ASTA HAUTAMÄKI

Genetic and Structural Variations Associated with the Activity of Exudative Age-Related Macular Degeneration Lesion

HELSINKI UNIVERSITY HOSPITAL AND
DEPARTMENT OF OPHTHALMOLOGY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN CLINICAL RESEARCH
UNIVERSITY OF HELSINKI
Genetic and structural variations associated with the activity of exudative age-related macular degeneration lesion

Asta Hautamäki

ACADEMIC DISSERTATION

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Unigrafia  
Helsinki 2017
What is essential is invisible to the eye.

Antoine de Saint-Exupéry
The Little Prince
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ORIGINAl PUBLICATIONS

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# ABBREVIATIONS

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A2E</td>
<td>N-retinylidene-N-retinylethanolamine</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>AREDS</td>
<td>Age-Related Eye Disease Study</td>
</tr>
<tr>
<td>ARMS2</td>
<td>age-related maculopathy susceptibility 2 gene (also called LOC387715)</td>
</tr>
<tr>
<td>BMP-4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>BRB</td>
<td>blood-retina barrier</td>
</tr>
<tr>
<td>C3</td>
<td>complement component 3</td>
</tr>
<tr>
<td>CARMS</td>
<td>Clinical Age-Related Maculopathy Staging system</td>
</tr>
<tr>
<td>CATT</td>
<td>Comparison of Age-Related Macular Degeneration Treatment trial</td>
</tr>
<tr>
<td>CFH</td>
<td>complement factor H</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CNV</td>
<td>choroidal neovascularization</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
<td>contrast sensitivity</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>FA</td>
<td>fluorescein angiography</td>
</tr>
<tr>
<td>Fab</td>
<td>region containing antigen binding site in an antibody</td>
</tr>
<tr>
<td>Fc</td>
<td>region containing complement and receptor binding site in an antibody</td>
</tr>
<tr>
<td>HIF-1 or -2A</td>
<td>hypoxia-inducible transcription factor 1 or 2A</td>
</tr>
<tr>
<td>HR</td>
<td>hazard ratio</td>
</tr>
<tr>
<td>Htra1</td>
<td>high-temperature requirement factor A1</td>
</tr>
<tr>
<td>ICGA</td>
<td>indocyanine green angiography</td>
</tr>
<tr>
<td>IL-1(β), -6, -8, or -10</td>
<td>interleukin-1(β), -6, -8, or -10</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IVAN</td>
<td>Inhibition of VEGF in Age-related choroidal Neovascularisation trial</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>NED</td>
<td>neuroepithelial detachment</td>
</tr>
<tr>
<td>OCT</td>
<td>optical coherence tomography</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCV</td>
<td>polypoidal choroidal vasculopathy</td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>PED</td>
<td>pigment epithelial detachment</td>
</tr>
<tr>
<td>ph/ms</td>
<td>photons per millisecond</td>
</tr>
<tr>
<td>PRN</td>
<td>pro re nata, as needed treatment strategy</td>
</tr>
<tr>
<td>RAP</td>
<td>retinal angiomatous proliferation</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
</tr>
<tr>
<td>RR</td>
<td>relative risk, risk ratio</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TAP</td>
<td>Treatment of Age-Related Macular Degeneration with Photodynamic Therapy trial</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VA</td>
<td>visual acuity</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor (refers to VEGF-A in this text)</td>
</tr>
<tr>
<td>VEGFR-1 or -2</td>
<td>vascular endothelial growth factor receptor 1 or 2</td>
</tr>
<tr>
<td>VIP</td>
<td>Verteporfin in Photodynamic Therapy trial</td>
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ABSTRACT

Vascular endothelial growth factor (VEGF) is a central mediator in the formation of new blood vessels both in physiological and pathological processes. In exudative age-related macular degeneration (AMD), a choroidal neovascularization (CNV) grows underneath the retinal pigment epithelium (RPE) or between the retinal layers. Agents inhibiting VEGF form the mainstay of present-day treatment of exudative AMD. Although the response to anti-VEGF agents is usually good, repeated monthly intravitreal injections are often required to maintain the results. Marked individual variations exist in the rate and magnitude of response to treatment. The reasons for these variations are still mostly unknown.

The aim of this study was to identify factors that affect the activity of the exudative AMD lesion and predict anatomic and functional treatment responses during anti-VEGF therapy. We analyzed the effects of CNV lesion characteristics determined with optical coherence tomography (OCT) scanning and angiographies, the effects of potential genetic predictors, variants of *interleukin-8* (IL-8), *VEGF*, and *erythropoietin* (EPO) genes, the effects of well-known risk factors for AMD: tobacco smoking, genetic risk variants of *complement factor H* (CFH), *ARMS2/LOC387715*, and *complement component 3* (C3) genes, and the variations in anterior chamber protein concentration.

We studied the initial treatment response after the first injections of anti-VEGF agent bevacizumab retrospectively in a material of 96 exudative AMD patients. Persisting macular fluid intra- or subretinally in OCTs was associated with a variant of IL-8 gene and with the type of CNV lesion. Larger size of CNV lesion at baseline and presence of cystic macular fluid after the initial treatment were associated with less visual acuity (VA) gain.

We analyzed the effects of the above-mentioned factors also on long-term treatment response in a prospective two-year follow-up. Fifty patients with exudative AMD were treated with bevacizumab at once-a-month visits if sub- or intraretinal fluid or a new hemorrhage was present in the lesion area. The genetic variant of *ARMS2/LOC387715* was associated with better VA outcomes. The number of needed retreatment injections was associated with smoking, baseline CNV lesion size in indocyanine green angiography (ICGA), and the risk variants of CFH and VEGF genes. Only the genetic factors retained their significance in multivariate analyses. The variant of IL-8 gene was significantly associated with the tendency of the lesion to have persisting intra- or subretinal fluid in OCTs at every follow-up visit. The risk variants of the IL-
VEGF and CFH genes had a marked cumulative effect on anatomic response.

In the two-year follow-up, we also measured protein concentration in the anterior chamber, flare, with laser flare photometry weekly during the first month and then monthly in both study and fellow eyes. We evaluated the value of flare in predicting disease activity and the associations between flare and changes in morphology of the exudative lesion. Flare values correlated with patients’ age and smoking behavior, the presence of cystic macular fluid in OCTs, and pseudophakia. Over the two years, flare values gradually rose in all eyes, but relatively less in the eyes with the classic type of CNV. The values also decreased during treatment-free periods. The values otherwise correlated poorly with CNV activity. However, the fellow eyes that developed exudative AMD later were found to have higher flare values at the beginning of follow-up compared with the eyes that remained free of exudative AMD features.

In the analyses of materials used in the short- and long-term follow-up studies, the genetic variant of IL-8 seemed to have an association with earlier onset of the exudative disease. We analyzed the association retrospectively in a larger patient material. Altogether 301 patients with exudative AMD, 72 patients with dry AMD, and 119 control subjects were included in the analysis. The variant of IL-8 gene was not associated with the prevalence of dry or exudative AMD, but an association with the earlier onset of exudative disease appeared to exist.

The genetic variant of IL-8 was the only genetic factor included in the analyses that had an association with the initial treatment response. It also had an effect on the anatomic outcome of the long-term treatment together with the variants of CFH and VEGF genes. CNV lesion characteristics affected both the functional and the anatomic outcome of the initial anti-VEGF treatment, but were less important in predicting long-term response. Anterior chamber protein concentration seemed to reflect some changes in the CNV lesion during the treatment, but cannot be used as a predictor of lesion activity or treatment response. The association found between the genetic variant of IL-8 and age of onset of exudative AMD increases the evidence that IL-8 may have an important role in the process leading to the growth of CNV in exudative AMD.
1 INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of visual impairment in developed countries. Early and intermediate forms of the disease are characterized by small aggregations of sub-pigment epithelial deposits, drusen, and retinal pigmentary changes. These forms may progress to late AMD, geographic atrophy, or exudative ("wet") AMD, the advanced forms of AMD accounting for the majority of visual impairment and blindness caused by AMD. The choroidal neovascular membrane forming the exudative AMD lesion can be targeted with both laser treatments and drug therapy, whereas no evidence-based medical treatments exist yet for the early forms of the disease or for geographic atrophy.

Direct thermal laser treatment was the first therapy preventing profound visual impairment caused by exudative AMD. It could, however, only be used for certain types of choroidal neovascularizations (CNVs) and led to the formation of an absolute scotoma corresponding to the treated area. Photodynamic therapy (PDT), a specific laser used with the intravenously administered sensitizing agent verteporfin, was approved for exudative AMD treatment in 2000. It was a major step forward in the treatment of central CNVs, not causing absolute scotoma formation. However, only a small proportion of the CNV lesions were eligible for this treatment, and both direct laser and PDT could typically only slow down the loss of visual acuity. Enormous numbers of patients were still losing vision due to exudative AMD.

The management of exudative AMD has radically changed after the discovery of the crucial role of VEGF in ocular neovessel formation and the first trials using intravitreally injected bevacizumab, an anti-VEGF agent developed and manufactured for intravenous cancer therapy. Anti-VEGF injections form the first treatment regimen with the potential to improve visual acuity that has already deteriorated due to exudative AMD. The main anti-VEGF agents used at the moment as intravitreal injections are ranibizumab (Lucentis®), aflibercept (Eylea®), and off-label bevacizumab (Avastin®). Individual variations exist in the response to these medications. The reasons behind these variations are not yet fully understood.

The analysis of the factors affecting the treatment outcome provides new information about the mechanisms underlying the pathological changes in anatomy and function caused by exudative AMD. It also may eventually lead to the development of new and better treatments for exudative AMD.
2 REVIEW OF THE LITERATURE

2.1 ANATOMY AND FUNCTION OF THE OUTER RETINA

Retinal pigment epithelium (RPE) is a monolayer of cells separated on its outer side from the choroidal vasculature by a basement membrane complex called Bruch’s membrane. On the inner side, it is in close contact with the outer segments of the photoreceptors. RPE cells are joined to each other with tight junctions, limiting the passage of fluid and solutes from the choroid to the inner structures of the eye. The formed barrier is called the outer blood-retina barrier (BRB). The tight junctions between the endothelial cells of retinal microvasculature form the inner BRB. The inner, apical sides of RPE cells have long microvilli that envelop the photoreceptor outer segments. Membranous discs containing the light-sensitive proteins are synthesized in the base of the photoreceptor outer segments. As the discs mature, they migrate distally, and the old discs are continually shed from the distal end of the outer segments to be phagocytosed by the RPE cells. Within the RPE, the phagosomes containing the shed discs merge with lysosomes, and the disc material is digested. Necessary fatty acids are recycled back to the photoreceptors, and waste material is extruded through the basal side of the RPE and Bruch’s membrane to be removed by the choroidal vasculature. The RPE cells contain also both antioxidant enzymes, diminishing the damaging effects of free radicals on the cell membranes, and growth factors, controlling function and repair of the surrounding tissues.9, 10

2.2 CLASSIFICATION OF AGE-RELATED MACULAR DEGENERATION, AMD

Early age-related changes in the macular area are characterized by small round yellowish sub-RPE aggregations of cellular and inflammatory debris, drusen, and alterations in the RPE cells, leading to hypo- and/or hyperpigmented areas. Late AMD comprises two major entities: geographic atrophy and exudative AMD. Geographic atrophy forms when a gradually growing area of RPE atrophy leads to the atrophy of the corresponding photoreceptor cells and choroidal vasculature. It is usually seen as an area of depigmented RPE with sharp margins. Exudative AMD develops when CNV membrane grows underneath the RPE or between the retinal layers.

Numerous classification systems for AMD have been developed based on the assessment of standardized stereoscopic 30-degree color fundus photographs. The main aim of the complex grading systems has been to standardize the nomenclature and categorization of the morphological changes for
epidemiological studies (the Wisconsin Age-Related Maculopathy Grading System,\textsuperscript{11} an International Classification and Grading System for Age-related Maculopathy and Age-related Macular Degeneration,\textsuperscript{12} and the Age-Related Eye Disease Study (AREDS), mainly based on the Wisconsin system\textsuperscript{13}). Simplified classifications for clinical purposes have been devised based on these complex systems: the Simplified Severity Scale for Age-Related Macular Degeneration based on AREDS,\textsuperscript{14} the Clinical Age-Related Maculopathy Staging system (CARMS),\textsuperscript{15} and the clinical classification system for age-related macular degeneration.\textsuperscript{16} The assessment of size, texture, and location of the drusen, the size and location of the pigmented abnormalities, and the total area covered by these changes in the complex systems have been replaced by the assessment of mere number and size of the drusen and the presence or absence of pigmentary changes in the simplified systems.

In the clinical classification system developed by the Macular Research Classification Committee in 2013, the area within two disc diameters of the fovea was assessed for the number and size of the drusen and the presence of pigmentary abnormalities related to AMD.\textsuperscript{16} Since minor changes are an extremely common clinical finding (at least one druse found in the macular area in up to 98.8\% of the American population over 49 years of age in the Beaver Dam Eye Study\textsuperscript{17}), the presence of only small drusen (diameter ≤63 μm) and/or pigmentary abnormalities were regarded as normal aging changes. Early AMD was defined as the presence of any medium-sized drusen (63–≤125 μm), but no pigmentary abnormalities. Intermediate AMD was defined as the presence of at least one large druse (>125 μm), or pigmentary abnormalities associated with at least one medium-sized druse. Late AMD was defined as the presence of geographic atrophy or lesions associated with neovascular AMD.\textsuperscript{16}

Indicators of exudative AMD have been determined as the presence of a serous or hemorrhagic pigment epithelial detachment (PED), serous or hemorrhagic sensory retinal detachment, visible subretinal or sub-RPE neovascular membrane, subretinal fibrous scar, or prior laser treatment for CNV.\textsuperscript{11-13, 18, 19}

Two major discrepancies exist between clinical practice and the criteria used in the classifications for the presence of exudative AMD. First, very often serous PED without any accompanying neuroepithelial detachment (NED) or retinal edema has no evidence of CNV in fluorescein angiography (FA) and is considered clinically neither to be of neovascular origin nor an indication for treatment. Second, the classifications are based on the analysis of photographs that may not show the very early signs of exudative AMD already clearly visible in optical coherence tomography (OCT) scans, nowadays forming the basis of diagnostics in macular diseases. Thus, estimated prevalences and incidences may be inaccurate measures of the numbers of patients needing treatment.
2.3 EPIDEMIOLOGY OF EXUDATIVE AMD

There are three major epidemiological studies on the prevalence, incidence, and risk factors for AMD in the populations with European ancestors: the Beaver Dam Eye Study (Wisconsin, USA), the Rotterdam Study (Netherlands), and the Blue Mountains Eye Study (Australia).

The Beaver Dam Eye Study and the Blue Mountains Eye Study used very similar study designs and classifications for fundus findings. In these studies, the prevalence of early or intermediate AMD in individuals aged under 55 years was quite low: large drusen were found in 2% and retinal pigment abnormalities in 7.3-7.5%. In individuals aged 75-86 years, findings were much more common: large drusen were present in 20-24% and pigment abnormalities in 20-26%.\(^{17,18}\) The Rotterdam Study included no individuals aged under 55 years and used a different classification system for the fundus findings. In individuals in the age group \(\geq 75\) years, the prevalence of large drusen was 16% and prevalence of pigment abnormalities 12%. Lower prevalences may reflect real differences in the genetic and risk factor profiles of the studied populations.\(^{19}\) Also the prevalence of exudative AMD was smaller in the Rotterdam Study than in the other two studies (Table 1). The steep increase of late AMD after the age of 75 years is, however, clear in all three studies. Table 2 shows the incidence rates of exudative AMD.

| Table 1. | Prevalence of exudative AMD (eAMD) and any late AMD (combined eAMD and geographic atrophy) in the Beaver Dam Eye Study, the Rotterdam Study, the Blue Mountains Eye Study, and the combined data.\(^{20}\) |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | Age group (years), % (n) | < 55 | 55-64 | 65-74 | 75-84 | \(\geq 85\) | All |
| Beaver Dam | eAMD | 0 | 0.3 (4) | 1.1 (13) | 4.6 (31) | 8.1 (3) | 1.1 (51) |
| n=4756 | any late AMD | 0 | 0.4 (5) | 1.4 (17) | 6.7 (45) | 13.5 (5) | 1.5 (72) |
| Blue Mountains | eAMD | 0 | 0.2 (2) | 0.5 (6) | 3.8 (24) | 14.1 (17) | 1.4 (49) |
| n=3585 | any late AMD | 0 | 0.2 (2) | 0.7 (8) | 5.4 (34) | 17.4 (21) | 1.8 (65) |
| Rotterdam | eAMD | - | 0.1 (2) | 0.3 (8) | 1.9 (26) | 7.2 (26) | 1.0 (62) |
| n=6411 | any late AMD | - | 0.1 (3) | 0.7 (16) | 3.2 (43) | 11.6 (42) | 1.6 (104) |
| Combined | eAMD | 0 | 0.2 (8) | 0.6 (27) | 3.1 (81) | 8.8 (46) | 1.1 (162) |
| n=14 753 | any late AMD | 0 | 0.2 (10) | 0.9 (41) | 4.6 (122) | 13.1 (68) | 1.6 (241) |

n, number of subjects with gradable photographs in each group
In the Finnish population, a study of 500 persons aged 70-95 years reported a similar prevalence of late AMD; exudative AMD was found in 3.8% and any late AMD in 8.2%.27

A meta-analysis of 25 epidemiological studies on prevalence of AMD in populations of European ancestry summarized the data obtained from a total of 57,173 subjects. Estimated prevalence of late AMD was 1.4% (95% CI, 1.0-2.0%) at the age of 70 years, rising to 5.6% (95% CI, 3.9-7.7%) at the age of 80, and to 20% (95% CI, 14-27%) at the age of 90. The increase in prevalence with age was similar in both sexes. However, the diagnosis of late AMD, mainly of exudative AMD, was slightly more common in women (OR, 1.13; 95% CI, 1.01-1.28).28 Based on a meta-analysis of global data, the prevalence of AMD seems to be higher in populations with European ancestors than in Asian and African populations. The largest difference in prevalence of late AMD was found between populations of European and African origin; in the age group of 80-84 years, prevalence of late AMD was 0.9% in subjects with African and 4.6% in subjects with European ancestors.29 In the future, the prevalence of AMD is expected to increase because of marked aging of the world’s population.

### 2.4 PATHOGENESIS OF EXUDATIVE AMD

Aging of RPE cells leads to the accumulation of intracellular material containing lipofuscin. Lipofuscin originates mainly from the undegradable products of photoreceptor outer segment metabolism, and interferes with normal RPE functions. Its major molecular component is N-retinylidene-N-retinylethanolamine (A2E), a byproduct of vitamin A recycling.30 A2E has been shown to increase permeability of plasma membranes. It is also
phototoxic, forming reactive oxygen species when illuminated with high-energy light.\textsuperscript{31} A2E has been demonstrated to both alter cholesterol metabolism, resulting in accumulation of cholesterol and cholesterol esters in RPE cells,\textsuperscript{32} and cause activation of the complement after photooxidation.\textsuperscript{33}

RPE dysfunction and changes in the permeability of Bruch’s membrane lead to the formation of drusen, and in some cases to the accumulation of drusenoid material between the neurosensory retina and RPE (reticular pseudodrusen or subretinal drusenoid deposits).\textsuperscript{34} Drusen material contains cholesterol, amyloid-β, apolipoproteins B and E, oxidative protein modifications, and a wide variety of proteins associated with inflammation and immune-mediated processes, including complement components (C3, C5 and C5b-9 complex), C-reactive protein (CRP), clusterin and vitronectin proteins, and also debris from RPE cells, macrophages, and choroidal dendritic cells. Chronic inflammation is thought to cause or increase the formation of drusen, and the drusen material may also further induce local immune reactions.\textsuperscript{35-38}

Exudative AMD develops when CNV membrane grows from the choroid through Bruch’s membrane into the space underneath the RPE, or between the RPE and the neurosensory retina. In a special form of exudative disease, retinal angiomatous proliferation (RAP), neovascularization is thought to arise from the retinal vessels and only later to form a choroidal CNV membrane.

The exact pathogenic mechanism that leads from the changes of early AMD to the growth of CNV are not yet fully known. However, connections linking lipofuscin and drusen material to inflammatory reactions exist, and the activated inflammatory signaling pathways also lead to neoangiogenesis.

\textbf{2.4.1 VASCULAR ENDOTHELIAL GROWTH FACTOR, VEGF}

In humans, the family of VEGFs is by now known to comprise five secreted glycoproteins with a highly conserved receptor binding structure: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. VEGF-A, referred to as VEGF in this text, has a major role in the regulation of angiogenesis and vascular permeability in humans. It is a proangiogenic factor that increases the permeability of blood vessels, impairs the integrity of BRB, and induces choroidal neovascularization. VEGF-B regulates angiogenesis and growth of myocytes in the heart and the metabolism and survival of vascular endothelial cells. It also seems to have neuroprotective effects and a role in neuronal growth and repair after injuries.\textsuperscript{39} VEGF-C and -D are factors regulating the formation of lymphatic vessels. Placental growth factor is expressed not only in the placenta but also at lower levels in many tissues, including the retina. It is produced by the RPE cells and has a regulatory role during the development of retinal vasculature,\textsuperscript{40} and may also participate in pathological angiogenesis.
In experimental CNV models in mice, inhibition of placental growth factor has been shown to inhibit the growth of laser-induced CNV.\textsuperscript{41, 42}

The human \textit{VEGF} gene has eight protein coding segments, exons. Alternative splicing of the exons leads to the formation of various isoforms with differing lengths measured in amino acids. The two major isoforms in humans are VEGF\textsubscript{121} and VEGF\textsubscript{165}, secreted as homodimeric proteins.\textsuperscript{43} In the eye, VEGF is produced by pericytes, endothelial cells, Müller cells, astrocytes, and RPE cells. A main regulator of VEGF expression is oxygen tension. The upregulation of transcription of the \textit{VEGF} gene in hypoxic conditions is mediated by the hypoxia-inducible transcription factor 1, HIF-1.\textsuperscript{44, 45} Induction of expression may also occur in non-hypoxic conditions in reaction to e.g. reactive oxygen species, various cytokines and growth factors, such as transforming growth factor beta (TGF-\(\beta\)), tumor necrosis factor alpha (TNF-\(\alpha\)), basic fibroblast growth factor, and interleukin (IL) -1, IL-1\(\beta\), and IL-8. Enhanced production of VEGF is regularly seen during wound healing and tissue repair, and also in RPE as a consequence of local inflammatory response.\textsuperscript{43, 46-48} Various constituents of drusen, e.g. complement components C3a and C5a, directly induce VEGF production in RPE cells, and others, e.g. amyloid-\(\beta\), may do so indirectly by increasing local inflammatory response.\textsuperscript{49-51}

Single nucleotide polymorphisms (SNPs) are common variations in the human genome, resulting in minor alterations in the structure and function of the coded proteins. If situated in or near the regions regulating the transcriptional activity of the gene, they may affect the volume of synthesis of the end-product. A common polymorphism of the \textit{VEGF} gene, rs699947 (-2578A/C), is situated in the region initiating the transcription process of the gene (promoter). The SNP has been connected to the regulation of transcriptional activity, and the C allele has been associated with higher VEGF production.\textsuperscript{52} Although it would be intriguing to speculate that individuals with genetically higher levels of basal VEGF production are more prone to develop neovascular AMD, no data exist to support this hypothesis.

\textbf{2.4.2 INTERLEUKIN-8, IL-8}

IL-8 (CXCL8) is a cytokine with powerful inflammatory and proangiogenic properties. It also acts as a chemoattractant to neutrophils and other granulocytes.\textsuperscript{53, 54} Various cell types secrete IL-8. In addition to cells of the immune system, also vascular endothelial cells and RPE cells produce IL-8 in response to inflammatory stimuli and other factors associated with AMD development and progression, e.g. CRP, TNF-\(\alpha\), and interleukin-1 (IL-1), oxidative stress, saturated fatty acids, preoxidized photoreceptor outer segments, and amyloid-\(\beta\).\textsuperscript{55-58, 59-63} IL-8 increases endothelial permeability and induces angiogenesis through both VEGF-dependent and -independent
pathways. IL-8 receptors CXCR1 and CXCR2 are expressed on endothelial cells. The activation of the CXCR2-receptor elevates the intracellular levels of VEGF and increases VEGF secretion. The activated CXCR2 has also been shown to be capable of forming a complex with the VEGF receptor, VEGFR-2, and to cause transactivation of the receptor without the presence of VEGF. Chronic oxidative stress leads to the production of higher levels of IL-8 in the RPE cells, stimulating both inflammation and angiogenesis. This, in turn, may have a role in the development of CNV. Intraocular IL-8 concentrations have been reported to be elevated in patients with exudative AMD, levels correlating with the size of an active CNV and with the macular volume after bevacizumab treatment.

IL-8 SNP rs4073 (-251A/T) is a promoter region polymorphism associated with the transcriptional activity of the IL-8 gene. Higher levels of circulating and mucosal IL-8 have been observed in patients carrying the A allele or the AA homozygous genotype. The SNP has been connected to an increased risk of various inflammatory diseases and tumors. The A allele in rs4073 has been associated with a poorer outcome of bevacizumab therapy in special types of ovarian and colorectal cancers. However, research on the IL-8 SNP haplotypes has shown that the SNP rs4073 might not be the real functional polymorphism responsible for the alterations in the transcriptional activity of the IL-8 gene, but is instead inherited in a strong linkage disequilibrium with another polymorphism, IL-8 rs22227306, which seems to be the main variant responsible for the interaction between the gene and the transcription factor binding complex. In a European population with Caucasian ancestors, 90% of those carrying an A allele in rs4073 also carried a T allele in rs22227306, and only 10% carried a C allele in that position. Only the haplotype carrying the T allele in IL-8 in rs22227306 was finally shown to be associated with the tendency of higher IL-8 expression. Marked ethnic differences in the linkage disequilibrium patterns may explain some contradictory reports; e.g. in a Chinese population, significantly increased promoter transcriptional activity has been associated with the T allele in IL-8 rs4073. The associations with the risk of various diseases may vary accordingly.

### 2.4.3 INTERLEUKIN-6, IL-6

IL-6 is a cytokine involved in both the acute and chronic phases of the inflammatory reaction, autoimmunity reactions, angiogenesis, and fibrogenesis. IL-6 has also been shown to induce VEGF expression in some cell lines. Higher levels of systemic IL-6 have been found to be associated with higher risk of AMD progression. Also, in a laser-induced CNV model in mice, both CNV growth and subretinal fibrosis development were significantly suppressed by blocking the IL-6 signaling.
2.4.4 BONE MORPHOGENETIC PROTEIN-4
It is quite clear the chronic inflammation and oxidative stress have the ability to induce signaling factors promoting neoangiogenesis. However, it is not in any way clear why some individuals develop CNV while others end up with geographic atrophy. A regulator of cellular differentiation and apoptosis, called bone morphogenetic protein-4 (BMP-4), has been hypothesized to have a role in the process that determines the end result of AMD development. This hypothesis is supported by findings showing that BMP-4 is highly expressed in RPE in eyes with geographic atrophy, while the expression is low in eyes with CNV lesion.\(^6^7, 8^7\) Possibly, a local proinflammatory microenvironment with high levels of TNF-α may suppress the expression of BMP-4 and lead to the formation of neovascularization.\(^8^8\) However, a causal relationship has not been established between BMP-4 levels and the final form of late AMD.

2.5 RISK FACTORS FOR EXUDATIVE AMD
In addition to the aforementioned large population-based studies, valuable information about the risk factors for AMD was provided by the Age-Related Eye Disease Study (AREDS), a randomized, double-masked, prospective trial of 4757 mostly Caucasian (96%) patients from the United States. The AREDS was designed to assess the natural course of AMD and the potential effects of various nutritional supplements on disease progression. In all of these studies, the main predictor of the risk of progression to late AMD was found to be the severity of AMD changes at the baseline ophthalmic investigation. Eyes with large drusen and pigmentary abnormalities were much more likely to develop late AMD than eyes without these changes. The risk was further modified by the patient’s age and race, smoking habits, and genetic and dietary factors.

2.5.1 AMD SEVERITY AT BASELINE
A simplified grading system based on the AREDS classification was developed to assess the individual risk of developing late AMD within the next 5 years. In this system, both eyes are assessed separately, and one risk factor is assigned for the presence of at least one large (>125 μm) druse and one factor for the presence of pigmentary abnormalities. One risk factor is assigned if intermediate-sized drusen exist in both eyes, but no large drusen are found. The risk factors are summed for the eyes, resulting in a score from 0 to 4. The estimate of a 5-year risk of late AMD varies according to the score: 0.4% for 0; 3% for 1; 12% for 2; 26% for 3; and 47% for 4.\(^1^4\) Due to differences in the classification systems used and categorizations of the data, comparisons between the AREDS and the other studies are somewhat difficult. However, the risks appear to be lower in the other population-based studies than in the AREDS. Using the same simplified grading system in the Australian Blue Mountains population, the risk levels were 0.1% for score 0; 2% for 1; 10% for
2; 10% for 3; and 35% for 4. In the European Rotterdam Eye Study population, the risk of late AMD in patients with intermediate drusen was 0.9% (2% in AREDS), and the 5-year risk of late AMD in patients with extensive intermediate or large drusen was 9.7% (27% in AREDS). The risk estimates appear to be the smallest in the American Beaver Dam Study population: in patients with large drusen, the 5-year progression rate to late AMD was 6%, compared with 14% in the Blue Mountains study and 17% in the AREDS.13, 21, 23, 24

Special characteristics, however, change the risk estimate considerably. The presence of noncentral geographic atrophy in one or both eyes at baseline increases the 5-year risk of late AMD, mainly of geographic atrophy involving the center of the macula, to about 70%. Similarly, the 5-year risk of second eye involvement in patients with late AMD in the first eye was high in all of the studies: 20-30% in the Blue Mountains population, 39% in the Rotterdam Study, and 55% in the AREDS.13, 21, 23, 24

Some estimates for the risk of developing exudative AMD come from the studies on the treatment of exudative AMD. In the Macular Photocoagulation Study, the overall rate of CNV development in the fellow eyes was 26% in 5 years. The risk factors for conversion were identified as the presence of at least five drusen, focal hyperpigmentation, any large drusen, and systemic hypertension. The 5-year risk estimates ranged from 7% in those without any of the risk factors to 87% in those with all four factors present at baseline examination. Corresponding rates in the AREDS ranged from about 10% to 45-55%. In the Submacular Surgery Trials, the 4-year cumulative incidence of CNV in the fellow eye was also quite similar, 37%.91

The presence of subretinal drusenoid deposits seems to be an independent risk factor for AMD progression, although not as significant as the presence of large drusen. Subretinal drusenoid deposits can be differentiated from the regular sub-RPE drusen with OCTs or by examining the blue channel of the color fundus photographs.34, 93

2.5.2 TOBACCO SMOKING
Tobacco smoking is a major risk factor for all AMD forms. The risk seems to correlate with the number of smoking pack-years [pack-year = (cigarettes per day) x (years of smoking) / (20 cigarettes per pack)]. The effect is reversible since ex-smokers have a significantly lower risk of developing AMD than current smokers, albeit they seem to reach the risk level of nonsmokers only several decades after quitting.95, 96

Tobacco smoke causes oxidative and inflammatory stress in microvascular environments and depletes antioxidants. The effect of tobacco smoke
chemicals on the impairment of the RPE function has been clearly shown in cellular and animal models.\textsuperscript{98-100} Hydroquinone, a benzene derivative component of the cigarette tar, has been shown to shift the balance between the growth factors in RPE towards the proangiogenic side.\textsuperscript{100} Also, nicotine has been demonstrated to both induce the proliferation of endothelial cells in vitro and promote the growth of experimental CNV in an animal model.\textsuperscript{101,102}

Pooled data from the Beaver Dam Eye Study, the Blue Mountains Eye Study, and the Rotterdam Study showed that the odds ratio (OR) for neovascular AMD was 4.6 for current smokers [95\% confidence interval (CI), 2.74-7.54], and 1.5 for ex-smokers (95\% CI, 0.97-2.4) compared with nonsmokers.\textsuperscript{20} In two large American follow-up studies, the Nurses' Health Study (31 843 women, 12-year follow-up) and the Physicians' Health Study (21 157 men, 7-year follow-up), about 2.4-fold increase in the incidence of AMD was seen in current smokers smoking at least 20 (men) or 25 (women) cigarettes per day compared with nonsmokers [relative risk (RR), 2.4; 95\% CI, 1.4-4.0 in women; RR, 2.5; 95\% CI, 1.6-3.8 in men]. In ex-smokers, the risk was 2-fold (RR, 2.0; 95\% CI, 1.2-3.4) that of nonsmokers in women (a trend shown in men during the shorter follow-up).\textsuperscript{103,104} Passive smoke exposure has also been found to increase the risk of AMD.\textsuperscript{95} The effect of smoking might not be restricted to the development of AMD; in the Macular Photocoagulation Study, a higher recurrence rate of neovascularization after laser treatment was found among smokers (85\%) than among nonsmokers (50\%).\textsuperscript{105}

The classification systems for smoking status used in the large studies are not identical. In some studies, the descriptions of methods suggest that any smoking ever was classified as smoking whereas usually smokers were determined as having smoked \( \geq 100 \) cigarettes in their lifetime or \( \geq 1 \) cigarette per day for a year. Also, the time required to pass between quitting and the time of the survey for being classified as an ex-smoker varies. Ex-smokers could be determined as those who just reported they were no longer smoking at the time of the interview or a defined period without smoking could be required, often \( \geq 1 \) year. To require some nonsmoking time to pass after quitting is justifiable, at least if the length of the nonsmoking period cannot be treated as a continuous variable in the analyses. In a mathematical model based on data from previous large studies among ex-smokers, the RR for developing exudative AMD had dropped only to approximately 0.8 in 7 years compared with current smokers (RR 1.0).\textsuperscript{96}

2.5.3 NUTRITION

Studies on nutritional factors have invariably found diets rich in lutein, zeaxanthin, zinc, and omega-3 fatty acids to decrease the risk of AMD. The results on the effects of \( \beta \)-carotene and vitamin E have been more controversial.
Lutein and zeaxanthin, dietary xanthophylls, are the major carotenoids comprising the macular pigment. They have antioxidant properties, filter short wavelength light, and increase membrane stability.\textsuperscript{106, 107} The inverse association between dietary lutein and zeaxanthin intake and both intermediate and late stage AMD is supported by the findings of many large studies.\textsuperscript{108-110} Lutein and zeaxanthin seem to be the carotenoids most strongly associated with reduced risk of AMD, in contrast to \(\beta\)-carotene, which seems to have a weaker, or even a harmful, effect on the risk of developing exudative AMD.\textsuperscript{108, 110}

Zinc acts as a cofactor for many enzymes, e.g. the antioxidant enzymes superoxide dismutase and catalase, and also in vitamin A metabolism.\textsuperscript{111} Lower risk of incident AMD was associated with zinc intake in the Blue Mountain Eye Study and with zinc intake as well as with intakes of vitamin E and the combination of vitamins C and E and \(\beta\)-carotene in the Rotterdam Study\textsuperscript{110, 112}

Omega-3 long-chain polyunsaturated fatty acids found in high concentrations in fish, vegetable oils, and nuts have a variety of anti-inflammatory, anti-angiogenic, anti-vasoproliferative, and neuroprotective effects that seem to prevent AMD.\textsuperscript{113} Both a diet rich in omega-3 fatty acids and fish intake at least twice a week have been shown to lower the risk of early and late AMD by approximately 30-50\%.\textsuperscript{114-119}

There are also some studies suggesting that a higher dietary glycemic index (estimate of the rise in blood glucose level after eating), higher total dietary fat intake, and high intake of omega-6 fatty acids are associated with higher risk of late AMD.\textsuperscript{115, 118, 120}

The AREDS studies, AREDS\textsubscript{1} and AREDS\textsubscript{2}, were designed to analyze the effects of antioxidant and mineral supplements on the risk of AMD progression. In AREDS\textsubscript{1}, participants were randomly assigned to one of four treatment groups receiving daily oral tablets of either: 1) zinc alone; 2) antioxidants alone; 3) a combination of antioxidants and zinc; or 4) a placebo. The original AREDS formulation contained 500 mg of vitamin C, 400 international units (IU) of vitamin E, 15 mg of \(\beta\)-carotene, 80 mg of zinc as zinc oxide, and 2 mg of copper as cupric oxide.\textsuperscript{121} In AREDS\textsubscript{2}, modification of the original supplement formulation was done, and four treatment groups were compared: 1) AREDS\textsubscript{1} formulation, 2) AREDS\textsubscript{1} + 10 mg of lutein and 2 mg of zeaxanthin, 3) AREDS\textsubscript{1} + 1000 mg of omega-3 fatty acids (350 mg of docosahexaenoic acid and 650 mg of eicosapentaenoic acid), and 4) AREDS\textsubscript{1} + 10 mg of lutein and 2 mg of zeaxanthin + 1000 mg of omega-3 fatty acids. Also, the effects of eliminating \(\beta\)-carotene and lowering the dose of zinc were analyzed. During the 5-year follow-up in AREDS\textsubscript{1} participants with early AMD (extensive small drusen, nonextensive intermediate-sized drusen, or pigment
abnormalities) had a very low rate of progression to late AMD, and no conclusions could be drawn about whether the supplementation had any effect on disease progression in patients with early AMD. The estimated probabilities of developing late AMD during the 5-year follow-up without the supplementation were 43% for participants with late AMD in the first eye, 27% for those with large drusen in both eyes or with paracentral GA in at least one eye, and about 6% for the other participants with intermediate AMD. The estimated risk reduction was 17% for those taking antioxidants alone, 21% for those taking zinc alone, and 25% for those taking antioxidants plus zinc. Thus, the supplementation used in the original formulation may benefit patients with late AMD in the first eye, large drusen in both eyes, or paracentral GA in at least one eye. The AREDS2 trial showed that adding omega-3 or lutein/zeaxanthin to the original formulation did not change its effect on the risk of late AMD. However, participants who took a formulation containing lutein/zeaxanthin without β-carotene had a slightly lower risk of late AMD than those taking the formulation with β-carotene. The effect was stronger in a subgroup of participants with low levels of lutein/zeaxanthin in their diet. Eliminating β-carotene or lowering the dose of zinc in the original formulation did not impact the effectiveness of the therapy.

The beneficial effect of a healthy diet rich in antioxidants and omega-3 fatty acids seems to be quite evident. On the other hand, using large doses of nondietary supplemental vitamins and antioxidants may have serious side-effects. In smokers, β-carotene has been shown to increase the risk of lung cancer. Evidence also exists that vitamin E supplementation comparable to that used in the AREDS1 formulation may cause an increased risk of prostate cancer. Based on the analyses of randomized antioxidant supplement trials in the Cochrane Database of Systematic Reviews it seems that treatments with supplements containing β-carotene, vitamin A, and vitamin E may, actually, increase mortality.

2.5.4 GENETIC FACTORS
It has been estimated that genetic factors explain about 45-70% of the variation in the overall severity of AMD. In 2005, a strong association was discovered between AMD and a single nucleotide polymorphism of the CFH gene, coding a regulatory factor of the alternative complement cascade. Many genetic association studies have since confirmed the association and also reported a higher risk of AMD to be associated with other SNPs affecting various factors involved in the activation and regulation of the alternative complement system, emphasizing the role of abnormal complement function and deficient control of inflammatory reactions in the disease process.

The 20 common genetic variants known to be associated with an increased risk of AMD (minor allele frequency ≥5%) are estimated to explain about 40-60%
of the total genetic contribution to AMD.\textsuperscript{138, 139} The products coded by the affected genes have variable functions in controlling and amplifying inflammatory responses, oxidative stress, and angiogenesis, the three main factors contributing to the progression of AMD.

2.5.4.1 Complement

The complement system is part of the innate, nonspecific immune system that provides an immediate defense against infections. Complement consists of a number of small proteins synthesized by the liver, tissue macrophages, blood monocytes, and epithelial cells, including RPE cells.\textsuperscript{140} When the system is activated, the inactive precursors of complement components in the blood circulation are cleaved by proteases to become active components, releasing cytokines to recruit inflammatory cells, and stimulating further cleavage and activation of inactive precursors. The end-result of the amplified cascade is the activation of cell-killing membrane attack complex, a transmembrane channel causing lysis of target cells.\textsuperscript{141}

There are three separate complement activation pathways, resulting in the generation of complement component 3 (C3) -convertase, which cleaves and activates the component C3 to C3a and C3b. The classical complement pathway typically requires antigen-antibody complexes for activation. The lectin pathway recognizes mannose-binding lectin or ficolin binding to certain sugars found on the surfaces of pathogens. In contrast to these other pathways, the alternative pathway is continuously activated at a low level due to the instability of C3 in aqueous environments, resulting in production of a C3b-like form of C3. C3b coats the surfaces of pathogens, enhancing their phagocytosis. Also, a surface-bound C3b is capable of binding other complement components to form a C3 convertase enzyme complex, which then cleaves more C3 to the active C3b form. C3a, on the other hand, is known to cause degranulation of mast cells, increase vascular permeability, and enhance IL-8 production.\textsuperscript{141, 142}

In the fluid phase, both the C3b that is generated from C3 by the C3 convertase and the C3b-like form of C3 produced by the spontaneous cleavage of the protein are rapidly inactivated by regulatory component factors I and H (CFH). The C3b binds, however, to both the surfaces of host cells and pathogens. Other regulatory proteins found on the host cell surfaces control the complement activation and help the alternative complement pathway to distinguish self from non-self.\textsuperscript{141}

Significantly increased risk of AMD has been associated with a polymorphism in \textit{CFH} (rs1061170 T→C, chromosome 1q32) that causes a tyrosine to histidine change (Y402H) in the CFH protein.\textsuperscript{130-132} In the Finnish population, OR for AMD was estimated to be 2.9 for the heterozygous (TC) and 9.3 for the
homozygous risk genotype (CC) compared with the homozygous non-risk genotype (TT) of CFH rs1061170.143 The risk variant of CFH has a reduced ability to bind to CRP and heparin.144,145 It also appears to have a weaker ability to bind to RPE and endothelial cells.145 The impaired regulatory function of CFH leads to uncontrolled complement activity, inflammatory damage of RPE cells, and accumulation of drusen material.149

A polymorphism in the C3 gene (rs2230199 C→G, R102G) results in substitution of a glycine for the original arginine, affecting the binding of the C3 to CFH. The ORs for AMD have been estimated to be 1.6-1.7 for the heterozygous (CG) and 2.6-3.2 for the homozygous risk genotype (GG) compared with the homozygous non-risk genotype (CC).135,136

Other polymorphisms of complement components modulating the risk of AMD include variants of Factor B, Complement Component 2, Complement Component 9, and Complement Factor I genes as well as a variant or a noncoding section of CFH.133,134,137,146

2.5.4.2 ARMS2 and Htra1

After the 1q32 coding for CFH, the second genetic region with a highly significant and consistent association with increased risk of AMD was the locus at 10q26. The region spans three neighboring genes: Pleckstrin Homology Domain-Containing Family A Member 1, Age-related Maculopathy Susceptibility 2 (ARMS2), also called LOC387715, and High-temperature Requirement Factor A1 (Htra1), also called PRSS11. A strong linkage disequilibrium exists across the whole region, and despite numerous attempts the gene having a causal association with AMD has not been definitively identified.

The pleckstrin homology domain-containing adapter protein is a plasma membrane protein binding phosphatidylinositol 3,4-bisphosphate and possibly forming signaling complexes in the plasma membrane. It may have a role in modifying local lymphocyte activation.147

ARMS2/LOC387715 encodes a protein of unknown function. The gene is expressed in the human retina,148 and the protein product has been located in the outer membrane of mitochondria,149 although others have reported its presence in the cytosol and secretion to the extracellular matrix.150,151 The SNP rs10490924 G→T showing a strong association with AMD maps to exon 1 of the gene and causes an alanine to serine change.134,147,148,152 In the Finnish population, the ORs for AMD were estimated to be 3.1 for the heterozygous (GT) and 12.1 for the homozygous risk genotype (TT) compared with the homozygous non-risk genotype (GG).143
*Htra1* encodes a heat-shock serine protease. The heat-shock proteins have molecular chaperone activity in bacteria. In mammals, the protein product seems to take part in the metabolism of type III collagen found e.g. in Bruch’s membrane and in the basement membranes of retinal vessels. The role of *Htra1* polymorphisms as genetic risk factors for AMD is controversial. The A allele in *IL-8* SNP rs4073 A→T was associated with an increased risk of AMD in Caucasian patients in a British study (OR, 1.2; 95% CI, 1.01-1.44). By contrast, in Chinese patients in Taiwan the T allele in *IL-8* rs2227306 C→T was associated with exudative AMD (OR, 2.2; 95% CI, 1.58-2.94), while no association was found with the *IL-8* rs4073 A→T. No data were provided, however, on the observed haplotype frequencies in that study. Similarly, in an Italian population the *IL-8* rs2227306 C→T was associated with an increased risk of exudative AMD (OR, 1.39; 95% CI, 1.19-1.62). In that study, four *IL-8* SNPs were analyzed: rs4073 A→T, rs2227306 C→T, rs2227346 C→T, and rs1126647 A→T. A complete linkage disequilibrium existed between the last three SNPs, and the fraction of samples inconsistent with a perfect linkage disequilibrium between all four SNPs was too small to analyze separately the effect of the rs4073 (5% of cases and 4% of controls). The higher risk of exudative AMD was associated with the haplotype ATTT for SNPs rs4073, rs2227306, rs2227346, and rs1126647.
2.6 EARLIER ONSET OF EXUDATIVE AMD

The earlier onset of exudative disease has been repeatedly associated with the established risk factors for AMD: tobacco smoking\textsuperscript{156, 161} and risk alleles in \textit{CFH} rs1061170\textsuperscript{156, 161-163} and \textit{ARMS2} rs10490924,\textsuperscript{156, 161-164}

Earlier onset of exudative AMD has also been reported in connection with a rare \textit{CFH} haplotype containing a missense mutation (c.3628C>T),\textsuperscript{165} with a homozygous risk genotype of VEGF receptor VEGFR-2 coding gene (\textit{KDR}, rs2071559),\textsuperscript{163} and with a genetic lack of glutathione S-transferase M1, an antioxidant enzyme in cellular detoxification.\textsuperscript{166} In addition to these, some suggestions have been made about the role of VEGF SNP rs699947,\textsuperscript{163} male sex,\textsuperscript{161, 163} alcohol consumption, and level of physical activity.\textsuperscript{161} Controversial data exist also on the influence of statin use.\textsuperscript{156, 167} Of these, only the missense mutation in \textit{CFH} has also been associated with a higher risk of developing AMD. The others may have some role in expediting the already initiated process of CNV growth, causing the disease to progress more rapidly and become symptomatic earlier.

In many of these studies, the association between the age of onset of disease and the studied genetic markers has been significant only with the homozygous risk genotypes, being less clear with the heterozygous genotypes.\textsuperscript{156, 162-164} This pattern differs from that seen in the studies analyzing the associations with the prevalence or incidence of AMD. The effect of smoking, however, seems to have more of a dose-response relationship also with the age of onset of exudative AMD.\textsuperscript{156, 161}

2.7 DIAGNOSIS OF EXUDATIVE AMD

Although patients with atrophic AMD may have symptoms of wavy outlines and blind spots in the central visual field when RPE elevations and cell death distort the retinal image, the progression of the changes is usually slow and symptoms less severe than those experienced with exudative lesions. In addition to the distortions of forms and sizes of visible objects, an exudative lesion causes a change in the perception of colors towards darker and less intense hues. Dark blind spots in the central visual field may appear especially if the fragile CNV vessels bleed. A decrease in visual acuity (VA) is inevitable without treatment. The disease is usually limited to the posterior pole of the eye and very rarely leads to total blindness, as the peripheral part of the visual field and the ability to see enough to move are preserved. The ability to read and recognize faces is usually affected the most. In clinical examination with biomicroscopy, retinal thickening, subretinal fluid, hard exudates, and hemorrhages are the most common findings, although grayish subretinal CNV membranes are also sometimes visible.
2.7.1 OPTICAL COHERENCE TOMOGRAPHY, OCT

OCT is the most important tool in the diagnosis of macular diseases in clinical practice. The technique is based on interferometry using near-infrared light, centered at 840 nm in the nowadays mostly used spectral domain or Fourier domain OCTs. The resolution of the cross-sectional images of the retinal layers is approximately 5 μm. The imaging technology is very efficient in discerning signs of CNV leakage: retinal thickening, intraretinal cysts, accumulating subretinal fluid (NED), presence of subretinal tissue, and PEDs (Figure 1). The normal retinal microvasculature cannot be visualized with the standard OCT technology. However, some clues to the type of CNV can be found also in OCTs. The classic CNVs (histological type 2 CNV, located between RPE and neuroretina) may appear as subretinal tissue, and occult CNVs (histological type 1, between Bruch’s membrane and RPE) as fibrovascular PEDs. In polypoidal choroidal vasculopathy (PCV), round or oval cavities underneath the RPE but over Bruch’s membrane can be seen in the areas corresponding to polypoidal lesions. These vessel cavities are often adherent to the outer surface of RPE in the area of PED. In RAP, the site of the anastomosis can be detected as an extension of tissue into a PED from the outer surface of RPE. Although OCTs may hint at the type of CNV in question, the classification of lesions is still based on angiographies.
2.7.2 ANGIOGRAPHY

Stereoscopic fluorescein angiography (FA) forms the gold standard for evaluation of retinal vasculature. The presence of leaking neovessels confirms the diagnosis of exudative AMD.

The interpretation of FA images of exudative lesions is mainly based on the criteria used in the Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) and Verteporfin in Photodynamic Therapy trials (VIP). According to these criteria, classic CNV is a bright area of quite well-demarcated and homogeneous fluorescence in the early phase of the angiography that is not related to RPE atrophy. Leakage further progresses during the later phases of angiography. Occult CNV has two differing patterns: fibrovascular PED and late leakage of an undetermined source. Fibrovascular PED is characterized as an area of RPE elevation with stippled or granular hyperfluorescence appearing within the first two minutes of the angiography, being less bright than the fluorescence of classic CNV. Staining and moderate leakage are present in the late phase. Late leakage of an undetermined source is not elevated and does not demonstrate hyperfluorescence in the early phase. Specled or punctate fluorescence with minimal leakage and poorly demarcated boundaries appears usually within 2 to 5 minutes.

CNV lesions can be further categorized according to their location in FA as extrafoveal, juxtafoveal or subfoveal lesions. Extrafoveal lesions remain at least 200 μm from the center of the fovea, juxtafoveal lesions are located 1 to 199 μm from the center of the fovea, and subfoveal lesions extend beneath the center of the foveal avascular zone.

In the original guidelines for the TAP and VIP trials, CNV lesions were classified as predominantly classic (at least 50% of the lesion area covered by classic CNV), minimally classic (classic CNV covering less than 50% of the lesion area), and occult with no classic lesions. According to this classification, the RAP type of lesions (also called type 3 CNVs) were usually defined as minimally classic CNVs, but have been treated as a separate entity since the discovery of their unique pathogenesis. Polypoidal choroidal vasculopathy (PCV) was not included in the subtypes of CNVs characterized in the guidelines either. Its diagnosis is primarily based on indocyanine green angiography (ICGA), whereas the original classification was based on FA. Still all lesions do not fit in the above-mentioned categories. If >50% of the lesion area consists of serous PED or hemorrhage, the classification used is “non-defined”.

ICGA serves as an important supplementary tool in exudative AMD diagnostics helping the visualization of the sub-RPE compartment. In contrast to FA, serous PEDs appear hypofluorescent in ICGA, and only CNVs stain, which helps to discern the nature of the PEDs in question. The classic CNVs are seen less clearly in ICGA than in FA, but occult CNVs are discerned well.
Occult lesions can be divided into subtypes according to their ICGA staining: hot spots (area of staining ≤1 optic disc diameter), plaques (>1 optic disc diameter) with either well- or ill-defined margins, or combinations of both.\textsuperscript{175} ICGA is irreplaceable in diagnosing PCV, which is defined as the presence of single or multiple hot spots of hyperfluorescence arising from the choroidal circulation within the first six minutes of ICGA. An associated branching vascular network of abnormal choroidal vessels located under the RPE may also be visible. The polypoidal dilatations may appear as a distinct lesion type or coexist with another CNV. PCV is much more common in Asian patients (in 24-55\% of all exudative AMD cases) than in Caucasian patients (in 4-10\% of cases).\textsuperscript{174} ICGA may also help in diagnosing the RAP type of lesions. Although the initial changes take place in retinal angiomaticus vessels, the retinochoroidal anastomosis appears as a hot spot in ICGA and fluorescence from the CNV membrane is clearly visible.

2.7.3 BLOOD-RETINA BARRIER DYSFUNCTION
Although measurement of the function of BRB is not currently used in the diagnostics of AMD, the technique may warrant further research.

In the exudative AMD lesion, CNV grows under the RPE or neurosensory retina. Both impaired integrity of the RPE and increased permeability of the walls of neovessels cause breakdown of the BRB. Changes in the integrity of BRB in diabetic retinopathy and branch and central vein occlusions as well as sub-Tenon triamcinolone and cryo treatments have been shown to be associated with alterations in the anterior chamber protein concentration (flare),\textsuperscript{176-180} which can be quantified with laser flare photometry.\textsuperscript{181-183} However, flare is not a direct measure of BRB function, and various other factors also affect the levels. Higher flare has been associated with older age.\textsuperscript{181, 183, 184} A true increase in protein concentration due to the breakdown of the blood aqueous barrier, and a decrease of the aqueous flow rate may cause higher flare with increasing age, although increased light scatter from the lenses with more cataract formation has been suspected to affect the measurements.\textsuperscript{181, 183, 185} In addition, higher flares have been measured in pseudophakic eyes than in phakic eyes.\textsuperscript{183, 186}

In earlier studies, higher flare has been associated with exudative AMD, and the values have been found to correlate with the size of the CNV lesion, but only in the advanced stage of the exudative process with fibrous proliferation and hemorrhage.\textsuperscript{187}

BRB dysfunction can also be detected with a retinal leakage analyzer measuring the amount of fluorescein leaking to the vitreous cavity during FA. In a study on factors predicting conversion from atrophic to exudative AMD,
BRB dysfunction detected with the retinal leakage analyzer had significant value as a predictor of progression to exudative disease. The finding of increased leakage was not, however, specific to the eyes developing exudative AMD during the three-year follow-up. Of course, some of these eyes may have converted to the exudative form of AMD later, after the three years of follow-up.

2.8 ANTI-VEGF TREATMENT

The anti-VEGF agents have almost completely replaced earlier options, direct laser and PDT, in exudative AMD treatment. Direct laser can still be used to treat certain types of extrafoveal CNVs and perhaps feeder vessels if the resulting scotoma is considered to be harmless. Evidence-based guidelines for treatment of PCV also include laser photocoagulation for extrafoveal lesions, and ICGA-guided PDT with or without anti-VEGF injections as an initial treatment for juxtafoveal and subfoveal lesions. In all other cases, repeated anti-VEGF injections are the recommended first-line treatment strategy for exudative AMD.

2.8.1 PEGAPTANIB SODIUM

Pegaptanib sodium (Macugen®, OSI Pharmaceuticals, Inc, Melville, NY and Pfizer, Inc, New York, NY, USA) was the first anti-VEGF agent approved for the treatment of exudative AMD as a 0.3 mg intravitreal injection, given every 6 weeks. The molecule is a polyethylene glycol-conjugated single strand of nucleic acid specifically binding and neutralizing VEGF isoform 165. The efficacy of the treatment measured as the number of patients losing less than 15 letters was significantly better than the efficacy of sham injections. Also, the effect was not restricted to certain lesion types, but the lesions larger than 12 optic disc areas were excluded from the trials. The net result of the treatment was, however, not improvement, but only a slower decline of VA, an effect similar to that of the PDT treatment.

2.8.2 BEVACIZUMAB

Bevacizumab (Avastin®, Genentech Inc., San Francisco, CA, USA) is a humanized monoclonal anti-VEGF antibody developed and manufactured for intravenous cancer therapy. The first reports on the effects of intravitreally injected bevacizumab in patients with exudative AMD were published in 2005 and 2006. The results were very promising, showing VA gain in most cases during a short follow-up. The most commonly used dose is 1.25 mg of bevacizumab (0.05 ml). The half-life of bevacizumab in nonvitrectomized human eyes (after an injection of 1.5 mg) is estimated to be 9.8 days.
Early studies in primates showed that a full-length antibody could penetrate the retina poorly, which led, for its part, to the development of ranibizumab, comprising smaller antibody fragments. In reality, however, the whole antibody worked well. The pathologically altered retina may be more easily penetrated than normal tissue, the structure of the human macular retina may differ from the retinal areas analyzed in the study, or a difference in the amount of antibody used may have affected the observations.

In comparison with ranibizumab, bevacizumab has been reported to cause more frequent inflammatory responses after the injections. Bevacizumab is synthesized in a Chinese hamster ovarian cancer cell line, and goes through a species- and cell-specific glycosylation, which leads to a higher immunogenic potential than the nonglycosylated protein product of the bacterial pathway used to manufacture ranibizumab. Bevacizumab is also produced for intravenous therapy and does not undergo as rigorous a purification procedure as the drugs meant for intraocular use. As a whole antibody containing both two antigen binding sites (Fab) and the tail region (Fc), bevacizumab has the ability to bind and interact with various Fc-gamma receptors and complement proteins. Chong and associates analyzed the postinjection reactions of over 16 000 bevacizumab injections. In their material, 44 patients developed a sterile inflammation within the first 7 days. Only three patients developed a recurrent inflammation with repeated treatment. In two patients, the subsequent reactions were more severe, suggesting that the response was specific to bevacizumab. The whole antibodies have also an ability to form aggregates when binding to antigens. The bevacizumab-VEGF aggregates seem to have a role in the thrombogenicity of systemic bevacizumab therapy in cancer patients. In intravitreal use, the effect seems to be less significant.

The risk of bacterial endophthalmitis is also, at least theoretically, somewhat higher with bevacizumab than with other available anti-VEGF agents due to the measures needed to divide a vial containing 4 ml or 16 ml of bevacizumab into smaller doses for intravitreal injections. If repackaging to 1 ml syringes is done, the procedure is vulnerable to contamination, causing a cluster of endophthalmitis. In a large retrospective cohort of over 383 000 injections, the risk of endophthalmitis did not, however, differ between the injections of ranibizumab distributed in single-use vials (0.025%; 95% CI, 0.015%-0.036%) and compounded bevacizumab (0.017%; 95% CI, 0.012%-0.021%).

The full-length antibodies are actively transported from inside the eye to the blood circulation. Significant systemic concentrations of bevacizumab have been detected after intravitreal injections. The serum concentration reaches a maximum at the 7th postinjection day. The systemic concentrations have also been found to rise from the first to the third postinjection period, indicating systemic accumulation. The levels of VEGF in serum have been observed to
be lower in patients treated with bevacizumab than in patients treated with ranibizumab. A more pronounced decrease was associated with more frequent retreatments. No significant difference in the occurrence of systemic adverse events has, however, been confirmed between these two drugs in a meta-analysis of pooled follow-up data from the Comparison of Age-Related Macular Degeneration Treatment Trial (CATT, n=1185) and the Inhibition of VEGF in Age-related choroidal Neovascularisation (IVAN, n=610) studies.

2.8.3 RANIBIZUMAB

The first on-label treatment for exudative AMD that increased VA was ranibizumab (Lucentis®, Genentech Inc., San Francisco, CA, USA, and Novartis Ophthalmics, Basel, Switzerland). It is a recombinant, humanized, monoclonal antibody Fab fragment that actively binds and inhibits all VEGF isoforms. It is derived from the same murine anti-VEGF antibody as bevacizumab. The dose used for exudative AMD treatment is 0.5 mg of ranibizumab (0.05 ml). The half-life of that dose is estimated to be 7.2 days in nonvitrectomized human eyes. It is slightly shorter than the half-life of bevacizumab, but the difference is probably compensated with the estimated 5- to 25-fold higher biological activity. Systemic ranibizumab levels reach a peak at 0.9 postinjection days. The systemic VEGF levels drop minimally compared with the drop caused by bevacizumab or aflibercept. Ranibizumab seems not to accumulate systemically.

The efficacies of bevacizumab and ranibizumab in exudative AMD treatment have been compared in two major clinical trials with large patient materials. In the CATT study, 1208 patients and in the IVAN study, 628 patients were randomly assigned to receive either bevacizumab or ranibizumab treatment for two years. No significant difference existed in the VA results between the two anti-VEGF agents. Studies conducted in Austria (317 patients, one year) and France (501 patients, one year) have confirmed this finding.

2.8.4 AFLIBERCEPT

Aflibercept (Eylea®, Regeneron Pharmaceuticals Inc, Tarrytown, NY, USA, and Bayer HealthCare AG, Leverkusen, Germany) is a newer anti-VEGF agent, a fusion protein that binds to all VEGF-A isoforms and also to VEGF-B and placental growth factor. It is a chimeric protein made by fusing the ligand binding domains of VEGF receptors, VEGFR-1 and VEGFR-2, with a Fc of human IgG. It has a high VEGF binding activity, estimated to be 140 times that of ranibizumab. The dose used for exudative AMD treatment is 2.0 mg of aflibercept (0.05 ml). Based on animal studies, the intravitreal half-life is estimated to be 1.5 times that of ranibizumab and 0.86 times that of bevacizumab. Maximum systemic concentration is reached at 1.1
postinjection days. It accumulates systemically the same way that bevacizumab does and seems to suppress the plasma VEGF levels even more efficiently than bevacizumab. Following intravenous administration, however, the half-life of aflibercept has been significantly shorter (5-6 days) than that of bevacizumab (20 days).

In the large phase II studies conducted in USA and Europe, 2 mg of aflibercept bimonthly, after three initial monthly loading doses, was as effective as a 0.5 mg monthly dose of ranibizumab in maintaining or improving VA during a one-year follow-up. These results led to the recommendation of bimonthly dosing of aflibercept after the initial three monthly injections, despite the individual variation in the intravitreal VEGF suppression time being shown to range from 41 to 109 days (with ranibizumab, from 26 to 69 days).

2.8.5 TREATMENT STRATEGIES

Injections of ranibizumab were given monthly in the first trials evaluating the treatment efficacy. Similar results with no less VA gain but with fewer injections have been reached using “as needed” (pro re nata, PRN) or “treat and extend” treatment strategies.

Two PRN regimens were thoroughly compared with monthly injections in the CATT and IVAN trials. In CATT, the PRN treatment was carried out without the widely recommended loading dose of three initial monthly anti-VEGF injections. The reinjection criteria were, however, very strict. Any fluid in OCT, persisting hemorrhage, or decrease in VA were indications for retreatment, given as single reinjections. In IVAN, the patients treated according to the PRN protocol received a loading dose of three initial monthly injections, and the retreatment was given in cycles of three additional monthly injections. Retreatment criteria, being somewhat less strict than the criteria used in CATT, were defined as any subretinal fluid, increasing intraretinal fluid, fresh blood, VA drop ≥10 letters, or dye leakage of more than 25% of the lesion circumference or growth of CNV in FA.

In the CATT trial, patients were randomly assigned to receive either bevacizumab monthly, ranibizumab monthly, bevacizumab as needed, or ranibizumab as needed. After the first year, patients who had received monthly treatment were rerandomized for the second year to continue the monthly treatment or to switch to PRN treatment with the same anti-VEGF agent. During the two years, no difference in VA results was observed between the two drugs, but the mean VA gain was greater in the patient group that has received monthly treatment (difference, -2.4 letters; CI from -4.8 to -0.1). Moreover, in the patients switched from monthly to PRN treatment for the second year, the VA decreased to a level not significantly different from the VA of those treated according to PRN strategy from the beginning. Thus, previous
monthly treatment did not have a sustained protective effect. The main difference between the two drugs was observed in their ability to render the macular retina completely fluidless: during the two years, the proportion of patients with dry macula was 14% among those receiving PRN bevacizumab compared with 46% among those receiving ranibizumab monthly.8,204

In the IVAN trial, no difference in VA response was observed between the drugs or between the treatment regimens during the two-year follow-up. Near VA and CS were, however, worse with discontinuous treatment. Fluid in OCT was more frequent in patients with PRN treatment compared with monthly injections. No statistically significant difference was observed between the two drugs.200, 201

In the analyses of pooled data from both of these trials, the PRN treatment was inferior to the monthly treatment, but no significant difference existed in the treatment response between the two drugs. However, in both studies a new geographic atrophy was detected more often in patients treated monthly, and in the CATT trial also in patients treated with ranibizumab than in those treated with bevacizumab.201, 204 Systemic adverse effects were more common with discontinuous treatment, and in CATT also associated with the use of bevacizumab.201, 204

The main problem with the anti-VEGF treatment is the enormous number of injections required, burdening both patients and the personnel of outpatient clinics. The monthly injections seem to be safer and more efficient than PRN treatment. However, the risk of endophthalmitis and geographic atrophy rises with more frequent injections, as do the costs of treatment, especially if ranibizumab or aflibercept is used. There are no large comparative trials yet comparing the efficacy and safety of the “treat and extend” strategy to the monthly regimen. Good results have been achieved with fewer injections in a smaller patient population.212

2.8.6 ANTI-VEGF INJECTIONS AND ANTERIOR CHAMBER FLARE

The anterior chamber flare levels after anti-VEGF injections have been studied by four research groups. Ziemssen and associates detected a non-significant rise in some study eyes at day one after an injection, no difference at week one, and a significant decrease at one month in patients with exudative AMD treated with bevacizumab.213 Kiss and associates showed a significant decrease at week one in patients with exudative AMD after an injection of 1.0 mg of bevacizumab (0.04 ml),214 but the baseline measurements of flare were done after a FA. During the angiography eyes are, however, exposed to repeated light stimulation that may affect the flare.215 Yeniad and associates did not find any differences in flare levels at days 1, 3, 7, and 30 after a bevacizumab
injection in patients having exudative AMD, non-proliferative diabetic retinopathy, or cystic macular edema caused by branch or central retinal vein occlusions, but the numbers of patients in each subgroup were quite small.216 The study by Blaha and associates compared the effects of bevacizumab, ranibizumab, and aflibercept on the anterior chamber flare in a mixed group of patients having exudative AMD, diabetic macular edema, or retinal vein occlusion. They showed that bevacizumab raised the flare values at day one after the injection, whereas aflibercept and ranibizumab caused no change. The subgroups were not analyzed separately.217

These reports have mainly concentrated on the flare as a sign of subclinical postinjection inflammation. However, the anti-VEGF agents are proteins that are as such measured by the flare meter. Thus, they may themselves have some effect on the measurements. The concentration of bevacizumab in the aqueous humor is known to peak at the first postinjection day.190 Moreover, the measured level of the flare is not only dependent on the concentration but also on the size of the protein in question. The molecular weights of the anti-VEGF agents differ considerably: bevacizumab 149 kDa, aflibercept 97 kDa, and ranibizumab 48 kDa. The short-term postinjection fluctuations in the flare are probably affected by the unequal sizes of the molecules and differences in their intraocular concentrations. The values may of course also reflect true differences in the inflammatory responses and changes in the BRB function caused by these agents.

2.9 FACTORS AFFECTING ANTI-VEGF TREATMENT RESPONSE

While most treated patients benefit from the anti-VEGF injections, some continue to lose vision despite the treatment or begin to lose the initially gained vision after a period of regular injections. A wide individual variation also exists in the number of needed injections when treatment is administered according to the PNR strategy.218, 219 Identifying the factors affecting treatment response and characteristics of patients and lesions that could predict a poor outcome would enable to development of alternative treatment strategies to avoid the loss of vision.

Treatment response to anti-VEGF injections can be divided both into functional and anatomic responses and into initial and long-term responses, which are not always correlated with each other. Functional response is usually determined as the change in VA or CS during the treatment. Distance VA is recommended to be measured using the logMAR scale, e.g. with the Early Treatment Diabetic Retinopathy Study (ETDRS) charts. With the ETDRS scale, a change >5 ETDRS letters is considered significant.220 The anatomic response is usually determined by the extent of recovery of the
normal macular morphology in OCTs. The initial treatment response is usually defined as the effect seen one month after the third injection (at month 4), and the long-term response as the effect seen thereafter. The analyzed predictors, for their part, can generally be classified as genetic markers, morphological lesion characteristics, such as lesion size or the presence of cystic intraretinal fluid, or other clinical factors, such as the patient’s VA at the beginning of treatment or tobacco smoking habits.

Numerous reports have been published on the factors affecting the treatment response in patients with exudative AMD. Variability of the treatment regimens and outcome measures used in these studies makes interpretation of the results quite challenging. Also, results may be affected by the differing genetic backgrounds of study populations and the variation in the characteristics of CNV lesions included in the analyses.

2.9.1 GENETIC FACTORS
No clear associations have been shown to exist between anti-VEGF treatment response and genetic factors, and the results of previous studies have been variable. The studies have mainly concentrated on the known genetic risk factors for AMD and the factors thought to directly affect the efficacy and binding of the anti-VEGF drugs.

2.9.1.1 Complement factor H
The risk genotype CC of the CFH rs1061170 has been variably associated with poorer VA gain and the number of needed re-injections. A meta-analysis by Chen and coworkers of nine primary research papers found that individuals with the homozygous CC genotype at rs1061170 were more likely to have a decreased response to anti-VEGF treatments compared with those carrying the TT genotype (OR, 1.7; 95% CI, 1.05-2.87).

In a Chinese study, CFH SNP rs800292 was observed to be associated with the initial VA gain with bevacizumab treatment, but the association was not supported by other reports in Asian or Caucasian populations.

2.9.1.2 ARMS2 and Htra1
Some studies have found the ARMS2 SNP rs10490924 to be associated with the VA gain during anti-VEGF treatment. Better VA responses during the initial treatment or in the first year of injections have been associated with the homozygous non-risk genotype, or with the heterozygous genotype compared with the homozygous risk genotype. Also, a cumulative effect of the risk alleles in ARMS2 rs10490924, CFH rs1061170, and VEGF rs699946
on the initial VA gain has been suggested to exist.\textsuperscript{163} However, many studies have not been able to confirm these associations.\textsuperscript{223, 232, 233, 236}

For \textit{Htra1} SNP rs11200638, reports exist of less VA gain with the homozygous risk genotype,\textsuperscript{231, 235} and a trend towards a better VA response with the heterozygous genotype.\textsuperscript{229} Again, however, other studies have not confirmed the association.\textsuperscript{233, 236}

Data from the two large studies (IVAN and CATT) analyzing the effects of anti-VEGF treatment regimens showed no clear genetic associations. However, in the CATT, a trend towards less decrease in foveal thickness during the first treatment year was found in patients carrying the homozygous risk genotype of \textit{CFH} rs1061170.\textsuperscript{237} Also, in the IVAN, the non-risk allele in \textit{ARMS2} rs10490924 showed a trend towards better response during the first year of treatment, determined as a change in total retinal thickness, which was the only outcome measure analyzed.\textsuperscript{238}

Hu and coworkers performed a systematic meta-analysis of 12 primary research papers, including the data from the IVAN and the CATT studies. The analysis showed that patients with the non-risk GG genotype of \textit{ARMS2} rs10490924 or the heterozygous GT genotype had somewhat better response to anti-VEGF treatment than those with the homozygous risk genotype TT (OR, 1.74; 95\% CI, 1.19-2.52). The association existed mainly in the East Asian populations.\textsuperscript{239}

\subsection{2.9.1.3 VEGF and VEGF receptor}

Of the numerous \textit{VEGF} SNPs analyzed four (rs699947, rs1413711, rs699946, and rs3025000) have been reported to have associations with the anti-VEGF treatment response. Two studies in Caucasian populations found that patients with the CC genotype of rs699947 were more likely to respond better to ranibizumab.\textsuperscript{163, 240} Conversely, in a Korean population, patients with the AA genotype had a better response.\textsuperscript{233} Further, some studies observed no association between the SNP and response.\textsuperscript{241-243} The genotype of \textit{VEGF} rs1413711 had an association with VA response in one study carried out in the UK,\textsuperscript{229} but the finding has not been verified in other studies.\textsuperscript{242, 244} The genotype of \textit{VEGF} rs699946 was associated with the VA response in a Japanese study,\textsuperscript{241} but the association was not confirmed in a Korean study.\textsuperscript{233} Also, while an association has been found between better VA gain and TT or TC genotype at \textit{VEGF} rs3025000, this has not yet been confirmed by other groups.\textsuperscript{245}

Furthermore, two \textit{VEGFR-2} SNPs, rs4576072 and rs6828477, have been suggested to be associated with VA outcome of ranibizumab treatment.\textsuperscript{246}
Patients with the CC or CT genotypes at either of these SNPs were more likely to respond better than patients expressing TT genotypes.

Data gathered from the CATT study were separately analyzed for associations between treatment outcomes and seven VEGFA SNPs (rs699946, rs699947, rs833069, rs833070, rs1413711, rs2010963, and rs2146323) and one VEGFR-2 SNP (rs2071559). Four VEGFA SNPs, rs699947, rs833070, rs1413711, and rs2146323, had an association with retinal thickness after one year of treatment. However, after adjusting for multiple comparisons the associations did not retain their significance and were not analyzed in more detail. No associations were found between the studied genetic markers and other treatment outcomes.247

2.9.1.4 Other genetic markers

In addition to the genetic markers associated strongly with the risk of developing AMD, and markers in the VEGF or VEGFR genes, factors connected to inflammatory and angiogenic pathways have been studied for associations with the treatment response. Some associations have been reported, although the findings have not been confirmed by other groups.

Wang and associates found a possible association between the SNP rs2285714 in the gene coding phospholipase A2. Phospholipase A2 is a family of enzymes having a role in glycerophospholipid metabolism, but also functioning as signaling molecules in inflammation and tissue injury, and having proangiogenic effects by affecting the levels of VEGF production.243

In a study of treatment response to ranibizumab, patients seemed more likely to have a favorable outcome if they were heterozygous for both polymorphisms CHF rs1061170 and frizzled homolog 4 rs10898563.227 The protein product of the latter gene seems to be involved in the development and maintenance of retinal blood vessels.248 However, the significance of the polymorphism has not been verified in other studies.163, 238

The allelic variants ε2, ε3 and ε4 of the apolipoprotein E gene (determined by genotypes at rs429358 and rs7412) may also have some effect on the initial VA gain during treatment. Patients with the ε4 variant were found to have significantly better visual outcomes than those with the ε2 variant.249 The apolipoprotein E ε4 isoforms have previously been suggested also to have a protective role in preventing AMD development and progression.250

Data from the IVAN study were used to analyze 485 SNPs. When the level of association with the change in total retinal thickness was assessed, several of the most significant SNPs were associated with the HIF-2A gene, supposedly regulating vascularity in hypoxic conditions.238 However, the CATT data
did not support any pharmacogenetic associations between these loci and the treatment outcomes.251

An interesting new candidate for modifying the anti-VEGF treatment response is neuropilin-1, a membrane-bound co-receptor binding to the VEGF/VEGFR complex that enhances signal transduction. It also potentially is a factor capable of promoting angiogenesis independently, without the presence of the VEGF/VEGFR complex.252 The SNP rs2070296 located in the Neuropilin-1 gene has been found to be associated with less initial VA gain with ranibizumab treatment. This SNP also had a combined effect with the VEGFR-2 rs4576072 on the long-term response at 6 and 12 months.253

2.9.2 CLINICAL AND MORPHOLOGICAL FACTORS

Probably the most robust factors affecting treatment outcome have been level of VA at the beginning of treatment, size of the CNV lesion, and age of the patient. In large studies, patients with better baseline VAs have had better VAs after treatment but less VA gain during treatment, probably due to a ceiling effect. Large lesion size at beginning of treatment has been a prognostic sign of poorer VA gain, and older patients have been less likely to gain VA during treatment than younger patients.218, 254-257 Also, a delay in the initiation of injection treatment has been shown to deteriorate VA outcome in several studies.258-261 A significant loss of VA occurs if the first anti-VEGF injection is delayed by 4 weeks or longer.258, 260

The CATT study analyzing the baseline factors predicting the long-term VA response indicated that, in addition to older age of the patient and larger size of the CNV, central elevation of RPE at baseline OCTs was associated with worse VA and less gain in VA at one and two years of follow-up. Lower baseline VA score was also associated with lower VA, but better gain. At one year, the predominantly or minimally classic lesion type (compared with the occult type), presence of geographic atrophy, and greater total foveal thickness were associated with worse VA, as well. In univariate analyses, the visual outcomes were affected by several OCT features, including retinal thickness, the presence of NED, the presence/thickness of subretinal tissue, the presence of intraretinal fluid, the presence of sub-RPE fluid, and the presence/thickness of RPE elevation. However, in multivariate analyses, the only factors independently associated with the response were greater total foveal thickness (associated with VA score) and RPE elevation (associated with both VA score and VA gain).256 The two-year results were quite similar. The significance of the presence of geographic atrophy was even more pronounced. It was also noted that the strongest predictor of VA outcome at one and two years was the initial VA response seen after three months of treatment. However, also the initial response predicted less than 50% of the variation in the long-term VA
responses, as a significant proportion of patients continued to gain or lose VA after the initial treatment period.\textsuperscript{257}

Factors identified as strong predictors of less favorable treatment outcome in several studies based on OCT findings have been the presence of PED (RPE elevation having a base of $\geq 400$ \textmu m and a height of $\geq 200$ \textmu m), the presence of intraretinal cystic fluid, especially as chronic cystic changes of duration $>3$ months, the presence of subretinal tissue (or hyperreflective material), and retinal thinning.\textsuperscript{262-269}

When the data from the large phase III trials assessing the effect of ranibizumab treatment on exudative AMD with two-year follow-ups (MARINA and ANCHOR) were re-analyzed to identify predictors differentiating patients losing VA from those gaining VA, it was found that in the patients losing VA the areas of RPE abnormality and CNV lesion increased more, and the increase was caused by the growing of either atrophic scar or CNV. VA loss was also associated with an increased incidence of RPE tears, although the tears were quite uncommon in general. However, these analyses were based on angiographic features of the lesions since OCTs were recorded at a very limited number of visits.\textsuperscript{218}

Various morphological characteristics have been reported to have associations with the anti-VEGF treatment outcomes. The presence of vitreomacular adhesion has been suggested to affect the VA results,\textsuperscript{270} differentiate non-responders from responders,\textsuperscript{271} and have an effect on the frequency of needed reinjections.\textsuperscript{272} An analysis on the CATT data revealed that eyes with a vitreomacular adhesion or traction required a greater number of anti-VEGF injections, on average two injections more over two years, than eyes with vitreomacular separation. At baseline, also a trend towards thicker subretinal tissue and greater total retinal thickness was seen in the eyes with vitreomacular traction. No difference in VA results was found. Interestingly, the exudative AMD patients with vitreomacular adhesion or traction were younger and more likely to be smokers.\textsuperscript{273} Also foveal autofluorescence patterns reflecting the metabolic function of RPE cells and possibly also the functional capacity of overlying photoreceptors have been suggested to predict the response. Increased central autofluorescence before the treatment was associated with worse VA after the initial treatment and less VA gain.\textsuperscript{274} Yet another OCT characteristic associated with poorer outcome of therapy is the development of outer retinal tubulations, branching structures with a round, ovoid, or tubular hyporeflective center and a hyperreflective border located within the outer retina. Although the structures are very rarely found in the central fovea, they were associated with worse VA at two years in the CATT data. The presence of outer retinal tubulations may reflect a greater degree of photoreceptor degeneration.\textsuperscript{275}
All in all, the pathological processes leading to neovascular growth, especially the underlying mechanisms determining the functional and anatomic responses of the lesions to the anti-VEGF treatment, are complex and remain largely obscure.
3 AIMS OF THE STUDY

I. To examine the effects of SNPs IL-8 (rs4073), VEGF (rs699947, rs2146323 and rs3025033), EPO (rs1617640), CFH (rs1061170), C3 (rs2230199), and ARMS2 (rs10490924) on the initial response to intravitreal bevacizumab treatment in patients with exudative AMD.

II. To analyze the associations of genetic variations IL-8 rs4073, VEGF rs699947, rs2146323, and rs3025033, CFH rs1061170, C3 rs2230199, and ARMS2 rs10490924 with the long-term response to bevacizumab treatment in patients with exudative AMD.

III. To investigate whether IL-8 rs4073 is associated with an earlier onset of exudative AMD.

IV. To explore the variations in anterior chamber protein concentration during the anti-VEGF treatment of exudative AMD and to analyze the associations between protein concentration and treatment response to bevacizumab in patients with exudative AMD.
4 MATERIALS AND METHODS

4.1 PATIENTS

Study I comprised combined data of 59 patients whose medical records were analyzed retrospectively and 37 patients enrolled in the prospective follow-up study (II) on the outcome of bevacizumab treatment of exudative AMD. The initial treatment response after the first bevacizumab injections was analyzed for associations with the studied genetic markers and clinical characteristics.

In study I, we analyzed the medical records of 304 patients who had received their first three intravitreal bevacizumab injections for exudative AMD at the Department of Ophthalmology, Helsinki University Central Hospital, Finland, between January 2007 and December 2008. Inclusion criteria were age >50 years, central lesions with intra- and/or subretinal fluid in OCTs at baseline, complete loading dose of three bevacizumab injections given within 5 months after the first injection, and complete six radial OCT scans available from the visits before and after the initial treatment. Exclusion criteria were any prior treatment of the study eye for exudative AMD, injections given as part of a combination therapy, polypoidal CNV, or RPE tear. Of the 133 patients fulfilling the criteria, only 59 agreed to donate a blood sample for genetic analyses. No significant difference existed between patients who provided a blood sample and those who did not in distributions of age, sex, CNV lesion type, response to treatment, VAs before or after treatment, or VA gain during treatment. One eye of each patient was included. If the patient received treatment for bilateral exudative AMD, the eye that was treated later was selected as the study eye, and if both eyes were treated simultaneously, the eye with better baseline VA was selected as the study eye. These criteria were set to maximize the probability of selecting the eye with as recent an onset of exudative disease as possible in bilateral cases, and to minimize the effects of more progressive cases affecting the results.

The total sample size required to detect a 20-25% difference in genotype frequencies between the groups was calculated to be about 100 patients. To better meet that goal, we used additional data gathered from the initial visits of patients enrolled to the two-year prospective follow-up study on the outcome of bevacizumab treatment in patients with exudative AMD (study II). These patients were recruited from the same patient population of the Helsinki University Central Hospital, Department of Ophthalmology, between May 2008 and May 2010. Inclusion criteria for the prospective study were as follows: age >50 years, a newly diagnosed exudative AMD without prior treatment, VA ≥20/200, and a subfoveal or juxtafoveal CNV lesion. Exclusion criteria were diabetic retinopathy, retinal vascular diseases, prior ocular
surgery except uncomplicated cataract surgery, AMD lesions with polypoidal CNVs, RAP, or hemorrhage >2.5 disc diameters. Of the 50 patients enrolled in the prospective study, 37 met the inclusion criteria for study I and together with the 59 original retrospectively evaluated patients formed the patient population (96 patients in total).

Studies II and IV analyzed the data collected from the two-year prospective follow-up study of 50 treatment-naive exudative AMD patients. Inclusion and exclusion criteria were as described above. One eye of each patient was included and none of the study patients with bilateral disease fulfilled the inclusion criteria with both eyes. The trial was registered in the Australian New Zealand Clinical Trials Registry (ACTR number 12608000223336).

In study III, all valid data from the previous and ongoing studies of our research group were included to investigate further the possible association of the risk genotype of IL-8 rs4073 and younger age of onset of the exudative disease. Altogether 492 Finnish subjects were included: 301 patients with exudative AMD, 72 patients with dry AMD, and 119 control subjects. All patients with exudative AMD, 90 control subjects, and 28 patients with dry AMD were enrolled between January 2003 and April 2010 in either study I or II or an earlier study on AMD genetics.\textsuperscript{143} The subjects were recruited from the Units of Medical retina (cases) and Cataract surgery (control subjects) of the Departments of Ophthalmology of the Helsinki (n=368), Oulu (n=6), and Tampere (n=1) University Hospitals, or private offices and outpatient clinics (n=44). Additionally, 44 diabetic patients were included as dry AMD patients in the analyses of IL-8 rs4073. They had large macular drusen visible in fundus photographs taken for screening of diabetic retinopathy between January 2006 and February 2007. Also, 29 men recruited between October 2004 and June 2007 were included as additional control subjects from an ongoing study of risk factors for AMD in a cohort of male executives and academic professionals, originally examined for cardiovascular health, cognition, and quality of life.\textsuperscript{276}

All subjects of the studies were Finnish (Caucasian). Demographic data of the participants are presented in Table 3. The study protocols adhered to the tenets of the Declaration of Helsinki and were approved by the local ethics committee. A written informed consent was obtained from all participants before enrollment. The subjects were interviewed to record their medical and smoking history and classified as never, ex-, or current smokers based on the information. Never-smokers were determined as having smoked less than one pack-year during their lifetime, and ex-smokers as having quit smoking at least 10 years ago. If a participant had ever smoked, the smoking pack-years were calculated. In non-smokers, the pack-years were determined to be zero. The reason for classifying the subjects as current smokers if they had quit smoking less than 10 years earlier was a previous finding that a relatively sharp
decline in the risk of neovascular AMD occurs during the first 10 years after quitting smoking. Thereafter, the decrease in the risk is slower. Compared with current smokers, the RR of neovascular AMD for ex-smokers had dropped to 0.7 at 10 years, but to reach a risk of 0.5 took more than 25 years.96

Table 3. Demographic data of study subjects and characteristics of CNV lesions in each study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study I n=96</th>
<th>Studies II and IV n=50</th>
<th>Study III n=492</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/Female</td>
<td>35 (36%)/61 (64%)</td>
<td>16 (32%)/34 (68%)</td>
<td>171 (35%)/321 (65%)</td>
</tr>
<tr>
<td>Age, years, mean (±SD)</td>
<td>76.7 (±7.1)</td>
<td>75.8 (±6.9)</td>
<td>75.8 (±6.5)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>52 (54%)</td>
<td>30 (60%)</td>
<td>282 (57%)</td>
</tr>
<tr>
<td>Ex</td>
<td>27 (28%)</td>
<td>14 (28%)</td>
<td>120 (24%)</td>
</tr>
<tr>
<td>Current</td>
<td>17 (18%)</td>
<td>6 (12%)</td>
<td>67 (14%)</td>
</tr>
<tr>
<td>Data missing</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>23 (5%)</td>
</tr>
<tr>
<td>Lesion type, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occult, no classic</td>
<td>60 (63%)</td>
<td>31 (63%)</td>
<td>129 (50%)*</td>
</tr>
<tr>
<td>Predominantly classic</td>
<td>8 (8%)</td>
<td>9 (18%)</td>
<td>39 (15%)</td>
</tr>
<tr>
<td>Minimally classic</td>
<td>14 (15%)</td>
<td>6 (12%)</td>
<td>53 (20%)</td>
</tr>
<tr>
<td>RAP</td>
<td>8 (8%)</td>
<td>0 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>Polypoidal</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (0.4%)</td>
</tr>
<tr>
<td>Non-defined</td>
<td>5 (5%)</td>
<td>4 (8%)</td>
<td>23 (9%)</td>
</tr>
<tr>
<td>Data missing</td>
<td>1 (1%)</td>
<td>0 (0%)</td>
<td>14 (5%)</td>
</tr>
<tr>
<td>CNV location, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subfoveal</td>
<td>82 (85%)</td>
<td>39 (78%)</td>
<td>NA</td>
</tr>
<tr>
<td>Juxtapfoveal</td>
<td>12 (13%)</td>
<td>10 (20%)</td>
<td>NA</td>
</tr>
<tr>
<td>Extrafoveal</td>
<td>2 (2%)</td>
<td>1 (2%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* % of the 259 patients with exudative AMD included in the analysis of age of onset
NA, data not analyzed

4.2 OPTHALMIC INVESTIGATIONS

In study I, the retrospectively analyzed data of 59 patients were gathered from medical records. The ETDRS charts at two meters were used for VA assessment at most visits. Only Snellen VAs were available for six baseline examinations and for two examinations after the initial treatment. These Snellen VAs were transformed to the ETDRS equivalents for the analyses. The
OCTs were taken with the Stratus OCT (Carl Zeiss Meditec, Inc., Dublin, CA, USA). Six radial scans were analyzed for the presence of cystic intraretinal fluid spaces (diameter ≥50 μm) and NED. FAs were available in all but one patient and ICGAs in 44 patients [Topcon Imagenet (Topcon Inc., Tokyo, Japan) or Heidelberg Retinal Angiograph (Heidelberg Engineering, Heidelberg, Germany)].

The 50 patients recruited to the prospective two-year study (data used in studies I, II, and IV) were followed up monthly. Between the baseline and the first monthly visit, 45 patients came for additional weekly visits; 37 patients were examined at week one, 36 at week two and 38 at week three. VA was assessed with ETDRS charts at two meters, using different charts for refraction and testing. Contrast sensitivity (CS) was assessed with the Pelli-Robson test chart at one meter. Anterior chamber protein concentration, flare (Kowa Laser Flare Meter FM-500, Kowa Company Ltd., Nagoya, Japan) was measured in both eyes at each visit after pupillary dilatation (0.5% tropicamide and 2.5% phenylephrine drops). Ten flare readings were obtained from each eye. Readings were discarded if the background measurements differed by more than 15%. After deleting the highest and lowest readings of each set of measurements, the mean of the remaining 7-8 values and its standard deviation were calculated for analysis. If the standard deviation was larger than the mean itself, the measurement was excluded as unreliable (only one set of measurements in the whole data). Eyes were examined with biomicroscopy both before the pupillary dilatation to assess the inflammatory reaction in the anterior chamber and after the dilatation to examine the status of the vitreous and retina. Radial and 3-dimensional OCT scans of both eyes (Spectral OCT/SLO, Opko/OTI, Miami, FL, USA) and a macular microperimetry of the study eye (Polar 3 pattern microperimetry program, Spectral OCT/SLO, Opko/OTI, Miami, FL, USA) were taken at every visit. FA and ICGA were recorded at baseline, and at one and two years [Topcon Imagenet (Topcon Inc., Tokyo, Japan) or Heidelberg Retinal Angiograph (Heidelberg Engineering, Heidelberg, Germany)]. All patients received an intravitreal bevacizumab injection at the baseline visit. Thereafter, the need for retreatment was evaluated at each follow-up visit. During the first year the criteria for retreatment were any sub- or intraretinal fluid in OCT (minimum diameter of a cyst requiring retreatment was set at 50 μm) or a new hemorrhage in biomicroscopy. During the second year the treatment was discontinued in those patients who had been given 14 injections for stationary intraretinal cysts until further progression was detected.

In study I, patients were classified as responders, partial responders, or nonresponders according to the changes seen in OCTs during the initial bevacizumab treatment (Figure 1 A-F, publication I). Those patients whose data was analyzed retrospectively were classified as responders if neither cysts nor NED were detected in the OCTs after the first three bevacizumab
injections, as partial responders if they had both NED and cysts before but only cysts after the injections or had only cysts before the treatment and a reduction of more than 70% in the total combined area of cysts in all six radial OCT scans was seen during the treatment, and as nonresponders if they had a persisting NED after the injections or the reduction in the area of cysts was less than 70%. The 37 patients included in study I from the prospective material were classified accordingly; patients who had received bevacizumab injections at all three initial visits and for whom remaining intra- or subretinal fluid was detected at the fourth visit were included as nonresponders or partial responders according to the criteria above (n=25). Patients with no detectable fluid in OCT at the fourth visit irrespective of the number of injections they had received during the first three visits were included as responders (n=12). Four of the responders had received three injections, six had received two, and two had received only the first injection after which the OCTs had remained dry.

The angiograms of the patients in studies I, II and IV were reviewed by two retina specialists (Professor Immonen and the writer) masked to the genotype of the patients. CNV lesion was measured including all CNV components: serous PED, hemorrhage, and blocked fluorescence (e.g. fibrous tissue covering underlying structures). The size of the CNV included occult and classic components. Lesions were classified as predominantly classic, minimally classic, occult, or RAP. Based on the ICGAs, the lesions were classified into four categories: hot spot, plaque, combined hot spot and plaque, or no hyperfluorescence. The area of the lesion in ICGA was defined as the area of late hyperfluorescence. In cases with no detectable hyperfluorescence, the area was determined to be zero. The two investigators agreed on lesion classification in 80% of cases, the remaining 20% of lesions were discussed to reach agreement. OCT scans and changes in fluid compartments were evaluated in a similar manner.

In study III, in control subjects and patients with dry AMD the macular changes were identified by analyzing fundus photographs graded according to the AREDS classification. Control subjects had no large drusen and at most minimal focal pigmented abnormalities within the radius of one disc diameter from the fovea. The combined area of pigmentary abnormalities was no larger than an area corresponding to a circle of diameter 250 μm, and up to five small (<63 μm) drusen were allowed. Sixty-five control subjects had neither pigmentary abnormalities nor drusen, 50 had only pigmentary abnormalities, three had small drusen, and one had both drusen and pigmentary abnormalities. Patients with dry AMD had at least 10 soft drusen >125 μm in size or confluent drusen over an area corresponding to >1 disc area within the radius of one disc diameter from the fovea, or they had central geographic atrophy related to AMD. In subjects with exudative AMD, the diagnosis was made by an experienced medical retina specialist. The age of onset of exudative
disease was determined as the age at diagnosis. Only patients with a recent onset of symptoms based on medical records and no substantial subretinal fibrosis detected in clinical examination or in angiography were included. It was possible to determine the age of onset of exudative AMD in the first affected eye for 259 of 301 patients. The diagnosis was considered to be delayed in 42 patients; they had had symptoms for longer than one year or subretinal fibrosis was detected, indicating a late stage of the disease. The diagnosis of exudative AMD was confirmed with FA in 253 patients. The CNV lesion classification was based on descriptions in the medical records and on re-evaluations of the angiographies when possible. In eight patients, the angiographies were recorded, but were not available for retrospective analysis and no classification of the lesion was found in the medical records. In six patients, FAs were not recorded; one of them had a RPE tear and four of them received no treatment because of poor VA.

4.3 GENETIC ANALYSES

The analyses of the polymorphisms were done by two independent research groups utilizing differing protocols for DNA extraction and genotyping. All methods were used as recommended by the manufacturers. Primer sequences and probes are shown in Table 4.

The analysis of IL-8 rs4073, CFH rs1061170, ARMS2 rs10490924, and C3 rs2230199 (studies I, II, and III) was done by using phenol-chloroform extraction for DNA, and polymerase chain reaction (PCR) -based genotyping with forward and reverse primers. The PCR thermocycling protocol consisted of 5 minutes (min) at 95°C, followed by 35 cycles of 30 seconds (s) at 95°C, 30 s at 53-58°C, and 1 min at 72°C. Final extension was set at 72°C for 10 min. For sequencing reactions, PCR products were digested with exonuclease I and ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). PCR products were then subjected to cycle sequencing reaction using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Products were purified with Performa DTR v3 filter plates (Edge Biosystems, Gaithersburg, MD, USA) to remove excess dye terminators. Products were resolved on an ABI 3730 capillary sequencer and analyzed using the Sequencer 4.10.1 software.

The analysis of EPO rs1617640 (study I) and VEGF rs699947, rs2146323, and rs3025033 (studies I and II) was performed by using the salting-out technique for DNA extraction and as described before for the EPO SNP,277 predesigned TaqMan® SNP Genotyping Assays, and the Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).
Table 4. Forward and reverse sequences of primers used for genotyping SNPs in IL-8, CFH, C3, and ARMS2, and probe sequences of predesigned TaqMan assays used for genotyping SNPs in EPO and VEGF genes.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 rs4073</td>
<td>5' TCATCCATGATCTTTGTTCTAA 3' forward</td>
</tr>
<tr>
<td></td>
<td>5' GGAAAACGCTGAGGTCAGA 3' reverse</td>
</tr>
<tr>
<td>CFH rs1061170</td>
<td>5' GTGCAAACCTTTGTAGTAA 3' forward</td>
</tr>
<tr>
<td></td>
<td>5' AAAGACATGAACATGCTAGGA 3' reverse</td>
</tr>
<tr>
<td>C3 rs2230199</td>
<td>5' ATCTTCTTGCCCCCTCTCAAG 3' forward</td>
</tr>
<tr>
<td></td>
<td>5' TGAAGCTCTCTTTTGGATTTTC 3' reverse</td>
</tr>
<tr>
<td>ARMS2 rs10490924</td>
<td>5' CATTCTGCTGCCGCGGGCGAA 3' forward</td>
</tr>
<tr>
<td></td>
<td>5' GCTGTTAAATGCAAGCTG 3' reverse</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SNP (TaqMan predesigned assay ID)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO rs1617640 (C___8786860_10)</td>
<td>GTCTCCTGCTCTGGAAATCTCACTC[A/C]</td>
</tr>
<tr>
<td></td>
<td>TCTGGCTCAGGTTTTCCAGAAGCCA</td>
</tr>
<tr>
<td>VEGF rs699947 (C___8311602_10)</td>
<td>GCCAGCTTGAAGCCAGACCTGGCA[A/C]</td>
</tr>
<tr>
<td></td>
<td>GATCTGGGTGGATAATCAGACTGAC</td>
</tr>
<tr>
<td>VEGF rs2146323 (C___1647372_10)</td>
<td>GGACTTACGTTAGTTTGGAGAAGA[A/C]</td>
</tr>
<tr>
<td></td>
<td>TTGCCCTATTCGAGGCAAGCTCAGAGA</td>
</tr>
<tr>
<td>VEGF rs3025033 (C___15985292_10)</td>
<td>CTTGGAGTGCTTCCTCCCTTCAGA[AG]</td>
</tr>
<tr>
<td></td>
<td>ATGTTCCTTGTCGTGAGCTG</td>
</tr>
</tbody>
</table>

4.4 STATISTICAL ANALYSES

In study I, PASW software version 18.0 (SPSS, Inc., Chicago, IL, USA) and plink-1.07 whole-genome association analysis toolset were used for statistical analyses. The differences in allele distributions and genotype frequencies between the responder groups were analyzed using Fisher’s exact test. Mann-Whitney U and Kruskal-Wallis tests were used for comparisons between distributions, and Spearman’s rho for measuring correlations between non-normally distributed continuous variables. Power calculations were performed with Russ Lenth’s Java Applets for Power and Sample Size computer software (http://homepage.stat.uiowa.edu/~rlenth/Power, accessed May 16, 2016). It was estimated that with a power of 0.8 and p-value of 0.05, differences of approximately 20% in allele frequencies between the groups could be detected. A p-value <0.05 was considered statistically significant. No correction was used for multiple comparisons. The simultaneous effects of genetic markers and CNV lesion characteristics on treatment response were estimated with multivariate modeling. The models were built with the Generalized Linear Model procedure in PASW with binary response distribution and logistic link function, including intercept and main effects.
In study II, IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA) was used for statistical testing. To analyze the univariate associations of the variables with the treatment response and the number of needed reinjections in the two-year data, the SPSS Generalized Estimating Equations procedure for repeated measurements was used with binary response distribution, logistic link function, and exchangeable correlation matrix. Multivariate models were built using the same Generalized Estimating Equations procedure, including intercept and main effects. Multivariate models estimating the effects in the aggregated data were built using the SPSS Generalized Linear Model procedure. Tests for non-normally distributed data were used in comparisons between the groups. A two-tailed p-value <0.05 was considered statistically significant, no correction for multiple comparisons was used. In the analyses of VA and CS, the last observation was carried forward in the cases of missing data. At two visits, the reinjection criteria were met, but the patient did not receive an injection. These visits were analyzed as if the patient had received treatment. Of the 50 patients enrolled, 47 completed the first and 43 the second year of follow-up. The three patients discontinuing the follow-up before the end of the first year completed 7 (discontinuation due to other medical problems), 8 (due to VA loss despite dry macula), and 9 visits (due to severe uveitis of the study eye after the 8th bevacizumab injection). Additionally, four patients discontinued after completing the first year of follow-up: for retinal detachment after 12 visits, for repeatedly rising eye pressure after mydriatic drops and need for cataract surgery after 13 visits, for non-response to the treatment after 13 visits, and for dense cataract formation after 16 visits. Missing data due to the dropouts can be considered as missing completely at random for five patients, and missing at random in the two cases of dropout due to unresponsiveness to treatment. The data of the first year of follow-up are therefore quite complete, but the results of the second follow-up year may be biased and not completely generalizable. The follow-up data were analyzed separately for one and two years, including all completed follow-up visits.

In studies III and IV, IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA) was used for statistical testing. Parametric and nonparametric tests were used in comparisons between the subgroups depending on whether the tested variables obeyed normal distribution or not.

In study III, the simultaneous effects of genetic markers and smoking on diagnosis and age of onset of exudative AMD were estimated first with multivariate models built with the Generalized Linear Model procedure in SPSS 22. To correct for differences in age distributions in the groups of control subjects and patients with exudative AMD, the effects of the studied variables were also estimated with Cox regression analysis, as suggested by van der Net and associates. The principle justifying the use of Cox regression in cross-sectional genetic association studies is the fact that the genotype status does
not change over time, and thus, the age of onset can be treated as the follow-up time. A two-tailed p-value <0.05 was considered statistically significant. It was estimated that 15% differences in allele frequencies between controls and exudative AMD patients, or between controls and all AMD patients, and a difference of 3 years in the age of onset could be detected with a power of 0.8 and a p-value of 0.05 (calculator for sample size provided at https://www.statstodo.com/ by StatsToDo Trading Pty Ltd, accessed May 16, 2016).

In study IV, the factors affecting the flare values were assessed. Data from the monthly visits during the two years were analyzed with the SPSS Linear Mixed Model procedure designed for repeated measurements. The models were corrected for interindividual differences in measurement levels. The univariate and multivariate associations of the studied factors with the level of flare were estimated using a scaled identity matrix as the repeated covariance type and including an individual-level random intercept. To meet the normality requirement of the response variable, a square root transformation was performed on the flare variable. All models were tested for interactions. A two-tailed p-value <0.05 was considered statistically significant.
5 RESULTS

5.1 INITIAL TREATMENT RESPONSE

After the initial bevacizumab treatment, of 96 patients, 38 were classified as responders (40%), 6 as partial responders (6%), and 52 as nonresponders (54%) based on the OCTs. During the treatment VA improved in 63% (60/96 patients), remained unchanged in 7% (7/96), and deteriorated in 30% (29/96). The OCT findings before and after treatment are presented in Table 2 of publication I.

No difference existed in the VA scores between the treatment response groups at baseline or after treatment. VA gain, however, was better in responders than in nonresponders (median change +5.5 letters, range from -29 to +35 letters vs. +1 letter, range from -8 to +7 letters, p=0.006, Mann-Whitney U). Gain in partial responders did not differ from that in responders or nonresponders. VA gain was not associated with the studied SNPs.

Patients with better baseline VAs had better VAs after treatment (r=0.66, p<0.001, Spearman’s rho), but also less VA gain during treatment (r=-0.315, p=0.002).

Cystic changes or NED in the baseline OCTs were not associated with VA outcomes. However, patients with cysts in OCTs after the initial treatment had lower VA than those without cysts (median 62 letters, range from 3 to 83 vs. median 70 letters, range from 27 to 85, p=0.003). They also had less VA gain (median -1 letter, range from -59 to +7 vs. median +4 letters, range from -29 to +53, p<0.001).

FAs taken no more than six months before the first injection were available in 95 of the 96 patients, and in one patient only the type and location of the lesion could be defined. A negative correlation existed between VA gain and the sizes of lesion (Spearman’s rho, r=-0.282, p=0.006) and of CNV (r=-0.276, p=0.007). Lesion size varied significantly between the groups with differing lesion types (p=0.001, Kruskal-Wallis, Figure 3, publication I), but the type of lesion itself was not associated with baseline VAs or VA outcomes.

ICGAs were available in 81 patients. The area of late hyperfluorescence could be measured in 64 patients, with the lesions classified as plaque or combined hot spot and plaque. Neither type nor size of lesion in ICGA correlated with VA response.
The initial anatomic treatment response was associated with the IL-8 rs4073 polymorphism. The homozygous AA genotype was carried by 25% of nonresponders (13/52), but by only 8% of responders (3/38), and the frequency of the A allele was higher in nonresponders than in responders (0.51 vs. 0.34, p=0.033, Fisher’s exact test). The difference was even more pronounced when partial responders were combined with responders. No differences in genotype or allelic frequencies of the other SNPs were observed between responder groups (Table 3, publication I).

Minimally classic lesions and lesions with RAP seemed to have a better anatomic response than other lesion types (Figure 2, publication I). Lesion size in FA was somewhat albeit not significantly larger in nonresponders than in responders (median area 8.3 mm², range from 0.6 to 23.2 mm² vs. median 5.0 mm², range from 0.6 to 20.7 mm², p=0.099, Mann-Whitney U). Presence of NED before (p=0.027) and cystic changes after the initial treatment (p=0.012) were associated with larger lesions. Type of lesion in ICGA did not correlate with treatment response, but the size of the lesion in ICGA mattered; smaller lesions were associated with better anatomic response (median area 0.9 mm², range from 0.0 to 5.3 mm² in responders vs. median 2.6 mm², range from 0.0 to 20.0 mm² in nonresponders, p=0.001, Mann-Whitney U).

No associations were found between treatment response and age, sex, smoking status, or smoking pack-years.

In multivariate analysis of the effects on anatomic response, the only variables reaching significance were lesion type in FA and homozygous AA genotype of IL-8 rs4073 (Table 4, publication I). Size of lesion in ICGA was not included in the model due to the low percentage of patients with a measurable lesion in ICGA.

### 5.2 LONG-TERM TREATMENT RESPONSE

Data from the two-year follow-up were analyzed to uncover any associations of the genetic factors and patient and lesion characteristics with the VA and CS results, number of needed reinjections, and presence of sub- or intraretinal fluid in OCTs.

The median number of reinjections was 9.5 (range 0-12) during the first year and 9 (range 0-12) during the second year. In three patients, the treatment was switched from bevacizumab to ranibizumab during the follow-up, in two patients by the patient’s request after 6 and 7 injections, respectively, and in one patient when a mild uveitis developed after 9 injections (ranibizumab used in 5.4% of reinjections, 43/801). During the follow-up a total of 943 injections
were given, 92 of them to the fellow eyes of 10 study patients with bilateral exudative AMD.

The VA and CS scores and their changes are presented in Table 2, publication II. VA and CS measurements correlated with each other at baseline (Spearman’s rho, r=0.567, p<0.001), and at the end of follow-up (r=0.514, p<0.001). Patients with better VA and CS at baseline had also better VA and CS after treatment (r=0.516, p<0.001 for VA, r=0.629, p<0.001 for CS), but less gain (r=-0.405, p=0.004 for VA, r=-0.489, p<0.001 for CS).

The only additional factor found to be associated with VA, or VA change during the two years was the polymorphism of ARMS2 rs10490924. At baseline, the TT homozygous patients had better VAs (median 76 letters, range from 61 to 84) than heterozygous patients (65 letters, range from 43 to 80) or GG homozygous patients (64, range from 37 to 75, p=0.016, Kruskal-Wallis). The VA gain during treatment was significantly better in patients with GG homozygous genotype (+10 letters, range from -31 to +23 letters) than in heterozygous (+3 letters, range from -33 to +15 letters) or TT homozygous patients (+0.5 letters, range from -53 to +7 letters after two years, p=0.008). This difference remained significant in multivariate modeling correcting for differences in baseline VAs (Generalized Linear Model, VA gain divided into groups “losing more than 5 letters”, “no change”, and “gaining more than 5 letters”).

The VEGF rs699947 was associated with the CS change during the two years, the CC homozygotes gaining less CS (median +0.0, range from -0.5 to +0.5, p=0.014, Mann-Whitney U) than heterozygotes (+0.15, range from -0.6 to +0.55) or AA homozygotes (+0.05, range from -0.05 to +0.75), but this difference did not retain its significance when analyzed together with the effect of baseline CS scores (Generalized Linear Model).

The number of follow-up visits at which cystic changes were detected in the OCTs correlated with less VA and CS gain during the first follow-up year (r=-0.441, p=0.001 for VA, r=-0.370, p=0.008 for CS, Spearman’s rho). The correlations did not persist in the second year. Baseline characteristics, cystic changes, or NED in OCTs or macular hemorrhage were not significantly associated with the number of needed reinjections or with the VA or CS outcomes, although a trend towards worse VA at the end of the first year was seen in patients having cysts in baseline OCTs (median 71 letters, range from 8 to 85 letters, with cysts at baseline vs. median 75, range from 35 to 84 letters, without cysts, p=0.056, Mann-Whitney U).

During the first year 27 of the 50 patients had sub- or intraretinal fluid in OCTs at every follow-up visit. The factors differentiating patients with persisting macular fluid from those who had at least one follow-up visit with no fluid
were analyzed. The factors associated with the disappearance of fluid were the
area of the baseline ICG lesion (median 1.05 mm², range from 0 to 13.3 mm²,
in responders vs. 2.5 mm², range from 0.5 to 11.9 mm², in nonresponders,
p=0.029, Mann-Whitney U), and the IL-8 rs4073 polymorphism (p=0.012,
Chi-square). In multivariate modeling, the only factor significantly associated
with persisting macular fluid was the homozygous AA genotype of IL-8 rs4073
(p=0.016, Generalized Linear Model). During the second year three more
patients had dry macula at least once. In the two-year data, only the
homozygous TT genotype of IL-8 rs4073 showed a trend towards more
frequent disappearance of macular fluid compared with the heterozygous and
AA homozygous genotypes (p=0.059, Chi-square).

The predictors of the number of needed reinjections were analyzed with
models built with the Generalized Estimating Equations procedure in SPSS.
In univariate analyses for the first year, more frequent reinjections were
associated with the area of late hyperfluorescence in the baseline ICGA
(p=0.027), the CC genotype of CFH rs1061170 (p=0.023), and the AA genotype
of VEGF rs699947 (p=0.019). In multivariate modeling, the only factor
retaining its significance was the area of late hyperfluorescence in the baseline
ICGA (p=0.029). When the two-year follow-up data were analyzed, the risk
factors for more frequent reinjections in univariate analyses were current
smoking (p=0.021), CC genotype of CFH rs1061170 (p=0.005), and AA
genotype of VEGF rs699947 (p=0.026). The area of late hyperfluorescence in
the baseline ICGA was less significant (p=0.058). In multivariate modeling,
the only significant predictors for more frequent reinjections were the CC
genotype of CFH rs1061170 and the A allele in VEGF rs699947.

Cumulative effects of the risk SNPs on the presence of intra- and subretinal
fluid in OCTs were also assessed. The proportion of follow-up visits at which
intra- or subretinal fluid was present in OCTs in relation to the total number
of visits for each patient was calculated and compared with the genotype
frequencies and with the cumulative sum of risk alleles A in IL-8 rs4073, A in
VEGF rs699947 and C in CFH rs1061170 (Figure 1, publication II). A
significant difference existed in the frequency of visits with fluid detected in
OCTs when the patients with zero to two risk alleles were compared with those
carrying at least three risk alleles (p=0.00002, Mann-Whitney U, Figure 1,
publication II). The other factors examined had no additional effects in this
model.

No associations existed between any measures of treatment outcome and age,
sex, or the other studied SNPs VEGF rs2146323, VEGF rs3025033, or C3
rs2230199. Exclusion of the visits at which bevacizumab treatment had been
switched to ranibizumab did not weaken any of the detected associations.
5.3 EARLIER ONSET OF EXUDATIVE DISEASE

To evaluate the possible association of IL-8 rs4073 with the age of onset of exudative AMD, we first analyzed the relationships of the genotypes of IL-8 rs4073, CFH rs1061170, ARMS2 rs10490924, and C3 rs2230199 and smoking behavior with the prevalence of any AMD or exudative AMD in the whole study population.

In this combined sample, the risk alleles C in CFH rs1061170 (p<0.0001, Pearson Chi-square) and T in ARMS2 rs10490924 (p<0.0001) and tobacco smoking (p<0.0001) were associated with both the prevalence of any AMD and exudative AMD, the same finding reported earlier for a subgroup of the patients included in this study. No associations were found between the genotypes of IL-8 rs4073 or C3 rs2230199 and the prevalences. In multivariate models, the effects of the SNPs and smoking remained almost identical both in the analyses carried out using the prevalence of any AMD as a dependent variable and in the analyses using the prevalence of exudative AMD as a dependent variable (Table 5).

The associations of the studied genotypes and smoking habits with the age of onset of exudative AMD were first analyzed with conventional multivariate modeling. The effects of the homozygous risk genotypes AA in IL-8 rs4073, CC in CFH rs1061170, and TT in ARMS2 rs10490924, as well as smoking were significant (Table 3, publication III).

To correct for the differences in age distributions in the groups of control subjects and patients with exudative AMD, the Cox regression model was used (Figure 1, publication III). Control subjects and patients with exudative AMD were compared to estimate the effects of the variables on the prevalence of exudative AMD (Figure 1, publication III, 1st column). For the cases, the age at onset of exudative AMD and for the control subjects the age at data collection was used as the time scale. Patients with dry AMD were not included as the age at onset of the disease in these cases could not be determined. The estimated hazard ratios (Table 6) were quite similar to effect sizes seen in the multivariate model.

The effects of the studied factors on the age of onset of exudative AMD were then analyzed including only the patients with exudative disease (Table 7). The homozygous risk genotypes AA in IL-8 rs4073 (p=0.03) and TT in ARMS2 rs10490924 (p= 0.001) as well as current smoking were associated with younger onset of exudative AMD. The heterozygous genotypes or ex-smoking did not increase the risk of developing exudative disease in this patient material. Also, the genotype of CFH rs1061170 was less significant in this analysis. A trend towards earlier onset of disease was seen only when the
genotypes CT and TT were combined and compared with the homozygous risk genotype CC (p=0.05, Figure 1, publication III, 2nd column).

We assumed that the frequency of a more delayed diagnosis of exudative AMD before the era of widely used OCTs and availability of more effective treatments could have been quite high. Therefore, a separate analysis of the data of patients diagnosed during the last five years of data collection was done (n=100, between April 2005 and April 2010). In this patient group, the effect of IL-8 rs4073 on the age at diagnosis was even clearer (HR=2.9, p=0.0003, Figure 1, publication III, 3rd column).

Table 5. Associations of the studied genetic variations and tobacco smoking on the prevalence of exudative AMD estimated with the Generalized Linear Models procedure in SPSS 22.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>B</th>
<th>SE</th>
<th>Lower</th>
<th>Upper</th>
<th>Wald Chi-Square</th>
<th>p-value</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>2.218</td>
<td>0.648</td>
<td>0.949</td>
<td>3.488</td>
<td>11.73</td>
<td>0.001</td>
</tr>
<tr>
<td>Ex</td>
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<td>0.311</td>
<td>1.545</td>
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<td>Never</td>
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<td>0.000</td>
<td>0.000</td>
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<tr>
<td>CFH rs1061170 genotype</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>2.338</td>
<td>0.429</td>
<td>1.497</td>
<td>3.178</td>
<td>29.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CT</td>
<td>1.132</td>
<td>0.334</td>
<td>0.477</td>
<td>1.787</td>
<td>11.48</td>
<td>0.001</td>
</tr>
<tr>
<td>TT</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>ARMS2 rs10490924 genotype</td>
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</tr>
<tr>
<td>TT</td>
<td>3.690</td>
<td>0.771</td>
<td>2.174</td>
<td>5.196</td>
<td>22.84</td>
<td>&lt;0.0001</td>
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<tr>
<td>GT</td>
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<td>0.282</td>
<td>0.822</td>
<td>1.926</td>
<td>23.80</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GG</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>GG</td>
<td>0.504</td>
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<td>-0.861</td>
<td>1.868</td>
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<tr>
<td>GC</td>
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<td>0.12</td>
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<tr>
<td>CC</td>
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<td>IL-8 rs4073 genotype</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>AA</td>
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<tr>
<td>AT</td>
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<td>-0.221</td>
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<td>0.22</td>
</tr>
<tr>
<td>TT</td>
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<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Intercept</td>
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<td>0.422</td>
<td>-2.728</td>
<td>-1.074</td>
<td>20.30</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

B, estimate of the effect size; SE, standard error of the estimate
The sum of homozygous risk genotypes of IL-8, CFH, and ARMS2 and tobacco smoking had a cumulative effect on the age of onset of exudative AMD (one factor assigned for each homozygous risk genotype and for current smoking). The sum of the risk factors correlated inversely with the age at diagnosis (r = -0.348, p < 0.0001, Spearman’s rho). Significant differences were observed between the patients with zero risk factors (84 patients, median age 77.6 years, range from 62.8 to 87.6 years) and the patients with one (107 patients, median age 74.5 years, range from 53.1 to 91.2 years, p = 0.0001), two to four (52 patients, median age 71.8 years, from 54.5 to 83.8 years, p < 0.0001), or three to four risk factors (6 patients, median age 68.3 years, range from 63.3 to 69.8 years, p = 0.0002, Figure 2, publication III).

The CNV lesion type had no association with the age of onset of exudative AMD. Sex had no effect in any of the analyses. No interactions between the variables were found in any of the statistical models used.

**Table 6. Hazard ratios for getting exudative AMD, both control subjects and patients with exudative AMD included calculated with the Cox Regression Model procedure in SPSS 22. The significance of each variable in the model, as well as the significances and hazard ratios for the genotype subgroups are presented.**

<table>
<thead>
<tr>
<th>Variables in the Equation</th>
<th>p-value</th>
<th>HR</th>
<th>95% CI for HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 rs4073</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT vs. TT</td>
<td>0.74</td>
<td>1.0</td>
<td>0.72 - 1.26</td>
</tr>
<tr>
<td>AA vs. TT</td>
<td>0.69</td>
<td>1.1</td>
<td>0.72 - 1.63</td>
</tr>
<tr>
<td>CFH rs1061170</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT vs. TT</td>
<td>0.011</td>
<td>1.7</td>
<td>1.13 - 2.55</td>
</tr>
<tr>
<td>CC vs. TT</td>
<td>&lt;0.0001</td>
<td>2.7</td>
<td>1.74 - 4.08</td>
</tr>
<tr>
<td>ARMS2 rs10490924</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GT vs. GG</td>
<td>0.003</td>
<td>1.6</td>
<td>1.16 - 2.13</td>
</tr>
<tr>
<td>TT vs. GG</td>
<td>&lt;0.0001</td>
<td>3.3</td>
<td>2.25 - 4.78</td>
</tr>
<tr>
<td>C3 rs2230199</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC vs. CC</td>
<td>0.065</td>
<td>1.3</td>
<td>0.98 - 1.74</td>
</tr>
<tr>
<td>GG vs. CC</td>
<td>0.42</td>
<td>1.4</td>
<td>0.63 - 3.00</td>
</tr>
<tr>
<td>Smoking</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex- vs. never smoker</td>
<td>0.042</td>
<td>1.4</td>
<td>1.01 - 1.83</td>
</tr>
<tr>
<td>Current vs. never smoker</td>
<td>&lt;0.0001</td>
<td>2.3</td>
<td>1.62 - 3.36</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval
Table 7. Hazard ratios for onset of exudative AMD, only patients with exudative AMD included, calculated with the Cox Regression Model procedure in SPSS 22. The significance of each variable in the model as well as the significances and hazard ratios for the genotype subgroups are presented.

<table>
<thead>
<tr>
<th>Variables in the Equation</th>
<th>p-value</th>
<th>HR</th>
<th>95% CI for HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 rs4073</td>
<td>0.025</td>
<td>0.9</td>
<td>0.67 - 1.20</td>
</tr>
<tr>
<td>AT vs. TT</td>
<td>0.45</td>
<td>1.6</td>
<td>1.04 - 2.40</td>
</tr>
<tr>
<td>AA vs. TT</td>
<td>0.033</td>
<td>1.6</td>
<td>1.04 - 2.40</td>
</tr>
<tr>
<td>CFH rs1061170</td>
<td>0.12</td>
<td>1.6</td>
<td>1.04 - 2.40</td>
</tr>
<tr>
<td>CT vs. TT</td>
<td>0.66</td>
<td>1.1</td>
<td>0.73 - 1.65</td>
</tr>
<tr>
<td>CC vs. TT</td>
<td>0.10</td>
<td>1.4</td>
<td>0.93 - 2.18</td>
</tr>
<tr>
<td>ARMS2 rs10490924</td>
<td>0.002</td>
<td>1.1</td>
<td>0.77 - 1.44</td>
</tr>
<tr>
<td>GT vs. GG</td>
<td>0.75</td>
<td>1.9</td>
<td>1.28 - 2.78</td>
</tr>
<tr>
<td>TT vs. GG</td>
<td>0.001</td>
<td>1.9</td>
<td>1.28 - 2.78</td>
</tr>
<tr>
<td>C3 rs2230199</td>
<td>0.20</td>
<td>1.3</td>
<td>0.97 - 1.72</td>
</tr>
<tr>
<td>GC vs. CC</td>
<td>0.08</td>
<td>1.3</td>
<td>0.60 - 2.85</td>
</tr>
<tr>
<td>GG vs. CC</td>
<td>0.50</td>
<td>1.3</td>
<td>0.60 - 2.85</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.022</td>
<td>1.0</td>
<td>0.73 - 1.33</td>
</tr>
<tr>
<td>Ex- vs. never smoker</td>
<td>0.93</td>
<td>1.6</td>
<td>1.13 - 2.31</td>
</tr>
<tr>
<td>Current vs. never smoker</td>
<td>0.009</td>
<td>1.6</td>
<td>1.13 - 2.31</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval

5.4 FLARE DURING BEVACIZUMAB TREATMENT

In the two-year follow-up data, the anterior chamber flare value was at baseline 7.8 photons/millisecond (ph/ms, SD, ±4.4) in the 50 study eyes, and 6.9 ph/msec (SD ±3.9) in the 49 fellow eyes. The mean flare values measured during the follow-up correlated strongly between the study and fellow eyes (8.2 ±4.0 in the study eyes and 7.4±4.0 in the fellow eyes, r=0.79, p<0.0001, Spearman’s rho) and with the baseline measurements (r=0.93, p<0.0001 in study eyes and r=0.86, p<0.0001 in fellow eyes). The mean flare values correlated moderately with the mean clinical grades of flare (r=0.34, p=0.15), and mean grades of cell count (r=0.34, p= 0.015) determined with biomicroscopy according to the international standardized classification.280 The clinical grade of flare was “none” in 84% of visits (992/1178), “faint” in 13% (156/1178), and “moderate” in 0.3% (3/1178), and the cell count “0” in 74% (869/1178), “0.5” in 23% (274/1178), and “1” in 0.9% (11/1178).
After the first bevacizumab injection, a rise in the flare values was detected in 24/35 study eyes at week one (p=0.034, Wilcoxon signed-ranks, median change +0.6 ph/ms, range from -6.5 to 4.0). The values decreased at week three in 22/36 eyes compared with baseline (p=0.275, median change -0.2, range from -6.3 to +2.8), but the decrease was significant only when compared with the values at week one (p=0.007) (Figure 1, publication IV).

In univariate analyses, higher values in the study eyes were associated with older age (p=0.006, Linear Mixed Models), higher number of smoking pack-years (p=0.019), pseudophakia (p=0.003, 20/50 study eyes), and the presence of cystic macular fluid in OCTs (p=0.041). In the study eyes, a gradual increase in the flare was observed during the follow-up, correlating inversely with the area of classic CNV (p=0.016). The levels of flare were analyzed in relation to the presence of intra- and/or subretinal fluid in OCTs, and simultaneously in relation to the need for reinjections, as fluid in OCTs served as a criterion for retreatment. The flares at the injection visits did not differ significantly from the flares measured at single visits when the macula was dry only once before the recurrence of macular fluid, or from the flares at the first visits of longer periods during which the macula remained dry. The flare decreased significantly, however, when the injection-free period lasted at least for two consecutive visits (p=0.005). The values did not change towards the end of a treatment-free period or between the last visit with dry macula and the first visit with recurring fluid or hemorrhage. Therefore, no change preceded the reappearance of fluid in OCTs. No correlation existed between the values of flare and the number of needed reinjections or the tendency of the lesion to dry during the treatment. The values did not correlate with the changes in VA or CS, the baseline CNV lesion size, or change in lesion size in FAs or ICGAs during the two years.

In the multivariate model, the effects of patient’s age, smoking pack-years, treatment-free periods lasting at least two visits, and length of follow-up remained significant predictors of the level of flare (Table 2, publication IV). The interaction between the length of follow-up and the area of classic CNV meant smaller increase in flare in eyes with larger areas of classic type CNV suggesting that BRB function deteriorated less in the eyes with classic CNV component (n=16) compared with the eyes without any classic CNV.

The gradual increase in flare during the follow-up was observed also in the fellow eyes (p=0.014). Higher values were associated with older age (p=0.007) and pseudophakia (p=0.019, 18/49 fellow eyes).

The fellow eyes were also analyzed for differences in flare in the following categories: remained dry (n=34), became exudative during follow-up (n=7), and had old exudative AMD lesion with scarring (no active treatment, n=6).
Two patients received anti-VEGF injections for both eyes for bilateral active exudative disease and were not included in these analyses. At baseline, no difference in flare existed between the categories (p=0.776, Mann-Whitney U). The seven fellow eyes that developed exudative AMD during the follow-up, however, had significantly higher mean flare values in the weekly measurements during the first month (p=0.009), and in the monthly measurements during the first 6 months (p=0.030) compared with the fellow eyes that remained free of exudative AMD during the whole follow-up (Figure 2). However, when the effect of age was taken into account with multivariate modeling (Generalized Linear Model) only the difference in the weekly measurements remained significant since these patients were also older. No predictive changes in flare were observed immediately preceding the conversion to exudative disease. The baseline AMD grade in the CARMS scale did not differ between the fellow eyes developing and not developing exudative AMD (p = 0.741, Mann-Whitney U).

Figure 2. Flare of fellow eyes during the first 6 months of follow-up.
6 DISCUSSION AND CONCLUSIONS

Although VEGF is the major proangiogenic factor in the eye, there are also other signaling molecules capable of causing neovascular growth and leakage. The amplified activation of these compensatory pathways is not surprising in situations where the primary trigger in the target tissue is still present and anti-VEGF agents inhibit the main response.

Older age of the patient, worse VA at the beginning of treatment, and larger size of the lesion have been robust predictors of worse treatment response, probably at least partly reflecting both advanced disease process and poor functional reserves. In clinical practice, it may also be helpful to identify those lesion characteristics in OCTs or angiographies that predict initial and long-term treatment outcomes. It remains largely unknown why in some patients chronic intraretinal cystic fluid persists or subretinal fibrosis develops, while in the others they do not.

In our short-term data (study I), in agreement with the results of earlier studies, VA response to the initial treatment was associated with the size of the CNV and with cystic macular fluid not disappearing after the first injections. Moreover, patients with better VAs at baseline had better VAs after the treatment, but also less VA gain.\textsuperscript{8, 201, 204, 218, 254-257} We could not find any additional predictors connected to the VA results. However, the anatomic response was associated with the IL-8 rs4073 promoter polymorphism linked to the regulation of the expression of the IL-8 gene and the levels of produced IL-8. Increased synthesis of IL-8 might be one of the adapting mechanisms of the retina to compensate for the iatrogenic lack of VEGF.

The lesion type in FA seemed to correlate with the initial anatomic response. The characteristics of choroidal neovascularization may affect the type, volume and persistence of fluid accumulating in the macula.\textsuperscript{282} Regardless of the true nature of this correlation, in multivariate analysis IL-8 rs4073 remained an independent predictor of the morphological treatment outcome.

Elevated intravitreal concentrations of IL-8 and other proangiogenic factors have been reported in patients with exudative AMD and proliferative diabetic retinopathy after bevacizumab injections.\textsuperscript{283, 283, 284} The same phenomenon has not been observed in patients with diabetic macular edema or venous occlusion.\textsuperscript{285-288} The cytokine environment in vascular diseases may, however, differ considerably from that existing in eyes with exudative AMD.

In the two-year follow-up data (study II), associations were found between the LOC387715 rs10490924 and VA gain, between persisting intra- or subretinal
fluid in macular OCTs and the IL-8 rs4073, and between the variants of VEGF rs699947 and CFH rs1061170 and the number of needed reinjections. The CFH rs1061170, IL-8 rs4073, and VEGF rs699947 had a marked cumulative effect on the frequency of fluid detected in OCTs during the two years.

The C allele in VEGF rs699947 has been connected to higher levels of secreted VEGF. Better response to anti-VEGF treatment has been variably associated with the genotype of VEGF rs699947. In our material, a small difference in CS gain was seen between the genotypes, but the effect did not remain significant in multivariate analysis. The A allele in VEGF rs699947 was more strongly associated with persisting fluid in OCTs and more frequently needed reinjections during the two-year follow-up. A possible association of abnormally thick macular retina (>212 μm) with the AA and AC genotypes can also be seen in the one-year results of the CATT study, especially if the distributions of allelic frequencies and thick retinas were examined separately (A allele vs. C allele, p=0.0007, Chi-square, calculated from the data provided in the article), but these associations were not analyzed in the original report.

The homozygous risk genotype CC of CFH rs1061170 was also found to be a risk factor for more frequent reinjections during the two-year follow-up. During the first year the area of late hyperfluorescence in the baseline ICGA had some effect, but its significance decreased during the second year, and in the two-year data while smoking seemed to affect the frequency of reinjections, the effect was not significant in multivariate analysis.

As in many previous studies, in our data cystic retinal changes in OCTs were associated with less VA and CS gain during the first year of treatment. The association did not, however, persist in the second year. At that stage, the VA and CS changes may reflect the development of other pathological changes such as atrophy of the RPE or the neuroretina or subretinal fibrosis.

The sum of risk alleles A in IL-8 rs4073, A in VEGF rs699947, and C in CFH rs1061170 was found to correlate with the presence of fluid in macular OCTs better than the allele frequencies separately. The combination of deficient regulation of the alternative complement pathway together with higher IL-8 production may lead to IL-8-stimulated angiogenesis and capillary leakage. In patients with the less producing VEGF genotype, the potential compensatory mechanisms, such as IL-8 production, may be more active. Low expression of VEGF gene may result in very low levels of retinal VEGF during the anti-VEGF therapy, and further blocking of the VEGF signaling pathway does not necessarily result in diminished CNV activity.

The association of the IL-8 rs4073 with younger age at diagnosis of exudative AMD (study III) suggests that IL-8 may have some role in the chronic
inflammatory process caused by the established risk factors for AMD in such a way that the patients who will get the exudative disease present with symptomatic CNV earlier.

The associations of IL-8 polymorphisms with the prevalence of AMD have been controversial. IL-8 SNPs have been variably associated with the prevalence of atrophic and exudative AMD. The differences in allelic frequencies in the previous studies have been smaller than the difference that we estimated we could detect with our sample size with a power of 0.8 (>13%, p-level 0.05). In our data, however, the AA genotype was actually more common in the control subjects than in AMD patients, although the difference was not significant (21.8% in controls, 15.0% in patients with exudative AMD, and 16.4% in patients with any AMD). The results suggest that the IL-8 polymorphism may have a stronger association with the tendency of CNVs to develop earlier, with more rapidly progressing exudative disease, or with more pronounced retinal edema, leading to more obvious symptoms earlier in the course of the disease than with the overall prevalence of AMD. The effect of the risk genotype would then be seen in the incidence or prevalence of exudative AMD only if the younger age groups were studied separately.

The possible connections between the anterior chamber protein concentration and the activity of CNV lesion were analyzed in study IV. The flare values were higher if cystic macular fluid was present in OCTs and lower if treatment-free periods lasted longer than one follow-up visit. The flare did not predict the activity of a CNV lesion or treatment response in any way.

The flare correlated with the age of the patient and with pseudophakia, as seen in previous studies. A new finding was that the values also seemed to correlate with smoking pack-years in the study eyes. Tobacco smoke causes oxidative stress and inflammatory reactions. The chemicals in tobacco smoke have been shown to impair RPE function and to negatively affect the blood-brain barrier function and integrity. These effects were, however, not seen in the fellow eyes. The reason for this is unknown, but the BRB function may need to be deteriorated before the effect of smoking is so clear that it is reflected in the levels of protein in the anterior chamber.

Previously, higher flare values have been found to correlate with the size of the CNV lesion in the advanced stage of the disease with fibrous proliferation and hemorrhage. We could not find any association between the size of the CNV lesion and flare, but all of our patients had a recently diagnosed and treatment-naïve lesion without fibrosis or significant hemorrhage. However, in our data, the area of classic CNV correlated with a relatively lower rise of flare during the two-year follow-up. The reason for the finding may be a relatively larger reduction in the leakage of the classic CNV membrane when scarring and fibrosis take place since in addition to the impairment of inner BRB function
caused by the neovessels, the classic type of CNV grows through the RPE, breaking also the outer BRB.

In our data, the flares of the study eyes increased at week one. The rise in protein concentration in our material may have been caused by the bevacizumab molecule itself or by an inflammatory response caused by the injected product or the procedure. Ziemssen and associates did not find any rise in the flare at week one. Differences in the results may reflect differences in the patient or CNV lesion characteristics or in the duration of the disease. Regardless of the reasons behind this elevation of flare, the values were already reverted to the baseline level at week three, and it may be assumed that the factors causing the rise at week one were no longer present at the first monthly visit. Over the two years, a similar gradual increase of flare was observed both in the study eyes and in the fellow eyes, probably reflecting either the increasing age of the patients or progression of RPE dysfunction. Both the recovery of the baseline level of flare before the first monthly visit and the similar rise in flare in study and fellow eyes support the assumption that the studied variations of flare were not caused by the treatment itself.

An interesting finding was the presence of higher flare during the early phase of follow-up in the fellow eyes that developed exudative AMD later during the follow-up. The finding possibly reflects the same changes of increased leakage before conversion to the exudative disease detected earlier with the retinal leakage analyzer. Changes in the BRB function seem to exist very early in the process leading to CNV growth. Possible explanations could be the presence of abnormally functioning vasculature existing before the neovessels are observed in the conventional angiograms, or a change in the inflammatory environment preceding the growth of CNV and affecting the levels of flare. The wide individual variations of the measured flare diminish its prognostic value in predicting the progression of AMD in clinical practice.

A limitation of our study is the relatively small patient populations. In addition, in the two-year follow-up, seven patients did not complete the second follow-up year, which may affect the evaluations of predictors of VA and CS outcomes. The generalizability of the results of the studies using only the data gathered from the two-year follow-up (II and IV), may also be affected by the study design requiring monthly visits. The participating patients were probably healthier and younger than the general exudative AMD patient population. The VA gains in our two-year follow-up were poorer than in the PRN bevacizumab treatment group in the CATT study. In our study, however, the baseline VAs were considerably higher than those in the CATT, probably accentuating the ceiling effect [at baseline, VA>82 letters in 2% (1/50 patients) vs. 0% in CATT; 68–82 letters in 52% (26/59) vs. 39% in CATT; 53–67 letters in 40% (20/50) vs. 36.2% in CATT, and VA<53 letters only 6% (3/50) vs. 24.9% in CATT].

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The associations of the genotypes connected to higher production of IL-8 with poorer anatomic response, persisting macular fluid, and younger onset of exudative disease suggest that IL-8 may be an important mediator in both CNV development and its persistence and leakage during bevacizumab treatment. The upregulation of the compensatory angiogenic mechanisms may have relevance in the cases that respond poorly to the anti-VEGF therapy. Reliable and noninvasive methods to measure the function of BRB in AMD patients would facilitate diagnosis of CNV growth during a very early phase of the exudative process, significantly improving the treatment results.
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“Forty-two!” yelled Loonquaw. "Is that all you've got to show for seven and a half million years' work?"

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