Systemic Changes in Neovascular Age-Related Macular Degeneration

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I. Singh A, Faber C, Falk M, Nissen MH, Hviid TV, Sørensen TL (2012) Altered expression of CD46 and CD59 on leukocytes in neovascular age-related macular degeneration. Am J Ophthalmol Jul:154:193-199. doi: 10.1016/j.ajo.2012.01.036. Epub 2012 Apr 27

II. Singh A, Falk M, Hviid TV, Sørensen TL (2013) Increased expression of CD200 on circulating CD11b+ monocytes in patients with neovascular age-related macular degeneration. Ophthalmology May;120:1029-37. doi: 10.1016/j.ophtha.2012.11.002. Epub 2013 Feb 12.

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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 choroido-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 choroidal 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 a far 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 [13]. 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].

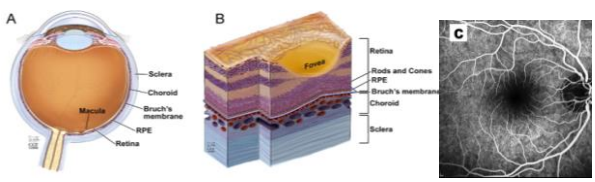

Figure 1.1. Anatomy of the macula. The macula lies in the visual axis, temporal to the optic nerve (A). The fovea lies centrally in the macula and is responsible for sharp, central vision (B). Adapted from [12]. Fundus angiography showing the macular region which coincides with the course of the major 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 pre-formulated 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 anaemia), 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) Pegatanib, 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 EDTRS 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 irresponsive to the drug [17,32,33]. 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. homozygosity for the CFH Y402H polymorphism located in the heparin 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].

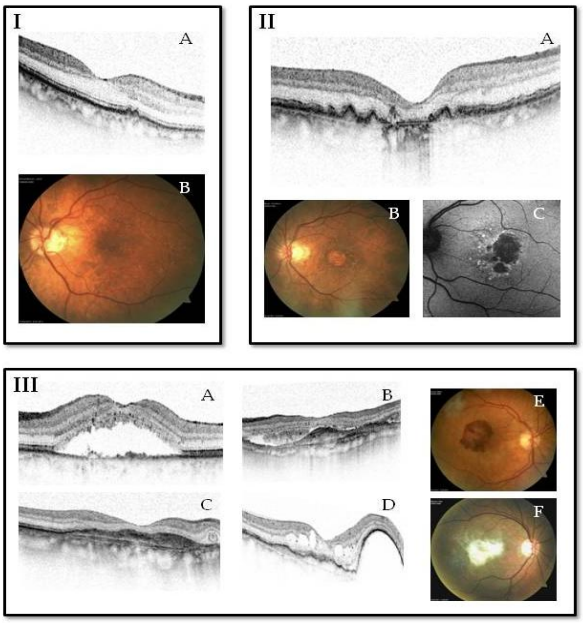

Figure 1.2. Clinical manifestations of age-related macular degeneration (AMD). **I)** Early AMD, cellular debris at the RPE-Bruch interface, drusen seen on SD-OCT (A) and fundus photography (B). **II)** Geographic atrophy, seen on SD-OCT (A), fundus photography (B), and fundus autofluorescence imaging (C). **III)** Wet AMD, serous detachment on SD-OCT (A), choroidal neovascular membrane in the subretinal space on SD-OCT (B), subretinal fibrosis on SD-OCT (C), intraretinal oedema adjacent to a pigment epithelial detachment on SD-OCT (D), macular haemorrhage on fundus photography (E), and disciform scar on fundus photography (F).

Immune privilege of the eye

Earlier, the eye was thought to be an immunologically privileged organ isolated from the systemic immune system. The term "immune privilege" was traditionally applied to a site in the body where foreign tissue grafts can survive for extended, often indefinite, periods of time, whereas similar grafts placed at regular (non-immune privileged) sites would result in rejection [55]. The purpose of immune privilege was thought to reflect evolutionary adaptation to protect tissues which are indispensable, but with limited ability to regenerate following immunological injury, e.g. the eye and central nervous system (CNS). Thus, exclusion of immune cells, e.g. leukocytes by specialised anatomical structures, such as the retina-blood or brain-blood barrier, was believed to be of fundamental importance in preserving immune privilege. This somewhat simplified view has since been challenged by studies showing that immune cell trafficking over the retinal barrier is essential for the homeostasis of the retinal environment [8]. Today, macrophages are not seen as "unwanted criminals" at a prohibited site, but as necessary modulators of inflammation having multiple, and often opposing, effects in the retina depending on local mechanisms and signals. In the CNS, infiltration of leukocytes and their interaction with microglia is seen as being crucial for neuroprotective and anti-inflammatory activities at the injury site. It has therefore been proposed that infiltrating cells fulfil specialised functions in the recovery process, where resident immune cells fail or do not react adequately [56,57].

The concept of "para-inflammation"

The immune system consists of a diverse group of specialised cells that are capable of detecting pathogens and activating effector mechanisms to control or destroy invading microorganisms, ultimately protecting the host against foreign organisms. In the absence of an attack by foreign organisms, other stressors, such as oxidised lipoproteins and free radicals, may provoke a tissue response which is lower in magnitude than a classic inflammatory response. Medhzitov proposed the term

"para-inflammation" to denote this milder form of inflammation, which lies intermediate 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 sustained 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 retina is known to have an endogenous immune system, which is coordinated by different cell types, including microglia, dendritic cells, perivascular 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]. According 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 mechanisms 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 (CRegs) 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 components 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 mutations in the complement factor H, a circulating regulator of the alternative pathway, and AMD [71-74]. Since then, similar genetic associations have been reported in other complement factors (B, C2, C3, and C5), but factor H remains, along with ARMS2 and HTRA1, the factor with the greatest impact on AMD risk. Moreover, altered blood levels of complement proteins have been reported in patients with AMD [75-81]. Recently, RPE-cells overlying affected areas in dry AMD has 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).

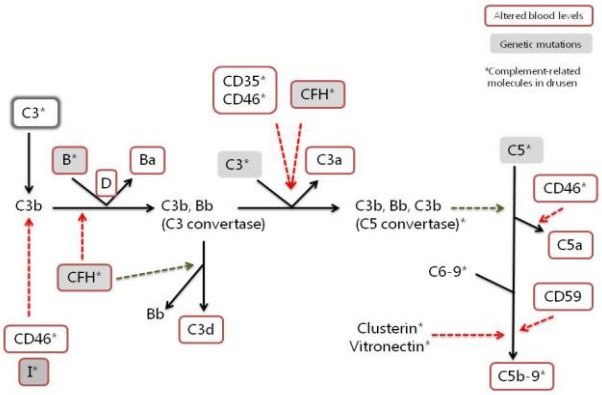

Figure 1.3. Schematic summary of current findings of regulating proteins in the complement pathway. Red dotted arrows: inhibition; Green dotted arrows: stimulation.

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 microenvironment [84]. Sometimes called the resident macrophages of the CNS or retina, microglia are functionally different from blood-derived macrophages, which originate from bone-marrow derived monocytes and enter the CNS and retina during pathological conditions. Microglia are believed to originate from macrophages produced by primitive haemopoiesis in the yolk sac [85]. Usually bone-marrow 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 plexiform 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 numbers of retinal microglia in mice, and along with tissue stress or injury, aging causes retinal microglia to undergo phenotypical changes whereby they become larger and less dendritic, which are typical morphological signs of activation [65]. While microglia are generally believed to return to their ramified phenotype and leave the subretinal space once the injury has subsided (e.g. when an active 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 microglia. The importance of subretinal accumulation of activated microglia in AMD is not clear, but could be both a symptom of inflammatory damage and a beneficial 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 [6,59,93,94]. Conversely, anti-VEGF therapy may induce a strong inhibitory effect on subretinal microglia migration 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-X3-C motif) ligand 1 (CX3CL1) 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. CD200 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 accelerated 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 DX109 (a CD200R agonist) led to reduction in the mean CNV size and reduced expression of the macrophage chemokine CCL2 [101]. The CD200:CD200 interaction has not been implicated in AMD before, but findings from studies on Alzheimer's and Parkinson's disease (which share some pathogenic mechanisms with AMD) have identified impairment of CD200:CD200R-mediated silencing of microglia and monocyte-derived macrophages as potential pathogenic mechanisms [102,103].

Vitamin D and AMD

Vitamin D3 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 -1α-hydroxylase (CYP27B1) in the kidney to the active form, 1,25-dihydroxyvitamin D. Circulating 1,25-dihydroxyvitamin D, bound 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 phosphate 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-supressor cell activity and downregulating T-helper and T-cytotoxic cells, monocytes, dendritic cells and natural killer cells [104-106]. Vitamin D also possesses properties which counter angiogenesis 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-β, plasminogen activator inhibitor, and several collagen isoforms, and increasing expression of antifibrotic factors, such as BMP7, a TGF-β1 antagonist, MMP8, 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].

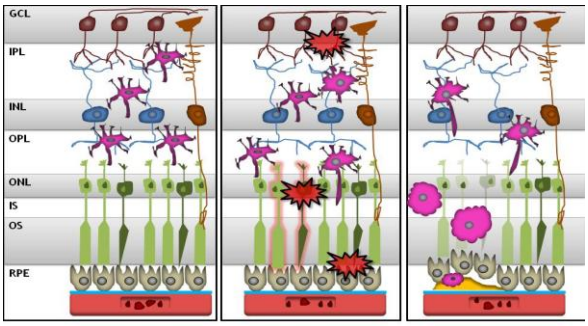

Figure 1.4 Three common phases of microglial activity in the retina. Left: In the normal retina, resident microglia (pink) are found primarily in the plexiform layers. In the resting state, they continously scan the local environment, phagocytose cell debris and secrete supporing factors. Center: Insults (red stars) leading to dysfunctions or degenerations in the RPE, photoreceptor layer, 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 (SJ-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 inflammatory/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 bowl of pipe 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 subsequently used to calculate the body mass index (kg/m^2). 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-expensive instruments which limits 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 investigators (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 (SD-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 intravenous 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 (CARMS) System by Seddon et al. [132] (Table 2.1).

GRADE OF MACULOPATHY	CLINICAL FEATURES
1.	No drusen or <10 small drusen without pigment abnormalities
2.	Approximately ≥10 small drusen or <15 intermediate drusen, or pigment abnormalities associated with ARM
3.	Approximately ≥15 intermediate drusen or any large drusen, drusenoid RPED
4.	Geographic atrophy
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.

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 epithelim; RPED= retinal pigment epithelial detachment. Small drusen <63µm 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 fossal 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 erogenous 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 CI8200 (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, Denmark. 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 x 10^6 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 monoclonal antibodies were added. Vial #1 consisted of antibodies to complement regulatory proteins (CD46, CD55, CD59) and specific leukocyte-markers (CD14, CD45). Vial #2 consisted of antibodies to microglia-inhibitory proteins (CD200, CD200R), 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 unfruitful. Moreover, we were restricted to using the fluorochrome ECD (Phycoerythrin-Texas Red/RED 613) due to our 5-colour flow cytometer (FC500 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 little impact on our findings. To control for non-specific binding, vials containing 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 monoclonal 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 in 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(OH2)D 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 ^{o}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 leukocytes using Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany). Samples were then sent for genotyping to LGC Genomics Ltd (Herts, United Kingdom) where genotyping was performed using in-house KASP^{TM} (Kompetetive Allele Specific Polymerase chain reaction) genotyping SNP-line system which subsequently resulted in KASP assays. LCG 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 in 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 wet 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.

The 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 $CD45^+CD46^+$ 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, immunohistochemical 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 CCR2 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 pathological CNV formation. Indeed, some mice with CCL2/CX3CR1 deficiency exhibit AMD-like retinal lesions and including CNV suggesting the notion that macrophages are beneficial in pathological 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. Drusen 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, inhibition 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 H_2O_2-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 fact that CReg-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 chemoattractant 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 were shown to increase the expression of alternative complement proteins in RPE cells (Luo 2013) [154-158]. In donor eyes with AMD, Ebrahimi and colleagues found decreased levels of CD46 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) [83]. In another study with donor eyes, RPE-CD46 expression was found to be reduced early in the process of GA [82]. In mice, laser-induced CNV was attenuated by adenovirus-delivered human CD59 into the subretinal space [159]. A

genetic association study of CD46, CD55 and CD59 recently carried out by Cirpriani and colleagues was unable to detect any association between these proteins and AMD [160]. However, this study cannot categorically rule out a potential association based on the confidence interval for the odds ratios. Moreover, the possibility of association with a common variant for poor linkage disequilibrium with the SNPs typed, or a rare variant that influences susceptibility to AMD cannot be excluded [160]. The complement system has also previously been implicated in the development of fibrosis in the subretinal space and other parts of the body. In a chorioretinal biopsy from a patient with subretinal fibrosis, complement proteins were deposited above the Bruchs membrane [161].

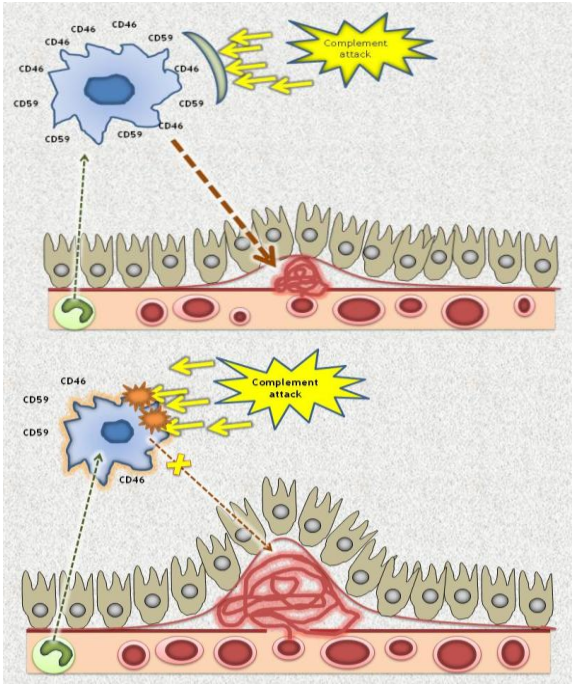

Figure 3.1. Top: Adequate expression of complement regulatory proteins (CD46, CD59) on cell surfaces protects macrophages from collateral damage from an ongoing complement attack. This allows macrophages to inhibit or restrict choroidal neovascularisation. Bottom: If complement regulatory protein expression is insufficient, macrophages may be damaged leading to unrestricted growth of CNV. Red dotted line: inhibition.

Conclusion

In summary, our data suggests that an inadequate regulation of the complement cascade may play a role in the pathogenesis of wet AMD and subretinal fibrosis. How complement dysregulation contributes to AMD development remains unknown, and plausible explanations are 1) increased vulnerability of CNV inhibiting macrophages that lack surface CRegs; or 2) a universal decrease in CReg expression leading to sublytic activation of the MAC, eventually leading to VEGF secretion by RPE cells. Our findings are in line with the existing knowledge on the area, and thus support the notion that complement dysregulation is associated with AMD.

4. The CD200:CD200R interaction – a possible explanation for choroidal neovascularisation? (Study II)

Lately, 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 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 sak 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 blood-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, since a lower expression of CD200 on monocytes would be expected to cause inadequate silencing of the retinal microglia, and hence uninhibited 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) Assumption: 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) Assumption: 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:CD200 receptor axis has not been implicated in AMD pathogenesis before, but there is some evidence to suggest its role in Alzheimer's disease, a neurological disease which shares many pathogenic parallels with AMD [102,168-171]. In AD, impairment of the CD200:CD200R mediated silencing of microglia and monocyte-derived macrophages has been identified as a potential pathogenic mechanism [102,103]. In donor human brains with AD, quantitative studies of CD200 protein and mRNA along with immunohistochemical analysis of brain sections revealed that the CD200:CD200R system was deficient, suggesting a role in maintaining chronic inflammation [102]. In animals with EAU, blocking the interaction between CD200R and CD200 was associated with increased CNS inflammation and neurodegeneration resulting in an aggravated clinical course [172]. A similar blockade in an experimental model of Parkinson's disease led to microglial activation and neurodegeneration [173]. Our findings of *increased* CD200 expression in AMD patients may, at first, seem to conflict with the above findings, but it is important to appreciate the systemic element in our study. Hence, the CD200 expression could very well differ in the two compartments: the retina or brain and the systemic circulation, semi-isolated by a membrane (BRB, BBB) which is a site of finely regulated leukocyte trafficking. There is no data available on the expression patterns of CD200 or CD200R in the retina during AMD.

Conclusion

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, is 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.

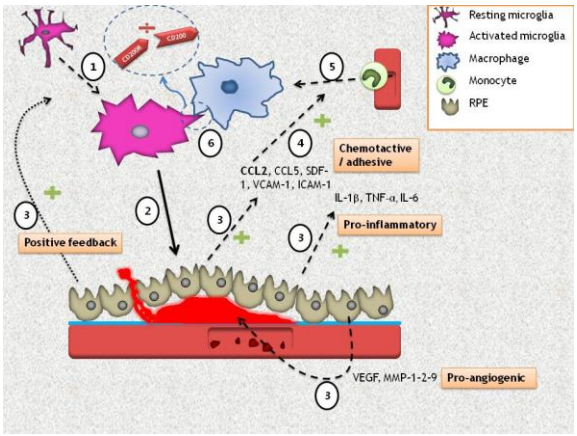

Figure 4.1. Schematic illustration of hypothesis assuming that microglia are detrimental in AMD and CD200 on circulating monocytes is upregulated as a protective mechanism. When resting microglia become activated (1) they affect the RPE-cells (2) to induce secretion of pro-inflammatory, pro-angiogenic, and chemotactice/adhesive factors (3). Moreover, a positive feedback mechanism further accentuates the activation of resting microglia (3). The chemotactive/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 modulates 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 (rs10877012), vitamin D receptor (rs2228570), 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 participants: 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 critiqued 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. We therefore explored 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-hydroxyvitamin 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 chemoattractant 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 $CD14^{+}CD46^{+}$ and $CD14^{+}CD59^{+}$ monocytes when compared to eye-healthy controls. Moreover, we found a lower frequency of $CD45^{+}CD46^{+}$ 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.

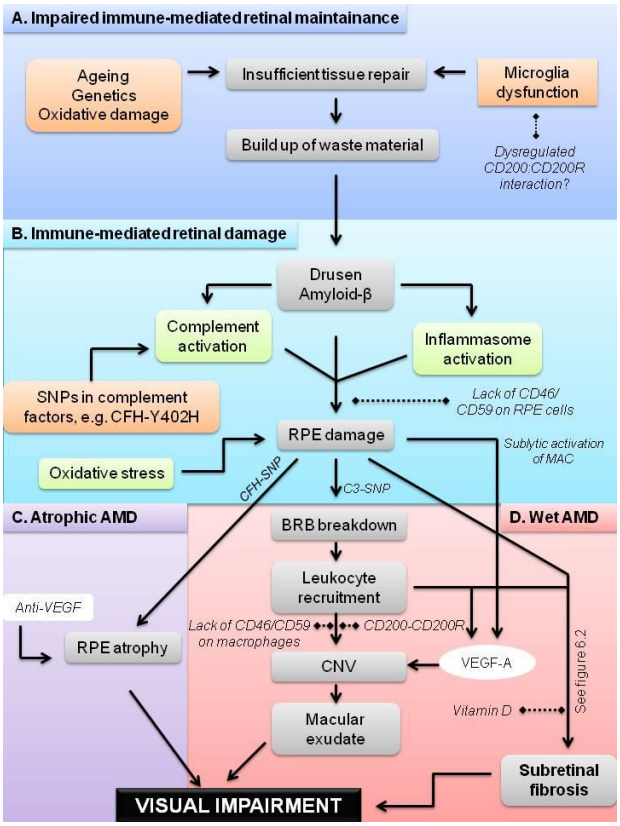

Figure 6.1. An simplified integrated model of AMD pathogenesis (adapted from [6]). The complement regulatory proteins, CD200, and vitamin D may inhibit several different steps in this proposed cascade.

Interpretational challenges

Interpretation of systemic alterations in a disease with a known predilection towards the macula can be challenging. The systemic differences observed may: 1) reflect a systemic pro-AMD profile which contributes to the disease process in the eye; 2) be secondary to the eye disease whereby feedback or signalling systems in the body up- or downregulate factors in the blood in order to create homeostasis in the eye; or 3) be products of blood-retinal barrier disruption resulting in spill-over of ocular factors into the systemic circulation. At this stage, it is not possible to conclusively infer the basis of the observed changes, or to assess whether the changes observed are caused by, or the cause of, the disease. Hence, it remains unknown whether AMD is a systemic disease with an ocular presentation, or vice-versa.

Possible basis of the clinical heterogeneity of AMD

AMD is a heterogenic and multifactorial disease with a variable phenotype. Early disease is characterised by drusen and pigment epithelial changes. In the AREDS 1 study, participants having extensive small drusen, pigment abnormalities, or at least 1 intermediate size druse, had a 1.3 % probability of progression to advanced AMD by year 5 [28]. This probability increased to 18% if extensive intermediate drusen, large drusen, or noncentral GA were present [28]. Advanced AMD was defined as either the wet form with or without subretinal fibrosis, or GA. Why do only some patients with early AMD progress to the advanced forms, and what determines whether the patient develops a choroidal neovascular membrane, geographic atrophy, or both? It is intuitive to assume that at some point during disease development, some events may trigger mechanisms which direct the further course towards either one of the two phenotypes (i.e. GA or wet AMD). This notion is supported by findings of concordance between families and concordance between the two eyes in the same patient [180,181]. Recently, analysis of single nucleotide polymorphisms in patients with different AMD phenotypes has revealed that SNPs in the C3 gene favours neovascular AMD whereas SNPs in the CFH has a predilection for GA (Oral presentation by Caroline Klaver, Euretina 2013). The differential expression of the VEGF gene may also facilitate the direction of disease, as increased secretion of VEGF by RPE-cells is a characteristic finding in wet AMD, and decreased VEGF expression results in atrophic changes around the RPE-choroid complex [182,183]. Another possible mechanism could be the RPE-secreted interleukin-18 (IL-18) which regulates RPE function, and determines whether the RPE cell succumbs to para-inflammation and dies (leading to retinal atrophy) while attempting to prevent an angiogenic response to an increasing age-related pro-inflammatory microenvironment [8]. In our studies 1 and 2, we did not have a sufficient number of patients with early or advanced, dry AMD; thus these two studies contribute little to the understanding of the pathogenic heterogeneity of AMD. However, study 3 compared 25-hydroxyvitamin 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 adaptive biological process that either leads to healing and resolution, or to a non-functioning mass of fibrotic tissue, known as a disciform 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 remodelling [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 epithelium/endothelium, and the signals which facilitate this transition are numerous, including TGF-β, tenascin-C, plasminogen activator inhibitor-1, and metalloproteinases [185-190].

In AMD, fibrosis adjacent to the RPE-layer disrupts normal retinal tissue architecture leading to permament 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, immunogloubulins and amyloid β [6]. The progressive accumulation of these factors induces inappropriate activation of diverse immune pathways, including classical and alternative complement pathways, the inflammasome 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/Th17 dominant response[184]. Facilitated by pro-fibrotic factors, such as macrophage and/or RPE cell-derived TGF-β, chronic inflammation favours proliferation, activation, migration and transdifferentation 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 $CD4^+$T-regulatory 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 uterine leiomyoma 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-9 [201]. A study on mouse and human mammary epithelial cell lines has shown that 1,25-dihydroxyvitamin D3 can inhibit tenascin-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 relationship 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 maculae 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.

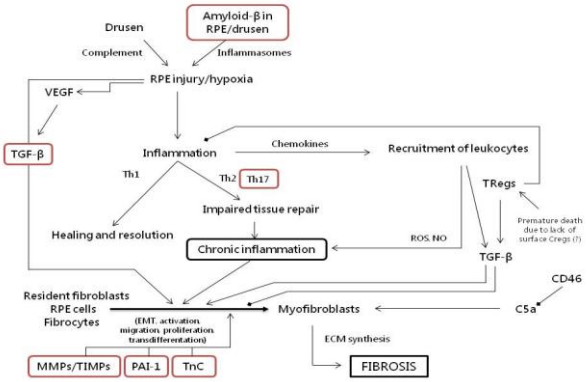

Figure 6.2. An overview of regulation of the fibrotic process by vitamin D and complement regulatory proteins. It is proposed that vitamin D displays its anti-inflammatory and anti-fibrotic effects by inhibiting various steps (marked in red boxes). The Tregs-derived transforming growth factor beta is believed to inhibit activation of myofibroblasts; thus, Tregs lacking surface protection from complement, i.e. lacking CD46 and CD59 may be more vulnerable to premature death. The lack of CD46, an inhibitor of the complement protein C5a, may promote fibrosis by activating fibroblasts. Tregs: T-regulatory cells; TGF-β: transforming growth factor beta; MMPs: matrix metalloproteinases; PAI-1: plasminogen activator inhibitor; TnC: tenascin-C; EMT: epithelial mesenchymal transition; ECM: extracellular matrix

The CD200:CD200R pathway is not previously described in the context of AMD. A better understanding of the role of microglia and macrophages/monocytes 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, randominised 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 that 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 heterogenic 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 as 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

1. Hutchinson J, Tay W. Symmetrical central choroido-retinal disease occurring in senile persons. J Ophthalmic Med Surg 1874; 8: 231-244.

2. Macular Photocoagulation Study Group. Argon laser photocoagulation for senile macular degeneration. Results of a randomized clinical trial. Arch Ophthalmol 1982; 100: 912-918.

3. Macular Photocoagulation Study Group. Changing the protocol: a case report from the Macular Photocoagulation Study. Control Clin Trials 1984; 5: 203-216.

4. Eyetech Study G. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina 2002; 22: 143-152.

5. Eyetech Study G. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. Ophthalmology 2003; 110: 979-986.

6. Ambati J, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. Nat Rev Immunol 2013; 13: 438-451.

7. Yanoff M, Duker Y. Ophthalmology 4th edition 2009. Elsevier (Textbook).

8. Forrester JV, Xu H. Good news-bad news: the Yin and Yang of immune privilege in the eye. Front Immunol 2012; 3: 338.

9. Bernstein MH, Hollenberg MJ. Fine structure of the choriocappillaris and retinal capillaries. Invest Ophthalmol 1965; 4: 1016-1025.

10. Chong NH, Keonin J, Luthert PJ, et al. Decreased thickness and integrity of the macular elastic layer of Bruch's membrane correspond to the distribution of lesions associated with age-related macular degeneration. Am J Pathol 2005; 166: 241-251.

11. Al-Hussaini H, Kam JH, Vugler A, et al. Mature retinal pigment epithelium cells are retained in the cell cycle and proliferate in vivo. Mol Vis 2008; 14: 1784-1791.

12. Yuan X, Gu X, Crabb JS, et al. Quantitative proteomics: comparison of the macular Bruch membrane/choroid complex from age-related macular degeneration and normal eyes. Mol Cell Proteomics 2010; 9: 1031-1046.

13. Kanski JJ. Clinical Ophthalmology, A Systematic Approach 7th edition 2011. Saunders (Textbook).

14. Sunness JS, Rubin GS, Applegate CA, et al. Visual function abnormalities and prognosis in eyes with age-related geographic atrophy of the macula and good visual acuity. Ophthalmology 1997; 104: 1677-1691.

15. Ferris FL, 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. Arch Ophthalmol 1984; 102: 1640-1642.

16. Pauleikhoff D . Neovascular age-related macular degeneration: Natural History and Treatment Outcomes. Retina 2005; 25: 1065-1084.

17. Eghoj MS, Sorensen TL. Tachyphylaxis during treatment of exudative age-related macular degeneration with ranibizumab. Br J Ophthalmol 2012; 96: 21-23.

18. Chakravarthy U, Evans J, Rosenfeld PJ. Age related macular degeneration. BMJ 2010; 340: c981.

19. Klein R. Race/ethnicity and age-related macular degeneration. Am J Ophthalmol 2011; 152: 153-154.

20. Owen CG, Fletcher AE, Donoghue M, et al. How big is the burden of visual loss caused by age related macular degeneration in the United Kingdom? Br J Ophthalmol 2003 87: 312-317.

21. Kawasaki R, Yasuda M, Song SJ, et al. The prevalence of age-related macular degeneration in Asians: a systematic review and meta-analysis. Ophthalmology 2010; 117: 921-927.

22. Woo JH, Sanjay S, Au Eong KG. The epidemiology of age-related macular degeneration in the Indian subcontinent. Acta Ophthalmol 2009; 87: 262-269.

23. Klein R, Rowland ML, Harris MI. Racial/ethnic differences in age-related maculopathy. Third National Health and Nutrition Examination Survey. Ophthalmology 1995; 102: 371-381.

24. Mitchell P, Smith W, Attebo K, et al. Prevalence of age-related maculopathy in Australia. The Blue Mountains Eye Study. Ophthalmology 1995; 102: 1450-1460.
25. Krishnan T, Ravindran RD, Murthy GV, et al. (2010) Prevalence of early and late age-related macular degeneration in India: the INDEYE study. Invest Ophthalmol Vis Sci 2010; 51: 701-707.

26. The Global Economic Cost of Visual Impairment. AMD Alliance International 2010; [online].

27. Rein DB, Wittenborn JS, Zhang X, et al. Forecasting age-related macular degeneration through the year 2050: the potential impact of new treatments. Arch Ophthalmol 2009; 127: 533-540.

28. Age-Related Eye Disease Study Research G. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. Arch Ophthalmol 2001; 119: 1417-1436.

29. The Age-Related Eye Disease Study 2 Research G. Lutein/Zeaxanthin for the Treatment of Age-Related Cataract: AREDS2 Randomized Trial Report No. 4. JAMA Ophthalmol 2013: 1-7.

30. Patel RD, Momi RS, Hariprasad SM. Review of ranibizumab trials for neovascular age-related macular degeneration. Semin Ophthalmol 2011; 26: 372-379.

31. Thomas M, Mousa SS, Mousa SA. Comparative effectiveness of aflibercept for the treatment of patients with neovascular age-related macular degeneration. Clin Ophthalmol 2013; 7: 495-501.

32. Binder S. Loss of reactivity in intravitreal anti-VEGF therapy: tachyphylaxis or tolerance? Br J Ophthalmol 2012; 96: 1-2.

33. Arjamaa O, Minn H. Resistance, not tachyphylaxis or tolerance. Br J Ophthalmol 2012; 96: 1153-1154.

34. Bakall B, Folk JC, Boldt HC, et al. Aflibercept Therapy for Exudative Age-related Macular Degeneration Resistant to Bevacizumab and Ranibizumab. Am J Ophthalmol 2013; 156: 15-22.

35. Cho H, Shah CP, Weber M, et al. Aflibercept for exudative AMD with persistent fluid on ranibizumab and/or bevacizumab. Br J Ophthalmol 2013; doi:10.1136/bjophthalmol-2013-303344

36. Yonekawa Y, Andreoli C, Miller JB, et al. (2013) Conversion to Aflibercept For Chronic Refractory Or Recurrent Neovascular Age-Related Macular Degeneration. Am J Ophthalmol 2013; 156:29-35.

37. Patel KH, Chow CC, Rathod R, et al. Rapid response of retinal pigment epithelial detachments to intravitreal aflibercept in neovascular age-related macular degeneration refractory to bevacizumab and ranibizumab. Eye (Lond) 2013; 27: 663-668.

38. Chakravarthy U, Wong TY, Fletcher A, et al. Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. BMC Ophthalmol 2010; 10: 31.

39. Parmeggiani F, Romano MR, Costagliola C, et al. Mechanism of inflammation in age-related macular degeneration. Mediators Inflamm 2012: 546786.

40. Hogan MJ. Role of the retinal pigment epithelium in macular disease. Trans Am Acad Ophthalmol Otolaryngol 1972; 76: 64-80.

41. Casswell AG, Kohen D, Bird AC. Retinal pigment epithelial detachments in the elderly: classification and outcome. Br J Ophthalmol 1985; 69: 397-403.

42. Bird AC. Bruch's membrane change with age. Br J Ophthalmol 1992; 76: 166-168.

43. Charbel Issa P, Chong NV, Scholl HP. The significance of the complement system for the pathogenesis of age-related macular degeneration - current evidence and translation into clinical application. Graefes Arch Clin Exp Ophthalmol 2011; 249: 163-174.

44. Thornton J, Edwards R, Mitchell P, et al. Smoking and age-related macular degeneration: a review of association. Eye (Lond) 2005; 19: 935-944.

45. Ting AY, Lee TK, MacDonald IM. Genetics of age-related macular degeneration. Curr Opin Ophthalmol 2009; 20: 369-376.

46. Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. JAMA 2006; 296: 301-309.
47. Lee S, Song SJ, Yu HG. Current smoking is associated with a poor visual acuity improvement after intravitreal ranibizumab therapy in patients with exudative age-related macular degeneration. J Korean Med Sci 2013; 28: 769-774.

48. Solberg Y, Rosner M, Belkin M. The association between cigarette smoking and ocular diseases. Surv Ophthalmol 1998; 42: 535-547.

49. Hammond BR, Jr., Wooten BR, Snodderly DM. Cigarette smoking and retinal carotenoids: implications for age-related macular degeneration. Vision Res 1996; 36: 3003-3009.

50. Kew RR, Ghebrehiwet B, Janoff A. Cigarette smoke can activate the alternative pathway of complement in vitro by modifying the third component of complement. J Clin Invest 1985; 75: 1000-1007.

51. Robbins RA, Nelson KJ, Gossman GL, et al. Complement activation by cigarette smoke. Am J Physiol 1991; 260: L254-259.

52. Kaushik S, Wang JJ, Flood V, et al. Dietary glycemic index and the risk of age-related macular degeneration. Am J Clin Nutr 2008; 88: 1104-1110.

53. Tan JS, Wang JJ, Flood V, et al. Dietary antioxidants and the long-term incidence of age-related macular degeneration: the Blue Mountains Eye Study. Ophthalmology 2008; 115: 334-341.

54. Margrain TH, Boulton M, Marshall J, et al. Do blue light filters confer protection against age-related macular degeneration? Prog Retin Eye Res 2004; 23: 523-531.

55. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 1948; 29: 58-69.

56. Benhar I, London A, Schwartz M. The privileged immunity of immune privileged organs: the case of the eye. Front Immunol 2012; 3: 296.

57. Shechter R, London A, Varol C, et al. Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. PLoS Med 2009; 6: e1000113.

58. Medzhitov R. Origin and physiological roles of inflammation. Nature 2008; 454: 428-435.

59. Chen M, Forrester JV, Xu H. Dysregulation in retinal para-inflammation and age-related retinal degeneration in CCL2 or CCR2 deficient mice. PLoS One 2011; 6: e22818.

60. Chen M, Zhao J, Luo C, et al. Para-inflammation-mediated retinal recruitment of bone marrow-derived myeloid cells following whole-body irradiation is CCL2 dependent. Glia 2012; 60: 833-842.

61. Xu H, Chen M, Reid DM, et al. LYVE-1-positive macrophages are present in normal murine eyes. Invest Ophthalmol Vis Sci 2007; 48: 2162-2171.

62. Xu H, Dawson R, Forrester JV, et al. Identification of novel dendritic cell populations in normal mouse retina. Invest Ophthalmol Vis Sci 2007; 48: 1701-1710.

63. Harman D. The free radical theory of aging. Antioxid Redox Signal 2003; 5: 557-561.

64. Chen M, Muckersie E, Forrester JV, et al. Immune activation in retinal aging: a gene expression study. Invest Ophthalmol Vis Sci 2010; 51: 5888-5896.

65. Xu H, Chen M, Forrester JV. Para-inflammation in the aging retina. Prog Retin Eye Res 2009; 28: 348-368.

66. Lachmann P. Complement before molecular biology. Mol Immunol 2006; 43: 496-508.

67. Ricklin D, Hajishengallis G, Yang K, et al. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010; 11: 785-797.

68. Jeong WI, Gao B. Innate immunity and alcoholic liver fibrosis. J Gastroenterol Hepatol 2008; 23 Suppl 1: S112-118.

69. Hageman GS, Mullins RF. Molecular composition of drusen as related to substructural phenotype. Mol Vis 1999; 5: 28.

70. Baudouin C, Peyman GA, Fredj-Reygrobellet D, et al. Immunohistological study of subretinal membranes in age-related macular degeneration. Jpn J Ophthalmol 1992; 36: 443-451.

71. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. Science 2005; 308: 385-389.

72. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci U S A 2005; 102: 7227-7232.

73. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 2005; 308: 419-421.

74. Edwards AO, Ritter R, 3rd, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. Science 2005; 308: 421-424.

75. Haas P, Aggermann T, Nagl M, et al. Implication of CD21, CD35, and CD55 in the pathogenesis of age-related macular degeneration. Am J Ophthalmol 2011; 152: 396-399 e391.

76. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. PLoS One 2008; 3: e2593.

77. Reynolds R, Hartnett ME, Atkinson JP, et al. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. Invest Ophthalmol Vis Sci 2009; 50: 5818-5827.

78. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. Arch Ophthalmol 2007; 125: 515-519.

79. Machalinska A, Dziedziejko V, Mozolewska-Piotrowska K, et al. Elevated plasma levels of C3a complement compound in the exudative form of age-related macular degeneration. Ophthalmic Res 2009; 42: 54-59.

80. Stanton CM, Yates JR, den Hollander AI, et al. Complement factor D in age-related macular degeneration. Invest Ophthalmol Vis Sci 2011; 52: 8828-8834.

81. Silva AS, Teixeira AG, Bavia L, et al. Plasma levels of complement proteins from the alternative pathway in patients with age-related macular degeneration are independent of Complement Factor H Tyr(4)(0)(2)His polymorphism. Mol Vis 2012; 18: 2288-2299.

82. Vogt SD, Curcio CA, Wang L, et al. Retinal pigment epithelial expression of complement regulator CD46 is altered early in the course of geographic atrophy. Exp Eye Res 2011; 93: 413-423.

83. Ebrahimi KB, Fijalkowski N, Cano M, et al. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. J Pathol 2013; 229: 729-742.

84. Kettenmann H, Hanisch UK, Noda M, et al. Physiology of microglia. Physiol Rev 2011; 91: 461-553.

85. Alliot F, Godin I, Pessac B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Dev Brain Res 1999; 117: 145-152.

86. Ajami B, Bennett JL, Krieger C, et al. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat Neurosci 2007; 10: 1538-1543.

87. Hickey WF, Vass K, Lassmann H. Bone marrow-derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. J Neuropathol Exp Neurol 1992; 51: 246-256.

88. Lee JE, Liang KJ, Fariss RN, et al. Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. Invest Ophthalmol Vis Sci 2008; 49: 4169-4176.

89. Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev 1995; 20: 269-287.

90. Brown DR. Role of microglia in age-related changes to the nervous system. ScientificWorldJournal 2009; 9: 1061-1071.

91. Mrak RE, Griffin WS. Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging 2005; 26: 349-354.

92. Langmann T. Microglia activation in retinal degeneration. J Leukoc Biol 2007; 81:1345-51.

93. Ma W, Zhao L, Fontainhas AM, et al. Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD. PLoS One 2009; 4: e7945.

94. Sakurai E, Anand A, Ambati BK, et al. Macrophage depletion inhibits experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 2003; 44: 3578-3585.

95. Couturier A, Bousquet E, Zhao M, et al. Anti-VEGF therapy inhibits microglial activation and migration in the retina. Invest Ophthalmol Vis Sci 2013; 54: E-abtract 5598

96. Kierdorf K, Prinz M. Factors regulating microglia activation. Front Cell Neurosci 2013; 7: 44.

97. Hoek RM, Ruuls SR, Murphy CA, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 2000; 290: 1768-1771.

98. Dick AD, Broderick C, Forrester JV, et al. Distribution of OX2 antigen and OX2 receptor within retina. Invest Ophthalmol Vis Sci 2001; 42: 170-176.

99. Wright GJ, Cherwinski H, Foster-Cuevas M, et al. (2003) Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. J Immunol 2003; 171: 3034-3046.

100. Dick AD, Carter D, Robertson M, et al. Control of myeloid activity during retinal inflammation. J Leukoc Biol 2003; 74: 161-166.

101. Horie S, Robbie SJ, Liu J. CD200R signaling inhibits pro-angiogenic gene expression by macrophages and supresses choroidal neovascularization. Scientific reports 2013; 3: 3072

102. Walker DG, Dalsing-Hernandez JE, Campbell NA, et al. Decreased expression of CD200 and CD200 receptor in Alzheimer's disease: a potential mechanism leading to chronic inflammation. Exp Neurol 2009; 215: 5-19.

103. Luo XG, Zhang JJ, Zhang CD, et al. Altered regulation of CD200 receptor in monocyte-derived macrophages from individuals with Parkinson's disease. Neurochem Res 2010; 35: 540-547.

104. Hayes CE, Nashold FE, Spach KM, et al. The immunological functions of the vitamin D endocrine system. Cell Mol Biol (Noisy-le-grand) 2003; 49: 277-300.

105. Guillot X, Semerano L, Saidenberg-Kermanac'h N, et al. Vitamin D and inflammation. Joint Bone Spine 2010; 77: 552-557.

106. Prietl B, Treiber G, Pieber TR, et al. Vitamin d and immune function. Nutrients 2013; 5: 2502-2521.

107. Steen B, Sejersen S, Berglin L, et al. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1998; 39: 2194-2200.

108. Artaza JN, Norris KC. Vitamin D reduces the expression of collagen and key profibrotic factors by inducing an antifibrotic phenotype in mesenchymal multipotent cells. J Endocrinol 2009; 200: 207-221.

109. Shokravi MT, Marcus DM, Alroy J, et al. Vitamin D inhibits angiogenesis in transgenic murine retinoblastoma. Invest Ophthalmol Vis Sci 1995; 36: 83-87.

110. Iseki K, Tatsuta M, Uehara H, et al. Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 of colon carcinogenesis induced by azoxymethane in Wistar rats. Int J Cancer 1999; 81: 730-733.

111. Halder SK, Goodwin JS, Al-Hendy A. 1,25-Dihydroxyvitamin D3 reduces TGF-beta3-induced fibrosis-related gene expression in human uterine leiomyoma cells. J Clin Endocrinol Metab 2011 96: E754-762.

112. Shen CL, Samathanam C, Graham S, et al. (2012) Green tea polyphenols and 1-alpha-OH-vitamin D(3) attenuate chronic inflammation-induced myocardial fibrosis in female rats. J Med Food 2012; 15: 269-277.

113. Holick MF. The role of vitamin D for bone health and fracture prevention. Curr Osteoporos Rep 2006; 4: 96-102.

114. Bischoff-Ferrari HA, Giovannucci E, Willett WC, et al. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. Am J Clin Nutr 2006; 84: 18-28.

115. Malabanan A, Veronikis IE, Holick MF. Redefining vitamin D insufficiency. Lancet 1998; 351: 805-806.

116. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008; 87: 1080S-1086S.

117. Graffe A, Milea D, Annweiler C, et al. Association between hypovitaminosis D and late stages of age-related macular degeneration: a case-control study. J Am Geriatr Soc 2012; 60: 1367-1369.

118. Millen AE, Voland R, Sondel SA, et al. Vitamin D status and early age-related macular degeneration in postmenopausal women. Arch Ophthalmol 2011; 129: 481-489.

119. Parekh N, Chappell RJ, Millen AE, et al. Association between vitamin D and age-related macular degeneration in the Third National Health and Nutrition Examination Survey, 1988 through 1994. Arch Ophthalmol 2007; 125: 661-669.

120. Morrison MA, Silveira AC, Huynh N, et al. Systems biology-based analysis implicates a novel role for vitamin D metabolism in the pathogenesis of age-related macular degeneration. Hum Genomics 2011; 5: 538-568.

121. Golan S, Shalev V, Treister G, et al. Reconsidering the connection between vitamin D levels and age-related macular degeneration. Eye (Lond) 2011; 25: 1122-1129.

122. Karlstetter M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: insights from novel mouse models. Immunobiology 2010; 215: 685-691.

123. Holick MF. Vitamin D deficiency. N Engl J Med 2007; 357: 266-281.

124. Freedman ND, Leitzmann MF, Hollenbeck AR, et al. Cigarette smoking and subsequent risk of lung cancer in men and women: analysis of a prospective cohort study. Lancet Oncol 2008; 9: 649-656.

125. Knudtson MD, Klein R, Klein BE. Physical activity and the 15-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. Br J Ophthalmol 2006; 90: 1461-1463.

126. Michigan A, Johnson TV, Master VA. Review of the relationship between C-reactive protein and exercise. Mol Diagn Ther 2011; 15: 265-275.

127. Schechtman KB, Barzilai B, Rost K, et al. Measuring physical activity with a single question. Am J Public Health 1991; 81: 771-773.

128. Burgisser P, Vaudaux J, Bart PA. Severe interference between retinal angiography and automated four-color flow cytometry analysis of blood mononuclear cells. Cytometry 2007; A 71: 632-636.

129. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. Surv Ophthalmol 1995; 39: 367-374.

130. Bressler NM, Bressler SB, West SK, et al. The grading and prevalence of macular degeneration in Chesapeake Bay watermen. Arch Ophthalmol 1989; 107: 847-852.

131. Klein R, Davis MD, Magli YL, et al. The Wisconsin age-related maculopathy grading system. Ophthalmology 1991; 98: 1128-1134.

132. Seddon JM, Sharma S, Adelman RA. Evaluation of the clinical age-related maculopathy staging system. Ophthalmology 2006; 113: 260-266.

133. Ferris FL, 3rd, Wilkinson CP, Bird A, et al. Clinical classification of age-related macular degeneration. Ophthalmology 2013; 120: 844-851.

134. Mollnes TE. Complement and biocompatibility. Vox Sang 1998; 74 Suppl 2: 303-307.

135. Seamans KM, Cashman KD. Existing and potentially novel functional markers of vitamin D status: a systematic review. Am J Clin Nutr 2009; 89: 1997S-2008S.

136. Hollis BW. Assessment of vitamin D status and definition of a normal circulating range of 25-hydroxyvitamin D. Curr Opin Endocrinol Diabetes Obes 2008; 15: 489-494.

137. Hollis BW. Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs. Am J Clin Nutr 2008; 88: 507S-510S.

138. Agborsangaya C, Toriola AT, Grankvist K, et al. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and androstenedione. Nutr Cancer 2010; 62: 51-57.

139. Crane IJ, Liversidge J. Mechanisms of leukocyte migration across the blood-retina barrier. Semin Immunopathol 2008; 30: 165-177.

140. Shechter R, London A, Schwartz M. Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. Nat Rev Immunol 2013; 13: 206-218.

141. Joly S, Francke M, Ulbricht E, et al. Cooperative phagocytes: resident microglia and bone marrow immigrants remove dead photoreceptors in retinal lesions. Am J Pathol 2009; 174: 2310-2323.

142. Raoul W, Auvynet C, Camelo S, et al. CCL2/CCR2 and CX3CL1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration. J Neuroinflammation 2010; 7: 87.

143. Tuo J, Bojanowski CM, Zhou M, et al. (2007) Murine ccl2/cx3cr1 deficiency results in retinal lesions mimicking human age-related macular degeneration. Invest Ophthalmol Vis Sci 2007; 48: 3827-3836.

144. Koch N, Jung M, Sabat R, et al. IL-10 protects monocytes and macrophages from complement-mediated lysis. J Leukoc Biol 2009; 86: 155-166.

145. Thurman JM, Renner B, Kunchithapautham K, et al. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. J Biol Chem 2009; 284: 16939-16947.

146. Kunchithapautham K, Rohrer B. Sublytic membrane-attack-complex (MAC) activation alters regulated rather than constitutive vascular endothelial growth factor (VEGF) secretion in retinal pigment epithelium monolayers. J Biol Chem 2011; 286: 23717-23724.

147. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 2006; 38: 458-462.

148. Fagerness JA, Maller JB, Neale BM, et al. Variation near complement factor I is associated with risk of advanced AMD. Eur J Hum Genet 2009; 17: 100-104.

149. Jakobsdottir J, Conley YP, Weeks DE, et al. C2 and CFB genes in age-related maculopathy and joint action with CFH and LOC387715 genes. PLoS One 2008; 3: e2199.

150. Spencer KL, Olson LM, Anderson BM, et al. C3 R102G polymorphism increases risk of age-related macular degeneration. Hum Mol Genet 2008; 17: 1821-1824.

151. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 2007; 357: 553-561.

152. Baas DC, Ho L, Ennis S, et al. The complement component 5 gene and age-related macular degeneration. Ophthalmology 2010; 117: 500-511.

153. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. Hum Mol Genet 2007; 16: 1986-1992.

154. Crabb JW, Miyagi M, Gu X, et al. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc Natl Acad Sci U S A 2002; 99: 14682-14687.

155. Anderson DH, Ozaki S, Nealon M, et al. Local cellular sources of apolipoprotein E in the human retina and retinal pigmented epithelium: implications for the process of drusen formation. Am J Ophthalmol 2001; 131: 767-781.

156. Mullins RF, Russell SR, Anderson DH, et al. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J 2000; 14: 835-846.

157. Hageman GS, Luthert PJ, Victor Chong NH, et al. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res 2001; 20: 705-732.

158. Johnson LV, Forest DL, Banna CD, et al. Cell culture model that mimics drusen formation and triggers complement activation associated with age-related macular degeneration. Proc Natl Acad Sci U S A 2011; 108: 18277-18282.

159. Cashman SM, Ramo K, Kumar-Singh R. A non membrane-targeted human soluble CD59 attenuates choroidal neovascularization in a model of age related macular degeneration. PLoS One 2011; 6: e19078.

160. Cipriani V, Matharu BK, Khan JC, et al. Genetic variation in complement regulators and susceptibility to age-related macular degeneration. Immunobiology 2012; 217: 158-161.

161. Palestine AG, Nussenblatt RB, Chan CC, et al. Histopathology of the subretinal fibrosis and uveitis syndrome. Ophthalmology 1985; 92: 838-844.

162. Combadiere C, Feumi C, Raoul W, et al. CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration. J Clin Invest 2007; 117: 2920-2928.

163. Chan-Ling T, Hughes S, Baxter L, et al. Inflammation and breakdown of the blood-retinal barrier during "physiological aging" in the rat retina: a model for CNS aging. Microcirculation 2007; 14: 63-76.

164. Guillemin GJ, Brew BJ. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. J Leukoc Biol 2004; 75: 388-397.

165. Aguzzi A, Barres BA, Bennett ML. Microglia: scapegoat, saboteur, or something else? Science 2013; 339: 156-161.

166. Ma W, Zhao L, Wong WT. Microglia in the outer retina and their relevance to pathogenesis of age-related macular degeneration. Adv Exp Med Biol 2012; 723: 37-42.

167. Caserta S, Nausch N, Sawtell A, et al. Chronic infection drives expression of the inhibitory receptor CD200R, and its ligand CD200, by mouse and human CD4 T cells. PLoS One 2012; 7: e35466.

168. Sivak JM. The aging eye: common degenerative mechanisms between the Alzheimer's brain and retinal disease. Invest Ophthalmol Vis Sci 2013; 54: 871-880.

169. Woo SJ, Park KH, Ahn J, et al. Cognitive impairment in age-related macular degeneration and geographic atrophy. Ophthalmology 2012; 119: 2094-2101.

170. Ohno-Matsui K. Parallel findings in age-related macular degeneration and Alzheimer's disease. Prog Retin Eye Res 2011; 30: 217-238.

171. Kaarniranta K, Salminen A, Haapasalo A, et al. Age-related macular degeneration (AMD): Alzheimer's disease in the eye? J Alzheimers Dis 2011; 24: 615-631.

172. Chitnis T, Imitola J, Wang Y, et al. Elevated neuronal expression of CD200 protects Wlds mice from inflammation-mediated neurodegeneration. Am J Pathol 2007; 170: 1695-1712.

173. Zhang S, Wang XJ, Tian LP, et al. CD200-CD200R dysfunction exacerbates microglial activation and dopaminergic neurodegeneration in a rat model of Parkinson's disease. J Neuroinflammation 2011; 8: 154.

174. Heaney RP. Barriers to optimizing vitamin D3 intake for the elderly. J Nutr 2006; 136: 1123-1125.

175. Petta S, Camma C, Scazzone C, et al. Low vitamin D serum level is related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C. Hepatology 2010; 51: 1158-1167.

176. Ramirez AM, Wongtrakool C, Welch T, et al. Vitamin D inhibition of pro-fibrotic effects of transforming growth factor beta1 in lung fibroblasts and epithelial cells. J Steroid Biochem Mol Biol 2010; 118: 142-150.

177. Jo YJ, Sonoda KH, Oshima Y, et al. Establishment of a new animal model of focal subretinal fibrosis that resembles disciform lesion in advanced age-related macular degeneration. Invest Ophthalmol Vis Sci 2011; 52: 6089-6095.

178. Van Geest RJ, Lesnik-Oberstein SY, Tan HS, et al. A shift in the balance of vascular endothelial growth factor and connective tissue growth factor by bevacizumab causes the angiofibrotic switch in proliferative diabetic retinopathy. Br J Ophthalmol 2012; 96: 587-590.

179. Grunwald JE, Daniel E, Huang J, et al. Risk of Geographic Atrophy in the Comparison of Age-related Macular Degeneration Treatments Trials. Ophthalmology 2014; 121: 150-161.

180. Klein ML, Mauldin WM, Stoumbos VD. Heredity and age-related macular degeneration. Observations in monozygotic twins. Arch Ophthalmol 1994; 112: 932-937.

181. Mann SS, Rutishauser-Arnold Y, Peto T, et al. The symmetry of phenotype between eyes of patients with early and late bilateral age-related macular degeneration (AMD). Graefes Arch Clin Exp Ophthalmol 2011; 249: 209-214.

182. Lopez PF, Sippy BD, Lambert HM, et al. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1996; 37: 855-868.

183. Saint-Geniez M, Kurihara T, Sekiyama E, et al. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. Proc Natl Acad Sci U S A 2009; 106: 18751-18756.

184. Wick G, Grundtman C, Mayerl C, et al. The immunology of fibrosis. Annu Rev Immunol 2013; 31: 107-135.

185. Nagaharu K, Zhang X, Yoshida T, et al. Tenascin C induces epithelial-mesenchymal transition-like change accompanied by SRC activation and focal adhesion kinase phosphorylation in human breast cancer cells. Am J Pathol 2011; 178: 754-763.

186. Takahashi Y, Sawada G, Kurashige J, et al. Tumor-derived tenascin-C promotes the epithelial-mesenchymal transition in colorectal cancer cells. Anticancer Res 2013; 33: 1927-1934.

187. Branton MH, Kopp JB. TGF-beta and fibrosis. Microbes Infect 1999; 1: 1349-1365.

188. Senoo T, Hattori N, Tanimoto T, et al. Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. Thorax 2010; 65: 334-340.

189. Fogo AB. Renal fibrosis: not just PAI-1 in the sky. J Clin Invest 2003 112: 326-328.

190. Radisky ES, Radisky DC. Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. J Mammary Gland Biol Neoplasia 2010; 15: 201-212.

191. Ahmad FK, Morley MG, Berry A, et al. Effects of ranibizumab on exudative disciform scars. Ophthalmic Surg Lasers Imaging 2009; 40: 366-372.

192. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008; 214: 199-210.

193. Liu RT, Gao J, Cao S, et al. Inflammatory mediators induced by amyloid-beta in the retina and RPE in vivo: implications for inflammasome activation in age-related macular degeneration. Invest Ophthalmol Vis Sci 2013; 54: 2225-2237.

194. Lee V, Rekhi E, Hoh Kam J, et al. Vitamin D rejuvenates aging eyes by reducing inflammation, clearing amyloid beta and improving visual function. Neurobiol Aging 2012; 33: 2382-2389.

195. Joshi S, Pantalena LC, Liu XK, Gaffen SL, et al. 1,25-dihydroxyvitamin D(3) ameliorates Th17 autoimmunity via transcriptional modulation of interleukin-17A. Mol Cell Biol 2011; 31: 3653-3669.

196. Hegyi Z, Zwicker S, Bureik D, et al. Vitamin D analog calcipotriol suppresses the Th17 cytokine-induced proinflammatory S100 "alarmins" psoriasin (S100A7) and koebnerisin (S100A15) in psoriasis. J Invest Dermatol 2012; 132: 1416-1424.

197. Chang JH, Cha HR, Lee DS, et al. 1,25-Dihydroxyvitamin D3 inhibits the differentiation and migration of T(H)17 cells to protect against experimental autoimmune encephalomyelitis. PLoS One 2010; 5: e12925.

198. Isik S, Ozuguz U, Tutuncu YA, et al. Serum transforming growth factor-beta levels in patients with vitamin D deficiency. Eur J Intern Med 2012; 23: 93-97.

199. Aschenbrenner JK, Sollinger HW, Becker BN, et al. 1,25-(OH(2))D(3) alters the transforming growth factor beta signaling pathway in renal tissue. J Surg Res 2001; 100: 171-175.

200. Halder SK, Osteen KG, Al-Hendy A. Vitamin D3 inhibits expression and activities of matrix metalloproteinase-2 and -9 in human uterine fibroid cells. Hum Reprod 2013; 28: 2407-2416.

201. Wasse H, Cardarelli F, De Staercke C, et al. 25-hydroxyvitamin D concentration is inversely associated with serum MMP-9 in a cross-sectional study of African American ESRD patients. BMC Nephrol 2011; 12: 24.

202. Gonzalez-Sancho JM, Alvarez-Dolado M, Munoz A. 1,25-Dihydroxyvitamin D3 inhibits tenascin-C expression in mammary epithelial cells. FEBS Lett 1998; 426: 225-228.

203. Wu-Wong JR, Nakane M, Ma J. Vitamin D analogs modulate the expression of plasminogen activator inhibitor-1, thrombospondin-1 and thrombomodulin in human aortic smooth muscle cells. J Vasc Res 2007; 44: 11-18.

204. Mansouritorghabe H, Bonakdaran S, Hossieni SJ. Correlation between Serum 25 hydroxy vitamin D level and plasminogen activator inhibitor 1 in type 2 diabetic patients. Endocr Metab Immune Disord Drug Targets 2013.

205. Do DV, Pieramici DJ, van Lookeren Campagne M, et al. A Phase Ia Dose-Escalation Study of the Anti-Factor D Monoclonal Antibody Fragment Fcfd4514s in Patients with Geographic Atrophy. Retina 2013; 34: 313-320.

206. Bora NS, Kaliappan S, Jha P, et al. CD59, a complement regulatory protein, controls choroidal neovascularization in a mouse model of wet-type age-related macular degeneration. J Immunol 2007; 178: 1783-1790.

207. Bora NS, Jha P, Lyzogubov VV, et al. Recombinant membrane-targeted form of CD59 inhibits the growth of choroidal neovascular complex in mice. J Biol Chem 2010; 285: 33826-33833.

208. Sweigard JH, Cashman SM, Kumar-Singh R . Adenovirus-mediated delivery of CD46 attenuates the alternative complement pathway on RPE: implications for age-related macular degeneration. Gene Ther 2011; 18: 613-621.

209. Manson JE, Bassuk SS, Lee IM, et al. The VITamin D and OmegA-3 TriaL (VITAL): rationale and design of a large randomized controlled trial of vitamin D and marine omega-3 fatty acid supplements for the primary prevention of cancer and cardiovascular disease. Contemp Clin Trials 2012; 33: 159-171.