.796 |
| rs8135665 | T | *SLC16A8* | 0.210 | 0.003 | 0.016 | 0.853 | 0.973 |

AF= Allele frequency, SE= Standard error.

*Location of the SNP as in Fritsche et al., 2016, †Refers to the AMD risk-increasing allele, ‡Analysis adjusted for AMD disease status, §Variant not found in the study cohort.

In agreement with the single variant analysis of *CFH* rs3753396, the haplotype H3 that gave the highest risk for higher systemic complement activation was not associated with AMD (*P*=0.80). Haplotype H6 carries the *CFHR4* rs6685931 complement-raising allele (C) but did not reach significance in the association with AMD (*P*=0.14); however the frequency of H6 was relatively low (3%). Haplotype H1-2, the most common haplotype carrying the *CFHR4* rs6685931 complement-raising allele (C), showed a strong risk-conferring association with AMD ($P=1.38\text{x}10^{-12}$, OR=1.318 CI=1.223-1.420) (Table 9, Fig. 4).

Finally, we determined whether other AMD-associated variants are associated with systemic complement activation levels. For this purpose, we extracted the 52 AMD-associated variants reported in the largest AMD study performed so far from the GWAS on complement activation levels.[9] However, no variants outside of the *CFH*/*CFHR* locus were found to be associated with systemic complement activation levels at the genome-wide significance level, nor at a suggestive significance level of $P<0.05/52=0.001$ (Table 10, available at www.aaojournal.org). Interestingly, a risk score based on the 52 AMD risk-conferring alleles associated with higher levels of complement activation ($P=0.043$, $\beta=0.004$, $SE(\beta)=0.002$). A similar risk score including only the variants located in or near complement genes was more strongly associated with higher levels of complement activation ($P=0.022$, $\beta=0.009$, $SE(\beta)=0.004$). This complement risk score included three nominally associated variants: two common variants located in the *CFH* and *C2*/*CFB*/*SKIV2L* loci: rs10922109 and rs116503776 respectively, and a rare variant located in the *CFI* gene: rs141853578 or p.Gly119Arg. However, the effects of these genetic risk scores are smaller compared to the single variant effects in the model for systemic complement activation described in Table 6.

# Discussion

We conducted a GWAS on systemic complement activation levels, evaluating for the first time in an unbiased approach the genetic risk factors involved in the activation of this essential component of the immune system. We identified and replicated two common variants, rs3753396 and rs6685931, that lead to higher systemic complement activation levels independently of age, sex, AMD disease status, triglycerides and body mass index. These two variants were included in a model for systemic complement activation, which explained 18.7% of its variability.

SNP rs3753396 (c.2016A>G, p.Gln672Gln) is a coding, synonymous variant located in exon 14 of the *CFH* gene, and therefore this variant, or the linked causal variant(s), may regulate complement activation levels through FH. Factor H is a key negative regulator of the AP and the amplification loop of the complement cascade, which is expressed constitutively in the liver and locally by other cell types, such as retinal pigment epithelial and endothelial cells.[28-30] Evidence to support the theory that rs3753396 exerts an effect on complement activation through FH comes from genetic studies on other diseases. SNP rs3753396 has been reported to be associated with atypical hemolytic uremic syndrome (aHUS), known to be caused by mutations in *CFH*.[31,32] Moreover, reduced susceptibility to meningococcal disease has also been associated with rs3753396. Meningococcal disease is caused by *Neisseria meningitides*, which binds FH to avoid complement-mediated killing.[33] SNP rs3753396 is in linkage disequilibrium (LD) with rs1065489, also located in *CFH* (c.2808G>T, p.Glu936Asp), which was proposed to be the causal variant for meningococcal disease based on *in-silico* pathogenicity predictions.[34]

SNP rs6685931 (c.59-4315T>C) is located in intron 1 of the *CFHR4* gene. Factor H related-4 (FHR-4) is a glycoprotein that, in contrast to the attenuating effects of FH, seems to promote complement activation. It binds the complement fluid-phase C3b and forms an additional AP C3 convertase (CFHR4-C3bBb), which is less susceptible to FH-mediated decay.35 However, since rs6685931 is in high LD ($r^2$>0.8) with several variants located in the *CFH* gene, either FH or FHR-4 could be responsible for the effects observed on complement activation.

We analyzed the association of genetic variants with systemic complement activation levels in a hypothesis-free manner. The results indicate that with our study design, the genetic variants with the largest effect on complement activation levels are rs3753396 and rs668593, located at the *CFH*/*CFHR* locus. Moreover, other previously associated variants in *CFH* and *C2*/*CFB* could be replicated.[18] Haplotype analysis at the *CFH*/*CFHR* locus revealed two haplotypes with stronger effects on complement activation levels compared to the individual SNPs. These findings suggest that additional variants at the *CFH*/*CFHR* locus play a role in the activation of the complement system. Indeed, several rare coding variants in the *CFH* gene have been shown to lead to increased complement activity.[10] Genetic variants in other genes that influence systemic complement activation levels may be uncovered with larger sample sizes which would allow for the detection of rarer variants and smaller effects. A compelling rare variant candidate which may merit further investigation is *CFI* rs141853578 (p.Gly119Arg), which was found nominally significant in our study. This variant has been previously associated with lower FI levels in plasma, and a lower ability to degrade C3d on the cell surface and C3b in the fluid phase.[36]

In this study, AMD was associated with systemic complement activation, which is an agreement with previous reports.[15-18,20] In our analysis, rs6685931 in *CFHR4* was associated with both systemic complement activation and AMD. Haplotype analyses were in line with these results; we observed that the complement-raising allele of SNP rs6685931 (C) was located mainly on the H1-2 haplotype, which associated with a higher risk for AMD development. Thus, this SNP and its linked haplotype could serve as a robust biomarker for complement activation in the context of AMD, and could be used to identify AMD patients that would benefit most from complement inhibiting therapies.

We noted that the rare haplotype H6 (with a frequency of 3%), also containing rs6685931, had a larger effect on complement activation levels compared to the single variant rs6685931. However, haplotype H6 was not significantly associated with AMD probably due to statistical power limitations. Studies with larger cohort sizes may clarify the role of the H6 haplotype in AMD, and may identify other rare haplotypes that associate with AMD and have larger effects on complement activation levels.

Strikingly, the genetic variant that was most strongly associated with systemic complement activation, rs3753396 in *CFH*, and its main haplotype (H3) did not associate with AMD. SNP rs3753396 and haplotype H3 have been, however, described to confer risk for aHUS development. aHUS is a complement system related disease that leads to systemic

thrombotic microangiopathy and renal endothelial injury.[32,37] This finding suggests that the effect of the haplotypes might be different systemically compared to the AMD disease site, possibly through a tissue-specific effect of the genetic variants. Consequently, systemic complement activation may not always reflect complement activation in the disease tissue and, therefore, it may not be the most appropriate measure for AMD studies. Genetic biomarkers such as SNP rs6685931 and haplotype H1-2 are a robust markers that, together with the C3d/C3 ratio, could serve as biomarkers for complement activity studies in AMD. This is supported by a recent study demonstrating that complement activation levels in aqueous humor are higher than in plasma samples of AMD patients.[38] As a consequence, the effect of rs6685931 and H1-2 on local complement activation might be even larger than the effect seen on systemic levels.

Our results could also further the understanding of other complement-related diseases, as well as be used in the context of personalized medicine involving FH supplementation therapy and other complement-targeting therapies.[39-41] Besides *Neisseria meningitidis*, a number of bacteria, fungi, parasites and viruses bind FH in order to avoid elimination by the alternative pathway of complement system.[42] Also, some cancer cells express FH in order to avoid being targeted by the immune system.[43-45] Other FH related diseases for which our results may be of interest include HUS, aHUS, encephalomyelitis, atherosclerosis, insulin resistance, IgA nephropathy, Alzheimer's disease, cisplatin nephropathy as well as severe dengue, for which variants in the *CFH* gene have been shown to be protective.[46]

In conclusion, we have identified two common variants located at the *CFH*/*CFHR* locus, rs3753396 and rs668593, which strongly influence systemic complement activation levels. Moreover, our haplotype studies suggest that other genetic variants in the *CFH*/*CFHR* locus influence systemic complement activation. Genetic and non-genetic factors identified in this and other studies explain up to 18.7% of the variability in systemic complement activation levels. The common variant rs6685931 in *CFHR4*, and its associated haplotype H1-2, could be used, together with other environmental factors as well as rare genetic variants, to select AMD patients that would benefit from complement inhibiting therapies.

# Methods

## Study population

In this study, we included 2,245 participants from the European Genetic Database (EUGENDA, www.eugenda.org). EUGENDA is a multicenter database for the clinical and molecular analysis of AMD collected at the Radboud University medical center in Nijmegen and at the University Hospital of Cologne. The study participants were separated in two cohorts: a discovery cohort who comprised 1,548 individuals and a replication cohort of 697 individuals.

The study was performed in accordance with the tenets of the Declaration of Helsinki (7th revision) and the Medical Research Involving Human Subjects Act (WMO). Approval of the local ethics committee of both University hospitals was obtained and written informed consents were acquired from all participants. All the individuals included in the study agreed to the performed serum measurements and genotyping. All participants were of European descent and over the age of 50 years. AMD and control status were assigned by multimodal image grading according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders. Age, gender, height and weight were obtained by standardized interviewer-assisted questionnaires.

## Serum complement and lipid measurements

Serum was obtained by a standard coagulation/centrifugation protocol, and within 1 hour after collection the samples were stored at –80°C.

Triglycerides and HDL cholesterol were measured using standard procedures by a clinical chemistry laboratory (Architect Analyzer, Abbott Diagnostics Hoofddorp, The Netherlands). Complement component C3 was assessed by radial immunodiffusion (or Mancini method) using mono specific polyclonal rabbit antisera, and C3d was measured by rocket electrophoresis, as previously described.[21] C3d is a fragment of C3 generated upon activation of the system and, therefore, a direct measurement of complement turnover.[4] Moreover, C3d has the longest half life of all C3 split products.[47] The C3d/C3 ratio is a sensitive way of assessing the activation of the complement system independently of the baseline individual C3 concentration.[48-50] The C3d/C3 ratio has been previously described to be a robust biomarker for complement activation in AMD studies.[19] The different measurements were performed for all samples in a single assay.

## Genotyping

Genomic DNA was extracted from peripheral blood samples using standard procedures. The discovery cohort was genotyped with a custom-designed HumanCoreExome array by Illumina within the International AMD Genetics Consortium (IAMDGC). All the details regarding the design of the array, annotation, imputation and quality control of the genotypic data have been previously described.[9]

Imputed lead variants in GWAS peaks that reached significance, rs6685931 and rs3130572, were confirmed by polymerase chain reaction and Sanger sequencing. SNP rs6685931 was evaluated in 12 individuals representing the three genotypes and a 100% of concordance with the imputed genotypes was achieved. SNP rs3130572 (chromosome 6) was located in a highly repetitive region and specific primers could not be designed, therefore, this SNP was excluded from further analysis.

In the replication cohort, *CFH* rs3753396 and *CFHR4* rs6685931 were genotyped using competitive allele-specific PCR assays according to the manufacturer instructions (KASP genotyping chemistry, LGC, Hoddesdon, UK).

## Statistical analysis

Natural log transformation was applied to normalize the skewed distribution of C3d/C3 measurements. A general linear model for ln(C3d/C3) including as independent variables the environmental factors collected was used to determine potential confounders. The R-squared and adjusted R-squared statistics were estimated for the model. Additionally, the R-squared statistic was estimated for each of the independent factors individually, performing separate models. Analyses were carried out using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA).

A power calculation for the GWAS was performed using the Genetic power calculator.[51] Association tests in the GWAS and replication analyses were performed by means of a linear Wald test from EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS) using allele dosages. Linear regression models adjusted for age, sex, BMI, triglycerides, clinic and the first two ancestry principal components were used. Manhattan and Q-Q plots were generated using the 'qqman' R package (version 0.1.2). The regional plots for chromosome 1, were generated using LocusZoom.[52]

Meta-analysis of fixed effects based on effect size estimates and standard errors was performed using METAL software (version 2-11-03-25).[53]

Evaluation of an interaction between the identified SNPs and clinic or AMD status was performed including an interaction parameter on the general linear model and assessing nominal significance.

Comparisons of systemic complement activation levels between the genotype groups were performed using a general linear model adjusted for age, BMI, triglycerides and clinic including both the discovery and the replication cohorts. SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA) was used for these analyses.

In order to estimate how much of the variation in systemic complement activation could be explained by the identified factors, general linear models for systemic complement activation were performed using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA). Only the 1,548 individuals from the discovery cohort were included in order to accommodate the *CFH* rs800292 and *C2* rs9332739 SNPs, which were not analyzed in the replication cohort. The adjusted R-squared statistic was estimated for the models.

Haplotype analysis was carried out on the 1,548 patients genotyped with exome-arrays using the haplo.glm function of the R library 'haplo.stats' (version 1.7.7). Analysis was

performed based on a general linear model adjusted for age, sex, BMI, triglycerides, clinic and the first two ancestry principal components.

Single variant and haplotype association analyses with AMD were performed on the 1,548 individuals of the discovery cohort. Single variant analyses were performed using a Firth bias-corrected likelihood-ratio test with EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS). Haplotype analyses were based on $X^2$ tests including haplotypes with a predicted probability ≥0.75 using SPSS software version 20.0 (IBM Software and Systems, Armonk, NY, USA).

Risk scores for AMD-associated variants were calculated as a sum of the number of AMD risk-increasing alleles. Two risk scores were calculated: the first risk score included the 52 AMD-associated variants described in Fritsche et al., 2016, and the second risk score included the 19 variants located in or near complement genes out of these 52. The variants included in the complement risk score were: rs10922109, rs570618, rs121913059, rs148553336, rs187328863, rs61818925, rs35292876 and rs191281603 from the *CFH* locus; rs10033900 and rs141853578 from the *CFI* locus; rs62358361 from the *C9* locus; rs116503776, rs144629244, rs114254831 and rs181705462 from the *C2*/*CFB*/*SKIV2L* locus; rs147859257 from the *TMEM97*/*VTN* locus; rs2230199, rs147859257 and rs12019136 from the *C3* locus. The risk scores were included in linear models for ln(C3d/C3) that included age, BMI, triglycerides and clinic as covariates, and the effect of the risk score was estimated. The 1,548 individuals of the discovery phase, genotyped with the HumanCoreExome array, were included in these analyses.

Figures including graphs were generated using Graphpad Prism 5.03 (GraphPad Software, La Jolla California USA).

# Additional information

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## Meeting Presentation

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# 3.2

# Complement factor H related 4 and age-related macular degeneration

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# Abstract

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in the Western world. However, the exact mechanisms underlying this disease are not fully understood yet. An over-activation of the complement system has been proven to be involved in the pathogenesis of AMD. Genetic studies have identified variants spanning the *CFH*/*CFHR* locus to be associated with AMD development, but the effect of these variants on protein expression or function of Factor H (FH) or the Factor H related proteins remains to be determined. A recent genome-wide association study on systemic complement activation in AMD described a SNP located in the *CFHR4* gene as the top associated variant. Therefore, we hypothesized that FHR-4 could be directly implicated in AMD pathogenesis.

In this study, we show that FHR-4 accumulates in the choriocapillaris, partially diffuses through the Bruch's membrane and can be detected in drusen. Additionally, FHR-4 activates the complement system by competing off FH and factor I binding to C3b. Whereas expression of FHR4 was not detected in the retinal pigment epithelium and choroid, evaluation of systemic FHR-4 shows elevated levels in advanced AMD cases compared to controls ($\beta$ = 0.21, CI = 0.12 – 0.30, $P = 7.1 \times 10^{-6}$). Systemic FH levels, however, do not show any difference ($P$ = 0.70). We also evaluated the AMD-associated variants at the *CFH*/*CFHR* locus for association with FHR-4 levels. The AMD-risk conferring alleles of rs10922109, rs187328863 and rs61818925 showed an association with increased FHR-4 levels independently of AMD status ($P = 6.5 \times 10^{-52}$, $P = 2.7 \times 10^{-5}$ and $P = 8.2 \times 10^{-18}$ respectively). The SNP rs570618, which is in high linkage disequilibrium with the p.Y402H variant, showed an association with lower FHR-4 levels ($P = 2.5 \times 10^{-14}$). Haplotype analysis of these variants revealed two haplotypes associated with AMD, which showed a protective effect (H2 and H3). The H2 and H3 haplotypes were also highly associated with decreasing FHR-4 levels, independently of AMD status ($\beta$ = -0.46, CI = -0.52 – -0.39, P = 4.7 x 10-42 and $\beta$ = -0.24, CI = -0.31 – -0.16, $P = 1.2 \times 10^{-9}$, respectively). A genome-wide association analysis on systemic FHR-4 levels revealed only the *CFH*/*CFHR* locus as significantly associated. Altogether, our findings suggest that FHR-4 is a new component of the complement cascade involved in the pathogenesis of AMD and a compelling new therapeutic target.

# Introduction

Age-related macular degeneration (AMD) is a highly prevalent cause of vision loss that is predicted to affect 196 million people worldwide by 2020[1]. The condition results in destruction of the central retina, i.e. the macula, where initial changes occur in the retinal pigment epithelium/Bruch's membrane (BrM)/choriocapillaris complex. In early AMD soft drusen are observed (Fig. 1a), which are accumulations of debris within BrM. Progress to late AMD is associated with visual loss and this can manifest as either geographic atrophy (commonly referred to as 'dry' AMD) or choroidal neovascularisation (or 'wet' AMD)[2].

AMD has a strong genetic basis and at least 34 genetic loci have been associated with this condition[3]. Several genes encoding components of the complement cascade are associated with AMD and the strongest effect is seen at the regulator of complement activation locus on chromosome 1 which encodes *CFH* and *CFHR1-5*[4,5]. The *CFH* gene encodes both factor H (FH) and a smaller splice variant called factor H-like protein 1 (FHL-1)[6,7]. While FH is the main blood borne regulator of complement activation, FHL-1 predominates in the BrM and extracellular matrix (ECM) in between the vessels of the choriocapillaris, i.e. the intercapillary septa (Fig. 1a)[4,8]. The identification of rare coding variants in *CFH* implicated this gene in AMD[9,10], but due to extensive linkage disequilibrium, the reason why common variants at the *CFH*/*CFHR* locus are associated with altered AMD risk remains elusive. Genetic variants located in the *CFHR4* gene and haplotypes delineated by single nucleotide polymorphism (SNPs) within and downstream of *CFHR4* have been shown to influence AMD disease risk[11,12]. Moreover, a genome-wide association study (GWAS) on systemic complement activation in the context of AMD has recently identified a variant in the *CFHR4* gene as the top SNP associated with higher systemic complement activation and a higher risk for AMD development (see chapter 3.1), which raises the possibility that this gene could be implicated in AMD. Furthermore, the factor H-related 4 (FHR-4) protein encoded by *CFHR4*, has been shown to promote complement activation by forming a platform for the assembly of alternative pathway C3 convertase[13,14]. Therefore, in this study we examined the role of FHR-4 in AMD.

# Results

## FHR-4 accumulation in the choriocapillaris drives complement activation

In order to assess whether FHR-4 localizes at the AMD disease site, immunohistochemistry experiments were performed on retinas of post-mortem donor eyes affected with AMD. Using a specific monoclonal antibody against FHR-4 (Extended data Fig. 1 and 2) we observed the protein accumulating in the choriocapillaris and Bruch's membrane (BrM), particularly localizing to the intercapillary septa: the extracellular matrix (ECM) between

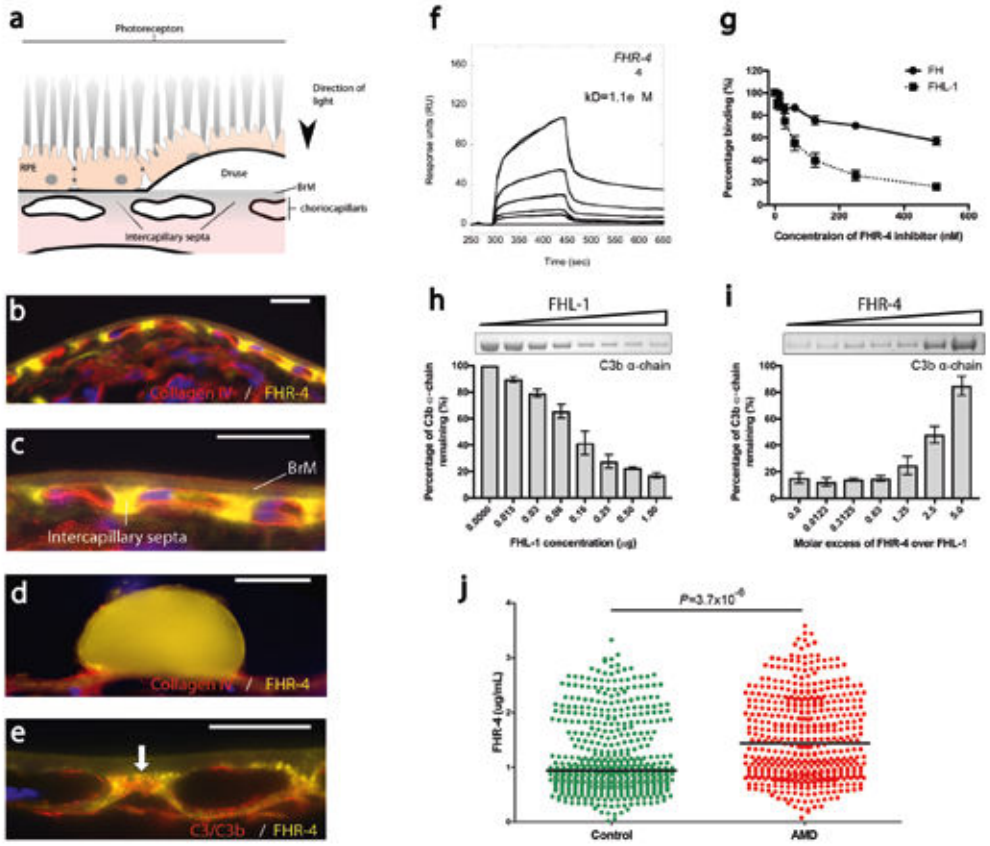


FIGURE 1 **Accumulation of FHR-4 in the eye inhibits C3b breakdown.**

**a)** Schematic diagram showing anatomical structures in the macula including the retinal pigment epithelium (RPE), the underlying Bruch's membrane (BrM), the choriocapillaris and the intercapillary septa (pillars of extracellular matrix that separate the fenestrated capillaries of the choriocapillaris); basement membranes are represented as black lines. Drusen, the hallmark lesions of early AMD, form within BrM, underneath the RPE basement membrane. **b-c)** Immunohistochemistry showing the localization of FHR-4 (yellow) which is particularly evident in the intercapillary septa of the choriocapillaris, but weak labeling is also seen within BrM: collagen IV staining is used to delineate basement membranes which define the inner and outer borders of BrM (red), DAPI labeling is in blue. **d)** FHR-4 is also localized in drusen. **e)** Both FHR-4 and C3/C3b localize in the intercapillary septa of the choriocapillaris (white arrow): scale bars 20μm. **f)** SPR analysis showing the binding of FHR-4 to immobilized C3b. **g)** Solid phase binding assays demonstrate that the binding of fluid phase FH or FHL-1 to immobilized C3b can be competed off with increasing concentrations of FHR-4. **h)** Measurement of factor H-like protein 1 (FHL-1) mediated breakdown of C3b by factor I (FI); in the presence of fixed concentrations of C3b and FI, increasing concentrations of FHL-1 result in increased breakdown of the C3b α-chain as measured by analyzing the intensity of Coomassie-blue stained bands following SDS-PAGE (Extended data Fig. 3). **i)** Optimal C3b breakdown conditions from (h) are repeated but now including increased concentrations of fluid-phase FHR-4, and an inhibition of FHL-1/FI-mediated C3b α-chain breakdown is observed. **j)** Mean systemic levels of FHR-4 are higher in subjects with AMD compared to non-AMD controls.

the fenestrated capillaries of the choriocapillaris (Fig. 1a-c). Weak staining for FHR-4 within BrM indicates that the protein can penetrate in some degree into the ECM (Fig. 1c), but diffusion experiments with BrM and purified protein indicated that it does not pass through completely (Extended data Fig. 3). In addition, staining of drusen showed immunoreactivity for FHR-4 (Fig. 1d). C3/C3b localized to the intercapillary septa of the choriocapillaris as well as FHR-4 (Fig. 1e). FHR-4 has previously been shown to bind C3b and form an alternative C3b convertase[13,15]. We confirmed that FHR-4 binds immobilized C3b (Fig. 1f) and demonstrated that FHR-4 can compete off the binding of both negative regulators of complement activation FH and FHL-1 to immobilized C3b in solid-phase binding assays (Fig. 1g).

Given that FHR-4 binds to C3b and out-competes the binding of FHL-1, the complement regulator responsible for protecting the intercapillary septa from aberrant complement activation[4,8], we investigated whether FHR-4 could inhibit C3b breakdown mediated by FHL-1 and FI, employing fluid-phase C3b breakdown assays where the cleavage of the C3b $\alpha$-chain was used as a readout. Using conditions in which ~80% C3b $\alpha$-chain breakdown is achieved in the absence of FHR-4, we found that adding increasing concentrations of FHR-4 resulted in a progressive inhibition of C3b $\alpha$-chain breakdown; where only ~50% C3b breakdown occurred at a 2.5 molar excess of FHR-4 over FHL-1(Fig. 1h and i and Extended data Fig. 4).

## Systemic FHR-4 levels are elevated in advanced AMD cases

Next we investigated the source of FHR-4 observed in the intercapillary septa. Transcription of the *CFHR4* gene was not detected in the RPE or in the choroid of human eyes (data not shown). Therefore, we concluded that the observed FHR-4 protein was derived from the systemic circulation and passed from the choriocapillaris into the surrounding ECM. Using a FHR-4 specific ELISA, we measured circulating FHR-4 concentration in a total of 484 advanced AMD patients (with geographic atrophy and/or choroidal neovascularization) and 522 examined controls, collected within two independent AMD studies (Cambridge and EUGENDA, Table 1). Patients with AMD showed a significant elevation of FHR-4 levels compared to controls, both in each study separately (Table 1) and in the meta-analysis (overall sex- and age-adjusted: $\beta = 0.21$, CI = 0.12 – 0.30, $P = 7.1 \times 10\text{-}6$; Table 1, Fig. 2), whilst there was no difference in FH levels between AMD patients and controls (overall sex- and age-adjusted $\beta = 0.01$, CI = -0.02 – 0.04, $P = 0.70$; Table 1, Fig. 1j).

## AMD-associated variants at the *CFH*/*CFHR* locus are associated with systemic FHR-4 levels

A recent large GWAS of AMD conducted by the International AMD Genomics Consortium reported 52 independently associated variants across 34 AMD risk loci, including 8 variants at the *CFH*/*CFHR* locus. We hypothesized that any of these 8 genetic variants (single or in combination) may be associated with systemic FHR-4 levels that in turn are increased in AMD patients.

TABLE 1 **Demographics of study cohorts and sex- and age-adjusted association of late AMD status with systemic FHR-4 and FH levels.**

| | Cambridge | | | Eugenda | | | Meta-analysis |
|---|---|---|---|---|---|---|---|
| | Controls | Cases | P | Controls | Cases | P | P |
| N | 214 | 304 | | 307 | 180 | | |
| Age, ys (SD) | 75.2 (8.0) | 74.1 (8.3) | | 70.0 (6.5) | 79.3 (8.6) | | |
| Male (%) | 36.5 | 47.0 | | 42.9 | 42.2 | | |
| FHR-4 levels, ug/ml* (95% CI) | 5.5 (4.9-6.2) | 6.6 (6.0-7.2) | 0.013 | 6.0 (5.6-6.3) | 7.2 (6.6-7.8) | $9.6 \times 10^{-5}$ | $3.7 \times 10^{-6}$ |
| FH levels, ug/ml* (95% CI) | 349.0 (338.9-359.4) | 348.6 (340.2-357.2) | 0.894 | 304.7 (297.3-312.2) | 308.7 (298.0- 319.8) | 0.339 | 0.487 |

SD = Standard deviation, CI = Confidence interval. FHR-4 and FH levels are expressed as geometric mean values (back-log transformed).

TABLE 2 **AMD-adjusted association of the 8 AMD variants at the *CFH*/*CFHR* locus with systemic FHR-4 levels.**

| | | | | | Cambridge | | | Eugenda | | | Meta-analysis | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IAMDGC association signal number | SNP | Position | Alleles | IAMDGC direction of allelic association | Beta | CI | P | Beta | CI | P | Beta | P |
| 1.5 | rs187328863 | 1:196380158 | C/T | + | 0.33 | 0.04 – 0.62 | 0.027 | 0.31 | 0.14 – 0.49 | $4.0 \times 10\text{-}4$ | 0.31 | $2.7 \times 10^{-5}$ |
| 1.4 | rs148553336 | 1:196613173 | T/C | - | 0.36 | -0.19 – 0.91 | 0.200 | 0.74 | 0 – 1.48 | 0.052 | 0.50 | 0.028 |
| 1.2 | rs570618 | 1:196657064 | T/G | + | -0.18 | -0.28 – -0.08 | 0.001 | -0.23 | -0.29 – -0.16 | $2.0 \times 10^{-11}$ | -0.21 | $2.5 \times 10^{-14}$ |
| 1.1 | rs10922109 | 1:196704632 | C/A | - | -0.40 | -0.51 – -0.29 | $2.4 \times 10^{-12}$ | -0.41 | -0.47 – -0.35 | $7.4 \times 10^{-35}$ | -0.41 | $6.5 \times 10^{-52}$ |
| 1.7 | rs35292876 | 1:196706642 | C/T | + | -0.05 | -0.53 – 0.43 | 0.836 | 0.32 | 0.05 – 0.58 | 0.020 | 0.23 | 0.053 |
| 1.3 | rs121913059 | 1:196716375 | C/T | + | Only 1 case heterozygote carrier | | | | - | | | - |
| 1.6 | rs61818925 | 1:196815450 | G/T | - | -0.24 | -0.35 – -0.13 | $1.9 \times 10^{-5}$ | -0.25 | -0.32 – -0.19 | $4.7 \times 10^{-13}$ | -0.25 | $8.2 \times 10^{-18}$ |
| 1.8 | rs191281603 | 1:196958651 | C/G | + | -0.56 | -1.71 – 0.60 | 0.344 | 0.32 | -0.06 – 0.69 | 0.098 | 0.23 | 0.199 |

IAMDGC=International AMD Genomics Consortium; SNP rs121913059, i.e., IAMDGC association signal number 1.3, was seen only in 1 case in the Cambridge cohort at heterozygous state and was not included in the analyses.

TABLE 3 Two-cohort meta-analysis of the association of haplotypes at the CFH locus with late AMD and systemic FHR-4 levels.

| | | | | | | | | Frequency | | Association with late AMD | | | Association with FHR-4 levels[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | rs187328863 | rs148553336 | rs570618 | rs10922109 | rs35292876 | rs61818925 | rs191281603 | Control | Case | OR | P | CI | Beta | CI | P |
| | 1.5 | 1.4 | 1.2 | 1.1 | 1.7 | 1.6 | 1.8 | | | | | | | | |
| H1 | C | T | T | C | C | G | C | 0.316 | 0.508 | Reference | | | Reference | | |
| H2 | C | T | G | A | C | T | C | 0.251 | 0.137 | 0.36 | $3.0 \times 10^{-13}$ | 0.28 – 0.48 | -0.46 | -0.52 – -0.39 | $4.7 \times 10^{-42}$ |
| H3 | C | T | G | A | C | G | C | 0.185 | 0.077 | 0.29 | $6.5 \times 10^{-14}$ | 0.21 – 0.40 | -0.24 | -0.31 – -0.16 | $1.2 \times 10^{-9}$ |
| H4 | C | T | G | C | C | T | C | 0.125 | 0.126 | 0.67 | 0.016 | 0.51 – 0.93 | 0.04 | -0.03 – 0.12 | 0.248 |
| H5 | C | T | G | C | C | G | C | 0.080 | 0.073 | 0.60 | 0.008 | 0.41 – 0.87 | 0.15 | 0.05 – 0.24 | 0.007 |
| H6 | T | T | T | C | C | G | C | 0.024 | 0.041 | 1.12 | 0.728 | 0.59 – 2.14 | 0.28 | 0.15 – 0.41 | $3.9 \times 10^{-5}$ |
| H7 | C | T | T | C | C | T | C | 0.013 | 0.018 | 0.70 | 0.362 | 0.32 – 1.51 | -0.34 | -0.57 – -0.10 | 0.005 |
| H8 | C | T | T | C | T | G | C | 0.006 | 0.020 | 1.99 | 0.169 | 0.75 – 5.28 | 0.12 | -0.08 – 0.32 | 0.249 |

[a] Adjusted for late AMD status

Association analyses with AMD status was performed using logistic regresion models and with FHR-4 levels using linear regression models.

One of the 8 variants is the highly penetrant rare variant p.R1210C. This variant was only present heterozygously in one individual belonging to the Cambridge cohort, and therefore was not included in the genetic association analyses. Out of the 7 evaluated variants, single-SNP association analyses revealed a strong association with FHR-4 levels, independently of AMD status, for four variants: SNPs rs10922109 ($\beta$ = -0.41, CI = -0.46 – -0.35, $P$ = 6.5 x $10^{-52}$), rs570618 ($\beta$ = -0.21, CI = -0.27 – -0.16, $P$ = 2.5 x $10^{-14}$), rs187328863 ($\beta$ = 0.32, CI = 0.17 – 0.47, P = 2.7 x $10^{-5}$) and rs61818925 ($\beta$ = -0.25, CI = -0.30 – -0.19, $P$ = 8.2 x $10^{-18}$). The AMD-risk alleles were associated with higher FHR-4 levels except rs570618, for which increasing disease risk allele G was associated with decreased FHR-4 levels. SNP rs148553336 showed only a nominally significant association with FHR-4 levels ($\beta$ = 0.50, CI = 0.05 – 0.94, $P$ = 0.028), which was not significant after correction for 7 tests and the other two SNPs, rs35292876 and rs191281603, were not associated ($P$ = 0.053 and $P$ = 0.199, respectively, Table 2).

## Haplotype analysis at the *CFH*/*CFHR* locus reveals haplotypes strongly associated both with AMD and FHR-4 levels

In order to determine the cumulative effect of several variants on the same haplotype, we extracted the haplotypes formed by the 7 AMD-associated variants at the *CFH*/*CFHR* locus using the phased genotype data produced within the GWAS of the IAMDGC study[3]. Haplotype-based analyses were performed to assess the association of the observed haplotypes with AMD and with FHR-4 levels in each cohort separately, and corresponding results were meta-analysed. We observed 8 haplotypes with an overall frequency greater than 1% in the two cohorts combined (Table 3). Using the most common haplotype CTTCCGC (H1; 50% in cases, 32% in controls) as reference, we found two AMD protective haplotypes, CTGACTC (H2; OR = 0.36 , CI = 0.28-0.48, $P$ = 3.0 x $10^{-13}$) and CTGACGC (H3; OR = 0.29, CI = 0.21-0.40, $P$ = 6.5 x $10^{-14}$). Haplotypes H2 and H3 were also highly associated with decreasing FHR-4 levels, independently of AMD status ($\beta$ = -0.46, CI = -0.52 – -0.39, $P$ = 4.7 x $10^{-42}$; $\beta$ = -0.24, CI = -0.31 – -0.16, $P$ = 1.2 x $10^{-9}$, respectively). These two haplotypes contain the AMD protective and FHR-4 lowering allele A of the top IAMDGC SNP rs10922109. While both haplotypes differ from the reference H1 also for the presence of FHR-4 lowering allele G of rs570618 (i.e., the second top IAMDGC SNP), only H2 contains additionally the FHR-4 lowering allele T of rs61818925. In addition, two other haplotypes showed an association with FHR-4 despite not being associated with AMD: TTTCCGC (H6) and CTTCCTC (H7). The haplotype H6 showed an association with higher levels of FHR-4 ($\beta$ = 0.28, CI = 0.15 – 0.41, $P$ = 3.9 x $10^{-5}$). This haplotype differed from the reference haplotype on the presence of the FHR-4-raising allele T in rs187328863. The rarer haplotype H7 showed an association with lower FH4-levels ($\beta$ = -0.34, CI = -0.57 – -0.10, $P$ = 0.005), and differed from the reference haplotype on the presence of the FHR-4-lowering allele T of rs61818925. The remaining haplotypes (H4, H5 and H8) did not show a significant association with FHR-4 levels after Bonferroni correction for multiple testing.

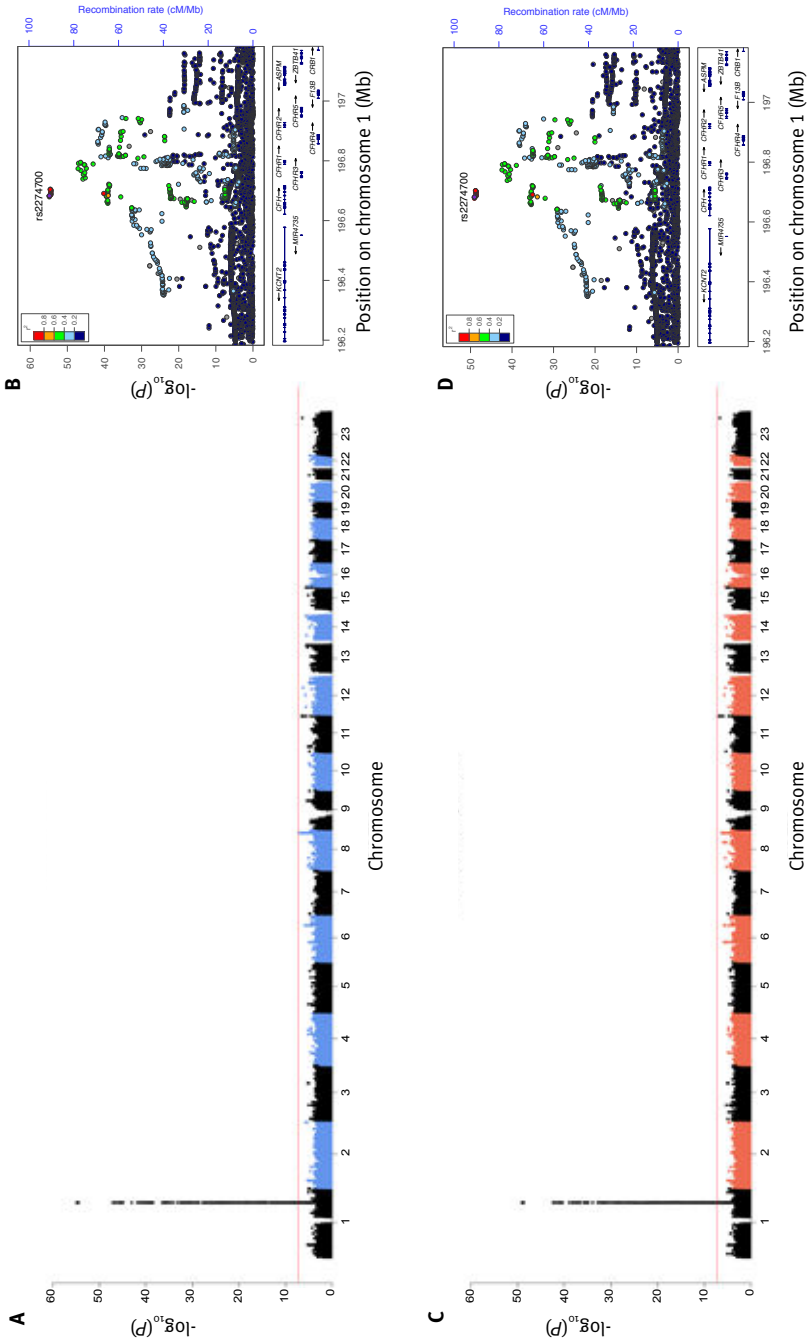


FIGURE 2 **GWAS on log-FHR-4 levels reveals a strong signal spanning the *CFH/CFHR* locus**

**a)** Results of the GWAS on FHR-4 levels meta-analysis (Cambridge and Eugenda cohorts). **b)** Locus zoom plot showing a detailed view of the associated loci in chromosome 1. The linkage disequilibrium calculations are based on the rs2274700 top variant. **c)** Results of the GWAS on FHR-4 levels meta-analysis (Cambridge and Eugenda cohorts) adjusted for AMD status. **d)** Locus zoom plot showing a detailed view of the associated loci in chromosome 1 in the GWAS adjusted for AMD status.

The linkage disequilibrium calculations are based on the rs2274700 top variant. The Manhattan plots (a and c) illustrate the -log(*P*-values) of each individual SNP tested for association. The horizontal red line indicates the threshold considered for significance (*P*-value<5x10$^{-8}$).

### No locus other than *CFH*/*CFHR* shows genome-wide level association with FHR-4 levels

To assess whether genetic variants associated with systemic FHR-4 levels exist at loci other than *CFH*/*CFHR*, we also performed a (hypothesis-free) GWAS for FHR-4 levels. The analysis was performed separately in the Cambridge and EUGENDA cohorts, and the results were combined in a meta-analysis. Genotype data on a total of 9,160,410 were available from the IAMDGC study for the 1,005 individuals included in this study.[3] This analysis had >80% power to detect associations of genetic variants with a minor allele frequency ≥5% explaining ≥3.9% of variance in FHR-4 levels.

Through linear regression models adjusted for age, sex and the first two ancestry principal components, we identified a total of 701 variants that reached genome-wide significance ($P < 5 \times 10^{-8}$) (Fig. 2, Supplementary Fig. 5). These variants were all located on chromosome 1 spanning the *CFH*/*CFHR* locus (chr1:196,240,335–197,275,153, according to the GRCh38 genome assembly). The top signals of association with FHR-4 levels were at SNPs rs7535263, rs10737680 and rs2274700 ($\beta = -0.195$, CI = -0.219 – -0.171, $P = 1.4 \times 10^{-55}$ for all three variants). These variants are located in the *CFH* gene (rs7535263, intron 9, c.1337-519G>A; rs10737680, intron 9, c.1337-3410A>C; rs2274700, exon 10, p.Ala473=, Figure 2). Sensitivity analysis adjusting additionally for AMD status revealed comparable results with the same top three associated SNPs rs7535263, rs10737680 and rs2274700 ($P = 8.9 \times 10^{-50}$ for all three variants; Fig. 2, Supplementary Fig. 5), and no other locus showed genome-wide significant associations.

## Discussion

In this study, we aimed to assess whether FHR-4 may play a role in AMD pathology. We found that FHR-4 localizes at the AMD disease site as it is present in drusen and in the BrM, specifically at the intercapillary septa. We did not detect FHR-4 expression in the RPE nor in the choroid, and the systemic circulation may be the source of the FHR-4 detected in the intercapillary septa. Systemic levels of FHR-4 were higher in AMD patients compared to controls; therefore FHR-4 levels could be used as a biomarker for the disease. This contrasts with the results for FH, for which differences in systemic levels between cases and controls were not detected. Several studies had previously evaluated systemic levels of FH and contradictory results had been published[16-24]. The largest studies performed so far were by Ansari et al., Silva et al. and Smailhodzic et al. Ansari et al. compared 382 cases vs. 201 controls and reported lower FH levels in AMD patients[20]. In contrast, Silva et al. analyzed 119 AMD patients and 152 controls and did not find a significant difference[24] in FH levels and Smailhodzic et al. analyzed 197 AMD patients and 150 controls and did not find a significant difference either[19]. Our study did not find an association between systemic FH levels and AMD status, either in the separate cohorts nor in the meta-analysis, which included 484 AMD patients and 394 controls. Overall, our findings suggest that systemic levels of FHR-4 (and not of FH) may be an important source of inflammation for AMD[25].

The results of our genetic analysis suggest that the elevated FHR-4 levels found in AMD patients may be in part related to genetic variability. Four AMD-associated variants at the *CFH*/*CFHR* locus were found to be associated with FHR-4 levels independently of AMD status. The AMD-risk conferring allele for the variants rs10922109, rs187328863 and rs61818925 showed increased FHR-4 levels. These SNPs locate in intron 15 of *CFH*, intron 12 of *KCNT2* (upstream of *CFH*) and in an intergenic region (upstream of *CFHR4*) respectively. Contrary to what we expected, one of the variants, rs570618, associated with lower FHR-4 levels. However, this variant is in high linkage disequilibrium with rs1061170 (p.Y402H) for which a damaging effect on FH function has been described[26]. To take into account the cumulative effect of these variants we carried out a haplotype analysis. Haplotypes H2 and H3 were protective for AMD and were associated with decreasing FHR-4 levels, independently of AMD status.

In conclusion, the immunohistochemistry experiments on human retinas, systemic FHR-4 measurements and genetic analyses presented in this study point towards a potential role of FHR-4 in AMD pathogenesis, through an increased activation of the complement system. As a consequence, inhibition of FHR-4 may represent a new therapeutic strategy for AMD.

# Methods

## Proteins and antibodies

Recombinant FHR-4 was made through the GenScript gene synthesis and protein expression service using their baculovirus-insect cell expression system, and was based on the published sequence for the FHR-4B variant of the *CFHR4* gene (UniProt identifier Q92496-3): the protein was designed to include a N-terminal 6x His tag and TEV cleavage site (see Extended Fig. 1). Recombinant FHL-1 was expressed in HEK293 cells as described previously[8]. Commercially available purified complement proteins used include C3b (VWR International, Lutterworth, UK, catalogue no. 204860), FH (Sigma-Aldrich, catalogue no. C5813), and FI (VWR International, catalogue no. 341280). Commercially available antibody against collagen IV was used (catalogue no: 600-401-106S, 2B Scientific Ltd., Oxford, UK).

## Generation of a FHR-4 monoclonal antibody

Mice were immunised subcutaneously (sc) with recombinant FHR-4 (~30µg/mouse; supplied by SC) in complete Freund's adjuvant; boosted 4 and 6 weeks later with FHR4 (dose as above), in incomplete Freund's adjuvant and test bled at 8 weeks.

The titre of anti-FHR-4 antibody was assessed by screening sera from individual mice in a capture ELISA. 96-well microtiter plates were coated with recombinant FHR4 (0.2µg/well

in carbonate buffer pH9.6) for 1 hour at 37˚C, blocked in 1% BSA in PBS/tween, washed three times in PBS/tween and blotted dry. A doubling dilution of serum from each mouse (in duplicate; 1:100 - 1:256,000) was added to the wells and incubated for 1 hour at 37˚C. Wells were washed three times in PBS/tween, then HRP-labelled anti-mouse IgG (Jacksons) diluted 1:1000 in PBS/tween was added to each well and incubated for 1 hour at 37˚C. Wells were washed as above, blotted dry then OPD substrate added to each well. When colour had developed sufficiently, the reaction was stopped by adding an equal volume of 10% $H_2SO_4$ to each well. Plates were read in an ELISA reader. Titre for a serum sample was the highest dilution giving an absorbance ›2x background (non-immune serum at the same dilution).

The mouse with the highest titre in the above assay was boosted intraperitoneally with FHR4 (30µg in PBS). The mouse was sacrificed 48 hours later and the spleen harvested aseptically.

Spleen cells were obtained by perfusion with RPMI in a sterile cabinet. Spleen cells were then fused with SP2 myeloma cells to generate hybridomas using a standard protocol. Cells were suspended in 100ml complete medium containing HAT (to select for hybridomas) and plated out, 100µl/well in 10 x 96-well flat-bottomed tissue culture plates that had been pre-conditioned by plating peritoneal macrophages 24 hours previously. Plates were placed in a 37˚C/5% $CO_2$ incubator and left undisturbed for 14 days.

Supernatant (50µl) was removed from each well and transferred to corresponding wells in a 96-well microtiter plate coated with FHR-4 as above; bound antibody was detected as above. Cells were harvested from wells that were positive in the screen, diluted to 10ml in complete medium containing HT, and re-cloned into macrophage-preconditioned 96-well plates.

After 10-14 days, wells containing visible clones were screened as above. Positive wells were simultaneously re-cloned and expanded to 24-well plates.

Once stable clones had been obtained and expanded in sufficient scale, antibodies were purified either on protein G or by salt precipitation. Purified antibodies were tested in western blotting against recombinant FHR-4 and human serum. Non-competitive pairs of antibodies were identified for ELISA development.

## Human eye tissue

Post-mortem donor eyes were obtained from the Manchester Eye Bank at the Royal Eye Hospital (Manchester, UK), within 48 hours from the time of death, after removal of the corneas for transplantation; these were classified and curated as part of the Manchester Eye Tissue Repository (ETR). No organs/tissues were procured from prisoners. Our research adhered to the tenets of the Declaration of Helsinki and in all cases, there was

prior informed consent for the eye tissue to be used for research obtained and held by the Manchester Eye Bank, and guidelines established in the Human Tissue Act of 2004 (UK) were followed. Ethical approval for the use of tissue in these experiments was granted by the Manchester Eye Tissue Repository ethics committee (ref. 15/NW/0932).

## Immunohistochemistry

Tissue sections (10 µm) were stained for the presence of endogenous FHR-4, collagen IV or C3/C3b as described previously[8]. Briefly, tissue sections were incubated with chilled (-20˚C) histological grade acetone (Sigma-Aldrich) and methanol (mixed 1:1) for 20 seconds before thorough washing with PBS. Tissue sections were blocked with 0.1% (w/v) BSA, 1% (v/v) goat serum, and 0.1% (v/v) Triton X-100 in PBS for 1 h at room temperature. After washing with PBS, tissue sections were incubated with Ab combinations of either 10 µg/ml of anti-FHR-4 (clone 150: see above) mixed with 1 µg/ml Collagen IV rabbit polyclonal antibody, or anti-FHR-4 mixed with 1 µg/ml anti C3 rabbit polyclonal antibody (catalogue no: 21337-1-AP, Proteintech Group, Inc, United States), for 16 h at 4ºC. Sections were washed and biotinylated anti-mouse IgG (Catalogue No. BA_9200, Vector laboratories, Inc) diluted 1:250 in PBS was applied for 1 hour to amplify the FHR-4 signal. Slides were subsequently washed and Alexa Fluor® 647 streptavidin (catalogue no: S32357, Invitrogen) diluted 1:250 in PBS and Alexa Fluor® 488-conjugated goat anti-rabbit Ab (Invitrogen, USA) diluted 1:500 in PBS were added for 2 h at room temperature. After washing DAPI was applied as a nuclear counterstain (at 0.3 mM for 5 min) prior to mounting with medium (Vectashield; H-1400, Vector Laboratories, Peterborough, UK) and application of a coverslip.

In the case of blank control sections the exact same protocol was followed but PBS replaced the primary antibody. To test antibody specificity in immunohistochemistry pre-adsorption experiments were performed whereby 10-fold molar excess of pure recombinant FHR-4 is premixed with the anti-FHR-4 Ab prior to application to the tissue sections. In all cases images were collected on a Zeiss Axioimager.D2 upright microscope using a 40x / 0.5 EC Plan-neofluar and 100x / 0.5 EC Plan-neofluar objective and captured using a Coolsnap HQ2 camera (Photometrics) through Micromanager software v1.4.23. Specific band pass filter sets for DAPI, FITC and Cy5 were used to prevent bleed through from one channel to the next. Images were then processed and analysed using Fiji ImageJ (http://imagej.net/Fiji/Downloads).

To prevent bleed-through of color from one channel to the next, specific band pass filter sets were used for DAPI, FITC, and Cy-5. All images were handled using ImageJ64 (version 1.40g; http://rsb.info.nih.gov/ij).

## Surface plasmon resonance

The binding of FHR-4 to immobilised C3b was measured by surface plasmon resonance (SPR) using a Biacore 3000 (GE Healthcare). The sensor surfaces were prepared by

immobilizing human C3b onto the flow cells of a Biacore series S carboxymethylated dextran (CM5) sensor chip (GE Healthcare) using standard amine coupling and included blank flow cells were no C3b protein was present. Experiments were performed at 25°C and a flow rate of 15 µl/min in PBS with 0.05% surfactant P20. FHR-4 was injected in triplicate at concentration ranging from 1 to 100µg/ml. Samples were injected for 150 seconds and dissociated for another 200 seconds and the chip was regenerated between injections with 1M NaCl for 1 min before chip is re-equilibrated into PBS with 0.05% surfactant P20 prior to the next injection. After subtraction of each response value from the blank cell, association and dissociation rate constants were determined by global data analysis. All curves were fitted using a 1:1 Langmuir association/dissociation model (BIAevaluation 4.1; GE Healthcare).

## Solid phase binding assays

Purified C3b was adsorbed onto the wells of microtiter plates (Nunc Maxisorb, Kastrup, Denmark) at 1µg/well in 100µl/well PBS for 16 h at room temperature. Plates were blocked for 90 minutes at 37°C with 300µl/well 1% (w/v) BSA in assay buffer (20mM HEPES, 130mM NaCl, 0.05% (v/v) Tween-20, pH 7.3). This standard assay buffer (SAB) was used for all subsequent incubations, dilutions and washes and all steps were performed at room temperature. A constant concentration of 100nM is made for either FH or FHL-1 in SAB and increasing concentrations of FHR-4 are used as a competitor, up to 500nM. FH/FHR-4 and FHL-1/FHR-4 mixes are incubated with the immobilized C3b for 4 hours. After washing, bound FH or FHL-1 protein was detected by the addition of 100µl/well of 0.5µg/ml OX23 antibody and incubated for 30 minutes followed by washing and a 30 minute incubation in 100µl of a 1:1000 dilution of AP-conjugated anti- mouse IgG (Sigma-Aldrich). Plates were developed using 100µl/well of a 1mg/ml disodium p-nitrophenylphosphate solution (Sigma-Aldrich) in 0.05 M Tris-HCl, 0.1 M NaCl, pH 9.3. The absorbance values at 405 nm were determined after 10 minutes of development at room temperature and corrected against blank wells (i.e., those with no immobilized C3b).

## Fluid phase C3b breakdown assays

The fluid phase cofactor activity of FHL-1 was measured by incubating purified FHL-1, C3b and FI together in a total volume of 20µl PBS for 15 minutes at 37°C. For each reaction 2µg C3b and 0.04µg FI were used with varying concentrations of FHL-1 ranging from 0.015µg to 1µg per reaction. The assay was stopped with the addition of 5µl 5x SDS reducing sample buffer and boiling for 10 minutes at 100°C. Samples were run on a 4-12% NuPAGE Bis Tris gel at 200V for 60 minutes in order to maximise the separation of the C3b breakdown product bands (see Extended data Fig. 4). Molecular weight markers used were Novex Sharp pre-stained protein standards (3.5-260kDa, Cat. No. LC5800, Life Technologies, Paisley, UK). The density of the 68kDa iC3b product band was measured using ImageJ64 (version 1.40g; rsb.info.nih.gov/ij) and used to track C3b breakdown efficiency of the FHL-1

proteins. For FHR-4 inhibition assays, the amount of FHL-1 used in the reaction is fixed at 1µg and increasing amounts of FHR-4 are added to create up to a 5-fold molar excess of FHR-4 over FHL-1. Otherwise the reactions are performed under the same condition as previously. In all cases averaged data from three separate experiments were used.

## Ussing chamber diffusion experiments

The macular region of enriched Bruch's membrane isolated from donor eyes was mounted in an Ussing chamber (Harvard Apparatus, Hamden, CT). Once mounted, the 5-mm-diameter macular area was the only barrier between two identical compartments (see Extended Fig. 3a). Both sides of Bruch's membrane were washed with 2 ml PBS for 5 min at room temperature. Fresh PBS was used in both the sample and diffusate chambers. To the sample chamber pure recombinant FHR-4, with a final concentration of 100µg/ml is added and the Ussing chamber was left at room temperature for 24 hours with gentle stirring in each compartment with magnetic stirrer bars to avoid generating gradients of diffusing protein. Samples from each chamber were analyzed on 4-12% NuPAGE Bis-Tris gels, run at 200V for 60 minutes. Either 20µl samples straight from each chamber were missed with 5µl 5x SDS loading buffer and run or 100µl samples were taken and concentrated using StrataClean beads (Agilent Technologies, Cheadle, U.K) by mixing the beads with each 100µl sample for 5 minutes at room temperature before centrifugation and removal of the supernatant. Beads were then re-suspended in 20µl neat 5x SDS loading buffer and loaded directly to the gel. Gels were stained with Instant Blue stain (Expedeon, Harston, UK) for 60 min at room temperature, before washing and storage in MiliQ water. Molecular weight markers used were Blue Prestained Protein Standards, Broad Range (11-190kDa, New England BioLabs, Hitchin, UK, catalogue no. P7706S). Diffusion experiments were performed on three separate donor BrM.

## FHR-4 measurements

The levels of FHR-4 were measured using an optimised in-house sandwich ELISA. Nunc-Immuno™ MaxiSorp™ 96-well plates were coated with 50µl/well of monoclonal anti-FHR4 antibody 4E9 at 5µg/ml (in 0.1M carbonate buffer pH9.6). After incubating for 1 hour at 37ºC, the plates were blocked with 2% BSA in PBS + 0.1%Tween-20 (PBST) for 1 hour at room temperature. After 3 washes of PBST, 50µl/well of purified FHR-4 protein diluted in 0.1% PBST was added in duplicate starting at 1µg/ml and serially diluted (1 in 2) down the plate. Test samples were added (50µl/well) in duplicate at a 1:40 dilution to the remaining wells, and plates were incubated at 37ºC for 1.5 hours. After 3 washes in PBST, 50µl/well of 1µg/ml of HRP-labelled anti-FHR-4 monoclonal antibody clone 17 was added and the plates were incubated for 1 hour at room temperature. After washing 3 times with PBST, 50µl/well of orthophenylenediamine (SIGMAFAST™ OPD, Sigma-Aldrich, UK) was added to develop the plates and the reaction was stopped after 5 minutes by adding an equal volume of 10% sulphuric acid. Absorbance was measured in a plate reader at 492 nm and protein concentrations were interpolated from standard curves plotted using GraphPadPrism5.

## Cambridge and EUGENDA study samples

The Cambridge AMD study is a case-control study of AMD with participants recruited from ophthalmic clinics in London, the southeast of England, and the northwest of England between 2001 and 2007[27]. All patients had at least 1 eye affected by choroidal neovascularization (CNV) and/or geographic atrophy (GA). Patients were excluded if they had greater than 6 diopters of myopic refractive error or evidence of other inflammatory or retinovascular disease (such as retinal vessel occlusion, diabetic retinopathy, or chorioretinitis) that could contribute to the development of or confound the diagnosis of maculopathy. Almost all of the controls were spouses or partners of index patients, and the remainder were friends of patients. All participants described their race/ethnicity as white rather than other on a recruitment questionnaire. Participants were examined by an ophthalmologist and underwent color stereoscopic fundus photography of the macular region. Images were graded at the Reading Centre, Moorfields Eye Hospital, London, using the International Classification of Age-related Maculopathy and Macular Degeneration[28].

Blood samples were obtained at the time of interview; EDTA samples were obtained for DNA extraction, and lithium-heparin plasma samples stored at –80°C were later used for FHR-4/FH measurements.

The European Genetic Database (EUGENDA) is a database for the clinical and molecular analysis of AMD. Patients from the EUGENDA cohort were recruited at the Department of Ophthalmology of the Radboud university medical center, Nijmegen, the Netherlands, and the University of Cologne, Germany. All the individuals were graded by classification of retinal images according to the standard protocol of the Cologne Image Reading Center by certified graders.[29] Only patients graded as late AMD were included in the study. Serum samples were obtained by a standard coagulation/centrifugation protocol, and within 1 hour after collection the samples were stored at –80°C.

For both studies ethical approval was obtained from either national or local ethics committees and adhered to the tenets of the Declaration of Helsinki. All participants provided written informed consent to undergo the clinical examination and epidemiological data collection, and to provide a blood sample for biochemical and genetic analyses.

## Genotype data

All Cambridge and EUGENDA individuals included in this study had been previously analyzed within a large GWAS of AMD carried out by the International AMD Genomics Consortium (IAMDGC) on 43,566 subjects (of which, 16,144 patients with advanced AMD and 17,832 control subjects of European ancestry)[3]. All DNA samples were genotyped with a custom-modified Illumina HumanCoreExome array at the Center for Inherited Disease Research (CIDR) and quality control and genotype imputation using the 1000 Genomes Project[30] reference panel were performed by the IAMDGC as described previously[3]. A total

of 12,023,830 directly genotyped (439,350) or imputed (11,584,480) quality-controlled variants were available for single-variant genetic association analyses. Phased genotype data as inferred within the IAMDGC study[3] were also available for haplotype-based association analyses.

## Association analyses of FHR-4/FH levels, SNPs, haplotypes and AMD

Stata software, version 13.1 (StataCorp), and *ipdmetan* and *mvmeta* commands were used for conducting 2-stage, fixed-effects meta-analyses of the available individual participant data from the 2 cohorts (Cambridge and EUGENDA). Heterogeneity across studies was assessed using the $I^2$ statistic. FHR-4 and FH levels were natural logarithmically transformed to ensure normality of the distribution. We assessed the association of late AMD status with FHR-4 and FH levels via Wald tests using linear regression models adjusted for age and sex. We also assessed the association of the 8 independently associated SNPs at the *CFH*/*CFHR* locus reported by the IAMDGC study[3] (rs10922109, rs570618, rs121913059, rs148553336, rs187328863, rs61818925, rs35292876, rs191281603) with FHR-4 and FH levels via Wald tests on the SNP genotypes coded as 0, 1 and 2 according to the number of minor alleles using linear regression models adjusted for late AMD status. Finally, we extracted data for the haplotypes formed by those independently associated SNPs using the phased genotype data produced within the IAMDGC study[3] and assessed the association of the observed haplotypes with AMD using logistic regression models and with FHR-4 and FH levels using linear regression models adjusted for late AMD status.

## GWAS meta-analysis of FHR-4/FH levels

We carried out GWASs of (natural logarithmically transformed) FHR-4 and FH levels in each cohort (Cambridge and EUGENDA) using linear regression models adjusted for age, sex and the first two ancestry principal components (as estimated within the IAMDGC study[3]). The GWASes were carried out using EPACTS software (http://genome.sph.umich.edu/wiki/EPACTS) and Wald tests were performed on the SNP genotypes coded as 0, 1 and 2 according to the number of minor alleles for the directly typed variants or allele dosages for the imputed variants. Genomic control correction[31] was applied if lambda was greater than 1. Effect size estimates and standard errors of single variants seen in both cohorts were subsequently combined in a fixed-effect meta-analysis using METAL[32]. Manhattan and Q-Q plots were generated using the *qqman* R package (version 0.1.2) (https://cran.r-project.org/web/packages/qqman/index.html). Sensitivity analyses adjusting the GWASes additionally for late AMD status were performed in an analogous manner.

# Extended data

```
      *       **     ***      ****
_____________ ____ _________ _______
HHHHHHGSSENLYFQGSSGQEVKPCDFPEIQHGGLYYKSLRRLYFPAAAGQSYSY
YCDQNFVTPSGSYWDYIHCTQDGWSPTVPCLRTCSKSDIEIENGFISESSSIYILNKE
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IQLKGKSDIKYYAKTGDTIEFMCKLGYNANTSVLSFQAVCREGIVEYPRCE
```
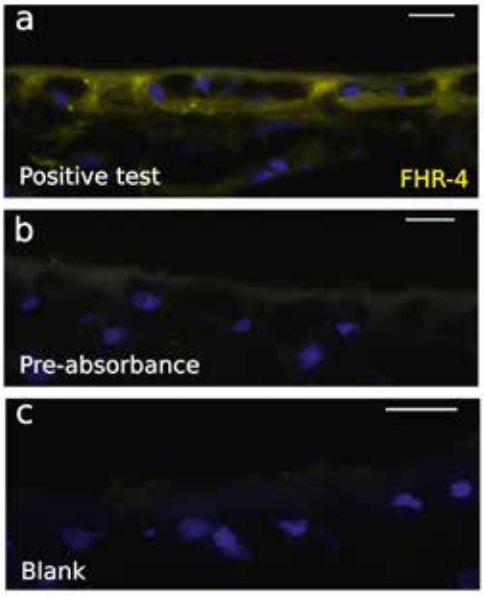
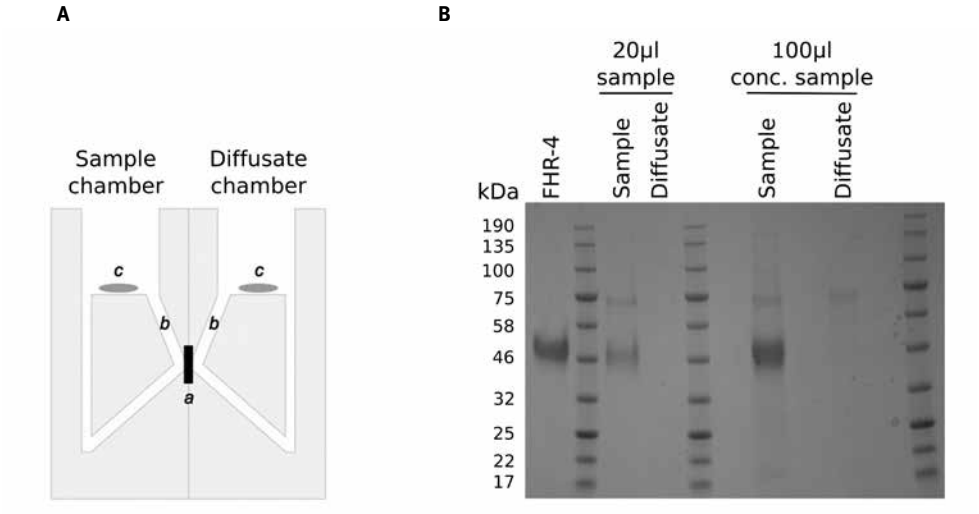

**EXTENDED DATA FIGURE 1 Sequence of FHR-4 recombinant protein.**

Recombinant FHR-4 gene synthesis was carried out by GenScript using their gene synthesis and protein expression service and is based on the published sequence for the FHR-4B variant of the *CFHR4* gene (UniProt identifier Q92496-3). The original recombinant protein included an N-terminal 6xHis tag (*) followed by, a linker region (**), and a TEV protease cleavage site (***). Removal of the N-terminal His tag results in two non-authentic N-terminal residues (****).



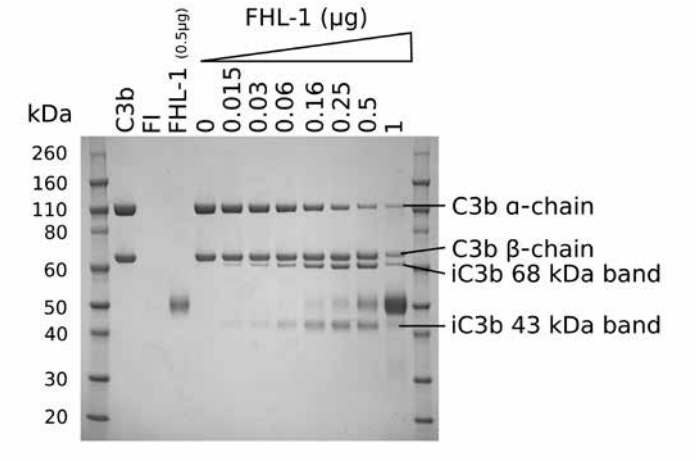

**EXTENDED DATA FIGURE 2 Pre-absorption test of anti-FHR-4 antibody.**

To test the tissue staining specificity of the anti-FHR-4 monoclonal antibody (clone 150), the normal 10µg/ml Ab mix used throughout the study **(a)** is pre-incubated with pure recombinant FHR-4 at a final concentration of 100µg/ml (i.e. 10-fold excess) **(b)**. Staining from the pre-absorption experiments are strikingly similar to the blank controls **(c)**, where no primary antibody is included.



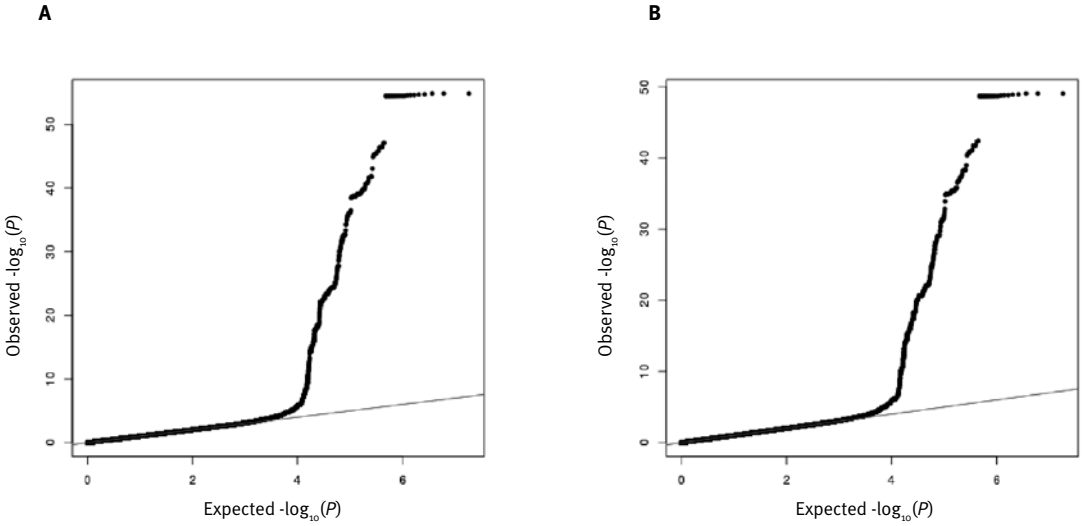

EXTENDED DATA FIGURE 3 **FHR-4 does not diffuse freely across Bruch's membrane.**

**a)** Enriched Bruch's membrane from donor eyes are placed inside a modified Ussing chamber where: a, is the enriched Bruch's membrane; b, are the sampling access points; and c, are magnetic stirrer bars to maintain flow around each chamber. **b)** samples from either the sample chamber or diffusate chamber are run on a 4-12% NuPage gel Bis-Tris gel and compared to a pure protein control (FHR-4). The gel shows 20µl samples taken and run directly from each chamber, as well as 100µl samples that have been concentrated prior to running on the gel. Gel is representative of three independent experiments.




EXTENDED DATA FIGURE 4 **FHL-1 mediated C3b breakdown assay.**

The ability of FI to cleave C3b in the fluid phase in the presence of a co-factor (FHL-1) is shown. Pure C3b (2µg), FI (0.04µg), and FHL-1 (0.5µg) control bands are shown. FI cannot cleave the α-chain of C3b without a co-factor (lane '0'), but with increasing concentration of FHL-1 the breakdown of the C3b α-chain into iC3b (seen as two bands at 68kDa and 43kDa) is observed. Gel is representative of three independent experiments.




**EXTENDED DATA FIGURE 5 Q-Q plot of the GWASes on log-FHR-4 levels.**

Shown as black dots are the observed *P*-values ($-\log_{10}(P)$) compared to those expected under the null hypothesis.

**A)** GWAS without adjustment for AMD status. In the meta-analysis, adjustment for the inflation factor of the different cohorts was conducted (EUGENDA $\lambda$=1.004, Cambridge $\lambda$=1.003). **B)** GWAS adjusted for AMD status. In the meta-analysis, adjustment for the inflation factor of the different cohorts was conducted (EUGENDA $\lambda$=1.005, Cambridge $\lambda$=1.005).

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# 4

# General discussion

Adapted from "Exploring the use of molecular biomarkers for precision medicine in age-related macular degeneration"

*Molecular Diagnosis and Therapy*, 2018 June; 22(3):315-343

At present, only advanced neovascular age-related macular degeneration (nvAMD) can be treated, by targeting vascular endothelial growth factor (VEGF). For geographic atrophy (GA), although several therapies are actively being developed, no established treatment is available to date. Also, progression of the disease cannot be halted, but it can be slowed down with the use of nutritional supplements.

Due to the heterogeneity in the AMD patient population, it is plausible that the effect of therapeutic interventions depends on the biological drivers of disease in each individual patient. In essence, the patient's genetic blueprint, in addition to demographic and lifestyle factors, is likely to influence how a patient responds to treatment. Consequently, the identification of biomarkers that can predict response to therapy in AMD could be used to improve AMD patient care, by tailoring medication to each patient's individual needs.

In this thesis, we have investigated what the influence of genetic biomarkers on treatment response to therapy in AMD is in chapters 2.1, 2.2 and 2.3. In chapter 3.1 we investigated the genetic drivers of complement activation, which is relevant in the context of novel treatment modalities for AMD. Finally, in chapter 3.2 we further explored the role of factor H related 4 (FHR-4) on AMD, as a potential new biomarker and target for therapy. In the Discussion, we will place these results in context and aim to provide an overview of the current literature investigating the association of biomarkers with response to supplements and anti-VEGF therapy. Moreover, we describe new therapeutic approaches undergoing clinical trials, and the potential use of biomarkers for patient selection for such new therapeutic approaches.

# 1 Molecular biomarkers for current therapeutic interventions for AMD

## 1.1 Dietary supplements for slowing disease progression

AMD-associated variants have been found to influence AMD progression and, for several years, there have been investigations into whether specific genotypes interact with the Age-Related Eye Disease Study (AREDS) supplementation affecting progression rates.[1] These studies sparked an intense debate in the field as different research groups arrived at different conclusions. In 2008 Klein, Seddon et al., suggested that response to AREDS supplements could be related to the *CFH* rs1061170 genotype.[2] The study evaluated 876 AREDS patients and found that for carriers of the CC genotype, dietary supplementation would have a smaller effect, possibly related to zinc consumption, but would still be beneficial. No interaction was found for the *ARMS2* rs10490924 SNP. In 2013, a second study that included 995 AREDS participants was published by Awh et al., also proposing a genotypic interaction,[3] and suggesting that improved outcomes could be obtained

after genotype selection. The authors described a deleterious interaction between *CFH* risk alleles (rs412852 and rs3766405) and supplementation with zinc, in which carriers of *CFH* risk alleles would progress to advanced AMD faster when taking zinc. Also, the authors claimed that individuals homozygous for the *CFH* and *ARMS2* risk alleles would not benefit from the AREDS formula. After these results, the AREDS Research Group attempted replication in a larger AREDS cohort of 1,237 AMD patients, but did not identify any interaction, and concluded that reduction in the risk of AMD progression after supplementation was seen in all genotype groups.[4] This study was followed by a series of contradictory results,[5-7] and intense argumentation.[8-10] However, the controversy seems to have been resolved: In a recent report, independent statistical research groups analyzed the data from the AREDS Research Group as well as from Awh and colleagues. Errors in the Awh et al., 2013 study were noted, and no interaction was reported between the *CFH* and *ARMS2* SNPs and treatment response after correction for multiple testing. Therefore, it was concluded that AMD patients should be offered dietary supplementation regardless of genotype.[11]

## 1.2 Anti-VEGF antibodies for choroidal neovascularization treatment

Since the first publication in 2007,[12] a vast number of studies have investigated associations of genetic variants with anti-VEGF treatment outcome in nvAMD. We reviewed the pharmacogenetic studies published to date, and provide a detailed overview of their study designs and conclusions in Table 1. Despite the large body of literature on this topic, with over 50 studies published, solid conclusions cannot be drawn. This is due to conflicting results and a high heterogeneity in study designs, which makes comparisons between studies challenging. Studies may involve ranibizumab treatment, bevacizumab treatment or both. Moreover, the definition of treatment response is highly variable: change in visual acuity, change in total retinal thickness, choroidal neovascularization (CNV) recurrence or number of injections are some of the variables used to measure treatment outcome. These variables are analyzed in a continuous or in a categorical manner, in which responders are compared to non-responders based on an arbitrary definition of response. Additionally, the studies evaluate response after the loading dose of 3 monthly injections or longer, and may therefore involve different treatment protocols. Also, correction for multiple testing is not applied in all studies, and the majority of studies do not provide a statistical power calculation.

TABLE 1 **Overview of pharmacogenetic studies for anti-VEGF treatment of neovascular age-related macular degeneration**

| Reference | N[a] | Ethnicity / country of origin | Design | Anti -VEGF | Treatment regimen | Treatment outcome definition(s) | Gene: SNPs | Conclusion |
|---|---|---|---|---|---|---|---|---|
| Brantley M A et al., 2007 | 86 | Caucasian | R | BVZ | 6-weekly inj. until no active CNV | VA after at least 6 months of treatment | *CFH* : rs1061170<br>*ARMS2*: rs10490924<br>*CFH+ARMS2*: rs1061170 + rs10490924 | *CFH* rs1061170 CC -› worse response |
| Lee A Y et al., 2009 | 156 | Caucasian | R | RNZ | PRN | VA after 6 , 9 months<br>N of inj. after 9 months<br>Interval between required inj. after 9 months<br>Interval between follow-up appointments after 9 months | *CFH* : rs1061170 | *CFH* rs1061170 CC -› worse response |
| Teper S J et al., 2010 | 90 | Caucasian | P | RNZ | 3 monthly inj. + SUSTAIN criteria | Change in VA after 12 months<br>Change in CSRT after 12 months<br>N of inj. after 12 months | *CFH* : rs1061170<br>*ARMS2*: rs10490924 | *ARMS2* rs10490924 TT-› worse response |
| Imai D et al., 2010 | 83 | Japanese | R | BVZ | 1 inj. + retreatment after recurrence of AMD | Responders vs. non-responders: responders if improvement in VA after 1, 3 months<br>Change in CRT after 1, 3 months<br>CNV recurrence after 1 , 3 months | *CFH*: rs800292, rs1061170, rs1410996<br>*HTRA1*: rs11200638<br>*VEGFA*: rs699947, rs1570360, rs2010963<br>*PEDF*: rs12150053, rs12948385, rs9913583, rs1136287 | *CFH* rs1061170 CT -› worse response than TT<br>*VEGFA* rs699947 C -› worse response<br>*PEDF* rs1136287 -› Not described |
| Nakata I et al., 2011 | 94 | NI (Japan) | R | BVZ | 1-3 inj., when remnant exudative changes 2nd and or 3rd injection monthly | VA after 12 months<br>Change in VA after 12 months<br>N of inj. after 12 months<br>N of recurrences after 12 months<br>Periods until recurrence after 12 months | *VEGFA*: rs699946, rs699947 | *VEGFA* rs69946 A -› worse response |
| Nischler C et al., 2011 | 197 | NI (Austria) | P | BVZ | 1 inj. + 6 weekly until no active CNV | N of treatments<br>CMT after treatment<br>VA after treatment<br>Reading VA after treatment<br>Gain or loss of ≥3 lines in distance VA<br>Gain or loss of ≥3 lines in reading VA | *CFH*: rs1061170 | *CFH* rs1061170 CC -› worse response |
| Wickremasinghe S S et al., 2011 | 168 | Caucasian | R | RNZ or BVZ and RNZ | No specific retreatment strategies (75% 3 monthly inj.) | Improved: 2 line gained in VA, stable: VA within 2 lines of baseline or decreased: reduction in VA of 2 lines or more | *APOE*: ε2, ε3 and ε4 | *APOE* ε4 allele -› better response |

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|---|---|---|---|---|---|---|---|---|
| Francis P J et al., 2011 | 44 | Caucasian | P | RNZ | Monthly "as needed" | Change in letters after 12 months | GWAS: Illumina 660-Quad SNP array: >480,000 SNPs | Candidate gene analysis: *CFH* rs1065489 AA -› worse response |
| Kloeckener-Gruissem B et al., 2011 | 243 eyes, 215 individuals | NI (Switzerland) | R | RNZ | PRN | Change in VA after 12 months, poor responders: ≤25th percentile, good responders: ≥75th percentile | *CFH*: rs1061170<br>*FZD4* : rs10898563 | *CFH* rs1061170 CC -› worse response<br>*CFH* rs1061170 CT and *FZD4* rs10898563 AG -› better response |
| Wang V M et al., 2012 | 106 | Caucasian | NI | BVZ/RNZ | 1 inj. + PRN for 12 months | Poor responder (based on VA, and OCT parameters) | 21 SNPs in IL23, *PLA2G12A*, *HIF1A*, *STAT3*, *VEGF*, *KDR* | No associations found (after correction for multiple testing) |
| McKibbin M et al., 2012 | 104 | Caucasian | R | RNZ | 3 monthly inj. + PRN | Gain of >5 letters vs. rest after 6 months<br>Change in VA after 6 months | *CFH* : rs1061170<br>*HTRA1*: rs11200638<br>*VEGFA*: rs1413711 | No association found (after correction for multiple testing) |
| Orlin A et al., 2012 | 150 | NI (USA) | R | BVZ/RNZ | 3 monthly inj. + T&E | After last visit, positive responders: improvement or no change in VA, negative responders: lose of VA or final VA 20/200 | *CFH*: rs1061170<br>*ARMS2*: rs10490924, rs3750848, del443ins54<br>*HTRA1*: rs3793917, rs11200638, rs932275 | No associations found |
| Smailhodzic D et al., 2012 | 420 eyes, 397 individuals | NI (The Netherlands, Germany, Canada) | R | RNZ | 3 monthly inj. + PRN | Change in VA after 3 months | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*VEGFA*: rs699947, rs833069<br>*KDR*: rs2071559, rs7671745<br>*LPR5*: rs3736228<br>*FZD4*: rs10898563<br>*CFH+ARMS2*: rs1061170 + rs10490924<br>*CFH+ARMS2+VEGFA*: rs1061170 + rs10490924 + rs699947 | *CFH* rs1061170 TT -› Better response<br>*CFH+ARMS2* 0 risk alleles -› Better response<br>*CFH+ARMS2* 1 or 2 risk alleles -› older age at first inj.<br>*CFH+ARMS2+VEGFA* less risk alleles -› Better response |
| Boltz A et al., 2012 | 185 eyes, 141 individuals | NI (Germany) | P | BVZ | PRN for 42 to 1182 days | Mean change in VA after treatment<br>N of treatments after treatment<br>Duration of the treatment | *VEGFA*: rs1413711, rs3025039, rs2010963, rs833061, rs699947, rs3024997, rs1005230 | No associations found |
| Menghini M et al., 2012 | 204 eyes, 194 individuals | NI (Switzerland) | R | RNZ | 3 monthly inj. + PRN or PRN | After 12, 24 months, good responders: ≥5 letter improvement, poor responders: ≥5 letters lose | *CFH*: rs1061170 | *CFH* rs1061170 CT -› better response<br>*CFH* rs1061170 CT/TT -› better response |

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|---|---|---|---|---|---|---|---|---|
| Tian J et al., 2012 | 144 | Chinese | P | BVZ | 6-weekly inj. | Change in letters after 3 months<br>Change in CRT after 3 months<br>Maximum lesion thickness after 3 months | *CFH*: rs800292, rs1061170, rs10801555, rs1410996<br>*ARMS2*: rs10490924<br>*HTRA1*: rs11200638<br>*VEGFA*: rs833069, rs3025039<br>*SERPING1*: rs1005510, rs2511989<br>*C3*: rs2230205, rs2250656 | *CFH* rs800292 CC -› worse response<br>*ARMS2* rs10490924 TT -› worse response<br>*HTRA1* rs11200638 AA -› worse response |
| Kang H K et al., 2012 | 75 | Korean | R | BVZ | 3 monthly inj. + PRN for 6 months/12 months | Change in VA after 3, 6, 12 months<br>Change in CRT after 3, 6, 12 months<br>N of additional inj. after loading dose | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*HTRA1*: rs11200638 | *ARMS2*: rs10490924 G -› worse response<br>*CFH* rs1061170 TC-› worse response than TT |
| Lazzeri S et al., 2013 | 64 | Italian | P | RNZ | 3 monthly inj. | Change in letters after 3 months | *VEGFA*: rs699947, rs1570360 | *VEGFA* rs699947 C -› better response |
| Dikmetas O et al., 2013 | 193 | Turkish | R | RNZ | PRN | At final examination, good response: ≥5 letters, bad response: decrease of ≥5 letters, very good response: ≥15 letters, very bad response: decrease in VA of ≥15 letters | *CFH*: rs1061170 | *CFH* rs1061170 C -› worse response |
| Chang W et al., 2013 | 102 | Korean | R | RNZ | 3 monthly inj. + PRN | Change in VA after 3, 6 months<br>Change in CSMT after 3, 6 months | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*HTRA1*: rs11200638<br>*VEGFA*: rs833069<br>*KDR*: rs2071559 | *VEGFA* rs833069 G -› better response |
| Kitchens J W et al., 2013 | 101 | NI (USA) | R | BVZ/RNZ | 3 monthly inj. + PRN for 6 months/12 months | Based on OCT, responder: no fluid after inj. for after least 1 month, non-responder: fluid present 1 month after the third inj.<br>Based on VA, responder: gained ≥3 lines after month 9, non-responder: not gain of ≥3 lines after month 9 | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*VEGFA*: rs699947, rs1570360, rs833060, rs36208049, rs833061, rs25648, rs59260042, rs2010963 | *ARMS2* rs10490924 TT -› worse response |
| Abedi F et al., 2013 | 224 | Caucasian | P | BVZ/RNZ | 3 monthly inj. + PRN | Change in VA after 12 months | *CFH*: rs800292, rs3766404, rs1061170, rs2274700, rs393955<br>*HTRA1*: rs11200638<br>*CFHR1-5*: rs10922153, rs16840639, rs6667243, rs1853883<br>*ARMS2*: rs3793917, rs10490924<br>*C3*: rs2230199, rs1047286<br>*C2*: rs547154<br>*CFB*: rs641153<br>*F13B*: rs6003 | *HTRA1* rs11200638 AA -› worse response<br>*ARMS2* rs10490924 TT -› worse response |

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|---|---|---|---|---|---|---|---|---|
| Hagstrom S A et al., 2013 | 834 | CATT (98% Caucasian), NI | P | BVZ/RNZ | PRN/monthly | VA after 12 months<br>Change in VA after 12 months<br>Proportion of patients with ≥15 letter increase after 12 months<br>Proportion of patients with a thin (<120µm), normal (120–212 µm), and thick (>212µm) retina after 12 months<br>Change in TFT after 12 months<br>N of inj. after 12 months | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*HTRA1*: rs11200638<br>*C3*: rs2230199 | No associations found |
| Abedi F et al., 2013 (2) | 201 | Caucasian | P | BVZ/RNZ | 3 monthly inj. + PRN for 12 months | Change in VA after 3, 6, 12 months<br>Responder vs. non-responder or stable for VEGFA rs3025000 at 12 months | *VEGFA*: rs3024994, rs3025000, rs3025042, rs3025047, rs3025035, rs3025030, rs3025010 | *VEGFA* rs3025000 T -› better response |
| Lotery A J et al., 2013 | 509 | IVAN, NI | P | BVZ/RNZ | 3 monthly inj. + PRN/ monthly | Change in TRT after 12 months or the preceding measurement nearest to this time point, responders ≥75th percentile, non responders ≤ 25th percentile | *CFH*: rs1061170<br>8: rs10898563<br>*ARMS2*: rs10490924<br>482 additional SNPs in candidate genes | No associations found (after correction for multiple testing) |
| Hautamäki A et al., 2013 | 96 | NI (Finland) | R (59), from a P study (37) | BVZ | 3 inj. in within 5 months | Responder, partial responder or non-responder based on neuroepithelial detachment, cystic changes and area of cysts | *IL8*: rs4073<br>*CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*C3*: rs2230199<br>*VEGFA*: rs699947, rs2146323, rs3025033<br>*EPO*: rs1617640 | *IL8* rs4073 A -› worse response |
| Habibi I et al., 2013 | 70 | Tunisian | P | BVZ | 6-weekly inj. until non-active CNV | Improvement in VA: 2-line gain after 6 months, stable vision: VA within 2 lines of baseline, decrease: reduction in VA of 2 lines or more | *CFH*: rs1061170 | No association found |
| Zhao L et al., 2013 | 223 eyes, individuals NI | Caucasian | P | BVZ/RNZ | 4 monthly inj. + PRN | Responders: ≥ 5 letters and resolution of intraretinal or subretinal fluid after 12 months, non-responders: rest of the patients | *VEGFA*: rs943080 | *VEGFA* rs943080 T -› worse response |
| Yuan D et al., 2013 | 168 | Han Chinese | P | RNZ | 3 monthly inj. + PRN | Change in VA after 6 months<br>Change in CRT after 6 months<br>Change in maximum lesion thickness after 6 months | *CFH*: rs1061170<br>*HTRA1*: rs11200638<br>*VEGFA*: rs1413711 | HTRA rs11200638 A -› worse response |
| Hagstrom S A et al., 2014 | 831 | CATT (98% Caucasian), NI | P | BVZ/RNZ | PRN/monthly | Change in TRT after the latest time point for which data were available through 1 year, responders: ≥75th percentile, non-responders: ≤ 25th percentile | *EPAS1*: rs6726454, rs7589621, rs9679290, rs12712973 | No associations found |
| Hermann M M et al., 2014 | 366 | NI (The Netherlands, Germany, Canada) | NI | RNZ | 3 monthly inj. + PRN | Change in VA after 3, 12 months | 126 SNPs in *VEGFA*, *VEGFB*, *VEGF-C*,*VEGFD*, *PGF*, *VEGR1*, *VEGFR2*, *VEGFR3*, *PEDF* | *KDR* rs4576072 C -› better response<br>*KDR* rs4576072 (C) and rs6828477 (C) 3 alleles -› better response |

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|---|---|---|---|---|---|---|---|---|
| Hagstrom SA et al., 2014 (2) | 835 | CATT (98% Caucasian), NI | P | BVZ/RNZ | 3 monthly inj. + PRN/ monthly | VA after 12 months<br>Change in VA after 12 months<br>TRT after 12 months<br>Change in TFT after 12 months<br>Presence of fluid on OCT<br>Presence of leakage on FA after 12 months<br>Change in lesion size after 12 months<br>N of inj. after 12 months | *VEGFA*: rs699947, rs699946, rs833069, rs833070, rs1413711, rs2010963, rs2146323<br>*KDR*: rs2071559<br>N of risk alleles | No associations found |
| Cruz-Gonzalez F et al., 2014 | 94 | Caucasian | P | RNZ | 3 monthly inj. + SUSTAIN criteria | Subjective improvement: patients measure their improvement after each inj. in a scale from 1 to 10, Subjective improvement ≥6<br>VA improvement: gain ≥ 5 letters<br>OCT improvement: improvement › 100 µmin in central subfield retinal thickness | *VEGFA*: rs699947, rs833061<br>*KDR*: rs2071559 | *VEGFA* rs8330611 CC -› better response<br>*VEGFA* rs699947 AA -› better response |
| Matsumiya W et al., 2014 | 120 | Japanese | R | RNZ | 3 monthly inj. | Anatomical resolution of the lesions after 3 months<br>Dry lesion in OCT/No exudative lesion<br>Change in VA after 3 months<br>Change in CRT after 3 months | *CFH*: rs1061170, rs800292<br>*HTRA1*: rs10490924<br>*CFH*: rs1061170 + rs800292 | *CFH* rs1061170 TT + rs800292 GG -› worse response |
| Park U C et al., 2014 | 273 | Korean | P | RNZ | 5 monthly inj. | Good response after month 5: visual improvement of ≥8 letters | 23 SNPs in 12 AMD genes | No associations found (after correction for multiple testing) |
| dos Reis Veloso C E et al., 2014 | 92 | Brazilian | P | RNZ | 3 monthly inj. + PRN | Change in VA after 1, 3, 6 and 12 months<br>Change in CRT after 1, 3, 6 and 12 months | *VEGFA*: rs1413711 | *VEGFA* rs1413711 CC -› Worse response |
| Hautamäki A et al., 2014 | 50 | Caucasian | P | BVZ | PRN | Change in CS after 24 months<br>Presence of intra/sub retinal fluid in OCT after 24 months<br>N of inj. after 24 months | *IL8*: rs4073<br>*VEGFA*: rs699947, rs2146323, rs3025033<br>*CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*C3*: rs2230199<br>*IL8* rs4073+VEGF rs699947+CFH rs1061170 | *VEGFA* rs699947 AA -› worse response<br>*ARMS2*: rs10490924 TT-› worse response<br>*IL8*: rs4073 AA -›worse response<br>*CFH* rs1061170 CC -› worse response<br>*IL8* rs4073 A+VEGF rs699947 A+*CFH* rs1061170 C 3-6 risk alleles -› worse response |
| Piermarocchi S et al., 2015 | 94 | Caucasian | P | RNZ | 3 monthly inj. + PRN | Change in VA after 12 months | *CFH*: rs1061170<br>*ARMS2*: rs10490924<br>*C3*: rs2230199 | *CFH* rs1061170 C -› worse response |

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|---|---|---|---|---|---|---|---|---|
| Medina F M C et al., 2015 | 25 | Brazilian | P | BVZ | 1 inj. | Improvement in VA and CRT is evaluated separately per genotype. Results are compared. | *CFH*: rs1061170 | *CFH* rs1061170 CC -› worse response |
| Hagstrom S A et al., 2015 | 1347 | CATT and IVAN, NI | P | BVZ/RNZ | PRN/monthly | Change in VA after 12 months | *KDR*: rs4576072, rs6828477<br>N of risk alleles in *KDR* rs4576072 + rs6828477 | No associations found |
| Kuroda Y et al., 2015 | 343 eyes, 326 individuals | Japanese | R | RNZ | 3 monthly inj. + PRN | Recurrence after 12 months<br>Change in VA after 12 months | *CFH*: rs1410996<br>*ARMS2*: rs10490923 | No associations found |
| Lorés-Motta L et al. ,2016 | 377 | NI (The Netherlands, Germany, Canada) | R | RNZ | 3 monthly inj. + PRN/TE | Change in VA after 3, 6 and 12 months | *NRP1*: rs2070296<br>N of risk alleles in KDR rs4576072 + *NRP1* rs2070296 | *NRP1* rs2070296 GA and AA -› worse response<br>3, 4, 5 risk alleles in *KDR* rs4576072 + NRP1 rs2070296 -› worse response |
| Bakbak B et al., 2016 | 109 | Turkish | P | RNZ | 3 monthly inj. + PRN | After 6 months, loss ›5 letters, loss or gain of VA between 5 letters or gain ›5 letters. | *APOE*: ε2, ε3 and ε4 | *APOE* ε4 allele -› better response |
| Lazzeri S et al., 2016 | 64 | NI (Italy) | P | RNZ | 3 monthly inj. + PRN | VA after 3, 12 months<br>Retinal sensitivity after 3, 12 months<br>TFT after 3, 12 months<br>N of inj. in the follow-up phase | *KDR*: rs2071559<br>*IL8*: rs4073 | *IL8* rs4073 AA -› worse response<br>*KDR* rs20715559 CC -› better response |
| Cruz-Gonzalez F et al., 2016 | 100 | Caucasian | P | RNZ | 3 monthly inj. + PRN | VA improvement: gain ≥ 5 letters<br>OCT improvement: gain › 100 µm in CSRT | *CFH*: rs1410996<br>*ARMS2*: rs10490923<br>*HTRA1*: rs11200638 | No associations found |
| Riaz M et al., | 661 (discovery: 285, replication: 376) | NI (Australia, The Netherlands, Germany, Canada) | R | RNZ | 3 monthly inj. + PRN/TE | Change in VA in after 3, 6 months, non-responder: lose ≥5 letters, responder: rest | GWAS: Illumina 4.3M SNP array: ›1,000,000 SNPs | *OR52B4* rs4910623 G -› worse response |
| Habibi I et al., 2016 | 90 | Tunisian | R | BVZ | 3 monthly inj. + PRN | After 12 months, good responder: reduction of ‹2 lines, poor responder: reduction of ≥2 lines | *CFH*: rs1061170<br>*C3*: rs2230199<br>*VEGFA*: rs2010963, rs3025039, rs699947<br>N of risk alleles in *CFH*, *C3* and *VEGFA*<br>*VEGFA* haplotypes | *VEGFA* haplotype rs2010963 G , rs3025039 T, rs699947 A -› worse response |

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|---|---|---|---|---|---|---|---|---|
| Chaudhary V et al., 2016 | 70 | Caucasian (68), Asian (2) | P | RNZ | 3 monthly inj. + PRN | Moderate vision gain: gain of ≥ 15 letters after 6 months<br>Change in CMT after 6 months<br>Change in VA after 6 months | *CFH*: rs1048663, rs3766405, rs412852, rs11582939, rs1066420<br>*C3*: rs2230199<br>*ARMS2*: rs10490924<br>mtDNA: A2917G<br>*CFH* haplotypes | *CFH* haplotype that reduces risk to AMD leads to better response |
| Kepez Yildiz B et al., 2016 | 109 | Turkish | R | NI | 3 monthly inj. | Good responder group and non-responder group based on VA and OCT parameters after 3 months | *CFH*: rs1061170<br>*VEGFA*: rs2146323, rs699947 | No associations found |
| Shah A R et al., 2016 | 72 | NI (USA) | R | BVZ/RNZ | PRN | Change in VA after 6, 12 months<br>Change in central foveal thickness after 6, 12 months | 20 SNPs in AMD genes<br>N of risk alleles in *CFH* rs1061170 and rs1410996<br>Number of risk alleles for *CFI* rs10033900 and *CFI* rare variants | *CFH* rs1061170 TT-› better response<br>0-2 risk alleles in *CFH* rs1061170 and rs1410996 -› better response |
| Bardak H et al., 2016 | 39 | NI (Turkey) | R | RNZ | 3 monthly inj. | Responders: absence of intraretinal or subretinal fluid, non-responder: presence.<br>Comparisons for each genotype | *ARMS2*: rs10490923 | *ARMS2* rs10490924 TT shows differences in response |
| Valverde-Megías A et al., 2017 | 103 | Caucasian | P | RNZ | 3 monthly inj. + PRN | N of inj. after 4 years | *CFH*: rs1061170<br>*ARMS2*: rs10490924 | *ARMS2* rs10490924 TT -› worse response |
| Yamashiro K et al., 2017 | 461 (discovery: 256, replication: 205) | NI (Japan) | P | RNZ | 3 monthly inj. + PRN | Dry macula after treatment,<br>Requirement for an additional treatment<br>VA changes after 12 months | GWAS: Illumina HumanOmni2.5–8 BeadChip Kit. Imputation followed: › 8,400,000 SNPs | No genome-wide level significant associations<br>No associations with $P < 5 \times 10^{-6}$ replicated<br>9 candidate SNPs:<br>ARMS2: rs10490924 G-› better response |
| Medina FMC et al., 2017 | 46 | Brazilian | R | BVZ/RNZ | PRN | Baseline and 12 months VA and CRT compared for each genotype separately | *CFH*: rs1061170 | *CFH* rs1061170 C -› worse response |
| Chapter 1.3 | 2,058 (discovery: 678, replication: 1,380) | Discovery European descent | R | BVZ/RNZ | 3 monthly inj. | Change in VA after 3 months | Custom-modified HumanCoreExome array (Illumina) by the IAMDGC. After imputation 28,930,739 variants | Rare protein altering variants in the *C10orf88* and *UNC93B1* genes -› worse response |

P = Prospective, R = Retrospective, BVZ = Bevacizumab, RNZ = Ranibizumab, N = Number, NI = Not indicated, Inj. = Injection(s), PRN = Pro re nata, TE = Treat-and-extend, CNV = Choroidal neovascularization, OCT = Optical coherence tomography, VA = Visual acuity, letters = Early treatment diabetic retinopathy study letters, CMT = Central macular thickness, CRT = Central retinal thickness, CS = Contrast sensitivity, TFT = Total foveal thickness, SNP = single nucleotide polymorphism, AMD = age-related macular degeneration, CSMT = Central subfield macular thickness.

[a] Used for analysis

At the onset of the field of pharmacogenetics in AMD, a natural target to explore was the main genetic variant associated with AMD: SNP rs1061170 in the *CFH* gene. Indeed, most of the studies have investigated this single nucleotide polymorphism (SNP), however conflicting results have been reported. Several studies have reported an association of this genetic variant with response to anti-VEGF treatment;[12-25] in all instances the AMD-risk-conferring allele (C) led to a worse outcome after therapy. However, others have not identified any association.[26-40] Three different meta-analyses have been carried out, all showing an association of rs1061170 with treatment response with a moderate level of significance.[41-43] The most recent and comprehensive study included a total of 2,963 individuals from 14 different studies and showed that patients homozygous for the AMD low risk allele (T) were more likely to have a better outcome compared to patients homozygous for the AMD high risk allele (C; OR=1.932, CI=1.125-3.173, *P*=0.017).[43] In the genome-wide association (GWAS) study we performed in chapter 2.3, this SNP was close to be nominally associated with visual acuity outcome after 3 months (n=678, *P*=0.059) and showed the same direction of the effect previously reported (the T allele led to a better outcome). Notably, the two studies based on the Comparison of AMD Treatments Trials (CATT) and the Inhibition of VEGF in Age-related choroidal Neovascularisation (IVAN) clinical trials did not find any association for this variant, nor for any other variant investigated, despite their relatively large sample sizes (n=834 and n=509, respectively).[33,34]

The SNPs in *ARMS2*/*HTRA1* (rs10490924 and rs11200638, which are in high linkage disequilibrium),[44] have also been widely evaluated for association with treatment outcome. A similar scenario emerged for these SNP's, where several studies reported an association in which the AMD-risk allele leads to worse response,[21,26,29,31,32,37,39,45] while others did not report an association.[12,14,21,22,24,27,30,33-35,40,46-49] Only one study, by Kang and colleagues, described better response for carriers of the AMD-risk allele in rs10490924, as they needed fewer bevacizumab injections after the loading dose.[19] A meta-analysis including 2,389 cases from 12 studies showed that patients homozygous for the AMD low risk allele in *ARMS2* rs10490924 (GG) have a higher chance of responding better to treatment compared to patients heterozygous (TG) or homozygous (TT) for the AMD high risk allele (OR=1.34, CI=1.01-1.77, *P*=0.039), although no significant difference was found on the allele level. Also, no differences were found when the analysis was limited to patients of European descent.[50] Another meta-analysis of 1,570 cases from 5 studies showed no association for the SNP rs11200638.[51] In the GWAS study described in chapter 2.3, this SNP was not associated with visual acuity outcome after 3 months (n=678, *P*=0.416). Most study designs evaluated treatment outcome after 3 - 12 months of treatment, but a recent study evaluated the effect of genetic variants after 4 years of anti-VEGF treatment. This study by Valverde-Megías et al. examined the rs1061170 *CFH* and rs10490924 *ARMS2* SNPs and reported that patients homozygous for the AMD-risk allele of the *ARMS2* SNP required more injections over this long-term follow-up period.[39]

Due to the nature of anti-VEGF therapy, the *VEGFA* gene and the *KDR* gene, encoding the main receptor for VEGF, were also considered candidates to be involved in anti-VEGF treatment response. Most of the SNPs investigated in these genes have recently been evaluated in a

meta-analysis. After evaluation of nine SNPs (rs699947, rs699946, rs833069, rs833061, rs2146323, rs1413711, rs2010963 and rs1570360 in *VEGFA*, and rs2071559 in *KDR*), anti-VEGF treatment was found to be more effective in patients homozygous for the *VEGFA* rs833061 minor allele C, compared to the remaining AMD patients (OR=2.362, 95% CI 1.41-3.95, *P*=0.001). This analysis was, however, limited in sample size including only 444 AMD patients from three independent studies.[52] Besides SNP rs2070296 in the neuropilin-1 (*NRP1*) gene, described in chapter 2.1 of this thesis,[53] other reported associations with treatment response include the *APOE* ε4 allele,[54,55] *IL8* rs4073,[21,35,56] and *PEDF* rs1136287;[14] those have been analyzed in only a limited number of studies and warrant replication analyses. In the GWAS described in chapter 2.3, only rs429358 in the *APOE* gene showed a nominal association with visual acuity treatment response (*P*=0.043), in which the C allele leads to better outcome after treatment. The other variants previously associated with treatment response were not replicated in our GWAS (n=678).

Three GWASes for anti-VEGF treatment response have been published to date.[45,57,58] The first study, by Francis, involved only 65 AMD patients. When evaluating only candidate genes, an association with visual acuity outcome was reported for *CFH* rs1065489, and an association with change in macular thickness was reported for *C3* rs2230205.[57] In the second study, described in chapter 2.2 of this thesis, a total of 285 AMD patients were included in the GWAS discovery phase and, followed by replication in an independent cohort of 376 AMD patients. In this study SNP rs4910623 located in the olfactory receptor gene *OR52B4* was described as a new variant associated with worse treatment outcome.[58] In the third study, which was recently published, Yamashiro et al. analyzed 461 AMD patients collected in a prospective study design, and in a discovery and replication setting. The discovery GWAS phase with 256 patients did not identify any genome-wide significant associations, and suggestive associations could not be replicated. In a candidate SNP analysis that included 9 variants, the G allele of *ARMS2* rs10490924 was associated with additional treatment requirement after the loading dose.[45] The GWAS study described in chapter 2.3 has a larger sample size compared to all the previous studies (n=678), and therefore more statistical power, however, it did not identify any genome-wide significant associations either for common variants. After replication and meta-analysis of the lead variants, rs12138564 located in the *CCT3* gene remained nominally associated with a better treatment outcome. This variants was nor analyzed in candidate studies nor reported in prior GWAS studies. This study analyzed for the first time the effect of rare genetic variants on treatment response showing a negative effect of rare protein-altering variants in *C10ORF88* and *UNC93B1* genes on treatment outcome.

In addition to the pharmacogenetic studies, other biomarkers have also been described to be associated with anti-VEGF treatment response in nvAMD. In aqueous humor, VEGF and interleukin-6 (IL-6) levels have been measured prior to treatment, and they seem to be indicative of the outcome.[59] Lai and colleagues reported that baseline aqueous VEGF levels associated with persistent angiographic leakage after 3 months of bevacizumab therapy.[59] In another study by Chalam and colleagues, correlations of VEGF and IL-6 levels with change in central subfield macular thickness after 3 monthly injections of bevacizumab treatment were described, with the correlation of IL-6 levels being the strongest.[60]

Studies in plasma and serum have also suggested potential systemic biomarkers. Kepez Yildiz et al. described higher levels of plasma IL-6 in good responders compared to non-responders.[61] Nassar and colleagues evaluated 16 inflammatory cytokines and found that high IL-17 and TNF-$\alpha$ serum levels were associated with favorable response to anti-VEGF therapy.[62] Lechner et al. described that plasma C3a levels were elevated in partial responders compared to complete responders; no differences were found for C4a and C5a levels.[63] Additionally, Kubicka-Trzaska and colleagues analyzed serum anti-retinal antibiodies and reported that a decrease in anti-retinal antibiodies levels after bevacizumab treatment correlated with functional and anatomical response.[64]

# 2 Molecular biomarkers for therapies in clinical trials

## 2.1 Complement inhibiting therapies

Complement inhibiting therapies will presumably be most effective in AMD patients in which the complement system is most over-activated. Several studies have evaluated levels of complement components and activation fragments, which may represent useful biomarkers for treatment response to complement inhibiting therapies in AMD. Systemic levels of complement activation fragments such as Ba, Bb, C3a, C3d, C5a as well as levels of complement components Factor B (FB) and complement factor D (FD) seem to be elevated in AMD patients compared to controls.[65-71] This is also the case for the C3d/C3 ratio, which is analyzed in chapter 3.1. Systemic levels of complement component C3 and complement factor I (FI) levels appear, however, not different between AMD patients and controls.[66,67,69,71-73] Factor H (FH) levels have been reported to be lower in AMD in some studies,[74,75] but others do not report a difference.[66,67,71,72,76] Specific complement component levels could therefore be used to identify AMD patients with high levels of complement activity. Nevertheless, a high variability in these systemic complement markers is found within the AMD and control groups, and the levels show a large overlap between cases and controls. Consequently, other markers may be needed as well to predict response. In a recent study including 31 nvAMD patients and 30 controls, differences in Ba and C3a levels in aqueous humor were detected, whereas in plasma these differences were not detected, probably due to the limited sample size. These results suggest that the difference in complement activation levels between patients and controls is larger locally in the eye than it is when measured systemically.[77]

Genetic variants located in or near the *CFH*, *CFI*, *C9*, *C2*/*CFB*, *C3* and *VNT* genes, encoding components of the complement system, are known to be associated with AMD.[78] Some of these genetic variants have shown to affect complement activation levels, and could therefore also be used as robust biomarkers for complement system activity in AMD.

We reviewed the reported associations between common AMD-associated variants and systemic complement system levels in Table 2. SNPs rs12144939 and rs1410996 in the CFH gene have been associated with the C3d/C3 ratio, and rs800292 has been associated with Ba and C3d levels and the C3d/C3 ratio.[69,79,80] Genetic variants in the *C2* and *CFB* genes have also been analyzed, and an association with complement activation fragments has been found for rs4151667 (with C3d/C3, Ba and FB), rs641153 (with C3d/C3), and rs9332739 (with Ba).[69,71,79,80] SNPs rs6795735 and rs2230199 in the *C3* gene seem to influence complement system activation as well. SNP rs6795735 associated with the C3d/C3 ratio, and rs2230199 with levels of C3d, C5a, and the C3d/C3 ratio.[67,69,79,80] The association of *ARMS2* rs10490924 with complement activation is inconclusive. While one study reported the SNP to influence C5a levels,[67] in another study it did not,[69] and a third study did not find an association with the C3d/C3 ratio.[79]

In chapter 3.1 we analyzed common variants distributed across the genome in a GWAS on complement activation. This allowed us to detect in a unbiased manner the location the strongest association signal in the genome and to analyze systematically the known AMD-associated variants. The previous associations described for *CFH* and *CFB*/*C2* were confirmed in our GWAS of systemic complement activation and no additional associations were detected. The associations of rs2230199 in *C3* and rs10490924 in *ARMS2* were, however, not confirmed. In previous studies, selected AMD-associated SNPs were analyzed. We analyzed for the first time the whole-genome in relation to complement activation. The strongest signal was surprisingly located in an SNP which did not associate with AMD and the AMD-associated variant that showed the strongest effect in the GWAS was rs6685931 located in the *CFHR4* gene. As a follow-up of these results, in chapter 3.2 we analyzed the role of FHR-4 in AMD. In this study, we found that AMD-associated variants as rs570618, rs10922109, rs187328863 and rs61818925 located in or near the *CFH* and *CFHR4* genes associated with FHR-4 levels.

Recently, rare coding variants in the *CFH*, *CFI*, *C3* and *C9* genes have been described in AMD patients, and have also been shown to have an effect on systemic levels of complement components. Carriers of *CFH* Arg127His,[81] Arg175Pro,[82] and Cys192Phe[83] variants showed reduced FH levels. In carriers of *CFI* Gly119Arg,[84] Gly188Ala,[73] and Ala240Gly variants,[85] reduced FI levels were observed. Carriers of the *C9* variant Arg$^{95}$Ter showed C9 levels below the detection level,[86] and in carriers of Pro167Ser,87 C9 levels were elevated. Other rare variants did not show an effect on systemic levels individually, but a functional effect on complement activation has been described. The effect of these rare variants has been recently reviewed by Geerlings and colleagues.[88] Rare coding variants, in particular those showing an effect on complement activation, may therefore also be useful to select patients for complement inhibiting treatments.

Besides genetic biomarkers, other biomarkers that associate with AMD and complement activity could also be used to identify AMD patients with an over-activated complement system. Other reported factors include low systemic triglyceride levels and high body mass index.[79]

TABLE 2 **AMD-SNPs associated with systemic levels of complement components**

| Gene | SNP | Study | Allele / Genotype tested | Complement measurement(s) | Direction of the effect | *P*-value |
|---|---|---|---|---|---|---|
| *CFH* | rs12144939 | Ristau et al., 2014 | T | C3d/C3 | - | $4.6x10^{-6}$ |
| | rs1410996 | Ristau et al., 2014 | T | C3d/C3 | - | $10^{-4}$ |
| | | Reynolds et al., 2009 | TT, CT and TT | Bb, C3a, C5a, FH | NA | |
| | rs800292 | Hecker et al., 2010 | G | Ba | + | $7.1x10^{-6}$ |
| | | Hecker et al., 2010 | G | C3d | + | 0.0013 |
| | | Ristau et al., 2014 | A | C3d/C3 | - | 0.003 |
| | | Paun et al., 2016 | A | C3d/C3 | - | 0.002 |
| | | Hecker et al., 2010 | G | FB, FD, FH/FHR-1 | NA | |
| | rs570618 | Chapter 3.2 | G | FHR-4 | - | $2.5x10^{-14}$ |
| | rs10922109 | Chapter 3.2 | A | FHR-4 | - | $6.5x10^{-52}$ |
| *KCNT2 (upstream of CFH)* | rs187328863 | Chapter 3.2 | T | FHR-4 | + | $2.7x10^{-5}$ |
| *CFHR4* | rs61818925 | Chapter 3.2 | T | FHR-4 | - | $8.2x10^{-18}$ |
| | rs6685931 | Lorés-Motta et al., in press | C | C3d/C3 | | $6.32x10^{-8}$ |
| *CFB* | rs4151667 | Hecker et al., 2010 | T | Ba | + | $3.9x10^{-6}$ |
| | | Ristau et al., 2014 | A | C3d/C3 | - | $1.0x10^{-5}$ |
| | | Paun et al., 2016 | A | C3d/C3 | - | $4.1x10^{-6}$ |
| | | Hecker et al., 2010 | T | FB, FD, FH/FHR-1, C5a, C3d | NA | |
| | | Smailhodzic et al., 2012 | TA | FB | - | <0.001 |
| | rs641153 | Paun et al., 2016 | A | C3d/C3 | - | 0.048 |
| | | Reynolds et al., 2009 | CT/TT | Bb, C3a, C5a, FH | NA | |
| *C2* | rs9332739 | Hecker et al., 2010 | G | Ba | + | $2x10^{-6}$ |
| | | Hecker et al., 2010 | G | FB, FD, FH/FHR-1, C5a, C3d | NA | |
| | | Reynolds et al., 2009 | CG/CC | Bb, C3a, C5a, FH | NA | |
| *C3* | rs6795735 | Ristau et al., 2014 | A | C3d/C3 | + | 0.04 |
| | rs2230199 | Reynolds et al., 2009 | CG/GG | C5a | + | 0.04 |
| | | Ristau et al., 2014 | G | C3d/C3 | + | 0.04 |
| | | Paun et al., 2016 | G | C3d/C3 | + | 0.035 |
| | | Hecker et al., 2010 | C | C3d | + | 0.039 |
| | | Hecker et al., 2010 | C | FB, FD, FH/FHR-1, C5a, Ba | NA | |
| | | Reynolds et al., 2009 | CG/GG | Bb, C3a, FH | NA | |
| *ARMS2* | rs10490924 | Reynolds et al., 2009 | GT/TT | C5a | + | 0.02 |
| | | Reynolds et al., 2009 | GT/TT | Bb, C3a, FH | NA | |
| | | Hecker et al., 2010 | NE | FB, FD, FH/FHR-1, C5a, Ba, C3d | NA | |

SNP = Single nucleotide polymorphism, NE = Not specified, NA = Not associated

## 2.2 New therapeutic approaches: Gene and cell based therapies

The high and increasing prevalence of AMD together with the limited therapeutic options have boosted research to develop new therapies.[89] These new therapeutic strategies make use of recent technological advances including gene therapy, stem cells and genome editing.

### 2.2.1 Gene therapy

Gene therapy introduces specific genetic material into the patient's cells, usually by means of a viral vector. The successful example of gene replacement therapy for the treatment of a monogenic retinal disease, Leber congenital amaurosis,[90] motivated the development of gene therapy clinical trials for AMD. In AMD, the focus is on promoting the expression of a therapeutic protein in retinal pigment epithelium (RPE) cells. Viral vectors are delivered intravitreally or subretinally. An overview of gene therapy clinical trials for AMD is presented in Table 3.

AAVCAGsCD59 is the only gene therapy trial targeting the complement system which is currently being tested for GA, and inhibits membrane attack complex (MAC) formation through CD59 expression. Other gene therapy trials target the neovascular form of AMD. AdGVPEDF.11D leads to expression of pigment epithelium-derived factor (PEDF), an anti-angiogenic protein that counteracts the effects of VEGF in the choroidal neovascularization process.[91] This therapy has not been further evaluated since the results of the phase I trial in 2006.[92] AAV2-sFLT01 and rAAV.sFLT-1 both express soluble vascular endothelial growth factor receptor 1 (sFLT-1), an antagonist for VEGF.[93] The results of the phase I trial of AAV2-sFLT01 have recently been published with positive safety data and toleration of the drug after 3 years.[94] rAAV.sFLT-1 has already been evaluated in phase IIa, however, the control and the treatment groups performed worse than ranibizumab alone.[95] OXB-201, also known as RetinoStat, leads to the expression of the anti-angiogenic proteolytic products angiostatin and endostatin.[96,97] Phase I has already been completed and no adverse events were observed,[98] therefore, long term-safety studies are on-going. Finally, RGX-314 encodes a soluble anti-VEGF protein and is currently being evaluated in phase I clinical trials.

Anti-angiogenic factors delivered using gene therapy might also show a variability in response, as has been described for the currently used anti-VEGF antibodies. Therefore, pharmacogenetic associations found for anti-VEGF therapy might also be useful to analyze in clinical trials of gene therapy for nvAMD.

In addition, research on gene therapy for supplementation of FH is currently ongoing,[99] and supplementation therapy for FI might be useful as carriers of rare variants show reduced FI levels. For this particular therapy, patient selection based on genotype will be required. Carriers of rare variants in *CFH* and *CFI* known to have strong effects on the protein function or levels would be the best candidates for inclusion in clinical trials.

TABLE 3 **Gene-therapy and stem-cell based therapies for AMD in clinical trials**

| **Gene therapy** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Drug** | **Gene expressed** | **Target** | **Clinical phase** | **Results** | **Reference** | **Clinicaltrials.gov identifier** | **Funding (clinicaltrials.gov)** |
| AdGVPEDF.11D | PEDF | NV AMD | Phase I | Completed: No serious adverse events and no dose-limiting toxicities, transient intraocular inflammation occurred in 25% of patients | Campochiaro PA et al., 2006 | NCT00109499 | GenVec |
| AAV2-sFLT01 | sFLT101 (domain 2 of Flt-1 linked to human IgG1-Fc) | NV AMD | Phase I | Completed: Safe and well tolerated at all doses, potential effect of baseline anti-AAV2 serum antibodies and transgene expression | Heier JS et al., 2017 | NCT01024998 | Genzyme, a Sanofi Company |
| OXB-201 (RetinoStat) | Angiostatin and endostatin | NV AMD | Phase I | Completed: Well-tolerated with no dose-limiting toxicities, reduction in leakage for 71% of participants, reduction in fluid in 1 patient | Campochiaro PA et al., 2007 | NCT01301443 | Oxford BioMedica |
| | | | Phase I | Ongoing (long term safety - 15 years) | | NCT01678872 | Oxford BioMedica |
| RGX-314 | sAnti-VEGF protein | NV AMD | Phase I | On going | | NCT03066258 | Regenxbio Inc. |
| AAVCAGsCD59 or HMR59 | CD59 | GA AMD | Phase I | On going | | NCT03144999 | Hemera Biosciences |
| rAAV. sFLT-1 | sFLT1 | NV AMD | Phase I | Completed: Safe and well tolerated after 36 months | Rakoczy EP et al., 2015; Constable IJ et al., 2017 | NCT01494805 | Lions Eye Institute, Perth, Western Australia |
| | | | Phase II | Phase 2a completed: Smaller improvement than ranibizumab alone | Constable IJ et al., 2016 | NCT01494805 | Lions Eye Institute, Perth, Western Australia |

**Stem cell therapy**

| Type of therapy | Target | Clinical phase | Results | Reference | Clinicaltrials.gov identifier/ UMIN_CTR identifier | Funding (clinicaltrials.gov) |
|---|---|---|---|---|---|---|
| Autologous BMSC | GA AMD | Phase I/II | Unknown (estimated completion date 2015) | | NCT02016508 | Al-Azhar University |
| Autologous BMSC | NV & GA AMD | Phase I/II | Completed | | NCT01518127 | University of Sao Paulo |
| HCNSSC | GA AMD | Phase I/II | Completed: Long term safety assessment terminated | | NCT01632527 | StemCells, Inc. |
| hESC-RPE | GA AMD | Phase I/II | Completed: Safe and possible activity of the cells | Schwartz SD et al., 2012; Schwartz SD et al., 2015 | NCT01344993 | Astellas Institute of Regenerative Medicine |
| Human umbilical tissue-derived cells (Palucorcel, CNTO-2476) | GA AMD | Phase I/IIa | Completed: Subretinal delivery associated with perforations and detachment, well tolerated, may lead to VA improvements | Ho AC et al., 2017 | NCT01226628 | Janssen Research & Development, LLC |
| Autologous BMSC | GA AMD | Phase I | Completed: Well tolerated | Park SS et al., 2014 | NCT01736059 | University of California, Davis |
| hESC-RPE (OpRegen) | GA AMD | Phase I/II | On going | | NCT02286089 | Cell Cure Neurosciences Ldt. |
| Somatic cell nuclear transfer hESC-RPE | GA AMD | Phase I | On going | | NCT03305029 | CHA University |
| hESC-RPE | GA AMD | Early phase I | On going | | NCT03046407 | Chinese Academy of Sciences |
| hESC-RPE | GA AMD | Early phase I | On going | | NCT02755428 | Chinese Academy of Sciences |
| hESC-RPE in suspension and seeded on a substrate | NV & GA AMD | Phase I/II | On going | | NCT02903576 | Federal University of Sao Paulo |
| Autologous BMSC | AMD | Not specified | On going | | NCT03011541 | MD Stem Cells |
| Autologous iPSC-RPE | GA AMD | Production of the cells | On going | | NCT02464956 | Moorfields Eye Hospital NHS Foundation Trust |
| hESC-RPE (Pf-05206388) | NV AMD | Phase I | Ongoing (long term safety, 4-year follow-up) | | NCT03102138 | Pfizer |

| | | | | | | |
|---|---|---|---|---|---|---|
| hESC-RPE on a parylene membrane (CPCB-RPE) | GA AMD | Phase I/II | On going | | NCT02590692 | Regenerative Patch Technologies, LLC |
| Autologous BMSCs | AMD | Not specified | On going | | NCT01920867 | Retina Associates of South Florida |
| hESC-RPE | AMD | Phase I | On going | | NCT02749734 | Southwest Hospital, China |
| hESC-RPE | GA AMD | Phase I/II | Ongoing (long term safety and tolerability) | | NCT02463344 | Astellas Institute for Regenerative Medicine |
| Somatic cell nuclear transfer hESC-RPE | GA AMD | Phase I/IIa | Ongoing (preliminary results: safe and tolerated, 2 patients included, one patient gained VA and the other maintained VA) | Song WK et al., 2015 | NCT01674829 | CHA Biotech CO., Ltd |
| hESC-RPE (ASP7317) | GA AMD | Phase Ib/II | Not open yet | | NCT03178149 | Astellas Institute for Regenerative Medicine |
| hESC-RPE | GA AMD | Phase I/II | Not open yet (evaluation of long term safety) | | NCT03167203 | Astellas Institute for Regenerative Medicine |
| Autologous fibroblast iPSC-RPE sheet | NV AMD | Phase I/II | Completed: Patient 1 after 1 year the cell sheet appears to be safe and remains intact, VA maintained. Patient 2 did not receive therapy due to concerns about genetic changes in the iPSCs and iPSC-derived RPE | Mandai M et al., 2017 | UMIN000011929 | RIKEN |
| Allogenic HLA-matched iPSC-RPE | NV AMD | Phase I | On going | | Unknown | RIKEN |

NV AMD = Advanced neovascular AMD, GA AMD = Advanced geographic atrophy AMD, BMSC = bone marrow-derived stem cells, hESC-RPE = Human embryonic stem cell-derived retinal pigmented epithelial cells, hCNSSC = Human central nervous system stem cells, iPSC-RPE = induced pluripotent stem cell-derived retinal pigmented epithelial cells, VA = visual acuity

### 2.2.2 Stem cell therapy

Another novel therapeutic approach with great potential for AMD is the use of stem cells, which are reprogrammed to the cell type of interest and transplanted to the patient. Transplantation of RPE cells derived from stem cells for AMD treatment is currently being evaluated in several clinical trials (Table 3). The first clinical trial started in 2011 and involved human embryonic stem cell (hESC) derived RPE cells (NCT01344993). The therapy was found to be safe with no tumorigenicity and showed potential effectiveness.[100,101] These results have been followed up with a new improved therapy (NCT03178149, NCT03167203) that is currently being evaluated by developers in the Astellas Institute for Regenerative Medicine. Other on-going clinical trials are also based on hESC derived RPE, however, their use requires immunosuppressive treatment, bearing risks,[102] and raising ethical concerns due to the use of embryonic cells.

More recently, the use of induced pluripotent stem cells (iPSC) has begun to be explored. One of the key benefits of this therapy is that immunosuppression is not needed, as the source is the patient's own somatic cells. However, it implies an increased cost of therapy, as it needs to be developed for each patient individually. The first clinical trial with iPSC (http://www.umin.ac.jp, UMIN000011929) has recently been performed at the Japanese research institute RIKEN, where a 70-year old AMD patient received a transplant of a sheet of autologous iPSC-RPE. After one year of follow-up, no adverse events had been detected and the patient's vision remained stable.[103] However, this trial has been stopped for the second patient enrolled due to genetic changes found in the generated iPSC.[104] This group has recently shifted their approach towards the use of allogenic human leukocyte antigen (HLA)-matched iPSC-RPE, and in March 2017, it was announced that the first patient received allogenic iPSC-RPE.[105] This approach would be less costly and would avoid the effect of the genetic AMD risk variants that the patients carry. Nevertheless, it would most likely imply the use of immunosuppressant drugs. Contrary to these promising results of the group in RIKEN, in a back-to-back publication, it was reported that autologous adipose tissue-derived stem cells were administered bilaterally to three AMD patients in a stem-cell clinic, leading to a severe visual loss in all cases.[106] These disastrous events highlight that even though stem cell therapy holds promise, strict regulations should be applied before any treatment with stem cells is administered to patients.

RPE stem cell therapy might be the best therapeutic option for advanced cases in which there is RPE degeneration, however, it involves the transplantation of new cells in a diseased environment and, as such, the survival of the new cells may depend on inflammation and oxidative stress levels in the host environment. The C3/C3 ratio as a marker of complement activation, malondialdehyde levels as a marker of lipid peroxidation, and homocysteine levels as an oxidative stress marker, are molecular biomarkers for AMD that may correlate with the success of such therapies.[107] Moreover, autologous iPSC might not be the best option for AMD patients carrying highly penetrant genetic variants, and hESC or HLA-matched iPSC may be more effective in these patients.

### 2.2.3 Genome editing

Genome editing through the use of the CRISPR/Cas9 system is a powerful tool for therapy development.[108] Recently, the first treatment with genome editing has been given to a man suffering from Hunter syndrome.[109] The first studies for AMD are being carried out in animal models. *In vivo* genome editing using subretinal injections of Cas9 ribonucleoproteins that target the VEGFA gene has shown to reduce CNV in an AMD mouse model.[110] Moreover, this system could be included in a viral vector and delivered to specific cells, as a form of gene therapy. In a mouse model of laser-induced CNV, viral delivery of the CRISPR/Cas9 system edited the *KDR* gene, abolishing angiogenesis.[111] In another recent study in mice, an adeno-associated virus vector delivering a small Cas9 targeting the *VEGFA* and *HIF1A* genes of RPE cells reduced the size of laser-induced CNV.[110] These studies represent the very first steps towards the use of genome editing for therapy purposes in AMD.

This technology opens up possibilities for correcting genetic variants that give a risk for the disease. Several rare variants associated with AMD confer a remarkable high risk, such as *CFH* Arg1210Cys (OR=31.8), *CFH* Arg53Cys (OR=22.54) and *CFI* Gly162Asp (OR=20.29),[78] and might be potential targets for genome editing therapies. Consequently, genetic analysis would become indispensable in patient selection for gene-editing therapies.

# 3 Conclusions and future perspectives

Based on the recent findings of Assel at al.,[11] dietary supplementation for slowing down disease progression should be prescribed to any AMD patient, irrespective of *CFH* and *ARMS2* genotypes. However, these findings are based on the AREDS dataset only, and future independent prospective studies would be beneficial to corroborate these results, as well as to further investigate if other genetic variants may interact with the formulation.

In regard to the pharmacogenetics of anti-VEGF treatment, results are not conclusive yet. Nonetheless, recurrent results from multiple studies suggest that SNP rs1061170 in *CFH* may influence response to treatment. This finding could potentially be explained by the effect of this SNP on faster disease progression.[112] However, this association was not detected in the analyses from the CATT and IVAN clinical trials,[33,34] therefore warranting further investigation. Additionally, the magnitude of the effect of this variant might not reach clinical utility and would need to be combined with other genetic variants or clinical parameters. Other compelling candidate genetic variants for further evaluation include *ARMS2* rs10490924, *VEGFA* rs833061, *OR52B4* rs4910623, *NRP1* rs2070296, *APOE* ε4 allele, *IL8* rs4073 and *PEDF* rs1136287. However, based on the results of our GWAS on visual acuity measures (chapter 2.3) and the GWAS of Yamashiro and colleagues on anatomical measures,[45] we propose that there is a limited contribution of common genetic variants to variability in nvAMD treatment response. We additionally evaluated for the first time the influence of rare variants on this treat and found rare variants in the *C10ORF88*

and *UNC93B1* genes with large effects on treatment outcome.[113] These are compelling candidates for replication studies as rare variants with large effects have the potential of being implemented in clinical practice for guidance of treatment protocols.

A key problem remains that the definition of response is not consistently defined across cohorts. In 2015, in order to provide a consensus, a committee of retinal specialists proposed definitions of good, poor and non-response based on a combination of anatomical and functional measurements.[114] These definitions should be adopted by researchers in future studies, which would enable study comparisons in a standardized framework. Analysis of the different outcome measures used for these definitions as continuous variables would be also highly valuable. Additionally, prospective studies with sufficient statistical power would allow sub-phenotype analyses, which may reveal new or stronger associations.

Biomarkers identified in aqueous humor samples are VEGF and IL-6, however, these samples are not taken routinely. IL-6, IL-17, TNF-$\alpha$ and C3a have been identified as potential systemic biomarkers, and therefore could be readily measured before treatment. Moreover, as baseline VEGF has been associated with response in aqueous humor samples, it could be further investigated as a systemic biomarker. Recent studies suggest that anti-VEGF treatment may lead to an increased risk of GA development.[115] Therefore, screening of genetic markers together with other biomarkers and clinical parameters for effective anti-VEGF therapy planning may become necessary. Clinical trials would however be needed before the screening of these biomarkers can be implemented in the clinic.

Complement therapies are being developed for the treatment of GA, and biomarkers for complement activity could be useful to identify the most suitable AMD patients for these therapies. Based on the results of chapter 3.2, FHR-4 could potentially be a new drug target, and also be used as a biomarker for complement activity in AMD. Moreover, levels of the specific target of each drug could be a useful biomarker. Therapies undergoing trials are targeting FD, C3, properdin and C5. FD levels have been seen to be higher in AMD patients compared to controls, and therefore, they could be a useful biomarker for this specific therapy. C3 levels do not differ between AMD and controls and properdin and C5 levels have not been evaluated. A comprehensive analysis of the complement system components in AMD (e.g. by mass spectrometry) could identify new potential biomarkers. However, our findings of chapter 3.1 show that genetic variants affecting systemic levels of complement activation may not have an influence in the eye. Therefore, how systemic measurements reflect the local situation at the disease site needs to be further investigated.

Additionally, AMD-associated SNPs that associate with systemic complement activation can be used as robust biomarkers. The added value of these genetic biomarkers is that, as they are associated with disease risk, they most probably reflect complement activity in the eye, whereas the overall systemic complement activation may not always be representative of the conditions at the disease site. rs12144939, rs1410996 and rs800292 in *CFH*, rs4151667, rs641153 and rs9332739 in *C2*/*CFB* and rs6795735 and rs2230199

in *C3* have been reported to be associated with systemic complement activation levels and replicated in our GWAS study on chapter 3.1, being therefore reliable markers. Additionally, these GWAS study revealed the strongest association for an AMD-associated variant in *CFHR4* rs6685931, representing a new compelling biomarker for the disease. Moreover, rare variants in the *CFH* gene (Arg127His, Arg175Pro and Cys192Phe), in the *CFI* gene (Gly119Arg, Gly188Ala and Ala240Gly) and in the *C9* gene (Arg95Ter and Pro167Ser) have been associated with altered FH, FI and C9 levels respectively. However, the magnitude of the effects of these genetic variants at the disease site still needs to be evaluated. Additionally, other AMD-associated variants for which a systemic effect has not been detected might have a local effect. Consequently, genetic studies on complement activation levels in aqueous humor samples or other local eye sample types are greatly needed. The identified genetic factors may be used alongside systemic complement activation levels and other environmental factors such as BMI and triglyceride levels to identify AMD patients with a burden of the complement system driving their disease. In this regard, we built a model of complement activation including AMD-associated factors in chapter 3.1, which may serve as a starting point for future studies.

Other new therapeutic approaches will most probably not be effective in for all AMD patients. As a consequence, a deeper molecular characterization of AMD patients including proteomics, metabolomics, transcriptomics and genomics is essential. Such in-depth characterization will help to understand the molecular drivers in each individual patient and to develop pharmac-omics, paving the way towards precision medicine in AMD.

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# Summary

Age-related macular degeneration (AMD) is the most common cause of blindness in the elderly and affects approximately 1 in 10 individuals of 85 years of age. AMD patients experience a loss of central vision, blurred vision and have less ability to discriminate colors. As a consequence, they are impaired in their daily life activities such as reading and face recognition, and may lose their independency in the retirement ages.

In AMD pathogenesis, degeneration of the central retina, termed the macula, occurs. This degeneration is associated with extracellular accumulation of debris called drusen, which can be easily identified by ophthalmologists as yellowish spots on photographs of the retina. Visual loss occurs in the advanced stage of AMD, which can be subdivided into two types: georgraphic atropy (GA) and choroidal neovascularization (CNV). In the GA type, degeneration of the outer segments of the retina occurs, and in the CNV type there is abnormal growth of exudative blood vessels from the choroid.

Both environmental and genetic factors play a role in AMD development, as it is a complex trait. The risk of AMD development increases exponentially with age, which is the strongest non-genetic risk factor. Genetic factors play an important role in AMD development. The largest genome-wide association study (GWAS) recently revealed 52 genetic variants that are associated with AMD. These variants locate in genes that are involved in neovascularization and the complement system among others, implicating these pathways in the molecular mechanisms of the disease.

The genetic background, demographics and lifestyle can influence how a patient responds to a specific treatment. Therefore, healthcare could be improved by taking into account the individual characteristics of each patient when prescribing medication. This emerging approach in medicine is termed precision medicine. For this purpose, genetic variants are robust and easily measurable biomarkers that can be used to predict response to a drug. The overarching goal of this thesis was to identify genetic biomarkers that can be used to adapt treatment options to the needs of each AMD patient.

The only therapeutic intervention available for advanced AMD is the use of anti-vascular endothelial growth factor (VEGF) antibodies to treat the CNV form. These antibodies block VEGF, the master regulator of the pathological CNV formation. Although these drugs have led to significant vision improvement for the AMD patients, the individual patient response is highly variable. Around 20% of the patients continue losing vision despite receiving treatment.

In **chapter 2** of this thesis, we aimed to identify genetic biomarkers that associate with anti-VEGF treatment response in advanced AMD patients with CNV. To that end, we first conducted a candidate gene analysis described in **chapter 2.1**, in which we analyzed four single nucleotide polymorphisms (SNPs) located in the neuropilin-1 gene (*NRP1*). NRP1 was a compelling candidate because it is a co-receptor of VEGF and enhances the transduction of downstream signaling. Moreover, it has been described to affect the evolution of CNV

formation in AMD and VEGF mediated vascular leakage. The results of this study suggest that the SNP rs2070296 associates with worse visual acuity treatment outcome. In our next studies, we moved into genome-wide association analyses, in which more than a million variants distributed across the genome were analyzed in a hypothesis-free approach GWAS. In **chapter 2.2**, we describe the results of a GWAS performed by comparing pools of DNA of responders and non-responders, carried out by the group of Prof. Baird at the Centre for Eye Research in Australia. In our replication cohort, we confirmed that SNP rs4910623 in the *OR52B4* gene was associated with treatment response. Finally, in **chapter 2.3**, we performed a larger GWAS on treatment response followed by a replication analysis, by leading a large collaboration of several groups belonging to the International AMD Genomics Consortium. In essence, we could not confirm any of the associations previously reported in literature, nor did we find an association with any of the 52 AMD-associated variants. However, in this study we evaluated for the first time rare genetic variation and found that rare protein-altering variants in the *C10orf88* and *UNC93B1* genes may associate with severe vision loss despite treatment.

For GA, the second type of advanced AMD, there is no available treatment to date. However, several therapies that target the complement system are currently being evaluated in clinical trials. Due to the high variability in complement activation levels among AMD patients, we hypothesized that patients with higher levels of complement activation will most benefit from complement-inhibiting therapies. Therefore, in **chapter 3** of this thesis we aimed to find genetic variants that associate with complement activation levels. In order to do that, we carried out a GWAS on systemic complement activation levels, described in **chapter 3.1**. This analysis revealed that complement activation was independently associated with the SNP rs3753396 located in the complement factor H gene (*CFH*) and the SNP rs6685931 located in the complement factor H related 4 gene (*CFHR4*). The SNP rs3753396 in *CFH* did not associate with AMD, which led us to the conclusion that the relationship between systemic and local complement activation in the eye merits further investigation. The SNP rs6685931 in *CFHR4* associated with AMD, and therefore may be used for selecting patients for complement-inhibiting therapies. This last finding led us to investigate the role of FHR-4 in AMD pathogenesis, as described in **chapter 3.2**. The results of biochemical studies and further genetic analyses have led us to pinpoint FHR-4 as a new complement component involved in AMD, and a new potential target for the treatment of AMD.

In **chapter 4** we reviewed the current status of literature and placed our results in context. For biomarkers of response to anti-VEGF drugs, definitions of response should be harmonized in order to facilitate study comparisons. Common genetic variants seem to have a limited effect on treatment response, and combining several variants may be needed to achieve clinical significance. Our results on the analysis of rare variants suggest stronger effects, although they warrant replication. For complement-inhibiting therapies, genetic variants in complement genes have shown association with systemic complement activation levels and are compelling biomarkers. The relationship between systemic and local levels of complement activity has therefore become highly relevant. A deep molecular characterization of AMD patients including proteomics, metabolomics, transcriptomics and genomics is a necessary next step to move forward towards precision medicine for AMD patients.

# Samenvatting

Leeftijdsgebonden maculadegeneratie (LMD) is de meest voorkomende vorm van blindheid onder ouderen en treft ongeveer 1 op 10 mensen die 85 jaar of ouder zijn. LMD patiënten ervaren een verlies van hun centrale gezichtsveld, het onscherp worden van hun zicht, en ze zijn minder goed in staat om kleuren te onderscheiden. In hun dagelijkse activiteiten ondervinden ze hier hinder van, bijvoorbeeld tijdens het lezen of bij het herkennen van gezichten. LMD beperkt ook de mate van onafhankelijkheid van mensen na hun pensioen. Bij patiënten met LMD vindt slijtage en afbraak plaats van het centrale deel van het netvlies, dat de macula wordt genoemd. Deze degeneratie is geassocieerd met een ophoping van afvalstoffen, drusen, die door een oogarts gemakkelijk te zien zijn als geelachtige puntjes op foto's van het netvlies. Verlies van zicht treedt in de late stadia van LMD op, waarbij twee vormen worden onderscheiden: droog (ook wel geografische atrofie - GA) en nat (ook wel neovasculaire LMD). Bij GA vindt er een degeneratie plaats van het netvlies. Bij de natte vorm van LMD dringen lekkende bloedvaatjes uit het vaatvlies het netvlies binnen.

Zowel omgevingsfactoren als genetische factoren spelen een rol in het ontstaan van LMD. Het risico om LMD te krijgen stijgt exponentieel naarmate men ouder wordt, wat leeftijd de sterkste niet-genetische risicofactor maakt. Genetische factoren spelen een belangrijke rol bij LMD. De grootste genetische associatie studie vond 52 genetische varianten die geassocieerd zijn met de ziekte. Deze varianten zitten in genen die betrokken zijn bij het ontstaan van nieuwe bloedvaten (neovascularisatie) en ook in genen die betrokken zijn bij een onderdeel van het afweersysteem (het complement systeem), wat erop duidt dat deze biologische systemen betrokken zijn bij LMD.

Hoe een patiënt reageert op een behandeling kan afhangen van zijn of haar genetische achtergrond en levensstijl. De gezondheidszorg zou verbeterd kunnen worden door rekening te houden met deze gegevens bij het voorschrijven van een behandeling. Deze benadering is sterk in opkomst en heet 'precision medicine', ofwel persoonsgerichte zorg. Kennis van de genetica levert gemakkelijk meetbare en robuuste biomarkers op die gebruikt kunnen worden om behandelsucces te voorspellen.

Het doel van dit proefschrift was om moleculaire biomarkers te vinden die behandelsucces kunnen voorspellen, om zo toekomstige behandelingen te kunnen optimaliseren voor de individuele patiënt.

Het enige geneesmiddel dat beschikbaar is voor natte LMD is de behandeling met een antistof die 'vasculair endotheel groei factor' remt (anti-VEGF). Deze antistoffen remmen VEGF, een belangrijke moleculaire aanjager van het proces waarbij nieuwe bloedvaten worden gevormd. Deze behandeling heeft een belangrijke bijdrage geleverd aan het behoud van gezichtsvermogen voor LMD patiënten, maar individuele patiënten reageren heel variabel op de behandeling. Ongeveer 20% van de patiënten reageert niet tot nauwelijks op de behandeling. In **hoofdstuk 2** van dit proefschrift hebben we onderzocht

welke genetische biomarkers associëren met het behandelsucces van anti-VEGF in LMD. Eerst hebben we vier genetische varianten in het kandidaat gen neuropilin-1 (*NRP1*) geanalyseerd in **hoofdstuk 2.1**. NRP1 was een interessante kandidaat omdat het een co-receptor is van VEGF en het moleculaire signaal versterkt. Daarnaast is het betrokken bij bloedvatnieuwvorming in LMD en lekkage van bloedvaten onder invloed van VEGF. Onze resultaten lieten zien dat een bepaalde genetische variant in *NRP1* (rs2070296) associeerde met een slechtere uitkomst na behandeling. In onze vervolgstudies zijn we over gegaan naar genoomwijde analyses, waarbij meer dan een miljoen genetische varianten over het hele genoom tegelijkertijd werden getest. In **hoofdstuk 2.2** beschrijven we de resultaten van een genoomwijde analyse, waarbij mensen die wel reageerden op de behandeling werden vergeleken met mensen die niet reageerden. Dit experiment werd gedaan in Australië, onder leiding van professor Paul Baird, van het Centre for Eye Research. In ons replicatiecohort konden we bevestigen dat een variant in *OR52B4* (rs4910623) betrokken was bij behandelsucces. Tenslotte hebben we in **hoofdstuk 2.3** zelf een grote genoomwijde associatie analyse uitgevoerd in samenwerking met verschillende groepen uit een groot internationaal samenwerkingsverband, het International AMD Genomics Consortium. We konden eerder gepubliceerde data niet bevestigen in deze studie, en we vonden ook geen associatie met de 52 varianten die betrokken zijn bij LMD. Wel vonden we in deze studie een mogelijke rol voor zeldzame genetische varianten, met name in de genen *C10orf88* en *UNC93B1*, in een slechte uitkomst na behandeling.

Voor de tweede vorm van LMD, de droge vorm, is op dit moment geen behandeling beschikbaar, maar verscheidene klinische studies zijn gaande waarin het complement systeem wordt geremd. Omdat de activiteit van het complement systeem sterk variabel is tussen LMD patiënten, hadden wij de hypothese dat patiënten met een verhoogde complement activiteit het meeste baat zouden hebben bij deze nieuwe behandeling. In **hoofdstuk 3** hadden wij daarom als doel om genetische factoren te vinden die geassocieerd zijn met complement activiteit. Wij voerden een genoomwijde associatie analyse uit (**hoofdstuk 3.1**) en vonden twee genetische varianten die sterk geassocieerd waren met complement activiteit (rs3753396 in het gen *CFH* en rs6685931 in het gen *CFHR4*). De variant rs3753396 was niet geassocieerd met LMD, wat er sterk voor pleit dat de complexe relatie tussen lokale complement activiteit in het oog en in het bloed nader onderzocht dient te worden. De variant rs6685931 in het gen *CFHR4* was wel geassocieerd met LMD, and kan derhalve gebruikt worden om patiënten te selecteren voor de complement remmende behandeling. Deze bevinding leidde er toe om de rol van FHR-4 in het ontstaan van LMD nader te onderzoeken, zoals beschreven in **hoofdstuk 3.2**. De resultaten van deze biochemische studies en aanvullende genetische analyses wezen erop dat FHR-4 mogelijk betrokken is bij LMD en dat het een nieuwe kandidaat is voor toekomstige behandelingen.

In **hoofdstuk 4** vatten we de actuele literatuur over dit onderwerp samen en plaatsten we onze eigen resultaten in deze context. Om biomarkers voor behandelsucces na anti-VEGF te vinden, zou eerst een eenduidige definitie van behandelsucces opgesteld moeten worden zodat verschillende studies met elkaar vergeleken kunnen worden. Veelvoorkomende genetische varianten lijken slechts een beperkt effect te hebben op behandelsucces, en het

combineren van genetische varianten is mogelijk nodig om een klinisch significant resultaat te behalen. Onze resultaten suggereren dat zeldzame genetische varianten mogelijk sterkere effecten hebben, maar deze bevindingen dienen gerepliceerd te worden in andere cohorten. In de studies waarin we zochten naar biomarkers voor complement activiteit vonden we enkele interessante kandidaten in complement genen. Het werd duidelijk dat de relatie tussen complement activiteit in het oog en in het bloed zeer relevant is en nader onderzocht dient te worden. Een gedetailleerde profilering van LMD patiënten, waarbij proteomics, metabolomics, transcriptomics en genomics worden samengenomen, is een belangrijke vervolgstap om persoonsgerichte zorg voor LMD patiënten te ontwikkelen.

# Resumen

La degeneración macular asociada a la edad (DMAE) es la causa más común de ceguera en la vejez y afecta aproximadamente a 1 de cada 10 personas de 85 años de edad. Los pacientes con DMAE experimentan una pérdida de visión central, visión borrosa y tienen una menor capacidad para discriminar colores. Como consecuencia, estos pacientes se ven perjudicados en sus actividades cotidianas, como la lectura y el reconocimiento facial, y pueden perder su independencia en edades de jubilación.

En la patogénesis de la DMAE se produce una degeneración de la parte central de la retina, denominada mácula. Esta degeneración está asociada con la acumulación extracelular de desechos llamados drusas, las cuales pueden ser identificadas como manchas amarillentas en fotografías de la retina. La pérdida de visión se produce en la etapa avanzada de la DMAE, que se puede subdividir en dos tipos: atrofia geográfica (forma seca) y neovascularización coroidea (forma húmeda). En la forma seca, se produce la degeneración de los segmentos externos de la retina mientras que en la forma húmeda hay un crecimiento anormal de vasos sanguíneos en la coroides, los cuales son exudativos.

Tanto factores ambientales como genéticos están involucrados en el desarrollo de DMAE ya que se trata de un fenotipo complejo. El riesgo de desarrollo de DMAE aumenta exponencialmente con la edad, siendo este el factor de riesgo no genético de mayor relevancia. Los factores genéticos juegan también un papel clave en el desarrollo de DMAE. El estudio de asociación del genoma completo (GWAS) más extenso llevado a cabo, reveló recientemente 52 variantes genéticas que están asociadas con la DMAE. Estas variantes se localizan, entre otros, en genes que están implicados en neovascularización y el sistema del complemento, implicando estas vías en los mecanismos moleculares de la enfermedad.

La manera en la que un paciente responde a un tratamiento específico puede variar dependiendo de la génetica, la demografía y el estilo de vida dicho paciente. Por ello, la atención médica podría mejorarse teniendo en cuenta las características individuales de cada paciente a la hora de prescribir medicamentos. Este enfoque emergente en medicina se denomina «medicina de precisión». Las variantes genéticas son biomarcadores robustos y fáciles de medir que pueden ser utilizados para predecir la respuesta a un fármaco y como consecuencia, son utilizados en el campo de la medicina de precisión. El objetivo general de esta tesis ha sido la identificación de biomarcadores genéticos que puedan utilizarse para adaptar las opciones de tratamiento a las necesidades de cada paciente con DMAE.

La única intervención terapéutica disponible para la DMAE avanzada consiste en anticuerpos contra el factor de crecimiento endotelial vascular (FCEV) para tratar la forma húmeda. Estos anticuerpos bloquean FCEV, que es el regulador principal de la formación patológica de neovascularización en la coroides. Aunque estos fármacos producen

una mejora significativa en la visión de los pacientes con la forma húmeda de DMAE, la respuesta individual de cada paciente es muy variable. De hecho, alrededor del 20% de los pacientes continúa perdiendo la visión a pesar de recibir tratamiento.

El objetivo del **capítulo 2** de esta tesis consiste en identificar biomarcadores genéticos de respuesta al tratamiento anti-FCEV en pacientes con la forma húmeda de DMAE. En primer lugar realizamos un análisis de genes candidatos descrito en el **capítulo 2.1**, en el cual analizamos cuatro polimorfismos de nucleótido único (SNPs) ubicados en el gen neuropilin-1 (*NRP1*). NRP1 es un correceptor de FCEV y mejora la transducción de la señalización aguas abajo. Además, NRP1 afecta el desarrollo de la neovascularización en la coroides y a la filtración vascular mediada por FCEV en DMAE. Los resultados de este estudio sugieren que el SNP rs2070296 está asociado con una peor respuesta al tratamiento en términos de agudeza visual. Es decir, tras el tratamiento, la mejora en agudeza visual de los pacientes con esta variante genética es menor en comparación con la de los pacientes que no son portadores de esta variante genética. En los siguientes estudios, ampliamos nuestro análisis al genoma completo y evaluamos más de un millón de variantes distribuidas a lo largo de todo el genoma sin tener ninguna hipótesis *a priori*. En el **capítulo 2.2** describimos los resultados de un GWAS realizado comparando ADN de pacientes que responden a la terapia con el ADN de pacientes que no responden. Este trabajo se llevo a cabo en el grupo del Prof. Baird en el Centro de Investigación de Ojos en Australia. En nuestra cohorte de replicación, confirmamos que el SNP rs4910623 en el gen *OR52B4* está asociado con la respuesta al tratamiento. Finalmente, en el **capítulo 2.3**, realizamos un GWAS sobre la respuesta al tratamiento seguido de un análisis de replicación en el que incluimos un mayor número de pacientes, liderando una gran colaboración de varios grupos pertenecientes al "International AMD Genomics Consortium". En esencia, no pudimos confirmar ninguna de las asociaciones previamente descritas, ni encontramos una asociación con ninguna de las 52 variantes asociadas con la DMAE. Sin embargo, en este estudio se incluyeron por primera vez variantes genéticas raras y encontramos que variantes genéticas raras que alteran las proteínas codificadas por los genes *C10orf88* y *UNC93B1* están asociadas con una pérdida de visión severa a pesar del tratamiento.

Para la forma seca, el segundo tipo de DMAE avanzada, no hay ningún tratamiento disponible hasta la fecha. Sin embargo, se están llevando a cabo ensayos clínicos que evalúan terapias que inhiben el sistema del complemento. Debido a que los pacientes con DMAE exhiben una gran variabilidad en los niveles de activación del complemento, planteamos la hipótesis de que los pacientes con la forma seca y los niveles más altos de activación del complemento serán los que más se beneficien de este tipo de terapias. Por ello, el propósito del **capítulo 3** de esta tesis ha sido identificar variantes genéticas asociadas con los niveles de activación del complemento. Con este fin, llevamos a cabo un GWAS sobre los niveles de activación sistémica del complemento, el cual está descrito en el **capítulo 3.1**. Este análisis reveló que la activación del complemento está asociada con el SNP rs3753396 localizado en el gen del factor H del complemento (*CFH*) y el SNP rs6685931 localizado en el gen numero 4 relacionado con el factor H del complemento (*CFHR4*). El SNP rs3753396 en el gen *CFH* no resultó estar asociado con la DMAE, lo que nos llevó a la

conclusión de que la relación entre la activación sistémica y local (en el ojo) merece mayor investigación. El SNP rs6685931 en el gen *CFHR4* está, por el contrario, asociado con la DMAE y por lo tanto se puede usar para seleccionar pacientes para terapias que inhiban el sistema del complemento. Este último hallazgo nos llevó a investigar el papel de FHR-4 en la patogénesis de la DMAE, como se describe en el **capítulo 3.2**. Los resultados de estudios bioquímicos y análisis genéticos sugieren que FHR-4 es un nuevo componente del complemento involucrado en la DMAE y que por lo tanto, podría ser suponer un nuevo objetivo para su tratamiento.

En el **capítulo 4** analizamos la literatura actual en este campo científico y pusimos nuestros resultados en contexto. En relación con los biomarcadores para la respuesta a los fármacos anti-FCEV, es necesario armonizar las definición de la respuesta al tratamiento para facilitar las comparaciones entre estudios. Las variantes genéticas comunes parecen tener un efecto minoritario en la respuesta al tratamiento. Por ello, tendrán que combinarse varias variantes para poder usarse como biomarcadores con aplicación clínica. Nuestros resultados en el análisis de variantes raras sugieren efectos de mayor magnitud, aunque es necesario analizar resultados en otras cohortes. Para las terapias que inhiben el sistema del complemento, las variantes genéticas que se asocian con los niveles de activación sistémica del complemento son biomarcadores convincentes. Aún así, es necesario analizar en más profundidad la relación entre la actividad del complemento a nivel sistémico y local. Para concluir, una caracterización molecular profunda de pacientes con DMAE, que incluya proteómica, metabolómica, transcriptómica y genómica, es un paso necesario para avanzar hacia la medicina de precisión en pacientes con DMAE.

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"*Eso de extrañar, la nostalgia y todo eso, es un verso. No se extraña un país, se extraña el barrio en todo caso, pero también lo extrañas si te mudas a diez cuadras. El que se siente patriota, el que cree que pertenece a un país, es un tarado mental. ¡La patria es un invento! ¿Qué tengo que ver yo con un tucumano o con un salteño? Son tan ajenos a mí como un catalán o un portugués. Estadísticas, números sin cara. Uno se siente parte de muy poca gente, tu país son tus amigos, y eso sí se extraña...*"
Adolfo Aristarain y Kathy Saavedra, Martín (Hache), 1997, Argentina

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Thank you **EyeTN team** (**Carla**, **Elena**, **Julio**, **Giulia**, **Simona**, **Riccardo**, **Raji** and our beloved postdoc **Vincent**) for the great moments and the 'funny' we had in every meeting, I could not have imagined it better. Thank you also to the **PIs of the EyeTN** network (**Anneke den Hollander**, **Bernard Wissinger**, **Carmel Toomes**, **Carmen Ayuso**, **Christian Hamel**, **Elfride De Baere**, **Frans Cremers**, **Rob Collin**, **Sandro Banfi**, **Susanne Kohl**, **Paul Ko Ferrigno**, **Viki Kalatzis** and specially to **Chris Inglehearn**); it was an honour to meet you all. **Paul** and **Carmen**, thank you very for hosting me in your labs, it was a very enjoyable and fruitful experience which definitely enriched my PhD learning process!

Thank you **Paul**, **Simon** and **Valentina** for the interesting discussions and collaborative efforts!

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Gracias **Roser Gonzàlez** por guiar mis primeros pasos en investigación y por alentar mis inquietudes científicas.

Gracias **Marta**, compañera y amiga, hace unos años mirábamos juntas las primeras secuencias y escuchábamos Manel! Ya he acabado la 'tesi'! Un pilar para celebrarlo, de 4 por lo menos!

**Anna**, **Amaia** y **Cris**, os guardo un cariño especial y cuando pienso en mi tesis también pienso en vosotras ya que todo esto empezó en las bibliotecas (y el bar!) de aquella facultad al sol . Amaia y Cris, os echo de menos!

**Àlex**, tú me animaste a esta aventura que comenzamos juntos, te quiero dar las gracias por tu apoyo y animarte también con la tuya que seguro que llegará pronto!

Thank you my habibis **Janneke** and **Osama** for the nice moments we spent together, and thank you **Asmaa**, الطيب وقلبك بشجاعتك كبير بعمق معجبة أنا !

Thanks to my friends in Nijmegen **Ale**, **Blan**, **Darío**, **Ellie**, **Ester**, **Lavi**, **Merce** and **Roque**. Thank you for the gezelligheid! These years were full of amazing experiences and touching moments! I would need quite some pages to thank you all individually for everything you gave me and they are all full of genetics stuff..., I see you somewhere in the world!

I arrived to the Netherlands to start this thesis work on September 15th, 2013.
I just took a flight.

Since 2014, there have been 10,645 deaths recorded in the Mediterranean sea. Many others have not been recorded. Those were people who desperately sought a new life, a life.

I would like, in all humility, to dedicate my work also to them.

# List of publications

**Factor H-Related Protein 4 Drives Complement Activation in Advanced Age-Related Macular Degeneration**
Cipriani V*, Lorés-Motta L*, He F, Fathalla, Tilakaratna V, McHarg S, Bayatti N, Acar IE[5], Hoyng CB, Fauser S, Moore AT, Yates JR, Morgan BP, de Jong EK[#], den Hollander AI[#], Bishop PN[#], Clark SJ[#]
* These authors contributed equally to this work. [#]These authors contributed equally to this work.

*Manuscript in preparation*

**Genome-Wide Association Study Reveals Variants in CFH and CFHR4 Associated with Systemic Complement Activation: Implications in Age-Related Macular Degeneration**
Lorés-Motta L*, Paun CC*, Corominas J, Pauper M, Geerlings MJ, Altay L, Schick T, Daha MR, Fauser S, Hoyng CB, den Hollander AI, de Jong EK.
* These authors contributed equally to this work.

July 2018, *Ophthalmology*

**Exploring the Use of Molecular Biomarkers for Precision Medicine in Age-Related Macular Degeneration**
Lorés-Motta L, de Jong EK, den Hollander AI.

June 2018, *Molecular Diagnosis and Therapy*

**Association of Genetic Variants With Response to Anti-Vascular Endothelial Growth Factor Therapy in Age-Related Macular Degeneration**
Lorés-Motta L, Riaz M, Grunin M, Corominas J, van Asten F, Pauper M, Leenders M, Richardson AJ, Muether P, Cree AJ, Griffiths HL, Pham C, Belanger MC, Meester-Smoor MA, Ali M, Heid IM, Fritsche LG, Chakravarthy U, Gale R, McKibbin M, Inglehearn CF, Schlingemann RO, Omar A, Chen J, Koenekoop RK, Fauser S, Guymer RH, Hoyng CB, de Jong EK, Lotery AJ, Mitchell P, den Hollander AI[#], Baird PN[#], Chowers I[#]
* These authors contributed equally to this work. [#]These authors contributed equally to this work.

May 2018, *JAMA Ophthalmology*

**Whole-Exome Sequencing in Age-Related Macular Degeneration Identifies Rare Variants in COL8A1, a Component of Bruch's Membrane**
Corominas J, Colijn JM, Geerlings MJ, Pauper M, Bakker B, Amin N, Lores Motta L, Kersten E, Garanto A, Verlouw JAM, van Rooij JGJ, Kraaij R, de Jong PTVM, Hofman A, Vingerling JR, Schick T, Fauser S, de Jong EK, van Duijn CM, Hoyng CB, Klaver CCW, den Hollander AI.

April 2018, *Ophthalmology*

**Complement C3 associates with incidence of diabetes, but no evidence of a causal relationship**
Borné Y, Muhammad IF, Lorés-Motta L, Hedblad B, Nilsson PM, Melander O, de Jong EK, Blom AM, den Hollander AI, Engström G.

September 2017, *The Journal of Clinical Endocrinology & Metabolism*

**GWAS study using DNA pooling strategy identifies association of variant rs4910623 in *OR52B4* gene with anti-VEGF treatment response in age-related macular degeneration**
Riaz M*, Lorés-Motta L*, Richardson AJ, Lu Y, Montgomery G, Omar A, Koenekoop RK, Chen J, Muether P, Altay L, Schick T, Fauser S, Smailhodzic D, van Asten F, de Jong EK, Hoyng CB, Burdon KP, MacGregor S, Guymer RH, den Hollander AI, Baird PN.
*These authors contributed equally to this work.

November 2016, *Scientific Reports*

**A genetic variant in *NRP1* is associated with worse response to ranibizumab treatment in neovascular age-related macular degeneration**
Lorés-Motta L, van Asten F, Muether PS, Smailhodzic D, Groenewoud JM, Omar A, Chen J, Koenekoop RK, Fauser S, Hoyng CB, den Hollander AI, de Jong EK.

January 2016, *Pharmacogenetics and Genomics*

**Combined genetic and high-throughput strategies for molecular diagnosis of inherited retinal dystrophies**
de Castro-Miró M, Pomares E, Lorés-Motta L, Tonda R, Dopazo J, Marfany G, Gonzàlez-Duarte R.

February 2014, *PLoS One*

# Radboud Institute for Molecular Life Sciences (RIMLS) portfolio

**L. Lorés de Motta**
**Department of Ophthalmology**
**Radboud Institute for Molecular Life Sciences**

**PhD period: 15-09-2013 – 15-09-2017**
**Promotors: Prof. A. I. Den Hollander, Prof. C. B. Hoyng**
**Co-promotor: Dr. E. K. De Jong**

| TRAINING ACTIVITIES | Year(s) | ECTS |
|---|---|---|
| **Courses & Workshops** | | |
| EyeTN Research Skills Training 1: Seminars | 2013,2014 | 0.45 |
| EyeTN Complementary Skills Training 1: Effective Research, Research Integrity and Social Media | 2013 | 0.5 |
| Graduate Course RIMLS | 2014 | 2.0 |
| EyeTN Research Skills Training 2: Quality System Course (Scientific Integrity course) | 2014 | 0.325 |
| Next Generation Sequencing Course (European Society of Human Genetics) | 2014 | 0.625 |
| EyeTN Complementary Skills Training 2: Communication and Interpersonal Skills | 2014 | 1.2 |
| Academic writing course (Radboud University) | 2015 | 1.0 |
| EyeTN Complementary Skills 3 Training: Exploring Career Opportunities | 2014 | 0.5 |
| EyeTN Complementary Skills 3 Training: Creative thinking | 2014 | 0.75 |
| EyeTN Research Skills 3: Biogazelle course, Real-time PCR | 2014 | 0.5 |
| Genetic epidemiology course (MED-5E005) | 2014 | 7 |
| Advanced Bioinformatics course, University of Tübingen | 2014 | 0.75 |
| EyeTN Complementary Skills 3 Training: Intellectual property | 2015 | 0.625 |
| Adobe Indesign workshop I and II | 2016 | 0.8 |
| Linux introduction course, Radboud umc | 2016 | 0.5 |
| **Seminars & Lectures** | | |
| NCMLS Neurodevelopmental Disease: From CNVs to Genes | 2013 | 0.1 |
| NCMLS lecture: Next Generation Human Genetics | 2013 | 0.1 |
| NCMLS lecture: Mechanisms and treatments for neurodevelopmental disorders | 2013 | 0.1 |
| Symposium on hereditary retinal dystrophies: From bench to bed | 2014 | 0.5 |
| Symposium Novel therapies for Retinal dystrophies and Master workshop | 2014 | 0.3 |
| Donders Theme 4 meeting on Statistical power | 2015 | 0.1 |
| RIHS lecture: An Editor's Perspective on Scientific Publishing | 2016 | 0.1 |
| Donders lecture: John O'Keefe | 2017 | 0.1 |
| Cybergenetica symposium , Koninklijke Nederlandse Akademie van Wetenschappen (KNAW) | 2016 | 0.1 |
| Radboud Research Rounds Sensory Disorders (x4) | 2015-2017 | 0.4 |
| **(Inter)national Symposia & Congresses** | | |
| EUGENDA Annual Retreat* | 2013-2016 | 1 |
| Radboud New Frontiers | 2013, 2015 | 1 |
| Dutch Ophthalmology PhD's (DOPS) retreat 2014#* | 2014, 2016 | 1 |
| ARVO (Association for Research in Vision and Ophthalmology) Annual meeting* | 2015, 2017 | 3 |
| International symposium on AMD, Baden Baden, Germany* | 2015 | 0.75 |
| European Society of Human Genetics Conference# | 2016 | 1.25 |
| Final Conference EyeTN* | 2016 | 1 |
| Coorganizing Final Conference EyeTN | 2016 | 2 |
| American Society of Human Genetics Conference# | 2016 | 1.75 |
| PhD retreat* | 2016 | 1.25 |
| Donders perception day# | 2016 | 0.75 |
| **Other** | | |
| Journal club | 2013-2016 | 3 |
| Sensory Disease (technical forum) | 2013-2017 | 4 |

| TEACHING ACTIVITIES | Year(s) | ECTS |
|---|---|---|
| **Lecturing** | | |
| Internship supervision (Ana Madalina Ion, Master's in molecular mechanisms of disease) | 2014 | 6.0 |
| Teaching Honors students | 2015 | 0.2 |
| Teaching BMW students | 2015 | 0.8 |
| Seminar on Pharmacogenetics of AMD at the 'Fundación Jimenez Díaz' | 2016 | 0.1 |
| 5KMP1: Moleculaire diagnostiek en therapeutisch perspectief voor leeftijdsgebonden macula degeneratie | 2016 | 0.2 |
| Internship supervision (Mathieu Lenders, HAN) | 2015 | 1 |
| DNA day teaching | 2016 | 0.4 |
| **Other** | | |
| Judge ESHG DNA-Day 2016 Essay contest | 2016 | 0.2 |
| Scientific publication revisions | 2016,2017 | 0.4 |
| **TOTAL** | **50.475** | |

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.

Institute for Molecular Life Sciences
Radboudumc

**Laura Lorés de Motta** was born on April 10, 1990 in Huesca, Spain. In 2008, after graduating with Honors from the Ramón y Cajal high school, she moved to Barcelona to study Biotechnology at the Autonomous University of Barcelona. During her Bachelor's degree, she developed a special interest in genetics and, upon completion in 2012, she enrolled in a Master's degree in Genetics and Genomics at the University of Barcelona. For her Master's project, Laura worked in the group of Prof. Roser Gonzàlez, where she developed a multiplex assay for *USH2A* screening, and identified several new mutations in families with Usher syndrome and retinitis pigmentosa. In 2013, she join the group of Prof. Anneke den Hollander at the Radboud University Medical Center in Nijmegen, the Netherlands for a PhD degree in a Marie Curie Initial Training Network (EyeTN). In her doctoral thesis, Laura studied the genetics and pharmacogenetics of the multifactorial ocular disease age-related macular degeneration (AMD). As part of this training, she moved to Leeds, United Kingdom to identify an Affimer for AMD treatment at Avacta Life Sciences, and to Madrid, Spain to perform rare variant analyses in the laboratory of Prof. Carmen Ayuso García at the Fundación Jiménez Díaz. She received the Association for Research in Vision and Ophthalmology (ARVO) Foundation Elizabeth Anderson Travel Grant to present her results at the ARVO annual meeting. She was also awarded with the best presentation award at the EyeTN conference and the Sensory Disease Talent Award from the Radboud University Medical Center. This thesis contains the work performed during her PhD studies and was completed in December 2017.