Systemic Changes in Neovascular Age-Related Macular Degeneration

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This review has been accepted as a thesis together with three previously published papers by University of Copenhagen 19 of December 2013 and defended on 25 of April 2014

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This PhD dissertation is based on the following three scientific papers:


1. BACKGROUND

Age-related macular degeneration is a chronic condition where degeneration of the photoreceptor-dense central retina leads to progressive visual impairment. First described in the medical literature as “symmetrical central chorioido-retinal disease occurring in senile persons” in 1874, the condition has been a significant cause of blindness in many people, as it was untreatable for more than a century [1]. In the 1980’s, ophthalmologists began attempting treatment with the use of thermal laser and photodynamic therapy, often with disappointing outcomes [2,3]. The introduction of anti-VEGF therapy for choroidal neovascularisation in 2004 was a major breakthrough, as it was effective in maintaining or even restoring vision in a significant proportion of patients [4,5]. Despite these encouraging advancements, no definitive or permanent cure for AMD has been discovered. Further research on the underlying pathogenic mechanisms is warranted if we are to find novel targets of therapy. Recent findings have emphasised that in order to understand the pathogenesis and clinical course of AMD better, one may need to look beyond the local environment of the retina [6]. The role of the immune system in AMD development is well-established and crucial for the development of AMD; hence, studying the modulators of the immune system may help answer vital questions related to the pathogenesis of AMD.

1.1. Anatomy of the human retina (Figure 1.1)

The human retina is a light-sensitive layer lining the inside of the posterior segment of the eye [7]. It consists of two distinct layers: 1) the neuroretina consisting of photoreceptor cells, neuronal cells, and glia cells; and 2) the retinal pigment epithelium (RPE) separated from the neuroretina by a virtual subretinal space. While the neuroretina serves to convert light into neural signals, the RPE constitutes the outer blood-retinal barrier (BRB), preventing extracellular fluid leaking into the subretinal space from the underlying choriocapillaris (a continuous layer of fenestrated capillaries), actively pumping fluid out of the subretinal space, and regulating trafficking of immune cells across the BRB [8]. Moreover, the RPE facilitates photoreceptor turnover by phagocytosis and lysosomal degradation of outer segments following shedding. The choriocapillaris arises from the branches of posterior ciliary arteries and supplies the photoreceptor-RPE complex and outer neuroretina, while the inner parts of the neuroretina are supplied by the central retinal artery [9]. Between the RPE and choriocapillaris lies a membrane which separates the two, known as the Bruch membrane. Integrity of the Bruch membrane appears to be important in suppressing invasion of vessels from the chorial circulation into the retina [10].

The macula (or macula lutea) is an area in the retina which measures about 5.5 mm in diameter and coincides with the course of the major temporal arcades. It is responsible for the central 15-20° of the visual field and provides the highest resolving power of the eye. Histologically, it differs from the peripheral retina by having more than one layer of neurons specialised in transmitting visual input to parts of the brain, known as retinal ganglion cells. Also, mature RPE cells in the peripheral, but not central retina are capable of proliferating and migrating to the central senescent regions [11]. In clinical practice, the macula can be observed by using direct or indirect ophthalmoscopy, often supplemented with an imaging modality which allows for a more detailed observation and recording.

1.2. Age-related macular degeneration

1.2.1. Clinical features (Figure 1.2)

AMD is a clinically heterogeneous disease with a variable presentation. The early stages, sometimes referred to as age-related maculopathy (ARM), are characterised by the presence of cellular debris at the interface between RPE and Bruch membrane (or between the RPE and neuroretina), known as drusen, and alterations in the RPE cells leading to hypo- or hyperpigmentation [12]. At this stage, the patient may be asymptomatic or complain of blurred vision, visual scotomas, decreased contrast sensitivity, abnormal dark adaptation or reading difficulties. Vision often becomes severely impaired if the disease progresses to the later stages of AMD, which may be either “dry” or “wet”, and are not mutually exclusive. In the dry form, also known as geographic atrophy or atrophic AMD, atrophic changes in the macula may lead to gradual and insidious loss of vision with central or pericentral scotomas over a course of months to years [14]. In the wet form, also known as neovascular or exudative AMD, accelerated and profound visual loss typically occurs as a result of choroidal neovascularisation, (CNV), where new vessels grow and invade the retina resulting in sub-RPE or subretinal haemorrhages, or fluid accumulation in or below the layers of the retina [15]. Although less common than its dry counterpart, wet AMD accounts for more than 80% of cases with severe visual loss or legal blindness resulting from AMD [15]. Without treatment, most patients will lose a significant proportion of their central vision with severe consequences for the patients, in terms of quality of life and their ability to perform tasks of everyday living [16]. In today’s era of anti-VEGF treatment, stabilisation or restoration of visual acuity is possible in the majority of cases with wet AMD, but a proportion of these patients will not respond positively to treatment as a result of various factors, including tachyphylaxis and fibrogenesis in the subretinal space. The latter permanently obliterates the retinal architecture often resulting in severe, irreversible visual loss [17].

Figure 1.1. Anatomy of the macula. The macula lies in the visual axis, temporal to the optic nerve (A). The fovea lies centrally in the macula and is responsible for sharp, central vision (B). Adapted from [12]. Fundus angiography showing the macular region which coincides with the course of the major temporal arcades (C).
1.2.2. EPIDEMIOLOGY
AMD is the leading cause of central visual impairment in persons aged 50 years and above in the Western world [6,18-22]. In the Third National Health and Nutrition Examination Survey, Klein et al. reported the prevalence of any AMD to be 9.2% in non-institutionalised persons above 40 years of age [23]. In the same year, the Blue Mountain Study by Mitchell’s group found the prevalence of end-stage AMD in 1.9% of the population, rising from 0% among people younger than 55 years of age to 18.5% among those 85 years of age or older [24]. In a more recent study by Owen et al., late-stage AMD was found to affect 2.4% of persons aged 50 years or above and 12.2% of persons aged 80 or above. AMD is also a very common cause of visual impairment in Asia with prevalence rates comparable with that reported from Western populations [21,25]. In absolute numbers, visual impairment as a result of AMD is estimated to affect more than 30 million individuals worldwide with an economic burden of more than USD 300 million annually [26]. The burden of AMD is expected to increase considerably in the coming years, calling for new and better developments in prevention and treatment of AMD [27].

1.2.3. TREATMENT
Presently, there is no definitive or permanent cure available for AMD. A combination of supplements has shown some effect in slowing the progression of AMD, and direct inhibition of VEGF in the eye is the mainstay of treatment for CNV. There are several registered trials looking specifically at treatment options for geographic atrophy, but so far no conclusive results have been published. Similarly, there is no effective treatment for prevention or reversal of subretinal fibrosis in AMD.

Preventive measures
The Age-Related Eye Disease Study was a prospective, multicentric, randomised clinical trial conducted between 1992 and 2006, which assessed the efficacy of a preformulated combination of antioxidants and minerals (15 mg of beta-carotene; 500 mg of vitamin C; 400 IU of vitamin E; 80 mg of zinc; and 2 mg of copper to prevent zinc-induced anemia), in reducing the risk of advanced AMD in high-risk patients, i.e. extensive intermediate size drusen, at least 1 large drusen, noncentral geographic atrophy in 1 or both eyes, or advanced AMD or vision loss due to AMD in 1 eye [28]. This combination was reported to reduce the risk of advanced AMD by 25% in high-risk patients and has since been used widely in patients at high risk of developing advanced AMD. AREDS-2 was launched in 2006 and was designed to assess whether adding DHA/EPA (omega-3 fatty acids) or lutein and zeaxanthin (macular xanthophylls) could further slow progression of vision loss from AMD. Adding DHA/EPA or lutein/zeaxanthin to the original AREDS formulation had no additional overall effect on the risk of advanced AMD. However, taking lutein/zeaxanthin instead of beta-carotene reduced the risk of advanced AMD slightly, compared to those who took AREDS with beta-carotene. Since beta-carotene is associated with a higher risk of lung cancer in current and former smokers, it may be better to replace it with lutein and zeaxanthin [29].

Treatment of manifest wet AMD
Once new vessels proliferate and invade through the Bruch’s membrane, the target of treatment becomes direct inhibition of VEGF in the eye. Currently, there are three FDA-approved drugs for use in patients with wet AMD: 1) Pegaptanib, approved in 2004, is a specific inhibitor of VEGF-165 and will not be discussed further as it is not routinely used to treat wet AMD anymore; 2) Ranibizumab, approved in 2006, is a recombinant, monoclonal, humanised antibody which inhibits VEGF-A [30]; and 3) Aflibercept, approved in 2011, is a fusion protein which, in addition to inhibiting VEGF-A, also inhibits VEGF-B and placental growth factor and is non-inferior to ranibizumab in treating wet AMD [31]. Clinical trials have shown that ranibizumab was effective in maintaining (i.e. losing less than 15 ETDRS letters) visual acuity in about 90% of patients with wet AMD, compared to about 53% who received sham and about 66% who received photodynamic therapy [30]. Additionally, about 30-40% of patients experience an improvement in visual acuity ≥ 15 letters compared to about 5% of sham-receiving patients. Some patients who benefit from ranibizumab initially develop resistance or tachyphylaxis after some time rendering themselves irresponsible to the drug [17,12,33]. Some of these patients with refractory or recurrent wet AMD may benefit from aflibercept [34-37].

1.2.4. PATHOGENESIS
The pathogenesis of AMD is complex, multifactorial and incompletely understood. As mentioned in the epidemiology section, advancing age is one of the most important risk factors in AMD [38]. Aging introduces several physiological changes in the retina, such as a reduction in the number and function of photoreceptors, ganglion- and RPE cells [39]. The Bruch’s membrane thickens and its biochemical properties change leading to dysfunction of the RPE-cell layer [40-42]. Moreover, increased aging is also associated with an accumulation of oxidative damage and immunological disturbances in the retina resulting in injury and malfunction [39]. While physiological aging is undoubtedly an important pathogenic factor in AMD, it is not sufficient to cause the disease, and genetic factors along with several environmental factors also play a role. The genetic component in AMD is suggested by the association between AMD and a positive family history as well as several single-nucleotide polymorphisms (SNPs). Most of the genes involved in AMD are regulators of complement pathways, indicating an important role of immune dysregulation in the pathogenesis of AMD [43]. Smoking is associated with a two- to three-fold increased risk of AMD [44], and when associated with a “pro-AMD” genetic profile (e.g. homozgyosity for the CFH Y402H polymorphism located in the hepary and C-reactive protein binding domain which may cause complement dysregulation [45]), the relative risk of AMD is increased by a factor 34 [46]. Moreover, failure to quit smoking after being diagnosed with wet AMD is associated with a significant worse response to anti-VEGF therapy [47]. The mechanisms through which smoking affects the eye are not fully understood and may be multiple. Firstly, smoking may reduce choroidal blood flow, promoting ischaemia, hypoxia, and micro-infarction, all of which render the macular susceptible to degenerative changes [48]. Smoking has also been shown to reduce the macular pigment optical density, which appears to protect the macula from damage [49]. Finally, smoking may interact with the complement pathway by activating the alternative pathway, perhaps by modifying C3 [50,51]. Other environmental and clinical risk factors include light exposure, previous cataract surgery, higher body mass index, cardiovascular disease, and high-glycaemic-index diet low on antioxidants [38,52-54].
“para-inflammation” to denote this milder form of inflammation, which lies inter-
mediate to basal states and classic inflammatory states, and may share common
underlying mechanisms and biomarkers with classical inflammatory states [58]. The
para-inflammatory process is basically a protective response which seeks to help
tissues adapt to noxious stimuli and restore homeostasis. However, para-
inflammation may become chronic and detrimental when overwhelmed by sus-
tained tissue injury or dysfunctional changes in the immune system, resulting in
tissue damage [58,59]. Indeed, when mice were exposed to chronic stress following
whole body irradiation, a para-inflammatory response was initiated, characterised
by increased infiltration of bone-marrow derived myeloid cells, enhanced expression
of chemokines, complement components, and microglial activation [60]. The reti
na is known to have an endogenous immune system, which is coordinated by different
cell types, including microglia, dendritic cells, peripheral macrophages and RPE
cells [61,62]. Thus, the concept of para-inflammation is also applicable to the retina,
especially the macula, since this region is functionally dependent on nonproliferative
cells and characterised by very high metabolism and oxidative stress [39]. Accord-
ting to Harman’s “free radical theory of aging”, AMD results from an imbalance between
free radical-induced tissue damage and the reparative processes of the host [63].
Moreover, aging introduces several changes in genes which are involved in the
immune response, such as genes regulating leukocyte activation, chemotaxis,
endocytosis, complement activation, phagocytosis, and myeloid cell differentiation
[64]. Hence, AMD may be considered a disease in which para-inflammatory mecha-
nisms fail as a result of genetic or environmental causes [65].

The complement system

The complement system consists of many plasma and membrane-bound proteins
that react in concert to opsonise pathogens and induce a series of inflammatory
responses that help fight infection [66,67]. In addition to protecting the body against
unwanted threats, the complement system is also involved in several non-
inflammatory processes, such as angiogenesis, tissue fibrosis, clearance of immune
complexes, and lipid metabolism [67,68]. Activation of C3, a central protein in the
complement cascade, is attained by one of three pathways (Figure 1.3). The ability to
distinguish between healthy, autologous cells and unhealthy, unwanted cells or
pathogens is necessary to avoid unnecessary collateral damage to autologous cells.
For this reason, healthy autologous cells express a variety of complement regulatory
proteins (CRggs) on their surfaces. Three proteins of this kind expressed ubiquitously
on human cells are CD46 (Membrane Cofactor Protein), CD55 (Decay Accelerating
Factor), and CD59 (Membrane Inhibitor of Reactive Lysis).

The role of the complement system in AMD was first proposed about two decades ago
following immunohistochemical detection of complement component 3d in
specimens of subretinal membrane from patients with wet AMD [69,70]. This
hypothesis was reinforced in 2005 with the discovery of a clear association between
specimens of subretinal membrane from patients with wet AMD [69] and altered blood levels of complement proteins [70]. Moreover, altered blood levels of complement proteins have been reported in patients with AMD [75-81]. Recently, RPE-cells overlying affected areas of AMD have been shown to have decreased expression of CD46 and CD59 [82,83]. Thus, even though the evidence linking complement dysregulation to AMD risk is strong, the underlying pathways through which these proteins increase risk of AMD are still only partly discovered (Figure 1.3).

Microglia in AMD

Microglial cells are specialised immune cells which reside in the brain and retina, and
are responsible for the initial detection of noxious stimuli arising in the local micro-
environment [84]. Sometimes called the resident macrophages of the CNS or retina,
microglia are functionally different from blood-derived macrophages, which origi-
nate from bone-marrow derived monocytes and enter the CNS and retina during
pathological conditions. Microglia are believed to originate from macrophages
present in the yolk sac [85]. Usually, blood-derived macrophages do not contribute to the mature microglial pool, suggesting that the microglial population is sustained by local progenitors [86,87].

In the healthy retina, microglia are distributed throughout the inner and outer
photoreceptor layers, where they carry out constant and dynamic surveillance of the
extracellular microenvironment [88]. Structurally, microglia are very adaptable,
changing their phenotype depending on location and role. This plasticity ensures
prompt microglial responses to tissue injury or stress without causing immunological
imbalance under normal circumstances [89]. However, maladaptive inflammatory
microglial responses have been implicated in the progression of various chronic
neurodegenerative diseases, such as Alzheimer’s disease [90]. The phenotypical
changes which cause microglia to become enlarged and activated may result from
accumulation of minor neuronal insults in the aging brain and/or oxidative damage
[91]. This may also be the case in the retina, which is regarded to be an outcrop of
the CNS, or vice versa (Figure 1.4). Indeed, aging is associated with increased num-
bers of retinal microglia in mice, and along with tissue stress or injury, aging causes
retinal microglia to undergo phenotypic changes whereby they become larger and
less dendritic, which are typical morphological signs of activation [65]. While micro-
glia are generally believed to return to their ramified phenotype and leave the
subretinal space once the injury has subsided (e.g. when an AMD lesion becomes inactive in atrophy or fibrosis), the response of aging microglia to injury is slower and less reversible fashion [92]. Moreover, microglia migrate from the inner retina to the subretinal space (the potential space between the photoreceptor outer segments and the apical surface of the RPE-cells) which is normally devoid of micro-
glia. The importance of microglial accumulation of activated microglia in AMD is not
clear, but could be both a symptom of inflammatory damage and a benefi-
tial response to injury, since infiltration of microglia/macrophages to sites of retinal
injury may promote neovascularisation, while impairment of this accumulation in
the subretinal space exacerbates retinal degeneration [65,93,94]. Conversely, anti-
vascularopathy may induce a strong inhibitory effect on subretinal inflammation
and on retinal and choroidal microglia activation, suggesting that the beneficial
effects of anti-VEGF therapy in wet AMD may exceed its vascular effects [95].

Microglial activity is under constant regulation, partly by the inhibitory cytokines
Chemokine (C-X-C motif) ligand 1 (CXCL1) and Chemokine (CC motif) ligand 2
(CCL2), which silence microglia after interacting with surface receptors (CX3CR1 and
CCR2), but also through the CD200:CD200R system, which has become a subject of
interest lately [96]. CD200 is a membrane glycoprotein that suppresses microglial
activity through interaction with its receptor, CD200R. CD200R is expressed on
myeloid cells, RPE, and neurons, while the expression of CD200R is restricted to
myeloid cells. Their only identified function to date is to interact with each other for
the activation of anti-inflammatory signalling in CD200R-expressing cells, including
microglia [97-99]. CD200 knockout mice show increased susceptibility to and acce-
erated onset of tissue autoimmunity, further supporting the notion that
CD200:CD200R interaction is required to downregulate the immune response [100]. Supressing pro-angiogenic macrophage activation in laser-induced CNV in mice using
CX10α (a CD200 agonist) led to reduction in the mean CNV size and reduced expression of the macrophage chemoattractant protein 1 [101]. The CD200:CD200R interaction has not been implicated in AMD before, but findings from studies on Alzheimer’s and Parkinson’s disease (which share some pathogenic mechanisms) have identified impairment of CD200:CD200R-mediated silencing of microglia and mono-
cyte-derived macrophages as potential pathogenic mechanisms [102,103].

Vitamin D and AMD

Vitamin D is a circulating steroid hormone which is acquired either from dietary
sources or via ultraviolet irradiation of 7-dehydrocholesterol in the epidermis. After
being metabolised in the liver to 25-hydroxyvitamin D, it is finally metabolised by the
enzyme 25-hydroxyvitamin D-1a-hydroxylase (CYP27B1) in the kidney to the active
form, 1,25-dihydroxyvitamin D. Circulating 1,25-dihydroxyvitamin D binds to vitamin D binding proteins (DBP), acts on target cells which house the intracellular
receptor, vitamin D receptor (VDR). Intestinal epithelial cells and osteoblasts are
the primary sites of VDR expression, where the interaction between the active form of
vitamin D and VDR mediates actions which promote intestinal calcium and phos-
phate uptake, and remodelling of skeletal mineral, respectively. The VDR is also
expressed in immune cells making vitamin D a modulator of the immune system.
Moreover, vitamin D exerts anti-inflammatory effects by enhancing T-suppressor cell
activity and downregulating T-helper and T-cytotoxic cells, monocytes, dendritic cells
and neutrophil killer cells [104-106]. Vitamin D also possesses properties which counter
atherosclerosis by reducing VEGF expression and proliferation of endothelial cells,
increasing the expression of platelet-derived growth factor, and inhibiting the
matrix-metallopeptidase 9 [107], and fibrosis by decreasing expression of the pro-
fibrotic factors, TGF-B, plasminogen activator inhibitor, and several collagen
isoforms, and increasing expression of antifibrotic factors, such as BMP7, a TGF-β1
antagonist, MMP9, a collagen breakdown inducer, and follistatin, an inhibitor of pro-
fibrotic factor myostatin [108-112].
Although there is no general consensus on optimal levels of vitamin D in humans, vitamin D deficiency is defined by most experts as a 25-hydroxyvitamin D level of less than 50 nmol/l [113-115]. However, levels of parathyroid hormone remain increased until 25-hydroxyvitamin D reaches 75 nmol/l; thus levels between 50 and 75 nmol/l may reflect insufficiency. Vitamin D deficiency or insufficiency is extremely common affecting an estimated 1 billion persons worldwide [116]. Of great concern is its association with numerous chronic conditions, including osteomalacia and rickets, cancer, autoimmune diseases, Alzheimer’s disease, multiple sclerosis, and depression. Vitamin D deficiency has also been associated with AMD, though with conflicting results [117-121].

Figure 1.4 Three common phases of microglial activity in the retina. Left: In the normal retina, resident microglia (pink) are found primarily in the plexiform layers. In the resting state, they continuously scan the local environment, phagocytose cell debris and secrete supporing factors. Center: insults (red stars) leading to dysfunctions or degenerations in the RPE, photoreceptor outer segments, and the ganglion cell later rapidly alert microglia. Right: Microglial migration to lesion sites and activation into amoeboid phagocytes. These effector cells may be protective or detrimental depending on their immunological phenotype and the local cytokine milieu. RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Adapted and modified from [122].

2. Methodology
2.1. Design, settings and participants
All three studies were case-control studies and took place at the Department of Ophthalmology, Copenhagen University Hospital Roskilde. The participants were either patients attending the department, or their relatives or staff members. Individuals with a diagnosis of cancer, systemic inflammatory or autoimmune diseases were not included in the study. Similarly, patients receiving systemic immune-modulating therapy (e.g. glucocorticosteroids) were excluded, as were patients who had received intravitreal anti-VEGF agents in either eye within the last 30 days. For study 3, the exclusion criteria were extended to include chronic kidney, liver, or parathyroid diseases, as these may influence plasma 25-hydroxyvitamin D levels [123].

2.2. Ethical considerations
All studies were conducted in accordance with the Declaration of Helsinki and were approved by the Regional Committee on Biomedical Research (Sr-142). The participants gave verbal and written consent prior to inclusion, and they retained the right to withdraw from the study at any given time. Those who wished to obtain insight into the results of our studies were provided with a summary in Danish after the studies were concluded. The data was treated in a confidential manner wherever possible.

2.3. Interview
Every participant was subjected to a standardised, structured interview where patient demographic and clinical data was explored in detail. The interview was conducted by one of three investigators with a medical background (AS, MF, YS). All known or suspected medical conditions (with special attention towards inflammato-ry/autoimmune diseases, cancer, cardiovascular and renal disease) were noted, and if in doubt, electronic or paper patient records were consulted. Participants were specifically asked if they had been feeling ill for the past 5 days, as a control for infection-related increases in C-reactive protein. Regular medication usage was noted, as was usage of over-the-counter drugs, vitamin and mineral supplements, antioxidant use, and natural remedies. Smoking history was recorded in order to group participants as current smokers, ever smokers, or never smokers [124]. Current smokers were either smoking at the time of inclusion, or reported quitting within the last 12 months of inclusion. Ever smokers had smoked more than 100 cigarettes during their lifetime. Never smokers had not smoked before, or smoked less than 100 cigarettes during their lifetime. One cigar or one bottle of wine was considered to be the equivalent of 3 cigarettes. None of the participants reported smoking water-pipes. Alcohol intake was quantified according to the updated guidelines from the Danish National Board of Health. Thus, an intake was said to be above the recommended level if usage was higher than 7 units of alcohol a week for women and 14 units a week for men. The participants were inquired about their body height and weight, and if in doubt, these were measured. The data was subse-quently used to calculate the body mass index (kg/m²). In order to minimise the risk of measurement bias, the interview was conducted prior to any definite information on the patients’ diagnoses, wherever possible.

Since regular physical activity has been associated with decreased incidence of wet AMD [125] and lower systemic levels of C-reactive protein [126], we asked all participants about physical activity. Several different questionnaires for quantifying physical activity exist, but many of these are complex, time-consuming instruments, which limit their usage due to practical issues. Schechtman et al. created a practical, concise and validated questionnaire with an age-adjusted association with BMI, HDL cholesterol status, and oxygen capacity in both sexes making it a useful, yet time-efficient alternative to other, more detailed questionnaires [127]. The single-question “Do you currently participate in any regular activity or programme, either on your own or in a formal class, designed to improve or maintain your physical fitness?” was translated to Danish and applied to all participants.

2.4. Retinal imaging
The participants underwent detailed imaging of both maculae using clinical examination and various imaging modalities. This ensured confident grading of all eyes and subsequent categorisation into subtypes. Grading was performed by two investiga-tors (AS, MF) independently of each other and when in doubt, a senior consultant (TLS) was consulted. All patients were dilated and assessed using ophthalmoscopic fundus examination using a 90 diopter lens, which allows visualisation of the macula, optic nerve and retinal blood vessels. Participants were further examined using Spectral-Domain Optical Coherence Tomography (SO-OCT), a non-contact medical imaging technology where reflected light is used to produce detailed cross-sectional and three-dimensional images of the eye; fundus autofluorescence imaging (FAF), that utilises the presence of lipofuscin and other fluorophores which accumulate in aging RPE cells and represent incomplete degradation of photoreceptor outer segments; and digital fundus colour photography, which produces high-quality colour images of the retina. In order to diagnose wet AMD with certainty and to exclude other conditions which may resemble AMD, a fluorescein angiography along with indocyanine green angiography was performed on all patients where a choroidal neovascular membrane was suspected. Since angiography requires intra-venous injection of dye, blood samples were always obtained before dye was given to avoid potential interference [128].

Several classification systems for AMD exist [129-133]. We used the most recent amongst these at the time of inclusion, the Clinical Age-Related Maculopathy Staging (CARRMS) System by Seddon et al. [132] (Table 2.1).

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<th>GRADE OF MACULOPATHY</th>
<th>CLINICAL FEATURES</th>
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<td>1. No drusen or &lt;10 small drusen without pigment abnormalities</td>
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<tr>
<td>2. Approximately ≥10 small drusen or ≥15 intermediate drusen, or pigment abnormalities associated with ARM</td>
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<td>3. Approximately ≥15 intermediate drusen or any large drusen, drusenoid RPED</td>
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<td>4. Geographic atrophy</td>
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<td>5. NV-AMD, including nondrusenoid PEDs, serous or retinal detachments, CNVM with subretinal or sub-RPE haemorrhages or fibrosis, or scars consistent with treatment of AMD.</td>
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Table 2.1. The Clinical Age-Related Maculopathy Grading System (adapted and modified from Seddon et al. [132]). NV-AMD = neovascular age-related macular degeneration; ARM= age-related maculopathy; CNVM= choroidal neovascular membrane; RPE= retinal pigment epithelium; RPED= retinal pigment epithelial detachment. Small drusen <65µm in diameter; intermediate drusen ≥63 but <125µm; large drusen ≥125µm in diameter. Drusen must be located within 2 disc diameters of the macula center.

2.5. Blood sampling and analysis
Venous blood was obtained from a cubital fossa vein. About 3-4 ml of blood was filled in four tubes: 1) two tubes containing ethylenediaminetetraacetic acid (EDTA) for white blood cell count, flow cytometry and genotyping; 2) one tube containing lithium-heparin for measurement of C-reactive protein (CRP); and 3) one gel tube for analysis of 25-hydroxyvitamin D. EDTA is more effective in inhibiting endogenous in vitro complement activation than heparin or citrate [134]. The tubes were kept at room temperature until preparation of blood samples began within 4 hours of phlebotomy. One EDTA tube was sent by post to the Kennedy Center in Glostrup, Denmark for gene extraction and preservation.

2.5.1. C-REACTIVE PROTEIN [STUDIES I & II]
High-sensitive plasma CRP was measured with latex immunoassay using Architect CIB200 (Abbott Laboratories, Chicago, IL, USA) with a detection limit of 0.2 mg/l. The analysis was performed at the Department of Clinical Biochemistry, Copenhagen University Hospital, Roskilde. Participants with pathologically elevated CRP-levels (≥10 mg/l) were excluded post-hoc, as levels above 10 mg/l are more likely to represent activation of the immune system due to other pathologies than AMD. If CRP was pathologically elevated without a plausible explanation (e.g. an
upper respiratory tract infection), we would also contact the patient, inform them about the result, and suggest them to consult their general practitioner.

2.5.2. White blood cell count (STUDIES I & II)

The white blood cell count (WBC) and differential count was performed using the Sysmex XE-5000 Hematology Analyzer (Kobe, Japan). The WBC was always performed prior to preparation for flow cytometry, and the total number of leukocytes was used to calculate the volume of blood to be used.

2.5.3. Flow cytometry (STUDIES I & II)

Preparation for flow cytometry commenced with a WBC count as previously described. Blood volume corresponding to 10×10⁶ cells was allocated to a 50 ml polypropylene tube. The erythrocytes were then destroyed by adding red blood cell lysis buffer and incubating for 5-10 minutes at room temperature in the dark. The dead erythrocytes and lysis buffer were removed by triple centrifugation at 500 G for 5 minutes each time. A solution corresponding to 500,000 cells was then transferred to two vials and monochromal antibodies were added. Vial #1 consisted of antibodies to complement regulatory proteins (CD46, CD55, CD59) and specific leucocyte-markers (CD14, CD45). Vial #2 consisted of antibodies to microglia inhibitory proteins (CD200, CD200L), an integrin (CD11b), and T cell markers (CD4, CD8). Initially, ECD-CD14 (monocyte-marker) was also added to the solution, but was later removed due to problems with interference with CD200R and CD200 resulting in almost no expression of these on monocytes. Thus, all flow-cytometric analyses in this study were performed without ECD-CD14. Attempts to correct this problem by using receptor blockade were unsuccessful. Moreover, we were restricted to using the fluorochrome ECD (Phycocyantrphin-Texas Red/RED 613) due to our 5-colour flow cytometer (FCS500 analyser, Beckman Coulter) and ECD-CD14 was not available in other clones. When monocytes were gated on forward scatter – side scatter, more than 90% stained positive for CD14. Thus, we would expect the absence of CD14 to have minimal impact on findings. To control for non-specific bindings of negative isotype controls were also included. After incubation at room temperature in the dark as per manufacturer’s recommendations, the solutions were again centrifuged and washed. During flow-cytometry, 100,000 events were recorded for vials containing mononuclear antibodies, and 30,000 events for vials containing negative isotype controls. Analysis was performed using the Kaluza software. Gating strategies are described in the manuscripts for studies I and II.

2.5.4. Plasma 25-hydroxyvitamin D (STUDY III)

Vitamin D status in participants was determined by analysing the inactive precursor 25-hydroxyvitamin D plasma. 25(OH)D in serum or plasma is considered to be the best marker for vitamin D status because it reflects combined dietary supply and dermal production [135]. While both 1,25(OH)2D and 25(OH)D are very stable in stored serum or plasma [136,137], the latter has the advantage of having a longer half-life (15 days vs. 15 hours) [137,138]. To measure plasma-25(OH)D, one venous blood sample (4 ml) was obtained in an evacuated gel tube and serum was isolated by centrifugation and stored at -80 °C. The samples were then transported to Copenhagen University Hospital Rigshospitalet where 25(OH)D2 and D3 were measured in plasma using liquid chromatography-tandem mass spectrometry.

2.5.5. DNA extraction and genotyping (STUDY III)

Venous blood for genotype analysis was posted to the Kennedy Center (Glostrup, Denmark) where genomic DNA was extracted from leucocytes using Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany). Samples were then sent for genotyping to LG Genomics Ltd (Herts, United Kingdom) where genotyping was performed using in-house KASP® (Kompetitive Allelic Specific Polymerase chain reaction) genotyping SNP-line system which subsequently resulted in KASP assays. LG Genomics Ltd carried out a number of blinded duplicate studies to determine the reproducibility of the KASP genotyping chemistry and found an error rate of less than 0.3%.

3. Are patients with wet AMD unable to regulate their complement cascade? (STUDY I)

Based on the observations that 1) single nucleotide polymorphisms in complement factors (e.g. CFH) are strongly associated with AMD; 2) markers of chronic complement activation (e.g. Ba and C3d) are elevated in AMD, and 3) complement components are found in or close proximity of AMD lesions, we hypothesised that inadequate regulation may underlie the pathogenesis of AMD. Therefore, we conducted a study to compare the levels of three complement regulatory proteins CD46 and CD55 which inhibit the cascade upstream of the membrane attack complex, MAC, and CD59 (which inhibits the formation of MAC) between patients with wet AMD and aged controls.

Following retinal examination and imaging, fresh venous blood was obtained and the frequency of the CD46, CD55, and CD59 was determined on monocytes, lymphocytes, and granulocytes using flow-cytometric analysis, as described in the methods section. This study included 35 patients with AMD and 30 control individuals without AMD. We observed a significantly lower frequency of CD14+CD46+ and CD14+CD59+ monocytes in patients with wet AMD. Patients with wet AMD also tended to have a lower frequency of CD45+CD59+ lymphocytes compared to controls. Since all monocytes and granulocytes expressed CD55 on their surfaces, we did not include these cells in the analysis of CD55 frequency. Interestingly, the absolute number of leukocytes positive for each marker did not differ between the groups.

Controlling for confounding variables

The patients with wet AMD were significantly older, and were more likely to smoke compared to the controls. Therefore, we performed multiple regression analysis to adjust for these factors and found that the differences in surface expression were not due to these factors.

2.5.6. Impact of subretinal fibrosis on surface expression

Since only some patients with wet AMD will have subretinal fibrosis at a given time, we also looked for differences in surface expression of Cregs between patients with wet AMD with or without fibrosis. Patients with subretinal fibrosis had a lower frequency of CD45+CD64+ lymphocytes compared to patients without subretinal fibrosis. A similar tendency was observed on CD14+ monocytes.

Even though there is ample evidence from genetic association studies, immunohistochimical studies of donor eyes, and studies of complement levels in blood to suggest a role of the complement system in AMD development, it still remains unknown how the over-activation of the complement system leads to AMD. While dysregulation of the alternative pathway, largely attributed to genetic studies, linking mutations in the CFH gene to AMD, has received special attention, the classical and lectin pathways appear also to be implicated. The Cregs CD46 and CD55 inhibit the complement cascade at different levels (Figure 1.2). All three pathways converge to form the terminal MAC, and the MAC is specifically inhibited by CD59. Thus, our findings support the notion that all three pathways of the complement cascade are involved.

Our findings may be explained by one of the following hypotheses: 1. Increased vulnerability of macrophages in a hostile environment (Figure 3.1). As previously discussed, some regulated trafficking across the blood-retina barrier has been reported [60,139-141]. Bone-marrow derived circulating monocytes (which express CR2 on their surfaces) may enter the eye in response to the chemokine CCL2 (also known as monocyte-chemoattractant protein-1), secreted by the RPE in response to aging, acute inflammation, or oxidative stress [142]. Once inside the eye, monocytes transform into macrophages which may be capable of inhibiting pathologic CNV formation. Indeed, some mice with CCL2/CR3CR1 deficiency exhibit AMD-like retinal lesions and inhibition of CNV suggesting that macrophages are beneficial in pathologic CNV [143]. Little is known about how these newly migrated macrophages survive in the retina where they are potentially threatened by the noxious pro-inflammatory environment. Drugen components are capable of activating the complement system; thus macrophages lacking their protective shields (i.e. CD46 and CD59) may be susceptible to damage. In fact, lack of CD59 on macrophages in an in vitro model made them more susceptible to injury from complement proteins [144].

2. Reduced universal expression of Cregs. Alternatively, our findings may reflect a universally reduced expression of CD46 and CD59 on cells, including the RPE. When immortalised human RPE cells (ARPE-19) were exposed to HO2-induced oxidative stress, the surface expression of CD46, CD55 and CD59 decreased, leading to increased complement activation [145]. This led to sublytic activation of the MAC which, in turn, increased regulated vEGF secretion by RPE cells, eventually leading to CNV formation [146]. While this hypothesis is supported by the observation that C5a expression was altered in cells belonging to the myeloid and the lymphoid lineage (albeit not significantly in the latter), we did not observe any difference in expression in granulocytes.

In addition to its role in regulating inflammation and neovascularisation, the complement system is also involved in the process of fibrogenesis. For instance, CD46 is an inhibitor of the complement protein C5a, an effective chemotactrant and leukocyte-activator which plays a role in fibrosis. Therefore, it could be proposed that infiltrating or resident cells, lacking sufficient CD46 are unable to inhibit over-secretion of C5a (as a result of uncontrolled complement activity) and thereby fail to inhibit fibrosis.

Our findings support the existing notion on the involvement of the complement system in the pathogenesis of AMD. Scholl et al. investigated plasma levels of complement proteins in a group of patients with various subtypes of AMD (early, GA, wet AMD) and found that patients had higher plasma levels of complement activation products (C3d, Ba, V3a, C5a) and factor B and D, but not H, C3, and C4, compared to controls. Reynolds et al. confirmed these findings, and also found reduced levels of plasma-CFH in patients with all types of AMD [77]. Single-nucleotide polymorphisms in the factor H gene are associated with an increased risk of AMD and may affect treatment response to anti-VEGF agents [71-74]. Also single-nucleotide polymorphisms in factor B, factor I, C2, C3, and C5 are implicated in AMD [147-153]. Complement proteins, including regulators, have been demonstrated in AMD tissue, specifically in drusen and activated macrophage supernatants was shown to increase the expression of alternative complement proteins in RPE cells (Luo 2013 [154-158]). In donor eyes with AMD, Ebrahim and colleagues found decreased levels of CD45 and CD59 in the RPE overlying drusen. A further decrease in expression was noted upon addition of oxidised low-density lipoproteins [triggers of the complement system] and inhibition of C5a receptors by C5a-receptor antagonists was shown to reduce the C5a expression was found to be reduced early in the process of GA (82] [83]. In another study with donor eyes, RPE-C5a expression was attenuated by adenosine-delivered human CD59 into the subretinal space [159]. A

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The only significant difference between the wet AMD patient group and control group was the age, which was significantly lower in the latter. When adjusting for age using multiple regression analysis, the difference in the frequency of CD11b+CD200- monocytes remained significant. Interestingly, while an age-related increment in the percentage of CD200+ and CD11b+CD200- monocytes was observed in controls, no such correlation was found in patients with wet AMD.

Discussion of findings

The role of inflammatory activation of microglia in the pathogenesis of neurodegenerative diseases has been studied extensively. Microglia are the resident immune cells in the retina located exclusively in the inner retinal layers under normal circumstances [65,162]. They are believed to arise from myeloid progenitors, which originate in the yolk sac and invade the CNS during embryogenesis [84]. Under normal conditions, the transmigration of monocytes and other leukocytes over the blood-retina-barrier is very limited. However, during considerable retinal stress or physiological aging [139,163], monocytes in the bloodstream can migrate across the BRB. In the brain, it is believed that once circulating monocytes have crossed the brain barrier, they may evolve into either perivascular or pial macrophages, microglia, or pericytes depending on the specific signals in the microenvironment [164]. The same may apply to the retina.

CD11b is a surface integrin which is expressed on most leukocytes, including monocytes. Functionally, it regulates leukocyte adhesion and migration to mediate the inflammatory response. Our findings suggest that circulating CD11b+ monocytes have higher levels of CD200 in AMD patients compared to controls. At first, this may seem paradoxical, as a lower expression of CD200 on monocytes would be expected to cause inadequate silencing of the retinal microglia, and hence uncontrolled microglial activity leading to a pro-inflammatory retinal environment. However, it is still debated whether microglia are causative or protective in the AMD, thus, it is not known if microglial silencing is detrimental or beneficial [165]. Presented below are two hypotheses which crudely assume that microglia are either detrimental (1) or beneficial (2). Needless to say, this division is for practical purposes only, as microglia are probably neither exclusively detrimental nor beneficial, but are capable of possessing either roles depending on local signals and environment.

1) Hypothesis: Microglia are detrimental and monocytes upregulate CD200 as a counter-action in disease (Figure 4.1).

The age-related activation and accumulation of microglia in the subretinal space and their association with AMD-associated lesions in mice and humans support the hypothesis that a disturbance in microglia activity and distribution perturbs tissue homeostasis and promotes chronic inflammation, leading eventually to development and progression of AMD [166]. Silencing of over-activated retinal microglia could, therefore, be a useful counter-mechanism initiated by upregulation of surface CD200 expression by bone-marrow derived monocytes. Leukocytes can upregulate CD200 through various mechanisms, including chronic inflammation [167]. The preferential upregulation of CD200 on CD11b+ cells suggests that only cells that are capable of passing the BRB upregulate CD200. This issue could not be addressed in the current study, as a longitudinal study would be required where systemic CD200 expression would be investigated over a period of time, to assess whether patients who develop AMD generally have higher levels of CD200, or if CD200 expression is upregulated as a consequence of the disease. Our findings suggest that the former would be more likely, since, in contrast to controls, patients with AMD lacked an age-related increment in CD200 expression.

2) Hypothesis: Microglia are beneficial and are down-regulated by increased expression of CD200 on infiltrating monocytes in disease

Assuming that microglia exert beneficial effects in AMD, an increased expression of CD200 on infiltrating immune cells may not be appropriate as this would inhibit the beneficial effects of microglia and result in perturbed retinal homeostasis and progression of disease. This increased expression would be disease-specific and not related to increasing age, as suggested by our findings.

The CD200:CD200R interaction – A possible explanation for choroidal neovascularisation?

Recently, microglia have been objects of great interest due to their age-related activation and accumulation in the subretinal space as well as their association with AMD-associated lesions. It is known, that the interaction between CD200 and CD200-receptor silences microglia, and thus, we wanted to investigate whether any alterations in the frequency of leukocytes expressing the CD200-ligand or the CD200-receptor could be detected in the blood of patients.

Following retinal examination and imaging, fresh blood was obtained for flow-cytometric analysis of CD200 and CD200R on CD11b+ monocytes, lymphocytes (including subsets), and granulocytes. In total, 106 participants were included in this study: 62 patients with wet AMD and 44 control participants. We observed that patients with wet AMD had a higher percentage of CD11b+CD200- monocytes compared to controls. This finding was not reproduced on CD11b+CD200+ cells, suggesting that the altered expression of CD200 was limited to CD11b+ monocytes. No intergroup difference was observed in the frequency of CD200R+ cells. The presence of subretinal fibrosis did not have any impact on the frequency of CD200 or CD200R (Figure 4.1). Moreover, we found an age-related increment in CD11b+CD200+ monocytes in controls, but not in wet AMD.

Controlling for confounding variables

The only significant difference between the wet AMD patient group and control group was the age, which was significantly lower in the latter. When adjusting for...
In summary, our study demonstrates an inverse relationship between circulating CD11b+CD200+ monocytes and wet AMD. The implications of this finding are unclear and depend to some extent on our assumptions regarding microglia activity. Thus, if microglia are detrimental in AMD, our findings may reflect a defensive response whereby circulating monocytes invade the retina to silence the microglia. However, if microglia are beneficial in AMD, an upregulation of CD200 on circulating monocytes may (unnecessarily) down-regulate the microglia. More research is required to understand the phenotypical and functional heterogeneity of microglia and monocytes which may provide explanations for our findings.

Figure 4.1. Schematic illustration of hypothesis assuming that microglia are detrimental in AMD and CD200 on circulating monocytes is upregulated as a protective mechanism. When resting microglia become activated (3) they affect the RPE cells (2) to induce secretion of pro-inflammatory, pro-angiogenic, and chemotactic/adhesive factors (3). Moreover, a positive feedback mechanism further accentuates the activation of resting microglia (3). The chemotactic/adhesive factors allow for infiltration (4) of immune cells, e.g. monocytes into the retina from the choroid, which upon invasion convert into macrophages (5). Assuming that these macrophages have increased expression of CD200, they interact with the CD200-receptors on microglia to downregulate these mentioned mechanisms (6).

5. Is vitamin D deficiency associated with AMD? (Study III)
Vitamin D deficiency is a widespread problem globally and is on the rise for various reasons, including increased urbanisation and indoor work resulting in less exposure to sunlight. Elders are more susceptible to vitamin D deficiency since the ability to produce vitamin D from sunlight is reduced with age [174]. Since vitamin D regulates the immune system and inhibits angiogenesis and fibrogenesis, it was intuitive to look for differences in vitamin D concentrations in patients with AMD. Moreover, genetic mutations in the vitamin D metabolism may cause relative deficiency of the active form of vitamin D, so we tested for four SNPs in the vitamin D metabolism: CYP27B1 (rs108707232), vitamin D receptor (rs2238270), and vitamin D binding proteins (rs4588, rs7041).

Following retinal examination and imaging, fresh venous blood was obtained and 25-hydroxyvitamin D2 and D3 were analysed in plasma. This study included 178 partici pants: 95 with wet AMD (CARMS 5), 12 with GA, 22 with early AMD, and 49 age-matched controls without AMD. Although we were unable to find any differences in 25-hydroxyvitamin D between the four groups mentioned above, we did find a significant difference in 25-hydroxyvitamin D between patients with or without subretinal fibrosis in wet AMD. Patients with subretinal fibrosis were also more likely to be vitamin D deficient, while patients without subretinal fibrosis were more likely to be vitamin D sufficient (p<0.006). The allele frequencies of the four SNPs was comparable in all subgroups, and were not associated with 25-hydroxyvitamin D concentrations.

Controlling for confounding variables
All groups were comparable with regards to age, sex distribution, smoking habits, alcohol consumption, body mass index, supplement usage, and physical activity. Patients with wet AMD with or without subretinal fibrosis were also comparable in terms of age and other variables. The difference in 25-hydroxyvitamin D between wet AMD patients with and without subretinal fibrosis remained significant even after adjusting for body mass index, supplement usage, and physical activity.

Discussion of findings
In 2007, a cross-sectional study reported a novel association between poor vitamin D status and early, but not late AMD [119]. This study has, however, been criticised for its small number of patients with advanced AMD (n=10). A similar finding was reported by Millen et al. who found that higher levels of vitamin D in blood were associated with a lower risk of early AMD in women younger than 75 years of age [118]. We did not observe any such decrease in plasma 25-hydroxyvitamin D in patients with early AMD. Our study was, however, limited by a relatively small number of participants with early AMD (n=22), and may not have been sufficient to detect a potential difference. Morrison et al. examined 481 extremely phenotypically discordant sibling pairs and found higher levels of vitamin D in the siblings unaffected by AMD compared to the siblings with AMD, but this difference did not reach statistical significance [120]. A recent cross-sectional study by Golan et al. reported no difference in p-25-hydroxyvitamin D levels between patients with non-specified AMD and control subjects [121]. While the study was well-powered with about 10,000 participants, the authors did not assign patients into clinical subgroups, which may have affected the final results. We propose that the clinical heterogeneity seen in AMD may reflect pathogenic heterogeneity. Thus, the underlying mechanisms and pathways responsible for the wet AMD may be different from the mechanisms involved in geographic atrophy. Disregard of this heterogeneity may explain why previous studies have provided conflicting results. Therefore, we explore the possibility that patients who had signs of subretinal fibrosis at presentation may lack the anti-fibrotic vitamin D. In accordance with our expectations, vitamin D levels were found to be negatively associated with subretinal fibrosis. This finding is supported by similar findings in other organs, where vitamin D deficiency has been associated with tissue fibrosis [111,175,176].

As described in the “Background” section, 1,25-dihydroxyvitamin D may shift the balance between profibrotic (TGF-β, PAI, and collagen isoforms) and antifibrotic (Follistatin, BMP7, MMP-8) factors. Damaged epithelial cells and macrophages are capable of releasing TGF-β, which promotes fibrosis by activating resident mesenchymal and epithelial cells, which then differentiate into collagen-producing myofibroblasts. In fact, both intrinsic and exogenous macrophages transform RPE cells into myofibroblasts (EMT), thereby giving rise to subretinal fibrosis [177]. In wet AMD, the RPE is exposed to stress and monocyte-derived macrophages enter the eye secondary to chemotactic signalling or from a damaged BRB [6]. Some degree of TGF-β is probably required for repair processes, but may need regulation to avoid accelerated fibrogenesis. Vitamin D may be the missing link here, preventing excessive scarring by inhibiting minimising or modulating TGF-β release.

Conclusion
In summary, our results indicate that vitamin D deficiency is an important entity to consider in patients with wet AMD. It may be associated with subretinal fibrosis which often complicates AMD leading to irreversible vision loss. Thus, detecting and preventing vitamin D deficiency could prove helpful when managing patients with wet AMD.

6. Final discussion and perspectives
AMD is a leading cause of visual impairment worldwide and with a growing elderly population, its prevalence and socioeconomic burden is expected to rise considerably in the coming years. Although the management and visual prognosis in wet AMD with a neovascular component has been revolutionised by the recent introduction of intravitreal anti-VEGF, the disease still poses a threat to patients and society. As discussed earlier, some patients with wet AMD do not respond sufficiently to anti-VEGF and continue to lose central vision. Patients with chronic macular manifestations, e.g. central scar formation or geographic atrophy, may be especially challenging to treat. Moreover, there are some concerns that anti-VEGF treatment may cause scar formation (due to an increase in the intravitreal connective tissue growth factor/VEGF ratio) and/or geographic atrophy [178,179]. Thus, there is a need to understand the underlying mechanisms responsible for the pathogenesis of AMD, as they may hold the secrets to better treatment options.

Summary of findings
Overall, we found evidence to suggest that immune dysregulation plays an important role in the pathogenesis of AMD. In agreement with our purpose and hypothesis, we found that patients with wet AMD have changes in their blood which differentiate them from persons with healthy maculae. Moreover, we report that the blood 25-hydroxyvitamin D levels are not similar in patients with different clinical phenotypes of AMD. These findings are briefly summarised below:

Patients with wet AMD were found to have a significantly lower frequency of CD14+CD45+ and CD14+CD59+ monocytes when compared to eye-healthy controls. Moreover, we found a lower frequency of CD45+CD106+ lymphocytes in patients with wet AMD and subretinal fibrosis when compared to patients with wet AMD without subretinal fibrosis. This finding underlines the role of the complement system in AMD and supports the notion that inadequate regulation of the complement system is involved in the pathogenesis of AMD.

Patients with wet AMD were found to have a significantly higher frequency of CD11b+CD200+ monocytes when compared to eye-healthy controls. In this case, the presence of subretinal fibrosis did not affect the results. We also found an age-related increment in CD11b+CD200+ monocytes in controls, but not in wet AMD. This study implicates the CD200:CD200R pathway in wet AMD.

The plasma 25-hydroxyvitamin D levels were similar across all CARMS groups; however, when comparing patients with wet AMD and subretinal fibrosis with those with wet AMD without subretinal fibrosis, we found significantly higher levels in the latter. This finding suggests that vitamin D deficiency may play a role in the development of subretinal fibrosis in wet AMD.

A hypothetical model of AMD pathogenesis which incorporates the findings made in this thesis is presented in figure 6.1.

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25-hydroxvitamin D levels across different subgroups of AMD, and the finding that vitamin D deficiency was associated with only one subgroup (wet, fibrotic AMD), supports the notion on heterogeneity as discussed above.

The possible role of complement regulatory proteins and vitamin D in fibrosis

Wound healing is a very complex and incompletely understood biological process that either leads to healing and resolution, or to a non-functioning mass of fibrotic tissue, known as a disform scar in the retina. The pathways involved in the wound repair or fibrotic process are similar throughout the body and occur in three distinct, but overlapping phases: initial inflammation, proliferation, and remodeling [184]. Although collagen deposition is an indispensable and often reversible part of wound healing, normal tissue repair can evolve into a progressive and irreversible stage where existing tissue is gradually replaced by excessive fibrotic connective tissue, i.e. components of extracellular matrix (ECM), such as collagen and fibronectin. Fibrosis may occur when the tissue injury is severe or repetitive, or when the wound-healing response becomes dysregulated [184]. An important feature in the process of fibrosis is activation of ECM-producing myofibroblasts, which are the key mediators of fibrotic tissue modelling (Gabbiani 2003). Activated myofibroblasts may arise from resident fibroblasts, bone-marrow fibrocytes, or epithelial/endothelial cells that signal when this transition is numerous, including TGF-β, tenasin-C, plasminogen activator inhibitor-1, and metalloproteinases (185-190).

In AMD, fibrosis adjacent to the RPE-layer disrupts normal retinal tissue architecture leading to permanent loss of structure and visual function. In clinical practice, development of subretinal fibrosis in the foveal region translates into omission or discontinuation of therapy (Danish Ophthalmological Society Guidelines), although anti-VEGF therapy may reduce retinal thickness without affecting visual acuity in patients with exudative disciform scars [191]. It is beyond the scope of this thesis to discuss the many established and postulated mechanisms involved in the pathogenesis of subretinal fibrosis; thus, the potential roles of lymphocytes (study 1) and vitamin D (study 3) are briefly discussed below and outlined in figure 6.2. Drusen are made up of proteins, lipids and cellular components, including complement factors and related proteins, immunoglobulins and amyloid β [5]. The progressive accumulation of these factors induces inappropriate activation of diverse immune pathways, including classical and alternative complement pathways, the inflammatory and Toll-like receptor signalling [6]. The inflammatory response may resolve resulting in effective healing in a Th1 dominant response, or it may fail to settle and progress to a chronic inflammatory state in a Th2/Treg dominant response [184]. Facilitated by pro-fibrotic factors, such as TGF-β1 and TGF-β3, chronic inflammation favours proliferation, activation, migration and transdifferentiation of fibroblasts, fibrocytes, and epithelial/endothelial cells to the collagen-secreting myofibroblasts [184,192]. Interestingly, TGF-β may have either pro-fibrotic or anti-fibrotic roles depending on the cellular source; macrophage-derived TGF-β shows wound-healing and pro-fibrotic properties, while TGF-β secreted by CD4+ regulatory T-cells is anti-inflammatory and anti-fibrotic [192]. Thus, if lymphocytes entering the eye were to express less complement regulatory proteins on their surface, premature death may alter the balance between macrophage- and lymphocyte-derived TGF-β; this could be a plausible explanation to why patients with subretinal fibrosis and wet AMD in study 1 were found to have a reduced expression of CD46 and CD59.

Vitamin D acts on multiple sites in the fibrotic pathway and is believed to inhibit fibrosis directly and/or indirectly through its anti-inflammatory properties (figure 6.2). Amyloid-β, a constituent of drusen, is known to stimulate inflammatory pathways in the RPE [193]. These may be subject to regulation by vitamin D, since in aged mice, administration of vitamin D3 significantly reduced retinal inflammation and levels of amyloid-β in the retina [194]. RPE injury or stress activate the immune system and repair mechanisms, which may be effective in a predominant Th1-response and lead to resolution, or may be ineffective in a predominant Th2 and Th17-response and lead to a state of chronic inflammation [184]. Vitamin D is known to regulate Th17-responses by suppressing its cytokine production and can thus avert a state of chronic inflammation [195-197]. As discussed, activation of myofibroblasts is a central step in fibrosis, and it appears that vitamin D can inhibit this step through various means. There are numerous factors which contribute to the activation of myofibroblasts, including TGF-β, MMPs, TNc, and PAI-1. There is some evidence to suggest that vitamin D has a negative effect on all of above. For example, vitamin D deficiency is associated with higher levels of TGF-β in humans [198], and vitamin D treatment has a significant reduction in bioactive renal TGF-β in rats [199]. In human urinary epithelia cells, 1,25-dihydroxyvitamin D3 reduces TGF-β3 induced fibrosis-related gene expression [111]. Another study on human uterine cells showed that 1,25-dihydroxyvitamin D3 could inhibit the expression and activities of MMP-2 and MMP-9 [200]. In patients with end-stage renal disease, 25-hydroxyvitamin D concentration is inversely associated with serum MMP-2 [201]. A study on mouse and human mammary epithelial cell lines has shown that 1,25-dihydroxyvitamin D3 can inhibit tenasin-C RNA expression [202]. In smooth muscle cells, 1,25-dihydroxyvitamin D3 downregulated the expression of PAI-1 mRNA and protein in a dose-dependent manner [203], although in a different study, no relation was reported between serum level of vitamin D and PAI-1 in diabetic patients [204]. Together, these studies suggest that vitamin D may prevent fibrosis.
through various strategies and at different steps in the fibrotic pathway (summarised in figure 6.2).

Perspectives for future research
The role of the complement system in AMD is well established, and clinical trials investigating the role of complement factors in AMD are currently being conducted [6]. Intravitreal inhibition of complement factor D has shown promising effects on the progression of GA [205]. A recent study of macular of AMD specimens, found that CD59 was decreased in the RPE overlying drusen and in GA, while CD46 was decreased in diseases areas of early AMD samples [83]. In mice models, intravitreal delivery of CD59 has shown to attenuate choroidal neovascularisation, and CD46 has shown to inhibit the alternative complement pathway in the RPE [159,206-208]. These studies have not been replicated in humans. As an extension of our study 1, it would be interesting to investigate the systemic expression of CD46 and CD59 on leukocytes in patients with dry forms of AMD. Moreover, expression of these (and other) complement regulators in other tissues in patients with AMD could help us understand whether the change in complement regulator expression is local or universal.

![Image](57x427 to 286x582)

The CD200:CD200R pathway is not previously described in the context of AMD. A better understanding of the role of microglia and macrophages in the aging eye is required. Studies are needed to assess the expression of CD200 and CD200R in the RPE-cells of healthy and AMD specimens. Mice studies would be especially helpful in understanding what happens to the CD200 and CD200R expression in the RPE-cells before, during, and after experimentally induced CNV. There is also a need to evaluate the differential expression of CD200/CD200R in subtypes of monocytes (incl. classical or non-classical) and lymphocytes. Since our study 2 only compared patients with wet AMD with controls, a similar study could be conducted to assess the CD200 and CD200R expression in the dry forms of AMD. Although it is difficult to predict at this moment, the CD200:CD200R pathway may ultimately prove to be an additional target for treatment of AMD in the future.

Currently, vitamin D supplementation is not part of the routine management of AMD. While our study 3 indicated that vitamin D deficiency was associated with subretinal fibrosis in wet AMD, it is too early to say whether supplementation with vitamin D in wet AMD may prevent progression to the fibrotic form. For this, larger scale, longitudinal studies and randomised placebo-controlled clinical trials are required. The Vitamin D and Omega-3 Trial (VITAL) is an ongoing large-scale, placebo-controlled, randomised clinical trial to investigate whether taking 2000 IU vitamin D3 or 1 gram Omega-3 fatty acids reduces the risk for developing cancer, heart disease, and stroke in people without a prior history of these illnesses. Furthermore, the effect of vitamin D3 or Omega-3 fatty acids on other medical conditions, including AMD will also be assessed [209].

Overall conclusion
In conclusion, our findings suggest that AMD has a systemic component, and that the systemic factors observed in AMD differ according to clinical phenotype. The novel approach used in this thesis has provided us with some interesting findings which are not backed up, but not opposed either, by previous findings in the field of ophthalmology. Therefore, much of the discussion is scenario-based and inspired by research from adjacent fields which share common pathogenic mechanisms with AMD, such as Alzheimer’s dementia, atherosclerosis, and fibrosis in tissues other than the eye. Nevertheless, I hope the research presented in this thesis will encourage new discussions and contribute to a better understanding of AMD.

In our study, some patients with low 25-hydroxyvitamin D levels did not present with subretinal fibrosis, while others with normal 25-hydroxyvitamin D levels had subretinal fibrosis on presentation; thus, other factors may also be responsible for the development of subretinal fibrosis, and these need to be identified if we are to prevent severe and irreversible vision loss in our patients.

Summary
Age-related macular degeneration (AMD) is a leading cause of visual impairment in the aged population worldwide. The mechanisms underlying this multifactorial and heterogeneous disease are complex and incompletely understood. There is increasing evidence to suggest that regulatory differences in the immune system are involved in the development of the various subtypes of AMD. The purpose of this thesis was to identify some of these potential differences in patients with early or late (wet, dry, or fibrotic) AMD. Specifically, we sought to determine differences in 1) expression of regulators of the complement pathway (CD46, CD55, and CD59) on circulating leukocytes; 2) expression of microglia-inhibitory proteins (CD200 and CD200R) on circulating leukocytes; and 3) plasma concentrations of 25-hydroxyvitamin D, a factor known to inhibit angiogenesis, fibrosis, inflammation, and oxidation.

All participants underwent a semi-structured interview and detailed retinal imaging. Fresh venous blood was obtained and the frequency of cells expressing the proteins in question was determined using flow cytometry. Plasma 25-hydroxyvitamin D was measured using liquid chromatography-tandem mass spectrometry. Also, genotyping was performed in order to determine the frequency of certain single-nucleotide polymorphisms in the vitamin D metabolism.

Patients with wet AMD were found to have a statistically significant lower frequencies of CD46 and CD59 on CD14 monocytes and higher frequencies of CD200 on CD11b monocytes compared to control individuals without AMD (p=0.0070 and p=0.047, respectively). Moreover, we found a lower frequency of CD46 on CD45 lymphocytes in patients with wet AMD and subretinal fibrosis compared to patients with wet AMD without fibrosis (p=0.010). Vitamin D status was not different across AMD subgroups; however, the presence of subretinal fibrosis in patients with wet AMD was associated with a statistically significant lower concentration of 25-hydroxyvitamin D (p=0.001).

Our results suggest that inadequate systemic immune modulation is an important pathogenic mechanism in the aetiology of AMD. Moreover, some differences in protein expression and vitamin D status appear to be related to the phenotypical diversity of AMD, proposing that different mechanisms may underlie the different subtypes of AMD.

References


