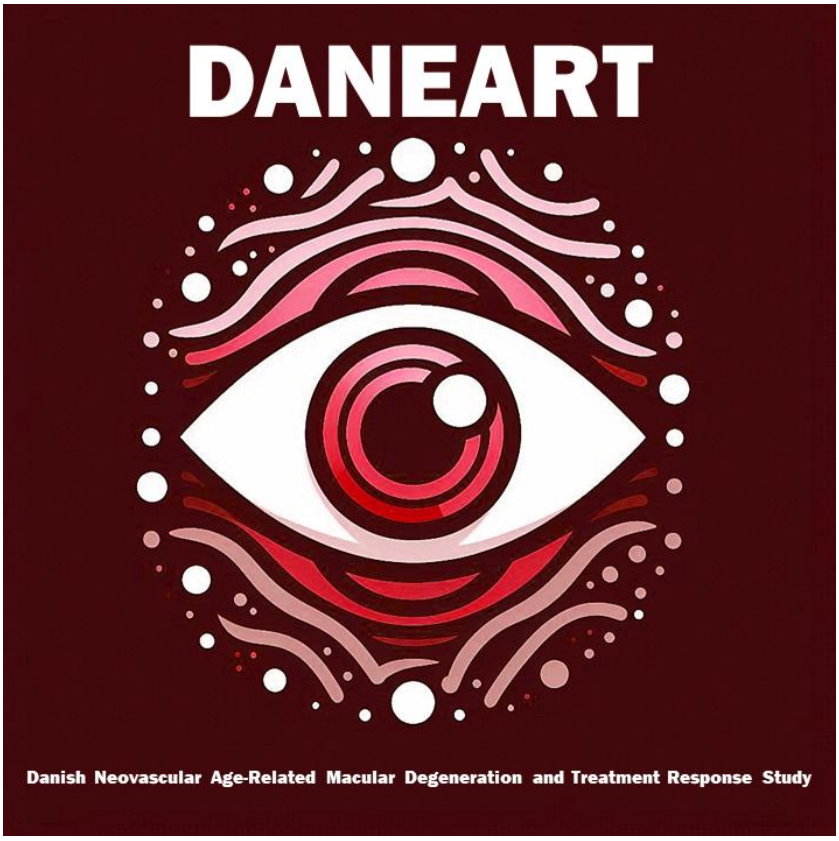
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES



PhD Thesis

Alexander Kai Thomsen

Development of Targeted Therapy to Patients with Age-Related Macular Degeneration

Principal supervisor: Torben Lykke Sørensen, MD, DMSc
Primary co-supervisor: Mogens Holst Nissen, MD, DMSc

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen: April 30th, 2025

Name of department: Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark,
Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

Author: Alexander Kai Thomsen, MD

Title: Development of Targeted Therapy to Patients with Age-related Macular Degeneration

Topic description: Investigation of systemic immune alterations in patients with age-related macular degeneration in relation to disease stage and treatment response.

Supervisors: Torben Lykke Sørensen, MD, Professor, DMSc, Chief Physician,
Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark,
Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.

Mogens Holst Nissen, MD, Professor, DMSc,
Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark,
Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.

Date: April 30th, 2025.

Cover image created with Microsoft Designer.

Table of contents

I. Preface

This PhD thesis was completed at the Faculty of Health and Medical Sciences, University of Copenhagen. The project was performed at the Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark, and the University of Copenhagen, Copenhagen, Denmark from September 2020 to April 2025.

I started the PhD project as a medical student, working on it part-time. It was a refreshing change from studying to have the opportunity to do research, and it provided a versatile, exciting, and at times, busy everyday life. When I started the project, I was very confused and did not know much about research. I felt like I was thrown in the deep end, which has been tough, but I have also learned a lot. I have learned from my mistakes and have developed a passion for pursuing further research.

I would like to thank all the research **participants** who took the time and allowed themselves to be examined in the name of research, so that we can expand our knowledge. I am deeply grateful for that.

I would like to thank my principal supervisor **Torben Lykke Sørensen**, who gave me the opportunity to carry out the project, even as a medical student with limited experience. Torben has always believed in me and given me the freedom to think for myself, run the project my way, and dive into side projects. Trust has been mutual, and our collaboration has been characterized by honesty, professional discussions, and cozy lunch breaks.

I would like to thank my fellow PhD students and friends **Kathrine Gotfredsen**, **Andrea Nedergaard Jensen**, **Birte Bay Højsted**, **Charlotte Liisborg**, and **Anders Sønderskov Jürs**, who have made the office a fun place. A big thanks to the research staff **Sophie Louise Kienle Vinther**, **Charlotte Thornbye Larsen**, **Gitte Henningsen**, and **Anni Margrethe Bæk** for your help and company in our research unit.

Thank you to the department's fantastic leadership, who have without hesitation helped me every time I had practical questions: **Ditte Erngaard**, **Lene Pia Mundt**, **Lisbeth Jelveh Sandfeld**, and **Gry Hansen**.

I would also like to thank my colleagues at the Institute of Immunology and Microbiology, University of Copenhagen, **Mogens Holst Nissen**, **Maria Abildgaard Steffensen**, and **Charlotte Vibeke Thorndahl**, who have helped me immensely in understanding immunology and laboratory work.

Marie Krogh Nielsen, **Mads Krüger Falk**, **Caroline Laugesen**, **Amalie Thomsen Nielsen**, **Bent Honoré**, and **Henrik Vorum** also deserve thanks for their help with research questions in everyday life and with the project.

Thank you to the Clinical Biochemical Department, Zealand University Hospital, Roskilde, and **Thomas Hviid**, **Nanna Jørgensen**, **Lisbeth Landt Hansen**, **Gitte Thomsen**, and **Liselotte Tinglev Jacobsen** for lending out your laboratory facilities and for your help whenever I had questions about flow cytometry.

Also, thanks to **SriniVas Sadda** and Doheny Eye Institute, University of Los Angeles, California, USA, whom I had the pleasure of visiting during my environmental change stay. I learned a lot about retinal imaging analysis from you and your group.

It has been a pleasure to supervise Danish and international bachelor's and master's students, who have brought youthful life to the office. I wish you all the best.

I would like to thank **every doctor**, **nurse**, **secretary**, and **SOSU assistant** in our department for their invaluable support in ensuring that all practical aspects ran smoothly.

Thank you to all the foundations that have supported the project financially. Without your support this research would not have been possible: Velux Foundation, Fight for Sight Denmark (Øjenforeningen), Synoptik Foundation, Region Zealand PhD Grant, Dagmar Marshall's Foundation, Grosserer L.F. Foght's Foundation, King Christian X's Foundation, Øjenfonden, Snedkermester Sophus Jacobsen og hustru's Foundation, and Lizzi and Mogens Staal's Foundation. The funding organizations had no role in the design or conduct of this research.

Thank you to the assessment committee **Morten Carstens Moe**, **Thomas Juhl Corydon**, and **Javad Nouri Hajari** for taking time out of your busy schedules to assess this PhD.

Thank you to my family who have always supported me.

This project was an amazing experience with so many interesting challenges and solutions.

Alexander Kai Thomsen

April 30th, 2025

II. List of papers

I Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response.
Thomsen AK, Steffensen MA, Hinnerskov JMV, Nielsen AT, Vorum H, Honoré B, Nissen MH, Sørensen TL.
Journal of Neuroinflammation. 2024;21(1):284. Published 2024 Nov 1.
doi: 10.1186/s12974-024-03273-7

II Plasma Interferon-gamma is Associated with Advanced Stage and Poor Treatment Response in Neovascular Age-Related Macular Degeneration.
Thomsen AK, Steffensen MA, Hinnerskov JMV, Nielsen AT, Vorum H, Honoré B, Nissen MH, Sørensen TL.
Aging and Disease. Published online 2025 Apr 9.
doi: 10.14336/AD.2024.1585

III Chemokine system changes drive age-related macular degeneration and influence treatment outcomes.
Thomsen AK, Steffensen MA, Nielsen AT, Vorum H, Honoré B, Nissen MH, Sørensen TL.
Investigative Ophthalmology and Visual Science. Accepted 2025 Apr 14.

IV Neutrophil-to-lymphocyte ratio is associated with treatment response in neovascular age-related macular degeneration.
Thomsen AK, Steffensen MA, Gotfredsen KG, Vorum H, Honoré B, Nissen MH, Sørensen TL.
American Journal of Ophthalmology International. In review, submitted 2025 Mar 25.

III. Abbreviations

AKT	Alexander Kai Thomsen
AMD	Age-related macular degeneration
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Allophycyanin
APC-Cy7	Allophycocyanin-cyanine 7
AREDS	Age-Related Eye Disease Study
ARMS2	Age-Related Maculopathy Susceptibility 2
ART	Automatic real time
BCVA	Best corrected visual acuity
C3	Complement component 3
CARMS	Clinical age-related maculopathy staging
CCL	C-C motif ligand
CCR	C-C motif receptor
CD	Cluster of differentiation
CFH	Complement factor H
CI95%	95% confidence interval
cMonocytes	Classical monocytes
Cregs	Complement regulatory proteins
CRP	C-reactive protein
CRT	Central retinal thickness
CX3CR	C-X3-X motif receptor
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DANEART	Danish Neovascular Age-Related Macular Degeneration and Treatment Response
EDTA	Ethylenediaminetetraacetic acid
ETDRS	Early Treatment of Diabetic Retinopathy Study
fcs	Flow cytometry standard data file
FITC	Fluorescein isothiocyanate
GA	Geographic atrophy
iAMD	Intermediate age-related macular degeneration
IFN-γ	Interferon-γ

IL	Interleukin
iMonocytes	Intermediate monocytes
IRF	Intraretinal fluid
LH	Lithium-heparin
nAMD	Neovascular age-related macular degeneration
NLR	Neutrophil-to-lymphocyte ratio
nMonocytes	Non-classical monocytes
OCT	Optical coherence tomography
OCTA	Optical coherence tomography angiography
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE/Cy7	Phycoerythrin-cyanine 7
PED	Pigment epithelium detachment
PerCP	Peridinin-chlorophyll-protein
RPE	Retinal pigment epithelium
rpm	Rounds per minute
rs	Reference single-nucleotide polymorphism
RT	Room temperature
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SRF	Subretinal fluid
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

IV. English summary

Age-related macular degeneration (AMD) is a retinal disease and one of the most common causes of impaired vision and blindness in the elderly. The pathogenesis is not fully understood, but aging, environmental factors, and genetics play a significant role. These factors also affect the aging immune system, and previous studies have shown that patients with neovascular AMD (nAMD) have increased activation and dysregulation of the systemic immune system.

In this PhD project, the Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study, we investigate whether specific immune changes are associated with treatment response and risk polymorphisms in nAMD patients. We also examine differences in these immune changes between healthy controls, intermediate AMD (iAMD), and nAMD patients.

In the DANEART study, 100 nAMD patients, 34 iAMD patients, and 61 healthy controls were included at baseline. The nAMD patients were followed for treatment response after a loading dose of three anti-vascular endothelial growth factor (VEGF) treatments and after one year. 94 nAMD patients completed the 1-year follow-up. The patients were examined using immunoassays and flow cytometry for systemic immune changes, as well as genetic analyses of AMD-associated risk polymorphisms.

We found that nAMD patients with suboptimal treatment responses had dysregulation of the complement cascade, decreased expression of complement regulatory protein CD35 on monocytes, increased T cell differentiation, elevated plasma concentration of interferon-γ (IFN-γ), decreased expression of chemokine receptors CXCR3 on CD4+ T cells, and CCR2 and CCR5 on monocyte subtypes, as well as higher neutrophil-to-lymphocyte ratio. iAMD patients had similar systemic changes, but to a lesser extent. Furthermore, AMD-associated risk polymorphisms were involved in the complement and chemokine systems in nAMD patients. Particularly, it seems that IFN-γ plays a central role, as this cytokine induces the complement cascade, inhibits complement regulatory proteins, and affects the chemokine system as well as the neutrophil-to-lymphocyte ratio.

These systemic immune changes may be the cause of chronic inflammation in the retina, potentially increasing VEGF levels or other proangiogenic mechanisms, leading to the suboptimal response to anti-VEGF treatment in nAMD patients. Future studies should investigate whether IFN-γ or its related molecular pathways could serve as new treatment targets for nAMD patients who exhibit suboptimal treatment responses.

V. Dansk resumé (Danish summary)

Aldersrelateret makuladegeneration (AMD) er en nethindesygdom og en af de hyppigste årsager til nedsat syn og blindhed hos ældre. Patogenesen er ikke fuldt klarlagt, men aldring, miljøfaktorer og genetik spiller en væsentlig rolle. Disse faktorer påvirker også det aldrende immunsystem, og tidligere studier har vist, at patienter med neovaskulær AMD (nAMD) har øget aktivering og dysregulering af det systemiske immunforsvar.

I dette ph.d.-projekt, det Danske Neovaskulær Aldersrelaterede Makuladegeneration og Behandlingsrespons (DANEART)-studie, undersøger vi, om specifikke immunforandringer er associeret med behandlingsrespons og risiko-polymorfismer hos nAMD-patienter. Vi undersøger også forskelle i disse immunforandringer mellem raske kontroller, patienter med intermediær AMD (iAMD) og nAMD-patienter.

I DANEART-studiet blev 100 nAMD-patienter, 34 iAMD-patienter og 61 raske kontroller inkluderet ved baseline. nAMD-patienterne blev fulgt for behandlingsrespons efter en loading-dose af tre anti-vaskulært endotelvækstfaktor (VEGF)-behandlinger og igen efter et år. 94 nAMD-patienter gennemførte 1-års opfølgningen. Patienterne blev undersøgt med immunoassays og flowcytometri for systemiske immunforandringer samt genetiske analyser af AMD-associerede risiko-polymorfismer.

Vi fandt at nAMD patienter med suboptimalt behandlingsrespons havde dysregulation af komplement-kaskaden, nedsat ekspression af komplement regulatorisk protein CD35 på monocytter, øget T celle differentiering, øget plasma koncentration af interferon-γ (IFN-γ), nedsat ekspression af kemokin-receptorer CXCR3 på CD4+ T celler, og CCR2 og CCR5 på monocyt-subtyper, samt højere neutrofil-lymfocyt-ratio. Patienter med iAMD havde lignende systemiske forandringer, men i mindre udtalt grad. Desuden var AMD-associerede risiko-polymorfismer involveret i komplement- og kemokin-systemet hos nAMD-patienter. Især virker det til, at IFN-γ spiller en central rolle, da dette cytokin inducerer komplement-kaskaden, hæmmer komplementregulerende proteiner og påvirker kemokin-systemet samt neutrofil-lymfocyt-ratioen. Disse systemiske immunforandringer kan være årsagen til den kroniske inflammation i nethinden, hvilket potentielt øger VEGF-niveauet eller andre angiogenese-fremmende mekanismer, som forårsager det suboptimale respons for anti-VEGF-behandling hos nAMD patienter. Fremtidige studier bør undersøge, om IFN-γ eller dets relaterede molekylære pathways kan fungere som nye behandlingsmål for nAMD-patienter, der udviser suboptimale behandlingsrespons.

1. Background

1.1. Human eye and retina

The eye is an extended part of the brain responsible for the sense of vision. Its architecture comprises structural and supportive elements that maintain the visual apparatus. Electromagnetic radiation within the human visual spectrum (photons) from the external environment is transformed into visual perception in the brain by traveling through the eye's refractive components, activating sensory cells and the visual cortex. The refractive elements include the cornea and the lens, with minor contributions from the aqueous humor and vitreous body, which focus light onto the retina. Here, photoreceptors are activated, generating changes in membrane potential that produce an electrochemical signal transmitted through the retina, optic nerve, and ultimately to the brain.[1]

The human retina has a complex microscopic anatomy. Its sensory cells include two types of photoreceptors: cones and rods. Cones are concentrated in the central retina and are responsible for color vision and visual acuity. Rods, predominantly located in the peripheral retina, are unable to differentiate colors but are more sensitive, making them critical for night vision. The retina also consists of multiple layers of specialized neurons, which relay signals to the brain. Together, these layers form the neuroretina, which architecture is essential for maintaining visual function, yet highly vulnerable to both internal and external damage.[2] Posterior to the neuroretina lies the retinal pigment epithelium (RPE), which absorbs most of the residual light, a critical function for sharp vision. Like all epithelia, the RPE is underlain by a basal membrane, called Bruch's membrane, which separates the retina from the choroid. The choroid contains a fine vascular network that supplies the retina with oxygen, nutrients, and waste clearance. The blood-retinal barrier consists of the RPE, Bruch's membrane, and tight junctions between endothelial cells in the retinal vasculature. This barrier protects the retina from pathogens and the systemic immune response, rendering the retina an immune-privileged tissue.[3]

Macroscopically, the retina comprises the following key elements: the optic disc, retinal blood vessels, macula, and fovea (Figure 1). The macula represents the central part of the retina, situated within the temporal vascular arcades, with the fovea at its center responsible for central vision. Central vision is essential for activities of daily living, such as reading, driving, and face recognition. Impairment of central vision significantly reduces quality of life.[4]

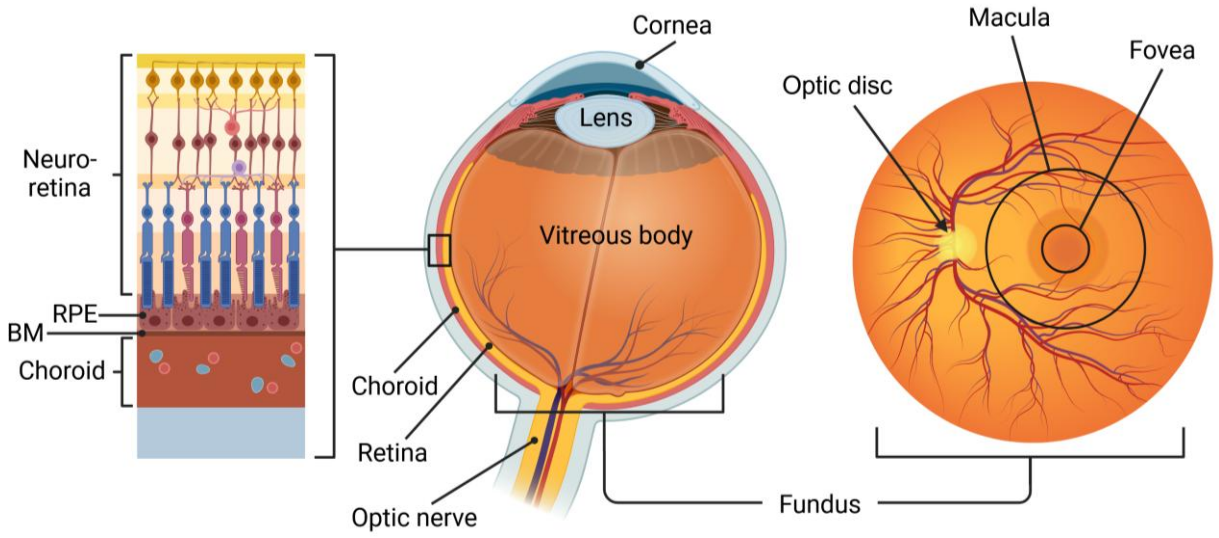


Figure 1. Key structures of the human eye and retina. RPE = retinal pigment epithelium, BM = Bruch's membrane. Own work, created with BioRender.

1.2. Age-related macular degeneration

Age-related macular degeneration (AMD) is a retinal disease characterized by degenerative changes of the macula, affecting photoreceptors, RPE, Bruch's membrane and choroid, causing decreased function of the central visual field. The early and intermediate stages present with the formation of drusen, extracellular deposits consisting of lipids, proteins and minerals. These stages cause minor symptoms and are rarely noticed by the patients, but they increase the risk of developing sight threatening late-stage AMD.[5]

1.2.1. Epidemiology

AMD is the most common cause of irreversible visual impairment and legal blindness in the Global North, affecting the elderly. The incidence of AMD increases exponentially with age in persons aged 50 years or older,[6] and it has been estimated that the prevalence of AMD will be 288 million globally in 2040 as a consequence of the increased elderly population.[7]

AMD is a multifactorial disease, and the pathogenesis has not been completely uncovered. Aging is the most important risk factor, but genetics, environmental factors and chronic inflammation also affect AMD development.[8] AMD overall and late-stage AMD have an estimated heritability of 46% and 71%, respectively.[9] Multiple genetic variants are independently associated with AMD, as identified through genome-wide association studies, revealing 52 single nucleotide polymorphisms (SNPs) across 34 loci.[10] The two most important SNPs are Complement Factor H (CFH) rs1061170 on chromosome 1q32 and Age-Related Maculopathy Susceptibility 2 (ARMS2)

rs10490924 on chromosome 10q26.[11] CFH acts as a key regulator of the complement system, while the function of ARMS2 remains unclear. Environmental factors also play a significant role, including smoking, diet, and physical activity. Smoking has been shown to increase the risk of AMD by 2- to 4-fold.[12]

1.2.2. Stages

Several classification systems exist for grading AMD, including AREDS,[13] CARMS,[14] and Beckman.[15] These systems share the common feature of being assessable through clinical examination or fundus imaging. They categorize the disease into dry AMD without exudation and wet AMD with exudation, as well as into early and late stages. In this thesis, the focus will be on the Beckman classification. This system divides AMD and the aging retina into the following stages: no age-related changes, normal age-related changes, early AMD, intermediate AMD (iAMD), and late AMD. Late AMD is further subdivided into neovascular AMD (nAMD) and geographic atrophy (GA) (Table 1).

AMD stage	Definition*
No apparent aging changes	No drusen No AMD pigmentary abnormalities†
Normal aging changes	Small drusen ≤63 µm No AMD pigmentary abnormalities†
Early AMD	Medium drusen >63 to ≤125 µm No AMD pigmentary abnormalities†
Intermediate AMD	Large drusen >125 µm and/or AMD pigmentary abnormalities†
Geographic atrophy	Atrophic lesions secondary to AMD
Neovascular AMD	Exudations and/or hemorrhages secondary to AMD

*Lesions are assessed within the macula.

†AMD pigmentary abnormalities = any hyper- or hypopigmentary abnormalities associated with medium or large drusen.

Table 1. Beckman classification of AMD stages. Own work, adapted from Ferris et al.[15]

In clinical practice, the diagnosis is typically suspected based on symptoms and medical history, and the diagnosis is supported by a series of diagnostic investigations to confirm the stage of the disease and for differential diagnostic purposes.

1.2.3. Diagnosis

Symptoms

Non-exudative AMD (including the early AMD, iAMD, and GA) progresses gradually over months to years, and symptoms might not be noticed until severe visual loss occurs. People living with early AMD will usually experience no symptoms. Patients with iAMD will rarely notice any symptoms but can have difficulty reading in low-light surroundings and might experience minor degrees of metamorphopsia and blurred vision. GA causes central blurred vision progression to absolute scotomas. Symptoms of nAMD occur more rapidly within days to weeks, with sudden central metamorphopsia and decline in vision starting with blurred vision leading to scotomas.[16]

Best corrected visual acuity

Best corrected visual acuity (BCVA) is assessed using a visual acuity chart, such as the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart. The patient is positioned 4 meters from the chart, and one eye is tested at a time with optimal refractive correction. The patient is instructed to identify the symbols, such as letters, and the examiner records how many out of five letters the patient correctly identifies. The test continues with increasingly smaller lines, featuring finer arcminute resolution, until the patient can correctly identify no more than two letters. If the patient is unable to see the top (largest) line at a distance of 4 meters, the distance is reduced to 1 meter. If the patient still cannot see the top line, alternative methods are employed in the following order: finger counting, hand motion detection, and light perception.

Patients with early or iAMD typically maintain normal visual acuity, whereas late-stage AMD often leads to significant deterioration.[17] However, in the case of geographic atrophy, the impact on visual acuity largely depends on the location, as foveal sparing is not uncommon, where the fovea remains intact well into the disease progression.[18]

Visualization of the retina

To visualize the eye's anatomy in vivo, a specialized microscope called a slit-lamp biomicroscope can be used. This allows the examinator to see finer details in and around the eye, including the clear elements and the posterior segment. Slit lamp biomicroscopy is examiner-dependent and

cannot be digitally stored. With color fundus photography, it is possible to capture an image of the retina's posterior pole, known as the fundus.

Drusen are a cardinal finding in AMD and can be observed as yellowish dot-like lesions in the macula, however larger confluent drusen might form. In iAMD and late-stage AMD, AMD pigmentary abnormalities can be observed, including hyperpigmentation, typically seen as dark spots, and hypopigmentation, typically seen as lighter zones. In geographic atrophy, thinned, atrophic regions in a map-like pattern are present, which has given this stage its name. In nAMD, hemorrhages in the macula and/or the presence of retinal fluid is observed (Figure 2).[17]

Optical coherence tomography

Spectral domain optical coherence tomography (OCT) is a rapid, non-invasive imaging technique that can visualize the depth of the retina. By directing a laser at the retina and analyzing the reflected signal, one-dimensional data (A-scan) can be generated. By performing multiple A-scans along a line, a two-dimensional cross-sectional image of the retina (B-scan) can be created, and by taking multiple B-scans at different heights, a three-dimensional representation of the retina can be achieved.[19]

The retinal layers can be identified with OCT, as they have different reflectivity. Key layers related to AMD diagnosis and progression include the neuroretina, RPE, Bruch's membrane, and choroid (Figure 3). Drusen appear as a homogeneous, medium reflective mass between the RPE and Bruch's membrane, causing an elevation of the RPE.[20] OCT factors found in iAMD patients that increases the risk of developing late AMD include presence of intraretinal hyperreflective foci, subretinal drusenoid deposits, hyporeflectivity within drusenoid lesions, double layer sign, and increased drusen volume (Figure 4).[20]

Also commonly seen in AMD are pigment epithelial detachments (PEDs). A PED is a larger elevation of the RPE from Bruch's membrane compared to drusen. One definition of a PED is an RPE elevation exceeding 400 μm in width and 75 μm in height, or a vertical height of RPE elevation exceeding 200 μm.[21] PEDs can vary in reflectivity depending on their content. In geographic atrophy, retinal thinning is observed, which can be graded from incomplete to complete atrophy (Figure 2).[22] OCT is effective in visualizing leaked fluid in nAMD, which appears hyporeflective. Pathological fluid in nAMD may be intraretinal fluid (IRF), which presents as cysts in the neuroretina, or subretinal fluid (SRF), which accumulates between the neuroretina and RPE (Figure 2). The amount of pathological fluid reflects the disease activity in nAMD.[23]

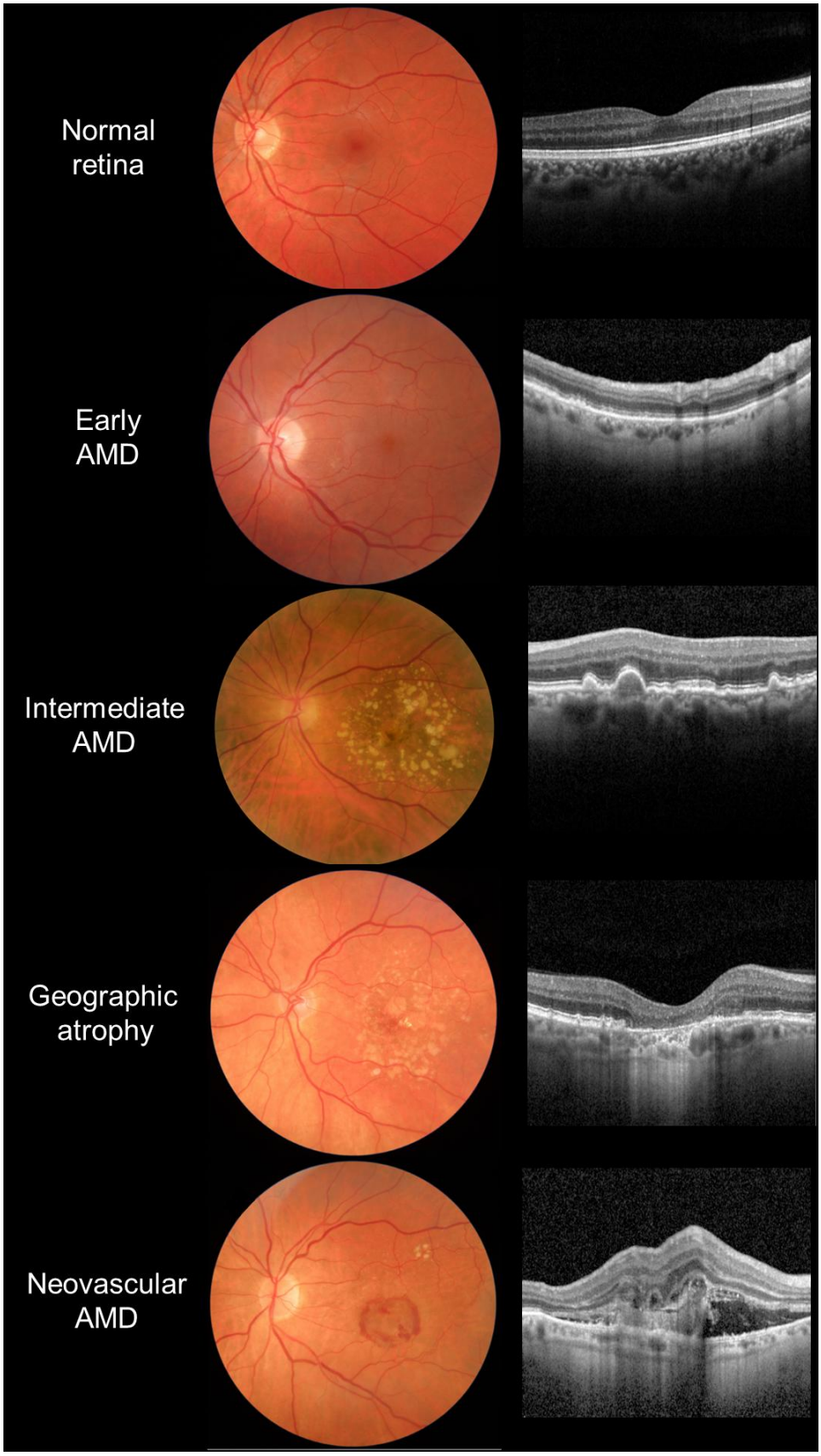


Figure 2. Fundus photography (left) and optical coherence tomography (right) of AMD stages. Own work.

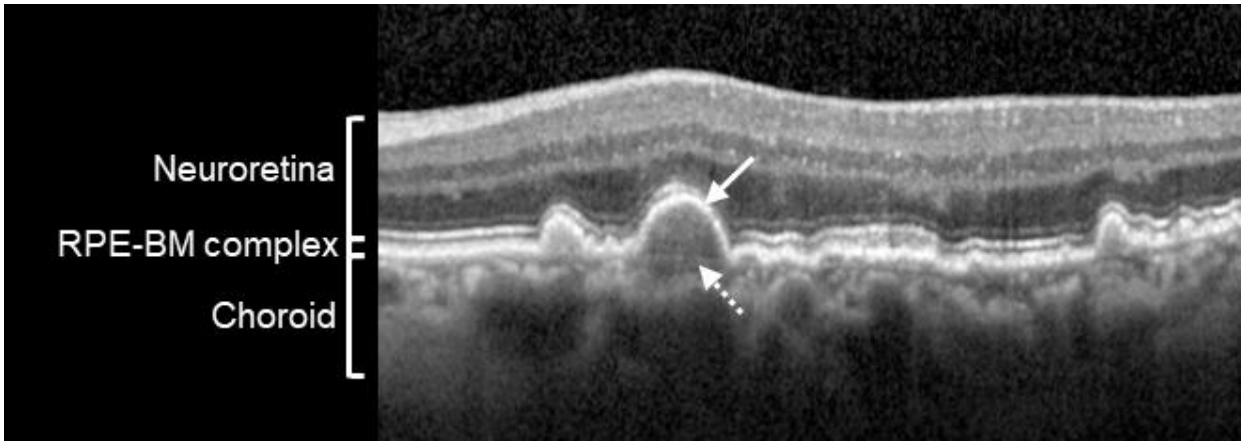


Figure 3. Optical coherence tomography of patients with intermediate AMD. The neuroretina constitutes the anterior part of the retina. The retinal pigment epithelium (RPE)-Bruch's membrane (BM) complex separates the neuroretina from the choroid. The RPE-BM complex will normally be tightly adherent, but drusen will separate the RPE (arrow) from the BM (dashed arrow). Own work.

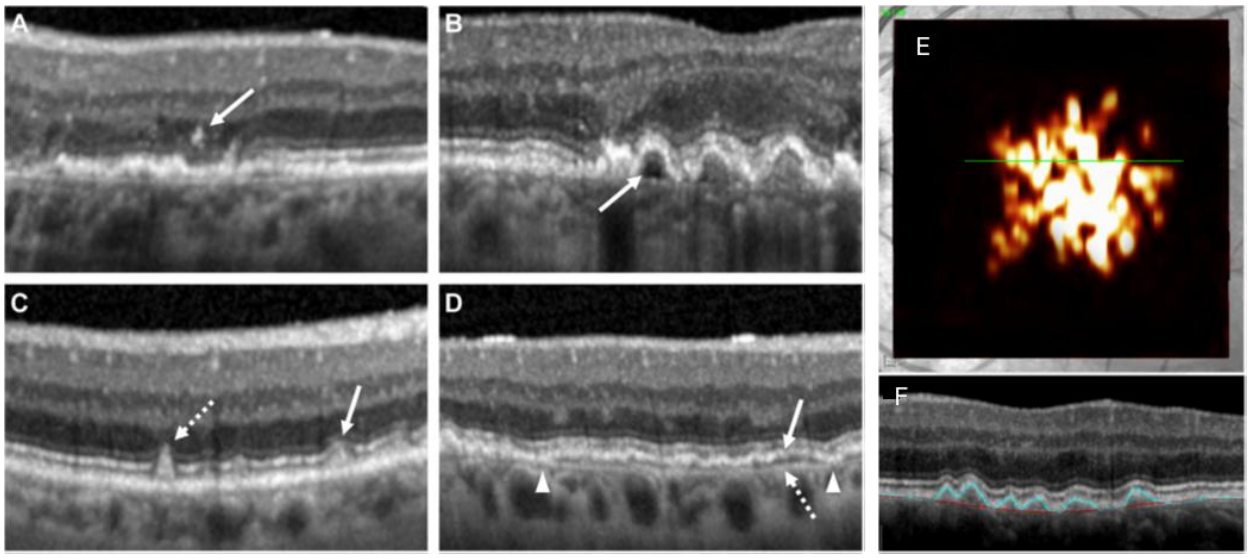


Figure 4. OCT risk factors in iAMD. (A) Intraretinal hyperreflective foci (arrow). (B) Hyporeflectivity within drusenoid lesion (arrow). (C) Subretinal drusenoid deposits stage 2 (solid arrow) and stage 3 (dashed arrow). (D) Double-layer sign (between arrowheads) being the retinal pigment epithelium (solid arrow) detached from Bruch's membrane (dashed arrow). (E) Drusen volume en face. (F) Drusen volume on B scan. Own work, published by Thomsen et al. Ophthalmology Retina (2024). Used with permission from Elsevier.[20]

Optical coherence tomography angiography

Using OCT technology, it is possible to visualize the retinal blood vessels, in a technique called OCT-angiography (OCTA). This allows for detailed imaging of blood vessels in all layers of the retina. Important layers of the retinal blood vessels include (1) the vascular plexuses of the inner retina, a network of concentric vessels not covering the fovea, (2) the avascular zone from the outer nuclear layer to Bruch's membrane, which is devoid of blood vessels, and (3) the choroid consisting of a fine meshwork of vessels. In nAMD pathological neovascularization is typically detected in the avascular zone on OCTA, which plays a key role in the diagnosis (Figure 5).[24]

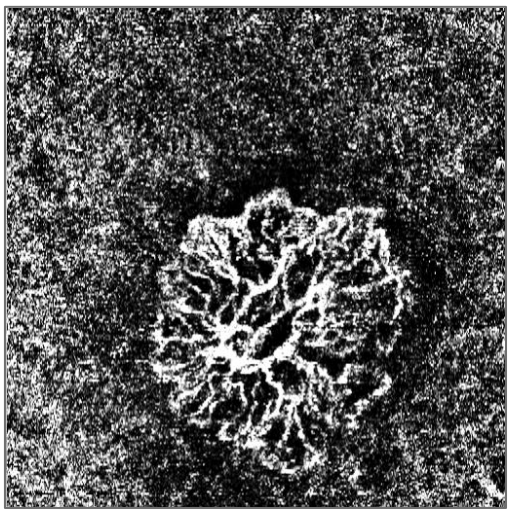


Figure 5. Macular neovascularization on OCTA in a patient with nAMD. Own work, adapted from master's thesis, Thomsen, University of Copenhagen (2021).

1.2.4. Treatment

Currently, no treatment options exists for iAMD, although high-dose antioxidant vitamins and zinc have been shown to reduce the risk of developing late-stage AMD within five years.[25] In 2023 the United States Food and Drug Administration approved the two complement inhibiting drugs pegcetacoplan and avacincaptad pegol for treatment of geographic atrophy. However, these have yet to be approved by the European Medicines Agency, due to the lack of long-term efficacy data.[26] Treatment for nAMD consists of repeated intravitreal injections with an anti-vascular endothelial growth factor (VEGF) antibody. Danish national guidelines recommend a loading dose of three injections at one-month intervals followed by personalized injection intervals scheduled based on the observe-and-plan regimen.[23] Until 2023 the recommended anti-VEGF antibody was aflibercept 2 mg, which binds to and thus inhibits VEGF-A, VEGF-B and placental growth factor. However,

it has since been replaced by the newly developed faricimab, which targets VEGF-A and angiopoietin-2, and has been shown to have a longer-lasting therapeutic effect.[27]

Since the treatment requires specialized personnel for the administration of the drug, patients must attend each treatment session in person, and both staff and facilities must be available. Patients will receive at least a loading dose, but the treatment may continue for the remainder of their lives. Furthermore, anti-VEGF antibodies are expensive and have been reviewed by the Danish Council for the Use of Expensive Hospital Medicines.[28] Consequently, this is a highly resource-intensive treatment for both the patient and the healthcare system.[29]

Intravitreal injections are an invasive procedure associated with the risk of complications, including endophthalmitis, which can lead to blindness.[16] Moreover, the treatment response can vary significantly between patients. We (Thomsen et al.) have previously evaluated the functional response in patients with nAMD. Functional response was defined based on visual acuity using the ETDRS chart. A good response was defined as an increase of 5 or more letters after treatment, partial response as a change of less than 5 letters (either an increase or decrease), and poor response as a loss of more than 5 letters. Following the loading dose, 36% of patients exhibited a good response, 52% partial response, and 12% poor response. After one year, 45% had a good response, 32% partial response, and 23% poor response.[23] Thus, there is a need for new treatment strategies to reduce the burden and to ensure that partially and poorly responding patients achieve adequate improvement in vision.

1.3. Inflammation and immunosenescence

1.3.1. Immune system and inflammation

The immune system is a complex system of tissues, cells, and signaling molecules that forms the foundation of the body's defense mechanisms and protection against external and internal factors. The immune system can be divided into the innate immune system, which can respond rapidly, and the adaptive immune system, which has the ability to learn and adapt but responds more slowly. These systems are highly integrated, and their roles in health and disease are active areas of research.[30]

Inflammation is the immune system’s response to harmful stimuli, such as pathogens, cellular damage, and toxins. It is a critical mechanism for protecting the body and maintaining homeostasis. Acute inflammation is primarily initiated through the activation of the innate immune system via pattern recognition receptors, which trigger a cascade of inflammatory pathways. Chronic inflammation is a prolonged and typically low-grade activation of both the innate and

adaptive immune systems, persisting in the absence of immediate injury or infection. This can result in damage to the body's own tissues, a phenomenon that plays a significant role in autoimmune diseases, cancers, and several age-related disorders.[30]

An important component of the innate immune system is the complement system, whose primary function is the formation of the cytolytic membrane attack complex (MAC), which induces osmotic lysis of target cells. However, the complement system also plays key roles in the recruitment of leukocytes and the opsonization of target cells for phagocytosis. The complement system operates as a cascade of reactions initiated by three pathways: the classical, lectin, and alternative pathways. All three pathways converge in the activation of C3 convertase, which cleaves the complement protein C3 into C3a and C3b, and the C3a/C3 ratio is thus an indicator of complement activation.[31] C3a acts as an anaphylatoxin, activating several inflammatory pathways, while C3b functions as an opsonin and contributes to the formation of C5 convertase. Cleavage of the complement protein C5 by C5 convertase is another central step in the complement cascade. The resulting products, C5a and C5b, have distinct functions: C5a serves as a proinflammatory anaphylatoxin, while C5b forms the first part of MAC.[32] The complement system is regulated in part by complement regulatory proteins (Cregs) on T cells and monocytes, which inhibits activation (Figure 6).[33]

Key cells of the adaptive immune system include T cells, which play essential roles in adaptive functions related to both homeostasis and inflammation. Naïve T cells are activated when their T cell receptors are stimulated by an antigen bound to a major histocompatibility complex on an antigen presenting cell. Activation also requires stimulation of costimulatory receptors and cytokines. This process enables T cells to recognize the antigen in the future and mount an inflammatory response upon re-exposure as memory and effector T cells. Mature T cells can be categorized into two main groups based on their surface markers, CD4 and CD8. CD4+ T helper cells are critical for the maturation and activation of other immune cells, while CD4+ regulatory T cells mediate immune tolerance as part of homeostasis. CD8+ cytotoxic T cells are responsible for destroying infected cells, cancer cells, and other damaged cells.[30]

Monocytes play vital roles in both the innate and adaptive immune systems. These cells are involved in cytokine secretion, phagocytosis, and antigen presentation. Monocytes can be recruited to sites of inflammation, where they differentiate into macrophages and dendritic cells. Monocytes are classified based on their surface markers CD14 and CD16 into classical (cMonocytes, $CD14^{high}CD16^{low}$), intermediate (iMonocytes, $CD14^{high}CD16^{high}$), and non-classical (nMonocytes, $CD14^{low}CD16^{high}$) subtypes. The majority of monocytes are cMonocytes, primarily functioning in

phagocytosis and inflammation, while iMonocytes and nMonocytes also contribute to antigen presentation and vascular patrolling.[34]

Neutrophils are granulocytes that constitute the majority of circulating leukocytes and represent a crucial component of the innate immune system. They act as the first line of defense in the immune response, where their primary functions include the phagocytosis of pathogens and apoptotic cells.[35] The complement system plays a key role in the activation and recruitment of neutrophils.[36,37] Additionally, neutrophils maintain a significant relationship with lymphocytes. They can activate T cells and contribute to their recruitment,[38,39] while T cells, in turn, can stimulate neutrophil activity, particularly through the secretion of interferon-γ (IFN-γ).[40] The neutrophil-to-lymphocyte ratio (NLR) is also considered an indicator of systemic inflammation.[41–43]

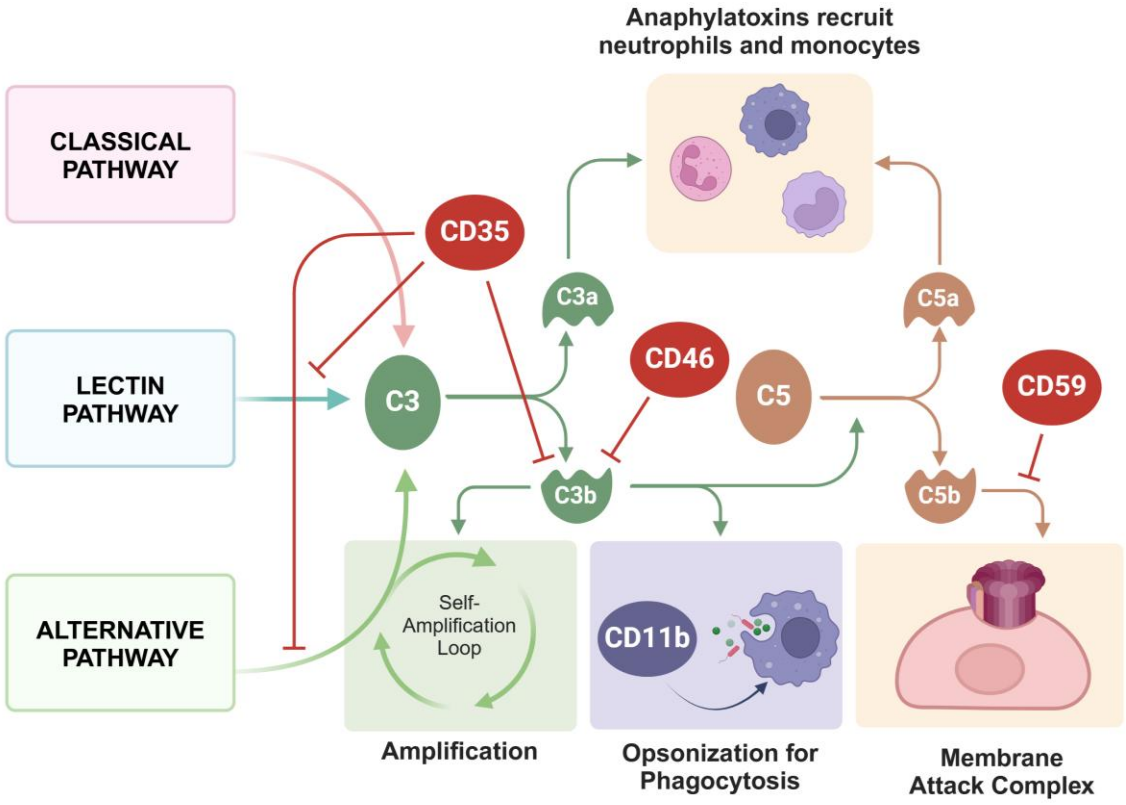


Figure 6. The complement system. The complement cascade can be initiated through three pathways, which converge at complement protein C3. These pathways lead to the formation of the membrane attack complex; however, complement proteins also serve additional functions, such as acting as anaphylatoxins that recruit leukocytes, self-amplification via C3 hydrolysis, and opsonization for phagocytosis. The complement cascade is regulated by complement regulatory proteins, including those bound to T cells and monocytes. Own work, created with BioRender.

1.3.2. Immunosenescence

Biological aging refers to the changes and degeneration of organ systems that occur as a consequence of chronological aging, genetics and environmental factors. Biological aging can therefore manifest very differently in two individuals of the same chronological age. Environmental factors include smoking, diet, and physical activity, which influence cellular stress.[44]

Biological aging also causes the immune system to undergo changes, a process termed immunosenescence. In this state, multiple functions of the immune system deteriorate, accompanied by an increase in proinflammatory activation known as inflammaging. Inflammaging is characterized as chronic, sterile, low-grade, asymptomatic activation of systemic inflammation. Under normal circumstances, the immune system remains vigilant for potential threats and is only activated when necessary.[45] Following activation, resolution of the inflammation is performed, thereby maintaining homeostasis. In inflammaging, however, there is a constant baseline proinflammatory activation, which affects both the inflammatory response and its resolution, leading to an imbalance in homeostasis.[46]

The innate immune system, including the complement system and neutrophils, are overactive in inflammaging.[37,47,48] Triggers of inflammaging include cellular senescence, partly caused by the accumulation of oxidative stress, which damages DNA and generates cellular debris. This leads to increased secretion of proinflammatory signaling molecules, including cytokines (such as IFN-γ, IL-1β, and IL-6) and chemokines.[49] Additionally, there is a shift in T-cell populations, where naïve T cells differentiate into central memory and effector memory T cells. T cell differentiation is also characterized by loss of expression of the costimulatory surface markers CD27 and CD28, as well as increased expression of CD56. This results in an upregulation of activity, including the cytotoxic functions of CD8+ T cells, which may cause tissue damage.[50–52] Increased NLR is also associated with aging of the immune system[53] (Figure 7). The changes associated with immunosenescence are reflected in altered risk profiles for the development of age-related chronic diseases.[51,52]

1.4. Inflammation in AMD

Histological studies have demonstrated local inflammatory activity in the retina, evidenced by the presence of complement proteins[54] and Cregs in AMD patients.[55] Similarly, increased concentrations of complement proteins[56] and cytokines have been detected in the aqueous humor.[57] However, there is substantial evidence suggesting that systemic inflammation plays a significant role in the retinal degeneration observed in AMD, and that these local changes might be a consequence of systemic alterations. This includes increased activation of the complement system,

with elevated plasma concentrations of complement proteins in plasma,[58] and decreased levels of Cregs on T cells and monocytes.[59,60] As mentioned in section 1.2.1 risk genotypes of SNPs in the CFH and ARMS2 genes significantly increases the risk of AMD development, and the CFH gene is highly influential in the complement system systemically.[58,61] It has also been reported that ARMS2 may play a role in expression levels of C-C motif receptor 5 (CCR5) on circulating CD8+ T cells.[62]

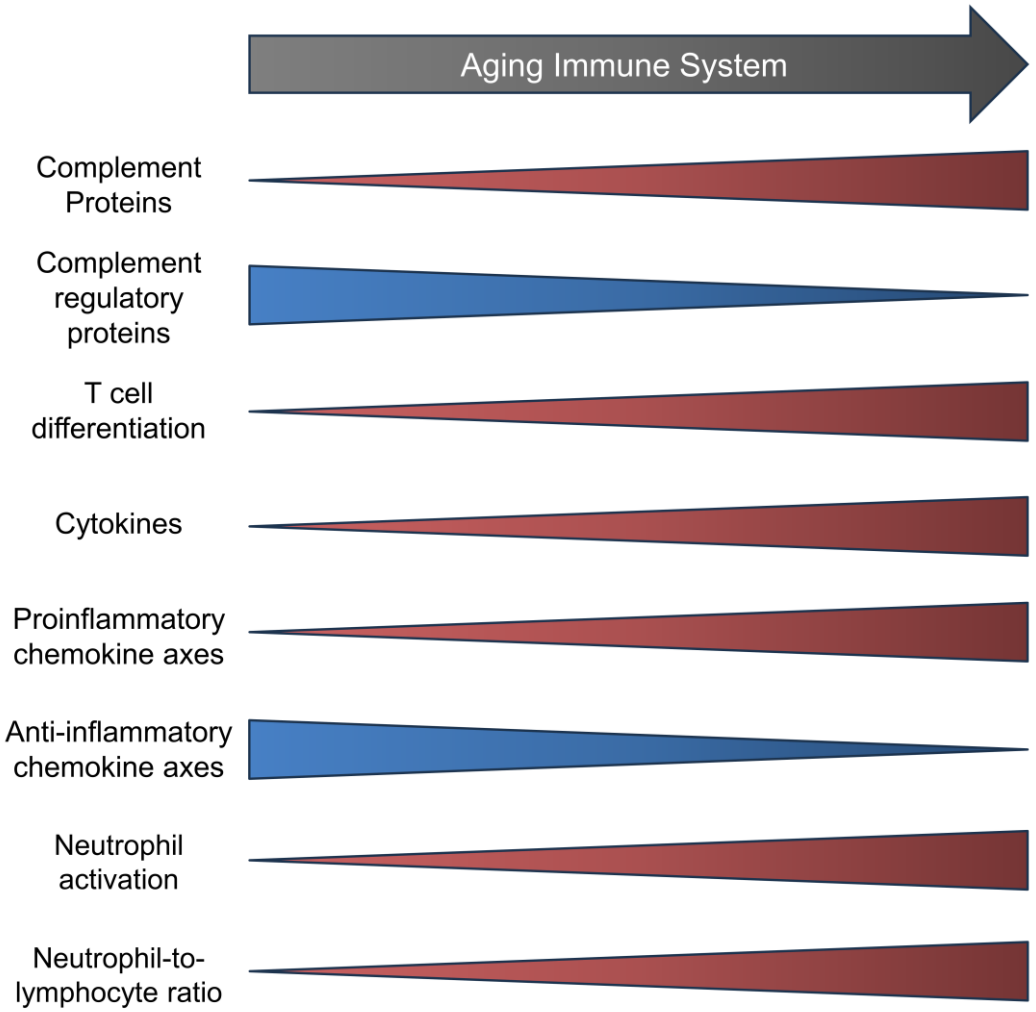


Figure 7. The aging immune system is characterized by increased chronic, systemic low-grade inflammation. This is manifested through multiple interconnected immune pathways. Own work.

Patients with AMD also have increased levels of inflammaging evident by increased plasma concentrations of proinflammatory cytokines[57,63] and increased levels of differentiated T cells.[50,51] The chemokine system has also been shown to be associated with AMD compared to healthy controls. Elevated proinflammatory plasma chemokines and chemokine receptors on T cells and

monocytes has been shown to be elevated in AMD,[64–67] as well as decreased levels of the anti-inflammatory chemokine-chemokine receptor axis C-X-C motif ligand 10 (CXCL10) - C-X-C motif receptor 3 (CXCR3).[68,69]

Alterations of the activation surface markers on circulating neutrophils have been reported in nAMD patients, which indicates an increase in systemic inflammation in these patients.[37,70] Likewise, the NLR has been shown to be increased in nAMD patients compared to healthy controls.[53]

1.5. Objectives and hypothesis

Since current treatment is suboptimal and treatment response is highly variable among patients, we wanted to investigate whether the systemic immune response plays a role in this varied response. Therefore, we formulated this hypothesis:

Do specific peripheral immune profiles influence treatment response in nAMD?

The specific systemic immune alterations studied were:

- Complement system: Plasma complement proteins and Cregs on circulating T cells and monocytes (Study I).
- Aging immune system: Circulating T cell differentiation and plasma cytokines (Study II).
- Chemokine system: Plasma chemokines and chemokine receptors on circulating T cells and monocytes (Study III).
- Neutrophils: NLR and activation surface markers on circulating neutrophils (Study IV).

Secondarily, genetic alterations of AMD risk polymorphisms CFH rs1061170 and ARMS2 rs10490924 (Study I-IV) were investigated in nAMD patients to determine whether these might influence immune profiles. The immune alterations were also investigated between AMD stages (healthy controls, iAMD, and nAMD patients) (Study I-IV).

The discoveries of this PhD study could lead to a better understanding of the role of systemic inflammation in treatment response and pathogenesis of nAMD. The findings may contribute to the identification of novel therapeutic targets for treating nAMD patients with suboptimal treatment response (partial and poor response).

2. Methods

2.1. Study design and ethics

This PhD project was conducted as the Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study, a clinical, prospective, cohort study, with consecutive enrolment of participants. As a single-center study all participants were included at the Department of Ophthalmology, Zealand University Hospital, Denmark between October 2020 and August 2022. The study was approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal no: SJ-768) and performed in adherence with the World Medical Association Declaration of Helsinki.[71] Verbal and written informed consent were obtained from all participants prior to inclusion.

2.2. Study population

The DANEART study population consists of treatment-naïve patients with nAMD, iAMD, and healthy controls. Exclusion criteria were age below 60 years, inflammatory, autoimmune, or infectious diseases, cancer, immunomodulatory treatment, active smoking, baseline plasma C-reactive protein (CRP) > 15 mg/L, vision-impairing conditions other than nAMD and iAMD, as well as prior treatment for nAMD. All participants were assessed at baseline, and nAMD patients underwent two additional research-specific examinations: a follow-up visit after the loading dose and a follow-up visit approximately one year later following three injections in accordance with the observe-and-plan regimen.[72]

The baseline assessment included an interview to address exclusion criteria, smoking status, medications, and comorbidities. This information was cross-checked with the patient's electronic health records. In addition, a comprehensive ophthalmological examination and blood sample collection were performed. At follow-up visits for nAMD patients, the ophthalmological examinations were repeated. Patients with nAMD were treated according to Danish national guidelines with aflibercept 2 mg without switching, following the observe-and-plan regimen.[27]

2.3. Ophthalmic examinations

All participants were examined for visual acuity, slit lamp biomicroscopy as well as multimodal retinal imaging by a retinal specialist to classify diagnosis. Multimodal imaging consisted of color fundus photography taken with the TopCon TRC NW8F (TopCon, Tokyo, Japan), OCT and OCTA with the Heidelberg Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg,

Germany). OCT scans were performed with a 20° × 15° scan field centered on the fovea consisting of 19 B-scans, 512 A-scans per B-scan, 25 frames automatic real time (ART). OCTA scans were performed with a 10° × 10° scan field consisting of 256 B-scans, 512 A-scans per B-scan, 7 frames ART. Healthy controls, iAMD patients, and nAMD patients were classified according to the Beckman criteria (section 1.2.2).

2.3.1. Classification of treatment response in nAMD

Treatment response was evaluated in nAMD patients according to the central retinal thickness (CRT) and retinal fluid on OCT, with the Heidelberg Eye Explorer Software (Heidelberg Engineering). CRT was defined as the average thickness within the central 1-mm diameter ETDRS circle, centered on the fovea. Retinal fluid included both IRF and SRF (section 1.2.3). Good treatment response was defined as total regression of IRF and SRF, resulting in a dry macula. Partial response was defined as persistence of IRF and/or SRF and a reduction in CRT. Poor response was defined as persistence of IRF and/or SRF and unchanged or increased CRT (Table 2, Figure 8). Treatment response was evaluated post-loading dose (initial treatment response) and after one year to determine the maintained response (1-year treatment response).

	Treatment response
Good	Total regression of IRF and SRF
Partial	Persistence of IRF and/or SRF and reduction of CRT
Poor	Persistence of IRF and/or SRF and unchanged or increased CRT

IRF = Intraretinal fluid
SRF = Subretinal fluid
CRT = Central retinal thickness

Table 2. Definition of treatment response in nAMD patients according to OCT factors. Table is adapted from own work previously published by Thomsen et al. Journal of Neuroinflammation (2024), CC-BY 4.0.[73]

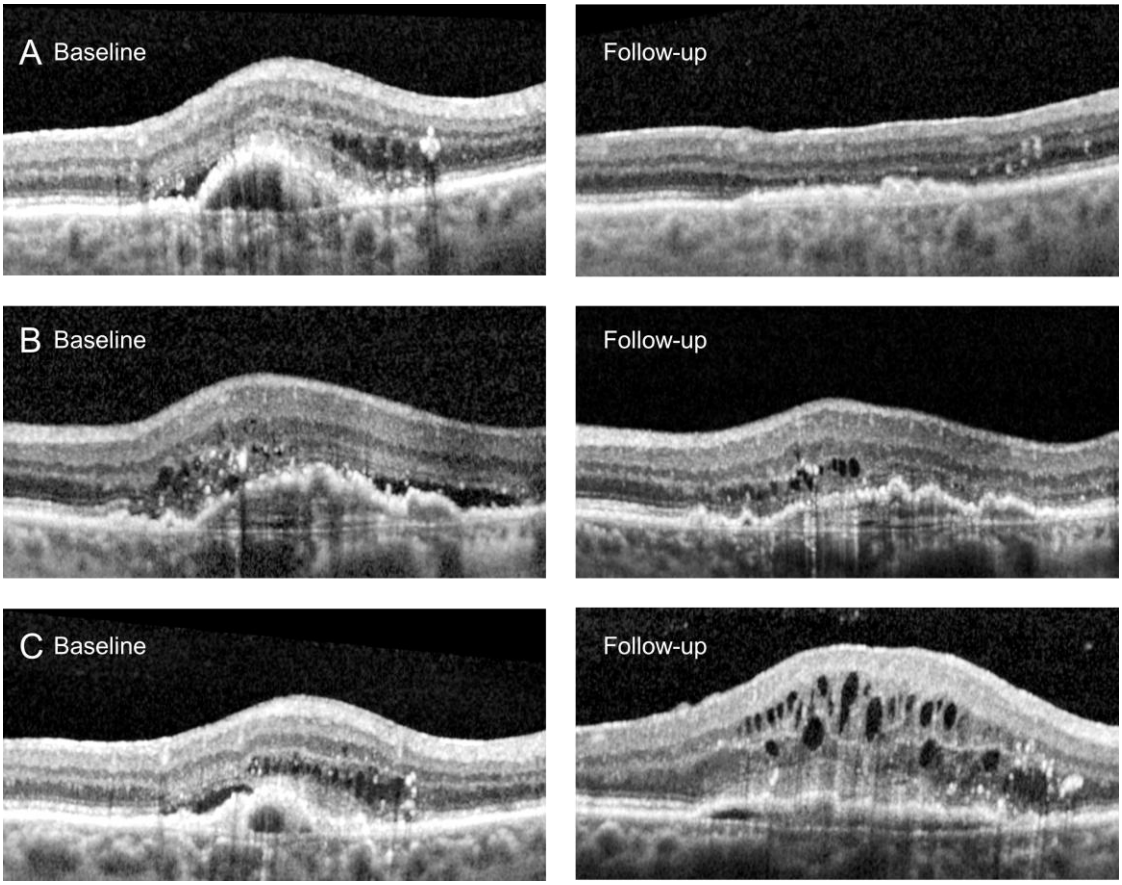


Figure 8. Examples of treatment response grading in nAMD. (A) Good response, (B) partial response, (C) poor response. Own work, previously published by Thomsen et al. Aging and Disease (2025), CC-BY 4.0.[74]

2.4. Blood sampling and analysis

Blood samples were collected from all participants at baseline from the antecubital vein drawn in ethylenediaminetetraacetic acid (EDTA) coated tubes, lithium-heparin (LH) coated tubes, and LH coated tubes with gel. The LH coated tubes with gel were used to measure plasma CRP concentrations through standard latex-enhanced immunoturbidimetry performed by the Department of Clinical Biochemistry at Zealand University Hospital.

2.4.1. Genotyping

Genotyping allows for the identification of the structure of genetic material. By identifying SNPs relevant to diseases, it is possible to assess risk profiles, which might help the patient and clinician in monitoring and treatment of the disease.

The Fluidigm dynamic array utilizes an integrated fluidic circuit that enables the precise and efficient combination of samples and reagents on a chip, which is mounted on a plastic interface equipped with microtubes. This setup ensures accurate mixing and efficient processing.

Polymerase chain reaction (PCR) is then performed on the prepared mixtures. Upon completion of the PCR, fluorescent imaging is used to capture data, which is subsequently analyzed to determine genotype calls (Figure 9).[75]

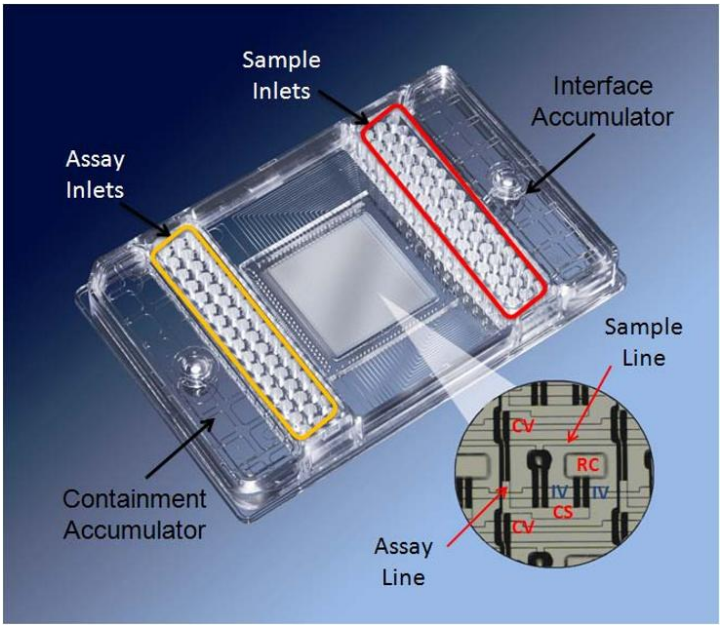


Figure 9. Fluidigm dynamic array. Published by Wang et al. BMC Genomics (2009), CC-BY 2.0.[75]

Study I, II, III, IV

As part of the DANEART study, patients with nAMD had full blood samples examined for the AMD risk SNPs CFH rs1061170 and ARMS2 rs10490924, performed by BioXpedia, Denmark. SNP analysis was performed using the Fluidigm GT192.24 Dynamic Array Integrated Fluidic Circuit (Fluidigm Corp., San Francisco, CA, USA) according to manufacturer guidelines. The genotype call data was analyzed using the Fluidigm SNP Genotyping analysis software v.4.5.1 with standard settings.

2.4.2. Immunoassays

Immunoassays can be used to measure plasma proteins, such as complement proteins and cytokines. Electrochemiluminescence immunoassays use plates consisting of wells coated with antibodies that bind to the analyte of interest. After the analyte is added and non-bound material is washed away, a fluorescein-labeled antibody is introduced, which also binds to the analyte. By applying electricity to the wells, the fluorescent label is stimulated to emit light, which can be measured by a specialized apparatus. The emitted light is proportional to the concentration of the analyte, as more labels are bound (Figure 10).

For the DANEART study, plasma was isolated from whole blood samples by centrifugation at 1500 × g for 15 minutes at 20 °C immediately after phlebotomy. The plasma was frozen at -80 °C within one hour and analyzed on a different day. Electrochemiluminescence immunoassays were performed using the MesoScale single- and multiplex platforms and analyzed on the MESO QuickPlex SQ 120MM instrument (MesoScale Discovery, Rockville, MD, USA).

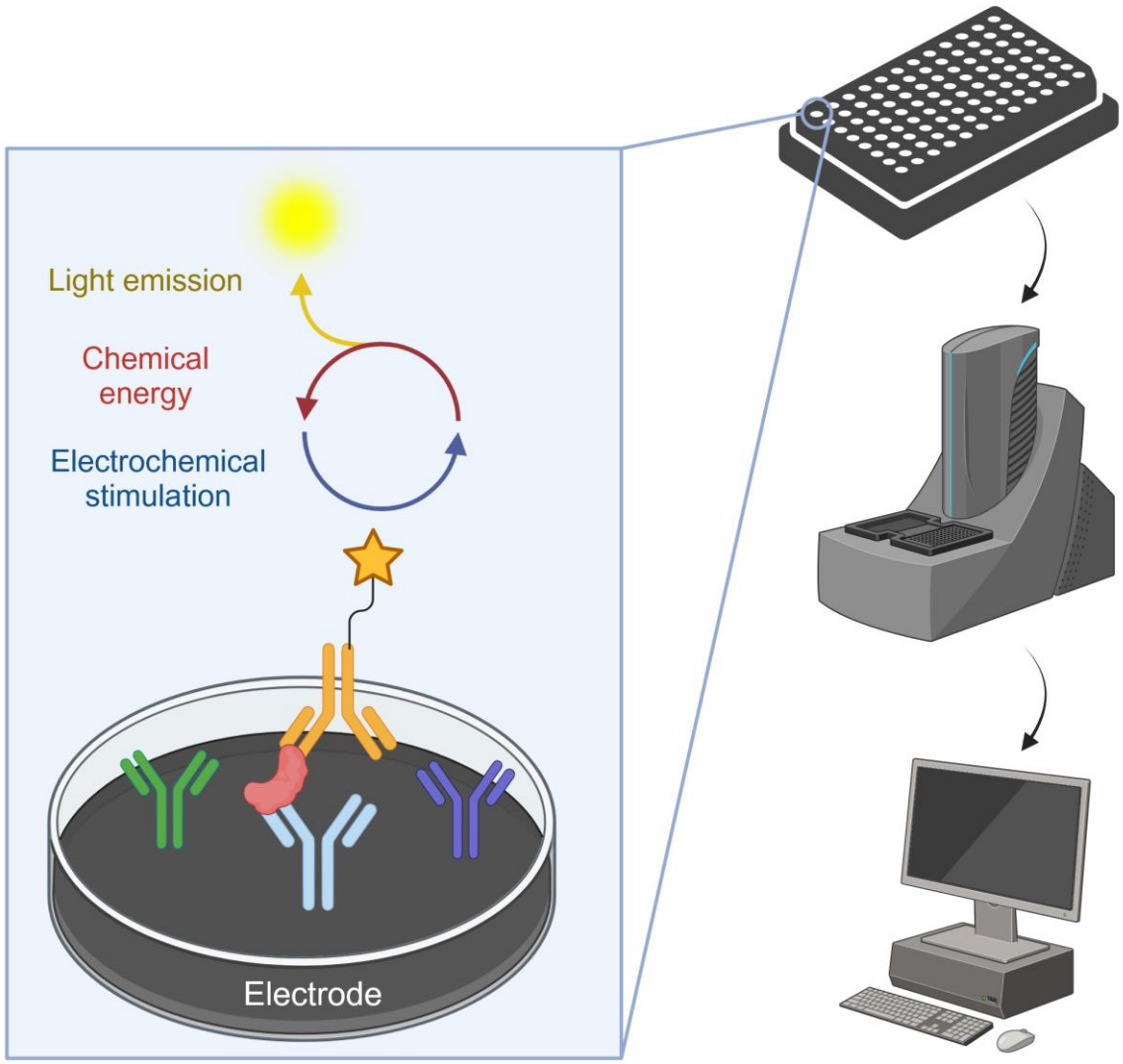


Figure 10. Electrochemiluminescence immunoassays. The wells of the MesoScale plate are coated with antibodies that bind to the analytes of interest. The samples are added to the wells, and excess material is washed away. The detection antibody is then added, binding to the analyte of interest, and excess antibody is washed away. The plate is placed in the MesoScale analysis instrument, which applies an electrical current through the electrode in each well. The detection antibody is electrochemically activated, generating chemical energy that emits light, which is then detected. The intensity of the emitted light indicates the concentration of the analyte. The data is stored digitally. Own work, created with BioRender.

Study I

For the first study, we aimed to analyze the complement proteins C3, C3a, C5a, and the C3a/C3 ratio in plasma of the DANEART population (healthy controls, iAMD patients, and nAMD patients). Plasma was isolated from EDTA-coated tubes, as this has been shown to be the most stable for complement proteins.[76] C3, C3a and C5a were analyzed using the single plex R-plex Human Complement C3 Assay (cat.no. K151XYR-2, MesoScale), R-plex Human Complement C3a Assay (cat.no. K151V0R-2, MesoScale), and R-plex Human Complement C5a Assay (cat.no. K151K4R-2, MesoScale), respectively. Samples were thawed and analyzed in duplicate in accordance with manufacturer guidelines performed by a single investigator (AKT):

- Plates were coated with a biotinylated capture antibody:
    - Diluted biotinylated capture antibody was added to each well of the plate.
    - Plates were incubated for one hour at room temperature (RT) with shaking (700-1000 rpm).
    - Wells were washed three times with wash buffer (0.05% Tween-20 in phosphate-buffered saline (PBS)).
- Preparation of calibrator standards:
    - The Stock Calibrator was diluted in 285 µl diluent (Calibrator 1).
    - 100 µl was transferred to Calibrator 2 diluted in 300 µl diluent.
    - This process was repeated a total of six times, creating seven calibrators with increased dilution. Calibrator 8 consisted of only Diluent 37.
- Diluting plasma samples:
    - Plasma for the C3 analysis was diluted 100,000 times on a mixing plate. Plasma for C3a was diluted 10,000 times. C5a was used undiluted.
- Preparation of plates:
    - Standard calibrators were added to the first two rows of wells in the plate (Calibrator 1 in wells A1 and B2, Calibrator 2 in wells A2 and B2, etc.).
    - Samples were added in duplicate to the remaining wells.
    - Plates were incubated for one hour at RT with shaking (700-1000 rpm), followed by washing three times with wash buffer.
    - Diluted detection antibody was added to the wells.
    - Plates were incubated for one hour at RT with shaking (700-1000 rpm).
    - MSD Gold read buffer B was added to each well.
- Plates were analyzed on the MESO QuickPlex SQ 120MM instrument.

To minimize plate variation affecting the results, a combination of healthy controls, iAMD, and nAMD patients was added to each plate. Transillumination bias is a phenomenon that can occur when a signal is very potent in one well and is partially read as signal in neighboring wells. To mitigate this, we semi-randomly mixed the order of the samples, ensuring there was no systematic tendency for the same group to be adjacent to each other.

Study II

For the second study, the concentrations of plasma cytokines were analyzed in the DANEART population. The ultrasensitive S-plex Human Proinflammatory Panel 1 (cat.no. K15396S-1, MesoScale) was used for analysis of IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17 and tumor necrosis factor (TNF)-α. The V-plex Human TH17 Panel 1 (cat.no. K15085D-1, MesoScale) was used for analysis of IL-22 and IL-27. Analyses were performed according to manufacturer guidelines by the Department of Clinical Immunology, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark. The protocol was similar to the R-plexes in Study I, however the S-plex and V-plex are multiplexes analyzing for multiple cytokines in a single well.

Study III

For the third study, the concentrations of plasma chemokines were analyzed in the DANEART population. Multiplex V-plexes were used for chemokine assays. C-C motif chemokine ligand (CCL) 2, CCL3, CCL4, CXCL8 and CXCL10 were analyzed with the V-plex Human Chemokine Panel 1 (cat.no. K151A9H-1, MesoScale), and CCL20 with V-plex Human TH17 Panel 1 (cat.no. K15085D-1, MesoScale). Analyses were performed according to manufacturer guidelines by the Department of Clinical Immunology, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark.

2.4.3. Flow cytometry

Flow cytometry is a technology that enables the analysis of individual cells, including their size, granularity, and surface markers. Each cell is illuminated by a laser, and the scattered light is detected and measured. Forward scatter corresponds to light scattered in the forward direction and is primarily indicative of cell size, while side scatter, measured at a 90-degree angle to the laser, reflects the cell's internal complexity or granularity. By staining cells with fluorescently labeled antibodies targeting surface markers of interest, it is possible to identify and quantify cells expressing these markers, such as CD4 conjugated to the red-fluorescent dye Peridinin-Chlorophyll-Protein (PerCP). Multiple antibodies can be added to the same sample, as the flow

cytometer is capable of detecting fluorescent signals at several distinct wavelengths (Figure 11). The resulting data is saved as a flow cytometry standard data file (fcs), which shows light signals converted into electronic signals, which are displayed in coordinate systems where each cell is represented as a dot. To analyze multiple cells for multiple surface markers, a Boolean gating strategy can be employed.[77]

Flow cytometry was performed on all DANEART participants by a single investigator (AKT). Multiple panels of antibody staining were used to analyze the blood samples, following a common protocol. Flow cytometry was initiated within 4 hours of phlebotomy to ensure sample integrity. Whole blood collected in EDTA-coated tubes was analyzed for leukocyte count using the Sysmex KX-21NTM (Sysmex Corp., Kobe, Japan) to calculate the required blood volume needed to obtain 1.0×10^6 leukocytes for analysis. Leukocytes were isolated by lysing erythrocytes through the addition of 1% red blood cell lysis buffer, followed by incubation in the dark at RT for 10 minutes. The leukocytes were washed to remove erythrocytes and debris by centrifuging the sample for 5 minutes at 20 °C at 500 × g, decanting the supernatant, and adding BD FACS isotonic buffer (BioLegend, San Diego, CA, USA). This washing process was repeated three times to ensure purity. The suspension of isolated leukocytes was transferred to flow cytometry tubes. Each panel of antibodies was added to a tube and incubated in the dark at RT for 20 minutes allowing for antibody binding. The stained leukocytes were washed a final time to remove excess antibodies. Flow cytometry was performed on the BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) with a gating threshold set to 100,000 singlet cells per sample. The fcs files were analyzed using FlowJo software (Tree Star, Ashland, OR, USA, v.10.10.0) with a Boolean gating strategy.

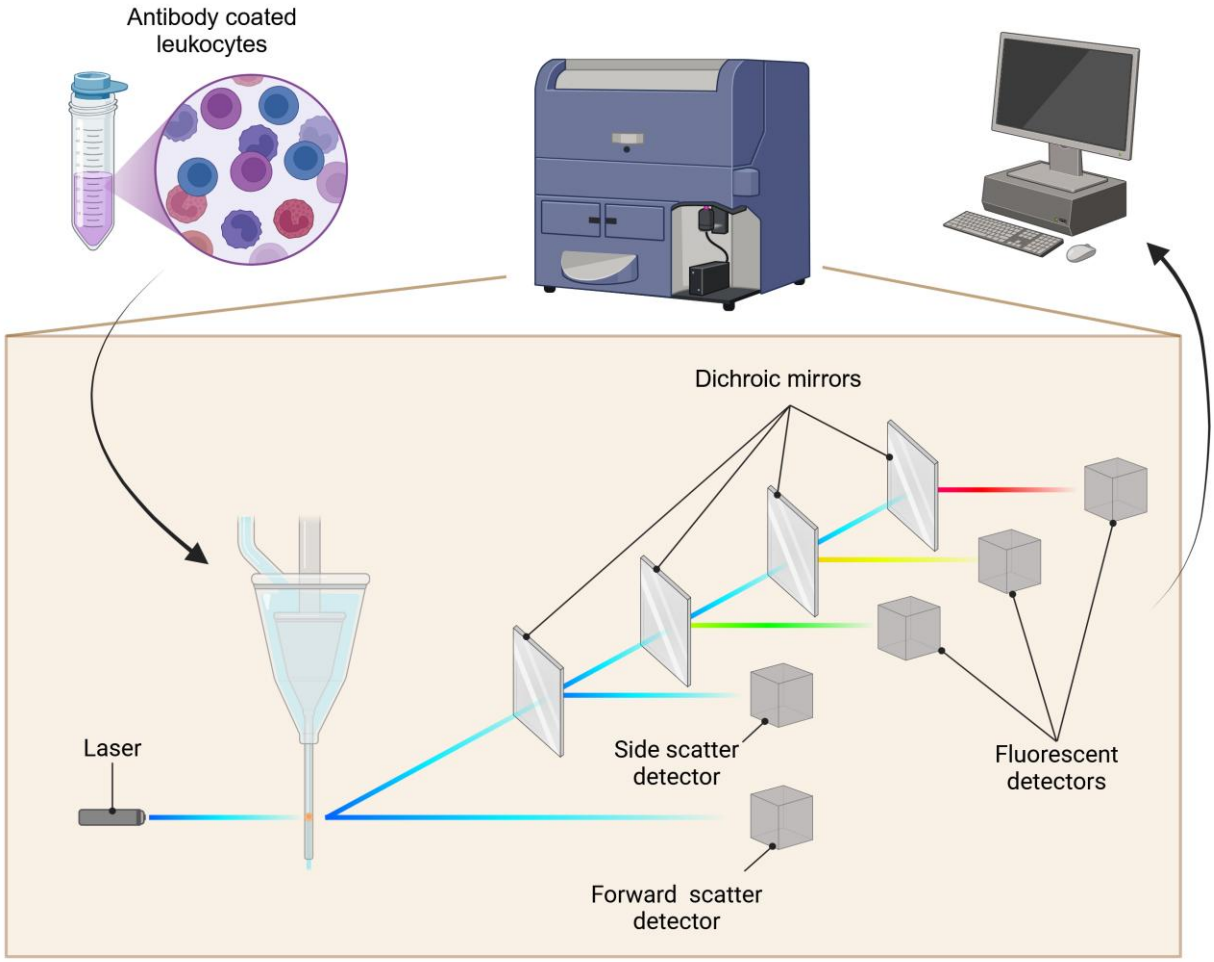


Figure 11. Flow cytometry. Isolated leukocytes coated with fluorescent antibodies are injected into the flow cytometer, where cells pass through a nozzle in a single-file stream. A laser beam shoots the cell and excites the fluorescent dye, which is scattered detected. The forward and side scatter are used to determine size and granularity, respectively. The emitted light is filtered through dichroic mirrors reflecting the relevant wavelengths to the appropriate fluorescent detectors to determine surface markers. Own work, created with Biorender.

Study I

For the first study, we examined the expression levels of Cregs CD35, CD46, CD59, and CD11b on T cells and monocytes. The antibodies used for staining can be found in Table 3.

The gating strategy consisted of determining lymphocytes and monocytes on the forward-side scatter plot. These were gated for singlet cells with the forward area-forward height scatter plot, thus excluding adherent cells. Lymphocytes were gated for CD4 and CD8 to determine CD4+ and CD8+ T cells. Monocytes were gated for CD14 and CD16 to determine the monocyte subset:

classical, intermediate and non-classical (section 1.3.1). The T cell and monocyte subsets were then gated for the expression levels of the Cregs (Figure 12).

Surface marker	Fluorochrome	Manufacturer	Catalog number
CD4	PerCP	R&D Systems	FAB3791C-100
CD8	Brilliant Violet	BioLegend	301048
CD14	Pacific Blue	BioLegend	325616
CD16	APC/Cy7	BioLegend	302018
CD35	APC	R&D Systems	FAB5748A
CD46	PE/Cy7	BioLegend	352408
CD59	FITC	BioLegend	304706
CD11b	APC/Cy7	BioLegend	301342

APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein.
R&D Systems, Minneapolis, MN, USA.
Biolegend, San Diego, CA, USA.

Table 3. Flow cytometry antibodies for Study I. Table is adapted from own work previously published by Thomsen et al. Journal of Neuroinflammation (2024), CC-BY 4.0.[73]

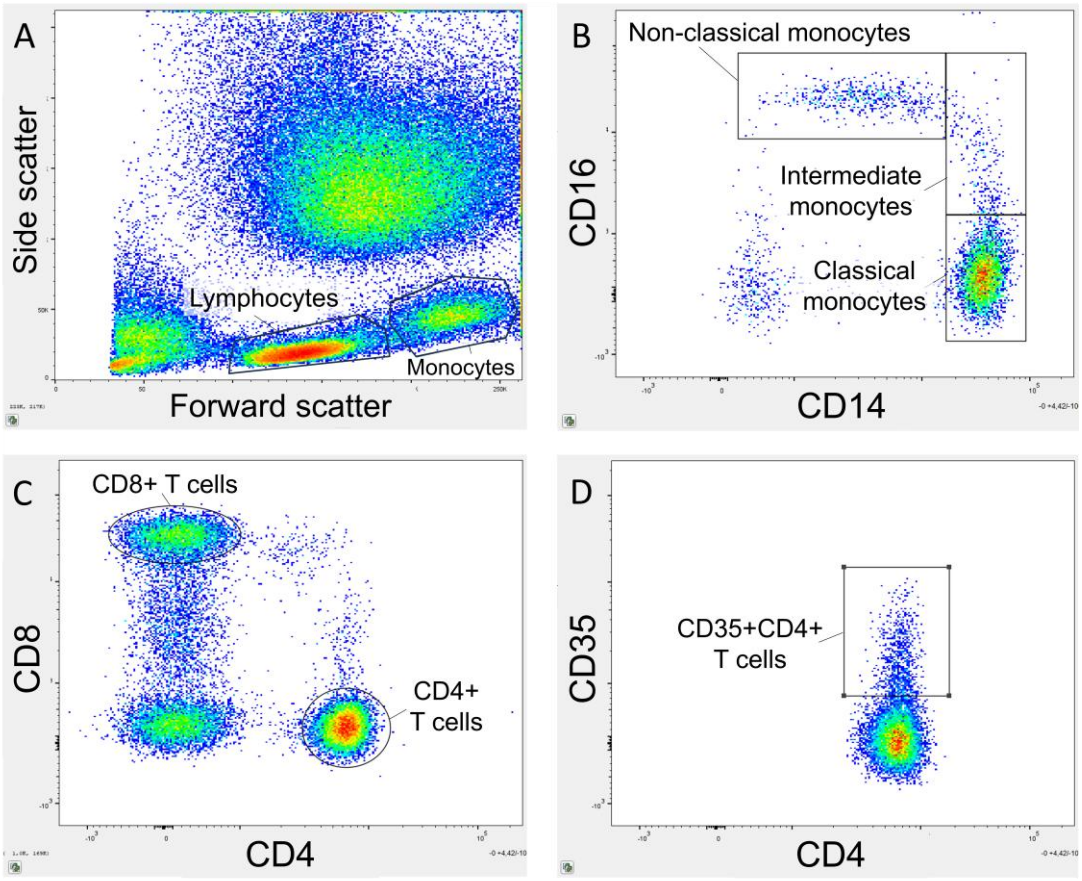


Figure 12. Flow cytometry gating strategy. (A) Lymphocytes and monocytes were identified on the forward-side-scatter. (B) Monocyte subsets were identified as classical ($CD14^{high}CD16^{low}$), intermediate ($CD14^{high}CD16^{high}$), and non-classical ($CD14^{low}CD16^{high}$). (C) CD4 + T cells and CD8 + T cells were identified among the lymphocytes. (D) Complement regulatory proteins were gated on the leukocyte subgroups with Boolean sequences, in this example CD35 on CD4 + T cells. Own work, published by Thomsen et al. Journal of Neuroinflammation (2024), CC-BY 4.0.[73]

Study II

For the second study, we examined the proportion of T cell differentiation (naïve, central memory, effector memory) and expression levels of costimulatory aging markers CD27, CD28, and CD56. The antibodies used for staining can be found in Table 4.

The CD4+ and CD8+ T cell subsets were identified similarly to Study I. Naïve T cells were defined as CD45RA+CD45RO-CCR7+, central memory as CD45RA-CD45RO+CCR7+, and effector memory as CD45RA-CD45RO+CCR7- T cells.[51,52,78,79] An example of a gating strategy can be seen in Figure 13.

Surface marker	Fluorochrome	Manufacturer	Catalog number
CD4	FITC	Abcam	ab59474
CD8	PerCP	BioLegend	300922
CD45RA	Pacific Blue	BioLegend	304123
CD45RO	PE/Cy7	BioLegend	304230
CCR7	Brilliant Violet	BioLegend	353232
CD27	PE	BioLegend	356406
CD28	APC	BioLegend	302912
CD56	APC-Cy7	BioLegend	300926

APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein.

Abcam, Cambridge, UK.

BioLegend, San Diego, CA, USA.

Table 4. Flow cytometry antibodies for Study II. Table is adapted from own work previously published by Thomsen et al. Aging and Disease (2025), CC-BY 4.0.[74]

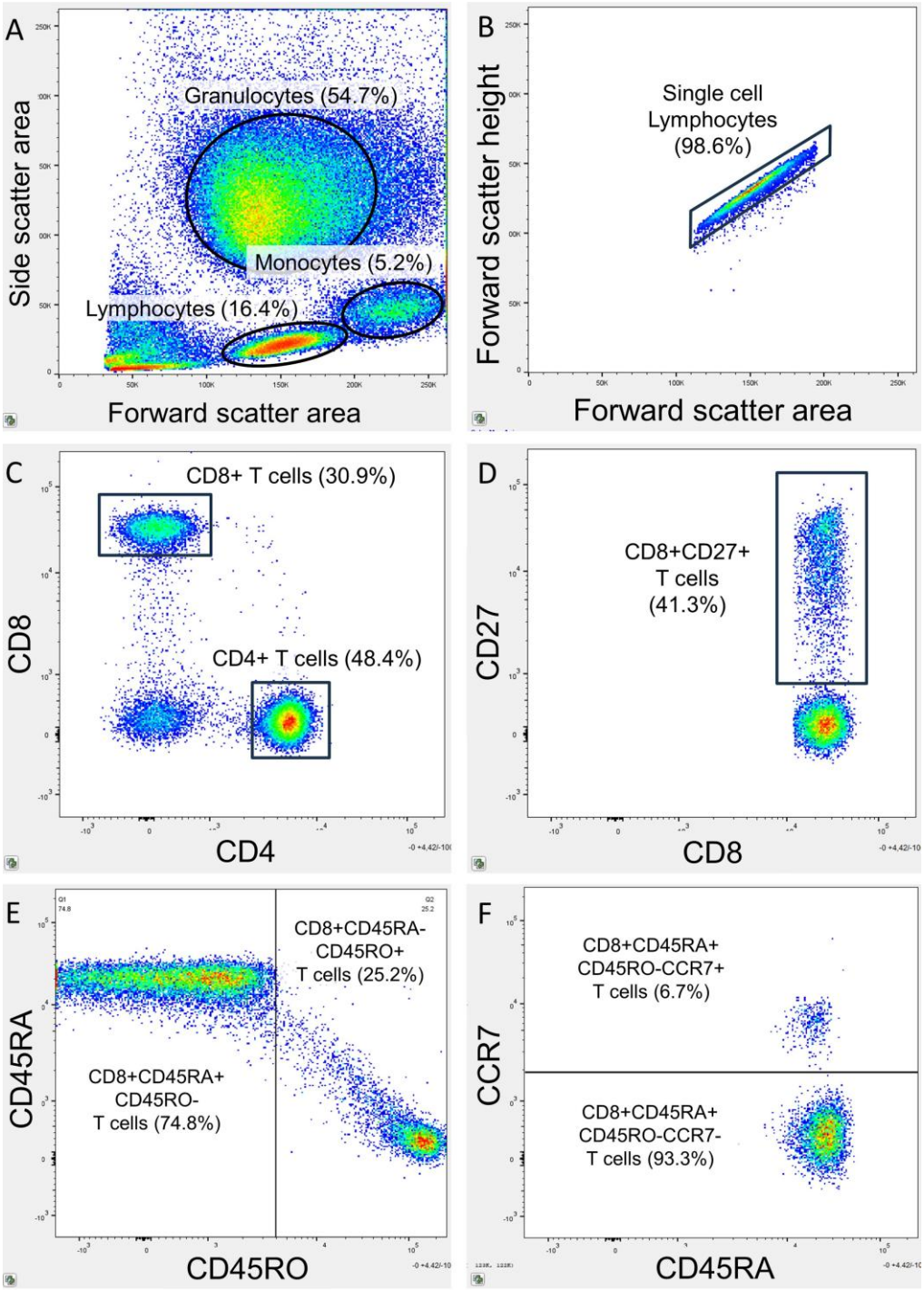


Figure 13. Flow cytometry gating strategy with Boolean sequences. (A) Lymphocytes were identified on the forward-side-scatter. (B) Singlet cell lymphocytes were gated. (C) CD4+ T cells and CD8+ T cells were identified among the singlet lymphocytes. (D) Co-stimulatory surface markers were identified on T cells, in this example CD27 expression on CD8+ T cells. (E) The expression of CD45RA and CD45RO was determined on T cells, in this example on CD8+ T cells. (F) The expression of CCR7 was determined on CD45RA+CD45RO- and CD45RA-CD45RO+ T cells, in this example on CD8+CD45RA+CD45RO- T cells. Own work, previously published by Thomsen et al. Aging and Disease (2025), CC-BY 4.0.[74]

Study III

For the third study, we examined the expression levels of CCR1, CCR2, CCR5, CCR6, and CXCR3 on T cells. Monocytes were analyzed for expression levels of CCR1, CCR2, CCR5, CXCR2, CXCR3, and C-X_3-C motif chemokine receptor (CX_3CR) 1. The antibodies used for staining can be found in Table 5.

T cell and monocyte subsets were identified, and the expression levels of each chemokine receptor were evaluated on each subset using gating strategy similar to that of Study I.

Surface marker	Fluorochrome	Manufacturer	Catalog number
CD4	PerCP	R&D Systems	FAB3791C
CD8	Brilliant Violet	BioLegend	301048
CD14	Pacific Blue	BioLegend	325616
CD16	APC-Cy7	BioLegend	302018
CCR1	APC	BioLegend	362908
CCR2	PE	R&D Systems	FAB151P
CCR5	FITC	R&D Systems	FAB182F
CCR6	FITC	BioLegend	353412
CXCR2	APC	BioLegend	320710
CXCR3	PE/Cy7	BioLegend	353720
CX_3CR1	FITC	BioLegend	341606

APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein.

R&D Systems, Minneapolis, MN, USA.

BioLegend, San Diego, CA, USA.

Table 5. Flow cytometry antibodies for Study III. Own work from unpublished manuscript (Appendix 7.3).

Study IV

For the fourth study, we examined the proportion of neutrophils and lymphocytes, as well as the expression levels of CD11a, CD11b, CD31, CD66b, CD162, and CD182 (CXCR2) on neutrophils. The antibodies used for staining can be found in Table 6.

Granulocytes and lymphocytes were gated on the forward-side scatter plot, and single cells were identified on the forward area-forward height scatter plot. Granulocytes were gated for CD14 and CD16 to identify neutrophils defined as CD14dimCD16+.[37] Neutrophils were gated for the activation surface markers using a gating strategy similar to Study I.

Surface marker	Fluorochrome	Manufacturer	Catalog number
CD14	FITC	BioLegend	400210
CD16	Brilliant Violet 510	Biolegend	302048
CD11a	PerCP	Biolegend	350608
CD11b	APC-Cy7	Biolegend	301342
CD31	Pacific Blue	Biolegend	303114
CD66b	PE/Cy7	Biolegend	304116
CD162	PE	Biolegend	328806
CD182	APC	Biolegend	320710

APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein.

BioLegend, San Diego, CA, USA.

Table 6. Flow cytometry antibodies for Study IV. Own work from unpublished manuscript (Appendix 7.4).

2.5. Statistics

Statistical analysis was performed with R software (R Foundation for Statistical Computing, Vienna, Austria, v.4.2.3). Normality of continuous data was assessed with histograms and the Shapiro-Wilk test. Normally distributed continuous patient characteristics were presented as mean and standard deviation (SD), and group comparisons were tested with analysis of variance (ANOVA). Categorical patient characteristics were presented as absolute number and percentages, and group comparisons were tested with Fischer's exact test. Logarithmic transformation was performed on non-normally distributed immune data to the fit assumption of normality. Group comparisons of immune data were performed with analysis of covariance (ANCOVA). This analysis adjusted for age, as age-related immune changes affect the immune profile (section 1.3.2.), and smoking status (never or previous), as active smoking increases the systemic immune activity.[80] In comparisons between healthy controls, iAMD patients and nAMD patients, healthy controls were chosen as reference group. In comparisons between nAMD treatment response groups, good responders were chosen as reference group. Results of the ANCOVA are presented in Study I as group means and SD, and in Study II, III, and IV as mean difference and 95% confidence intervals (CI95%) in percentages (not percentage points). Comparisons between high and low risk genotypes of the CFH and ARMS2 SNPs were analyzed with Welch two sample t-test in nAMD patients.

Correlation networks were created to analyze the relationships and patterns between immune markers. These can indicate disease mechanisms and immune system dynamics. The correlation networks consist of nodes representing immune markers, which are connected by edges (lines) representing statistically significant correlation coefficients. The thickness and color of the edges indicate the correlation coefficient, which was set at a threshold of $|r| > 0.4$ (Study I and III). Spiderweb charts were created to show relative differences in immune marker levels in compartments with statistically significant differences. The highest group value corresponds to 100%, and the other group values as a relative of this magnitude (Study II).

P values were adjusted for false discovery rate in Study II and IV (section 4.1). A *P* value < 0.05 was interpreted as statistically significant.

Sample size calculations for healthy controls, iAMD, and nAMD patients were based on prior immunological studies on AMD patients, using an α level of 0.05, a power of 80%, and an effect size of 20%, yielding a minimum requirement of 30 participants per diagnostic group.[51,60,62,63] Direct power calculations for treatment response analysis were not feasible due to the lack of studies examining these immune markers in nAMD treatment response. Recruitment of nAMD patients continued until 100 participants were enrolled.

3. Results

3.1. Summary of Study population results

The DANEART study population consisted of 100 nAMD patients, 34 iAMD patients, and 61 healthy controls. 94 patients with nAMD completed the 1-year follow-up. Five participants died, and 1 was excluded due to lack of following the treatment plan. The treatment response in nAMD patients was evaluated post-loading dose (initial treatment response) and after one year. The distribution of the initial treatment response groups was 61 (65%) good, 26 (28%) partial, and 7 (7%) poor responders. The distribution of 1-year treatment response groups was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. There were no statistically significant differences in patient characteristics between treatment response groups.[23] Neither were there any significant differences between healthy controls, iAMD and nAMD patients, except nAMD patients were significantly older than healthy controls (Appendix 7.1).

3.2. Summary of Study I results

Aim

This study investigated differences in systemic levels of complement proteins and Cregs between nAMD treatment response groups, as well as between healthy controls, iAMD, and nAMD patients. Plasma concentrations of complement proteins C3, C3a, and C5a were measured, and the C3a/C3 ratio was calculated. Expression levels of Cregs CD35, CD46, and CD59 were investigated on CD4+ and CD8+ T cells. Expression levels of Cregs CD35, CD46, CD59, and CD11b were investigated on monocytes and monocyte subsets. Association between CFH rs1061170 and ARMS2 rs10490924 genotypes and these immune markers were studied in nAMD patients.

Results

This study showed that nAMD patients with a partial initial treatment response had a significantly decreased plasma concentration of C3a and C5a. There was a tendency of poor initial responders to have elevated C3, C3a, and C3a/C3 ratio, however not statistically significant. There were no significant differences between Cregs on T cells or monocytes between initial treatment groups. Partial 1-year treatment responders had a significantly decreased proportion of CD35+ monocytes and CD35+ cMonocytes, but showed no significant differences in plasma complement protein concentrations. nAMD patients overall had a significantly elevated plasma concentration of C3

and C3a, as well as C3a/C3 ratio compared to healthy controls. Patients with iAMD also had significantly elevated plasma concentrations of C3a compared to healthy controls. Furthermore, nAMD patients had elevated proportions of CD4+CD46+ T cells and CD59+ iMonocytes. The correlation networks indicated differences in initial treatment response groups. It seemed that complement proteins had a more central function in partial and poor initial responders compared to good responders. Partial initial responders had the most complex network, indicating increased regulation. In contrast, partial 1-year responders seemed to have a simpler correlation network compared to good and poor 1-year responders. Patients with iAMD seemed to have a more complex network compared to healthy controls and nAMD patients.

Elevated levels of C3, C3a/C3 ratio, CD8+CD35+ T cells, and CD46+ cMonocytes were associated with the high-risk genotypes CC/CT in the CFH rs1061170 SNP in nAMD patients. Decreased proportions of CD46+ nMonocytes were associated with the high risk CFH genotype (Appendix 7.1).

3.3. Summary of Study II results

Aim

This study investigated differences in systemic levels of T cell differentiation and cytokines between nAMD treatment response groups, as well as between healthy controls, iAMD, and nAMD patients. The proportion of naïve, central memory and effector memory, CD27+, CD28+, and CD56+ T cells was investigated in CD4+ and CD8+ T cell compartments. Plasma concentrations of IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17, IL-22, IL-27, and TNF-α were measured. Association between CFH rs1061170 and ARMS2 rs10490924 genotypes and these immune markers were studied in nAMD patients.

Results

Plasma concentrations of IFN-γ were significantly increased in nAMD patients with poor initial treatment response compared to good initial responders. Partial initial responders also tended to have elevated IFN-γ concentrations, however not statistically significant. There was a tendency for nAMD patients with poor initial treatment response to have increased proportions of effector memory CD8+ T cells and CD8+CD56+ T cells, however not statistically significant. Patients with nAMD had a significantly higher proportion of central memory and effector memory CD8+ T cells compared to healthy controls. Plasma cytokines IFN-γ, IL-1β, IL-2, and IL-10 were also significantly elevated in nAMD patients compared to healthy controls. Patients with iAMD had significantly elevated proportions of CD8+CD56+ T cells compared to healthy controls.

Spiderweb charts revealed that every proinflammatory aging-marker seemed to be increased and all anti-inflammatory markers seemed to be decreased in nAMD patients compared to healthy controls and iAMD patients, except CD8+CD56+ T cells, which was elevated in iAMD patients. A pattern between the initial treatment response groups' spiderweb charts was less clear but seemed to differ between the groups.

No significant associations between the immune markers and CFH rs1061170 and ARMS2 rs10490924 genotypes were found in nAMD patients (Appendix 7.2).

3.4. Summary of Study III results

Aim

This study investigated differences in systemic levels of chemokines and chemokine receptors between nAMD treatment response groups, as well as between healthy controls, iAMD, and nAMD patients. Plasma concentrations of chemokines CCL2, CCL3, CCL4, CCL20, CXCL8, and CXCL10 were measured. Expression levels of chemokine receptors CCR1, CCR2, CCR5, CCR6, and CXCR3 were investigated on CD4+ and CD8+ T cells. Expression levels of chemokine receptors CCR1, CCR2, CCR5, CXCR2, CXCR3, and CX_3CR1 were investigated on monocytes and monocyte subsets. Association between CFH rs1061170 and ARMS2 rs10490924 genotypes and these immune markers were studied in nAMD patients.

Results

The proportion of CD4+CXCR3+ T cells, CCR5+ cMonocytes, and CCR2+ nMonocytes were significantly decreased in nAMD patients with poor initial treatment response compared to good initial responders. There was a tendency of poor initial responders to have increased concentrations of CXCL10, however not statistically significant. Partial initial responders had significantly decreased levels of CCR2+ cMonocytes compared to good initial responders. Patients with nAMD and poor 1-year treatment response had significantly lower proportions of CD4+CXCR3+ T cells and CCR2+ nMonocytes, while partial 1-year responders had significantly lower proportions of CCR5+ monocytes compared to good 1-year responders. Patients with nAMD overall had significantly elevated levels of CCL3, CCL4, and CD4+CCR1+ T cells, and significantly lower levels of CCL2, CD8+CXCR3+ T cells, CX_3CR1+ cMonocytes, and CCR2+ iMonocytes compared to healthy controls. Patients with iAMD had significantly lower levels of CCL2, CX_3CR1+ cMonocytes, CCR2+ iMonocytes, and CX_3CR1+ iMonocytes.

Correlation networks between initial treatment response seemed to differ between the groups. Partial and poor initial responders appeared to have more complex chemokine correlation networks compared to good responders, reflecting the increased regulation.
Correlation networks between initial treatment response seemed to differ between the groups. Poor and partial initial responders appeared to have more complex chemokine correlation networks compared to good responders, reflecting the increased regulation.
Elevated levels of CCL4, CD4+CCR2+ T cells, CD8+CCR2+ T cells, and CXCR2+ iMonocytes were associated with the high-risk genotypes CC/CT in the CFH rs1061170 SNP in nAMD patients. Elevated levels of CX_3CR1+ monocytes and cMonocytes, and CCR1+ nMonocytes, as well as decreased levels of CCR1+ iMonocytes were associated with the high-risk genotypes (TT/TG) in the ARMS2 rs10490924 SNP in nAMD patients (Appendix 7.3).

3.5. Summary of Study IV results

Aim

This study investigated differences in systemic levels of NLR and activation surface marker expression on neutrophils between nAMD treatment response groups, as well as between healthy controls, iAMD, and nAMD patients. The NLR was calculated as singlet neutrophil percentage divided by singlet lymphocyte percentage. Expression levels of activation surface markers CD11a, CD11b, CD31, CD66b, CD162, and CD182 were investigated on neutrophils. Association between CFH rs1061170 and ARMS2 rs10490924 genotypes and these immune markers were studied in nAMD patients.

Results

The NLR was significantly elevated in poor 1-year treatment responders compared to good 1-year treatment responders. There was a tendency of poor initial treatment responders to have increased NLR compared to good initial responders, however not statistically significant. Patients with nAMD had significantly elevated NLR compared to healthy controls. No significant differences were found between treatment response groups and activation surface marker expression on neutrophils. nAMD patients overall had a significantly higher proportion of CD11a+, CD11b+, CD31+, CD66b+, CD162+, and CD182+ neutrophils compared to healthy controls. No significant associations between the immune markers and CFH rs1061170 and ARMS2 rs10490924 genotypes was found in nAMD patients. (Appendix 7.4).

4. Discussion

This PhD project investigated systemic immunoregulatory plasma proteins and surface markers on circulating leukocytes to explore their relation to treatment response in patients with nAMD, as well as AMD stage (healthy controls, iAMD, and nAMD patients). A further objective was to investigate the relationship between AMD risk polymorphisms and these immune markers in nAMD patients. These aims were based on prior research highlighting systemic immune alterations in nAMD patients compared to healthy controls, and by the limited understanding of how these immune changes relate to treatment response in nAMD.

We found that patients with nAMD who exhibited poor initial treatment response demonstrated a non-significant trend toward elevated plasma concentrations of complement protein C3, C3a, and an increased C3a/C3 ratio. This observation aligns with the hypothesis that enhanced systemic activation of the complement cascade may promote a proinflammatory microenvironment in the retina, fostering angiogenesis and diminishing the efficacy of anti-VEGF therapy. Supporting this hypothesis, we also observed significantly elevated concentrations of these complement proteins in nAMD patients compared to healthy controls.

Unexpectedly, patients with a partial initial treatment response exhibited significantly lower plasma concentrations of C3a and C5a. This finding suggests that this subgroup may exhibit a phenotypically distinct complement activation profile, more similar to that of healthy controls, potentially contributing to their reduced responsiveness to anti-VEGF treatment. Furthermore, partial 1-year responders were characterized by significantly lower expression levels of CD35 on monocytes overall and cMonocytes. CD35+ monocytes inhibits the complement cascade by clearing complement proteins C3b and C4b,[81] thus decreased inhibition of these opsonins in partial responders might cause inflammatory dysregulation. This dysregulation may result in a diminished angiostatic response to intravitreal anti-VEGF therapy. Additionally, nAMD patients showed reduced expression of CD4+CD46+ T cells and CD59+ iMonocytes, suggesting a dysregulation of complement activation, which could be involved in nAMD pathogenesis as CD4+CD46+ T cells are involved in the regulation of inflammation by secreting the anti-inflammatory cytokine IL-10.[82] CD59 inhibits MAC formation. MAC causes cell lysis, and decreased inhibition of MAC might cause retinal degeneration.[83] Interestingly, these changes were not observed in poor treatment responders, indicating that the complement system may play a less prominent role in this subgroup, although correlation networks revealed that poor and partial responders might have a more complex regulation of the complement system compared to good responders.

Cregs on T cells can modulate the complement system, while the complement system itself can influence T cell differentiation.[84] Proinflammatory cytokines also regulate the complement cascade.[85] Aging of the immune system is characterized by a systemic increase in T cell differentiation and plasma concentration of cytokines, among other factors. Differentiated memory T cells secrete proinflammatory cytokines,[86] and CD8+CD56+ T cells secrete IFN-γ.[87] This increases the systemic activation of the immune system in a low-grade chronic inflammatory state, inflammaging.[45]

We found patients with nAMD who exhibited poor treatment response showed a tendency towards increased T cell differentiation in the forms of CD8+ effector memory T cells and CD8+CD56+ T cells, consistent with the elevated CD8+ T cell differentiation observed in nAMD patients compared to healthy controls. Furthermore, nAMD patients demonstrated higher plasma concentrations of the cytokines IFN-γ, IL-1β, IL-2, and IL-10. Among these, poor responders displayed significantly elevated IFN-γ levels compared to good responders. IFN-γ is a potent proinflammatory cytokine that promotes the activation of proinflammatory M1 macrophages. These macrophages drive inflammation by secreting cytokines such as IL-1β and IL-6, which exhibit synergistic proinflammatory effects with IFN-γ, thereby amplifying the inflammatory response,[88] and induces release of proinflammatory chemokines.[89] IFN-γ is also involved in upregulation of the complement cascade[90] and inhibits CD35.[91] Additionally, IFN-γ has been shown to induce the production of reactive oxygen species in RPE cells, leading to damage of the blood-retina barrier.[92] IFN-γ further enhances the cytotoxic potential of CD8+ T cells by upregulating antigen processing, presentation and costimulatory molecules, as well as growth inhibition of T helper type 2 cells,[93] which may contribute to increased angiogenesis.[94] Moreover, IFN-γ stimulates RPE cells to produce VEGF, a key driver of neovascularization.[95] In poor initial responders, IFN-γ may therefore play a crucial role in maintaining a proangiogenic retinal environment, potentially contributing to reduced treatment efficacy.

The aging immune profile of CD8+ T cell differentiation and plasma cytokines, visualized as spiderweb charts, demonstrated that nAMD patients exhibited advanced immunosenescence compared to healthy controls. Thus, biological aging of the immune system may partly explain why some elderly individuals develop nAMD, while others remain unaffected.

The chemokine system plays a central role in chemotaxis, facilitating the recruitment of leukocytes, such as monocytes, T cells, and neutrophils, to sites of inflammation.[96] Beyond this, it is highly integrated into the regulation of other immune pathways, including T cell differentiation[97] and angiogenesis.[98]

We found nAMD patients with poor initial and 1-year treatment responses had decreased proportions of CD4+CXCR3+ T cells, alongside a trend toward elevated CXCL10 levels in poor initial responders. CXCL10 is also called IFN-γ induced protein 10 because IFN-γ stimulate the secretion of this chemokine.[89] CXCL10 in turn binds and leads to internalization of the anti-angiogenic chemokine receptor CXCR3 on leukocytes.[99] Dysregulation of the CXCL10-CXCR3 axis has been linked to angiogenesis, mediated by VEGF signaling.[100] This reduction of anti-inflammatory CD4+CXCR3+ T cells might contribute to the reduced anti-VEGF response in poor responders.

Additionally, lower proportions of pro-angiogenic CCR5+ and CCR2+ monocytes were observed in poor and partial responders, potentially disrupting inflammation resolution and angiogenesis regulation. Since CCR2 and CCR5 recruit monocytes that differentiate into macrophages involved in these processes, their depletion may result in chronic inflammation and persistent VEGF production.[101] IFN-gamma has also been suggested to reduce the expression of CCR2 on monocytes,[102] but increases expression of CCR5 on monocytes.[103] Correlation networks also seemed to suggest a more complex regulation of the chemokine system in partial and poor responders.

The expression levels of chemokine receptor CXCR2 (CD182) was also significantly elevated on neutrophils in nAMD patients compared to healthy controls, along with other neutrophil activation markers. Neutrophils are integrated in lymphocyte activation, and vice versa, particularly for T cells. Neutrophils are involved in cytokine mediated T cell activation, like IL-1β and IL-6[39,104] and antigen presentation,[105] as well as in T cell differentiation.[106] Neutrophils can secrete IFN-γ,[40] and lymphocytes can activate neutrophils by IFN-γ stimulation.[40] The number of circulating lymphocytes is also influenced by neutrophils, as increased neutrophil count has been shown to decrease lymphocyte count,[107] and the NLR is an indicator of systemic inflammation.[108–110]

We observed a significantly elevated NLR in poor 1-year responders, with a similar trend in poor initial responders. This aligns with our findings of increased NLR in nAMD patients compared to healthy controls, which has also been reported previously.[53] A previous study also found an association between NLR and initial treatment response defined as a reduction of CRT of 100 µm.[111] As an indicator of systemic inflammation, NLR is also implicated in angiogenesis.[112,113] Neutrophils secrete VEGF,[114] and when the blood-retina barrier is compromised, this can promote retinal neovascularization.[57] We found increased expression levels of activation surface markers on neutrophils in nAMD patients, but this did not seem to influence treatment response. Thus, the ratio rather than the immunophenotype of neutrophils might be the cause of an increased angiogenic retinal environment resistant to anti-VEGF.

Genetics play an important role in AMD development and treatment response.[11,115] We hypothesized that high-risk polymorphisms in the CFH and ARMS2 genes might be involved in nAMD pathogenesis by influencing the systemic levels of proinflammatory agents of the immune system. We found that the high-risk genotype (CC/CT) of CFH rs1061170 was associated with increased levels of C3a, C3a/C3 ratio, CD46+ cMonocytes, CCL4, CD4+CCR2+ T cells, CXCR2+ iMonocytes, and decreased levels of CD46+ nMonocytes. The high-risk genotype (TT/TG) of ARMS2 rs10490924 was associated with increased levels of CX_3CR1+ monocytes and cMonocytes, CCR1+ nMonocytes, and decreased levels of CCR1+ iMonocytes. No significant differences were found between these SNPs and the other studied immune markers. These findings suggest that the complement and chemokine systems may be modulated by genetic factors in nAMD patients. However, the overall understanding remains incomplete, underscoring the complexity and multifactorial nature of nAMD pathogenesis. This condition likely involves numerous interrelated pathways, many of which extend beyond the scope of those assessed in this study, emphasizing the need for further investigation to fully elucidate the underlying mechanisms.

Patients with iAMD typically exhibit few or no symptoms, which may explain the limited immunological research conducted on this group. In this study, we found that iAMD patients had significantly elevated plasma C3a levels compared to healthy controls, along with a seemingly more complex regulation of the complement system, as assessed by correlation networks.

Additionally, iAMD patients displayed higher proportions of CD8+CD56+ T cells compared to healthy controls, as well as lower proportions of CX_3CR1+ cMonocytes and iMonocytes, and CCR2+ iMonocytes. These findings suggest that iAMD is associated with systemic immune dysregulation, though to a lesser extent than in nAMD.

Most iAMD patients do not progress to nAMD. Therefore, the iAMD patient group is heterogeneous, comprising individuals who will and will not develop nAMD. This heterogeneity may explain the observed increase in immune activation and dysregulation, which remains below the levels seen in nAMD. Patients who eventually progress to nAMD may elevate the average levels of these markers, while those who do not develop nAMD lower it.

Overall, many of the significant differences found in this PhD project are biologically minor, primarily the expression levels of surface markers on leukocytes. This was expected and confirms that AMD is characterized by low-grade systemic inflammation.

We have previously found that initial treatment response can predict 1-year outcomes in nAMD,[23] however these groups differ in immunological baseline phenotype. This discrepancy might be caused by the variability after one year. The initial treatment protocol of the loading dose is

identical in all included nAMD patients, the maintained treatment follows an individualized plan, which differs according to treatment response.[72]

4.1. Methodological strengths and limitations

Methodological strengths of this study include the consecutive inclusion of patients in a real-world, prospective setting, ensuring the collection of clinically relevant data. Consecutive inclusion also reduces the risk of selection bias, seasonal biases, such as those related to allergies,[116] or patient treatment delays.[117,118] Additionally, all diagnoses were made by retinal specialists, enhancing diagnostic accuracy. A major strength is the inclusion of treatment-naïve patients, which eliminates the risk of excluding patients with suspended treatment caused by disease stabilization or untreatable deterioration and eliminates potential confounding effects of prior anti-VEGF treatment on the systemic immune system. The 1-year follow-up was determined after three injections approximately after one year, which may have slightly compromised the precision of the exact 1-year follow-up. However, this approach ensured a more clinically representative assessment of treatment response, preventing an underestimation of clinical efficacy that might otherwise occur just before a scheduled injection in the observe-and-plan algorithm.

Flow cytometry was performed by a single investigator, ensuring consistent analysis and eliminating the risk of inter-examiner bias. Immunoassays were conducted using MesoScale plates, with samples from all groups distributed across plates to minimize systematic and inter-plate variability bias.[119] Additionally, all samples were analyzed in duplicates, reducing the risk of measurement errors and enhancing data reliability.

This study has several limitations. The observational design prevents definitive conclusions regarding causality. In prospective studies, selection bias may arise, such as if the most vulnerable or severely ill patients are less likely to participate, potentially confounding diagnosis and treatment response analysis. Additionally, loss to follow-up may introduce further selection bias for the same reason,[120] however this was not observed in this project.[23] A notable limitation is that healthy controls were not age-matched, however this was accounted for with statistical adjustments. We attempted to recruit partners of patients, as they often share similar age, socioeconomic status, and lifestyle factors.[63,121] However, due to COVID-19 lockdown restrictions at the department throughout the entire inclusion period, relatives were not allowed in the clinic, making this approach challenging. The lockdown may also have influenced patient healthcare-seeking behavior and treatment intervals.[122] Interestingly, a previous study from our department found that nAMD patients received fewer injections during lockdown but had better visual outcomes compared to pre-lockdown, potentially reflecting increased prioritization of patients

with acute treatment needs.[123] Another potential limitation is that the study was conducted in a high-volume clinic, where delays in treatments within the observe-and-plan regimen may have occurred. The generalizability of the findings is affected by exclusion criteria, including active smoking, elevated CRP, and immunomodulating disease and treatment. These patients represent a significant subgroup, and their exclusion raises uncertainty about whether the results would apply to them.

Further limitations include the assessment of disease progression and treatment response. A more detailed OCT scan with additional B-scans could have improved detection accuracy, as retinal fluid may be missed between scans. Additionally, baseline ophthalmic factors that could influence treatment response, such as MNV type,[124] fibrosis,[125] and geographic atrophy[126] were not accounted for. The categorical classification of treatment response is another limitation, as this might obscure subtle differences. For example, patients might experience a substantial reduction in CRT and retinal fluid yet still be classified as partial if any fluid remains at follow-up.

A power calculation was performed to determine the study population comparing AMD stages (healthy controls, iAMD, and nAMD patients). However, no power calculation was performed for treatment response in nAMD, as no directly comparable studies exist. A number of 100 nAMD patients was chosen to evaluate treatment response in the scopes of this PhD project. The relatively small number of poor responders may have masked potential significant findings. However, a post-hoc power analysis was not conducted due to its limited validity.[127] While this prevents definitive conclusions regarding potential false-negative results, the positive findings remain robust. The duration of patient inclusion was limited by the timeframe of this PhD project, and further inclusion could have increased the statistical power. Statistical adjustments for multiple testing were applied in Studies II and IV but not in Studies I and III. In exploratory studies, such corrections are recommended, whereas in confirmatory studies with prior supporting evidence, adjustments may not be necessary.[128] Since Studies I and III build upon existing evidence, adjustment was deemed unnecessary. However, multiple testing correction reduces statistical power, and as inflammatory changes in AMD are subtle due to its low-grade chronic inflammation,[44] conservative corrections could obscure meaningful differences.

5. Conclusions and future perspectives

A leading cause of vision loss in the elderly is nAMD, which significantly impairs daily activities and adversely impacts mental health. Intraocular anti-VEGF injections enhance visual acuity and improve retinal morphology in most nAMD patients. However, a substantial proportion of

individuals with nAMD exhibit persistent disease activity or progressive deterioration despite treatment. There are currently no alternative or additional treatment options for these patient groups.

In this PhD project we aimed to identify specific systemic immune system differences in nAMD patients with different treatment responses, which could serve as potential therapeutic targets for nAMD patients with suboptimal treatment response (partial and poor response).

We found multiple differences suggesting an advanced proinflammatory state in nAMD patients with suboptimal treatment response. IFN-γ seems to play a central role in these patients, as this cytokine induces and is induced by differentiated T cells, which were also increased. IFN-γ induces the complement cascade and inhibits Cregs affected in these patients. IFN-γ induces CXCL10 increased in poor responders, which decreases CXCR3 expression on CD4+ T cells. IFN-γ itself also induces CCR5 and inhibits CCR2, which were affected in suboptimally responding nAMD patients (partial and poor responders). The NLR was increased in 1-year poor treatment responders, which IFN-γ partly regulates by inducing and activating neutrophils and lymphocytes. These systemic immune factors may play a crucial role in promoting chronic inflammation and excessive angiogenesis in the retinal microenvironment, potentially leading to elevated VEGF levels or the activation of additional, yet unidentified molecular pathways. Such immune dysregulation could create a proinflammatory and proangiogenic state that exacerbates disease progression and interferes with the efficacy of anti-VEGF therapy. Consequently, this pathological retinal environment may be a key contributor to the diminished treatment response observed in these patients, highlighting the need for a deeper understanding of immune-mediated mechanisms and the development of alternative therapeutic strategies (Figure 14).

We also observed advanced biological aging and proinflammation of the systemic immune system in nAMD patients compared to healthy controls. Specifically, we found increased activation of the complement system, indicated by elevated plasma concentrations of complement proteins and reduced proportions of Cregs; advanced CD8+ T cell differentiation and elevated plasma cytokine levels; greater dysregulation of the chemokine system; and an increased NLR. Patients with iAMD had similar tendencies but less prominent.

The genetic influence of CFH and ARMS2 polymorphisms influence the complement and chemokine system in nAMD patients, however not T cell differentiation, cytokines, or NLR. This demonstrates that nAMD pathology is complex and multifactorial, involving multiple pathways not studied in this PhD.

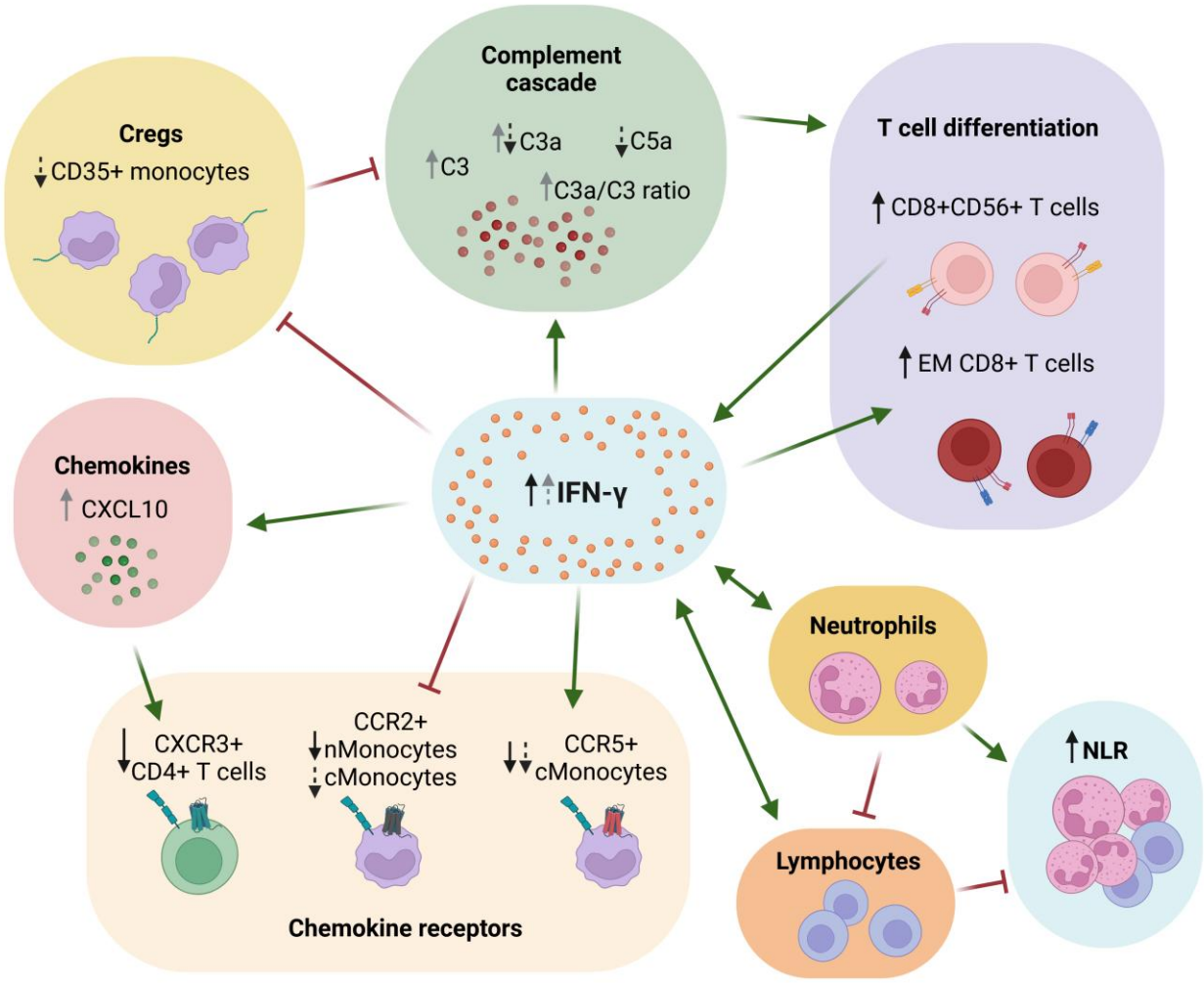


Figure 14. Systemic inflammation in relation to treatment response in nAMD patients, as found in the DANEART study. Multiple pathways involving the complement system, aging immune system, chemokine system, and NLR are associated with treatment response in nAMD patients. Plasma IFN-γ plays a central role in these affected pathways. Solid black arrow = significant increase/decrease in poor responders; solid gray arrow = trending increase/decrease in poor responders; dashed black arrow = significance increase/decrease in partial responders; dashed gray arrow = trending increase/decrease in partial responders; green arrow = stimulation/secretion; red flat-head arrow = inhibition. Own work, created with BioRender.

We have demonstrated that the systemic immune system profiles differ between nAMD treatment response groups, with IFN-γ playing a central role. Future studies should investigate whether IFN-γ or its related molecular pathways could serve as novel treatment targets for nAMD patients who exhibit suboptimal treatment responses. Such research should aim to understand the role of IFN-γ in disease progression and therapeutic resistance, exploring its potential to modulate inflammatory

and angiogenic processes in the retina. Additionally, studies should assess the feasibility and clinical impact of targeting these pathways in combination with intravitreal anti-VEGF injections. This could lead to better treatment for this patient group increasing visual function, and thus health and quality of life (Figure 15).

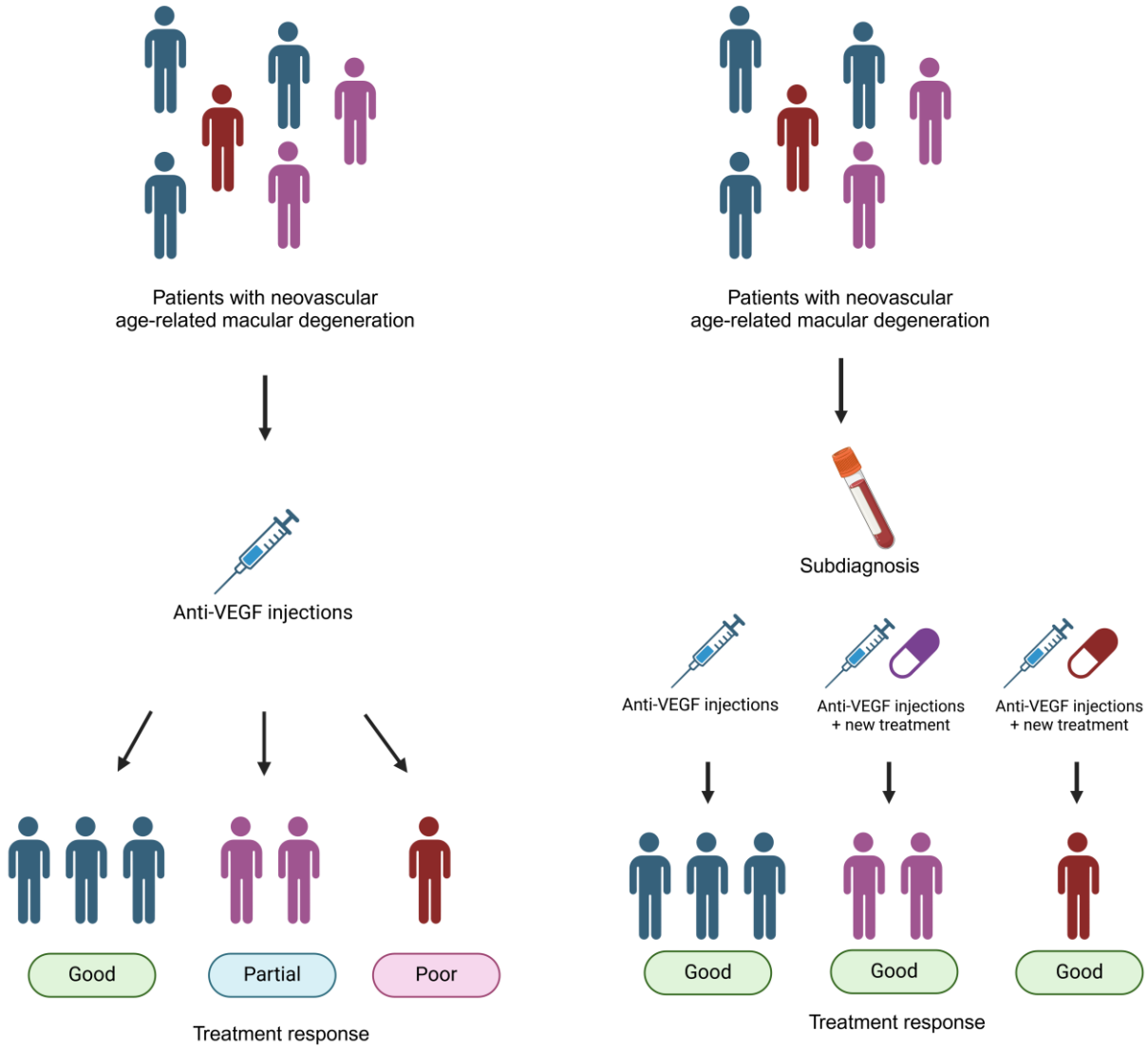


Figure 15. Future perspectives for personalized treatment in nAMD. Currently, all patients receive the same treatment targeting ocular VEGF despite significant variations in response. By implementing subdiagnosis strategies, tailored systemic treatments can be applied, leading to improved outcomes across different patient groups. Own work, created with BioRender.

6. References

1. Hall JE, Guyton AC. *Guyton and Hall Textbook of Medical Physiology*. 12th ed. Saunders/Elsevier; 2011.

2. Brüel, Annemarie, Christensen, Erik Ilsø, Eld, Mikkel, et al. *Genesers Histologi*. 2nd ed. Munksgaard; 2020.

3. Chen M, Luo C, Zhao J, Devarajan G, Xu H. Immune regulation in the aging retina. *Progress in Retinal and Eye Research*. 2019;69:159-172. doi:10.1016/j.preteyeres.2018.10.003

4. Palbo A, Roed Rasmussen ML, Hansen MS, Subhi Y. Association between visual impairment and suicide: A systematic review and meta-analysis. *Acta Ophthalmologica*. 2024;102(3):263-273. doi:10.1111/aos.15730

5. Fleckenstein M, Keenan TDL, Guymer RH, et al. Age-related macular degeneration. *Nat Rev Dis Primers*. 2021;7(1):31. doi:10.1038/s41572-021-00265-2

6. Rudnicka AR, Jarrar Z, Wormald R, Cook DG, Fletcher A, Owen CG. Age and Gender Variations in Age-related Macular Degeneration Prevalence in Populations of European Ancestry: A Meta-analysis. *Ophthalmology*. 2012;119(3):571-580. doi:10.1016/j.ophtha.2011.09.027

7. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *The Lancet Global Health*. 2014;2(2):e106-e116. doi:10.1016/S2214-109X(13)70145-1

8. Mitchell P, Liew G, Gopinath B, Wong TY. Age-related macular degeneration. *The Lancet*. 2018;392(10153):1147-1159. doi:10.1016/S0140-6736(18)31550-2

9. Seddon JM. The US Twin Study of Age-Related Macular Degeneration: Relative Roles of Genetic and Environmental Influences. *Arch Ophthalmol*. 2005;123(3):321. doi:10.1001/archopht.123.3.321

10. Fritsche LG, Igl W, Bailey JNC, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nat Genet*. 2016;48(2):134-143. doi:10.1038/ng.3448

11. Smailhodzic D, Klaver CCW, Klevering BJ, et al. Risk Alleles in CFH and ARMS2 Are Independently Associated with Systemic Complement Activation in Age-related Macular Degeneration. *Ophthalmology*. 2012;119(2):339-346. doi:10.1016/j.ophtha.2011.07.056

12. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration. *Ophthalmology*. 2001;108(4):697-704. doi:10.1016/S0161-6420(00)00580-7

13. The age-related eye disease study system for classifying age-related macular degeneration from stereoscopic color fundus photographs: the age-related eye disease study report number 6. *American Journal of Ophthalmology*. 2001;132(5):668-681. doi:10.1016/S0002-9394(01)01218-1

14. Seddon J, Sharma S, Adelman R. Evaluation of the Clinical Age-Related Maculopathy Staging System. *Ophthalmology*. 2006;113(2):260-266. doi:10.1016/j.ophtha.2005.11.001

15. Ferris FL, Wilkinson CP, Bird A, et al. Clinical Classification of Age-related Macular Degeneration. *Ophthalmology*. 2013;120(4):844-851. doi:10.1016/j.ophtha.2012.10.036

16. Fleckenstein M, Schmitz-Valckenberg S, Chakravarthy U. Age-Related Macular Degeneration: A Review. *JAMA*. 2024;331(2):147. doi:10.1001/jama.2023.26074

17. Bek T, Sørensen TL. Age-related Macular Degeneration. *Ugeskr Laeger*. 2023;185(48):V05230316.

18. Sayegh RG, Sacu S, Dunavölgyi R, et al. Geographic Atrophy and Foveal-Sparing Changes Related to Visual Acuity in Patients With Dry Age-Related Macular Degeneration Over Time. *American Journal of Ophthalmology*. 2017;179:118-128. doi:10.1016/j.ajo.2017.03.031

19. Hee MR. Optical Coherence Tomography of the Human Retina. *Arch Ophthalmol*. 1995;113(3):325. doi:10.1001/archopht.1995.01100030081025

20. Thomsen AK, Gøttsche LF, Hinnerskov JMV, Falk MK, Sørensen TL. Microperimetry and Structural Risk Factors on OCT in Intermediate Age-Related Macular Degeneration. *Ophthalmology Retina*. Published online March 2024:S2468653024000885. doi:10.1016/j.oret.2024.02.016

21. Cho HJ, Yoon W, Yoon J, et al. Development of Intraretinal Fluid in Neovascular Age-Related Macular Degeneration During Anti–Vascular Endothelial Growth Factor Treatment. *American Journal of Ophthalmology*. 2022;234:6-14. doi:10.1016/j.ajo.2021.07.026

22. Sadda SR, Guymer R, Holz FG, et al. Consensus Definition for Atrophy Associated with Age-Related Macular Degeneration on OCT. *Ophthalmology*. 2018;125(4):537-548. doi:10.1016/j.ophtha.2017.09.028

23. Thomsen AK, Fasih-Ahmad S, Sadda S, Sørensen TL. Initial treatment response can predict one-year treatment outcomes in neovascular age-related macular degeneration treated according to the observe-and-plan regimen. *Acta Ophthalmologica*. Published online November 27, 2024:aos.16801. doi:10.1111/aos.16801

24. Spaide RF, Fujimoto JG, Waheed NK, Sadda SR, Staurenghi G. Optical coherence tomography angiography. *Progress in Retinal and Eye Research*. 2018;64:1-55. doi:10.1016/j.preteyeres.2017.11.003

25. 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(10):1417. doi:10.1001/archopht.119.10.1417

26. Chew EY. Complement inhibitors for the treatment of geographic atrophy. *The Lancet*. 2023;402(10411):1396-1398. doi:10.1016/S0140-6736(23)01844-5

27. Medicinrådets behandlingsvejledning vedrørende lægemidler til våd aldersrelateret makuladegeneration. https://medicinraadet.dk/media/qvofq20p/baggrund-for-

medicinr%C3%A5dets-behandlingsvejledning-vedr-l%C3%A6gemidler-til-v%C3%A5d-amd-vers-3-0-x.pdf.

28. Rådet for anvendelse af dyr sygehusmedicin. https://rads.dk/behandlingsvejledninger/oejensygdomme/.

29. Mantel I, Niderprim SA, Gianniou C, Deli A, Ambresin A. Reducing the clinical burden of ranibizumab treatment for neovascular age-related macular degeneration using an individually planned regimen. *Br J Ophthalmol*. 2014;98(9):1192-1196. doi:10.1136/bjophthalmol-2013-304556

30. Abbas AK, Lichtman AH, Pillai S, Baker DL, Baker A. *Basic Immunology: Functions and Disorders of the Immune System*. Fifth edition. Elsevier; 2016.

31. Nürnberger W, Bhakdi S. Plasma C3d/C3 quotient as a parameter for in vivo complement activation. *Journal of Immunological Methods*. 1984;74(1):87-91. doi:10.1016/0022-1759(84)90370-3

32. Armento A, Ueffing M, Clark SJ. The complement system in age-related macular degeneration. *Cell Mol Life Sci*. 2021;78(10):4487-4505. doi:10.1007/s00018-021-03796-9

33. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol*. 2009;9(10):729-740. doi:10.1038/nri2620

34. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*. 2011;11(11):762-774. doi:10.1038/nri3070

35. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175. doi:10.1038/nri3399

36. Schmidt T, Brodesser A, Schnitzler N, et al. CD66b Overexpression and Loss of C5a Receptors as Surface Markers for Staphylococcus aureus-Induced Neutrophil Dysfunction. Akiyama T, ed. *PLoS ONE*. 2015;10(7):e0132703. doi:10.1371/journal.pone.0132703

37. Krogh Nielsen M, Hector SM, Allen K, Subhi Y, Sørensen TL. Altered activation state of circulating neutrophils in patients with neovascular age-related macular degeneration. *Immun Ageing*. 2017;14(1):18. doi:10.1186/s12979-017-0100-9

38. Bickel M. The role of interleukin-8 in inflammation and mechanisms of regulation. *J Periodontol*. 1993;64(5 Suppl):456-460.

39. Hashizume M, Higuchi Y, Uchiyama Y, Mihara M. IL-6 plays an essential role in neutrophilia under inflammation. *Cytokine*. 2011;54(1):92-99. doi:10.1016/j.cyto.2011.01.007

40. Ellis TN, Beaman BL. Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology*. 2004;112(1):2-12. doi:10.1111/j.1365-2567.2004.01849.x

41. Huang Z, Fu Z, Huang W, Huang K. Prognostic value of neutrophil-to-lymphocyte ratio in sepsis: A meta-analysis. *The American Journal of Emergency Medicine*. 2020;38(3):641-647. doi:10.1016/j.ajem.2019.10.023

42. Forget P, Khalifa C, Defour JP, Latinne D, Van Pel MC, De Kock M. What is the normal value of the neutrophil-to-lymphocyte ratio? *BMC Res Notes*. 2017;10(1):12. doi:10.1186/s13104-016-2335-5

43. Socorro Faria S, Fernandes Jr PC, Barbosa Silva MJ, et al. The neutrophil-to-lymphocyte ratio: a narrative review. *ecancer*. 2016;10. doi:10.3332/ecancer.2016.702

44. Rozing MP, Durhuus JA, Krogh Nielsen M, et al. Age-related macular degeneration: A two-level model hypothesis. *Progress in Retinal and Eye Research*. 2020;76:100825. doi:10.1016/j.preteyeres.2019.100825

45. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Annals of the New York Academy of Sciences*. 2000;908(1):244-254. doi:10.1111/j.1749-6632.2000.tb06651.x

46. Xia S, Zhang X, Zheng S, et al. An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. *Journal of Immunology Research*. 2016;2016:1-12. doi:10.1155/2016/8426874

47. Zhuang Y, Lyga J. Inflammaging in Skin and Other Tissues - The Roles of Complement System and Macrophage. *IADT*. 2014;13(3):153-161. doi:10.2174/18715281136661405221120003

48. Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. *Cell Mol Life Sci*. 2016;73(9):1765-1786. doi:10.1007/s00018-016-2147-8

49. Chen L, Deng H, Cui H, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*. 2018;9(6):7204-7218. doi:10.18632/oncotarget.23208

50. Faber C, Singh A, Krüger Falk M, Juel HB, Sørensen TL, Nissen MH. Age-related Macular Degeneration Is Associated with Increased Proportion of CD56+ T Cells in Peripheral Blood. *Ophthalmology*. 2013;120(11):2310-2316. doi:10.1016/j.ophtha.2013.04.014

51. Liisborg C, Skov V, Kjær L, Hasselbalch HC, Sørensen TL. Retinal drusen in patients with chronic myeloproliferative blood cancers are associated with an increased proportion of senescent T cells and signs of an aging immune system. *Aging*. 2021;13(24):25763-25777. doi:10.18632/aging.203803

52. Subhi Y, Nielsen MK, Molbech CR, et al. T-cell differentiation and CD56+ levels in polypoidal choroidal vasculopathy and neovascular age-related macular degeneration. *Aging*. 2017;9(11):2436-2452. doi:10.18632/aging.101329

53. Niazi S, Krogh Nielsen M, Sørensen TL, Subhi Y. Neutrophil-to-lymphocyte ratio in age-related macular degeneration: a systematic review and meta-analysis. *Acta Ophthalmologica*. 2019;97(6):558-566. doi:10.1111/aos.14072

54. Jensen EG, Jakobsen TS, Thiel S, Askou AL, Corydon TJ. Associations between the Complement System and Choroidal Neovascularization in Wet Age-Related Macular Degeneration. *IJMS*. 2020;21(24):9752. doi:10.3390/ijms21249752

55. Ebrahimi KB, Fijalkowski N, Cano M, Handa JT. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. *The Journal of Pathology*. 2013;229(5):729-742. doi:10.1002/path.4128

56. Schick T, Steinhauer M, Aslanidis A, et al. Local complement activation in aqueous humor in patients with age-related macular degeneration. *Eye*. 2017;31(5):810-813. doi:10.1038/eye.2016.328

57. Tan W, Zou J, Yoshida S, Jiang B, Zhou Y. The Role of Inflammation in Age-Related Macular Degeneration. *Int J Biol Sci*. 2020;16(15):2989-3001. doi:10.7150/ijbs.49890

58. Scholl HPN, Issa PC, Walier M, et al. Systemic Complement Activation in Age-Related Macular Degeneration. Toland AE, ed. *PLoS ONE*. 2008;3(7):e2593. doi:10.1371/journal.pone.0002593

59. Singh A, Faber C, Falk M, Nissen MH, Hviid TVF, Sørensen TL. Altered Expression of CD46 and CD59 on Leukocytes in Neovascular Age-Related Macular Degeneration. *American Journal of Ophthalmology*. 2012;154(1):193-199.e2. doi:10.1016/j.ajo.2012.01.036

60. Hinnerskov, JMV. *Specific Systemic Immune Changes in Patients with Geographic Atrophy and Their Relation to Progression (PhD Thesis).* University of Copenhagen

61. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. *Human Molecular Genetics*. 2010;19(1):209-215. doi:10.1093/hmg/ddp472

62. Martinez Villarruel Hinnerskov J, Krogh Nielsen M, Kai Thomsen A, et al. Chemokine Receptor Profile of T Cells and Progression Rate of Geographic Atrophy Secondary to Age-related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2024;65(1):5. doi:10.1167/iovs.65.1.5

63. Subhi Y, Krogh Nielsen M, Molbech CR, et al. Plasma markers of chronic low-grade inflammation in polypoidal choroidal vasculopathy and neovascular age-related macular degeneration. *Acta Ophthalmol*. 2019;97(1):99-106. doi:10.1111/aos.13886

64. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. CX3CL1/CX3CR1 and CCL2/CCR2 Chemokine/Chemokine Receptor Complex in Patients with AMD. Sennlaub F, ed. *PLoS ONE*. 2014;9(12):e112473. doi:10.1371/journal.pone.0112473

65. Grunin M, Burstyn-Cohen T, Hagbi-Levi S, Peled A, Chowers I. Chemokine Receptor Expression in Peripheral Blood Monocytes from Patients with Neovascular Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2012;53(9):5292. doi:10.1167/iovs.11-9165

66. Krogh Nielsen M, Subhi Y, Molbech CR, Falk MK, Nissen MH, Sørensen TL. Chemokine Profile and the Alterations in CCR5-CCL5 Axis in Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2020;61(4):28. doi:10.1167/iovs.61.4.28

67. Subhi Y, Krogh Nielsen M, Molbech CR, Sørensen TL. Altered proportion of CCR2 $^{+}$ and CX3CR1 $^{+}$ circulating monocytes in neovascular age-related macular degeneration and

polypoidal choroidal vasculopathy. *Clinical Exper Ophthalmology*. 2018;46(6):661-669. doi:10.1111/ceo.13152

68. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. Dysregulation of CXCR3 Expression on Peripheral Blood Leukocytes in Patients With Neovascular Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2014;55(7):4050. doi:10.1167/iovs.14-14107

69. Liisborg C, Skov V, Kjær L, Hasselbalch HC, Lykke Sørensen T. Lower CXCR3 expression in both patients with neovascular AMD and advanced stages of chronic myeloproliferative blood cancers. Yassin MA, ed. *PLoS ONE*. 2022;17(6):e0269960. doi:10.1371/journal.pone.0269960

70. Lechner J, Chen M, Hogg RE, et al. Alterations in Circulating Immune Cells in Neovascular Age-Related Macular Degeneration. *Sci Rep*. 2015;5(1):16754. doi:10.1038/srep16754

71. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. *JAMA*. 2013;310(20):2191. doi:10.1001/jama.2013.281053

72. Subhi Y, Schneider M, Hajari JN, La Cour M. Injection burden and treatment intervals of aflibercept in observe-and-plan regimen for neovascular age-related macular degeneration. *Acta Ophthalmologica*. 2024;102(7):821-827. doi:10.1111/aos.16709

73. Thomsen AK, Steffensen MA, Villarruel Hinnerskov JM, et al. Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response. *J Neuroinflammation*. 2024;21(1):284. doi:10.1186/s12974-024-03273-7

74. Thomsen, AK, Steffensen, MA, Hinnerskov, JMV, et al. Plasma Interferon-gamma is Associated with Poor Treatment Response in Neovascular Age-Related Macular Degeneration. *Aging and disease*. doi:10.14336/AD.2024.1585

75. Wang J, Lin M, Crenshaw A, et al. High-throughput single nucleotide polymorphism genotyping using nanofluidic Dynamic Arrays. *BMC Genomics*. 2009;10(1):561. doi:10.1186/1471-2164-10-561

76. Brandwijk RJMGE, Michels MAHM, Van Rossum M, et al. Pitfalls in complement analysis: A systematic literature review of assessing complement activation. *Front Immunol*. 2022;13:1007102. doi:10.3389/fimmu.2022.1007102

77. McKinnon KM. Flow Cytometry: An Overview. *CP in Immunology*. 2018;120(1). doi:10.1002/cpim.40

78. Moro-García MA. When aging reaches CD4+ T-cells: phenotypic and functional changes. *Front Immunol*. 2013;4. doi:10.3389/fimmu.2013.00107

79. Larbi A, Fulop T. From “truly naïve” to “exhausted senescent” T cells: When markers predict functionality. *Cytometry Pt A*. 2014;85(1):25-35. doi:10.1002/cyto.a.22351

80. Arnson Y, Shoenfeld Y, Amital H. Effects of tobacco smoke on immunity, inflammation and autoimmunity. *Journal of Autoimmunity*. 2010;34(3):J258-J265. doi:10.1016/j.jaut.2009.12.003

81. Oliveira LC, Kretzschmar GC, Dos Santos ACM, et al. Complement Receptor 1 (CR1, CD35) Polymorphisms and Soluble CR1: A Proposed Anti-inflammatory Role to Quench the Fire of “Fogo Selvagem” Pemphigus Foliaceus. *Front Immunol*. 2019;10:2585. doi:10.3389/fimmu.2019.02585

82. Astier AL. T-cell regulation by CD46 and its relevance in multiple sclerosis. *Immunology*. 2008;124(2):149-154. doi:10.1111/j.1365-2567.2008.02821.x

83. Couves EC, Gardner S, Voisin TB, et al. Structural basis for membrane attack complex inhibition by CD59. *Nat Commun*. 2023;14(1):890. doi:10.1038/s41467-023-36441-z

84. Kwan W hong, Van Der Touw W, Heeger PS. Complement regulation of T cell immunity. *Immunol Res*. 2012;54(1-3):247-253. doi:10.1007/s12026-012-8327-1

85. Laufer J, Oren R, Farzam N, Goldberg I, Passwell J. Differential Cytokine Regulation of Complement Proteins in Human Glomerular Epithelial Cells. *Nephron*. 1997;76(3):276-283. doi:10.1159/000190192

86. Martin MD, Badovinac VP. Defining Memory CD8 T Cell. *Front Immunol*. 2018;9:2692. doi:10.3389/fimmu.2018.02692

87. Bergström I, Backteman K, Lundberg A, Ernerudh J, Jonasson L. Persistent accumulation of interferon-γ-producing CD8+CD56+ T cells in blood from patients with coronary artery disease. *Atherosclerosis*. 2012;224(2):515-520. doi:10.1016/j.atherosclerosis.2012.07.033

88. Jiang K, Cao S, Cui JZ, Matsubara JA. Immuno-modulatory Effect of IFN-gamma in AMD and its Role as a Possible Target for Therapy. *J Clin Exp Ophthalmol*. 2013;Suppl 2:0071-0076. doi:10.4172/2155-9570-S2-007

89. Madhurantakam S, Lee ZJ, Naqvi A, Prasad S. Importance of IP-10 as a biomarker of host immune response: Critical perspective as a target for biosensing. *Current Research in Biotechnology*. 2023;5:100130. doi:10.1016/j.crbiot.2023.100130

90. Mitchell TJ, Naughton M, Norsworthy P, Davies KA, Walport MJ, Morley BJ. IFN-gamma up-regulates expression of the complement components C3 and C4 by stabilization of mRNA. *J Immunol*. 1996;156(11):4429-4434.

91. Schlesinger LS, Horwitz MA. Phagocytosis of Mycobacterium leprae by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacterium. *J Immunol*. 1991;147(6):1983-1994.

92. Yang D, Elner SG, Bian ZM, Till GO, Petty HR, Elner VM. Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. *Experimental Eye Research*. 2007;85(4):462-472. doi:10.1016/j.exer.2007.06.013

93. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*. 2004;75(2):163-189. doi:10.1189/jlb.0603252

94. Lee H, Schlereth SL, Park EY, Emami-Naeini P, Chauhan SK, Dana R. A Novel Pro-Angiogenic Function for Interferon-γ–Secreting Natural Killer Cells. *Invest Ophthalmol Vis Sci*. 2014;55(5):2885. doi:10.1167/iovs.14-14093

95. Nagineni CN, Kommineni VK, William A, Detrick B, Hooks JJ. Regulation of VEGF expression in human retinal cells by cytokines: Implications for the role of inflammation in age-related macular degeneration. *Journal Cellular Physiology*. 2012;227(1):116-126. doi:10.1002/jcp.22708

96. Zlotnik A, Yoshie O. The Chemokine Superfamily Revisited. *Immunity*. 2012;36(5):705-716. doi:10.1016/j.immuni.2012.05.008

97. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. *Nat Immunol*. 2001;2(2):102-107. doi:10.1038/84205

98. Cecchinato V, Martini V, Pirani E, Ghovehoud E, Uguccioni M. The chemokine landscape: one system multiple shades. *Front Immunol*. 2023;14:1176619. doi:10.3389/fimmu.2023.1176619

99. Colvin RA, Campanella GSV, Sun J, Luster AD. Intracellular Domains of CXCR3 That Mediate CXCL9, CXCL10, and CXCL11 Function. *Journal of Biological Chemistry*. 2004;279(29):30219-30227. doi:10.1074/jbc.M403595200

100. Yates-Binder CC, Rodgers M, Jaynes J, Wells A, Bodnar RJ, Turner T. An IP-10 (CXCL10)-Derived Peptide Inhibits Angiogenesis. Proost P, ed. *PLoS ONE*. 2012;7(7):e40812. doi:10.1371/journal.pone.0040812

101. Spahn JH, Kreisel D. Monocytes in Sterile Inflammation: Recruitment and Functional Consequences. *Arch Immunol Ther Exp*. 2014;62(3):187-194. doi:10.1007/s00005-013-0267-5

102. Penton-Rol G, Polentarutti N, Luini W, et al. Selective inhibition of expression of the chemokine receptor CCR2 in human monocytes by IFN-gamma. *J Immunol*. 1998;160(8):3869-3873.

103. Hariharan D, Douglas SD, Lee B, Lai JP, Campbell DE, Ho WZ. Interferon-γ Upregulates CCR5 Expression in Cord and Adult Blood Mononuclear Phagocytes. *Blood*. 1999;93(4):1137-1144. doi:10.1182/blood.V93.4.1137

104. Sabbione F, Keitelman IA, Shiromizu CM, et al. Regulation of human neutrophil IL-1β secretion induced by Escherichia coli O157:H7 responsible for hemolytic uremic syndrome. Baumler AJ, ed. *PLoS Pathog*. 2023;19(12):e1011877. doi:10.1371/journal.ppat.1011877

105. Hänsch GM, Wagner C. Expression of MHC class II antigen and coreceptor molecules in polymorphonuclear neutrophils. *Chem Immunol Allergy*. 2003;83:45-63. doi:10.1159/000071556

106. Bert S, Nadkarni S, Perretti M. Neutrophil-T cell crosstalk and the control of the host inflammatory response. *Immunol Rev*. 2023;314(1):36-49. doi:10.1111/imr.13162

107.Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327-336. doi:10.1172/JCI57990

108.Tamhane UU, Aneja S, Montgomery D, Rogers EK, Eagle KA, Gurm HS. Association Between Admission Neutrophil to Lymphocyte Ratio and Outcomes in Patients With Acute Coronary Syndrome. *The American Journal of Cardiology*. 2008;102(6):653-657. doi:10.1016/j.amjcard.2008.05.006

109.Torun S, Tunc BD, Suvak B, et al. Assessment of neutrophil-lymphocyte ratio in ulcerative colitis: A promising marker in predicting disease severity. *Clinics and Research in Hepatology and Gastroenterology*. 2012;36(5):491-497. doi:10.1016/j.clinre.2012.06.004

110.Sengul EA, Artunay O, Kockar A, et al. Correlation of neutrophil/lymphocyte and platelet/lymphocyte ratio with visual acuity and macular thickness in age-related macular degeneration. *Int J Ophthalmol*. 2017;10(5):754-759. doi:10.18240/ijo.2017.05.16

111.Gökce SE, Çelik A, Başkan C. The role of blood neutrophil lymphocyte ratio in predicting the initial response to anti-VEGF treatment in neovascular AMD patients. *Ir J Med Sci*. 2024;193(1):517-521. doi:10.1007/s11845-023-03379-6

112.Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. *Int J Exp Pathol*. 2009;90(3):222-231. doi:10.1111/j.1365-2613.2009.00641.x

113.Christoffersson G, Vågesjö E, Vandooren J, et al. VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. *Blood*. 2012;120(23):4653-4662. doi:10.1182/blood-2012-04-421040

114.Gong Y, Koh DR. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. *Cell Tissue Res*. 2010;339(2):437-448. doi:10.1007/s00441-009-0908-5

115.Brantley MA, Fang AM, King JM, Tewari A, Kymes SM, Shiels A. Association of Complement Factor H and LOC387715 Genotypes with Response of Exudative Age-Related Macular Degeneration to Intravitreal Bevacizumab. *Ophthalmology*. 2007;114(12):2168-2173. doi:10.1016/j.ophtha.2007.09.008

116.Lee YC, Ju HJ, Kwon JW, Bae JM. Seasonality of allergic diseases: Real-world evidence from a nationwide population-based study. *Immun Inflamm Dis*. 2020;8(3):360-362. doi:10.1002/iid3.316

117.Argha A, Savkin A, Liaw ST, Celler BG. Effect of Seasonal Variation on Clinical Outcome in Patients with Chronic Conditions: Analysis of the Commonwealth Scientific and Industrial Research Organization (CSIRO) National Telehealth Trial. *JMIR Med Inform*. 2018;6(1):e16. doi:10.2196/medinform.9680

118.Tsiropoulos GN, Amaxilati E, Tranou M, et al. The Seasonality of Retinal Detachment: Peaks, Troughs, and Global Trends. *Life*. 2025;15(2):190. doi:10.3390/life15020190

119.Bastarache JA, Koyama T, Wickersham NE, Ware LB. Validation of a multiplex electrochemiluminescent immunoassay platform in human and mouse samples. *J Immunol Methods*. 2014;408:13-23. doi:10.1016/j.jim.2014.04.006

120.Cleland JGF, Torp-Pedersen C, Coletta AP, Lammiman MJ. A method to reduce loss to follow-up in clinical trials: informed, withdrawal of consent. *Eur J Heart Fail*. 2004;6(1):1-2. doi:10.1016/j.eheart.2003.12.001

121.Krogh Nielsen M, Subhi Y, Molbech CR, et al. Patients with a fast progression profile in geographic atrophy have increased CD200 expression on circulating monocytes: CD200 expression in geographic atrophy. *Clin Experiment Ophthalmol*. 2019;47(1):69-78. doi:10.1111/ceo.13362

122.Fung THM, Kuet ML, Patel MK, Puri P. Addressing COVID-19 fear to improve clinic attendance for patients with wet age-related macular degeneration. *Acta Ophthalmol*. 2021;99(2):e285. doi:10.1111/aos.14520

123.Bagi Nordsten C, Rasmussen BK, Li X, Thomsen AK, Sørensen TL. Impact of COVID -19 restrictions on the treatment of neovascular age-related macular degeneration. *Acta Ophthalmologica*. 2024;102(3). doi:10.1111/aos.15766

124.Izquierdo-Serra J, Martin-Pinardel R, Moll-Udina A, et al. Macular Neovascularization Type Influence on Anti-VEGF Intravitreal Therapy Outcomes in Age-Related Macular Degeneration. *Ophthalmol Retina*. 2024;8(4):350-359. doi:10.1016/j.oret.2023.10.022

125.Armendariz BG, Chakravarthy U. Fibrosis in age-related neovascular macular degeneration in the anti-VEGF era. *Eye*. 2024;38(17):3243-3251. doi:10.1038/s41433-024-03308-6

126.Amaro MH, Roller AB. Intravitreal ranibizumab and bevacizumab therapy for choroidal neovascularization in age-related macular degeneration with extensive pre-existing geographic atrophy. *Arq Bras Oftalmol*. 2012;75(4):273-276. doi:10.1590/s0004-27492012000400011

127.Heinsberg LW, Weeks DE. Post hoc power is not informative. *Genet Epidemiol*. 2022;46(7):390-394. doi:10.1002/gepi.22464

128.Boulesteix AL, Hoffmann S. To adjust or not to adjust: it is not the tests performed that count, but how they are reported and interpreted. *BMJ Med*. 2024;3(1):e000783. doi:10.1136/bmjmed-2023-000783

7. Appendices

7.1. Study I

7.2. Study II

7.3. Study III

7.4. Study IV

7.1. Study I

Journal of Neuroinflammation

RESEARCH

Open Access

Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response

Alexander Kai Thomsen[1,2*], Maria Abildgaard Steffensen[3], Jenni Martinez Villarruel Hinnerskov[1,2], Amalie Thomsen Nielsen[1,2], Henrik Vorum[4,5], Bent Honoré[4,6], Mogens Holst Nissen[2,3] and Torben Lykke Sørensen[1,2]

Abstract

Background Dysregulation of the complement system is involved in development of age-related macular degeneration (AMD). The complement cascade is regulated by membrane bound complement regulatory proteins (Cregs) on mononuclear leukocytes among others. This study aims to investigate systemic complement proteins and Cregs in AMD stages and their association with treatment response in neovascular AMD (nAMD).

Methods In this clinical prospective study, treatment-naïve patients with nAMD, intermediate AMD (iAMD) and healthy controls were recruited and systemic complement proteins C3, C3a and C5a were investigated with electrochemiluminescence immunoassays, and Creg expression (CD35, CD46 and CD59) on T cells (CD4 + and CD8+) and monocytes (classical, intermediate and non-classical) investigated with flow cytometry. Treatment response in nAMD patients was evaluated after loading dose and after one year, and categorized as good, partial or poor. Complement proteins and Creg expression levels were compared between healthy controls, iAMD and nAMD, as well as between good, partial and poor nAMD treatment response groups. Polymorphisms in the CFH and ARMS2 genes were analyzed and compared to complement proteins and Creg expression levels in nAMD patients.

Results One hundred patients with nAMD, 34 patients with iAMD and 61 healthy controls were included. 94 nAMD patients completed the 1-year follow-up. Distribution of treatment response in nAMD was 61 (65%) good, 26 (28%) partial, and 7 (7%) poor responders. The distribution of 1-year treatment response was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. The concentrations of systemic C3, C3a, and the C3a/C3-ratio were significantly increased in patients with nAMD compared to healthy controls ($P < 0.001$, $P = 0.002$, and $P = 0.035$, respectively). Systemic C3 was also increased in iAMD compared to healthy controls ($P = 0.031$). The proportion of CD46 + CD4 + T cells and CD59 + intermediate monocytes were significantly decreased in patients with nAMD compared to healthy controls ($P = 0.018$ and $P = 0.042$, respectively). The post-loading dose partial treatment response group had significantly lower concentrations of C3a and C5a compared to the good response group ($P = 0.005$ and $P = 0.042$,

*Correspondence:
Alexander Kai Thomsen
alext@regionsjaelland.dk

Full list of author information is available at the end of the article

respectively). The proportion of CD35 + monocytes was significantly lower in the 1-year partial response group compared to the 1-year good response group ($P = 0.039$). High-risk CFH genotypes in nAMD patients was associated with increased C3a, C3a/C3-ratio, and expression levels of CD35 + CD8 + T cells and CD46 + classical monocytes, while expression level of CD46 + non-classical monocytes was decreased.

Conclusion Elevated concentrations of systemic complement proteins were found in patients with iAMD and nAMD. Decreased Creg expression levels were found in patients with nAMD. Partially responding nAMD patients had a dysregulated complement system and Cregs compared to good responders.

Keywords Neovascular age-related macular degeneration, Intermediate age-related macular degeneration, Complement, Complement regulatory proteins, T cells, Monocytes, Treatment response, Inflammation, CFH, ARMS2, Genetics

Introduction

Age-related macular degeneration (AMD) is a leading cause of visual impairment and blindness in the elderly [1]. The preliminary stage of the disease is intermediate AMD (iAMD) characterized by macular drusen, but few clinical symptoms. Two types of late-stage AMD can develop from iAMD, called neovascular AMD (nAMD) and geographic atrophy. Neovascular AMD is fast-progressing and can cause symptoms of metamorphopsia and central scotomas within weeks to months [2]. Treatment of nAMD consists of repeated injections with an anti-vascular endothelial growth factor (VEGF) antibody, that can stabilize the disease and, in some cases, even reverse the symptoms completely. However, treatment response differs greatly between individuals and some patients will continue to experience visual deterioration [3]. Thus, VEGF might not be the only mediating factor for neovascularization secondary to AMD. In patients with insufficient response to anti-VEGF treatment, it may be beneficial to add an additional therapy targeting a different pathway [4].

The cause of AMD is multifactorial and is not yet completely elucidated. Environmental factors, genetics and chronic low-grade inflammation play significant roles in the pathophysiology [5–8]. Age-related chronic inflammation and dysregulated immune responses with involvement of both the innate and adaptive immune systems have been shown to increase the risk of developing AMD [9]. Alterations in the complement system have previously been shown to be associated with development and stages of AMD [10–15]. The complement system is responsible for enhancing the ability of antibodies and phagocytic cells to clear pathogens and damaged cells. It functions through a cascade of protein activations that lead to pathogen opsonization, promotion of inflammation, and direct lysis of pathogens by forming the membrane attack complex (MAC). Complement protein C3 is central in this cascade by cleavage into C3a, which acts as an anaphylatoxin activating multiple inflammatory pathways, and C3b, which acts as an opsonin and forms part of the MAC. C3b is also involved in creating C5 convertase that cleaves C5 into C5a and C5b further downstream in the complement cascade. C5a, like C3a, is an anaphylatoxin, which activates phagocytosis in immune cells like monocytes [16].

Dysregulation of complement regulatory proteins (Cregs) has also been found in patients with nAMD [17–19]. CD35 (Complement Receptor 1) and CD46 (Membrane Cofactor Protein) are involved in regulation of complement activation on T cells and monocytes [20]. CD59 (MAC inhibiting protein) inhibits the formation of MAC, thus protecting cells from lysis [20]. CD11b (Integrin α-M) is expressed on monocytes, among other cells, and facilitates adhesion and migration in inflammatory sites. Furthermore, CD11b is part of Complement Receptor 3 involved in complement mediated phagocytosis [21, 22].

Genetic predisposition is a key factor in AMD risk and more than half of AMD heritability is associated with genes related to the complement cascade [16, 23]. Polymorphisms in the complement factor H (CFH) gene has been extensively studied and the single nucleotide polymorphism (SNP) CFH rs1061170 has been shown to be strongly associated with AMD [24–28]. The polymorphism Age-Related Maculopathy susceptibility 2 (ARMS2) rs10490924 is also a major genetic risk factor for AMD, although the function of the ARMS2 protein remains largely unknown [24–27, 29].

This study aims to investigate the association between systemic complement protein concentrations and AMD stage (healthy controls, iAMD and nAMD), as well as the association between systemic Creg expression levels on mononuclear leukocytes (T cells and monocytes) and AMD stage. Because of previous findings of dysregulated complement proteins and Cregs in nAMD, we further sought to investigate the association of these proteins and treatment response in nAMD patients. Furthermore, we explored whether these complement proteins and Cregs were associated with the risk polymorphisms CFH rs1061170 and ARMS2 rs10490924 in nAMD patients. This may further our understanding of AMD pathophysiology and potentially reveal new therapeutic targets.

Methods

Study Design and participants

The Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study is a prospective cohort study investigating immunological profiles of patients with nAMD, iAMD and healthy controls. The study is a single-center study conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal no: SJ-768) and performed in adherence with the Declaration of Helsinki. Verbal and written informed consent were obtained from all participants prior to inclusion.

Treatment-naïve patients with nAMD, patients with iAMD and healthy controls were consecutively enrolled in this study. Exclusion criteria were age younger than 60 years, inflammatory, autoimmune, cancer and infectious diseases, use of immunomodulating treatment, active smoking, plasma C-reactive protein > 15 mg/L, vision-affecting disorders other than nAMD and iAMD, and previous treatment for nAMD.

Healthy controls and patients with iAMD were examined at baseline, while patients with nAMD were examined at baseline and two follow-up examinations. Patients with nAMD had diagnosis and disease severity evaluated at baseline, while progression was evaluated at follow-ups post-loading dose and after one year. All nAMD patients were treated according to the observe-and-plan regimen with aflibercept as per Danish national guidelines [30].

Clinical investigations and medical interview

All participants were examined by a retinal specialist for best corrected visual acuity (BCVA), slit-lamp biomicroscopy, color fundus photography, spectral domain optical coherence tomography (OCT), and OCT angiography. Diagnosis of nAMD and iAMD was based on multimodal imaging. Participants were interviewed regarding their medical history, medications and smoking habits, which was crosschecked with the electronic health records.

Table 1 Definitions of treatment responses in nAMD patients evaluated on optical coherence tomography scans

	Treatment response
Good	Total regression of IRF and SRF
Partial	Persistence of IRF and/or SRF and reduction of CRT
Poor	Persistence of IRF and/or SRF and unchanged or increased CRT

IRF: Intraretinal fluid

SRF: Subretinal fluid

CRT: Central retinal thickness

Grading disease and treatment response

Healthy controls were examined with the same thorough examination as patients to confirm they were indeed ophthalmologically normal. AMD stage was classified according to the Beckman criteria [31]. Eyes with presence of large drusen (diameter > 125 µm) or pigmentary abnormalities associated with at least medium drusen (> 65–125 µm) were classified as iAMD. Eyes with neovascularization and exudative changes on multimodal imaging were classified as nAMD.

OCT scans were graded to determine disease severity and treatment response in patients with nAMD. These scans were evaluated for presence of intra- and subretinal fluid, and central retinal thickness (CRT). Patients with nAMD were classified according to treatment response post-loading dose and after one year, according to criteria previously described [3, 4]. In brief, good response was classified as total regression of retinal fluid, partial response as persistence of retinal fluid and a reduction of CRT, and poor response as persistence of retinal fluid and unchanged or increased CRT (Table 1). The eye with nAMD was chosen as the study eye. In cases of bilateral nAMD, the right eye was chosen.

Blood sampling

Blood sampling and flow cytometry were performed at baseline. Blood was sampled from the antecubital vein in tubes coated with ethylenediamine-tetraacetic acid (EDTA) for flow cytometry and complement protein assays, as well as lithium-heparin with gel for plasma C-reactive protein.

Flow cytometry

Flow cytometry preparations were initiated within 4 h of phlebotomy. Leukocyte count was performed on Sysmex KX-21NTM (Sysmex Corporation, Kobe, Japan) to calculate blood volume sufficient to obtain 1.0×10^6 leukocytes for analysis. To lyse erythrocytes a 1% lysis buffer (BioLegend, San Diego, CA, USA) was added to the blood sample and stored at room temperature in the dark for 10 min. Cells were washed three times in a process of adding BD FACS Flow isotonic buffer, centrifugation at 500 × g for five minutes, followed by decantation of the supernatant. The isolated leukocytes were then resuspended in isotonic buffer and monoclonal fluorescent antibodies were added (Supplementary Table 1, Additional File 1) and incubated for 20 min at room temperature in the dark. The stained leukocytes were washed and resuspended in isotonic buffer a last time before being analyzed on the BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) with a gating size of 100.000 singlet cells analyzed per sample. The flow cytometry data was analyzed with FlowJo software (Tree Star, Ashland, OR, USA, v.10.10.0). Gating strategy

consisted of identifying lymphocytes and monocytes on a forward-side scatter plot, followed by singlet cells on a forward area-forward height scatter plot. Lymphocytes were gated for CD4 and CD8 to identify CD4+T cells and CD8+T cells. These cells were then gated for the surface membrane Cregs CD35, CD46, and CD59. Monocytes were gated for CD16 and CD14 to identify classical ($CD14^{high}CD16^{low}$), intermediate ($CD14^{high}CD16^{high}$) and non-classical ($CD14^{low}CD16^{high}$) monocytes. Monocytes and monocyte subgroups were gated for CD35, CD46, CD59 and CD11b (Fig. 1).

Cytokine assays

The plasma concentrations of complement proteins C3, C3a, and C5a were quantified with immunoassays. The EDTA coated tubes for the assays were centrifuged at 1500 × g for 15 min at 20 °C immediately after phlebotomy. The plasma was isolated, frozen at -80 °C within 1 h and analyzed a different day. The assays were performed with the commercially available electrochemiluminescence R-plex immunoassays (Mesoscale Discovery, Rockville, MD, USA). Blood samples were thawed and analyzed in duplicate according to manufacturer guidelines. The specific assays used, can be found in Supplementary Table 2, Additional File 1. The C3a/C3-ratio was calculated as a measure of complement activation [32].

Genotyping

Genotyping of the SNPs CFH rs1061170 and ARMS2 rs10490924 was performed on EDTA full blood from nAMD patients. These tubes were frozen at -80 °C immediately after phlebotomy and analyzed a different day.

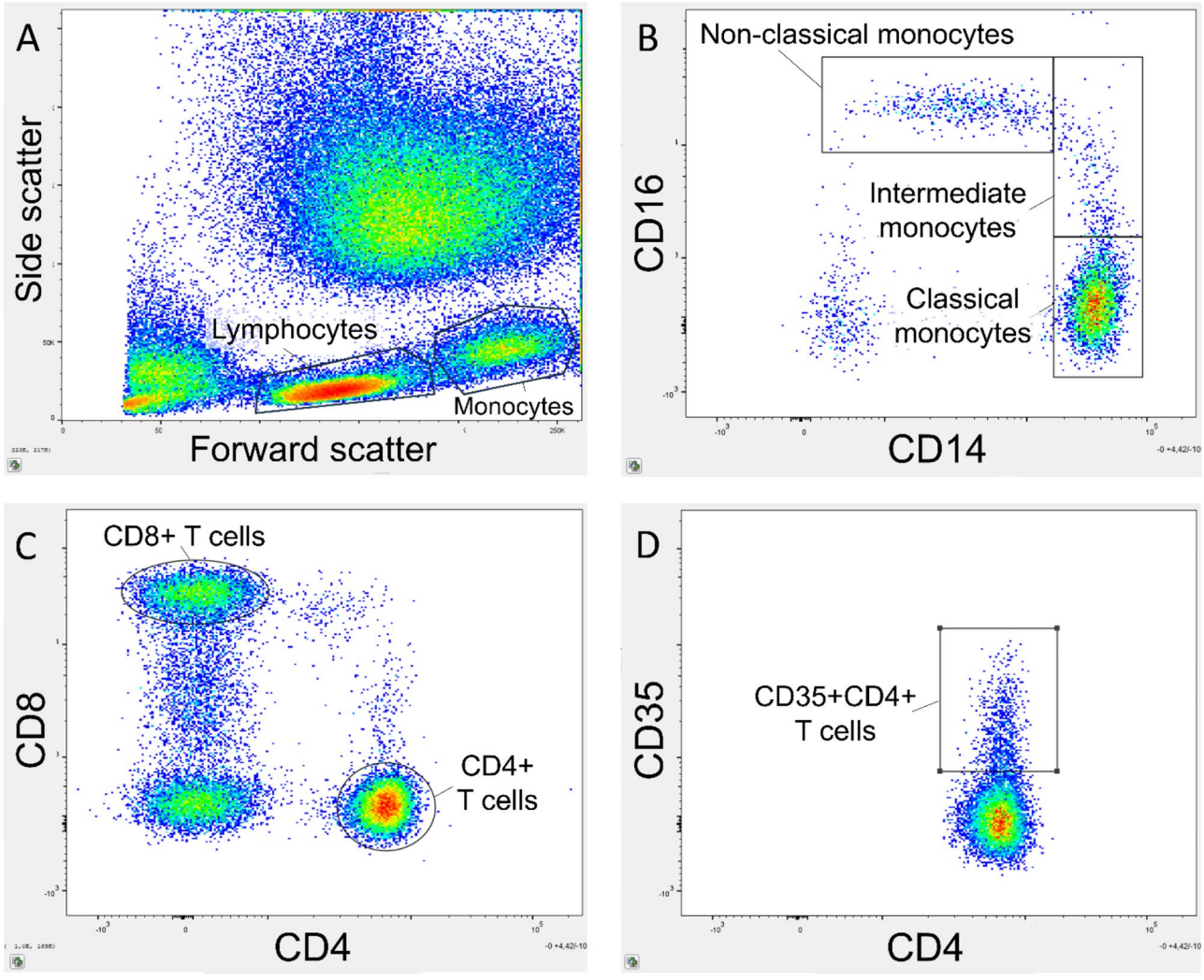


Fig. 1 Flow cytometry gating strategy. (**A**) Lymphocytes and monocytes were identified on the forward-side-scatter. (**B**) Monocyte subsets were identified as classical ($CD14^{high}CD16^{low}$), intermediate ($CD14^{high}CD16^{high}$), and non-classical ($CD14^{low}CD16^{high}$). (**C**) CD4+T cells and CD8+T cells were identified among the lymphocytes. (**D**) Complement regulatory proteins were gated on the leukocyte subgroups with Boolean sequences, in this example CD35 on CD4+T cells

Genomic DNA extraction and SNP analyses were performed by BioXpedia, Denmark. Using the Fluidigm GT192.24 Dynamic Array Integrated Fluidic Circuit (Fluidigm Corp., San Fransisco, CA, USA) according to the manufacturer's protocol. The data was analyzed with the Fluidigm SNP Genotyping analysis software v.4.5.1 with standard settings.

Statistics

Statistical analysis was performed with R software version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria). Normally distributed data is reported as mean and standard deviation (SD). Analysis of covariance (ANCOVA) was performed to evaluate the differences of complement proteins and Cregs according to diagnosis and treatment response groups. Healthy controls were chosen as reference group in the diagnosis analysis, and good responders as reference group in the treatment response analyses. All ANCOVA analyses were adjusted for age, as age-related changes of immunosenescence is a well described phenomenon [9], and smoking (never or previous smoker) as it has been shown that smoking increases systemic proinflammation [33]. Logarithmic transformation was applied as appropriate in cases of a positive skewness to fit assumption of normality. Correlation networks were created showing nodes, representing the complement proteins and Cregs, connected by edges (lines) of statistically significant correlations. The thickness of the edges indicates the absolute correlation coefficient with a threshold of >0.4. These networks can help visualize the complex correlations of the complement cascade and regulation. Association between genotypes and complement proteins, and Cregs were performed using Welch two sample t-test. A P value < 0.05 was interpreted as statistically significant. As the analyzed parameters are related, non-independent factors, a statistical adjustment for multiple testing might be too conservative and was not performed [34].

Table 2 Patient characteristics

	Diagnosis			
	Healthy Controls ($n=61$)	iAMD ($n=34$)	nAMD ($n=100$)	P value
Age, years, mean (SD)	73 (7)	75 (8)	80 (6)	< 0.001
Hypertension, n (%)	30 (49)	19 (56)	64 (64)	0.17
Hypercholesterolemia, n (%)	17 (28)	5 (15)	28 (28)	0.28
Cardiovascular disease, n (%)	20 (33)	8 (24)	37 (37)	0.37
Type 2 diabetes, n (%)	7 (12)	1 (3)	6 (6)	0.27
Smoking status, n (%)				
Never	27 (44)	17 (50)	38 (38)	0.43
Previous	34 (56)	17 (50)	62 (62)	

BCVA: Best corrected visual acuity

ETDRS: Early treatment of diabetic retinopathy

Results

Study Population

A total of 100 patients with nAMD, 34 patients with iAMD and 61 healthy controls were included. Healthy controls were significantly younger than nAMD patients, however age was adjusted for in subsequent analyses. There were no significant differences in other participant characteristics (Table 2).

Of the 100 nAMD patients, 94 completed the 1-year follow-up. Five participants died and one was excluded due to inability of following the treatment plan. The nAMD patients treated with anti-VEGF injections responded differently to treatment. The distribution of post-loading dose treatment response in patients with nAMD was 61 (65%) good, 26 (28%) partial, and 7 (7%) poor responders. The distribution of 1-year treatment response was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. Baseline median (IQR) visual acuity of initial treatment response was 64 (23), 62.5 (19.5), and 58 (13) ETDRS letters for good, partial and poor responders, respectively ($P=0.61$). Mean (SD) number of injections were 5.8 (1.4), 6.8 (1.9) and 6.3 (1.8) for good, partial and poor responders after one year ($P=0.14$).

Complement proteins and Cregs Association to diagnosis

The concentration of complement protein C3 differed significantly between healthy controls and patients with nAMD (Fig. 2A). The concentration of C3 in nAMD patients was 862 µg/ml (SD, 375), which was significantly higher than 656 µg/ml (SD, 262) in healthy controls ($P<0.001$). Concentrations of C3a also differed significantly between nAMD and iAMD compared to healthy controls. Patients with nAMD had a C3a concentration of 548 ng/ml (SD, 238) significantly higher than 401 ng/ml (SD, 100) of healthy controls ($P=0.002$). Likewise, iAMD patients had a significantly higher C3a concentration of 467 µg/ml (SD, 124) compared to healthy controls ($P=0.031$). There was no significant difference between C3 in healthy controls and iAMD patients, or between C5a between the treatment groups. The mean (SD) C3a/C3-ratio in nAMD patients was 0.078% (0.009%), which was significantly higher than 0.073% (0.006%) in healthy controls ($P=0.035$) (Fig. 3A).

The expression of the Creg CD46 was slightly, but significantly, decreased on CD4+T cells in nAMD patients compared to healthy controls. The proportion of CD46+CD4+T cells was 97.0% (SD, 1.9) in nAMD patients and 97.7% (SD, 1.0) in healthy controls ($P=0.018$). The proportion of CD59 on intermediate monocytes in nAMD patients was 26.8% (SD, 17.5), which was significantly lower than 34.2% (SD, 15.1) in healthy controls ($P=0.042$). Healthy controls did not differ significantly from iAMD or nAMD patients in Creg

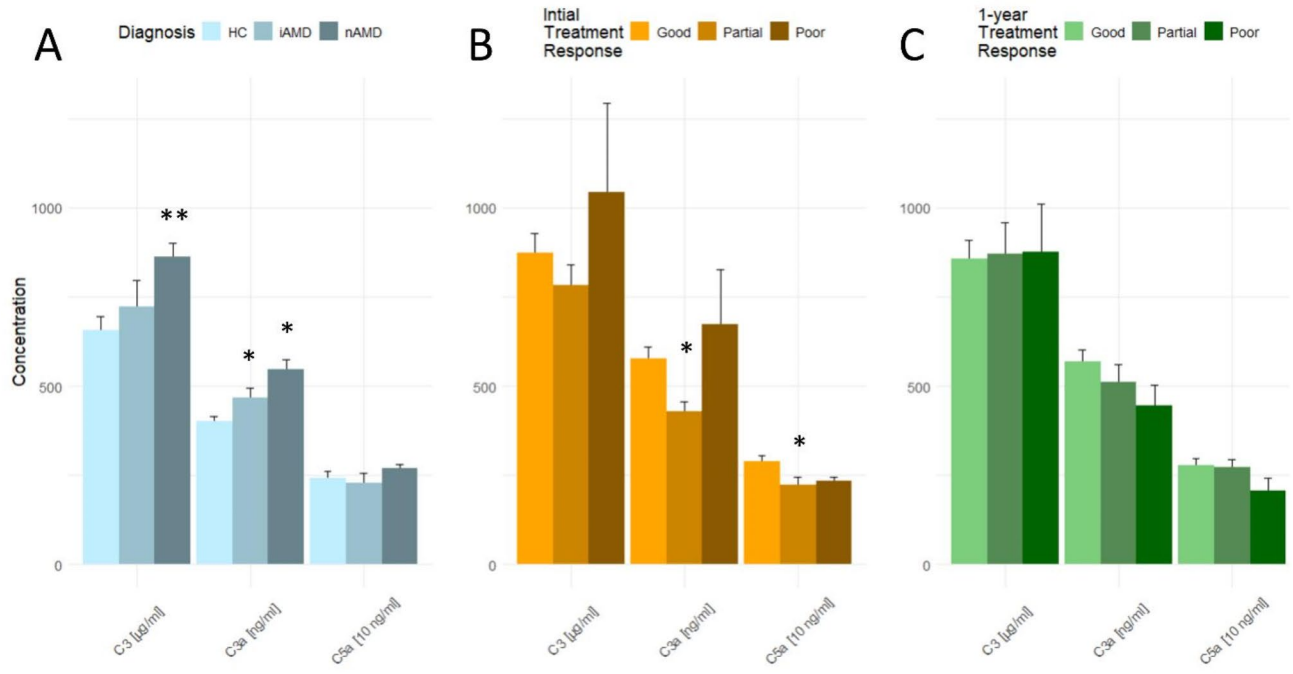


Fig. 2 Concentration of complement proteins according to (**A**) Diagnosis, (**B**) Initial treatment response of nAMD patients, (**C**) 1-year treatment response of nAMD patients. HC = healthy controls; iAMD = intermediate AMD; nAMD = neovascular AMD. * $P < 0.05$; ** $P < 0.001$ compared to reference group (healthy controls or good treatment response)

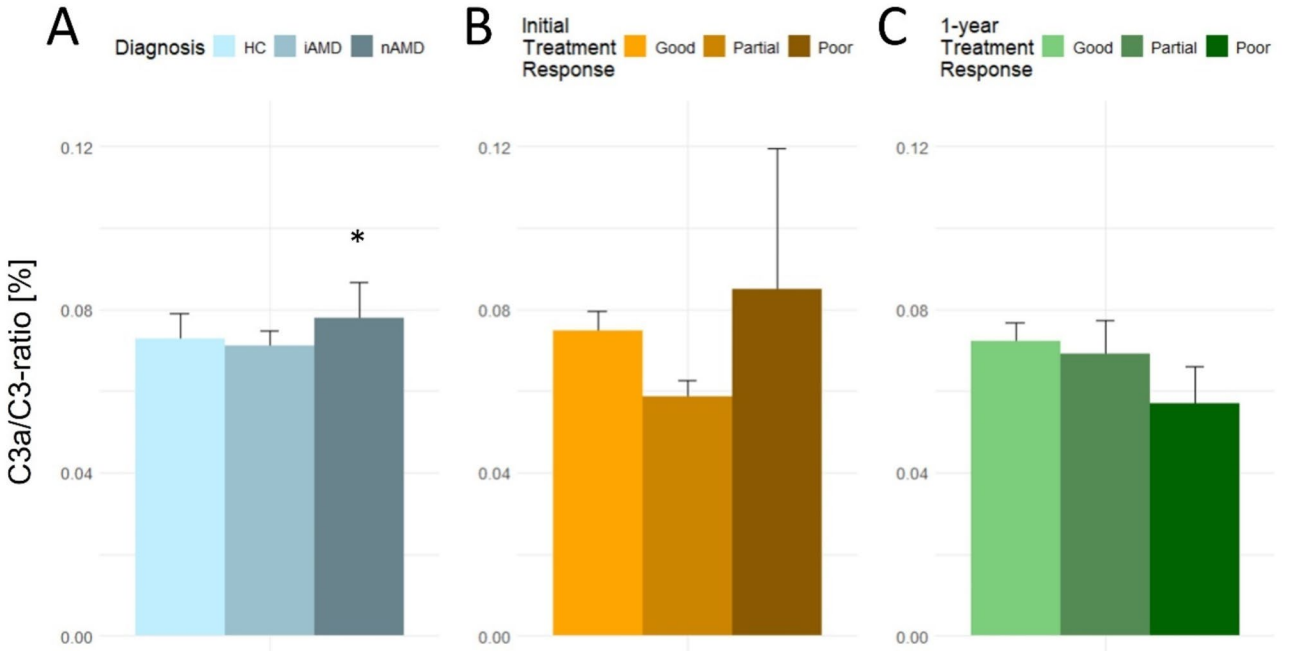


Fig. 3 Complement C3a/C3-ratio [%] according to (**A**) Diagnosis, (**B**) Initial treatment response of nAMD patients, (**C**) 1-year treatment response of nAMD patients. HC = healthy controls; iAMD = intermediate AMD; nAMD = neovascular AMD. * $P < 0.05$; ** $P < 0.001$ compared to reference group (healthy controls or good treatment response)

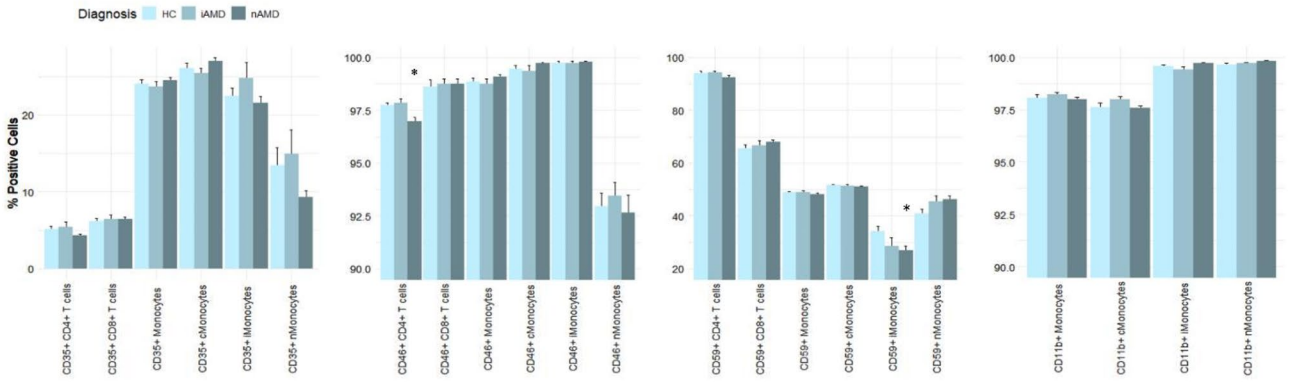


Fig. 4 Proportion of complement regulatory proteins according to diagnosis. HC = healthy controls; iAMD = intermediate AMD; nAMD = neovascular AMD; cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * $P < 0.05$; ** $P < 0.001$ compared to reference group (healthy controls)

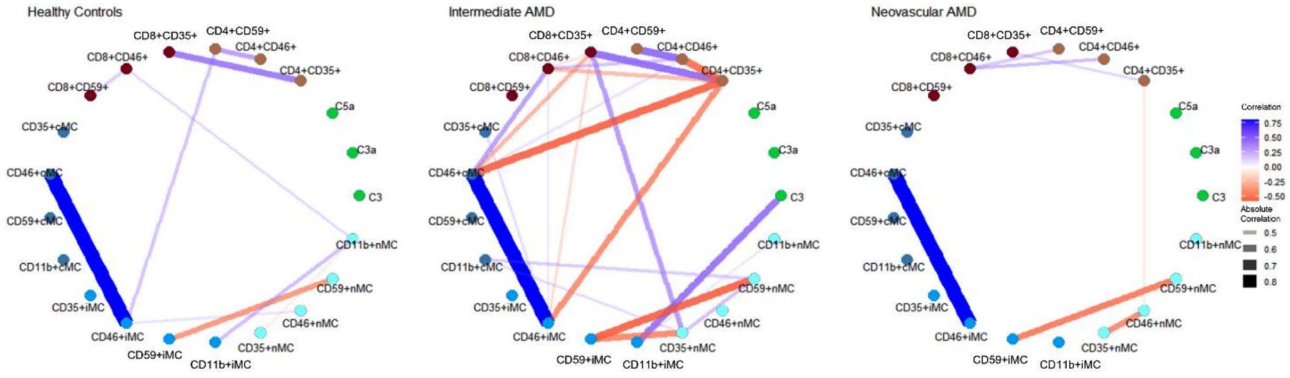


Fig. 5 Correlation network of complement proteins and complement regulatory proteins according to diagnosis, showing statistically significant correlations ($P < 0.05$) with a threshold of absolute correlation coefficient > 0.4

proportion of CD35, CD46, CD59 and CD11b on T cells or monocytes, other than the two aforementioned (Fig. 4).

The correlations of complement proteins and Cregs seems to differ between healthy controls, iAMD and nAMD, showing unique phenotypes of the complement and complement regulatory systems. Especially patients with iAMD seemed to have a more complex network of correlations between complement proteins and Cregs, while healthy controls and nAMD had fewer significant correlations (Fig. 5).

Complement proteins and Cregs Association to initial treatment response of nAMD patients

Concentrations of complement proteins C3a and C5a differed in nAMD patients' initial treatment response group. Patients with a partial initial treatment response had a C3a concentration of 429 ng/ml (SD, 127), which was significantly lower than the good initial treatment response with a concentration of 576 ng/ml (SD, 252) ($P=0.005$). Patients in the partial initial treatment response group also had a significantly lower concentration of C5a compared to good initial treatment response (mean (SD) concentration of 22.2 ng/ml (11.2) and 29.0 ng/ml (11.6), respectively, $P=0.010$). There was a trend toward higher concentrations of C3 and C3a in patients with poor initial treatment response, however not statistically significant. No significant difference was found between C5a concentrations of poor and good initial responders (Fig. 2B). No significant difference in C3a/C3-ratio was found between treatment response groups (Fig. 3B).

A tendency of a higher proportion of CD46+non-classical monocytes in poor responders appears in Fig. 6, however not significant ($P=0.46$). The expression levels of the Cregs CD35, CD46, CD59 and CD11b did not differ significantly between initial treatment response groups on CD4+T cells, CD8+T cells, monocytes or monocyte subgroups (Fig. 6).

The correlation between complement proteins and Cregs seemed to show intergroup differences between each initial treatment response group. Noticeably, complement proteins seemed to play a more central role in partial and poor responders compared to good responders. Partial responders have the most complex network with the most significant correlations (Fig. 7).

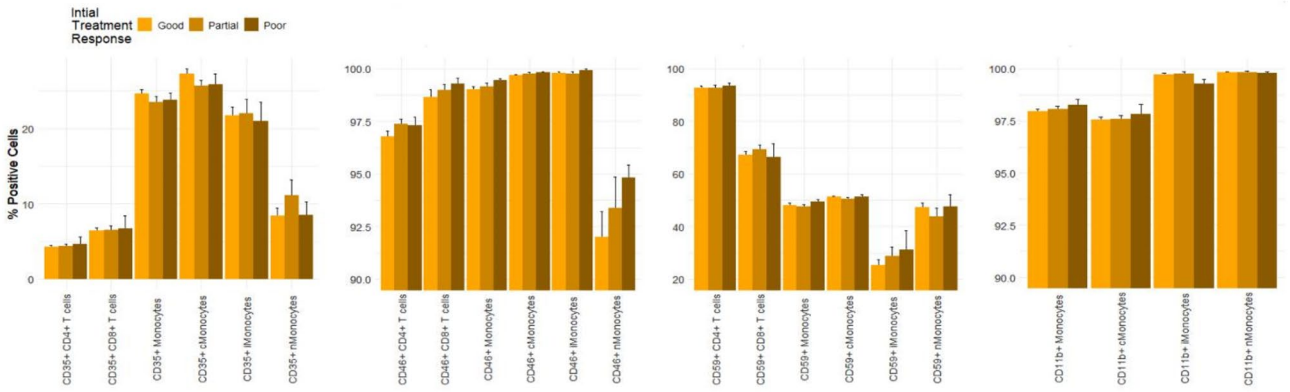


Fig. 6 Proportion of complement regulatory proteins according to initial treatment response in neovascular AMD patients. cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * $P < 0.05$; ** $P < 0.001$ compared to reference group (good treatment response)

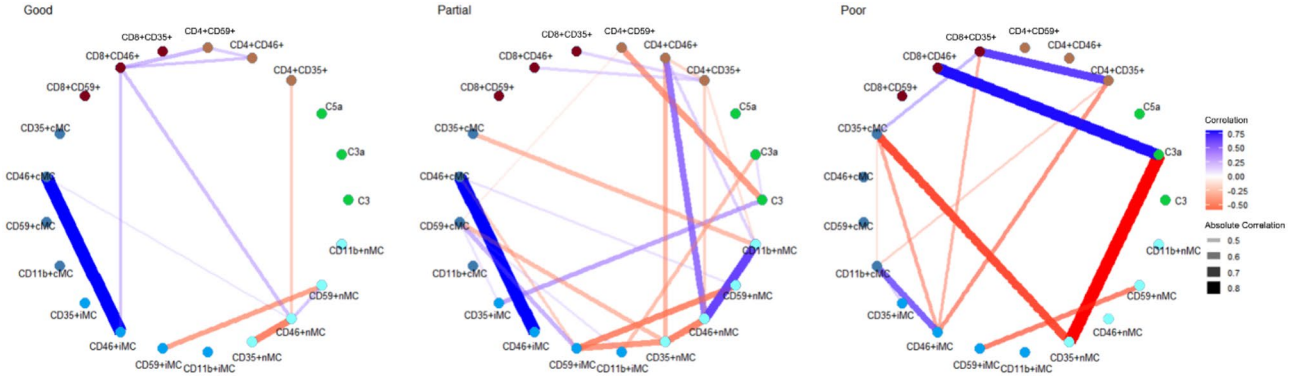


Fig. 7 Correlation network of complement proteins and complement regulatory proteins according to initial treatment response in neovascular AMD patients, showing statistically significant correlations ($P < 0.05$) with a threshold of absolute correlation coefficient > 0.4

Complement proteins and Cregs Association to 1-year treatment response of nAMD patients

Complement proteins and C3a/C3-ratio did not differ significantly between 1-year treatment groups (Figs. 2C and 3C).

Patients with partial 1-year treatment response differed significantly in proportion of CD35+monocytes and CD35+classical monocytes. The percentage of CD35+monocytes in the partial 1-year treatment response group was 23.2% (SD, 3.6), significantly lower than 25.1% (SD, 3.9) of good 1-year treatment response ($P = 0.039$). The percentage of CD35+classical monocytes in the partial 1-year treatment response group was 25.2% (SD, 4.0), significantly lower than 27.9% (SD, 4.9) in the good 1-year treatment response group ($P = 0.019$). There was no significant difference between poor and good 1-year responders in the proportions of CD35+monocytes or CD35+classical monocytes. Neither were there any significant differences between other Cregs in the 1-year treatment response groups (Fig. 8). There was a tendency of a lower proportion of CD46+non-classical monocytes in the partial response group, however not significant ($P = 0.29$).

The correlation networks seemed to differ uniquely between 1-year treatment response groups. The partial 1-year treatment response group was the simplest, while good and poor were more complex, in contrast to the correlation networks in the initial treatment response groups (Fig. 9).

Complement proteins and Cregs Association to genotypes in nAMD patients

Complement protein C3a and the C3a/C3-ratio were significantly elevated in nAMD patients carrying the high-risk CFH rs1061170 genotypes (Table 3). The proportion of CD35+CD8+T cells and CD46+classical monocytes were significantly elevated in high-risk genotypes, while the proportion of CD46+non-classical monocytes was significantly lower in the high-risk genotypes (Table 3). There were no significant differences in complement proteins or Cregs according to ARMS2 rs10490924 genotype (Table 4).

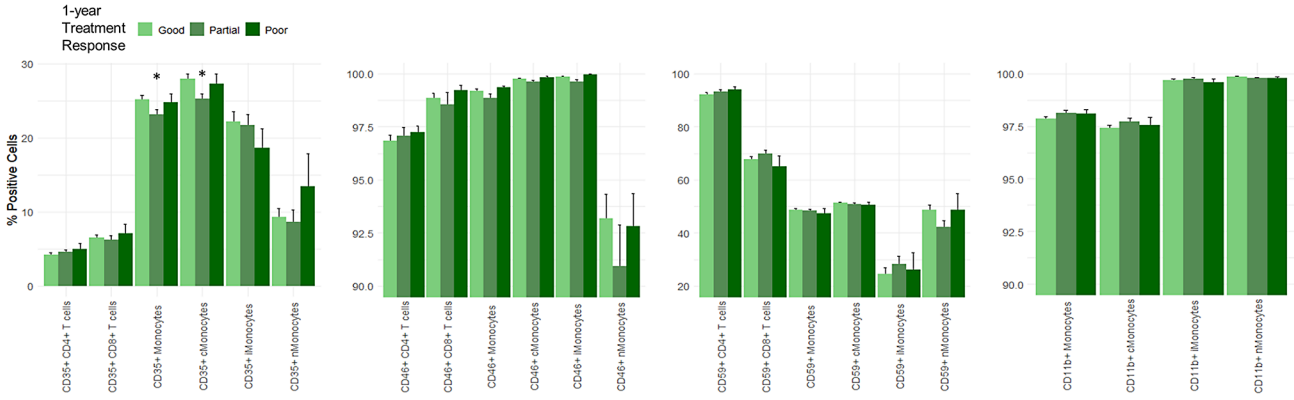


Fig. 8 Proportion of complement regulatory proteins according to 1-year treatment response in neovascular AMD patients. cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * $P < 0.05$; ** $P < 0.001$ compared to reference group (good treatment response)

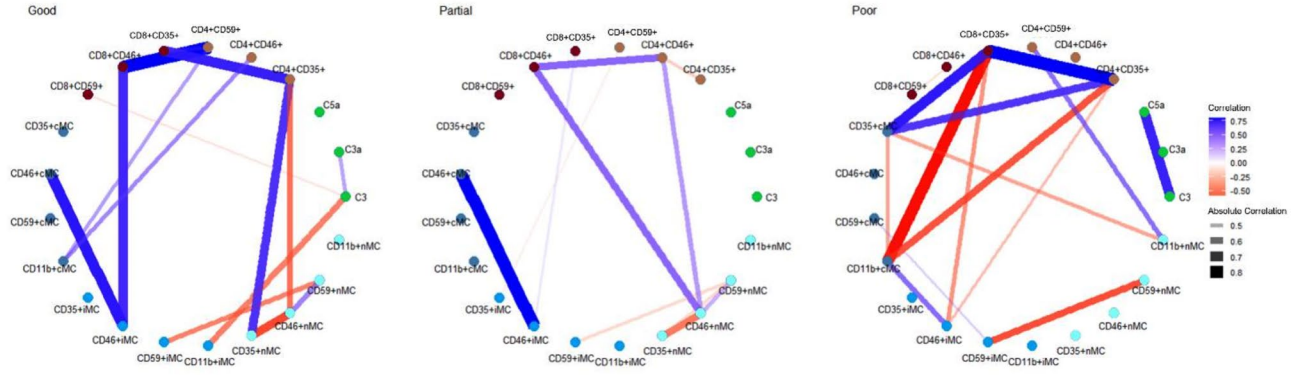


Fig. 9 Correlation network of complement proteins and complement regulatory proteins according to 1-year treatment response in neovascular AMD patients, showing statistically significant correlations ($P < 0.05$) with a threshold of absolute correlation coefficient > 0.4

Discussion

This prospective cohort study aimed to analyze differences in plasma concentration of complement proteins and proportions of membrane Cregs on T cells and monocytes in patients with nAMD and iAMD compared to healthy controls. The association between complement proteins, Cregs and treatment response in nAMD was also evaluated at post-loading dose and after one year, as well as genotypes of CFH and ARMS2 in nAMD patients.

In this study we found that circulating C3 was significantly elevated in nAMD and iAMD patients compared to healthy controls. C3a and the C3a/C3-ratio was also significantly elevated in nAMD patients compared to healthy controls. This suggests an elevated activation of the complement system in patients with AMD, and especially in nAMD patients. Elevated levels of C3 and C3a are associated with increased systemic inflammation by facilitating opsonization, leading to phagocytosis, recruitment of inflammatory cells and antibodies [35, 36]. We did not find C5a to be elevated in iAMD og nAMD patients. A previous study investigating C3 and C5a levels in nAMD patients found a significantly elevated concentration of C5a compared to healthy controls, but did not find any difference in C3 concentrations [24]. Another study investigating C3a levels found patients with nAMD and dry AMD, including both intermediate AMD and geographic atrophy, had significantly elevated levels of C3a compared to a control group [13]. In studies not differentiating between AMD phenotypes compared to healthy controls, C3a and C5a in AMD patients was found significantly increased, while C3 was non-significantly different in the AMD group in one study [11], and C5a significantly increased, while C3 and C3a were non-significantly different in another study [10]. These differing results might be attributed to the different complement measurement protocols. Increased complement activation has also been found in patients with geographic atrophy [25, 37, 38]. The C3 inhibitor pegcetacoplan and C5 inhibitor avacincaptad pegol has been FDA-approved as they are shown to significantly halt the atrophy progression in patients with geographic atrophy secondary to AMD. These drugs are administered intravitreally, and a systemic treatment has yet to be approved [39, 40], but indicates that the complement

Table 3 Complement proteins and complement regulatory proteins stratified according to CFH rs1061170 genotype

	CFH, rs1061170		
Complement proteins, mean (SD)	**CC/CT (high risk), *n* = 82**	**TT (low risk), *n* = 18**	**P value**
C3 [µg/ml]	876 (398)	800 (243)	0.58
C3a [ng/ml]	573 (251)	435 (111)	**0.0072**
C5a [ng/ml]	27.09 (11.07)	26.10 (14.65)	0.60
C3a/C3-ratio [%]	0.074 (0.037)	0.058 (0.018)	**0.011**
Complement regulatory proteins, mean (SD)			
CD35 + CD4 + T cells [%]	4.47 (1.83)	3.60 (1.05)	0.071
CD35 + CD8 + T cells [%]	6.75 (2.98)	4.96 (3.58)	**0.024**
CD35 + Monocytes [%]	24.26 (3.82)	25.60 (4.15)	0.27
CD35 + Classical monocytes [%]	26.54 (4.47)	29.41 (5.32)	0.077
CD35 + Intermediate monocytes [%]	22.01 (9.04)	19.16 (6.78)	0.28
CD35 + Non-classical monocytes [%]	9.74 (8.26)	6.67 (4.28)	0.27
CD46 + CD4 + T cells [%]	96.94 (2.00)	97.19 (1.72)	0.61
CD46 + CD8 + T cells [%]	98.82 (2.30)	98.43 (2.68)	0.60
CD46 + Monocytes [%]	99.12 (0.96)	99.01 (0.53)	0.52
CD46 + Classical monocytes [%]	99.76 (0.26)	99.54 (0.38)	**0.043**
CD46 + Intermediate monocytes [%]	99.87 (0.26)	99.49 (0.70)	0.050
CD46 + Non-classical monocytes [%]	92.21 (9.03)	94.76 (3.20)	**0.043**
CD59 + CD4 + T cells [%]	92.70 (5.34)	91.99 (7.15)	0.68
CD59 + CD8 + T cells [%]	67.05 (8.76)	71.10 (10.27)	0.16
CD59 + Monocytes [%]	48.09 (5.29)	48.77 (1.85)	0.27
CD59 + Classical monocytes [%]	51.12 (2.53)	51.43 (2.12)	0.59
CD59 + Intermediate monocytes [%]	26.95 (17.91)	26.19 (16.65)	0.98
CD59 + Non-classical monocytes [%]	45.92 (14.43)	48.46 (14.47)	0.28
CD11b + Monocytes [%]	98.04 (0.68)	97.81 (0.94)	0.36
CD11b + Classical monocytes [%]	97.63 (0.84)	97.31 (1.26)	0.33
CD11b + Intermediate monocytes [%]	99.69 (0.48)	99.84 (0.25)	0.065
CD11b + Non-classical monocytes [%]	99.83 (0.24)	99.88 (0.14)	0.19

Bold values indicate statistical significance ($P < 0.05$)

Table 4 Complement proteins and complement regulatory proteins stratified according to ARMS2 rs10490924 genotype

	ARMS2, rs10490924		
Complement proteins, mean (SD)	**TT/TG (high risk), *n* = 45**	**GG (low risk), *n* = 55**	**P value**
C3 [µg/ml]	837 (409)	893 (330)	0.32
C3a [ng/ml]	509 (216)	597 (257)	0.057
C5a [ng/ml]	26.38 (12.16)	27.58 (11.25)	0.62
C3a/C3-ratio [%]	0.067 (0.028)	0.076 (0.041)	0.30
Complement regulatory proteins, mean (SD)			
CD35 + CD4 + T cells [%]	4.15 (1.39)	4.54 (2.11)	0.51
CD35 + CD8 + T cells [%]	6.28 (3.12)	6.66 (3.18)	0.46
CD35 + Monocytes [%]	26.22 (3.64)	24.80 (4.19)	0.54
CD35 + Classical monocytes [%]	26.65 (4.40)	27.47 (5.08)	0.46
CD35 + Intermediate monocytes [%]	20.86 (8.11)	22.37 (9.48)	0.47
CD35 + Non-classical monocytes [%]	7.08 (5.96)	7.71 (6.91)	0.62
CD46 + CD4 + T cells [%]	97.03 (1.78)	96.93 (2.17)	0.81
CD46 + CD8 + T cells [%]	98.84 (1.14)	98.66 (1.33)	0.71
CD46 + Monocytes [%]	99.17 (0.63)	99.02 (0.98)	0.41
CD46 + Classical monocytes [%]	99.75 (0.22)	99.69 (0.27)	0.28
CD46 + Intermediate monocytes [%]	99.82 (0.18)	99.78 (0.12)	0.60
CD46 + Non-classical monocytes [%]	92.92 (5.97)	92.29 (7.71)	0.59
CD59 + CD4 + T cells [%]	93.22 (4.14)	91.77 (7.09)	0.21
CD59 + CD8 + T cells [%]	68.12 (9.32)	67.26 (8.90)	0.70
CD59 + Monocytes [%]	48.46 (3.24)	47.90 (6.39)	0.47
CD59 + Classical monocytes [%]	50.96 (2.47)	51.44 (2.43)	0.35
CD59 + Intermediate monocytes [%]	24.80 (17.34)	29.33 (17.86)	0.071
CD59 + Non-classical monocytes [%]	49.06 (13.46)	42.99 (14.95)	0.22
CD11b + Monocytes [%]	98.03 (0.68)	97.97 (0.80)	0.66
CD11b + Classical monocytes [%]	97.62 (0.89)	97.53 (0.97)	0.65
CD11b + Intermediate monocytes [%]	99.65 (0.34)	99.79 (0.21)	0.11
CD11b + Non-classical monocytes [%]	99.82 (0.22)	99.86 (0.23)	0.42

system might be an important target for AMD in general. The elevated C3a/C3-ratio in nAMD patients suggests increased activation of the complement system, as C3 is converted to C3a by the C3 convertase upon activation [16]. Other ratios between a C3 activation fragment and C3 can be used as an estimation of complement activation. Multiple studies have found an association between C3d/C3-ratio and AMD [28, 41] and specifically nAMD [24], which is comparable to the findings of this current study. The role of C3 in nAMD has been demonstrated in C3 knockdown mice, that did not develop neovascularizations after laser photocoagulation [42], and local C3a increase on inducing neovascularization after laser photocoagulation in mice [43]. C3 has also been found in histological specimens of choroidal neovascularizations in human nAMD patients [44].

The proportion of CD46+CD4+T cells and CD59+intermediate monocytes were significantly decreased in nAMD patients compared to healthy

controls. CD46 has an important role as an inactivator of the central complement fragments C3b and C4b [45]. The decreased proportion of CD46+CD4+T cells in nAMD patients could cause a dysregulation of the complement cascade, as these patients will have an increased activation of the complement system, including C3 and C3a, leading to phagocytosis and inflammation [46]. Although the difference was slight, AMD is a disease characterized by chronic low-grade inflammation, and these slight changes present during many years might be part of the cause [47–50]. CD46+CD4+T cells have also been shown to be important in regulation of inflammation, producing the anti-inflammatory cytokine interleukin-10 [51]. CD59 inhibits the activation of MAC and nAMD patients with decreased CD59+intermediate monocytes might thus have an increased activation of MAC, causing cell lysis [52]. Intermediate monocytes play an important proinflammatory role, although not yet fully elucidated [53]. This dysregulation of the complement system and the increased complement proteins systemically could cause increased inflammation manifested in the retina [9]. Retinal inflammation leads to tissue damage causing the development of drusen and degeneration of the blood-retinal-barrier consisting of Bruch's membrane, the retinal pigment epithelium (RPE) and microvascular endothelium. With this degeneration, oxygenation of the retina might be compromised and the rescue mechanism of macular neovascularization can come into effect [54, 55]. Also, the transportation of waste products might be compromised causing debris to accumulate, forming drusen [56]. The complement proteins and Cregs C3, C3a, C5a, CD35 and CD46 have been found in drusen, while CD59+RPE cells was found reduced overlying drusen in immunohistochemical analyses [57–60]. A previous study also found that the proportion of CD46+and CD59+leukocytes were lower in nAMD patients compared to healthy controls [18]. We were not able to replicate the findings of Haas et al., who reported that patients with nAMD had a significantly higher proportion of CD35+leukocytes compared to healthy controls [19]. Neither did we find increased proportions of CD11b+monocytes in nAMD patients compared to healthy controls reported by Subhi et al. [17]. This may be due to differences in the flow cytometry protocol and gating strategy of the blood samples.

To our knowledge, this is the first study to investigate complement proteins and Cregs in association to treatment response in nAMD. Since the introduction of intraocular anti-VEGF injections, the prevalence of blindness caused by nAMD has significantly decreased. A clinical challenge is however, that many patients respond partially or poorly to this treatment, the reason being largely unknown. We hypothesized that the neovascularization in these patients is mediated by a different signal than ocular VEGF, which might be a dysregulated complement system. Thus, we expected the concentrations of systemic complement proteins to be increased and Creg expression levels to be decreased in patients with partial and poor treatment response compared to good responders. Surprisingly, in this study we find that systemic C3a and C5a were significantly decreased at baseline in nAMD patients with a partial initial (post-loading dose) treatment response compared to a good response. This might suggest an alternative mechanism, possibly indicating dysregulation and subsequent depletion of complement proteins. This depletion could impair the immune system's ability to manage inflammation and repair tissue effectively, contributing to the persistence and progression of nAMD despite treatment.

We did however find nAMD patients with partial 1-year treatment response had lower proportion of CD35+classical monocytes and total monocytes at baseline compared to good responders, in agreement with our hypothesis. CD35 inhibits the complement cascade by removing opsonized antigens [61]. Thus, patients responding partially to anti-VEFG after one year might have long lasting dysregulation of the complement system causing inflammatory changes in the retina. As systemic low-grade age-related inflammation is known to cause inflammatory tissue damage in multiple diseases, like Alzheimer's disease, chronic kidney disease, cardiovascular diseases, and diabetes mellitus [9, 62, 63], so can chronic activation and dysregulation of the complement system cause retinal damage leading to AMD [64]. Patients responding partially to treatment, with altered CD35 expression, might have more low-grade inflammation causing a proinflammatory milieu in the retina, thus not responding ideally [65]. As the statistical tests are adjusted for age, the low-grade inflammation might be a sign of immunosenescence and biological aging, which can be caused by genetics and environmental factors [2, 9, 66–68].

Correlation networks show the phenotypically different interactions between complement proteins and Cregs between healthy controls, iAMD and nAMD patients. Especially iAMD patients seem to have a more complex network than healthy controls and nAMD patients. This might be because iAMD patients are a more heterogeneous group. Patients with iAMD have a 27% risk of developing late-stage AMD within 5 years, while a large proportion never develops late AMD [69]. In this group, there might thus be patients with the same immunological phenotype as nAMD patients that have not yet developed nAMD, as well as geographic atrophy, which might have yet another unique correlation network. Part of the iAMD group might also have a specific relationship not similar to late-stage profiles. For initial treatment response in nAMD patients, it seems that complement

proteins play a more central role in poor and partial responders. Furthermore, the partial group seems to have the most complex correlation network, which like iAMD might be caused by being a mixture of potential good and poor late responders. Complement proteins seemed to play a more central role in partial and poor responders compared to good responders. As the correlation networks suggest after the 1-year treatment response, the partial group seems to have simplified, as they may be settled in their final treatment response group after one year, while good and poor 1-year networks have become more complex, as a result of less extreme phenotypes, that takes longer to settle in treatment outcomes. This is similar to a study investigating the correlation networks in nAMD treatment response of chemokine receptors [70].

Genetic susceptibility plays a major role in development of AMD. The two main risk SNPs are the CFH rs1061170 and ARMS2 rs10490924 [24–29]. CFH acts as an important regulator of the complement system controlling the alternative pathway and accelerating the decay of this pathways C3 convertase [66, 71]. Decreased levels of CFH will lead to failure to downregulate the spontaneous activation of C3 [72]. ARMS2 polymorphisms has been suggested to be involved in the activation of the complement system with a genetic interaction between CFH and ARMS2 in AMD patients [24], although the exact function of the ARMS2 protein is yet to be determined [29]. We find that the concentration of C3a was elevated in nAMD patients carrying the high-risk CFH rs1061170 genotype. The C3a/C3-ratio was also elevated in these high-risk carriers, which is in agreement with the previous studies, that find the C3d/C3-ratio was associated with the high-risk alleles of this CFH SNP [24, 41]. Thus, the increased complement activation of nAMD patients could be explained by the CFH genotype associated with nAMD development, which is also demonstrated previously [11, 73]. In this study, we found that a decreased expression level of CD46+non-classical monocytes in nAMD patients with the high-risk CFH genotypes, corresponding to the increased complement activation. Surprisingly, we found increased expression levels of CD46+classical monocytes and CD35+CD8+T cells which would suggest a higher complement regulation in these patients. The regulatory properties of these particular cell types might be complex and interlinked [65]. We did not find any association between ARMS2 genotype and complement proteins or Cregs. A previous study did not find an association between C3 or C3a and ARMS2 but did find an association with C5a in a population of AMD patients and healthy controls [10].

As nAMD patients respond differently to treatment, planning an individualized treatment strategy might improve visual outcomes for these patients. Predicting the response is essential in such a planning process, and determining the individual profile of circulating complement proteins and Cregs might be useful in such predictions. Furthermore, the complement system and Cregs may possibly be targets for new therapeutics in addition to the current intraocular anti-VEGF treatment. It might be beneficial for patients with iAMD and high risk of developing late AMD [74] to be treated before occurrence of these vision impairing states. C3 could potentially be a target for this treatment. However, the elevated complement proteins and decreased Creg levels were associated with partial treatment response within the nAMD group, and a complement inhibitory treatment might cause potential good responders to respond partially. Patients with iAMD treated this way might also be at risk of becoming partial responders. The dysregulation of the complement system is complex and influenced by genetic factors in patients with AMD.

Limitations of this study include the observational study design, which precludes any definitive conclusions about causality. Furthermore, the relatively small number of nAMD patients with poor response might have hidden significant correlations. The categorical nature of the treatment response classification is a limitation, as especially the partial response group includes all patients with decreased CRT and persistent retinal fluid. Thus, patients with major quantities of retinal fluid at baseline, that have persistence of minor cysts will still be in the partial group. There was a significant age difference between the groups, leading to all ANCOVA analyses being adjusted for age, although age-matched groups would have been more ideal. The EDTA coated tubes for immunoassays were centrifuged at 20 °C, rather than 4 °C, which might have led to some complement activation [75].

In conclusion, patients with nAMD and iAMD have elevated levels of complement proteins, and nAMD have decreased levels of Cregs compared to healthy controls. Patients with nAMD, who respond partially to anti-VEGF treatment have a dysregulation of the complement system and Cregs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-024-03273-7.

Supplementary Material 1

Acknowledgements

We thank Charlotte Vibeke Thorndahl for laboratory assistance.

Author contributions

AKT, TLS, MHN, MAS, BH and HV conceptualized the study. AKT led the data collection and data analysis with inputs and help from TLS, MHN, MAS and ATN. AKT led the interpretation of data and wrote the first draft of the manuscript. All named authors contributed to the critical revision of the manuscript. AKT and TLS obtained funding with help from MAS, MHN, HV and

BH. TLS and MHN led study supervision. All named authors read and approved the final manuscript.

Funding
This work was supported by the Velux Foundation (00024226), Region Zealand PhD Grant (R29-A1337), Synoptik Foundation, Fight for Sight Denmark (Øjenforeningen), Grosserer L.F. Foghts Foundation, and Lizzi and Mogens Staal Foundation. The funding organizations had no role in the design or conduct of this research.
Open access funding provided by Copenhagen University

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal no: SJ-768) and performed in adherence with the Declaration of Helsinki. Verbal and written informed consent were obtained from all participants prior to inclusion.

Competing interests
BH and HV have obtained research funding from Bayer to a study not related to the present. All other authors declare no financial or non-financial conflicts of interest.

Author details
[1]Department of Ophthalmology, Zealand University Hospital, Sygehusvej 10, Roskilde 4000, Denmark
[2]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
[3]Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark
[4]Department of Clinical Medicine, Aalborg University Hospital, Aalborg, Denmark
[5]Department of Ophthalmology, Aalborg University Hospital, Aalborg, Denmark
[6]Department of Biomedicine, Aarhus University, Aarhus, Denmark

Received: 30 July 2024 / Accepted: 23 October 2024
Published online: 01 November 2024

References

1. Wong WL, Su X, Li X, Cheung CMG, Klein R, Cheng C-Y, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. Lancet Glob Health. 2014;2:e106–16. https://doi.org/10.1016/S2214-109X(13)70145-1.
2. Fleckenstein M, Keenan TDL, Guymer RH, Chakravarthy U, Schmitz-Valckenberg S, Klaver CC, et al. Age-related macular degeneration. Nat Rev Dis Primer. 2021;7:31. https://doi.org/10.1038/s41572-021-00265-2.
3. Amoaku WM, Chakravarthy U, Gale R, Gavin M, Ghanchi F, Gibson J, et al. Defining response to anti-VEGF therapies in neovascular AMD. Eye. 2015;29:721–31. https://doi.org/10.1038/eye.2015.48.
4. Thomsen AK, Krogh Nielsen M, Liisborg C, Sørensen T. Interleukin-8 promoter polymorphism – 251 A/T and Treatment Response in Neovascular Age-related Macular Degeneration. Clin Ophthalmol 2024;Volume 18:537–43. https://doi.org/10.2147/OPTH.S448794
5. Andriessen EM, Wilson AM, Mawambo G, Dejda A, Miloudi K, Sennlaub F, et al. Gut microbiota influences pathological angiogenesis in obesity-driven choroidal neovascularization. EMBO Mol Med. 2016;8:1366–79. https://doi.org/10.15252/emmm.201606531.
6. SanGiovanni JP, SanGiovanni PM, Sapieha P, De Guire V. miRNAs, single nucleotide polymorphisms (SNPs) and age-related macular degeneration (AMD). Clin Chem Lab Med CCLM 2017;55. https://doi.org/10.1515/cclm-2016-0898
7. Hata M, Hata M, Andriessen EMMA, Juneau R, Pilon F, Crespo-Garcia S, et al. Early-life peripheral infections reprogram retinal microglia and aggravate neovascular age-related macular degeneration in later life. J Clin Invest. 2023;133:e159757. https://doi.org/10.1172/JCI159757.
8. Hata M, Andriessen EMMA, Hata M, Diaz-Marin R, Fournier F, Crespo-Garcia S, et al. Past history of obesity triggers persistent epigenetic changes in innate immunity and exacerbates neuroinflammation. Science. 2023;379:45–62. https://doi.org/10.1126/science.abj8894.
9. Rozing MP, Durhuus JA, Krogh Nielsen M, Subhi Y, Kirkwood TBL, Westendorp RGJ, et al. Age-related macular degeneration: a two-level model hypothesis. Prog Retin Eye Res. 2020;76:100825. https://doi.org/10.1016/j.preteyeres.2019.100825.
10. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma Complement Components and activation fragments: associations with Age-Related Macular degeneration genotypes and phenotypes. Investig Opthalmology Vis Sci. 2009;50:5818. https://doi.org/10.1167/iovs.09-3928.
11. Scholl HPN, Issa PC, Walier M, Janzer S, Pollok-Kopp B, Börncke F, et al. Systemic complement activation in Age-Related Macular Degeneration. PLoS ONE. 2008;3:e2593. https://doi.org/10.1371/journal.pone.0002593.
12. Heesterbeek TJ, Lechanteur YTE, Lorés-Motta L, Schick T, Daha MR, Altay L, et al. Complement activation levels are related to Disease Stage in AMD. Investig Opthalmology Vis Sci. 2020;61:18. https://doi.org/10.1167/iovs.61.3.18.
13. Machalińska A, Dziedziejko V, Mozolewska-Piotrowska K, Karczewicz D, Wiszniewska B, Machaliński B. Elevated plasma levels of C3a complement compound in the Exudative form of age-related Macular Degeneration. Ophthalmic Res. 2009;42:54–9. https://doi.org/10.1159/000219686.
14. Karlstetter M, Kopatz J, Aslanidis A, Shahraz A, Caramoy A, Linnartz-Gerlach B, et al. Polysialic acid blocks mononuclear phagocyte reactivity, inhibits complement activation, and protects from vascular damage in the retina. EMBO Mol Med. 2017;9:154–66. https://doi.org/10.15252/emmm.201606627.
15. Schick T, Steinhauer M, Aslanidis A, Altay L, Karlstetter M, Langmann T, et al. Local complement activation in aqueous humor in patients with age-related macular degeneration. Eye. 2017;31:810–3. https://doi.org/10.1038/eye.2016.328.
16. Armento A, Ueffing M, Clark SJ. The complement system in age-related macular degeneration. Cell Mol Life Sci. 2021;78:4487–505. https://doi.org/10.1007/s00018-021-03796-9.
17. Subhi Y, Krogh Nielsen M, Molbech CR, Oishi A, Singh A, Nissen MH, et al. CD11b and CD200 on circulating monocytes differentiate two angiographic subtypes of Polypoidal Choroidal Vasculopathy. Investig Opthalmology Vis Sci. 2017;58:5242. https://doi.org/10.1167/iovs.17-22479.
18. Singh A, Faber C, Falk M, Nissen MH, Hviid TVF, Sørensen TL. Altered expression of CD46 and CD59 on leukocytes in Neovascular Age-Related Macular Degeneration. Am J Ophthalmol. 2012;154:193–e1992. https://doi.org/10.1016/j.ajo.2012.01.036.
19. Haas P, Aggermann T, Nagl M, Steindl-Kuscher K, Krugluger W, Binder S. Implication of CD21, CD35, and CD55 in the pathogenesis of age-related Macular Degeneration. Am J Ophthalmol. 2011;152:396–e3991. https://doi.org/10.1016/j.ajo.2011.02.017.
20. Kim DD, Song W-C. Membrane complement regulatory proteins. Clin Immunol. 2006;118:127–36. https://doi.org/10.1016/j.clim.2005.10.014.
21. Singh A, Falk MK, Hviid TVF, Sørensen TL. Increased expression of CD200 on circulating CD11b + monocytes in patients with neovascular age-related Macular Degeneration. Ophthalmology. 2013;120:1029–37. https://doi.org/10.1016/j.ophtha.2012.11.002.
22. Subhi Y, Krogh Nielsen M, Molbech CR, Krüger Falk M, Singh A, Hviid TVF, et al. Association of CD11b $^{+}$ monocytes and anti–vascular endothelial growth factor injections in treatment of Neovascular Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy. JAMA Ophthalmol. 2019;137:515. https://doi.org/10.1001/jamaophthalmol.2019.0010.
23. Seddon JM, The, US Twin Study of Age-Related Macular Degeneration. Relative roles of genetic and environmental influences. Arch Ophthalmol. 2005;123:321. https://doi.org/10.1001/archopht.123.3.321.
24. Smailhodzic D, Klaver CCW, Klevering BJ, Boon CJF, Groenewoud JMM, Kirchhof B, et al. Risk alleles in CFH and ARMS2 are independently Associated with systemic complement activation in Age-related Macular Degeneration. Ophthalmology. 2012;119:339–46. https://doi.org/10.1016/j.ophtha.2011.07.056.
25. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, 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. 2005;102:7227–32. https://doi.org/10.1073/pnas.0501536102.
26. Klein RJ, Zeiss C, Chew EY, Tsai J-Y, Sackler RS, Haynes C, et al. Complement factor H polymorphism in Age-Related Macular Degeneration. Science. 2005;308:385–9. https://doi.org/10.1126/science.1109557.

27. Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, et al. Complement factor H variant increases the risk of age-related Macular Degeneration. Science. 2005;308:419–21. https://doi.org/10.1126/science.1110359.
28. Lorés-Motta L, Paun CC, Corominas J, Pauper M, Geerlings MJ, Altay L, et al. Genome-Wide Association Study Reveals Variants in CFH and CFHR4 Associated with systemic complement activation. Ophthalmology. 2018;125:1064–74. https://doi.org/10.1016/j.ophtha.2017.12.023.
29. Kozhevnikova OS, Fursova AZh, Derbeneva AS, Nikulich IF, Tarasov MS, Devyatkin VA, et al. Association between polymorphisms in CFH, ARMS2, CFI, and C3 genes and response to Anti-VEGF treatment in Neovascular Age-Related Macular Degeneration. Biomedicines. 2022;10:1658. https://doi.org/10.3390/biomedicines10071658.
30. https://medicinraadet.dk/anbefalinger-og-vejledninger/behandlingsvejledninger-og-laegemiddelrekommandationer/vad-amd-aldersrelateret-makuladegeneration n.d
31. Ferris FL, Wilkinson CP, Bird A, Chakravarthy U, Chew E, Csaky K, et al. Clinical classification of Age-related Macular Degeneration. Ophthalmology. 2013;120:844–51. https://doi.org/10.1016/j.ophtha.2012.10.036.
32. Nürnberger W, Bhakdi S. Plasma C3d/C3 quotient as a parameter for in vivo complement activation. J Immunol Methods. 1984;74:87–91. https://doi.org/10.1016/0022-1759(84)90370-3.
33. Krogh Nielsen M, Hector SM, Allen K, Subhi Y, Sørensen TL. Altered activation state of circulating neutrophils in patients with neovascular age-related macular degeneration. Immun Ageing. 2017;14:18. https://doi.org/10.1186/s12979-017-0100-9.
34. Perneger TV. What's wrong with Bonferroni adjustments. BMJ. 1998;316:1236–8. https://doi.org/10.1136/bmj.316.7139.1236.
35. Wu M, Jia B, Li M. Complement C3 and activated fragment C3a are involved in complement activation and anti-bacterial immunity. Front Immunol. 2022;13:813173. https://doi.org/10.3389/fimmu.2022.813173.
36. Schartz ND, Tenner AJ. The good, the bad, and the opportunities of the complement system in neurodegenerative disease. J Neuroinflammation. 2020;17:354. https://doi.org/10.1186/s12974-020-02024-8.
37. Xu H, Chen M. Targeting the complement system for the management of retinal inflammatory and degenerative diseases. Eur J Pharmacol. 2016;787:94–104. https://doi.org/10.1016/j.ejphar.2016.03.001.
38. Desai D, Dugel PU. Complement cascade inhibition in geographic atrophy: a review. Eye. 2022;36:294–302. https://doi.org/10.1038/s41433-021-01765-x.
39. Heier JS, Lad EM, Holz FG, Rosenfeld PJ, Guymer RH, Boyer D, et al. Pegcetacoplan for the treatment of geographic atrophy secondary to age-related macular degeneration (OAKS and DERBY): two multicentre, randomised, double-masked, sham-controlled, phase 3 trials. Lancet. 2023;402:1434–48. https://doi.org/10.1016/S0140-6736(23)01520-9.
40. Jaffe GJ, Westby K, Csaky KG, Monés J, Pearlman JA, Patel SS, et al. C5 inhibitor Avacincaptad Pegol for Geographic Atrophy due to age-related Macular Degeneration. Ophthalmology. 2021;128:576–86. https://doi.org/10.1016/j.ophtha.2020.08.027.
41. Ristau T, Paun C, Ersoy L, Hahn M, Lechanteur Y, Hoyng C, et al. Impact of the Common Genetic associations of Age-Related Macular Degeneration upon systemic complement component C3d levels. PLoS ONE. 2014;9:e93459. https://doi.org/10.1371/journal.pone.0093459.
42. Bora PS, Sohn J-H, Cruz JMC, Jha P, Nishihori H, Wang Y, et al. Role of complement and complement membrane Attack Complex in Laser-Induced Choroidal Neovascularization. J Immunol. 2005;174:491–7. https://doi.org/10.4049/jimmunol.174.1.491.
43. Nozaki M, Raisler BJ, Sakurai E, Sarma JV, Barnum SR, Lambris JD, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci. 2006;103:2328–33. https://doi.org/10.1073/pnas.0408835103.
44. Lommatzsch A, Hermans P, Weber B, Pauleikhoff D. Complement factor H variant Y402H and basal laminar deposits in exudative age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol. 2007;245:1713–6. https://doi.org/10.1007/s00417-007-0649-7.
45. Riley-Vargas RC, Gill DB, Kemper C, Liszewski MK, Atkinson JP. CD46: expanding beyond complement regulation. Trends Immunol. 2004;25:496–503. https://doi.org/10.1016/j.it.2004.07.004.
46. Kirschfink M. Controlling the complement system in inflammation. Immunopharmacology. 1997;38:51–62. https://doi.org/10.1016/S0162-3109(97)00057-X.
47. Donoso LA, Kim D, Frost A, Callahan A, Hageman G. The role of inflammation in the Pathogenesis of Age-related Macular Degeneration. Surv Ophthalmol. 2006;51:137–52. https://doi.org/10.1016/j.survophthal.2005.12.001.
48. Behnke V, Wolf A, Langmann T. The role of lymphocytes and phagocytes in age-related macular degeneration (AMD). Cell Mol Life Sci. 2020;77:781–8. https://doi.org/10.1007/s00018-019-03419-4.
49. Akhtar-Schäfer I, Wang L, Krohne TU, Xu H, Langmann T. Modulation of three key innate immune pathways for the most common retinal degenerative diseases. EMBO Mol Med. 2018;10:e8259. https://doi.org/10.15252/emmm.201708259.
50. Martinez Villarruel Hinnerskov J, Krogh Nielsen M, Kai Thomsen A, Steffensen MA, Honoré B, Vorum H, et al. Chemokine Receptor Profile of T Cells and Progression Rate of Geographic Atrophy Secondary to age-related Macular Degeneration. Investig Opthalmology Vis Sci. 2024;65:5. https://doi.org/10.1167/iovs.65.1.5.
51. Astier AL. T-cell regulation by CD46 and its relevance in multiple sclerosis. Immunology. 2008;124:149–54. https://doi.org/10.1111/j.1365-2567.2008.02821.x.
52. Couves EC, Gardner S, Voisin TB, Bickel JK, Stansfeld PJ, Tate EW, et al. Structural basis for membrane attack complex inhibition by CD59. Nat Commun. 2023;14:890. https://doi.org/10.1038/s41467-023-36441-z.
53. Kapellos TS, Bonaguro L, Gemünd I, Reusch N, Saglam A, Hinkley ER, et al. Human monocyte subsets and phenotypes in Major Chronic Inflammatory diseases. Front Immunol. 2019;10:2035. https://doi.org/10.3389/fimmu.2019.02035.
54. Grossniklaus HE, Green WR. Choroidal neovascularization. Am J Ophthalmol. 2004;137:496–503. https://doi.org/10.1016/j.ajo.2003.09.042.
55. Lückoff A, Caramoy A, Scholz R, Prinz M, Kalinke U, Langmann T. Interferon-beta signaling in retinal mononuclear phagocytes attenuates pathological neovascularization. EMBO Mol Med. 2016;8:670–8. https://doi.org/10.15252/emmm.201505994.
56. Zarbin MA. Age-related Macular Degeneration: review of Pathogenesis. Eur J Ophthalmol. 1998;8:199–206. https://doi.org/10.1177/112067219800800401.
57. Nita M, Grzybowski A, Ascaso FJ, Huerva V. Age-related Macular Degeneration in the aspect of chronic low-Grade inflammation (Pathophysiological ParaInflammation). Mediators Inflamm. 2014;2014:1–10. https://doi.org/10.1155/2014/930671.
58. Ebrahimi KB, Fijalkowski N, Cano M, Handa JT. Decreased membrane complement regulators in the retinal pigmented epithelium contributes to age-related macular degeneration. J Pathol. 2013;229:729–42. https://doi.org/10.1002/path.4128.
59. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002;134:411–31. https://doi.org/10.1016/S0002-9394(02)01624-0.
60. Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, et al. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc Natl Acad Sci. 2002;99:14682–7. https://doi.org/10.1073/pnas.222551899.
61. Oliveira LC, Kretzschmar GC, Dos Santos ACM, Camargo CM, Nisihara RM, Farias TDJ, et al. Complement receptor 1 (CR1, CD35) Polymorphisms and soluble CR1: a proposed anti-inflammatory role to quench the fire of Fogo Selvagem Pemphigus Foliaceus. Front Immunol. 2019;10:2585. https://doi.org/10.3389/fimmu.2019.02585.
62. Boren E, Gershwin ME. Inflamm-aging: autoimmunity, and the immune-risk phenotype. Autoimmun Rev. 2004;3:401–6. https://doi.org/10.1016/j.autrev.2004.03.004.
63. Ferrucci L, Fabbri E. Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat Rev Cardiol. 2018;15:505–22. https://doi.org/10.1038/s41569-018-0064-2.
64. Chen J, Wang W, Li Q. Increased Th1/Th17 responses contribute to low-Grade inflammation in Age-Related Macular Degeneration. Cell Physiol Biochem. 2017;44:357–67. https://doi.org/10.1159/000484907.
65. Liisborg C, Skov V, Kjær L, Hasselbalch HC, Sørensen TL. Patients with MPNs and retinal drusen show signs of complement system dysregulation and a high degree of chronic low-grade inflammation. eClinicalMedicine. 2022;43:101248. https://doi.org/10.1016/j.eclinm.2021.101248.
66. Zipfel PF, Lauer N, Skerka C. The role of complement in AMD. In: Lambris JD, Adamis AP, editors. Inflamm. Retin. Dis. Complement Biol. Pathol. Volume 703. New York, NY: Springer New York; 2010. pp. 9–24. https://doi.org/10.1007/978-1-4419-5635-4_2.
67. Micklisch S, Lin Y, Jacob S, Karlstetter M, Dannhausen K, Dasari P, et al. Age-related macular degeneration associated polymorphism rs10490924 in ARMS2 results in deficiency of a complement activator. J Neuroinflammation. 2017;14:4. https://doi.org/10.1186/s12974-016-0776-3.

68. Ersoy L, Ristau T, Hahn M, Karlstetter M, Langmann T, Dröge K, et al. Genetic and environmental risk factors for age-related Macular Degeneration in persons 90 years and older. Investig Opthalmology Vis Sci. 2014;55:1842. https://doi.org/10.1167/iovs.13-13420.
69. Randomized A, 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. https://doi.org/10.1001/archopht.119.10.1417.
70. Bjerregaard T, Krogh Nielsen M, Molbech CR, Subhi Y, Sørensen TL. Treatment failure in neovascular age-related macular degeneration is associated with a complex chemokine receptor profile. BMJ Open Ophthalmol. 2019;4:e000307. https://doi.org/10.1136/bmjophth-2019-000307.
71. Khandhadia S, Cipriani V, Yates JRW, Lotery AJ. Age-related macular degeneration and the complement system. Immunobiology. 2012;217:127–46. https://doi.org/10.1016/j.imbio.2011.07.019.
72. Rodriguezdecordoba S, Esparzagordillo J, Goicoecheadejorge E, Lopeztrascasa M, Sanchezcorral P. The human complement factor H: functional roles, genetic variations and disease associations. Mol Immunol. 2004;41:355–67. https://doi.org/10.1016/j.molimm.2004.02.005.
73. Hecker LA, Edwards AO, Ryu E, Tosakulwong N, Baratz KH, Brown WL, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. Hum Mol Genet. 2010;19:209–15. https://doi.org/10.1093/hmg/ddp472.
74. Thomsen AK, Gøttsche LF, Hinnerskov JMV, Falk MK, Sørensen TL. Microperimetry and Structural Risk factors on OCT in Intermediate Age-Related Macular Degeneration. Ophthalmol Retina 2024:S2468653024000885. https://doi.org/10.1016/j.oret.2024.02.016
75. Brandwijk RJMGE, Michels MAHM, Van Rossum M, De Nooijer AH, Nilsson PH, De Bruin WCC, et al. Pitfalls in complement analysis: a systematic literature review of assessing complement activation. Front Immunol. 2022;13:1007102. https://doi.org/10.3389/fimmu.2022.1007102.

Publisher's note

Additional File 1

Supplementary Table 1. Flow Cytometry Antibodies.

	CD4	**CD8**	**CD14**	**CD16**	**CD35**	**CD46**	**CD59**
Fluorochrome	PerCP	Brilliant Violet 510	Pacific Blue	APC/Cy7	APC	PE/Cy7	FITC
Manufacturer	R&D Systems	BioLegend	BioLegend	BioLegend	R&D Systems	BioLegend	BioLegend
Catalog number	FAB3791C-100	301048	325616	302018	FAB5748A	352408	304706
APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein. R&D Systems, Minneapolis, MN, USA. Biolegend, San Diego, CA, USA.							

Supplementary table 2. Immunoassays for complement components.

	C3	**C3a**	**C5a**
Immunoassay	R-plex Human Complement C3 Assay	R-plex Human Complement C3a Assay	R-plex Human Complement C5a Assay
Catalog number	K151XYR-2	K151V0R-2	K151K4R-2
Manufacturer: Mesoscale Discovery, Rockville, MD, USA			

7.2. Study II

Aging and Disease
www.aginganddisease.org
Early access date: April 9, 2025
http://dx.doi.org/10.14336/AD.2024.1585

Original Article

Plasma Interferon-gamma is Associated with Poor Treatment Response in Neovascular Age-Related Macular Degeneration

Alexander Kai Thomsen[1,2*], Maria Abildgaard Steffensen[3], Jenni Martinez Villarruel Hinnerskov[1,2], Amalie Thomsen Nielsen[1,2], Henrik Vorum[4,5], Bent Honoré[4,6], Mogens Holst Nissen[2,3], Torben Lykke Sørensen[1,2]

[1]Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark. [2]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark. [3]Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark. [4]Department of Clinical Medicine, Aalborg University, Aalborg, Denmark. [5]Department of Ophthalmology, Aalborg University Hospital, Aalborg, Denmark. [6]Department of Biomedicine, Aarhus University, Aarhus, Denmark.

[Received November 30, 2024; Revised February 19, 2025; Accepted February 21, 2025]

ABSTRACT: Age-related macular degeneration (AMD) is a leading cause of visual impairment and blindness in the elderly. Aging is the most important risk factor for AMD, and the aging immune system seems to be involved in pathogenesis. This study investigates the systemic aging immune profile in relation to AMD stage and treatment response. Treatment-naïve patients with neovascular AMD (nAMD), intermediate AMD and healthy controls were included in this prospective study. Participants were examined for systemic aging immune profiles and compared to AMD stage, as well as initial and one-year treatment response in nAMD patients. Flowcytometry was performed to determine T cell differentiation (naïve, central memory and effector memory) and expression of costimulatory markers (CD27, CD28, CD56). Cytokine assays were performed to measure the concentrations of plasma cytokines IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17, IL-22, IL-27, TNF-α. Polymorphisms of CFH and ARMS2 genes were compared in nAMD patients. Patients with nAMD had significantly higher proportions of central and effector memory CD8+ T cells compared to controls (both $P < 0.036$). nAMD patients had significantly elevated concentrations of IFN-γ, IL-1β, IL-2, and IL-10 (all $P < 0.05$). nAMD patients with poor initial treatment response had a significantly higher concentration of plasma IFN-γ compared to good responders ($P = 0.026$). Patients with nAMD had a more advanced systemic aging immune profile with higher levels of T cell differentiation and plasma cytokines compared to controls. Poor initial response had elevated levels of plasma IFN-γ compared to good responders in nAMD.

Key words: age-related macular degeneration, immunosenescence, T cells, cytokines, treatment response, systemic inflammation

INTRODUCTION

Age-related macular degeneration (AMD), the most common cause of irreversible vision loss and blindness, is a chronic, multifactorial disease with a pathophysiology driven by aging, genetic susceptibility and environmental factors [1–4]. The onset of AMD can vary greatly but will usually occur after age 50, with the incidence increasing exponentially with increasing age [5]. One late stage is the development of exudative neovascularizations secondary to AMD, where the key driver is vascular endothelial growth factor (VEGF). Neovascular AMD (nAMD) is

***Correspondence should be addressed to:** Dr. Alexander Kai Thomsen, MD, Department of Ophthalmology, Zealand University Hospital, Sygehusvej 10, 4000 Roskilde, Denmark; e-mail: alext@regionsjaelland.dk.

treated with repeated intravitreal anti-VEGF injections, which since its introduction has reduced the incidence of blindness caused by AMD greatly [6]. However, treatment response in nAMD patients can vary considerably with a significant proportion of patients experiencing deterioration of visual function regardless of anti-VEGF treatment, and these patients might benefit from additional treatment acting on an additional target [7]. Preceding late-stage AMD is intermediate AMD (iAMD), which is characterized by macular drusen and pigmentary abnormalities. Patients with iAMD are at increased risk of developing late-stage AMD, but far from all do [8, 9].

The reason for the varying onset of AMD, stage of AMD, and treatment response of nAMD might lie in the interplay of aging, genetics and environmental factors affecting the individual's biological aging. An important factor in biological aging is aging of the immune system characterized by dysfunction and dysregulation of the innate and adaptive immune system [10]. Aging of the immune system is associated with chronic low-grade systemic inflammation, including a remodeling of the T cell differentiation profile, a central component in adaptive immunity. The number of naïve T cells decreases with age as they differentiate into central memory and effector memory T cells. Hallmarks of aging T cell differentiation include loss of the costimulatory receptors CD27 and CD28 [11, 12], as well as upregulation of cytotoxic activity, evident by the increase of the activation marker CD56 [12–14]. Dysregulation of aging T cells can cause tissue damage and production of proinflammatory cytokines. Cytokines play an essential role in inflammation, by leading to recruitment and activation of cytotoxic and phagocytic leukocytes, as well as activating the complement cascade. Especially interferon (IFN)-γ and interleukin (IL)-1β has potent proinflammatory properties and dysregulation of these cytokines might lead to destruction of healthy tissue [15]. This chronic inflammation might cause a dysregulated response in the retina secondary to local accumulation of cellular damage, which could lead to the degeneration and neovascularization present in AMD [12, 16–20].

Genetic susceptibility plays an important role in AMD and treatment response. Two important genetic variations related to increased AMD risk are the complement factor H (CFH) rs1061170 single nucleotide polymorphism (SNP) and the Age-Related Maculopathy susceptibility 2 (ARMS2) rs10490924 SNP [3, 21–24].

Our group has previously found increased levels of effector memory T cells in patients with iAMD and nAMD [12], and increased proportions of CD27-, CD28- and CD56+ T cells in nAMD patients [19, 25]. Furthermore, our and other groups have shown a shift in systemic cytokine profiles, characterized by increased levels of proinflammatory cytokines in AMD patients compared to healthy controls [16, 18, 20, 26, 27]. Our group has also found associations between increased systemic inflammation and treatment response in nAMD [28].

Based on these previous findings, we investigate the degree of immune system aging in AMD subtypes by comparing the systemic profiles of circulating T cell differentiation and proinflammatory cytokines in nAMD and iAMD patients compared to healthy controls. Furthermore, we study the association between these profiles and treatment response to anti-VEGF in patients with nAMD. This might contribute to the understanding of the pathogenesis and reveal novel treatment targets of AMD.

MATERIALS AND METHODS

Study Design and Participants

The Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study is a prospective cohort study investigating systemic immune phenotypes of patients with AMD of different subtypes. The study is conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark as a single-center study approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal no: SJ-768) and performed in adherence with the Declaration of Helsinki. Verbal and written informed consent were obtained from all participants prior to inclusion.

Details of the design of the DANEART study have been reported previously [28]. In brief, treatment-naïve nAMD patients, iAMD patients and healthy controls were included. Exclusion criteria were age < 60 years, inflammatory, autoimmune, cancer and infectious diseases, use of immunomodulating treatment, active smoking, plasma C-reactive protein (CRP) > 15 mg/L, vision-affecting disorders other than nAMD and iAMD, and previous treatment for nAMD.

All participants were examined at baseline. Patients with nAMD were treated according to Danish national guidelines with a loading dose consisting of three intravitreal anti-VEGF (aflibercept, 2 mg) injections with a month's interval and continued with an individualized treatment plan following the observe-and-plan regimen [29]. Patients with nAMD were examined at the time of diagnosis (baseline), after loading-dose to determine the initial treatment response, and after one year to determine the maintained treatment response. The examination consisted of best corrected visual acuity, slit-lamp biomicroscopy as well as multimodal imaging with color

fundus photography, spectral domain optical coherence tomography (OCT), and OCT angiography.

AMD subtype was classified according to the Beckman criteria [30], and treatment response in nAMD patients was graded based on retinal fluid and central retinal thickness (CRT) on OCT. Good responders had total regression of intra- and subretinal fluid, partial responders had persistence of fluid and decreased CRT, and poor responders had persistence of fluid and unchanged or increased CRT [31, 32] (Fig. 1).

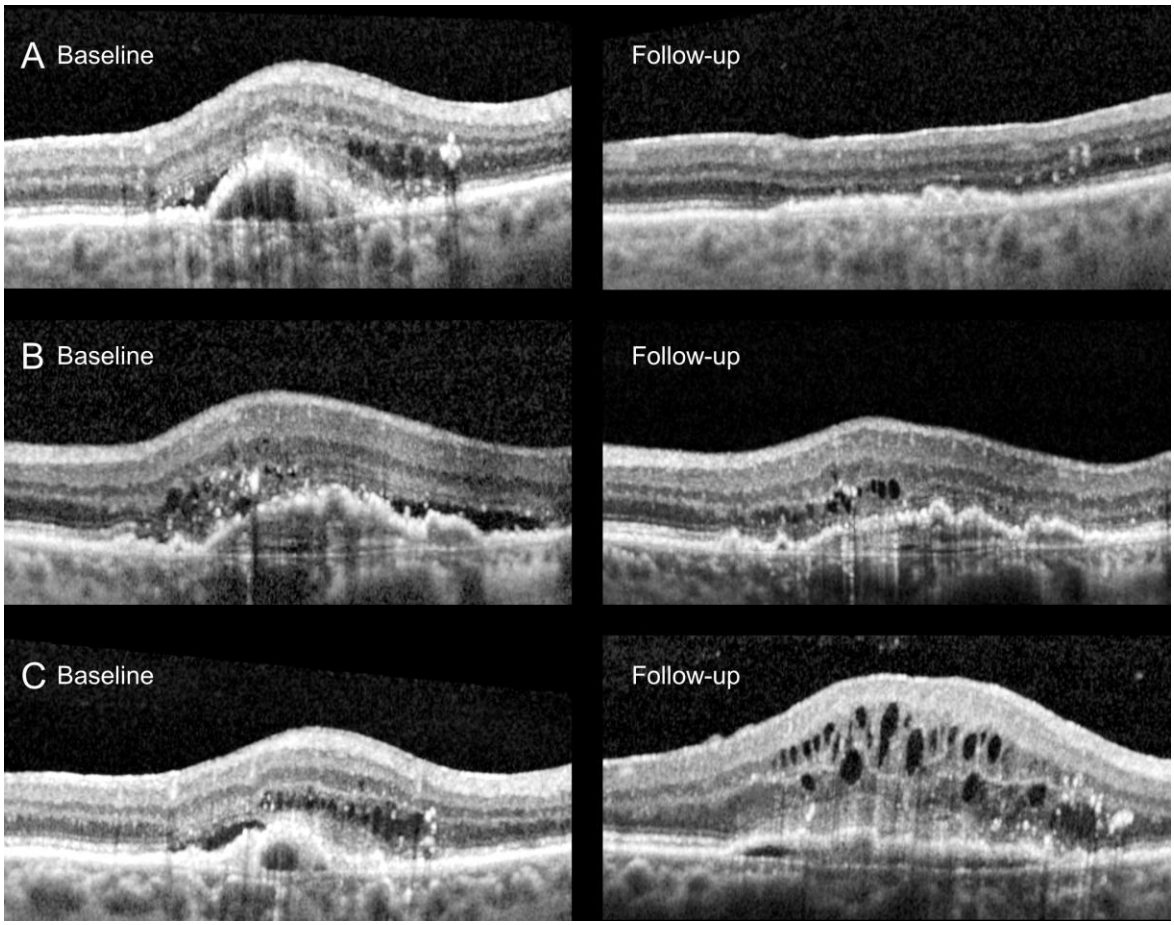


Figure 1. **Examples of treatment response grading in neovascular age-related macular degeneration (nAMD).** At baseline retinal fluid is present in all nAMD patients as intraretinal cysts or subretinal fluid detaching the neuroretina from the retinal pigment epithelium. **(A)** Good response with absence of retinal fluid at follow-up, **(B)** Partial response with persistence of retinal fluid and thinning of central retinal thickness (CRT), and **(C)** poor response, with persistence of retinal fluid and thickening of CRT.

Flow cytometry

To identify the T cell differentiation profile and costimulatory markers at baseline, peripheral blood was sampled to be analyzed with flow cytometry. Blood was sampled from the antecutibal vein in ethylenediamine tetraacetic acid (EDTA) coated tubes and lithium-heparin coated tubes for quantification of plasma CRP concentration. Details of the flow cytometry protocol can be found in Supplementary table 1. Each blood sample was prepared and stained with a panel of monoclonal fluorescent antibodies using fluorescein isothiocyanate (FITC) CD4 (Abcam, cat.no. ab59474), peridinin-chlorophyll-protein (PerCP) CD8 (Biolegend, cat.no. 300922), Brilliant Violet 510 CCR7 (Biolegend, cat.no. 353232), Pacific Blue CD45RA (Biolegend, cat.no. 304123), phycoerythrin-cyanine7 (PE/Cy7) CD45RO (Biolegend, cat.no. 304230), phycoerythrin (PE) CD27 (Biolegend, cat.no. 356406), allophycocyanin (APC) CD28 (Biolegend, cat.no. 302912), and allophycocyanin-cyanine 7 (APC-Cy7) CD56 (Biolegend, cat.no. 300926). Flow cytometry was performed on the BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) with a gating size of 100,000 singlet cells. FlowJo analytical software (Tree Star, Ashland, OR, USA, v.10.10.0) was used for the flow cytometric analyses. Gating strategy

consisted of identifying lymphocytes on a forward-side scatter plot, followed by gating singlet cells identified on a forward area-forward height scatter plot. Lymphocytes were gated for CD4 and CD8 to identify CD4+ and CD8+ T cells, respectively. T cells were gated for CD45RA, CD45RO and CCR7 to classify the T cell differentiation profile. T cells were classified as naïve (CD45RA+ CD45RO-CCR7+), central memory (CD45RA-CD45RO+CCR7+), and effector memory (CD45RA-CD45RO+CCR7-) [11, 12, 19, 33]. T cells were also gated for the costimulatory markers CD27, CD28 and CD56 (Fig. 2). An example of the gating strategy can be found in Figure 3.

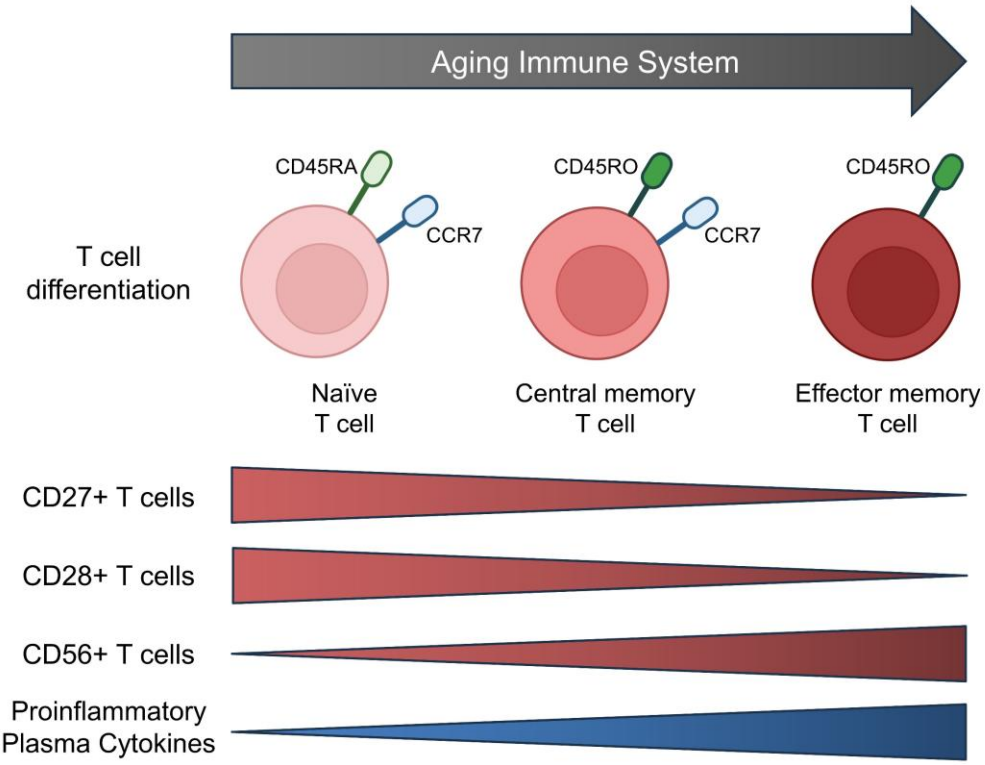


Figure 2. The aging immune system determined by T cell differentiation, expression of costimulatory markers on T cells, and plasma concentration of proinflammatory cytokines. An advanced aging profile is characterized by increased levels of central memory and effector memory T cells, lower levels of the costimulatory markers CD27 and CD28, higher levels of the costimulatory marker CD56, and higher levels of proinflammatory cytokines. Made with BioRender.

Cytokine assays

The plasma concentrations of cytokines were quantified with immunoassays. Immediately after phlebotomy, the EDTA coated tubes for the cytokine assays were centrifuged at 1500 × g for 15 minutes. The supernatant was isolated and frozen at -80°C within 1 hour. The cytokine assays were performed on a different day using the commercially available electrochemiluminescence ultrasensitive immunoassay S-plex (Proinflammatory Panel 1 human, cat.no. K15396S-1) analyzing for IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-17 and tumor necrosis factor (TNF)-α, and V-plex (TH17 Panel 1 human, cat.no. K15085D-1) analyzing for IL-22 and IL-27 (Mesoscale Discovery, Rockville, MD, USA). Plasma samples were analyzed in duplicate using the standardized protocol according to manufacturer guidelines.

Genotyping

Details on genotyping have been reported previously [28]. In brief, the single nucleotide polymorphisms CFH rs1061170 and ARMS2 rs10490924 genotyping was performed on EDTA full blood in patients with nAMD. The blood was frozen at -80°C immediately after phlebotomy and analyzed a different day by BioXpedia, Denmark using the Fluidigm GT192.24 Dynamic Array Integrated Fluidic Circuit (Fluidigm Corp. San Fransisco, CA, USA) according to manufacturer guidelines.

Statistics

Statistical analysis was performed with R software version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria). Group comparisons were analyzed with

analysis of covariance (ANCOVA) adjusted for age and smoking status (never or previous smoker) between AMD subtypes and nAMD treatment response groups. A logarithmic transformation was applied on the T cell differentiation profiles and plasma cytokines to normalize distributions. Normality was tested with histograms and the Shapiro-Wilk test. In the analysis of AMD subtypes, healthy controls were chosen as reference group. In the analysis of nAMD treatment response, good responders were chosen as reference group. Results of the ANCOVA are presented as mean difference and 95% confidence interval (CI95%) in percentages. Associations between genotypes and T cell differentiation, costimulatory markers and cytokines were analyzed with Wilcoxon rank sum test and presented as median and interquartile range (IQR) in nAMD patients. *P* values were adjusted for multiple testing using the false discovery rate (FDR) method for each compartment. A *P* value < 0.05 was interpreted as statistically significant.

Sample size calculations of healthy controls, iAMD and nAMD patients were based on previous immunological studies of patients with AMD, with an α level of 0.05, power of 80%, and effect size of 20%, resulting in a minimum of 30 in each diagnosis group [12, 19, 20]. A direct power calculation on the treatment response analysis was not possible, as no studies have investigated T cell differentiation and these cytokines in nAMD treatment response. Recruitment of nAMD patients continued until reaching 100 participants.

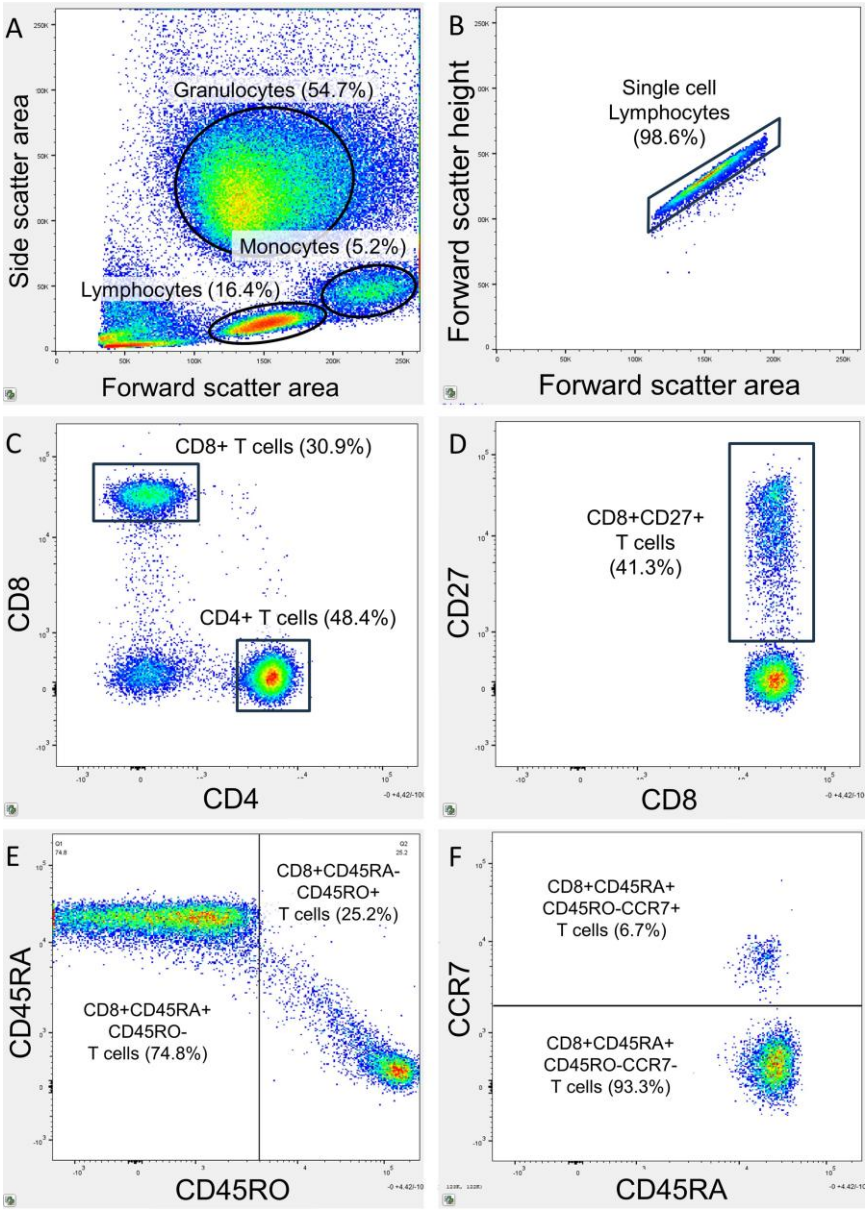


Figure 3. Flow cytometry gating strategy with Boolean sequences. (A) Lymphocytes were identified on the forward-side-scatter. **(B)** Singlet cell lymphocytes were gated. **(C)** CD4+ T cells and CD8+ T cells were identified among the singlet lymphocytes. **(D)** Co-stimulatory surface markers were identified on T cells, in this example CD27 expression on CD8+ T cells. **(E)** The expression of CD45RA and CD45RO was determined on T cells, in this example on CD8+ T cells. **(F)** The expression of CCR7 was determined on CD45RA+CD45RO- and CD45RA-CD45RO+ T cells, in this example on CD8+CD45RA+ CD45RO- T cells.

RESULTS

In the DANEART study, 100 patients with nAMD, 34 patients with iAMD and 61 healthy controls were included. Healthy controls, iAMD patients and nAMD patients had a mean (SD) age of 73 (7), 75 (8) and 80 (6), respectively. Patients with nAMD were significantly older, which was adjusted for in the group comparisons. There were no significant differences in co-morbidities or smoking status (never or previous smoker). Patients with nAMD responded differently to anti-VEGF treatment and of the 100 nAMD patients, 94 completed the 1-year follow-up. The initial (post-loading dose) treatment response of nAMD patients was distributed as 61 (65%) good, 26 (28%) partial, and 7 (7%) poor responders. The distribution of 1-year treatment response was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. There were no significant differences in age, baseline visual acuity or number of injections between the groups after one year [28].

The median and range of the aging immune system markers in the population can be found in Table 1.

Table 1. Median and range of aging immune system markers, including proportions of circulating T cells and concentrations of plasma cytokines in the study population (n = 195).

Aging Immune System Marker	Median (range)
CD8+ T cell differentiation	
CD8+ Naïve [%]	1.8 (0.1 – 20.5)
CD8+ Central memory [%]	12.4 (1.3 – 57.7)
CD8+ Effector memory [%]	28.9 (3.7 – 76.9)
CD8+CD27+ [%]	58.9 (10.3 – 97.5)
CD8+CD28+ [%]	48.7 (6.3 – 95.8)
CD8+CD56+ [%]	13.1 (1.0 – 66.0)
CD4+ T cell differentiation	
CD4+ Naïve [%]	13.4 (2.2 – 33.9)
CD4+ Central memory [%]	28.6 (7.1 - 60.9)
CD4+ Effector memory [%]	12.2 (4.1 – 61.2)
CD4+CD27+ [%]	90.4 (13.7 – 98.8)
CD4+CD28+ [%]	98.0 (30.4 – 98.8)
CD4+CD56+ [%]	66.5 (41.4 – 92.5)
Plasma cytokines	
IFN-γ [fg/ml]	844 (195 – 12,594)
IL-1β [fg/ml]	150 (15 – 595)
IL-2 [fg/ml]	182 (46 – 5133)
IL-6 [fg/ml]	3178 (566 – 24,974)
IL-10 [fg/ml]	812 (86 – 3955)
IL-12 [fg/ml]	913 (255 – 5237)
IL-17 [fg/ml]	607 (135 – 7557)
IL-22 [fg/ml]	1489 (14 – 3147)
IL-27 [fg/ml]	2234 (1625 – 8146)
TNF-α [fg/ml]	874 (263 – 12,414)

Aging Immune System and AMD stage

The T cell differentiation profile differed significantly between patients with nAMD and healthy controls. The proportion of central memory CD8+ T cells was significantly elevated in nAMD patients compared to healthy controls (mean difference: 31%, CI95%: 7%-54%, P = 0.036, ANCOVA with FDR correction for 6 tests). Likewise, the proportion of effector memory CD8+ T cells was significantly higher than healthy controls (mean difference: 54%, CI95%: 31%-77%, P < 0.001, ANCOVA with FDR correction for 6 tests). The proportion of the costimulatory marker CD56 on CD8+ T cells was significantly increased in patients with iAMD compared to healthy controls (mean difference: 43%, CI95%: 14%-80%, P = 0.036, ANCOVA with FDR correction for 6 tests). nAMD patients also tended to have an elevated proportion of CD8+CD56+ T cells compared to healthy controls, however not statistically significant (mean difference: 31%, CI95%: 3%-58%, P = 0.064, ANCOVA with FDR correction for 6 tests). Costimulatory markers CD27 and CD28 on CD8+ T cells tended to be lower in AMD patients, but this was not statistically significant (Figure 4A). There were no significant differences in the CD4+ T cell differentiation profile (Fig. 4B).

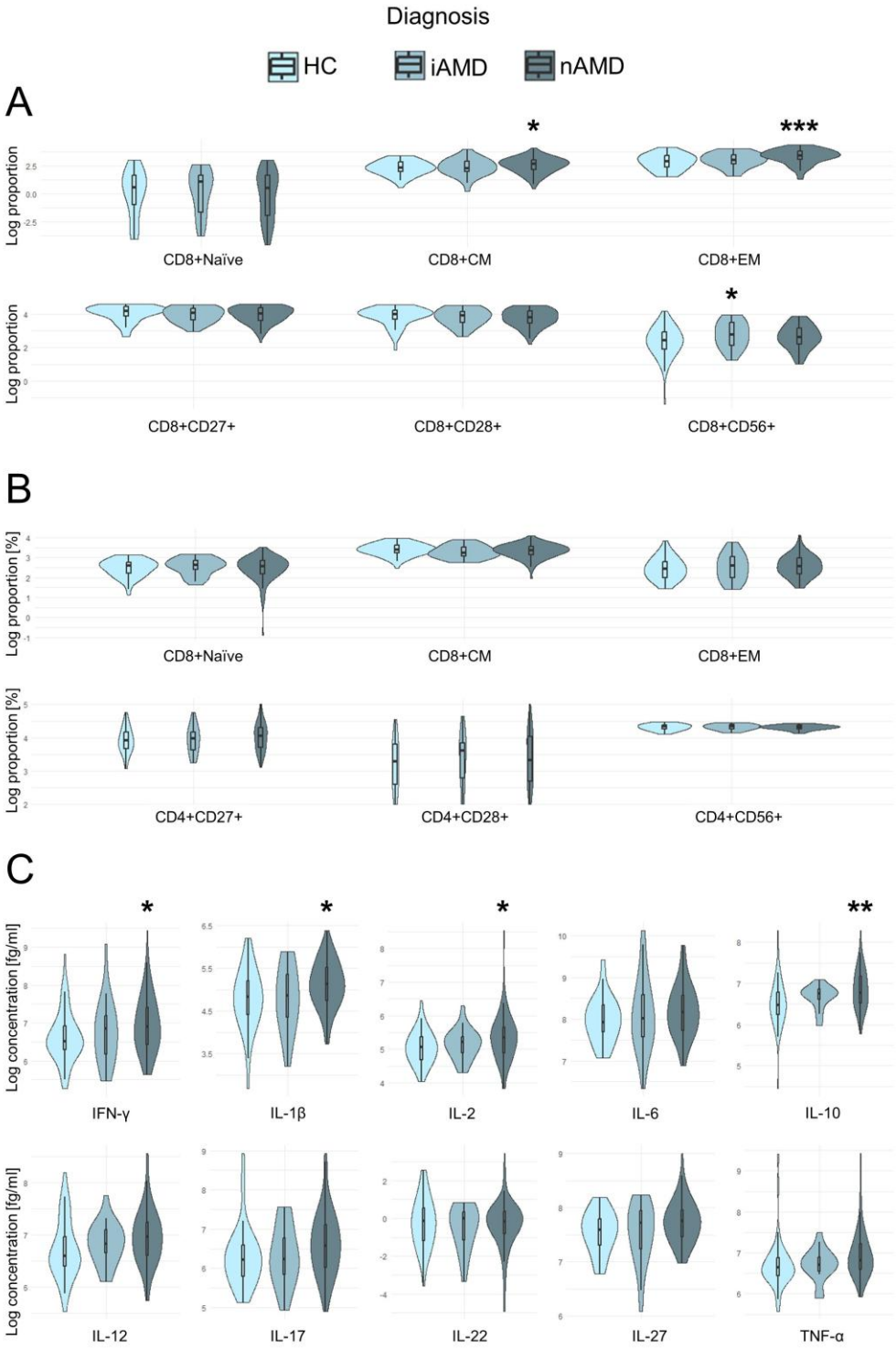


Figure 4. **Violin plots of T cell differentiation, costimulatory markers, and cytokines across AMD stages. (A)** logarithm of the proportion of CD8+ T cell differentiation profile and costimulatory markers, **(B)** logarithm of the proportion of CD4+ T cell differentiation profile and costimulatory markers, **(C)** logarithm of the concentration of systemic plasma cytokines. The number of healthy controls, iAMD patients and nAMD patients was 61, 34, and 100, respectively. HC = healthy controls; iAMD = intermediate AMD; nAMD = neovascular AMD; CM = central memory; EM = effector memory. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to the reference group (healthy controls) adjusted for age and smoking status with false discovery rate correction.

The cytokine profile was significantly altered in nAMD patients. Patients with nAMD had a significantly elevated plasma concentration compared to healthy controls of IFN-γ (mean difference: 36%, CI95%: 7%-65%, *P* =0.038, ANCOVA with FDR correction for 10 tests), IL-1β (mean difference: 36%, CI95%: 11%-60%, *P* = 0.021), IL-2 (mean difference: 31%, CI95%: 7%-55%, *P* = 0.038), and IL-10 (mean difference: 33%, CI95%: 14%-53%, *P* =0.009). nAMD patients had non-significantly tendencies of elevated IL-12 (mean difference: 20%, CI95%: 1%-40%, *P* = 0.077, ANCOVA with FDR correction for 10 tests), and IL-27 (mean difference: 18%, CI95%: 2%-35%, *P* = 0.055, ANCOVA with FDR correction for 10 tests) compared to healthy controls. No significant differences were found between nAMD patients and health controls of IL-6, IL-17, IL-22, or TNF-α plasma concentrations, or iAMD and healthy controls of any cytokine (Fig. 4C).

Aging Immune System and Treatment Response in nAMD Patients

The treatment response to anti-VEGF injections in nAMD patients was evaluated after the loading dose (initial treatment response) and after one year. nAMD patients with a poor initial treatment response had a tendency to have higher proportions of effector memory CD8+ T cells and CD8+CD56+ T cells, however not significant. (Fig. 5A).

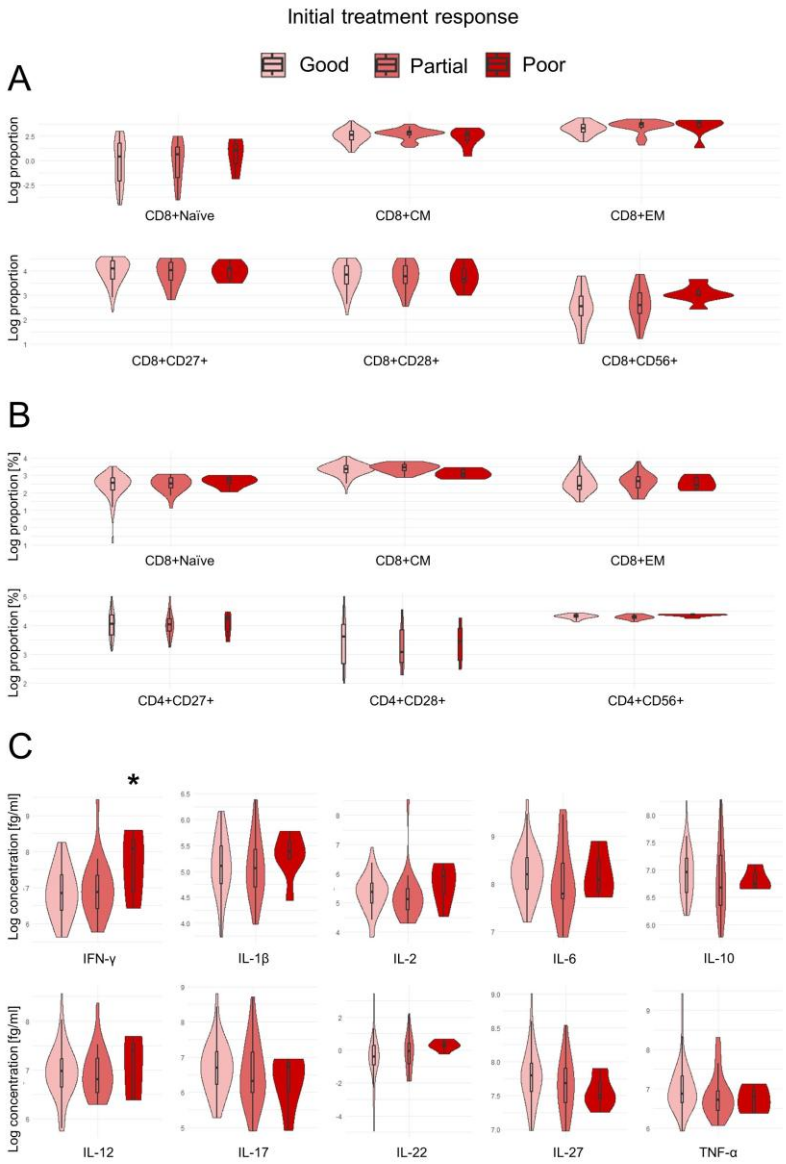


Figure 5. Violin plots of T cell differentiation, costimulatory markers, and cytokines across initial treatment response of nAMD patients. (A) logarithm of the proportion of CD8+ T cell differentiation profile and costimulatory markers, **(B)** logarithm of the proportion of CD4+ T cell differentiation profile and costimulatory markers, **(C)** logarithm of the concentration of systemic plasma cytokines. The number of individuals in the initial treatment response groups categorized as good, partial, and poor was 61, 26, and 7, respectively. CM = central memory; EM = effector memory. * $P < 0.05$; ** $P < 0.01$ compared to the reference group (good responders) adjusted for age and smoking status with false discovery rate correction.

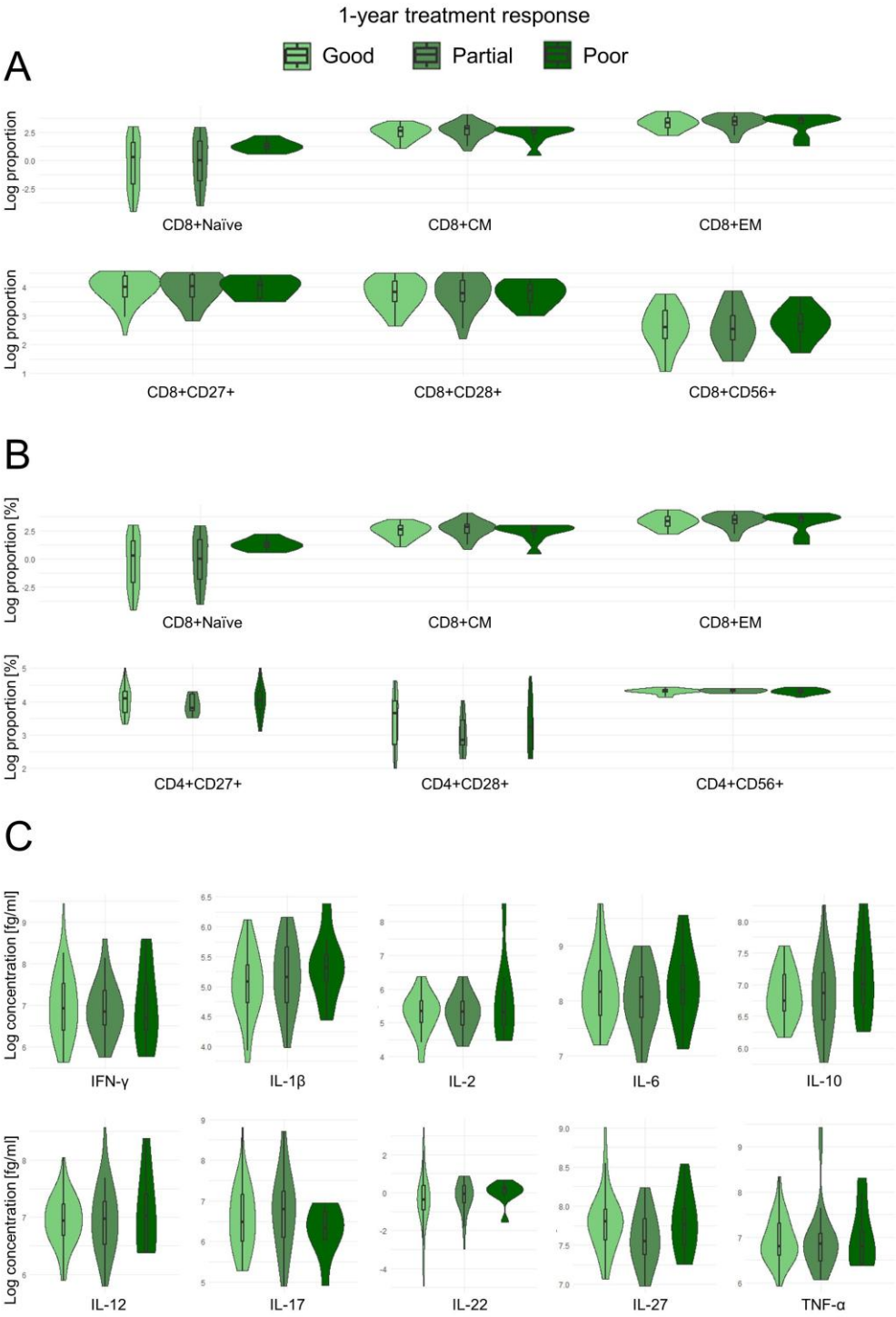


Figure 6. **Violin plots of T cell differentiation, costimulatory markers, and cytokines across 1-year treatment response of nAMD patients. (A)** logarithm of the proportion of CD8+ T cell differentiation profile and costimulatory markers, **(B)** logarithm of the proportion of CD4+ T cell differentiation profile and costimulatory markers, **(C)** logarithm of the concentration of systemic plasma cytokines. The number of individuals in the 1-year treatment response groups categorized as good, partial, and poor was 50, 33, and 11, respectively. CM = central memory; EM = effector memory. No statistically significant differences were found compared to the reference group (good responders) adjusted for age and smoking status with false discovery rate correction.

Plasma concentrations of IFN-γ were significantly elevated in poor initial responders compared to good initial responders (mean difference: 96%, CI95%: 35%-149%, *P* =0.026, ANCOVA with FDR for 10 tests). Partial initial responders also tended to have increased IFN-γ concentration, however not statistically significant (Fig. 5C).

There were no significant differences between 1-year treatment response groups. There was a non-significant tendency of increased concentrations of IL-2, IL-10, and IL-12 in the poor 1-year responders, and decreased levels of IL-22 in partial and poor 1-year responders compared to good 1-year responders (Fig. 6).

Aging immune profile charts

To visualize the aging immune profiles of the AMD stages and nAMD treatment response groups, spiderweb charts were created. These charts show the relative differences in expression levels and concentrations for each group, with the highest group value corresponding to 100%, and the other group values as a relative to this magnitude. Spiderweb charts were plotted for the compartments that included statistically significant group differences before adjustments for multiple testing, being the CD8+ T cell compartment and plasma cytokines between AMD stages and nAMD initial treatment response groups.

Patients with nAMD seemed to have a more advanced profile of the aging immune system compared to healthy controls and iAMD patients. nAMD patients had the highest (most peripheral) level of differentiated CD8+ T cells and most of the plasma cytokines. In addition, this group had the lowest levels of CD27 and CD28 on CD8+ T cells. Patients with iAMD also seemed to have a more advanced aging immune profile compared to healthy controls, but less than nAMD patients. iAMD patients had a higher level of CD56 and differentiated CD8+ T cells, higher level of most of the plasma cytokines, and lower level of CD27 and CD28 compared to healthy controls (Fig. 7A).

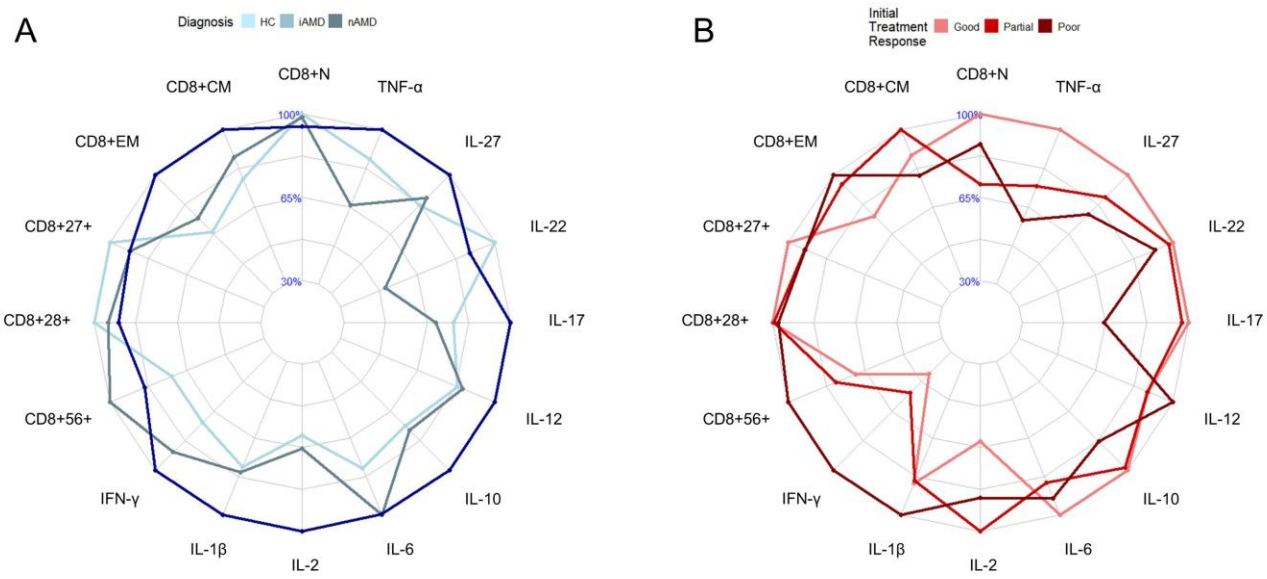


Figure 7. **Aging immune profile charts. (A)** AMD stages and **(B)** initial treatment response groups of neovascular AMD patients. The number of healthy controls, iAMD patients and nAMD patients was 61, 34, and 100, respectively. The number of individuals in the initial treatment response groups categorized as good, partial, and poor was 61, 26, and 7, respectively. HC = healthy controls; iAMD = intermediate AMD; nAMD = neovascular AMD; N = naïve; CM = central memory; EM = effector memory.

The aging immune profiles of initial treatment response in nAMD patients also seemed to differ between groups. The largest relative differences between good and poor initial treatment responders seemed to be poor responders with higher levels of effector memory and CD56+CD8+ T cells, IFN-γ, IL-1β and IL-12, and lower levels of naïve CD8+ T cells, IL-10, IL-17, IL-22, IL-27 and TNF-α. Partial initial responders seemed to have a mix of higher, lower or in between levels compared to the other groups (Fig. 7B).

Aging Immune Profile and Risk Genotypes in nAMD Patients

Among the 100 included nAMD patients, 82 participants carried the high-risk CFH rs1061170 genotypes (CC or CT), while 18 carried the low-risk genotype (TT). 45 nAMD patients carried the high-risk ARMS2, rs10490924 genotypes (TT or TG), while 55 carried the low-risk genotype (GG). The proportion of CD8+CD28+, CD8+ naïve, and CD8+ central memory T cells were

elevated in nAMD patients carrying the high-risk CFH rs1061170 genotypes, however not statistically significant after adjusting for multiple testing (all P = 0.062, Wilcoxon rank sum test with FDR correction for 6 tests). No statistically significant differences in plasma cytokines were found across CFH genotypes (Table 2). No significant differences were found in T cell differentiation or plasma cytokines across ARMS2 genotypes (Supplementary Table 2).

Table 2. T cell senescence and plasma cytokines stratified according to CFH rs1061170 genotype.

	CFH, rs1061170			
	CC/CT (high risk), n = 82	**TT (low risk), n = 18**	***P* value***	**Adjusted *P* value†**
CD8+ T cell differentiation, median (IQR)				
CD8+ Naïve T cells [%]	2.3 (5.3)	0.2 (3.7)	**0.029**	0.062
CD8+ Central memory T cells [%]	15.1 (11.2)	7.9 (12.1)	**0.031**	0.062
CD8+ Effector memory T cells [%]	30.7 (22.0)	28.6 (31.6)	0.87	0.87
CD8+CD27+ T cells [%]	58.1 (40.4)	38.4 (36.9)	**0.046**	0.069
CD8+CD28+ T cells [%]	48.2 (33.2)	32.0 (20.2)	**0.012**	0.062
CD8+CD56+ T cells [%]	13.6 (12.4)	14.1 (20.4)	0.42	0.50
CD4+ T cell differentiation, median (IQR)				
CD4+ Naïve T cells [%]	13.4 (9.0)	12.7 (6.5)	0.83	0.98
CD4+ Central memory T cells [%]	28.5 (13.4)	31.9 (11.4)	0.64	0.98
CD4+ Effector memory T cells [%]	12.8 (10.6)	12.2 (8.6)	0.67	0.98
CD4+CD27+ T cells [%]	94.8 (14.5)	94.7 (19.0)	0.79	0.98
CD4+CD28+ T cells [%]	99.6 (10.9)	98.8 (8.1)	0.62	0.98
CD4+CD56+ T cells [%]	65.9 (17.1)	66.2 (10.2)	0.98	0.98
Cytokines, median (IQR)				
IFN-γ [fg/ml]	983 (1189)	1017 (778)	0.63	0.90
IL-1β [fg/ml]	172 (139)	160 (90)	0.73	0.90
IL-2 [fg/ml]	215 (154)	196 (114)	0.17	0.50
IL-6 [fg/ml]	3547 (2715)	3738 (3165)	0.81	0.90
IL-10 [fg/ml]	947 (604)	733 (479)	0.19	0.50
IL-12 [fg/ml]	1075 (657)	900 (649)	0.25	0.50
IL-17 [fg/ml]	651 (651)	1157 (1033)	0.077	0.50
IL-22 [fg/ml]	828 (992)	1089 (1208)	0.22	0.50
IL-27 [fg/ml]	2323 (975)	2424 (2100)	0.73	0.90
TNF-α [fg/ml]	906 (583)	916 (713)	0.97	0.97

* Wilcoxon rank sum test.
† *P* values with false discovery rate corrections in each compartment.
Bold values indicate statistical significance ($P < 0.05$).

DISCUSSION

This study aimed to investigate whether the aging immune profiles, characterized as T cell differentiation and plasma cytokine levels, differed between AMD stages compared to healthy controls. An additional aim was to investigate differences in these profiles and treatment response in nAMD patients evaluated after the loading dose (initial response) and after one year.

We found an elevated proportion of differentiated CD8+ T cells in patients with nAMD. These patients had a significantly higher level of T cell differentiation characterized by elevated proportions of central memory and effector memory CD8+ T cells compared to healthy controls. We also found that iAMD patients had a significantly higher proportion of CD8+CD56+ T cells compared to healthy controls, and an overall tendency of advanced T cell differentiation. The concentration of multiple proinflammatory plasma cytokines was also significantly increased in nAMD patients compared to healthy controls. These results suggest that nAMD patients have advanced aging of the immune system and chronic inflammation, which may play a role in the pathogenesis of the disease.

Previous studies have found similar results regarding T cell differentiation in nAMD patients. A study found nAMD patients had a higher proportion of effector memory CD8+ T cells compared to patients with myeloproliferative neoplasms without drusen, a condition with increased systemic inflammation and incidence of AMD [12]. Other studies found the proportion of CD8+CD56+ T cells was increased in nAMD patients

compared to healthy controls [19, 25]. The differentiation of CD8+ T cells is especially important, as this compartment is most affected by aging [34]. Memory CD8+ T cells have cytotoxic properties and can cause tissue damage if dysregulated [35] and have been found to be associated with retinal degeneration [36, 37]. A previous study also found an increased proportion of cytotoxic CD8+ T cells in eyes with drusen [38].

Another important function of memory CD8+ T cells is secretion of proinflammatory cytokines [39]. Increased concentrations of the proinflammatory cytokines IFN-γ, IL-1β, and IL-2, as well as anti-inflammatory cytokine IL-10 was found in nAMD patients in our study, and might be important in nAMD development [16–18]. Previous studies also find nAMD patients to have increased levels of systemic IFN-γ [40], IL-1β [20] and IL-10 [20, 26], while other studies find no difference or decreased levels of IL-2 [41]. The increase in the proinflammatory cytokines might be involved in the pathogenesis of nAMD as systemic IFN-γ promotes a shift towards the proinflammatory M1 macrophages [15], IL-1β stimulates retinal inflammation and neovascularization upon entrance to the choroidal vessels following activation by inflammasomes [42], and IL-2 is involved in the migration of retinal pigment epithelial cells and synthesis of extracellular material [44]. The increased levels of the anti-inflammatory cytokine IL-10, might be explained by the age-related increase [43, 44] which stimulates a shift towards pro-angiogenic macrophages [45], and might thus be a rescue mechanism to obtain homeostasis in the increased proinflammatory milieu [46]. We did not find a significant difference between AMD patients and healthy controls in IL-6 or TNF-α plasma concentrations, that has been previously reported [20, 26, 47, 48]. This disagreement may be caused by differing methods of performing cytokine assays or the inclusion of current smokers. We find that iAMD patients tended to have a more advanced aging immune profile, however not statistically significant. We speculate that this observation may result from iAMD patients being a heterogeneous group of patients who will develop nAMD later in life and those who remain with iAMD. Thus, the iAMD patients with a similar profile to the nAMD patients may be more likely to develop nAMD, whereas iAMD patients who do not progress may possess a distinct profile more closely resembling that of healthy controls.

The CFH and ARMS2 genotypes are strongly associated with development of AMD and has been shown to be associated with increased activation of the complement cascade systemically [21, 49, 50]. However, we found no correlations between the CFH rs1061170 or ARMS2 rs10490924 SNPs and T cell differentiation or plasma cytokine levels in nAMD patients. This suggests that the risk genotypes of these SNPs are unlikely to contribute to the systemic changes observed in this study. These findings underscore the multifactorial nature of AMD, where multiple pathways interplay in the disease's development.

To our knowledge, this is the first study to investigate the systemic levels of T cell differentiation and proinflammatory cytokines according to treatment response in nAMD patients. The treatment response was grouped in good, partial and poor assessed post-loading dose (initial response) and after one year. We find that nAMD with a poor initial response had a significantly higher plasma concentration of IFN-γ compared to good initial responders. The diminished therapeutic response to anti-VEGF might especially lie in the effects of IFN-γ. This cytokine has potent proinflammatory properties, causing an inflammatory stress reaction promoting angiogenesis [51]. This might create a retinal angiogenic environment, promoting and maintaining the neovascularization in poor treatment responders, that anti-VEGF might not be able to counter [52]. We do not find any statistically significant differences between the aging immune profile and 1-year treatment response groups. This is likely due to the great variability after one year, where an individualized treatment plan is followed, opposed to the identical treatment protocol of the loading dose [53]. There might thus be conflicting factors that obscure the effects of the aging immune profiles.

Chronic, low-grade inflammation that develops with aging is a hallmark of the aging immune system often referred to as inflammaging. Inflammaging is a consequence of biological aging, resulting from chronological aging, genetics and environmental factors [54]. These are the very risk factors for AMD, and our results suggest that inflammaging might be the reason why some people develop nAMD [16, 17], and why some nAMD patients respond poorly to treatment. Systemic inflammaging can manifest and cause tissue damage in several organ systems [55–57] and is associated with multiple age-related diseases, such as Alzheimer's disease, cardiovascular diseases, diabetes and cancer [58–62]. Likewise, systemic inflammatory changes of multiple pathways have been identified in AMD patients. This includes increased activation of the complement system [28, 49, 50], T helper cells [63], monocyte activation [64–66], CRP [24, 67], chemokines and chemokine receptors [68–70], as well as systemic inflammation in mouse models [71–73]. The pathways of the immune system interact to a great extent [16, 74] and our findings of an advanced aging immune profile fits in the larger picture of the pathophysiology caused by inflammaging. The advancement of the systemic aging of the immune system might cause the retinal damage, by increasing the proinflammatory milieu locally in the retina [4], or as a

two-level model, where age-related stochastic accumulation of molecular damage in the retina combined with the proinflammatory host response causes AMD [16]. In cultured RPE cells, CD56+ T cells were shown to secrete IFN-γ, which induced the production of VEGF from the RPE cells [75]. In another RPE cell culture study, IFN-γ and IL-1β induced reactive oxygen species in RPE cells, which caused oxidative stress leading to protein, DNA and RNA damage [76]. A human study found an increased VEGF expression in RPE cells in response to IFN-γ and IL-1β [77]. These damages and proangiogenic environment at the RPE barrier causes choroidal endothelial cell migration and proliferation, which can lead to the neovascularizations seen in nAMD [4].

The variability in treatment response to anti-VEGF injections in nAMD patients has been a challenge since the introduction of the treatment [53]. However, numerous measures have been developed to create a personalized treatment, including the individualized planning of injection intervals. These regimens include the treat-and-extend and observe-and-plan regimens, which adjust the intervals depending on the previous response [53, 78]. Personalized medicine continues to drive significant advancements across various fields [79], and the time has also come to nAMD [80]. Patients with poor response might benefit from additional or alternative treatment acting on an alternative target than ocular VEGF. Our results suggest that nAMD patients have a more advanced systemic T cell differentiation and higher plasma cytokines levels. Specifically, poor responders exhibit elevated plasma IFN-γ levels, which could serve as a potential predictor of this response and as a target for novel treatments to be explored in future studies.

This study is limited by its observational design, which prevents drawing definitive conclusions about causality. The categorical classification of treatment response was based on a system designed for clinical implementation [31, 32], however the categorical nature limits the ability to capture nuanced differences. The relatively few patients in the poor response group were also a limitation, as potential significant correlations might be hidden.

In conclusion, the advanced aging of the immune system was present in patients with nAMD and to a lesser extent in iAMD patients compared to healthy controls. Poor initial response in nAMD patients was associated with elevated plasma concentrations of IFN-γ.

Acknowledgements

This work was supported by VELUX FONDEN (00024226), Region Zealand PhD Grant (R29-A1337), Synoptik Foundation, Fight for Sight Denmark (Øjenforeningen), Grosserer L.F. Foghts Foundation, and King Christian X's Foundation. The funding organizations had no role in the design or conduct of this research.

Conflict of interest statement

BH and HV have obtained research funding from Bayer for a study not related to the present. All other authors declare no financial or non-financial conflicts of interest.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2024.1585.

References

[1] Scholz R, Langmann T (2016). Gut flora connects obesity with pathological angiogenesis in the eye. EMBO Mol Med, 8:1361–1363.

[2] Hata M, Andriessen EMMA, Hata M, Diaz-Marin R, Fournier F, Crespo-Garcia S, et al. (2023). Past history of obesity triggers persistent epigenetic changes in innate immunity and exacerbates neuroinflammation. Science, 379:45–62.

[3] Fritsche LG, Igl W, Bailey JNC, Grassmann F, Sengupta S, Bragg-Gresham JL, et al. (2016). A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. Nat Genet, 48:134–143.

[4] Fleckenstein M, Keenan TDL, Guymer RH, Chakravarthy U, Schmitz-Valckenberg S, Klaver CC, et al. (2021). Age-related macular degeneration. Nat Rev Dis Primers, 7:31.

[5] Rudnicka AR, Kapetanakis VV, Jarrar Z, Wathern AK, Wormald R, Fletcher AE, et al. (2015). Incidence of Late-Stage Age-Related Macular Degeneration in American Whites: Systematic Review and Meta-analysis. American Journal of Ophthalmology, 160:85-93.e3.

[6] Bloch SB, Larsen M, Munch IC (2012). Incidence of Legal Blindness from Age-Related Macular Degeneration in Denmark: Year 2000 to 2010. American Journal of Ophthalmology, 153:209-213.e2.

[7] Thomsen AK, Krogh Nielsen M, Liisborg C, Sørensen T (2024). Interleukin-8 Promoter Polymorphism −251 A/T and Treatment Response in Neovascular Age-related Macular Degeneration. OPTH, Volume 18:537–543.

[8] (2001). 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, 119:1417.

[9] Thomsen AK, Gøttsche LF, Hinnerskov JMV, Falk MK, Sørensen TL (2024). Microperimetry and Structural Risk Factors on OCT in Intermediate Age-Related Macular Degeneration. Ophthalmology Retina, 8(8):786-793

[10] Liu Z, Liang Q, Ren Y, Guo C, Ge X, Wang L, et al. (2023). Immunosenescence: molecular mechanisms and diseases. Sig Transduct Target Ther, 8:200.
[11] Moro-García MA (2013). When aging reaches CD4+ T-cells: phenotypic and functional changes. Front Immunol, 10:4:10
[12] Liisborg C, Skov V, Kjær L, Hasselbalch HC, Sørensen TL (2021). Retinal drusen in patients with chronic myeloproliferative blood cancers are associated with an increased proportion of senescent T cells and signs of an aging immune system. Aging, 13:25763–25777.
[13] Lemster BH, Michel JJ, Montag DT, Paat JJ, Studenski SA, Newman AB, et al. (2008). Induction of CD56 and TCR-Independent Activation of T Cells with Aging. The Journal of Immunology, 180:1979–1990.
[14] Van Acker HH, Capsomidis A, Smits EL, Van Tendeloo VF (2017). CD56 in the Immune System: More Than a Marker for Cytotoxicity? Front Immunol, 8:892.
[15] Jing Z Cui SC (2011). Immuno-modulatory Effect of IFN-gamma in AMD and its Role as a Possible Target for Therapy. J Clin Exp Ophthalmol. doi: 10.4172/2155-9570-S2-007.
[16] Rozing MP, Durhuus JA, Krogh Nielsen M, Subhi Y, Kirkwood TBL, Westendorp RGJ, et al. (2020). Age-related macular degeneration: A two-level model hypothesis. Progress in Retinal and Eye Research, 76:100825.
[17] Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K (2016). Inflammation and its role in age-related macular degeneration. Cell Mol Life Sci, 73:1765–1786.
[18] Tan W, Zou J, Yoshida S, Jiang B, Zhou Y (2020). The Role of Inflammation in Age-Related Macular Degeneration. Int J Biol Sci, 16:2989–3001.
[19] Subhi Y, Nielsen MK, Molbech CR, Oishi A, Singh A, Nissen MH, et al. (2017). T-cell differentiation and CD56+ levels in polypoidal choroidal vasculopathy and neovascular age-related macular degeneration. Aging, 9:2436–2452.
[20] Subhi Y, Krogh Nielsen M, Molbech CR, Oishi A, Singh A, Nissen MH, et al. (2019). Plasma markers of chronic low-grade inflammation in polypoidal choroidal vasculopathy and neovascular age-related macular degeneration. Acta Ophthalmol, 97:99–106.
[21] Smailhodzic D, Klaver CCW, Klevering BJ, Boon CJF, Groenewoud JMM, Kirchhof B, et al. (2012). Risk Alleles in CFH and ARMS2 Are Independently Associated with Systemic Complement Activation in Age-related Macular Degeneration. Ophthalmology, 119:339–346.
[22] Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, et al. (2005). A common haplotype in the complement regulatory gene factor H (*HF1/CFH*) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA, 102:7227–7232.
[23] Klein RJ, Zeiss C, Chew EY, Tsai J-Y, Sackler RS, Haynes C, et al. (2005). Complement Factor H Polymorphism in Age-Related Macular Degeneration. Science, 308:385–389.
[24] Hong T, Tan AG, Mitchell P, Wang JJ (2011). A Review and Meta-analysis of the Association Between C-Reactive Protein and Age-related Macular Degeneration. Survey of Ophthalmology, 56:184–194.
[25] Faber C, Singh A, Krüger Falk M, Juel HB, Sørensen TL, Nissen MH (2013). Age-related Macular Degeneration Is Associated with Increased Proportion of CD56+ T Cells in Peripheral Blood. Ophthalmology, 120:2310–2316.
[26] Krogh Nielsen M, Subhi Y, Molbech CR, Falk MK, Nissen MH, Sørensen TL (2019). Systemic Levels of Interleukin-6 Correlate With Progression Rate of Geographic Atrophy Secondary to Age-Related Macular Degeneration. Invest Ophthalmol Vis Sci, 60:202–208.
[27] Behnke V, Wolf A, Langmann T (2020). The role of lymphocytes and phagocytes in age-related macular degeneration (AMD). Cell Mol Life Sci, 77:781–788.
[28] Thomsen AK, Steffensen MA, Villarruel Hinnerskov JM, Nielsen AT, Vorum H, Honoré B, et al. (2024). Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response. J Neuroinflammation, 21:284.
[29] Thomsen AK, Fasih-Ahmad S, Sadda S, Sørensen TL (2024). Initial treatment response can predict one-year treatment outcomes in neovascular age-related macular degeneration treated according to the observe-and-plan regimen. Acta Ophthalmologica, aos.16801.
[30] Ferris FL, Wilkinson CP, Bird A, Chakravarthy U, Chew E, Csaky K, et al. (2013). Clinical Classification of Age-related Macular Degeneration. Ophthalmology, 120:844–851.
[31] Thomsen, Alexander Kai, Fasih-Ahmad, Sohaib, Sadda, SriniVas, Sørensen, Torben Lykke (2024). Initial treatment response can predict one-year treatment outcomes in neovascular age-related macular degeneration treated according to the observe-and-plan regimen. Acta Ophthalmol, in press.
[32] Amoaku WM, Chakravarthy U, Gale R, Gavin M, Ghanchi F, Gibson J, et al. (2015). Defining response to anti-VEGF therapies in neovascular AMD. Eye, 29:721–731.
[33] Larbi A, Fulop T (2014). From "truly naïve" to "exhausted senescent" T cells: When markers predict functionality. Cytometry Pt A, 85:25–35.
[34] Goronzy JJ, Weyand CM (2019). Mechanisms underlying T cell ageing. Nat Rev Immunol, 19:573–583.
[35] Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B (2000). Response of naïve and memory CD8+ T cells to antigen stimulation in vivo. Nat Immunol, 1:47–53.
[36] Lee KS, Lin S, Copland DA, Dick AD, Liu J (2021). Cellular senescence in the aging retina and developments of senotherapies for age-related macular degeneration. J Neuroinflammation, 18:32.
[37] Karahan M, Hazar L, Erdem S, Ava S, Dursun ME, Demirtaş AA, et al. (2021). Is there a relationship between hematological inflammatory parameters and age-related macular degeneration? Ophthalmol Eye Dis, 13:25158414211010550.

[38] Conedera FM, Runnels JM, Stein JV, Alt C, Enzmann V, Lin CP (2023). Assessing the role of T cells in response to retinal injury to uncover new therapeutic targets for the treatment of retinal degeneration. J Neuroinflammation, 20:206.
[39] Martin MD, Badovinac VP (2018). Defining Memory CD8 T Cell. Front Immunol, 9:2692.
[40] Litwińska Z, Sobuś A, Łuczkowska K, Grabowicz A, Mozolewska-Piotrowska K, Safranow K, et al. (2019). The Interplay Between Systemic Inflammatory Factors and MicroRNAs in Age-Related Macular Degeneration. Front Aging Neurosci, 11:286.
[41] Nassar K, Grisanti S, Elfar E, Lüke J, Lüke M, Grisanti S (2015). Serum cytokines as biomarkers for age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol, 253:699–704.
[42] Marneros AG (2023). Role of inflammasome activation in neovascular age-related macular degeneration. FEBS J, 290:28–36.
[43] Kelly J, Khan AA, Yin J, Ferguson TA, Apte RS (2007). Senescence regulates macrophage activation and angiogenic fate at sites of tissue injury in mice. J Clin Invest, 117:3421–3426.
[44] Almanan M, Raynor J, Ogunsulire I, Malyshkina A, Mukherjee S, Hummel SA, et al. (2020). IL-10–producing Tfh cells accumulate with age and link inflammation with age-related immune suppression. Sci Adv, 6:eabb0806.
[45] Dace DS, Khan AA, Kelly J, Apte RS (2008). Interleukin-10 Promotes Pathological Angiogenesis by Regulating Macrophage Response to Hypoxia during Development. PLoS ONE, 3:e3381.
[46] Couper KN, Blount DG, Riley EM (2008). IL-10: The Master Regulator of Immunity to Infection. The Journal of Immunology, 180:5771–5777.
[47] Nahavandipour A, Krogh Nielsen M, Sørensen TL, Subhi Y (2020). Systemic levels of interleukin-6 in patients with age-related macular degeneration: a systematic review and meta-analysis. Acta Ophthalmologica, 98:434–444.
[48] Rajeswaren V, Wagner BD, Patnaik JL, Mandava N, Mathias MT, Manoharan N, et al. (2024). Elevated tumor necrosis factor alpha and vascular endothelial growth factor in intermediate age-related macular degeneration and geographic atrophy. Front Ophthalmol, 4:1356957.
[49] Scholl HPN, Issa PC, Walier M, Janzer S, Pollok-Kopp B, Börncke F, et al. (2008). Systemic Complement Activation in Age-Related Macular Degeneration. PLoS ONE, 3:e2593.
[50] Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM (2009). Plasma Complement Components and Activation Fragments: Associations with Age-Related Macular Degeneration Genotypes and Phenotypes. Invest Ophthalmol Vis Sci, 50:5818.
[51] Lee H, Schlereth SL, Park EY, Emami-Naeini P, Chauhan SK, Dana R (2014). A Novel Pro-Angiogenic Function for Interferon-γ–Secreting Natural Killer Cells. Invest Ophthalmol Vis Sci, 55:2885.
[52] Faber C, Juel HB, Jensen BAH, Christensen JP, Prause JU, Thomsen AR, et al. (2019). Chemokine Expression in Murine RPE/Choroid in Response to Systemic Viral Infection and Elevated Levels of Circulating Interferon-γ. Invest Ophthalmol Vis Sci, 60:192.
[53] Mantel I, Niderprim S-A, Gianniou C, Deli A, Ambresin A (2014). Reducing the clinical burden of ranibizumab treatment for neovascular age-related macular degeneration using an individually planned regimen. Br J Ophthalmol, 98:1192–1196.
[54] Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. (2000). Inflamm-aging: An Evolutionary Perspective on Immunosenescence. Annals of the New York Academy of Sciences, 908:244–254.
[55] Franceschi C, Campisi J (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 69:S4–S9.
[56] Singh A, Schurman SH, Bektas A, Kaileh M, Roy R, Wilson DM, et al. (2024). Aging and Inflammation. Cold Spring Harb Perspect Med, 14:a041197.
[57] Agrawal R, Hu A, Bollag WB (2023). The Skin and Inflamm-Aging. Biology, 12:1396.
[58] Liberale L, Badimon L, Montecucco F, Lüscher TF, Libby P, Camici GG (2022). Inflammation, Aging, and Cardiovascular Disease. Journal of the American College of Cardiology, 79:837–847.
[59] Ferrucci L, Fabbri E (2018). Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nat Rev Cardiol, 15:505–522.
[60] Kosyreva A, Sentyabreva A, Tsvetkov I, Makarova O (2022). Alzheimer's Disease and Inflammaging. Brain Sciences, 12:1237.
[61] Baechle JJ, Chen N, Makhijani P, Winer S, Furman D, Winer DA (2023). Chronic inflammation and the hallmarks of aging. Molecular Metabolism, 74:101755.
[62] Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. (2015). Neuroinflammation in Alzheimer's disease. Lancet Neurol, 14:388–405.
[63] Singh A, Subhi Y, Krogh Nielsen M, Falk MK, Matzen SMH, Sellebjerg F, et al. (2017). Systemic frequencies of T helper 1 and T helper 17 cells in patients with age-related macular degeneration: A case-control study. Sci Rep, 7:605.
[64] Subhi Y, Krogh Nielsen M, Molbech CR, Oishi A, Singh A, Nissen MH, et al. (2017). CD11b and CD200 on Circulating Monocytes Differentiate Two Angiographic Subtypes of Polypoidal Choroidal Vasculopathy. Invest Ophthalmol Vis Sci, 58:5242.
[65] Hector SM, Sørensen TL (2017). Circulating monocytes and B-lymphocytes in neovascular age-related macular degeneration. OPTH, Volume 11:179–184.
[66] Roubeix C, Nous C, Augustin S, Ronning KE, Mathis T, Blond F, et al. (2024). Splenic monocytes drive pathogenic subretinal inflammation in age-related macular degeneration. J Neuroinflammation, 21:22.
[67] Nielsen MK, Subhi Y, Falk M, Singh A, Sørensen TL, Nissen MH, et al. (2023). Complement factor H Y402H polymorphism results in diminishing CD4+ T cells and increasing C-reactive protein in plasma. Sci Rep, 13:19414.

[68] Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL (2014). CX3CL1/CX3CR1 and CCL2/CCR2 Chemokine/Chemokine Receptor Complex in Patients with AMD. PLoS ONE, 9:e112473.

[69] Liisborg C, Skov V, Kjær L, Hasselbalch HC, Lykke Sørensen T (2022). Lower CXCR3 expression in both patients with neovascular AMD and advanced stages of chronic myeloproliferative blood cancers. PLoS ONE, 17:e0269960.

[70] Martinez Villarruel Hinnerskov J, Krogh Nielsen M, Kai Thomsen A, Steffensen MA, Honoré B, Vorum H, et al. (2024). Chemokine Receptor Profile of T Cells and Progression Rate of Geographic Atrophy Secondary to Age-related Macular Degeneration. Invest Ophthalmol Vis Sci, 65:5.

[71] Lunding BS, Bassi MR, Christensen JP, Thomsen AR, Sørensen TL, Vorum H, et al. (2024). Systemic infection in aged mice causes upregulation of crystallin alpha A in the RPE/choroid. Experimental Eye Research, 245:109984.

[72] Khan AM, Steffensen MA, Paskeviciute E, Abduljabar AB, Sørensen TL, Vorum H, et al. (2024). Neuroretinal degeneration in a mouse model of systemic chronic immune activation observed by proteomics. Front Immunol, 15:1374617.

[73] Paskeviciute E, Chen M, Xu H, Honoré B, Vorum H, Sørensen TL, et al. (2023). Systemic virus infection results in CD8 T cell recruitment to the retina in the absence of local virus infection. Front Immunol, 14:1221511.

[74] Daëron M (2022). The immune system as a system of relations. Front Immunol, 13:984678.

[75] Liu B, Faia L, Hu M, Nussenblatt RB (2010). Pro-angiogenic effect of IFNgamma is dependent on the PI3K/mTOR/translational pathway in human retinal pigmented epithelial cells. Mol Vis, 16:184–193.

[76] Yang D, Elner SG, Bian Z-M, Till GO, Petty HR, Elner VM (2007). Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. Experimental Eye Research, 85:462–472.

[77] Nagineni CN, Kommineni VK, William A, Detrick B, Hooks JJ (2012). Regulation of VEGF expression in human retinal cells by cytokines: Implications for the role of inflammation in age-related macular degeneration. Journal Cellular Physiology, 227:116–126.

[78] Soliman MK, Tuli N, Lee TK, Britton WA, Tuli R (2021). Visual and anatomical outcomes associated with treat-and-extend administration of intravitreal aflibercept for neovascular age-related macular degeneration. Int J Retin Vitr, 7:54.

[79] Goetz LH, Schork NJ (2018). Personalized medicine: motivation, challenges, and progress. Fertility and Sterility, 109:952–963.

[80] Souied EH, Leveziel N (2012). Toward Personalized Medicine for Age-Related Macular Degeneration. American Journal of Ophthalmology, 154:427–428.

Supplementary Table 1. Flow Cytometry Protocol

Flow cytometry preparations were initiated within 4 hours of phlebotomy.
Leukocyte count was performed on Sysmex KX-21NTM (Sysmex Corporation, Kobe, Japan) to calculate blood volume sufficient to obtain 1.0×10^6 leukocytes for analysis.
1% lysis buffer was added to the blood sample to lyse erythrocytes.
Blood sample was stored at room temperature in the dark for 10 minutes.
Cells were washed by adding BD FACS Flow isotonic buffer to the sample, centrifuging at 500 × g for five minutes, followed by decantation of the supernatant. This step was repeated a total of three times.
The isolated leukocytes were resuspended in isotonic buffer.
Monoclonal fluorescent antibodies were added.
The sample was incubated for at room temperature in the dark for 20 minutes.
The stained leukocytes were washed and resuspended in isotonic buffer a last time.
The sample was analyzed on the BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) with a gating size of 100.000 singlet cells analyzed per sample.

Supplementary Table 2. T cell senescence and plasma cytokines stratified according to ARMS2 rs10490924 genotype.

	ARMS2, rs10490924			
	TT/TG (high risk), n = 45	GG (low risk), n = 55	P value*	Adjusted P value†
CD8+ T cell differentiation, median (IQR)				
CD8+ Naïve T cells [%]	2.4 (4.9)	1.6 (7.5)	0.24	0.49
CD8+ Central memory T cells [%]	15.1 (10.1)	13.6 (14.1)	0.80	0.94
CD8+ Effector memory T cells [%]	27.9 (25.1)	34.2 (14.0)	0.15	0.49
CD8+CD27+ T cells [%]	66.9 (38.0)	54.9 (43.4)	0.32	0.49
CD8+CD28+ T cells [%]	53.5 (30.4)	41.3 (40.2)	0.33	0.49
CD8+CD56+ T cells [%]	13.4 (12.9)	14.1 (14.4)	0.94	0.94
CD4+ T cell differentiation, median (IQR)				
CD4+ Naïve T cells [%]	13.6 (6.7)	12.6 (11.7)	0.76	0.76
CD4+ Central memory T cells [%]	27.8 (13.5)	30.4 (11.1)	0.13	0.76
CD4+ Effector memory T cells [%]	11.2 (10.1)	13.6 (11.2)	0.51	0.76
CD4+CD27+ T cells [%]	88.3 (13.0)	88.9 (20.2)	0.74	0.76
CD4+CD28+ T cells [%]	98.0 (8.6)	97.8 (17.0)	0.42	0.76
CD4+CD56+ T cells [%]	66.0 (14.1)	65.5 (17.2)	0.69	0.76
Cytokines, median (IQR)				
IFN-γ [fg/ml]	810 (1228)	1002 (909)	0.43	0.90
IL-1β [fg/ml]	161 (116)	170 (151)	0.69	0.90
IL-2 [fg/ml]	214 (161)	204 (122)	0.73	0.90
IL-6 [fg/ml]	3837 (3163)	3143 (2418)	0.37	0.90
IL-10 [fg/ml]	830 (698)	1008 (577)	0.23	0.90
IL-12 [fg/ml]	1046 (717)	1061 (603)	0.70	0.90
IL-17 [fg/ml]	649 (774)	796 (799)	0.63	0.90
IL-22 [fg/ml]	814 (1081)	916 (896)	0.90	0.90
IL-27 [fg/ml]	2380 (1028)	2323 (1389)	0.87	0.90
TNF-α [fg/ml]	983 (487)	897 (788)	0.41	0.90

* Wilcoxon rank sum test.

† *P* values with false discovery rate corrections in each compartment.

Bold values indicate statistical significance ($P < 0.05$).

7.3. Study III

Title

Chemokine System Changes Drive Age-Related Macular Degeneration and Influence Treatment Outcomes

Running title

Chemokine System and AMD Treatment Response

Authors

Alexander Kai Thomsen[1,2],
Maria Abildgaard Steffensen[3],
Amalie Thomsen Nielsen[1,2],
Henrik Vorum[4,5],
Bent Honoré[4,6],
Mogens Holst Nissen[2,3],
Torben Lykke Sørensen[1,2]

Affiliations

1. Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark.
2. Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.
3. Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark.
4. Department of Clinical Medicine, Aalborg University, Aalborg, Denmark
5. Department of Ophthalmology, Aalborg University Hospital, Aalborg, Denmark.
6. Department of Biomedicine, Aarhus University, Aarhus, Denmark.

Corresponding author

Alexander Kai Thomsen, MD, Department of Ophthalmology, Zealand University Hospital, Sygehusvej 10, 4000 Roskilde, Denmark; e-mail: alext@regionsjaelland.dk; telephone: +45 47323900; fax: +45 46321055.

Word count: 3748

Funding information

This work was supported by VELUX FONDEN (00024226), Region Zealand PhD Grant (R29-A1337), Fight for Sight Denmark (Øjenforeningen), Synoptik Foundation, Grosserer L.F. Foghts Foundation, and Snedkermester Sophus Jacobsen og hustru Astrid Jacobsens Fond. The funding organizations had no role in the design or conduct of this research.

Conflicts of interest

A.K. Thomsen, None; **M.A. Steffensen**, None; **A.T. Nielsen**, None; **H. Vorum**, Bayer (F); **B. Honoré**, Bayer (F); **M.H. Nissen**, None, **T.L. Sørensen**, None.

Abstract

Purpose: The chemokine system is associated with age-related macular degeneration (AMD), shown in previous studies. In this study, we investigate these chemokines and chemokine receptors and their association with treatment response in neovascular AMD (nAMD), and association to intermediate AMD (iAMD).

Methods: In this prospective cohort study, patients with nAMD, iAMD, and healthy controls were included. The initial and 1-year treatment response was evaluated in nAMD patients. Plasma chemokine concentrations of CCL2, CCL3, CCL4, CCL20, CXCL8, and CXCL10 were measured with immunoassays. Chemokine receptor expression levels of CCR1, CCR2, CCR5, CCR6, CXCR2, CXCR3 and CX_3CR1 on circulating T cells and monocytes were measured with flow cytometry. Correlation network analyses were performed of the chemokine system. Genotyping for CFH and ARMS2 risk polymorphisms was performed in nAMD patients.

Results: nAMD patients with poor initial treatment response had significantly lower proportions of CD4+CXCR3+, CCR5+ classical monocytes and CCR2+ non-classical monocytes compared to good initial responders (all $P < 0.05$). nAMD patients with poor 1-year treatment response had significantly lower CD4+CXCR3+ and CCR2+ non-classical monocytes compared to good 1-year responders (both $P < 0.05$). Correlation networks revealed a more complex regulation in partial and poor initial treatment responders. Multiple chemokines and chemokine receptors significantly correlated with the risk genotypes of CFH and ARMS2.

Conclusion: nAMD patients with poor treatment response had dysregulation of the chemokine system. The chemokine system might be a potential target of novel treatment in nAMD. Further studies are needed to clarify the chemokine system's role in nAMD treatment response.

Introduction

Age-related macular degeneration (AMD) is a multifactorial disease and a common cause of visual impairment and blindness in the elderly, yet the treatment options are limited.[1] In the late stage of neovascular AMD (nAMD), characterized by the formation of macular neovascularization (MNV), the pro-angiogenic vascular endothelial growth factor (VEGF) plays a key role. Treatment consists of repeated intravitreal anti-VEGF injections which have improved visual outcomes for this patient group greatly. However, treatment response differs significantly between patients, and a considerable number will respond poorly with loss of vision despite treatment.[2] Novel treatment for the patients poorly responding to anti-VEGF injections are needed to halt the deterioration of vision and life quality of this patient group.[3] No treatment options exist for patients with intermediate AMD (iAMD).[4]

Chronic low-grade inflammation is a key factor in the pathogenesis of AMD.[5,6] Immunosenescence, a pro-inflammatory shift in the immune profile with aging, contributes to the angiogenic changes present in AMD patients.[7] A key component of the immune system is the chemokine system. Chemokines are cytokines that primarily induce chemotaxis, guiding leukocytes to sites of inflammation. Leukocytes, such as T cells and monocytes, have surface receptors for chemokines and will react upon stimulation by migrating to these sites and initiate their inflammatory and angiogenic response.[8] The chemokine system is also affected by aging with increased systemic concentrations of pro-angiogenic chemokines,[9] as well as alterations in expression levels of chemokine receptors on T cells[10] and monocytes.[11] Our group and others have previously found that systemic chemokines are associated with AMD, including C-C motif chemokine ligand 2 (CCL2), CCL3, CCL4,[12–14] as well as chemokine receptors C-C motif chemokine receptor type 1 (CCR1),[15] CCR2,[15,16] CCR5,[17–19] CCR6,[18] C-X-C motif chemokine receptor type 3 (CXCR3),[18–21] and C-X_3-C motif chemokine receptor type 1 (CX_3CR1).[16,17,22] These chemokines and receptors regulate inflammation, which might contribute to the increased systemic inflammation observed in AMD.[5] Bjerregaard and colleagues also found a significantly increased levels of CD14+CCR1+ monocytes and decreased level of CD8+CCR3+ T cells in circulation between good and poor treatment responders in nAMD patients.[23]

Genetic predisposition is also associated with AMD development. Single nucleotide polymorphisms of the complement factor H (CFH) and age-related maculopathy susceptibility 2 (ARMS2) genes have been shown to be associated with stage of disease.[24–26] It has also been suggested that these polymorphisms are associated with treatment response,[27] although this remains a subject of debate.[28]

Based on these prior findings, we investigated the relationship between the chemokine system and AMD stage (healthy, iAMD and nAMD), as well as treatment response in nAMD patients. Furthermore, the association between chemokines and chemokine receptors on mononuclear leukocytes and the risk genotypes CFH rs1061170 and ARMS2 rs10490924 in nAMD patients was evaluated. These findings will contribute to

the understanding of the chemokine system in iAMD and nAMD patients. Novel treatment targets might be identified in nAMD patients with partial and poor treatment response to anti-VEGF injections.

Methods

Study Design and Participants

The Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study is a prospective cohort study. Systemic inflammatory and angiogenic biomarkers are analyzed and compared between AMD stages, as well as treatment response groups of nAMD patients. The study was conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal no: SJ-768) and performed in adherence with the Declaration of Helsinki. Verbal and written informed consent were obtained from all participants prior to inclusion.

Details on study design of the DANEART study has previously been reported,[6] and are summarized below. Healthy controls, patients with iAMD and treatment-naïve nAMD were consecutively enrolled. Exclusion criteria were age younger than 60 years, active infections and cancer, inflammatory and autoimmune diseases, use of immunomodulating treatment, active smoking, plasma C-reactive protein (CRP) > 15 mg/L, previous anti-VEGF treatment, other vision-affecting diseases than iAMD and nAMD.

Participants were examined at baseline, and nAMD patients were additionally examined at two follow-up visits. To evaluate the initial treatment response, nAMD patients were examined after a loading dose of three intravitreal anti-VEGF injections with monthly intervals, and the maintained treatment response after one year. Patients were treated according to Danish national guidelines with aflibercept, without switching, starting with a loading dose followed by individualized planning based on the observe-and-plan regimen.[2] Participants were examined by retinal specialists in the clinic for best corrected visual acuity, slit-lamp biomicroscopy, color fundus photography, spectral domain optical coherence tomography (OCT) and OCT angiography. Classification of AMD stage was evaluated according to the Beckman criteria,[29] and treatment response in nAMD patients was graded according to persistence of intra- and subretinal fluid, and central retinal thickness on OCT. Good responders had total regression of retinal fluid, partial responders had persistence of fluid and decreased CRT, and poor responders had persistence of fluid and unchanged or increased CRT (Table 1).[6,30]

Participants were examined for plasma chemokine concentrations and their receptor's expression level on T cells and monocytes (Table 2). Genotyping was also performed on nAMD patients.

Chemokine assays

All participants had blood sampled from the antecubital vein in lithium-heparin coated tubes and ethylenediamine tetraacetic acid (EDTA) coated tubes.
Plasma chemokine concentrations were measured with immunoassays from the lithium-heparin coated tubes. These were first centrifuged at 1500G for 15 minutes immediately after phlebotomy. The supernatant was isolated and frozen at -80°C within 1 hour to be analyzed a different day. Chemokine assays were performed with electrochemiluminescence immunoassay V-plex analyzing for CCL2, CCL3, CCL4, CXCL8, and CXCL10, and V-plex analyzing for CCL20 (Mesoscale Discovery, see details in Supplementary table S1). Duplicate analyses were performed according to manufacturer guidelines.

Flow cytometry

Chemokine receptors were identified on T cells and monocytes with flow cytometry. The EDTA coated tubes were used for flow cytometry, which was initiated within 4 hours after phlebotomy. To determine the necessary blood volume for obtaining 1.0×10^6 leukocytes for analysis, a leukocyte count on full blood was performed on the Sysmex KX-21NTM (Sysmex Corporation, Kobe, Japan). The determined volume was isolated, and erythrocytes were lysed at room temperature in the dark for 10 minutes with a 1% lysis buffer (BioLegend, San Diego, CA, USA). Cells were washed by adding BD FACS Flow isotonic buffer, centrifuged at 500G for five minutes, followed by decantation of the supernatant. The washing was repeated a total of three times. The isolated leukocytes were resuspended in isotonic buffer, stained with monoclonal fluorescent antibodies (see details in Supplementary table S2) and incubated for 20 minutes at room temperature in the dark. The stained leukocytes were washed in isotonic buffer and analyzed on the BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) with a gating size of 100,000 cells. Flow cytometry data was analyzed using FlowJo (Tree Star, Ashland, OR, USA, v.10.10.0). Gating was performed with Boolean sequences by identifying singlet lymphocytes and monocytes. Lymphocytes were gated for CD4 and CD8 to identify CD4+ and CD8+ T cells. Monocytes were gated for CD14 and CD16 to identify classical ($CD14^{high}CD16^{low}$), intermediate ($CD14^{high}CD16^{high}$) and non-classical ($CD14^{low}CD16^{high}$) subtypes. T cells were analyzed for the expression levels of CCR1, CCR2, CCR5, CCR6, and CXCR3, as these chemokine receptors on T cells have been suggested to be implicated in AMD pathogenesis.[17–21] Monocytes were analyzed for the expression levels of CCR1, CCR2, CCR5, CXCR2, CXCR3, and CX_3CR1, as these chemokine receptors on monocytes have been suggested to be implicated in AMD pathogenesis.[15,16,22,23] An example of the gating strategy can be found in Figure 1.

Genotyping

A detailed description of the genotyping has been reported previously.[6] In brief, genotyping was performed in nAMD patients on full blood for the single nucleotide polymorphisms CFH rs1061170 and ARMS2

rs10490924. The blood was frozen at -80°C within 1 hour to be analyzed another day. Genotyping was performed by BioXpedia, Aarhus, Denmark with the Fluidigm GT192.24 Dynamic Array Integrated Fluidic Circuit (Fluidigm Corp. San Fransisco, CA, USA) according to manufacturer guidelines.

Statistics

Analysis of covariance (ANCOVA) was performed in group comparisons adjusted for age and smoking status (never or previous smoker). Histograms and the Shapiro-Wilk test were performed to evaluate the normality of the distributions. Logarithmic transformation was performed to normalized skewed data for ANCOVA. Healthy controls were chosen as reference group in the AMD stage comparisons (healthy controls, iAMD, and nAMD) using ANCOVA. Good treatment response was chosen as reference group in nAMD treatment response comparisons (good, partial, and poor) using ANCOVA. Results of ANCOVA are presented as mean difference and 95% confidence interval (CI95%) in percentages. Visual acuity group comparisons were tested with analysis of variance (ANOVA) and reported as mean and standard deviation (SD).

The chemokine-chemokine receptor interactions are visualized and described with correlation networks, with nodes representing the variables and the correlation as edges connecting them. Correlations were determined from Pearson's correlation coefficient with a threshold of $|r| > 0.4$ and $P < 0.05$. Association between genotypes and chemokines and chemokine receptors in nAMD patients was analyzed with Welch two sample t-test.

The CXCL8 concentration measurement was in 30.2% of cases below the lower limit of detection (LLOD) defined as 2.5 SD above the blank control adjusted for the dilution factor, with a value of approximately 135 pg/ml. Thus, CXCL8 was analyzed as a binary variable being over or under the LLOD. Correlations between CXCL8 and genotype were tested with Fisher's exact test. A P value < 0.05 was interpreted as statistically significant. Statistical analyses were performed with R software version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Study population

In the DANEART study, 61 healthy controls, 34 iAMD patients, and 100 nAMD patients were included, of which 94 nAMD patients completed the 1-year follow-up. Among patients with nAMD, the initial response distribution was as follows: 61 (65%) were good responders, 26 (28%) were partial responders, and 7 (7%) were poor responders. The 1-year response distribution was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. Mean (SD) number of injections after 1 year was 6.06 (1.78). Visual acuity between treatment response groups did not differ significantly at any timepoint. Mean (SD) baseline BCVA of good, partial, and poor responders was 59.7 (15.7), 62.6 (14.3), and 57.7 (12.1) ETDRS letters, respectively. Mean

(SD) post-loading dose BCVA for good, partial, and poor responders was 64.2 (14.7), 65.3 (15.6), and 51.8 (15.0), respectively. Mean (SD) 1-year BCVA for good, partial, and poor responders was 66.75 (11.9), 65.2 (11.9), and 64.8 (17.5), respectively ($P > 0.05$ for all group comparisons). The full details of the characteristics of these participants have been described previously.[6]

Chemokine system and diagnosis

Levels of the individual chemokines and chemokine receptors differed significantly according to diagnosis. CCL3 (mean difference: 20.8%, CI95%: 2.4% to 39.2%, $P = 0.027$), CCL4 (mean difference: 26.8%, CI95%: 9.6% to 44.1%, $P = 0.002$), and CD4+CCR1+ (mean difference: 20.7%, CI95%: 3.2% to 38.1%, $P = 0.021$) were significantly elevated in nAMD patients compared to healthy controls. CCL2 (mean difference: -13.5%, CI95%: -24.3% to -2.5%, $P = 0.016$), CD8+CXCR3+ (mean difference: -2.9%, CI95%: -5.7% to -0.1%, $P = 0.041$), CX_3CR1+ classical monocytes (mean difference: -0.001%, CI95%: -0.003% to -0.000%, $P = 0.015$), and CCR2+ intermediate monocytes (mean difference: -24.2%, CI95%: -38.5 to -10.1, $P < 0.001$) were significantly decreased in nAMD patients compared to healthy controls. CCL2 (mean difference: -16.2%, CI95%: -31.0 to -1.3%, $P = 0.032$), CX_3CR1+ classical monocytes (mean difference: -0.002%, CI95%: -0.003% to -0.001%, $P = 0.004$), CCR2+ intermediate monocytes (mean difference: -59%, CI95%: -76.2% to -42.1%, $P < 0.001$), and CX_3CR1+ intermediate monocytes (mean difference: -1.2%, CI95%: -0.8% to -1.5%, $P < 0.001$) were significantly decreased in iAMD patients compared to healthy controls (Fig. 2). All results of the correlations between the chemokine system and diagnosis can be found in Supplementary Fig. S1.

Chemokine system and treatment response of nAMD patients

Levels of chemokine system components were compared across nAMD treatment response groups (good, partial, and poor) with good responders as control group. Poor initial treatment response in nAMD patients had significantly decreased proportions of CD4+CXCR3+ (mean difference: -9.2%, CI95%: -18.1% to -0.2%, $P = 0.046$), CCR5+ classical monocytes (mean difference: -36.2%, CI95%: -67.1% to -4.4%, $P = 0.029$) and CCR2+ non-classical monocytes (mean difference: -38.8%, CI95%: -73.9% to -4.2%, $P = 0.031$) compared to good initial responders. Partial initial treatment response was significantly associated with lower proportions of CCR2+ classical monocytes (mean difference: -1.8%, CI95%: -2.5% to -0.1%, $P = 0.044$) compared to good initial responders (Fig. 3). There was a tendency of increased CXCL10 in poor initial responders compared to good initial responders (mean difference: 41.2%, CI95%: -4.5% to 86.8%, $P = 0.076$).

Poor 1-year treatment response was significantly associated with CD4+CXCR3+ (mean difference: -9.7, CI95%: -18.0% to -1.2%, $P = 0.025$), and CCR2+ non-classical monocytes (mean difference: -34.3%, CI95%: -66.9% to -1.4%, $P = 0.042$) compared to good 1-year responders. Partial 1-year treatment response was significantly associated with CCR5+ monocytes (mean difference: -15.2%, CI95%: = -27.5% to -2.2%,

P = 0.026) compared to good 1-year response (Fig.4). There was a tendency of increased CXCL10 in poor 1-year responders compared to good 1-year responders (mean difference: 35.2%, CI95%: -4.6% to 75.1%, *P* = 0.082). All results of the correlations between the chemokine system and treatment response can be found in Supplementary Figure S2 and S3.

Correlation networks

To describe the interactions between chemokines and chemokine receptors on CD4+ T cells, CD8+ T cells, and monocytes, we performed a correlation network analysis for each treatment response group. The link between analytes can highlight interactions, and centrality can describe the importance of the analytes in the groups.

Correlation networks between initial treatment response seemed to differ between the groups. Partial and poor initial responders appeared to have more complex chemokine correlation networks compared to good responders (26, 25 and 10 connections, respectively), reflecting the increased regulation (Fig. 5).

The 1-year treatment response groups did not seem to differ greatly in complexity, but unique correlations were found in each group (Fig. 6). The correlation networks between good initial and good 1-year responders appear similar, whereas those of partial and poor responders differ notably. These findings suggest that chemokine correlation networks may be more effective in predicting good responses compared to partial or poor responses.

The positive correlations between CCL3, CCL4 and CCL20 were consistent in all treatment response groups both initially and after one year. These might thus add negligible information regarding prediction of treatment response.

Single nucleotide polymorphisms

There was a significant association between the high-risk CFH rs1061170 genotypes (CC or CT) and elevated levels of CCL4 (*P* = 0.028), CD4+CCR2+ T cells (*P* = 0.002), CD8+CCR2+ T cells (*P* = 0.002), and CXCR2+ intermediate monocytes (*P* = 0.049) (Table 3). The high-risk ARMS2 rs10490924 genotypes (TT/TG) was also significantly associated with elevated expression levels of CX_3CR1+ monocytes and classical monocytes (*P* = 0.013 and *P* = 0.010, respectively), CCR1+ non-classical monocytes (*P* = 0.036, respectively), as well as decreased levels of CCR1+ intermediate monocytes (*P* = 0.049), although these differences were quite minor (Table 4).

Discussion

Angiogenesis is a crucial factor in nAMD pathogenesis and treatment. The MNV formation in these patients is highly mediated by stimulation from VEGF and current treatment aims to inhibit this pro-angiogenic growth factor. The chemokine system has important angiogenic properties and has previously been shown to

be affected in AMD patients.[5]

The chemokine system is a highly complex and integrated system.[31] Chemokine receptors are dynamic and adapt to environmental stimuli, with ligand bias and allosteric regulation causing activation of multiple pathways.[32] Additionally, the system modulates immune responses and T cell differentiation, influencing inflammation, angiogenesis, wound healing, and scar formation.[33–35]

Previous studies have found increased levels of systemic pro-angiogenic chemokines and receptors in nAMD patients. However, these studies have investigated few ligand-receptor axes[22], chemokines in isolation[12,14] or chemokine receptors in isolation.[15–17,20,21,23] In the same regard, the association between the chemokine system and treatment response in nAMD patients is sparsely investigated,[22,23] as well as in iAMD patients.[13,20,22]

In this study, we find plasma concentrations of CCL3 and CCL4 to be significantly increased in nAMD patients compared to healthy controls. Zhou et al. did not find a significant difference of CCL3 between nAMD patients and healthy controls, but did observe a significant difference for CCL4.[12] Activated monocytes and T cells can secrete CCL3 and CCL4, which can recruit monocytes and T cells,[36] and has been shown to be increased with aging.[37] We find increased levels of circulating CD4+CCR1+ T cells in nAMD patients compared to healthy controls. CCL3 can activate CD4+CCR1+ T cells, which will be recruited to sites of inflammation and promote their pro-inflammatory properties.[8] This includes inducing a pro-angiogenic environment, which may contribute to the neovascularization present in nAMD. The proportion of the anti-inflammatory CD8+CXCR3+ T cells was decreased in nAMD patients, which causes a dysregulation of inflammation and angiogenesis, which has also been observed previously by Falk et al.[20]

We also find decreased levels of plasma CCL2 and CCR2+ intermediate monocytes in iAMD and nAMD patients compared to healthy controls. The CCL2-CCR2 axis is involved in the chemotaxis and inflammation of monocytes. The downregulation of this axis suggests that there might be a decreased activation of circulating monocytes systemically.[38] Similarly CX_3CR1+ classical monocytes were statistically significantly decreased in iAMD and nAMD patients, as well as CX_3CR1+ intermediate monocytes in iAMD patients, suggesting these cells might be especially involved in iAMD pathogenesis.

Patient treatment response varies and patients with poor and partial response need improved treatment options. Because of the previously found alterations of the chemokine system and the difference found in this study between nAMD patients and healthy controls, we investigated whether differences of specific chemokines and chemokine receptors are associated with treatment response. nAMD patients with poor initial and 1-year treatment response had lower proportions of CD4+CXCR3+ T cells. There was also a tendency of poor initial treatment responders to have elevated levels of the ligand CXCL10. The lower proportions of the anti-angiogenic circulating CD4+CXCR3+ T cells and tendency of increased CXCL10 might cause angiogenesis. As CXCL10 binds to CXCR3, leading to the internalization of the receptor,[39] this

observation is consistent with the expected outcome. Dysregulation and blocking of CXCR3 has been shown to cause angiogenesis, and the CXCL10-CXCR3 axis mediates VEGF-induced angiogenesis.[40] Thus, treatment with anti-VEGF injections might not be sufficient to dampen the pro-angiogenic environment of the retina in poor responders. We have previously reported that interferon-γ is elevated in poor treatment responders, which induces the production of CXCL10.[41]

Proportions of the pro-angiogenic CCR5+ classical and CCR2+ non-classical monocytes were lower in poor initial responders, as well as CCR2+ monocytes in partial responders. Similarly, proportions were decreased of CCR2+ non-classical monocytes in poor 1-year responders, as well as CCR5+ monocytes in partial 1-year responders. The lower proportions of CCR2 and CCR5 might contribute to the development of nAMD by disrupting the balance of inflammation resolution and angiogenesis in the retina. CCR2 and CCR5 recruit monocytes, where they differentiate into macrophages that resolve inflammation and regulate angiogenesis.[42] A reduction of CCR2 in the poor and partial responders might lead to insufficient macrophage-mediated clearance causing chronic inflammation and angiogenesis in the retina. This would lead to increased VEGF production, and the conventional anti-VEGF injections might not be potent enough to reduce the leakage and regression of the MNV.

Most of the statistically significant differences found in this study are minor and might have limited biological significance. As AMD is characterized by chronic low-grade inflammation, it was expected that individual chemokines would be only slightly affected. Thus, correlation networks were generated, which can reveal immune regulation dynamics in complex integrated systems. The correlation networks showed substantial differences between the groups. Initial treatment response groups of nAMD patients showed different correlation phenotypes, where partial and poor initial responders seemed to have a more complex correlation network compared to good responders. The complexity indicates a more pro-inflammatory regulation in these groups, which could cause the dysregulation of angiogenesis systemically. A definitive pattern after one year was less apparent, and the chemokine correlation profile might thus be of most value post-loading dose. This was expected, as patients will be subjected to differing treatment intervals and confounding factors after one year, rather than the similar initial treatment course.

The two main risk polymorphisms for developing AMD, CFH rs1061170 and ARMS2 rs10490924, are also important in activation of the complement system.[24] In this study, we evaluated the effect of these polymorphisms on the chemokine system in nAMD patients. We found the CFH risk genotypes were associated with elevated levels of CCL4, CD4+CCR2+ T cells, CD8+CCR2+ T cells, and CXCR2+ intermediate monocytes. The ARMS2 risk genotype was associated with CX_3CR1+ monocytes and classical monocytes, as well as CCR1+ intermediate monocytes and non-classical monocytes. This adds to our understanding that genetic susceptibility is involved in the low-grade inflammation in nAMD, caused by the chemokine system, at least in part. The differences were statistically significant but were minor. However,

genetic risk factors in chemokine receptor genes CX_3CR1 and CCR3 has previously been identified in AMD, which supports the primary role of the chemokine system in AMD pathogenesis.[43,44]

Limitations of this study includes the observational design, which is unable to determine whether these differences in the chemokine system are causal or reflect an adaptation to the condition. The categorical groupings build on previous classifications focused on clinical implementation;[1,2,45] however, this approach restricts the capture of subtle differences. The relatively small number of patients in the poor response group was also a limitation, as it may obscure potentially significant correlations. Since the analyses in this study build upon existing evidence of specific dysfunctional pathways in the chemokine system in nAMD, it might not be necessary to adjust for multiple testing.[46] However, given the large number of statistical tests, further investigation is required to confirm the significance of the chemokine system in the treatment response of nAMD.

Future studies should validate these findings in other study populations to robustly establish the described associations. Investigating changes in the chemokine system before and after treatment would enhance clinical relevance by providing insights into the dynamics of these pathways and their role in treatment response.

In conclusion, our findings underscore the critical role of the chemokine system in nAMD pathogenesis and treatment response. Dysregulation of chemokine-mediated inflammation and angiogenesis may contribute to disease progression and therapeutic resistance. Further studies are warranted to explore targeted therapies that modulate the chemokine system to improve outcomes in nAMD patients, particularly those with poor or partial response to anti-VEGF treatment.

References

1. Amoaku WM, Chakravarthy U, Gale R, et al. Defining response to anti-VEGF therapies in neovascular AMD. *Eye*. 2015;29(6):721-731. doi:10.1038/eye.2015.48

2. Thomsen AK, Fasih-Ahmad S, Sadda S, Sørensen TL. Initial treatment response can predict one-year treatment outcomes in neovascular age-related macular degeneration treated according to the observe-and-plan regimen. *Acta Ophthalmologica*. Published online November 27, 2024:aos.16801. doi:10.1111/aos.16801

3. Palbo A, Roed Rasmussen ML, Hansen MS, Subhi Y. Association between visual impairment and suicide: A systematic review and meta-analysis. *Acta Ophthalmologica*. 2024;102(3):263-273. doi:10.1111/aos.15730

4. Souied EH, Leveziel N. Toward Personalized Medicine for Age-Related Macular Degeneration. *American Journal of Ophthalmology*. 2012;154(3):427-428. doi:10.1016/j.ajo.2012.05.022

5. Rozing MP, Durhuus JA, Krogh Nielsen M, et al. Age-related macular degeneration: A two-level model hypothesis. *Progress in Retinal and Eye Research*. 2020;76:100825. doi:10.1016/j.preteyeres.2019.100825

6. Thomsen AK, Steffensen MA, Villarruel Hinnerskov JM, et al. Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response. *J Neuroinflammation*. 2024;21(1):284. doi:10.1186/s12974-024-03273-7

7. Liu Z, Liang Q, Ren Y, et al. Immunosenescence: molecular mechanisms and diseases. *Sig Transduct Target Ther*. 2023;8(1):200. doi:10.1038/s41392-023-01451-2

8. Zlotnik A, Yoshie O. The Chemokine Superfamily Revisited. *Immunity*. 2012;36(5):705-716. doi:10.1016/j.immuni.2012.05.008

9. Chaudhary JK, Danga AK, Kumari A, Bhardwaj A, Rath PC. Role of chemokines in aging and age-related diseases. *Mechanisms of Ageing and Development*. Published online December 2024:112009. doi:10.1016/j.mad.2024.112009

10. Mo R, Chen J, Han Y, et al. T Cell Chemokine Receptor Expression in Aging. *The Journal of Immunology*. 2003;170(2):895-904. doi:10.4049/jimmunol.170.2.895

11. Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol*. 2010;11(1):30. doi:10.1186/1471-2172-11-30

12. Zhou H, Zhao X, Chen Y. Plasma Cytokine Profiles in Patients With Polypoidal Choroidal Vasculopathy and Neovascular Age-Related Macular Degeneration. *Asia-Pacific Journal of Ophthalmology*. 2022;11(6):536-542. doi:10.1097/APO.0000000000000577

13. Palestine AG, Wagner BD, Patnaik JL, et al. Plasma C-C Chemokine Concentrations in Intermediate Age-Related Macular Degeneration. *Front Med*. 2021;8:710595. doi:10.3389/fmed.2021.710595

14. Agrawal R, Balne PK, Wei X, et al. Cytokine Profiling in Patients With Exudative Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy. *Invest Ophthalmol Vis Sci*. 2019;60(1):376. doi:10.1167/iovs.18-24387

15. Grunin M, Burstyn-Cohen T, Hagbi-Levi S, Peled A, Chowers I. Chemokine Receptor Expression in Peripheral Blood Monocytes from Patients with Neovascular Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2012;53(9):5292. doi:10.1167/iovs.11-9165

16. Subhi Y, Krogh Nielsen M, Molbech CR, Sørensen TL. Altered proportion of CCR2 $^{+}$ and CX3CR1 $^{+}$ circulating monocytes in neovascular age-related macular degeneration and polypoidal choroidal vasculopathy. *Clinical Exper Ophthalmology*. 2018;46(6):661-669. doi:10.1111/ceo.13152

17. Choi YJ, Lim D, Byeon SH, Shin EC, Chung H. Chemokine Receptor Profiles of T Cells in Patients with Age-Related Macular Degeneration. *Yonsei Med J*. 2022;63(4):357. doi:10.3349/ymj.2022.63.4.357

18. Martinez Villarruel Hinnerskov J, Krogh Nielsen M, Kai Thomsen A, et al. Chemokine Receptor Profile of T Cells and Progression Rate of Geographic Atrophy Secondary to Age-related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2024;65(1):5. doi:10.1167/iovs.65.1.5

19. Krogh Nielsen M, Subhi Y, Molbech CR, Falk MK, Nissen MH, Sørensen TL. Chemokine Profile and the Alterations in CCR5-CCL5 Axis in Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2020;61(4):28. doi:10.1167/iovs.61.4.28

20. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. Dysregulation of CXCR3 Expression on Peripheral Blood Leukocytes in Patients With Neovascular Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2014;55(7):4050. doi:10.1167/iovs.14-14107

21. Singh A, Subhi Y, Krogh Nielsen M, et al. Systemic frequencies of T helper 1 and T helper 17 cells in patients with age-related macular degeneration: A case-control study. *Sci Rep*. 2017;7(1):605. doi:10.1038/s41598-017-00741-4

22. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. CX3CL1/CX3CR1 and CCL2/CCR2 Chemokine/Chemokine Receptor Complex in Patients with AMD. Sennlaub F, ed. *PLoS ONE*. 2014;9(12):e112473. doi:10.1371/journal.pone.0112473

23. Bjerregaard T, Krogh Nielsen M, Molbech CR, Subhi Y, Sørensen TL. Treatment failure in neovascular age-related macular degeneration is associated with a complex chemokine receptor profile. *BMJ Open Ophthalmol*. 2019;4(1):e000307. doi:10.1136/bmjophth-2019-000307

24. Smailhodzic D, Klaver CCW, Klevering BJ, et al. Risk Alleles in CFH and ARMS2 Are Independently Associated with Systemic Complement Activation in Age-related Macular Degeneration. *Ophthalmology*. 2012;119(2):339-346. doi:10.1016/j.ophtha.2011.07.056

25. 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 USA*. 2005;102(20):7227-7232. doi:10.1073/pnas.0501536102

26. Klein RJ, Zeiss C, Chew EY, et al. Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science*. 2005;308(5720):385-389. doi:10.1126/science.1109557

27. Chang F, Huang C, Yang C, et al. Genetics in neovascular age-related macular degeneration susceptibility and treatment response to ANTI-VEGF intravitreal injection: A case series study. *Clinical Exper Ophthalmology*. 2024;52(6):655-664. doi:10.1111/ceo.14388

28. Fleckenstein M, Mitchell P, Freund KB, et al. The Progression of Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Ophthalmology*. 2018;125(3):369-390. doi:10.1016/j.ophtha.2017.08.038

29. Ferris FL, Wilkinson CP, Bird A, et al. Clinical Classification of Age-related Macular Degeneration. *Ophthalmology*. 2013;120(4):844-851. doi:10.1016/j.ophtha.2012.10.036

30. Thomsen AK, Krogh Nielsen M, Liisborg C, Sørensen T. Interleukin-8 Promoter Polymorphism −251 A/T and Treatment Response in Neovascular Age-related Macular Degeneration. *OPTH*. 2024;Volume 18:537-543. doi:10.2147/OPTH.S448794

31. Dyer DP, Medina-Ruiz L, Bartolini R, et al. Chemokine Receptor Redundancy and Specificity Are Context Dependent. *Immunity*. 2019;50(2):378-389.e5. doi:10.1016/j.immuni.2019.01.009

32. D'Agostino G, García-Cuesta EM, Gomariz RP, Rodríguez-Frade JM, Mellado M. The multilayered complexity of the chemokine receptor system. *Biochemical and Biophysical Research Communications*. 2020;528(2):347-358. doi:10.1016/j.bbrc.2020.02.120

33. Yates CC, Hebda P, Wells A. Skin Wound Healing and Scarring: Fetal Wounds and Regenerative Restitution. *Birth Defects Research Pt C*. 2012;96(4):325-333. doi:10.1002/bdrc.21024

34. Cecchinato V, Martini V, Pirani E, Ghovehoud E, Uguccioni M. The chemokine landscape: one system multiple shades. *Front Immunol*. 2023;14:1176619. doi:10.3389/fimmu.2023.1176619

35. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. *Nat Immunol*. 2001;2(2):102-107. doi:10.1038/84205

36. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell–dendritic cell interaction. *Nature*. 2006;440(7086):890-895. doi:10.1038/nature04651

37. Thorsen SU, Eising S, Mortensen HB, et al. Systemic Levels of CCL 2, CCL 3, CCL 4 and CXCL 8 Differ According to Age, Time Period and Season among Children Newly Diagnosed with type 1 Diabetes and their Healthy Siblings. *Scand J Immunol*. 2014;80(6):452-461. doi:10.1111/sji.12240

38. Pozzi S, Satchi-Fainaro R. The role of CCL2/CCR2 axis in cancer and inflammation: The next frontier in nanomedicine. *Advanced Drug Delivery Reviews*. 2024;209:115318. doi:10.1016/j.addr.2024.115318

39. Colvin RA, Campanella GSV, Sun J, Luster AD. Intracellular Domains of CXCR3 That Mediate CXCL9, CXCL10, and CXCL11 Function. *Journal of Biological Chemistry*. 2004;279(29):30219-30227. doi:10.1074/jbc.M403595200

40. Yates-Binder CC, Rodgers M, Jaynes J, Wells A, Bodnar RJ, Turner T. An IP-10 (CXCL10)-Derived Peptide Inhibits Angiogenesis. Proost P, ed. *PLoS ONE*. 2012;7(7):e40812. doi:10.1371/journal.pone.0040812

41. Thomsen, AK, Steffensen, MA, Hinnerskov, JMV, et al. Plasma Interferon-gamma is Associated with Poor Treatment Response in Neovascular Age-Related Macular Degeneration. 2025;17. doi:10.14336/AD.2024.1585

42. Spahn JH, Kreisel D. Monocytes in Sterile Inflammation: Recruitment and Functional Consequences. *Arch Immunol Ther Exp*. 2014;62(3):187-194. doi:10.1007/s00005-013-0267-5

43. Combadière 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(10):2920-2928. doi:10.1172/JCI31692

44. Sharma NK, Gupta A, Prabhakar S, Singh R, Bhatt AK, Anand A. CC chemokine receptor-3 as new target for age-related macular degeneration. *Gene*. 2013;523(1):106-111. doi:10.1016/j.gene.2013.03.052

45. Thomsen AK, Gøttsche LF, Hinnerskov JMV, Falk MK, Sørensen TL. Microperimetry and Structural Risk Factors on OCT in Intermediate Age-Related Macular Degeneration. *Ophthalmology Retina*. Published online March 2024:S2468653024000885. doi:10.1016/j.oret.2024.02.016

46. Boulesteix AL, Hoffmann S. To adjust or not to adjust: it is not the tests performed that count, but how they are reported and interpreted. *BMJ Med*. 2024;3(1):e000783. doi:10.1136/bmjmed-2023-000783

Acknowledgements

This work was supported by VELUX FONDEN (00024226), Region Zealand PhD Grant (R29-A1337), Synoptik Foundation, Fight for Sight Denmark (Øjenforeningen), Grosserer L.F. Foghts Foundation, and King Christian X's Foundation. The funding organizations had no role in the design or conduct of this research.

Conflict of interest statement

BH and HV have obtained research funding from Bayer for a study not related to the present. All other authors declare no financial or non-financial conflicts of interest.

	Treatment response
Good	Total regression of IRF and SRF
Partial	Persistence of IRF and/or SRF and reduction of CRT
Poor	Persistence of IRF and/or SRF and unchanged or increased CRT

IRF = Intraretinal fluid
SRF = Subretinal fluid
CRT = Central retinal thickness

Table 1. Definitions of treatment responses to intravitreal anti-VEGF injections.

Chemokine	**Chemokine Receptor**
CCL3	CCR1, CCR5
CCL4	CCR5
CCL20	CCR6
CCL2	CCR2
CXCL8	CXCR2
CXCL10	CXCR3
*	CX_3CR1

Table 2. Investigated chemokines and chemokine receptor pairs. CC = C-C motif, CXC = C-X-C motif, CX_3C = C-X_3-C motif, L = ligand, R = receptor, *The ligand of CX_3CR1 is CX_3CL1, which was not analyzed in this study.

	CFH, rs1061170		
Plasma chemokines, mean (SD)	CC/CT (high risk), n = 82	TT (low risk), n = 18	*P* value
CCL3 [pg/ml]	18.35 (1.70)	15.45 (1.44)	0.96
CCL4 [pg/ml]	105.78 (1.58)	79.18 (1.40)	**0.028**
CCL20 [pg/ml]	15.57 (1.96)	15.42 (2.80)	0.46
CCL2 [pg/ml]	175.03 (1.34)	165.31 (1.28)	0.64
CXCL10 [pg/ml]	1379.69 (1.76)	1198.63 (1.65)	0.41
CXCL8 [% above lower detection limit]	66.67%	81.25%	0.37*
CD4+ T cells [%], mean (SD)			
CCR1+	1.78 (1.71)	1.60 (1.59)	0.56
CCR2+	1.27 (1.25)	0.80 (0.80)	**0.041**
CCR5+	2.89 (2.16)	3.13 (1.77)	0.28
CCR6+	26.14 (10.02)	26.49 (8.00)	0.88
CXCR3+	48.49 (11.12)	46.92 (8.95)	0.55
CD8+ T cells [%], mean (SD)			
CCR1+	24.90 (1.54)	27.59 (1.75)	0.73
CCR2+	3.50 (1.79)	1.53 (2.10)	**0.002**
CCR5+	17.43 (2.65)	18.92 (3.48)	0.73
CCR6+	9.87 (2.94)	5.23 (1.62)	0.72
CXCR3+	71.18 (10.63)	71.24 (8.31)	0.98
Monocytes [%], mean (SD)			
CCR1+	97.25 (1.01)	97.21 (1.01)	0.46
CCR2+	78.82 (4.55)	77.92 (3.53)	0.39
CCR5+	2.84 (1.31)	3.03 (1.45)	0.71
CXCR2+	84.40 (1.12)	83.24 (1.14)	0.97
CXCR3+	64.14 (4.70)	65.02 (4.80)	0.51
CX_3CR1+	98.52 (1.02)	98.85 (1.01)	0.23
Classical Monocytes [%], mean (SD)			
CCR1+	97.25 (1.01)	97.21 (1.01)	0.46
CCR2+	87.05 (3.83)	87.64 (3.43)	0.54
CCR5+	95.55 (1.02)	95.45 (1.02)	0.73
CXCR2+	88.04 (3.25)	87.94 (4.93)	0.94
CXCR3+	63.62 (5.24)	64.61 (5.69)	0.53
CX_3CR1+	99.37 (0.30)	99.46 (0.28)	0.27
Intermediate Monocytes [%], mean (SD)			
CCR1+	86.83 (1.07)	86.59 (1.06)	0.79
CCR2+	76.44 (1.15)	74.39 (1.12)	0.23
CCR5+	8.53 (1.76)	8.38 (1.77)	0.94
CXCR2+	84.44 (1.08)	81.51 (1.07)	**0.049**
CXCR3+	65.47 (7.01)	64.38 (4.33)	0.42
CX_3CR1+	99.32 (0.60)	99.38 (0.45)	0.76
Non-classical Monocytes [%], mean (SD)			
CCR1+	48.45 (9.98)	48.61 (9.55)	0.95
CCR2+	4.08 (1.54)	4.22 (1.43)	0.53
CCR5+	8.12 (1.61)	6.29 (1.61)	0.068
CXCR2+	8.90 (2.00)	7.34 (3.32)	0.30
CXCR3+	68.43 (1.10)	68.13 (1.06)	0.51
CX_3CR1+	90.59 (1.09)	93.31 (1.06)	0.12
Bold values indicate statistical significance ($P < 0.05$). Statistical test: Welch two sample t-test *Fisher's exact test			

Table 3. Chemokines and chemokine receptor levels stratified according to CFH, rs1061170 genotype.

	ARMS2, rs10490924		
Plasma chemokines, mean (SD)	TT/TG (high risk), n = 45	GG (low risk), n = 55	*P* value
CCL3 [pg/ml]	14.75 (1.88)	21.79 (1.45)	0.20
CCL4 [pg/ml]	99.78 (1.64)	102.80 (1.51)	0.88
CCL20 [pg/ml]	17.15 (1.99)	13.53 (2.20)	0.30
CCL2 [pg/ml]	172.11 (1.31)	174.86 (1.39)	0.66
CXCL10 [pg/ml]	1300.27 (1.80)	1408.53 (1.69)	0.50
CXCL8 [% above lower detection limit]	70.00	68.63	0.99*
CD4+ T cells [%], mean (SD)			
CCR1+	1.66 (0.77)	1.86 (1.40)	0.76
CCR2+	1.28 (1.66)	1.08 (0.51)	0.61
CCR5+	2.86 (1.70)	3.02 (2.56)	0.35
CCR6+	27.16 (8.97)	24.98 (10.50)	0.28
CXCR3+	47.86 (9.96)	48.69 (11.80)	0.71
CD8+ T cells [%], mean (SD)			
CCR1+	25.29 (1.46)	25.41 (1.66)	0.61
CCR2+	3.35 (1.90)	3.17 (1.65)	0.99
CCR5+	18.87 (2.18)	16.18 (3.46)	0.14
CCR6+	9.97 (2.12)	8.01 (1.64)	0.26
CXCR3+	71.13 (10.57)	71.27 (9.95)	0.95
Monocytes [%], mean (SD)			
CCR1+	97.28 (1.02)	97.19 (1.01)	0.94
CCR2+	78.55 (3.98)	78.76 (4.73)	0.81
CCR5+	2.77 (1.37)	3.01 (1.31)	0.24
CXCR3+	63.91 (4.16)	64.75 (5.32)	0.40
CXCR2+	84.70 (1.09)	83.57 (1.15)	0.80
CX_3CR1+	98.81 (1.00)	98.27 (1.02)	**0.013**
Classical Monocytes [%], mean (SD)			
CCR1+	97.29 (1.01)	97.19 (1.02)	0.94
CCR2+	87.07 (3.88)	87.25 (3.64)	0.82
CCR5+	95.44 (1.02)	98.44 (1.02)	0.66
CXCR3+	63.41 (4.57)	64.26 (6.12)	0.45
CXCR2+	88.06 (3.38)	87.98 (3.81)	0.91
CX_3CR1+	99.45 (0.30)	99.30 (0.27)	**0.010**
Intermediate Monocytes [%], mean (SD)			
CCR1+	85.64 (1.05)	88.24 (1.07)	**0.049**
CCR2+	76.46 (1.15)	75.66 (1.13)	0.63
CCR5+	8.14 (1.75)	8.97 (1.77)	0.39
CXCR3+	64.73 (6.33)	65.99 (7.01)	0.36
CXCR2+	83.44 (1.08)	84.60 (1.07)	0.42
CX_3CR1+	99.34 (0.64)	99.32 (0.49)	0.34
Non-classical Monocytes [%], mean (SD)			
CCR1+	50.33 (9.81)	46.14 (9.54)	**0.036**
CCR2+	4.20 (1.47)	3.99 (1.58)	0.39
CCR5+	7.68 (1.62)	7.96 (1.63)	0.83
CXCR3+	67.75 (6.34)	69.16 (5.86)	0.29
CXCR2+	8.42 (2.10)	8.90 (2.01)	0.43
CX_3CR1+	91.33 (1.09)	90.67 (1.08)	0.64
Bold values indicate statistical significance ($P < 0.05$). Statistical test: Welch two sample t-test *Fisher's exact test			

Table 4. Chemokines and chemokine receptor levels stratified according to ARMS2, rs10490924 genotype.

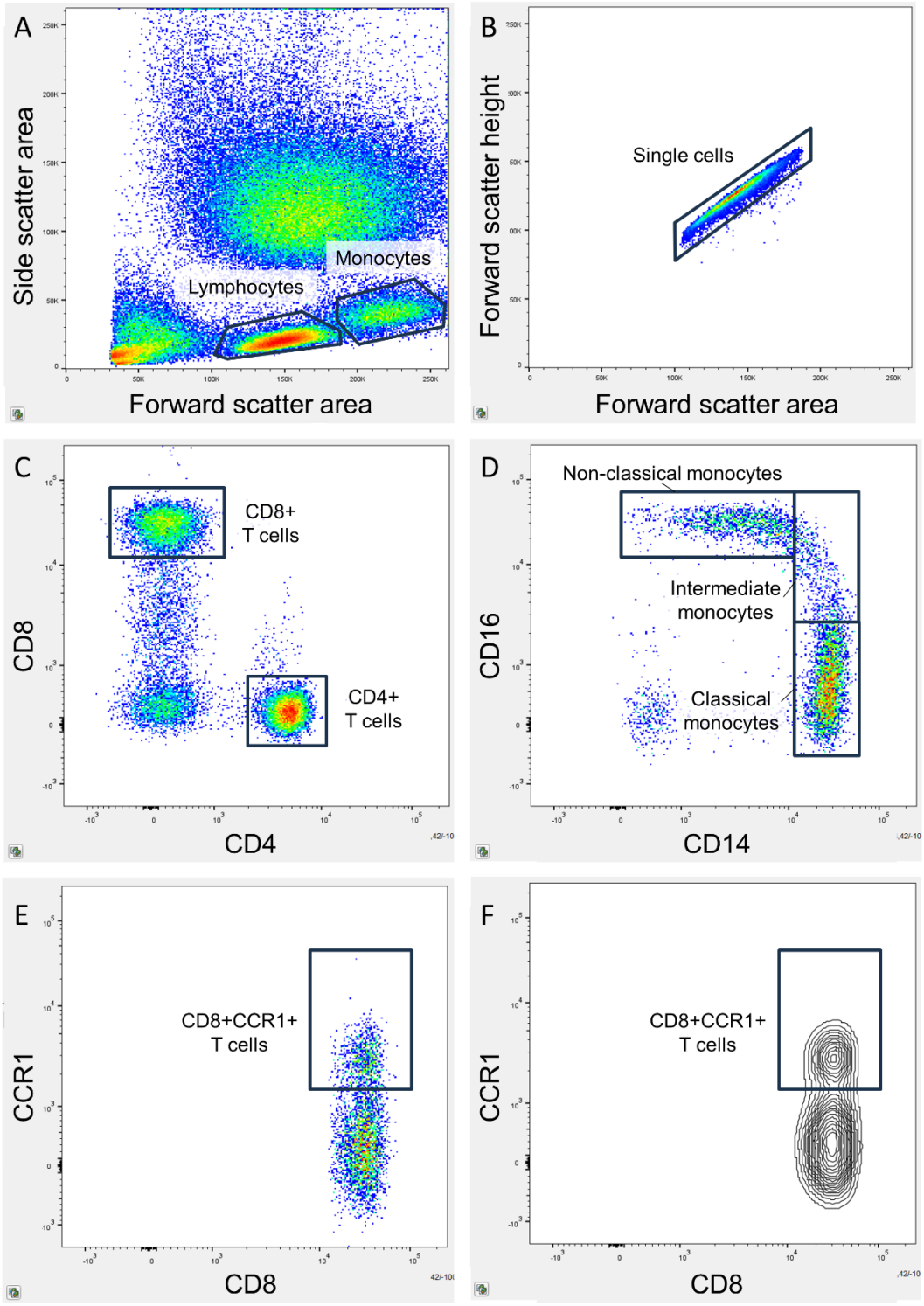


Figure 1. Flow cytometry gating strategy. (A) Lymphocytes and monocytes were identified on the forward-side-scatter plot. (B) Singlet cells were identified on the forward area-forward height plot. (C) Lymphocytes were gated for CD4 and CD8 to identify CD4+ and CD8+ T cells. (D) Monocytes were gated for CD14 and CD16, and monocyte subsets were identified as classical ($CD14^{high}CD16^{low}$), intermediate ($CD14^{high}CD16^{high}$) and non-classical ($CD14^{low}CD16^{high}$). (E) Chemokine receptors were gated, in this example CCR1 on CD8+ T cells using the pseudocolor plot and (F) contour plot.

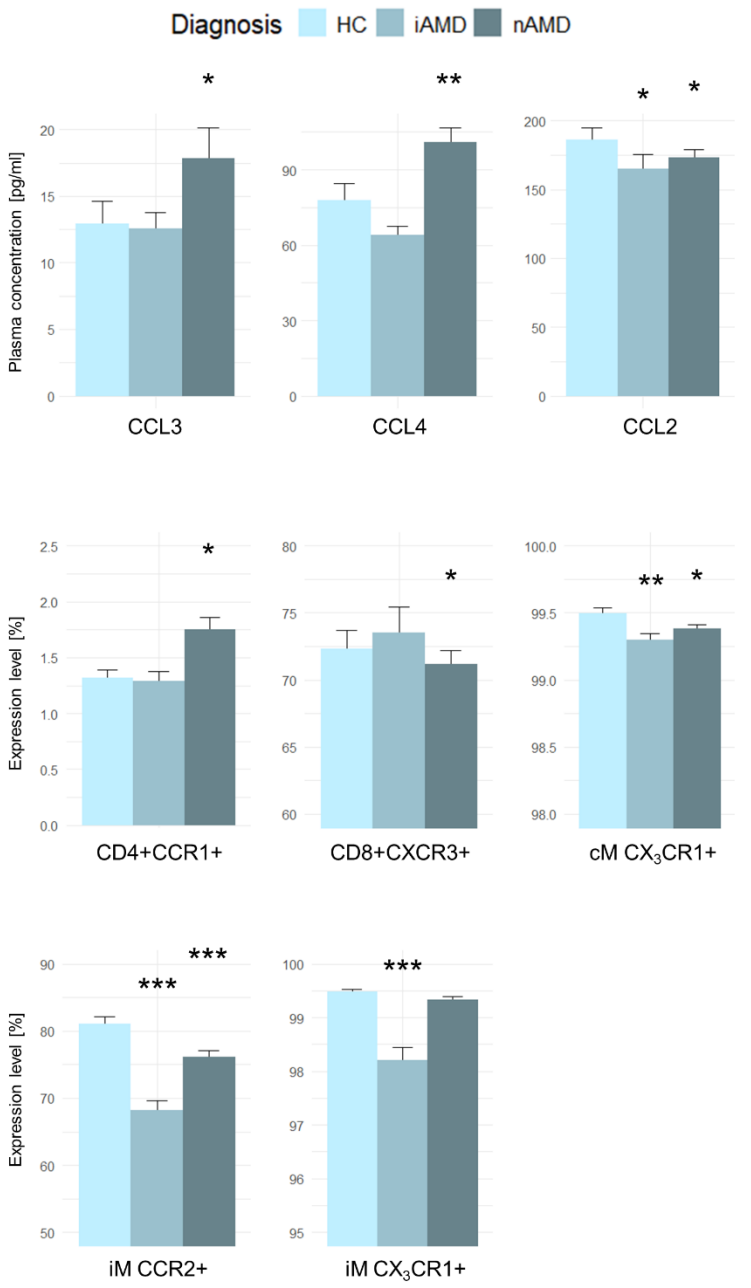


Figure 2. Chemokine and chemokine receptor levels significantly associated with diagnosis. HC = healthy controls; iAMD = intermediate age-related macular degeneration; nAMD = neovascular age-related macular degeneration; cM = classical monocytes; iM = intermediate monocytes. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to the reference group (healthy controls) adjusted for age and smoking status.

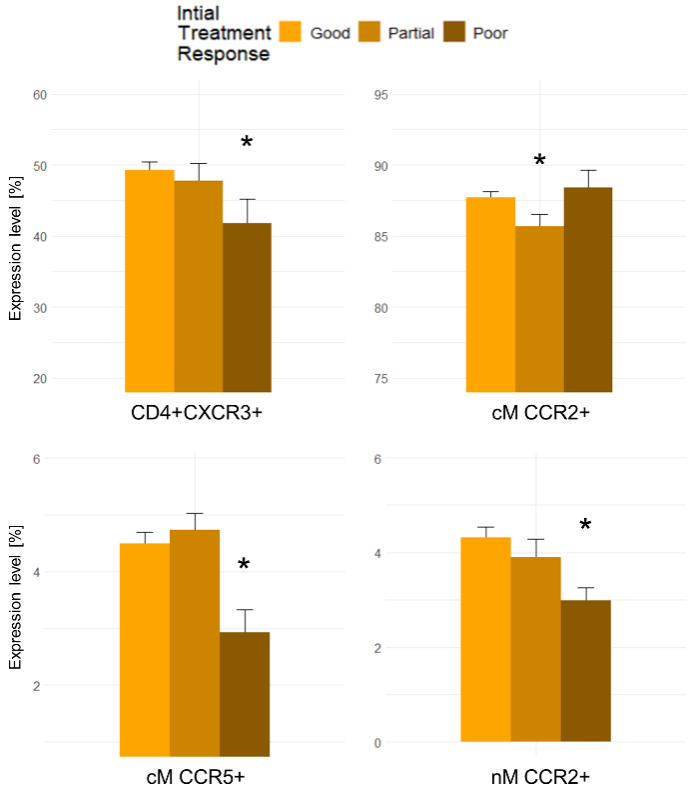


Figure 3. Chemokine receptor levels significantly associated with initial treatment response. cM = classical monocytes; nM = non-classical monocytes. * $P < 0.05$ compared to the reference group (good responders) adjusted for age and smoking status.

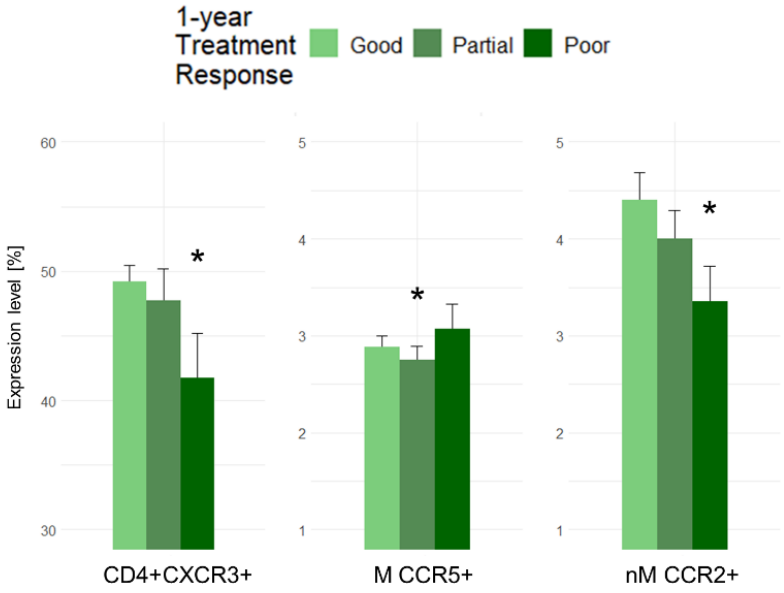


Figure 4. Chemokine receptor levels significantly associated with 1-year treatment response. M = monocytes; nM = non-classical monocytes. * $P < 0.05$ compared to the reference group (good responders) adjusted for age and smoking status.

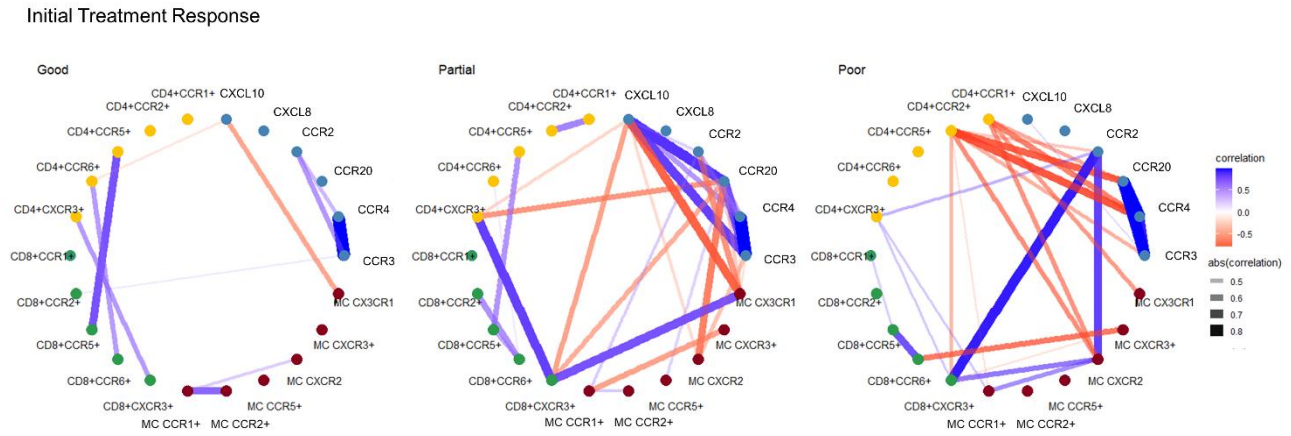


Figure 5. Correlation networks of the chemokine system according to initial treatment response in patients with neovascular age-related macular degeneration. Chemokines and chemokine receptors (nodes) are connected by correlations (edges), with a threshold of $|r| > 0.4$ and $P < 0.05$. MC = monocytes.

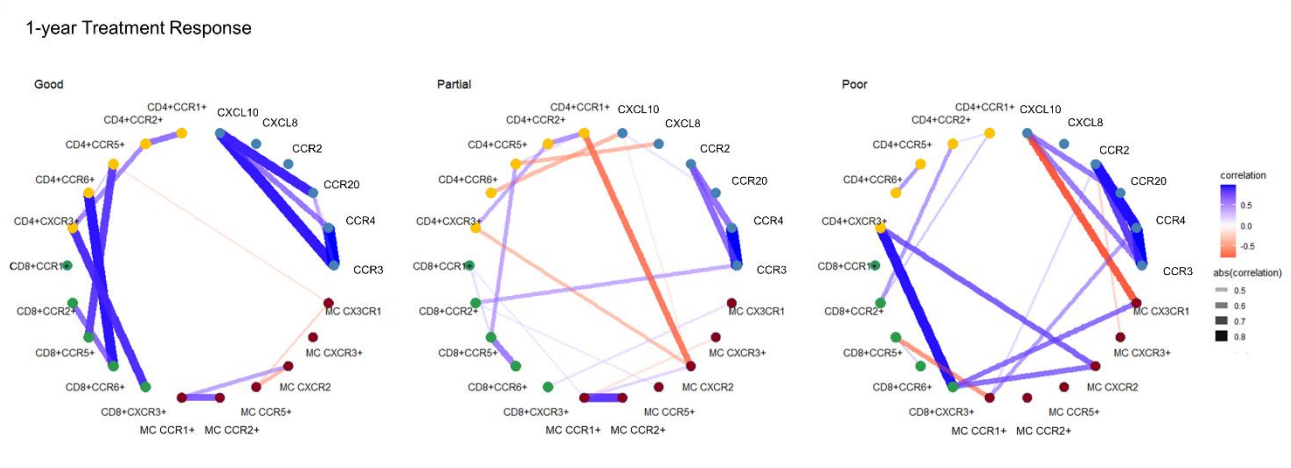


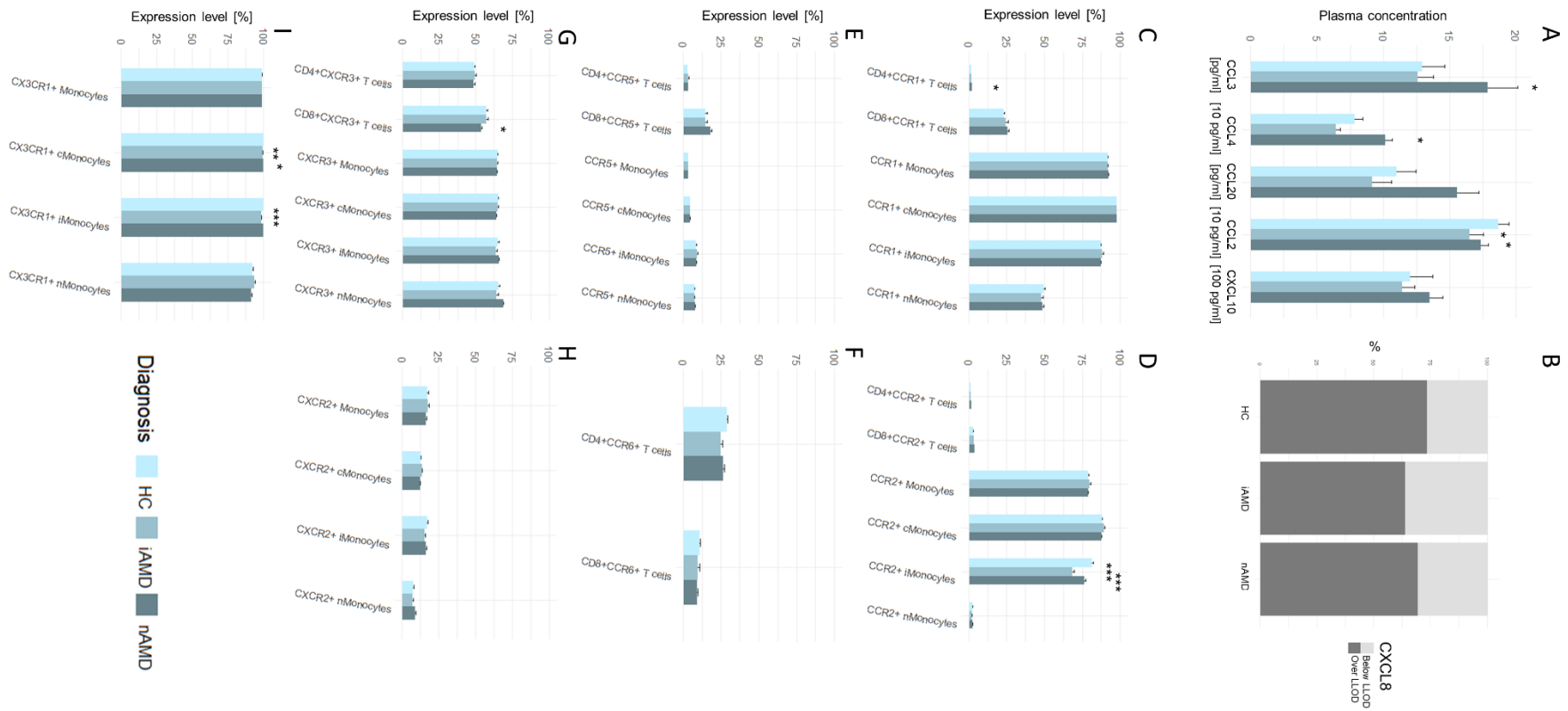
Figure 6. Correlation networks of the chemokine system according to 1-year treatment response in patients with neovascular age-related macular degeneration. Chemokines and chemokine receptors (nodes) are connected by correlations (edges), with a threshold of $|r| > 0.4$ and $P < 0.05$. MC = monocytes.

Supplementary table S1. Chemokine assays.

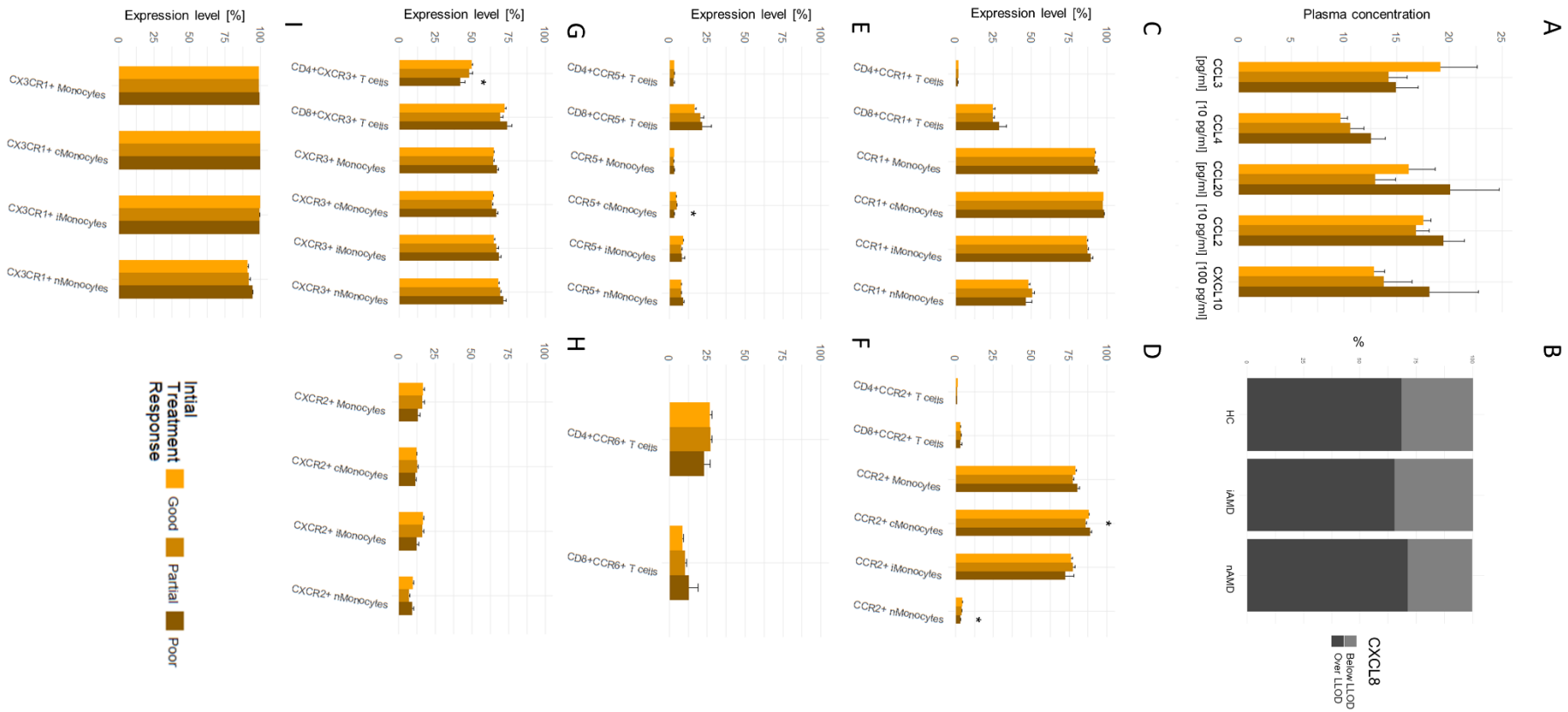
Analytes	**CCL2, CCL3, CCL4, CXCL8, CXCL10**	**CCL20**
Cytokine assay	V-PLEX Chemokine Panel 1 (human)	V-PLEX TH17 Panel 1 (human)
Catalog number	K151A9H-1	K15085D-1
Abbreviations: CCL = C-C motif chemokine ligand, CXCL = C-X-C motif chemokine ligand. Manufacturer: Mesoscale Discovery, Rockville, MD, USA.		

Supplementary table S2. Flow cytometry antibodies.

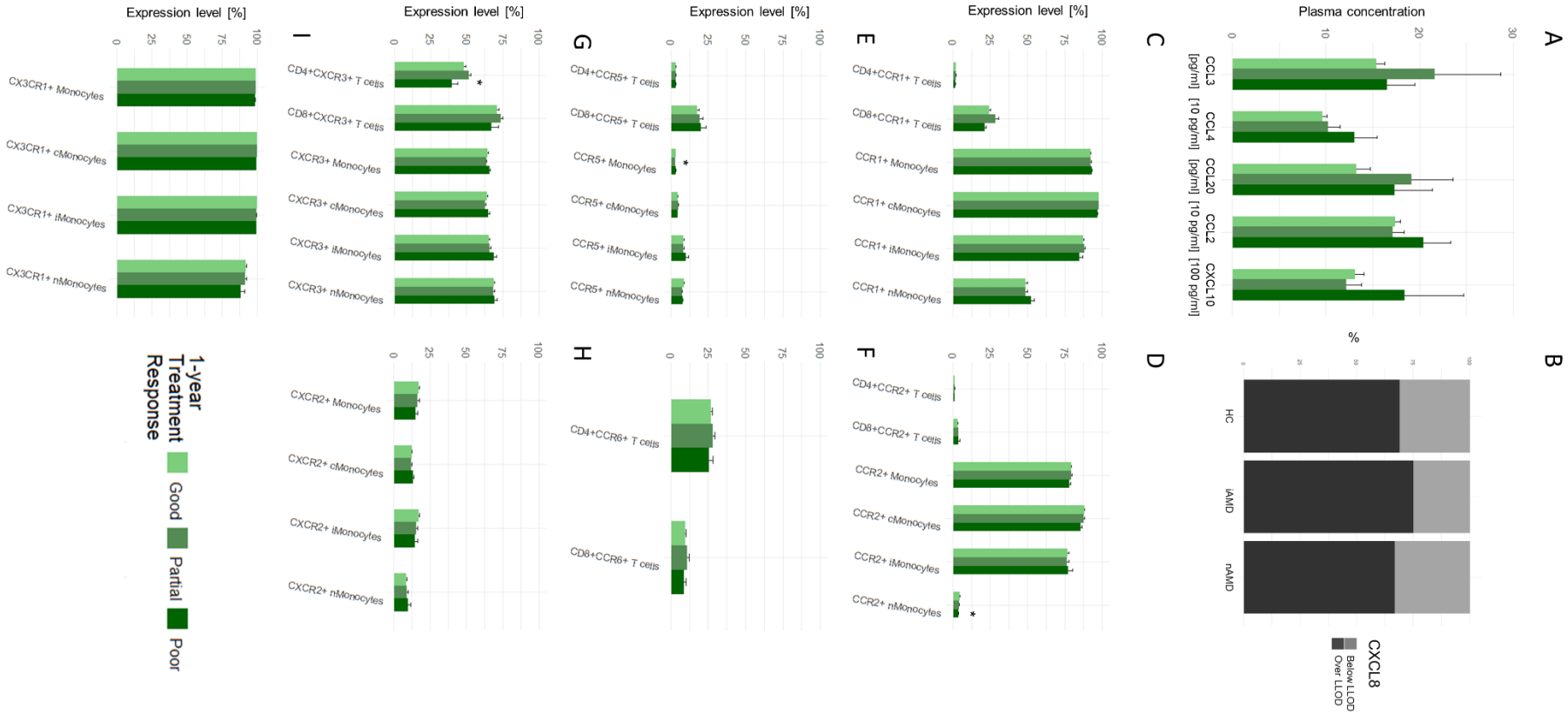
Surface marker	**Fluorochrome**	**Manufacturer**	**Catalog number**
CD4	PerCP	R&D Systems	FAB3791C
CD8	Brilliant Violet	BioLegend	301048
CD14	Pacific Blue	BioLegend	325616
CD16	APC-Cy7	BioLegend	302018
CCR1	APC	BioLegend	362908
CCR2	PE	R&D Systems	FAB151P
CCR5	FITC	R&D Systems	FAB182F
CCR6	FITC	BioLegend	353412
CXCR2	APC	BioLegend	320710
CXCR3	PE/Cy7	BioLegend	353720
CX_3CR1	FITC	BioLegend	341606
APC= Allophycyanin, APC/Cy7 = Allophycocyanin-cyanine 7, FITC = Fluorescein isothiocyanate, PE = Phycoerythrin, PE/Cy7 = Phycoerythrin-cyanine 7, PerCP = Peridinin-chlorophyll-protein. R&D Systems, Minneapolis, MN, USA. BioLegend, San Diego, CA, USA.			



Supplementary figure S1. *Chemokine and chemokine receptor levels according to diagnosis. HC = healthy controls; iAMD = intermediate age-related macular degeneration; nAMD = neovascular age-related macular degeneration; LLOD = lower level of detection; cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * P < 0.05; ** P < 0.01; *** P < 0.001 compared to the reference group (healthy controls) adjusted for age and smoking status.*



Supplementary figure S2. *Chemokine and chemokine receptor levels according to initial treatment response. LLOD = lower level of detection; cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * P < 0.05 compared to the reference group (good responders) adjusted for age and smoking status.*



Supplementary figure S3. *Chemokine and chemokine receptor levels according to 1-year treatment response. LLOD = lower level of detection; cMonocytes = classical monocytes; iMonocytes = intermediate monocytes; nMonocytes = non-classical monocytes. * P < 0.05 compared to the reference group (good responders) adjusted for age and smoking status.*

7.4. Study IV

Title

Association of Neutrophil-to-Lymphocyte Ratio and Neutrophil Activation with Treatment Response in Neovascular AMD.

Running title

Neutrophils and AMD Treatment Response

Authors

Alexander Kai Thomsen[1,2], alexanderkaithomsen@gmail.com
Maria Abildgaard Steffensen[3], mariaas@sund.ku.dk
Kathrine Gotfredsen[1,2], kathg@regionsjaelland.dk
Henrik Vorum[4,5], henrik.vorum@rn.dk
Bent Honoré[4,6], bh@biomed.au.dk
Mogens Holst Nissen[2,3], mhni@sund.ku.dk
Torben Lykke Sørensen[1,2], tlso@regionsjaelland.dk

Affiliations

1. Department of Ophthalmology, Zealand University Hospital, Roskilde, Denmark.
2. Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.
3. Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark.
4. Department of Clinical Medicine, Aalborg University, Aalborg, Denmark
5. Department of Ophthalmology, Aalborg University Hospital, Aalborg, Denmark.
6. Department of Biomedicine, Aarhus University, Aarhus, Denmark.

Corresponding author

Alexander Kai Thomsen, MD, Department of Ophthalmology, Zealand University Hospital, Sygehusvej 10, 4000 Roskilde, Denmark; e-mail: alexanderkaithomsen@gmail.com; telephone: +45 47323900; fax: +45 46321055.

Abstract

Background: The neutrophil-to-lymphocyte ratio (NLR) and alterations of activation surface markers on circulating neutrophils have been suggested to be involved in the pathogenesis of age-related macular degeneration (AMD). The aim of this study is to investigate the relationship between NLR, activation surface markers on neutrophils, AMD stage, and treatment response in neovascular AMD (nAMD).

Methods: Treatment-naïve nAMD patients, intermediate AMD (iAMD) patients, and healthy controls were consecutively enrolled in this prospective study. Treatment response in nAMD patients was evaluated after the loading phase and after one year. NLR and activation surface markers on circulating neutrophils (CD11a, CD11b, CD31, CD66b, CD162, and CD182) were examined with flow cytometry. NLR and activation surface markers were compared between healthy controls, iAMD, and nAMD patients, as well as between nAMD patients with good, partial, and poor treatment response. Polymorphisms in the CFH and ARMS2 genes were analyzed and compared to NLR and the surface markers in nAMD patients.

Results: NLR was significantly elevated in nAMD patients compared to healthy controls ($P < 0.001$). nAMD patients with poor 1-year treatment response had a significantly higher NLR compared to good 1-year treatment responders. Expression levels of all studied activation surface markers on circulating neutrophils were elevated in nAMD patients compared to healthy controls (all $P < 0.05$).

Conclusion: Elevated NLR was associated with a poor 1- year treatment response. The NLR and expression levels of activation surface markers on circulating neutrophils were significantly elevated in treatment-naïve nAMD patients compared to healthy controls.

Keywords

age-related macular degeneration; treatment response; inflammation; neutrophils; neutrophil-to-lymphocyte ratio; genetics

1. Introduction

Age-related macular degeneration (AMD) is a retinal disease affecting the central vision and is the most common cause of visual impairment and blindness in the Global North.[1] The disease is characterized by an age-associated degeneration of the macula, the central part of the retina.[2] Intermediate AMD (iAMD) is the preliminary stage of late AMD. There are few symptoms related to iAMD, but these patients are at increased risk of developing the vision affecting late stage neovascular AMD (nAMD).[3] Macular neovascularizations (MNV) present in nAMD cause leakage and hemorrhage leading to prompt deterioration of visual function. nAMD is treated with intravitreal anti-vascular endothelial growth factor (VEGF) injections, which greatly improves vision by inhibiting the neovascularization, thus reducing the leakage and hemorrhage of the retina.[4] However, a significant portion of the patients respond sub-optimally to this treatment and will continue to be visually impaired or even experience further visual deterioration.[5,6] Individualized treatment options for these patients are needed and might lie in alternatives to ocular VEGF inhibition.[7]

The pathogenesis of AMD is largely unknown, however increasing evidence suggest that the aging immune system plays a key role.[8] Aging of the systemic immune system and immunological dysfunction have been studied extensively in AMD, affecting both the innate and adaptive immune systems.[9–12] The complement system, which is part of the innate immune system, has previously been shown to be associated with AMD and treatment response in nAMD.[13] The complement system is involved in recruitment and activation of neutrophils.[14,15] Neutrophils constitute the majority of circulating leukocytes, responsible for multiple aspects of the innate immune system, including phagocytosis of pathogens and apoptotic cells.[16] Aging of the immune system includes alterations of the activation surface marker expression on neutrophils,[17–19] which are associated with multiple age-related diseases such as Alzheimer's disease,[20] cardiovascular disease,[21] and diabetes.[22] The activation surface markers on neutrophils CD11a, CD11b, CD31, CD66b. CD162, and CD182 have previously been studied in nAMD.[15,23] The neutrophil-to-lymphocyte ratio (NLR), an indicator of systemic inflammation,[24–26] is associated with a proinflammatory and proangiogenic systemic state of immunity.[26,27] A meta-analysis by Niazi et al. found that NLR is strongly associated with nAMD compared to healthy controls.[28]

Genetic predisposition is also associated with AMD stage and treatment response, including the single nucleotide polymorphisms (SNPs) of the complement factor H (CFH) and age-related maculopathy susceptibility 2 (ARMS2) genes.[29–31]

Based on the aforementioned observations in nAMD, this study investigates whether the NLR and activation surface markers on circulating neutrophils are associated with treatment response in nAMD patients. Furthermore, the study explores correlations between these factors in patients with iAMD and nAMD compared to healthy controls. The association between the risk genotypes of the CFH rs1061170 and ARMS2 rs10490924 SNPs and nAMD patients is also evaluated.

2. Methods

2.1 Study design and participants

The Danish Neovascular Age-Related Macular Degeneration and Treatment Response (DANEART) study is a prospective cohort study investigation focused on characterizing systemic immune phenotypes in patients with AMD across different subtypes. This single-center study is conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark, and has received approval from the Regional Committee of Ethics in Research for the Region of Zealand, Denmark (Journal No: SJ-768, clinical trial number: not applicable). All procedures are conducted in compliance with the Declaration of Helsinki. Informed consent, both verbal and written, was obtained from all participants prior to their enrollment.

The detailed design and methodology of the DANEART study have been described previously [13]. In summary, the study includes treatment-naïve nAMD patients, iAMD patients, and healthy controls. Participants were excluded if they met any of the following criteria: age under 60 years, a history of inflammatory, autoimmune, cancer, or infectious diseases, current use of immunomodulatory treatments, active smoking, plasma C-reactive protein (CRP) levels exceeding 15 mg/L, vision-impairing conditions other than nAMD or iAMD, or prior treatment for nAMD.

All participants underwent a comprehensive baseline examination. The examination protocol included assessments of best-corrected visual acuity, slit-lamp biomicroscopy, and multimodal imaging comprising color fundus photography, spectral-domain optical coherence tomography (OCT), and OCT angiography. Patients diagnosed with nAMD received treatment in accordance with Danish national guidelines, which included an initial loading phase of three intravitreal anti-VEGF injections (aflibercept, 2 mg) administered at monthly intervals. Following this, an

individualized treatment strategy based on the observe-and-plan regimen was implemented.[32] nAMD patients were evaluated at diagnosis (baseline), after completing the loading phase to assess the initial treatment response, and again after one year to evaluate the maintained response.

2.2 Grading disease and treatment response

Disease grading was based on the Beckman criteria.[33] Healthy controls were classified as no AMD or normal aging changes. iAMD patients were classified as having drusen >125 μm in diameter or pigmentary abnormalities associated with drusen >65-125 μm. nAMD patients were classified as having exudative neovascularizations secondary to AMD.

Treatment response in nAMD patients were classified according to morphological changes following anti-VEGF injections. Good response was classified as having total regression of intra- and subretinal fluid, partial response as having persistence of fluid and decreased central retinal thickness (CRT), and poor response as having persistence of fluid and increased or unchanged CRT.[13]

2.3 Flow cytometry

Peripheral blood was sampled in ethylenediamine tetraacetic acid (EDTA) coated tubes for flow cytometry to identify NLR and the expression of activation surface markers on neutrophils at baseline. Blood in lithium-heparin coated tubes were sampled to measure the plasma CRP concentration. Flow cytometry was initiated within 4 hours of phlebotomy. Leukocyte count was performed on the Sysmex KX-21NTM (Sysmex Corporation, Kobe, Japan) to assess sufficient blood volume to achieve 1.0×10^6 leukocytes for analysis. Erythrocytes were lysed by adding 1% lysis buffer and stored at room temperature in the dark for 10 minutes. Cells were washed three times by adding BD FACS Flow isotonic buffer to the sample, centrifuging at 500G for five minutes and decanting the supernatant. The isolated leukocytes were resuspended in isotonic buffer and monoclonal fluorescent antibodies were added: fluorescein isothiocyanate (FITC) CD14 (Biolegend, cat.no. 400210), Brilliant Violet 510 CD16 (Biolegend, cat.no. 302048), peridinin-chlorophyll-protein (PerCP) CD11a (Biolegend, cat.no. 350608), allophycocyanin-cyanine 7 (APC-Cy7) CD11b (Biolegend, 301342), Pacific Blue CD31 (Biolegend, 303114), phycoerythrin-cyanine7 (PE/Cy7) CD66b (Biolegend, cat.no. 304116), phycoerythrin (PE) CD162 (Biolegend, cat.no. 328806), allophycocyanin (APC) CD182 (Biolegend, cat.no. 320710). Samples were incubated at room temperature in the dark for 20 minutes. The stained cells were washed and

resuspended in isotonic buffer. Samples were analyzed on BD FACS Canto II Flow Cytometer (BD, Bioscience, San Jose, CA, USA). Gating size was set to 100,000 cells per sample.

Flow Cytometry Standard files were analyzed with FlowJo software (Tree Star, Ashland, OR, USA, v.10.10.0). Granulocytes and lymphocytes were identified on the forward-side scatter plot. Singlet cells were identified on forward area-forward height scatter plots to exclude adherent cells. Singlet granulocytes were gated for CD14 and CD16 to identify neutrophils, defined as $CD14^{dim}CD16^{+}$. Neutrophils were gated based on the expression of the activation markers CD11a, CD11b, CD31, CD66b, CD162, and CD182. An example of the gating strategy can be found in Figure 1. The NLR was calculated as percentage of singlet neutrophils divided by percentage of singlet lymphocytes.

2.4 Genotyping

Details on genotyping in the DANEART study has been described previously.[13] In brief, genotyping was performed on EDTA full blood in nAMD patients for the SNPs CFH rs1061170 and ARMS2 rs10490924. Following collection, the blood samples were immediately frozen at -80°C and later analyzed by BioXpedia, Denmark. The analysis was performed using the Fluidigm GT192.24 Dynamic Array Integrated Fluidic Circuit (Fluidigm Corp., San Francisco, CA, USA) following the manufacturer's instructions.

2.5 Statistics

Statistical analysis was performed with R software (R Foundation for Statistical Computing, Vienna, Austria, v.4.2.3). Logarithmic transformation was performed on NLR and expression levels of neutrophil surface markers to normalize distributions for analysis. Normality was tested with histograms and Shapiro-Wilk test. Group comparisons were tested with analysis of covariance (ANCOVA) adjusted for age and smoking status (never or previous). In comparisons between diagnoses, healthy controls were chosen as reference group. In comparisons between treatment response groups, good responders were chosen as reference group. Results of the ANCOVA are presented as mean difference and 95% confidence interval (CI95%) in percentages.
Comparisons between high and low risk genotypes of CFH and ARMS2 SNPs were analyzed with Welch two sample t-test in nAMD patients. *P* values were adjusted for multiple testing with false discovery rate (FDR) corrections. A P value < 0.05 was interpreted as statistically significant.

3. Results

3.1 Study population

In the DANEART study, 61 healthy controls, 34 iAMD patients, and 100 nAMD patients were included, of which 94 nAMD patients completed the 1-year follow-up. Among patients with nAMD, the initial (post-loading phase) response distribution was as follows: 61 (65%) were good responders, 26 (28%) were partial responders, and 7 (7%) were poor responders. The 1-year response distribution was 50 (53%) good, 33 (36%) partial, and 11 (11%) poor responders. The details of the characteristics of these participants have been described previously.[13]

3.2 Neutrophil-to-lymphocyte ratio

The median (range) of NLR in the study population was 4.8 (1.2 – 28.7) (Table 1). Patients with nAMD had a significantly higher NLR compared to healthy controls (mean difference: 52.9%, CI95%: 28.3% to 82.3%, $P < 0.001$). NLR did not differ significantly between iAMD patients and healthy controls ($P = 0.56$) (Fig. 2). We stratified nAMD patients according to initial and 1-year treatment response. nAMD patients with poor initial treatment response had a tendency of higher NLR compared to good initial responders, however not statistically significant (mean difference: 24.5%, CI95%: -21.8% to 98.2%, $P = 0.35$). Patients with poor 1-year treatment response had a significantly higher NLR compared to good 1-year responders (mean difference: 65%, CI95%: 9.3% to 150.6%, $P = 0.018$) (Fig. 2).

3.3 Neutrophil surface marker expression

The proportions of neutrophils expressing the activation surface markers can be found in Table 1. Patients with nAMD had significantly higher proportions of neutrophils expressing the surface markers CD11a (mean difference: 15.0%, CI95%: 2.0% to 31.7%, $P = 0.045$), CD11b (mean difference: 33.7%, CI95%: 14.7% to 55.8%, $P = 0.002$), CD31 (mean difference: 22.5%, CI95%: 3.0% to 45.7%, $P = 0.037$), CD66b (mean difference: 17.3%, CI95%: 2.1% to 34.8%, $P = 0.037$), CD162 (mean difference: 22.0%, CI95%: 1.6% to 46.4%, $P = 0.040$), and CD182 (mean difference: 23.1%, CI95%: 7.8% to 40.5%, $P = 0.007$) compared to healthy controls(Fig. 3). There were no significant differences between nAMD treatment response groups initially or after one year.

3.4 Single nucleotide polymorphisms

Patients with nAMD underwent genotyping for two SNPs in the AMD associated risk loci CFH and ARMS2. The proportion of $CD11a^+$ neutrophils trended to be higher in patients with the high-risk CFH rs1061170 genotypes (CC/CT) compared with the low-risk genotype (TT) ($P = 0.033$), however not statistically significant after FDR correction ($P = 0.20$). The proportion of $CD162^+$ neutrophils trended to be higher in patients with the high-risk ARMS2 genotype (TT/TG) compared to the low-risk genotype (GG) ($P = 0.032$), however not statistically significant after FDR correction ($P = 0.23$). There was no statistically significant association between NLR or the expression levels of activation surface markers on neutrophils and CFH genotype (Table 2) or ARMS2 genotype (Table 3).

4. Discussion

This study aimed to investigate NLR and neutrophil expression levels of activation surface markers in relation to AMD stage (healthy controls, iAMD, nAMD) and treatment response in nAMD patients. To our knowledge, this is the first study to assess these factors over a 1-year follow-up period in nAMD patients. We found that the NLR was significantly elevated in treatment-naïve patients with nAMD compared to healthy controls. Furthermore, we showed that nAMD patients with poor 1-year treatment response had a significantly elevated NLR compared to good 1-year responders.

Neutrophils and lymphocytes are essential cells of the immune system, each playing distinct yet interconnected roles. Neutrophils primarily function within the innate immune system, while lymphocytes are key mediators of adaptive immunity. Despite this distinction, complex regulatory interactions exist between these cell types. Chemotactic signals, including interleukin (IL)-8, facilitate their coordinated recruitment.[34] Neutrophils can activate lymphocytes, particularly T cells by secreting cytokines such as IL-6 and IL-1β,[35,36] and by antigen presentation.[37] Conversely, lymphocytes release interferon-γ, a cytokine that can further stimulate neutrophil activity.[38] It has also been shown that elevated neutrophil levels can lead to a reduction in lymphocyte counts.[39,40] The ratio between these cells will thus influence the magnitude of the immune response,[26] and NLR has been shown to be a reliable indicator of systemic inflammation.[18,41,42] It is well established that an increased systemic activation and dysregulation is present in AMD patients.[8–12,43–45] The elevated NLR observed in this study contributes to the theory that AMD may be a systemic inflammatory disease. This is in accordance with multiple previous studies that also find elevated NLR in nAMD

patients.[17,18,23,46–48] We did not find a significant difference between NLR in iAMD patients and healthy controls, which is in accordance with the results of Ojaghi et al.[49] Increased NLR has also been associated with other chronic inflammatory diseases such as arthritis,[50] cardiovascular disease,[41,51] and chronic obstructive pulmonary disease,[52] as well as diabetes[53] and diabetic retinopathy.[54]

One study has previously investigated NLR in relation to initial treatment response in nAMD and found that NLR could predict a reduction of CRT of 100 μm.[55] In accordance with this, we found a tendency of poor initial responders to have elevated NLR. The discrepancy might be caused by the differing definitions of treatment response. We did however find a significant association between NLR and 1-year treatment response comparing poor and good responders. Neutrophils have been demonstrated to play an active role in angiogenesis,[56,57] including retinal neovascularization in laser-induced murine models.[58] Their involvement in MNV formation has been proposed, potentially due to compromised blood-retinal barrier integrity.[45] Neutrophils can also promote angiogenesis by releasing the proangiogenic VEGF.[59] If increased levels of circulating neutrophils reflect increased levels locally, we speculate that the proangiogenic properties of circulating neutrophils might be causing the poor treatment response in nAMD patients, evident by the increased NLR. Therefore, we speculate that anti-VEGF injections might be insufficient in reducing this proinflammatory environment in these poor responders. The NLR may serve as a potential predictor of treatment response, and target for novel therapeutic approaches to be investigated in future studies.

In this study, we found the proportion of circulating neutrophils expressing the activation surface markers CD11a, CD11b, CD31, CD66b, CD162, and CD182 were significantly elevated in nAMD patients compared to healthy controls. During inflammation, neutrophils will be recruited to sites of inflammation partly by stimulation from IL-8, which stimulates the chemokine receptor CD182 (also known as C-X-C motif receptor 2 (CXCR2)).[60] Here, the neutrophils will initiate rolling facilitated by CD162.[61] The neutrophils will adhere to the endothelial cells activated by CD11a, CD11b and CD66b among other surface proteins.[62–64] CD31 on neutrophils are involved in the trans-endothelial migration, enabling these cells to perform their inflammatory functions.[65] nAMD patients exhibited elevated expression of all studied activation surface markers compared to healthy controls. Given that nAMD is characterized by low-grade systemic inflammation,[8] these differences, though subtle, may influence neutrophil activation. A previous study investigating the same activation surface markers in nAMD patients also found increased expression levels of CD66b

on neutrophils, but lower expression levels of CD162 and CD182.[15] This discrepancy might be due to differing flow-cytometric gating strategies and population size. Lechner et al. also found increased proportions of $CD11b^+$ neutrophils.[23] The increased activation of circulating neutrophils might contribute to the pathogenesis of nAMD, as these patients have an increased systemic inflammatory and proangiogenic state, causing damage locally in the retina.[8] We speculate that the age-related low-grade inflammation caused by neutrophils, as well as other pathways including the complement system,[13,66] chemokine system,[67,68] monocytes,[9,69] and T cell differentiation[70,71] a destruction of the RPE barrier[45] which in turn causes the retinal degeneration and neovascularization in nAMD. Future studies are needed to explore the mechanisms underlying the increased activation of circulating neutrophils and their impact on retinal pathology in nAMD. We did not find a significant difference in activation surface markers between treatment response groups, either initially or after one year. This suggests that neutrophil composition may be less critical in determining treatment response, with NLR potentially playing a more significant role.

To our knowledge, this is the first time the association between the AMD risk genotypes CFH and ARMS2 and neutrophil biomarkers has been studied. We did not find any significant association between the SNPs CFH rs1061170 and ARMS2 rs10490924 and their effect on NLR or activation surface markers on neutrophils in nAMD patients. These findings highlight the complex, multifactorial nature of AMD, which cannot be attributed to one single gene or immune pathway.

Limitations of this study include the observational design, which makes us unable to determine the causality of our findings. While the categorical groupings are derived from previous classifications intended for clinical application,[6,7] they limit the ability to detect subtle differences. Additionally, the small sample size in the poor response group posed a limitation, potentially masking significant correlations.

In conclusion, we find NLR was elevated in nAMD patients with poor 1-year treatment response. We also find that NLR and activation surface markers on circulating neutrophils are elevated in nAMD patients compared to healthy controls. These results support the theory that systemic inflammation plays a key role in the development of nAMD and treatment response in these patients.

5. References

1. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *The Lancet Global Health*. 2014;2(2):e106-e116. doi:10.1016/S2214-109X(13)70145-1

2. Fleckenstein M, Keenan TDL, Guymer RH, et al. Age-related macular degeneration. *Nat Rev Dis Primers*. 2021;7(1):31. doi:10.1038/s41572-021-00265-2

3. Thomsen AK, Gøttsche LF, Hinnerskov JMV, Falk MK, Sørensen TL. Microperimetry and Structural Risk Factors on OCT in Intermediate Age-Related Macular Degeneration. *Ophthalmology Retina*. Published online March 2024:S2468653024000885. doi:10.1016/j.oret.2024.02.016

4. Bloch SB, Larsen M, Munch IC. Incidence of Legal Blindness From Age-Related Macular Degeneration in Denmark: Year 2000 to 2010. *American Journal of Ophthalmology*. 2012;153(2):209-213.e2. doi:10.1016/j.ajo.2011.10.016

5. Thomsen AK, Krogh Nielsen M, Liisborg C, Sørensen T. Interleukin-8 Promoter Polymorphism −251 A/T and Treatment Response in Neovascular Age-related Macular Degeneration. *OPTH*. 2024;Volume 18:537-543. doi:10.2147/OPTH.S448794

6. Amoaku WM, Chakravarthy U, Gale R, et al. Defining response to anti-VEGF therapies in neovascular AMD. *Eye*. 2015;29(6):721-731. doi:10.1038/eye.2015.48

7. Thomsen AK, Fasih-Ahmad S, Sadda S, Sørensen TL. Initial treatment response can predict one-year treatment outcomes in neovascular age-related macular degeneration treated according to the observe-and-plan regimen. *Acta Ophthalmologica*. Published online November 27, 2024:aos.16801. doi:10.1111/aos.16801

8. Rozing MP, Durhuus JA, Krogh Nielsen M, et al. Age-related macular degeneration: A two-level model hypothesis. *Progress in Retinal and Eye Research*. 2020;76:100825. doi:10.1016/j.preteyeres.2019.100825

9. Subhi Y, Krogh Nielsen M, Molbech CR, et al. CD11b and CD200 on Circulating Monocytes Differentiate Two Angiographic Subtypes of Polypoidal Choroidal Vasculopathy. *Invest Ophthalmol Vis Sci*. 2017;58(12):5242. doi:10.1167/iovs.17-22479

10. Subhi Y, Krogh Nielsen M, Molbech CR, et al. Plasma markers of chronic low-grade inflammation in polypoidal choroidal vasculopathy and neovascular age-related macular degeneration. *Acta Ophthalmol*. 2019;97(1):99-106. doi:10.1111/aos.13886

11. Krogh Nielsen M, Subhi Y, Molbech CR, Falk MK, Nissen MH, Sørensen TL. Systemic Levels of Interleukin-6 Correlate With Progression Rate of Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2019;60(1):202-208. doi:10.1167/iovs.18-25878

12. Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. *Cell Mol Life Sci*. 2016;73(9):1765-1786. doi:10.1007/s00018-016-2147-8

13. Thomsen AK, Steffensen MA, Villarruel Hinnerskov JM, et al. Complement proteins and complement regulatory proteins are associated with age-related macular degeneration stage and treatment response. *J Neuroinflammation*. 2024;21(1):284. doi:10.1186/s12974-024-03273-7

14. Schmidt T, Brodesser A, Schnitzler N, et al. CD66b Overexpression and Loss of C5a Receptors as Surface Markers for Staphylococcus aureus-Induced Neutrophil Dysfunction. Akiyama T, ed. *PLoS ONE*. 2015;10(7):e0132703. doi:10.1371/journal.pone.0132703

15. Krogh Nielsen M, Hector SM, Allen K, Subhi Y, Sørensen TL. Altered activation state of circulating neutrophils in patients with neovascular age-related macular degeneration. *Immun Ageing*. 2017;14(1):18. doi:10.1186/s12979-017-0100-9

16. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175. doi:10.1038/nri3399

17. Ilhan N, Daglioglu MC, Ilhan O, et al. Assessment of Neutrophil/Lymphocyte Ratio in Patients with Age-related Macular Degeneration. *Ocular Immunology and Inflammation*. 2015;23(4):287-290. doi:10.3109/09273948.2014.921715

18. Sengul EA, Artunay O, Kockar A, et al. Correlation of neutrophil/lymphocyte and platelet/lymphocyte ratio with visual acuity and macular thickness in age-related macular degeneration. *Int J Ophthalmol*. 2017;10(5):754-759. doi:10.18240/ijo.2017.05.16

19. Pinna A, Porcu T, D'Amico-Ricci G, et al. Complete Blood Cell Count–Derived Inflammation Biomarkers in Men with Age-Related Macular Degeneration. *Ocular Immunology and Inflammation*. 2019;27(6):932-936. doi:10.1080/09273948.2018.1485960

20. Zenaro E, Pietronigro E, Bianca VD, et al. Neutrophils promote Alzheimer's disease–like pathology and cognitive decline via LFA-1 integrin. *Nat Med*. 2015;21(8):880-886. doi:10.1038/nm.3913

21. Silvestre-Roig C, Braster Q, Ortega-Gomez A, Soehnlein O. Neutrophils as regulators of cardiovascular inflammation. *Nat Rev Cardiol*. 2020;17(6):327-340. doi:10.1038/s41569-019-0326-7

22. Giovenzana A, Carnovale D, Phillips B, Petrelli A, Giannoukakis N. Neutrophils and their role in the aetiopathogenesis of type 1 and type 2 diabetes. *Diabetes Metabolism Res*. 2022;38(1):e3483. doi:10.1002/dmrr.3483

23. Lechner J, Chen M, Hogg RE, et al. Alterations in Circulating Immune Cells in Neovascular Age-Related Macular Degeneration. *Sci Rep*. 2015;5(1):16754. doi:10.1038/srep16754

24. Huang Z, Fu Z, Huang W, Huang K. Prognostic value of neutrophil-to-lymphocyte ratio in sepsis: A meta-analysis. *The American Journal of Emergency Medicine*. 2020;38(3):641-647. doi:10.1016/j.ajem.2019.10.023

25. Forget P, Khalifa C, Defour JP, Latinne D, Van Pel MC, De Kock M. What is the normal value of the neutrophil-to-lymphocyte ratio? *BMC Res Notes*. 2017;10(1):12. doi:10.1186/s13104-016-2335-5

26. Socorro Faria S, Fernandes Jr PC, Barbosa Silva MJ, et al. The neutrophil-to-lymphocyte ratio: a narrative review. *ecancer*. 2016;10. doi:10.3332/ecancer.2016.702

27. Templeton AJ, McNamara MG, Šeruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2014;106(6):dju124. doi:10.1093/jnci/dju124

28. Niazi S, Krogh Nielsen M, Sørensen TL, Subhi Y. Neutrophil-to-lymphocyte ratio in age-related macular degeneration: a systematic review and meta-analysis. *Acta Ophthalmologica*. 2019;97(6):558-566. doi:10.1111/aos.14072

29. 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 USA*. 2005;102(20):7227-7232. doi:10.1073/pnas.0501536102

30. Klein RJ, Zeiss C, Chew EY, et al. Complement Factor H Polymorphism in Age-Related Macular Degeneration. *Science*. 2005;308(5720):385-389. doi:10.1126/science.1109557

31. Smailhodzic D, Klaver CCW, Klevering BJ, et al. Risk Alleles in CFH and ARMS2 Are Independently Associated with Systemic Complement Activation in Age-related Macular Degeneration. *Ophthalmology*. 2012;119(2):339-346. doi:10.1016/j.ophtha.2011.07.056

32. Subhi Y, Schneider M, Hajari JN, La Cour M. Injection burden and treatment intervals of aflibercept in observe-and-plan regimen for neovascular age-related macular degeneration. *Acta Ophthalmologica*. 2024;102(7):821-827. doi:10.1111/aos.16709

33. Ferris FL, Wilkinson CP, Bird A, et al. Clinical Classification of Age-related Macular Degeneration. *Ophthalmology*. 2013;120(4):844-851. doi:10.1016/j.ophtha.2012.10.036

34. Bickel M. The role of interleukin-8 in inflammation and mechanisms of regulation. *J Periodontol*. 1993;64(5 Suppl):456-460.

35. Hashizume M, Higuchi Y, Uchiyama Y, Mihara M. IL-6 plays an essential role in neutrophilia under inflammation. *Cytokine*. 2011;54(1):92-99. doi:10.1016/j.cyto.2011.01.007

36. Sabbione F, Keitelman IA, Shiromizu CM, et al. Regulation of human neutrophil IL-1β secretion induced by Escherichia coli O157:H7 responsible for hemolytic uremic syndrome. Baumler AJ, ed. *PLoS Pathog*. 2023;19(12):e1011877. doi:10.1371/journal.ppat.1011877

37. Hänsch GM, Wagner C. Expression of MHC class II antigen and coreceptor molecules in polymorphonuclear neutrophils. *Chem Immunol Allergy*. 2003;83:45-63. doi:10.1159/000071556

38. Ellis TN, Beaman BL. Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology*. 2004;112(1):2-12. doi:10.1111/j.1365-2567.2004.01849.x

39. Shau HY, Kim A. Suppression of lymphokine-activated killer induction by neutrophils. *J Immunol*. 1988;141(12):4395-4402.

40. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327-336. doi:10.1172/JCI57990

41. Tamhane UU, Aneja S, Montgomery D, Rogers EK, Eagle KA, Gurm HS. Association Between Admission Neutrophil to Lymphocyte Ratio and Outcomes in Patients With Acute Coronary Syndrome. *The American Journal of Cardiology*. 2008;102(6):653-657. doi:10.1016/j.amjcard.2008.05.006

42. Torun S, Tunc BD, Suvak B, et al. Assessment of neutrophil-lymphocyte ratio in ulcerative colitis: A promising marker in predicting disease severity. *Clinics and Research in Hepatology and Gastroenterology*. 2012;36(5):491-497. doi:10.1016/j.clinre.2012.06.004

43. Krogh Nielsen M, Subhi Y, Molbech CR, Falk MK, Nissen MH, Sørensen TL. Chemokine Profile and the Alterations in CCR5-CCL5 Axis in Geographic Atrophy Secondary to Age-Related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2020;61(4):28. doi:10.1167/iovs.61.4.28

44. Armento A, Ueffing M, Clark SJ. The complement system in age-related macular degeneration. *Cell Mol Life Sci*. 2021;78(10):4487-4505. doi:10.1007/s00018-021-03796-9

45. Tan W, Zou J, Yoshida S, Jiang B, Zhou Y. The Role of Inflammation in Age-Related Macular Degeneration. *Int J Biol Sci*. 2020;16(15):2989-3001. doi:10.7150/ijbs.49890

46. Kurtul BE, Ozer PA. The Relationship between Neutrophil-to-lymphocyte Ratio and Age-related Macular Degeneration. *Korean J Ophthalmol*. 2016;30(5):377. doi:10.3341/kjo.2016.30.5.377

47. Karahan M, Hazar L, Erdem S, et al. Is there a relationship between hematological inflammatory parameters and age-related macular degeneration? *Ophthalmol Eye Dis*. 2021;13:25158414211010550. doi:10.1177/25158414211010550

48. Hu WW, Huang YK, Huang XG. Comparison of Peripheral Blood Inflammatory Indices in Patients with Neovascular Age-related Macular Degeneration and Haemorrhagic Polypoidal Choroidal Vasculopathy. *Ocular Immunology and Inflammation*. 2023;31(5):935-939. doi:10.1080/09273948.2022.2071742

49. Ojaghi H, Poorsheykhian S, Najafi A, Iranpour S. The role of blood related inflammatory factors on age-related macular degeneration (AMD). *Immun Ageing*. 2024;21(1):35. doi:10.1186/s12979-024-00440-5

50. Fu H, Qin B, Hu Z, et al. Neutrophil- and Platelet-to-Lymphocyte Ratios are Correlated with Disease Activity in Rheumatoid Arthritis. *Clin Lab*. 2015;61(03+04/2015). doi:10.7754/Clin.Lab.2014.140927

51. Yayla Ç, Açikgöz SK, Yayla KG, et al. The association between platelet-to-lymphocyte ratio and inflammatory markers with the severity of aortic stenosis. *Biomark Med*. 2016;10(4):367-373. doi:10.2217/bmm-2015-0016

52. Paliogiannis P, Fois AG, Sotgia S, et al. Neutrophil to lymphocyte ratio and clinical outcomes in COPD: recent evidence and future perspectives. *Eur Respir Rev*. 2018;27(147):170113. doi:10.1183/16000617.0113-2017

53. Mertoglu C, Gunay M. Neutrophil-Lymphocyte ratio and Platelet-Lymphocyte ratio as useful predictive markers of prediabetes and diabetes mellitus. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2017;11:S127-S131. doi:10.1016/j.dsx.2016.12.021

54. Ulu SM, Dogan M, Ahsen A, et al. Neutrophil-to-Lymphocyte Ratio as a Quick and Reliable Predictive Marker to Diagnose the Severity of Diabetic Retinopathy. *Diabetes Technology & Therapeutics*. 2013;15(11):942-947. doi:10.1089/dia.2013.0097

55. Gökce SE, Çelik A, Başkan C. The role of blood neutrophil lymphocyte ratio in predicting the initial response to anti-VEGF treatment in neovascular AMD patients. *Ir J Med Sci*. 2024;193(1):517-521. doi:10.1007/s11845-023-03379-6

56. Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. *Int J Exp Pathol*. 2009;90(3):222-231. doi:10.1111/j.1365-2613.2009.00641.x

57. Christoffersson G, Vågesjö E, Vandooren J, et al. VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. *Blood*. 2012;120(23):4653-4662. doi:10.1182/blood-2012-04-421040

58. Tsutsumi-Miyahara C, Sonoda KH, Egashira K, et al. The relative contributions of each subset of ocular infiltrated cells in experimental choroidal neovascularisation. *Br J Ophthalmol*. 2004;88(9):1217-1222. doi:10.1136/bjo.2003.036392

59. Gong Y, Koh DR. Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. *Cell Tissue Res*. 2010;339(2):437-448. doi:10.1007/s00441-009-0908-5

60. Delobel P, Ginter B, Rubio E, Balabanian K, Lazennec G. CXCR2 intrinsically drives the maturation and function of neutrophils in mice. *Front Immunol*. 2022;13:1005551. doi:10.3389/fimmu.2022.1005551

61. Moore KL, Patel KD, Bruehl RE, et al. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *The Journal of cell biology*. 1995;128(4):661-671. doi:10.1083/jcb.128.4.661

62. Lakschevitz FS, Hassanpour S, Rubin A, Fine N, Sun C, Glogauer M. Identification of neutrophil surface marker changes in health and inflammation using high-throughput screening flow cytometry. *Experimental Cell Research*. 2016;342(2):200-209. doi:10.1016/j.yexcr.2016.03.007

63. Futosi K, Fodor S, Mócsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology*. 2013;17(3):638-650. doi:10.1016/j.intimp.2013.06.034

64. Yang C, Li L, Ye Z, et al. Mechanisms underlying neutrophils adhesion to triple-negative breast cancer cells via CD11b-ICAM1 in promoting breast cancer progression. *Cell Commun Signal*. 2024;22(1):340. doi:10.1186/s12964-024-01716-5

65. Woodfin A, Voisin MB, Nourshargh S. Recent developments and complexities in neutrophil transmigration: *Current Opinion in Hematology*. 2010;17(1):9-17. doi:10.1097/MOH.0b013e3283333930

66. Scholl HPN, Issa PC, Walier M, et al. Systemic Complement Activation in Age-Related Macular Degeneration. Toland AE, ed. *PLoS ONE*. 2008;3(7):e2593. doi:10.1371/journal.pone.0002593

67. Falk MK, Singh A, Faber C, Nissen MH, Hviid T, Sørensen TL. CX3CL1/CX3CR1 and CCL2/CCR2 Chemokine/Chemokine Receptor Complex in Patients with AMD. Sennlaub F, ed. *PLoS ONE*. 2014;9(12):e112473. doi:10.1371/journal.pone.0112473

68. Martinez Villarruel Hinnerskov J, Krogh Nielsen M, Kai Thomsen A, et al. Chemokine Receptor Profile of T Cells and Progression Rate of Geographic Atrophy Secondary to Age-related Macular Degeneration. *Invest Ophthalmol Vis Sci*. 2024;65(1):5. doi:10.1167/iovs.65.1.5

69. Hector SM, Sørensen TL. Circulating monocytes and B-lymphocytes in neovascular age-related macular degeneration. *OPTH*. 2017;Volume 11:179-184. doi:10.2147/OPTH.S121332

70. Faber C, Singh A, Krüger Falk M, Juel HB, Sørensen TL, Nissen MH. Age-related Macular Degeneration Is Associated with Increased Proportion of CD56+ T Cells in Peripheral Blood. *Ophthalmology*. 2013;120(11):2310-2316. doi:10.1016/j.ophtha.2013.04.014

71. Liisborg C, Skov V, Kjær L, Hasselbalch HC, Sørensen TL. Retinal drusen in patients with chronic myeloproliferative blood cancers are associated with an increased proportion of senescent T cells and signs of an aging immune system. *Aging*. 2021;13(24):25763-25777. doi:10.18632/aging.203803

6. Declarations

Ethics approval and consent to participate

This study was conducted at the Department of Ophthalmology, Zealand University Hospital, Denmark approved by the Regional Committee of Ethics in Research of the Region of Zealand, Denmark (journal number: SJ-768, clinical trial number: not applicable) and performed in adherence with the Declaration of Helsinki. Verbal and written informed consent were obtained from all participants prior to inclusion.

Availability of data and materials

De-identified data are available from the corresponding author upon reasonable request.

Competing interests

BH and HV have obtained research funding from Bayer to a study not related to the present. All other authors declare no financial or non-financial conflicts of interest.

Funding

This work was supported by the Velux Foundation (00024226), Region Zealand PhD Grant (R29-A1337), Synoptik Foundation, Fight for Sight Denmark (Øjenforeningen), and Grosserer L.F. Foghts Foundation. The funding organizations had no role in the design or conduct of this research.

Author contributions

AKT, TLS, MHN, MAS, BH and HV conceptualized the study. AKT led the data collection and data analysis with inputs and help from TLS, MHN, MAS, and KG. AKT led the interpretation of data and wrote the first draft of the manuscript. All named authors contributed to the critical revision of the manuscript. AKT and TLS obtained funding with help from MAS, MHN, HV and BH. TLS and MHN led study supervision. All named authors read and approved the final manuscript.

7. Tables

	Median (range)
Neutrophil-lymphocyte-ratio	4.8 (1.2 – 28.7)
Neutrophil surface marker expression [%]	
$CD11a^+$	98.8 (97.5 – 99.7)
$CD11b^+$	99.0 (97.2 – 99.7)
$CD31^+$	99.3 (97.2 – 99.8)
$CD66b^+$	99.1 (97.9 – 99.7)
$CD162^+$	99.2 (96.7 – 99.7)
$CD182^+$	98.2 (94.9 – 99.3)

Table 1. Median and range for neutrophil-to-lymphocyte ratio and neutrophil surface markers of the study population (n = 195).

	CFH, rs1061170		
	CC/CT (high risk), n = 82	TT (low risk), n = 18	*P* value*
Neutrophil-to lymphocyte ratio, mean (SD)	7.19 (4.02)	5.85 (3.41)	0.35
Neutrophil surface marker expression			
$CD11a^+$, mean (SD), [%]	98.88 (0.47)	98.69 (0.39)	0.20
$CD11b^+$, mean (SD), [%]	99.05 (0.42)	98.97 (0.44)	0.49
$CD31^+$, mean (SD), [%]	99.22 (0.40)	99.05 (0.58)	0.46
$CD66b^+$, mean (SD), [%]	99.08 (0.31)	98.97 (0.47)	0.59
$CD162^+$, mean (SD), [%]	99.14 (0.51)	98.84 (0.79)	0.36
$CD182^+$, mean (SD), [%]	98.34 (0.62)	98.19 (0.57)	0.46
*Welch two sample t-test with false discovery rate correction.			

Table 2. Neutrophil-to-lymphocyte ratio and neutrophil surface marker expression levels stratified according to CFH rs1061170 AMD high-risk (CC/CT) and low-risk (TT) genotypes in nAMD patients.

	ARMS2, rs10490924		
	TT/TG (high risk), n = 45	GG (low risk), n = 55	*P* value*
Neutrophil-to lymphocyte ratio, mean (SD)	7.64 (3.78)	6.42 (4.02)	0.32
Neutrophil surface marker expression			
$CD11a^+$, mean (SD), [%]	98.79 (0.56)	98.92 (0.37)	0.77
$CD11b^+$, mean (SD), [%]	99.02 (0.45)	99.05 (0.41)	0.84
$CD31^+$, mean (SD), [%]	99.18 (0.48)	99.20 (0.40)	0.84
$CD66b^+$, mean (SD), [%]	99.09 (0.38)	99.04 (0.30)	0.65
$CD162^+$, mean (SD), [%]	99.24 (0.41)	98.98 (0.65)	0.23
$CD182^+$, mean (SD), [%]	99.24 (0.65)	98.34 (0.59)	0.84
*Welch two sample t-test with false discovery rate correction.			

Table 3. Neutrophil-to-lymphocyte ratio and neutrophil surface marker expression levels stratified according to ARMS2 rs10490924 AMD high-risk (TT/TG) and low-risk (GG) genotypes in nAMD patients.

8. Figures

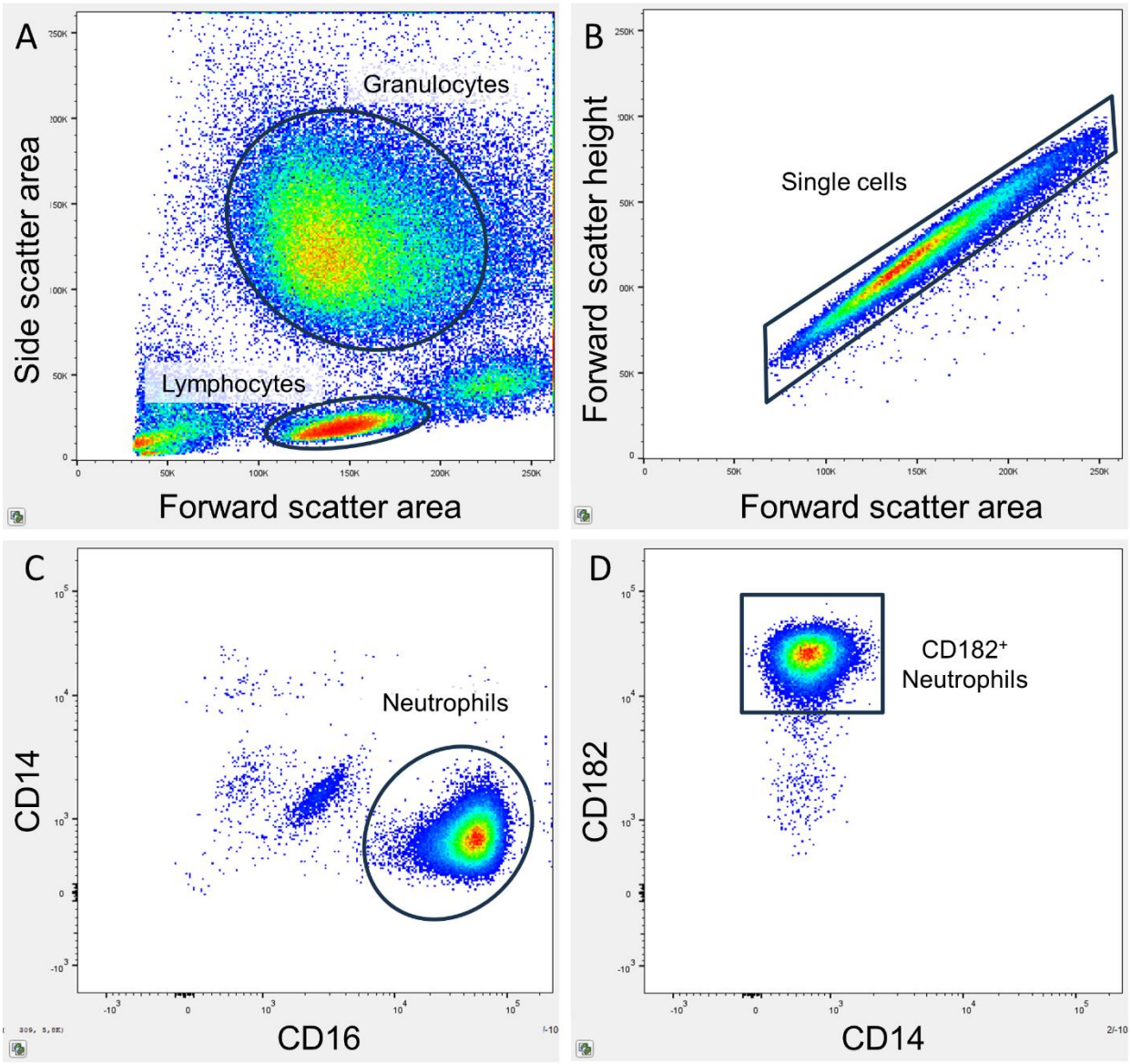


Figure 1. Flow cytometry gating strategy with Boolean sequences. (A) Granulocytes and lymphocytes were identified on the forward-side-scatter. (B) Singlet cells were gated. (C) Neutrophils were identified as $CD14^{dim}CD16^{+}$ singlet granulocytes. (D) Activation markers were identified on neutrophils, in this example CD182.

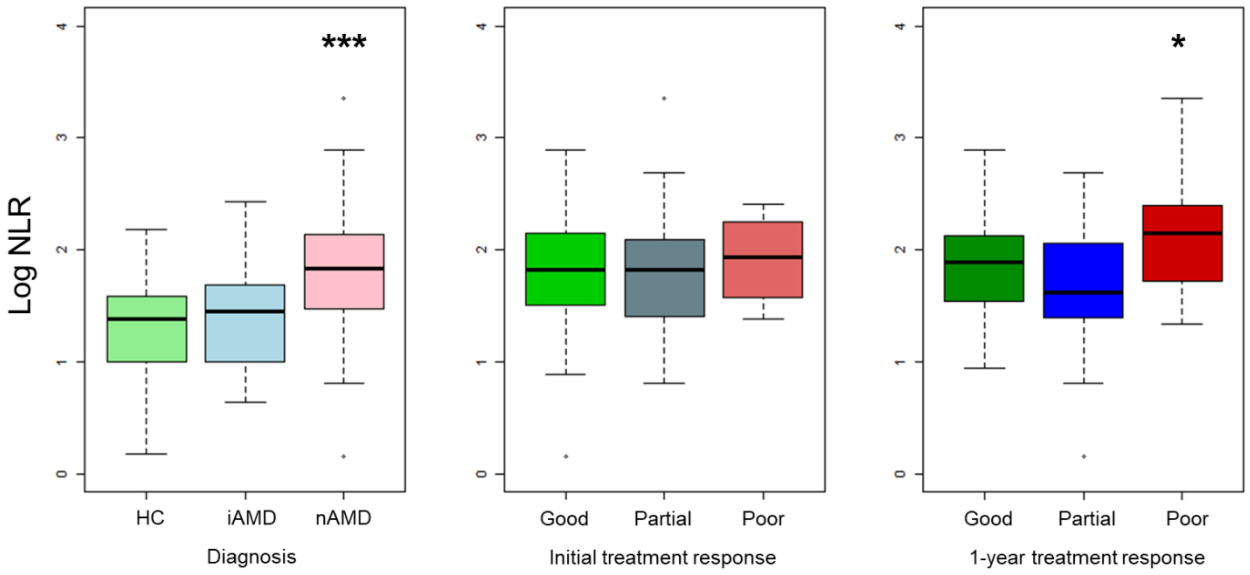


Figure 2. Logarithm of the neutrophil-to-lymphocyte ratio compared between (A) diagnosis groups, (B) initial treatment response groups of nAMD patients, and (C) 1-year treatment response groups of nAMD patients. NLR = neutrophil-to-lymphocyte ratio, HC = healthy controls, iAMD = intermediate age-related macular degeneration, nAMD = neovascular age-related macular degeneration. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to the reference group (healthy controls or good treatment response) adjusted for age and smoking status with false discovery rate corrections.

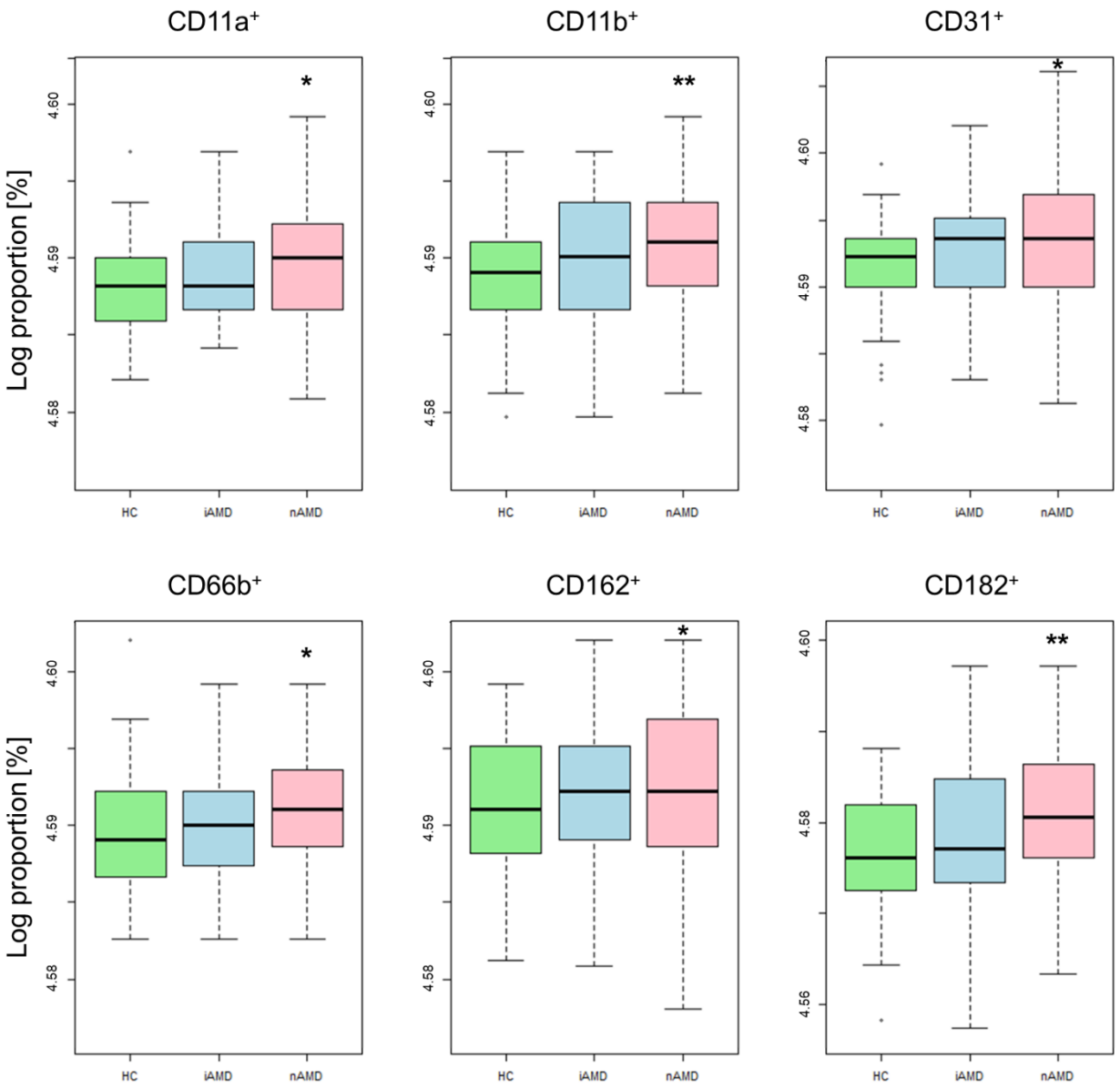


Figure 3. Logarithm of the proportion of neutrophils expressing the studied surface markers. HC = healthy controls, iAMD = intermediate age-related macular degeneration, nAMD = neovascular age-related macular degeneration. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to the reference group (healthy controls) adjusted for age and smoking status with false discovery rate corrections.