LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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AGE-RELATED MACULAR DEGENERATION: NEW IMMUNOGENETIC BIOMARKERS AND ASSOCIATIONS WITH TREATMENT EFFICACY

Doctoral Dissertation
Natural Sciences,
Biology (N 010)

Kaunas, 2022

Dissertation has been prepared at the Laboratory of Ophthalmology of Neuroscience Institute of Lithuanian University of Health Sciences during the period of 2017–2022.

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Dissertation will be defended at the open session of the Biology Research Council on the 16th of June 2022 at 2 p. m. in the Auditorium No. A-203 of the Centre for the Advanced Pharmaceutical and Health Technologies of Lithuanian University of Health Sciences.

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LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

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AMŽINĖ GELTONOSIOS DĖMĖS DEGENERACIJA: NAUJI IMUNOGENETINIAI ŽYMENYS IR JŲ SĄSAJA SU GYDYMO EFEKTYVUMU

Daktaro disertacija
Gamtos mokslai,
biologija (N 010)

Kaunas, 2022

Disertacija rengta 2017–2022 metais Lietuvos sveikatos mokslų universitete, Neuromokslų instituto Oftalmologijos laboratorijoje.

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Disertacija bus ginama viešame biologijos mokslo krypties tarybos posėdyje 2022 m. birželio 16 d. 14 val. Lietuvos sveikatos mokslų universiteto Naujausių farmacijos ir sveikatos technologijų centro A-203 auditorijoje.

Adresas: Sukilėlių pr. 13, LT-50166 Kaunas, Lietuva.

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ABBREVIATIONS

AGE	–	Advanced glycosylation end product
AIC	–	akaike information criterion
AMD	–	age-related macular degeneration
AREDS	–	Age Related EYE Disease Study
BCVA	–	Best-corrected visual acuity
BrM	–	Bruch's membrane
CAD	–	coronary heart disease
CEU	–	Utah Residents with Northern and Western European ancestry population
CHD	–	Coronary heart disease
CI	–	Confidence interval
CNA	–	Choroidal neovascularization
CNTF	–	ciliary neurotrophic factor
COL10A1	–	Collagen type X alpha-1
COL8A1	–	Collagen type VIII alpha-1
D'	–	Linkage disequilibrium coefficient
EDTA	–	Ethylenediaminetetraacetic acid
GA	–	Geographic atrophy
HDL	–	high-density lipoprotein
HDL-C	–	low-density lipoprotein cholesterol
hRVECs	–	human retinal vascular endothelial cells
HWE	–	Hardy-Weinberg equilibrium
IL-10	–	Interleukin 10
IL-9	–	Interleukin 9
IQR	–	interquartile range
IQR	–	tarpkvartilinis plotis
JAK	–	Janus kinase
LDL	–	low-density lipoprotein
LDL-C	–	low-density lipoprotein cholesterol
LS	–	Linkage disequilibrium
LSMU	–	Lithuanian University of Health Sciences
MGB	–	minor groove binder
NF-kB	–	specific nuclear factor kappa-light-chain-enhancer of activated B cells
NO	–	Nitric oxide
OCT	–	optical coherent tomography
OCT-A	–	optical coherence tomography angiography
OR	–	Odds ratio

p	–	significance level
PCR	–	Polymerase chain reaction
PCV	–	polypoidal choroidal vasculopathy
PDT	–	photodynamic therapy
r^2	–	Squared correlation coefficient of the haplotype frequencies
RAD51B	–	RAD51D Paralog B
RPE	–	Retinal pigment epithelium
RSO	–	Reactive oxygen species
RSV	–	respiratory syncytial virus
RT-PCR	–	real-time polymerase chain reaction
SNP	–	Single nucleotide polymorphism
STAT	–	Signal Transducer and Activator of Transcription
TG	–	triglycerides
TNF-α	–	tumour necrosis factor-alpha
TRIB1	–	Tribbles pseudokinase 1
VEGF-A	–	vascular endothelial growth factor A
VEGFA	–	vascular endothelial growth factor A gene
VEGF-R2/KDR	–	receptor for vascular endothelial grow factor 2 / kinase insert domain receptor
vs. (lot. *versus*)	–	compare
χ^2	–	Chi-square test

INTRODUCTION

Age-related macular degeneration (AMD) is a progressive neurodegenerative disease of the macula that leads to loss of visual function and eventual blindness. The macula is the part of the retina where the photoreceptors responsible for visual acuity and colour perception are concentrated [1]. Mostly disease is diagnosed in older people (60 years and older) [2], but the first signs of the disease can appear in people as young as 40 years old [3]. AMD causes a decrease in visual acuity leading to decreased quality of life; it is difficult to recognize faces or distinguish colours [4]. According to the Age-Related Eye Disease Study (AREDS), AMD can be classified into early, intermediate, and advanced AMD [5]. Although early AMD is much more common and diagnosed earlier; exudative AMD can cause blindness in up to 80 % of patients [6]. While the pathophysiology of AMD is not clearly understood, age and especially ageing is strongly associated with the AMD, especially in the developed countries where the overall people survival rate is highly increasing, leading to elevated age-related disease development [7]. The previous study showed that the number of people with early AMD would increase to 21.5 million and late AMD to 4.8 million in Europe. Such a dramatic increase is expected for the world to be up to 288 million in 2040 [8].

The eye ageing processes include drusogenesis and oxidative stress as the essential processes leading to AMD [9]. Drusen or lipid, protein and collagen deposits and reactive oxygen species (ROS), accumulated between the retinal pigment epithelium (RPE) and Bruch's membrane (BrM) in the retina [10], causes chronic inflammation leading to irreversible damage to RPE cells and photoreceptors in the macula [11,12], as well as the breakdown of the blood ocular barrier, which is a normal function in protecting the eye [13]. Also, the accumulated substances stimulate the RPE to release large amounts of various inflammatory factors. Long-term exposure to these factors leads to degeneration and atrophy of photoreceptors and RPE cells in the retina [14].

Previous studies discovered that impaired inflammatory response stimulates the release of a high amount of proangiogenic mediators including vascular endothelial growth factor A (VEGF-A), which triggers progressive angiogenesis [15–17]. On the other hand, new, weak, permeable, and leaky blood vessels in the choroid cause local oedema leading to the development of exudative AMD [17]. While only the exudative AMD is already treated with anti-VEGF inhibitors, in recent years, the VEGF-A and its signaling pathway have been targeted for the most effective therapy development [18], including the other molecules included in VEGF-A pathway [15].

It was revealed that inflammatory factors, including IL-10 and IL-9, are potentially related to the pathogenesis of AMD. They also uncovered their function and regulation via specific nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways, encouraging the new treatment of exudative AMD [19].

The essential risk factors of AMD development are lifestyle habits like smoking, alcohol consumption or insufficient diet [20], but the genetic factors remain the most significant risk factors for AMD and account for about 70 % of them. Moreover, developing genetic studies and innovative testing methods encourage us to search for the potential biomarkers which could help improve the early diagnosis of AMD and target the molecules for the possible treatment strategies.

As the genetic factors are the most important risk factors for AMD, many studies focus on single nucleotide polymorphisms (SNPs) for the biomarker search. We also chose to reveal the associations between the SNPs in *IL-9* and *IL-10,* collagen coding genes *COL8A1* and *COL10A1*, lipid transport associated gene *TRIB1* [21], oxidative stress and DNA damage linked gene *RAD51B* [22] and progressive angiogenesis associated gene *VEGFA* as new biomarkers for the AMD development and exudative AMD treatment efficacy. We think that selected SNPs and IL-10, VEGF-A and VEGF-R2/KDR serum levels can have a significant role in AMD development and exudative AMD treatment.

Study aim and objectives

The aim of study:

The aim of this study was to identify new immunogenetic blood-based molecular markers in patients with age-related macular degeneration and to evaluate the associations of the biomarkers with exudative age-related macular degeneration treatment efficacy.

Objectives:

1. To determine the associations between *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226) and *COL10A1* (rs1064583) genetic variants and early and exudative age-related macular degeneration development.
2. To determine the associations between *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884), *IL-10* (rs1800871, rs1800872 and rs1800896) genetic variants, IL-9 and IL-10 serum levels and

early, exudative and atrophic age-related macular degeneration development.

3. To determine the associations between *VEGFA* (rs1570360, rs699947, rs3025033 and rs2146323A) genetic variants, VEGF-A and VEGF-R2/KDR serum levels with early and exudative age-related macular degeneration development.
4. To determine the associations between *RAD51B*, *TRIB1*, *COL8A1*, *COL10A1*, *IL-9, IL-10* and *VEGFA* genetic variants, IL-10, VEGF-A and VEGF-R2/KDR serum levels and exudative age-related macular degeneration treatment with anti-VEGF injections efficacy.

Novelty and the relevance of the study

Considering the inflammatory pathogenesis in AMD development, we identified new potential biomarkers for AMD:

1. *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotypes A-G-C-G-G and G-A-T-A-T were associated with 2 and 12-fold decreased odds of early AMD, respectively.
2. *VEGFA* rs3025033 G allele and *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotype A-A-G-A are associated with 2-fold decreased odds of exudative AMD. Moreover, *VEGFA* rs3025033 G allele associations remain significant only in females with exudative AMD.
3. *RAD51B* rs2588809 TT genotype, compared to CC genotype, is associated with 7-fold increased odds of early AMD development in females.

According to the databases, our study showed for the first time that the T allele in *IL-10* rs1800896 is associated with lower IL-10 serum levels, and the C allele in *VEGFA r*s699947 is associated with elevated VEGF-A serum concentrations in patients with exudative AMD.

Moreover, for the first time, such significant associations between immunogenetic markers and exudative AMD treatment efficacy were determined:

1. *RAD51B* rs8017304 minor allele is associated with increased central macular thickness (CMT) in exudative AMD before anti-VEGF treatment; *TRIB1* rs4351379 minor allele is associated with more decreased CMT after six months of treatment.
2. *IL-9* rs1859430, rs2069870 and rs2069884 minor alleles are associated with worse best-corrected visual acuity (BCVA) before anti-VEGF treatment; *IL-9* rs2069885 minor allele is associated with

better improvement of BCVA after six months of anti-VEGF treatment.

3. *VEGFA* rs699947 allele C is associated with worse BCVA before and after 3 and 6 months of anti-VEGF treatment and higher CMT after six months of anti-VEGF treatment.

We believe that identified immunogenetic markers or biomarker complexes could become prognostic tools and help assure the most effective treatment for AMD in the future.

1. LITERATURE REVIEW

1.1. Age-related macular degeneration

AMD is generally described as a multifactorial, progressive, neurodegenerative disease of the macula that leads to loss of visual function and eventual blindness. The macula is the central part of the retina where the photoreceptors responsible for visual acuity and colour perception are concentrated [1]. While the disease is diagnosed in older people (60 years and older) [2], the first signs of the disease can appear in people as young as 40 years old [3]. AMD causes a decrease in visual acuity; it is difficult to recognize faces, distinguish colours and decrease the overall quality of life [4]. The Age-Related Eye Disease Study (AREDS) classification system can be used for AMD diagnosis and classification [5]:

1. Early AMD consists of a combination of multiple small drusen (protein and lipid deposit) formation between the RPE and BrM [23] and several intermediate (63–124 μm in diameter) drusen, or retinal pigment epithelial abnormalities; may not cause any symptoms.
2. The intermediate form is described as a presence of at least one large (≥125 μm in diameter) drusen, numerous medium-sized drusen, or GA without extension to the center of the macula with mild symptoms as mild blurriness in their central vision or trouble seeing in low lighting or may not cause any symptoms.
3. The advanced AMD is divided into:
   - dry/atrophic AMD with the GA of the RPE;
   - neovascular or exudative AMD, which is diagnosed when choroidal neovascularization with detachments in the RPE haemorrhages and/or scars appear and cause progressive blurring or other central vision impairments [24].

Although early AMD is much more common, exudative AMD can cause blindness in up to 80 % of patients [6].

1.2. AMD prevalence

The exponential ageing of the population is a current global problem affecting human health, including eye diseases. AMD is known to affect about 10 % of people over 65 years and more than 25 % of people over 75 years in developed countries [23], and early AMD is also diagnosed in 4 % of people under 60 years and more than 30 % of people over 85 years [25]. The earlier study projection shows that the number of people with early AMD

will increase to 21.5 million and late AMD to 4.8 million in Europe. A dramatic increase is expected for the whole world to be up to 288 million in 2040 [8].

About 65 % of people with AMD are women, and only 35 % are men. This ratio could be explained by the higher overall survival rate of women and longer life expectancy leading to age-related diseases such as AMD [7].

The incidence of AMD also varies significantly between different ethnic groups: The majority of people with AMD in the United States are Caucasian, but the incidence among Latin Americans is expected to increase sixfold by 2050 [7]. Wong et al. also studied different racial and ethnic groups in the United States and found that AMD is more common in people of European descent (12–30 %), with incidence increasing with age [2].

On the other hand, Wong and co-authors found that the incidence of AMD was similar in ethnic groups of Latin Americans (10.4 %), Africans (7.5 %) and Asians (7.4 %) [2].

Klein and co-authors found that the incidence of AMD was only 7.3 % in white Europeans and 2.4 % in African Americans [26]. The differences in the incidence of AMD observed between racial or ethnic groups indicate the importance of environmental and genetic factors in the development of AMD.

1.3. AMD pathogenesis

The pathophysiology of AMD is not clearly understood yet. It has been associated with environmental factors [27, 28] and genetic factors [29], metabolite profile [30] and even alterations in the microbiome [31]. Drusogenesis and oxidative stress are still the leading processes in AMD [9]. The accumulation of molecular debris, including lipid and protein particles and reactive oxygen species (ROS), leading to chronic inflammation, causes irreversible damage to RPE cells and photoreceptors in the macula [11,12] as well as the breakdown of the blood ocular barrier, which is a normal function in protecting the eye [13].

Drusen are described as tiny particles of lipid, protein and collagen deposits that accumulate between the RPE and BrM in the retina [10] (Fig. 1.3.1).

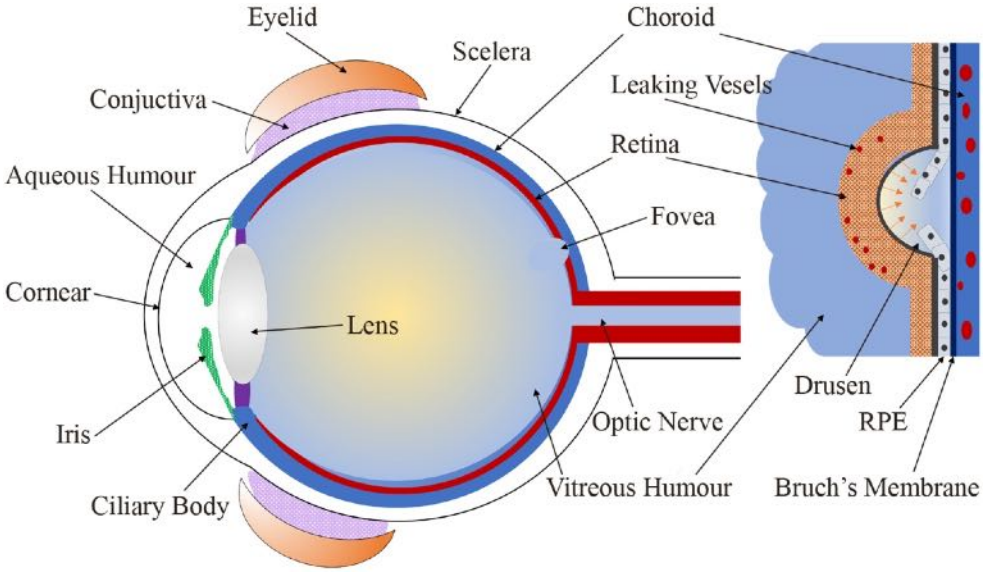

Fig. 1.3.1. *Drusogenesis*

Drusen or lipid, protein or collagen accumulates between the RPE and Bruch's membrane in the retina and damage choriocapillaris, which start leaking and cause local oedema leading to acute visual loss. Adapted by Maeva [32].

The RPE controls fluid transport between the choriocapillaris and retina, including lipid transport and metabolism and oxygen transport [33]. The BrM is a five-layer extracellular matrix between the RPE and the choroid. It regulates metabolic exchange between RPE cells and blood flow out of the choroid through the semi-permeable filtration barrier [34]. Any change in the structure of the BrM can affect the dysfunction of the RPE and the outer retina [35]. One of the most biologicaly important processes that can affect the BrM is ageing: age-related processes push for the accumulation of incompletely digested phospholipids [36] and, in combination with oxidative stress, lead to lipid peroxidation [37] and lysosomal defects in the outer segments of photoreceptors [38].

Oxidative stress caused by reactive ROS, nitric oxide (NO), oxidized lipoproteins, advanced glycosylation end products (AGEs) and apoptotic cells leads to ocular inflammation [39–41]. These accumulated substances also cause the RPE to release large amounts of various inflammatory factors. Long-term exposure to these factors leads to degeneration and atrophy of photoreceptors and RPE cells in the retina [14]. During inflammation, components of the complement system are activated, and their prolonged accumulation impairs the RPE and promotes the activation of inflammatory cells (leukocytes, microglial cells, macrophages) [42]. When macrophage

recruitment to the site of inflammation is impaired, the accumulating metabolites stimulate the release of a high amount of proangiogenic mediators such as vascular endothelial growth factor A (VEGF-A), which trigger progressive angiogenesis [19, 39, 43, 44]. New but weak, permeable and leaky blood vessels in the choroid cause local oedema leading to acute visual loss with haemorrhage and fibrotic scarring [45]. These pathological processes are responsible for the breakdown of BrM and the extracellular matrix, leading to the development of exudative AMD [43].

1.4. AMD etiology and risk factors

AMD is thought to have a multifactorial aetiology: environmental factors, genetic factors, lifestyle, gender and age. The risk of developing AMD increases with age. In people older than 75 years, the risk rises threefold compared to people between 65 and 74 years [46]. Women older than 75 are more likely to be diagnosed with AMD than men [46]. People who smoke ten packs of cigarettes per year increase the risk of developing exudative AMD. In the UK population, the risk of developing AMD was calculated, taking into account other risk factors such as socioeconomic factors, alcohol consumption and cardiovascular disease in smokers and non-smokers aged over 75 years. AMD occurred twice as often in smokers as in non-smokers; ex-smokers had a slightly increased risk, while those who had stopped smoking 20 years or more ago had no increased risk [47].

Some studies show a corrneelation between the colour of the iris and the manifestation of AMD. The lighter colour is associated with the higher the likelihood of developing the disease, but researchers have not considered other possible risk factors [48]. Some twin studies [49, 50] have been conducted that showed higher concordance in identical twins than in fraternal twins. A positive family history of AMD increases the impact of developmental risk by a factor of 2 to 27 [51, 52]. Systemic disease is a significant risk factor for the development of AMD. The incidence of AMD has been studied in patients who had hypertension, dyslipidaemia and cardiovascular diseases such as myocardial infarction and angina [53]. Cholesterol is a component of drusen [54], so dyslipidaemia is a risk factor for the development of atherosclerosis, which exacerbates AMD. One study showed that HDL and total cholesterol levels correlated positively with AMD development [55].

Besides mentioned AMD risk factors, as much as 70 % of them are genetic and genetic factors involved in lipid metabolism, inflammation and neovascularization are currently being widely studied with AMD development.

1.5. AMD treatment

In the pathogenesis of AMD, not all molecular mechanisms have been explored. The possible treatments do not cure the cause and only stop the progression of symptoms, such as improving visual acuity. None of the currently proposed methods can cure or change the course of this disease. Although the anti-VEGF drugs used to treat the exudative form of AMD are considered a therapeutic achievement because of their positive effect on visual acuity, other ways to treat this disease are still being sought. There is still no treatment for the dry form of AMD, but intensive research is being conducted to change the current situation [56].

1.5.1. Methods for the treatment of the exudative form of AMD

In 2000, the treatment method for wet AMD was approved – photodynamic therapy using intravascular light-sensitive verteporfin (benzoporphyrin derivative; Visudine) and low-power red laser (689 nm). It fundamentally changed the approach to treating untreatable choroidal neovascularisation (CNV). The essence of the treatment was the newly formed vessel occlusion. However, side effects are also possible, such as impaired visual acuity in the occlusion areas and renewal of neovascularisation three months after photodynamic therapy [57]. This method is rarely used as monotherapy but is usually preferred as part of combined therapy. If monotherapy with anti-VEGF drugs is ineffective, anti-VEGF medicines and/or corticosteroids are combined [57]. Currently, there are three major anti-VEGF drugs in ophthalmology: Bevacizumab (Avastin), Ranibizumab (Lucentis) and Aflibercept (Eyles). These drugs are injected into the vitreous under sterile conditions and according to a predetermined schedule. According to recent studies, the cumulative effect of high-risk alleles results in the younger age of onset of neovascular AMD and poor response to intravitreal ranibizumab treatment. Identifying high-risk markers would help clinicians select the best treatment [58].

1.5.2. Methods for the treatment of the dry form of AMD

Currently, there is no approved treatment for the dry form of AMD. Numerous clinical trials have been conducted to find the right drug, but no optimal variant has been found for a long time. The test drugs have been divided into groups according to their mechanism of action, which reduces oxidative stress, modulators of the visual cycle (fenretinide – blocks retinol-binding protein), neuroprotective agents (ciliary neurotrophic factor (CNTF) – protects photoreceptors from degeneration; Tandospirone – protects photore-

ceptors and RPE from oxidative stress), drugs that reduce toxic by-products (RN6G – reduces beta-amyloid accumulation in drusens), drugs that inhibit inflammation (ILuvien – immunosuppressive glucocorticosteroid, is currently approved for the treatment of diabetic macular oedema; FCFD4514S (Lampalizumab) – inhibits complement activation and chronic inflammatory processes in tissues), vascular enhancers (MC-1101 – improves choroidal blood flow), stem cells (MA09-hRPE – transplanted human RPE embryonic cells; itMSC-DryAMD – ischaemia-tolerant mesenchymal cells; HuCNS-SC – the stem cells of the central nervous system) [59]. Currently, there is insufficient information to develop evidence-based recommendations for the potential drug safety and efficacy of a complementary inhibitor for the prevention and treatment of AMD [56].

1.6. AMD prevention

It must be remembered that carotenoids – lutein and zeaxanthin – are essential in preventing AMD [60]. They are concentrated in the macula and form macular pigment [61]. Macular pigment may protect against the development of AMD because it has physical properties that philtre out blue light and act as a local antioxidant [62, 63]. Epidemiological studies have shown that the risk of AMD decreases when lutein and zeaxanthin are taken [64–67]. The metabolism of these lipophilic molecules is closely linked to cholesterol metabolism [62]. Lutein and zeaxanthin are absorbed in intestinal fat and transported to the retina in the presence of low-density lipoproteins (LDL) and high-density lipoproteins (HDL), respectively [68]. They act as antioxidants – limiting oxidative damage to retinal cells, including protection against AMD progression [62], and altering lipid metabolism, overall with increased AMD risk factors that may influence macular pigment accumulation [69].

1.7. Single nucleotide polymorphisms and proteins' role in AMD

1.7.1. Collagen

Collagen accounts for one-third of the total protein content in the body [70]. Collagens are essential supporting proteins of the extracellular matrix, which is found in connective tissue and the membrane of epithelial tissue [71]. There are two groups of collagens according to the structure they form, for example, fibrillar collagens (types I, II, III and V) and non-fibrillar – basement membrane collagen (type IV) [72]. Collagen performs several functions in cells: Several collagens play an essential role in cell adhesion,

vital for maintaining normal tissue structure and function [73]. Most importantly, collagen is a significant component of the extracellular matrix [74].

1.7.2. Collagen type VIII alpha-1 (*COL8A1*) gene

The *COL8A1* gene is located on chromosome 3q12.1 [75]. It encodes one of the two alpha chains of collagen type VIII, a central component of the basement membranes of corneal endothelial cells, including BrM, choroidal stroma and blood vessel endothelia, which play a role in maintaining vascular integrity and structure [76, 77] BrM and new blood vessel formation play a key role in the pathogenesis of AMD [78]. Recent studies have discovered an intronic variant in the *COL8A1* gene (rs13095226) that suggests an association with advanced AMD [33, 79–83].

1.7.3. Collagen type X alpha-1 (*COL10A1*) gene

Another member of the collagen gene family is the *COL10A1* gene, which is located on chromosome 6q22.1 [84] and encodes the alpha chain of type X collagen. Hypertrophic chondrocytes express this short-chain collagen during endochondral ossification. *COL10A1* expression was shown to be significantly downregulated in patients with osteoarthritis [85], but the expression was higher in various solid tumours and correlated with tumour vasculature [86]. The novel variant near *FRK/COL10A1* (rs1999930) has been linked to the advanced development of AMD [80], suggesting that *COL10A1* is a potential locus for future association studies.

1.7.4. RAD51D Paralog B (RAD51B)

The RAD51B protein is involved in the homologous recombinative repair of DNA double-strand breaks by promoting the activity of the central recombinase [87]. The absence of RAD51B protein is thought to interfere with the formation of the RAD51 nucleoprotein filament, which is the first step in homologous recombination repair [88].

1.7.5. *RAD51B* gene

The *RAD51B* gene is located on chromosome 14q24.1 [89]. While oxidative stress has been associated with various types of DNA damage that play an essential role in ageing and age-related diseases [90,91], only a few studies [92, 93] have shown a significant link between DNA damage associated with oxidative stress and the development of AMD. In addition, recent studies have identified new genetic variations in the *RAD51B* gene that may be related to AMD [22, 81, 82, 94].

1.7.6. Tribbles pseudokinase 1 (TRIB1)

TRIB1 is mainly found in the nucleus of a cell. These g-protein-coupled receptor-induced proteins play a role in the mitogen-activated protein kinases (MAPK)-related signalling cascade [95, 96], which mediates cell proliferation, differentiation and apoptosis [97] and may also regulate lipid metabolism through this pathway [21]. It has also been suggested that TRIB1 expression is regulated by inflammatory stimuli [98], one of AMD's pathogenic mechanisms.

1.7.7. *TRIB1* gene

The *TRIB1* gene is located on chromosome 8q24.13 [99]. A SNP in the *TRIB1* gene (rs6987702) is associated with AMD in the African-American and Mexican-American populations [100], suggesting a significant role of *TRIB1* in the development of AMD, which was also considered in our study.

1.7.8. Interleukin 9 (IL-9)

IL-9 belongs to the IL-2Rγc-chain family and acts as a pleiotropic cytokine in inflammatory processes [101]. T lymphocytes, or more specifically Th2, have been described as the primary source of IL-9 production [102]. However, further studies have found other cell types, including Th9, mast cells, innate lymphoid cells, NK cells and even Foxp3+ Tregs, and mucin-producing cells eosinophils can also produce IL-9 [103–105]. In addition, Dardalhon et al. have identified unique T cells that produce both IL-9 and IL-10, leading to tissue inflammation [106]. Previously, IL-9 had been described as a growth factor for only T and mast cells [107, 108]. It is known that IL-9 can promote the growth and function of erythroid progenitor cells, foetal thymocytes, myeloid progenitor cells and human megakaryoblastic leukaemic cell lines [109]. The behaviour of IL-9 is regulated by the specific IL-9 receptor (IL-9R), which consists of two subunits: the alpha chain (IL-9Rα) and the common gamma chain receptor. IL-9 binds the IL -9Rα subunit and forms the IL-9R heterocomplex. Due to the lack of specific enzymatic activity, the JAK/STAT pathway must be activated, and JAK is the initiator of the subsequent phosphorylation cascades [110]. Previous inflammation-related studies were reviewed and showed the pathogenic role of IL-9 in the development of inflammatory diseases [109].

In addition, IL-9 has also been previously investigated in several AMD studies [111–114]. Still, only Lin et al. showed that the cytokine IL-9 was overexpressed in stimulated RPE cells, which may be related to AMD development [19].

1.7.9. *IL-9* gene

The *IL-9* gene is located on chromosome 5q31.1 [115]. Several *IL-9* gene SNPs have already been identified as potential biomarkers for inflammatory diseases: atopic dermatitis, asthma, respiratory syncytial virus (RSV) infection and pituitary adenoma [116–121], and an SNP in the IL-9 gene has also been associated with IL-9 expression [122]. Given the confirmed role of inflammation and IL-9 in the development of AMD, five *IL-9* SNPs (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) were selected for the current study. Otherwise, no *IL-9* SNPs have been analyzed in AMD according to the databases.

1.7.10. Interleukin 10 (IL-10)

IL-10 belongs to the IL-10 family and regulates the inflammatory response as an anti-inflammatory cytokine [123]. Macrophages are the primary source of IL-10 is. However, other immune cells: monocytes, dendritic cells, B lymphocytes, T helper 1 (Th1) and Th2 lymphocytes, mast cells, NK cells, cytotoxic T cells, granulocytes such as neutrophils and eosinophils can secrete this interleukin [124, 125]. As a pleiotropic cytokine, IL-10 inhibits antigen-presenting cells via inhibiting the expression of major histocompatibility complex (MHC) class II molecules. It also suppresses the expression of other interleukins (IL-1, IL-6, IL-8, IL-12) and tumour necrosis factor-alpha (TNF-α). In addition, IL-10 promotes proliferation, activation and differentiation in B cells and helps prevent cell apoptosis [124,126]. The immunosuppressive IL-10 function is mediated by the heterodimeric IL-10 receptors: IL-10R1 and IL-10R2. IL-10R1 primarily ligates to IL-10 and dimerizes with IL-10R2, leading to activation of the JAK/STAT signaling pathway. Phosphorylated STAT3 molecules enter the nucleus and induce changes in the expression of immunomodulatory genes, leading to inhibition of secreted pro-inflammatory cytokines and downstream immune responses and regulating the activity of growth factors such as VEGF-A [127, 128]. The changes in IL-10 levels have been reported as a significant pathophysiological modulator in many diseases and have been studied previously [126]. Previous studies have also shown that elevated IL-10 levels may be associated with the development of choroidal neovascularization [129, 130] or overall AMD [131, 132]. Another study also published opposite results [133].

1.7.11. *IL-10* gene

The *IL-10* gene is located on chromosome 1q32.1 [134]. The three most studied SNPs in the *IL-10* promoter region were selected for our study: -592 A/C (rs1800872), -819 C/T (rs1800871) and -1082 G/A (rs1800896), as they

have been described as the most important genetic variants [135]. These genetic alterations have been associated with IL-10 levels [126], and several diseases: RSV bronchiolitis or oesophageal cancer in the Chinese population [136], and AMD [69, 70]. While one study did not find associations between IL-10 -592 A/C, -819 C/T or -1082 G/A polymorphisms and advanced AMD [137], another study found IL-10 -1082 associations with AMD [138].

1.7.12. Vascular endothelial grow factor A (VEGF-A) and receptor for vascular endothelial grow factor 2/ kinase insert domain receptor (VEGF-R2/KDR)

VEGF-A regulates angiogenesis in vascular endothelium by binding two types of VEGF family receptors: VEGF-R1, encoded by the FMS-related tyrosine kinase 1; FLT1 gene and VEGF-R2, encoded by the kinase insert domain receptor; KDR gene [139]. One of high-affinity receptor tyrosine kinases, VEGF-R2, is a primary angiogenic receptor associated with VEGFA-stimulated vascular permeability [140, 141], while VEGF-R1 is targeted for endogenous VEGF-A inhibition [140, 142].

While only exudative AMD is already treated with anti-VEGF inhibitors, in recent years, VEGF-A and its signaling pathway have been targeted for the most effective therapeutic development [18], including the other molecules based on the pathogenic processes in which they are involved [15]. Serum VEGF-A levels are being studied in depth in the development of AMD. Elevated VEGF-A levels in exudative AMD [112, 143] and patients with total AMD have been reported previously [16, 144, 145].

VEGF-R2 levels have only been analyzed in a few studies, suggesting different roles of VEGF-R2 in AMD pathogenesis. Recently, the opposing roles of serum VEGF-R2 levels in AMD have been reported [146–148].

1.7.13. *VEGFA* gene

The *VEGFA* gene is located on chromosome 6p21.1 [149]. A wide range of *VEGFA* SNPs have been studied concerning the development of AMD, demonstrating different roles of SNPs in AMD pathogenesis [16, 145, 150–156], and response to treatment [157–164]. Considering the significant associations with AMD in previous studies, we decided to investigate the effects of four SNPs in *VEGFA* on AMD in the Lithuanian population.

In the overall, the genetics play a crucial role in many diseases including AMD, so it is important to highlight the most important pathways of AMD pathogenesis and analyse it's components which could help to find the best targets for the future studies, especially including the possible markers for AMD treatment development.

2. METHODS

The present Lithuanian population-based case-control study was performed at the Laboratory of Ophthalmology, Neuroscience Institute, Lithuanian University of Health Sciences, Kaunas, Lithuania.

2.1. Ethics

The recent study was conducted according to the guidelines of the Declaration of Helsinki, and protocol was approved by Kaunas Regional Biomedical Research Ethics Committee, Lithuanian University of Health Sciences (No. BE-2-/48) (Supplementary material 1). All participants were informed about the study structure and objectives. The written informed consent form was obtained from all study subjects.

2.2. Study design and structure

2.2.1. Study Design

The study groups consisted of subjects who were admitted for the ophthalmological evaluation to the Ophthalmology Department, Hospital of Lithuanian University of Health Sciences Kauno klinikos during the period from 2014 to 2021. An ophthalmological evaluation was performed for all the study subjects, and data about general health and other diseases were obtained during an examination by a family doctor and gathered from medical records.

2.2.2. Study Structure

The whole study was divided into four individual studies (Study I–Study IV) (Fig. 2.2.2.1).

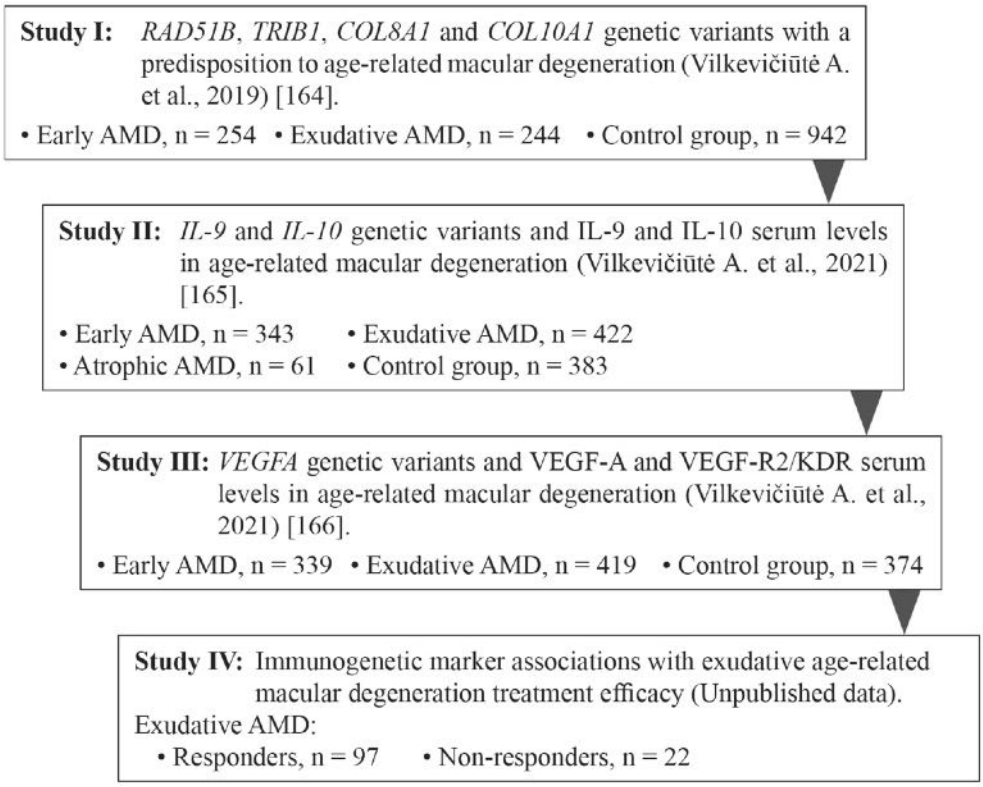

Fig. 2.2.2.1. *Study structure*

The thesis consists of four studies. Study I describes the associations between *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), and *COL10A1* (rs1064583) gene variants and early and exudative AMD development [165]. Study II describes associations between IL-9 (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and IL-10 (rs1800871, rs1800872 and rs1800896) genetic variants and early, exudative, and atrophic AMD development [166]. New study subjects were added into patients' groups, and younger controls were excluded from the control group. Also, this study includes IL-9 and IL-10 serum concentration analysis in 19 control subjects, 20 patients with early AMD, 20 atrophic AMD, and 26 exudative AMD. Study III describes the associations between *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genetic variants and early and exudative AMD development [167]. The same study subjects as in Study II were analysed. Only the atrophic AMD group and several subjects were excluded because of insufficient DNA. Moreover, this study includes VEGF-A and VEGF-R2 serum concentration analysis in 19 control subjects and 20 patients with exudative AMD. All genetic variants were analysed using TaqMan genotyping assays and serum protein concentrations measured using the ELISA method. Study IV describes the associations between genetic variants and protein concentrations from Study I–Study III and response to exudative AMD treatment with intravitreal anti-VEGF injections. Overall, the response to treatment was evaluated for 119 exudative AMD patients.

2.3. Ophthalmological evaluation

Present study subjects were evaluated by slit-lamp biomicroscope as described in our previous publication [166]. All AMD patients underwent optical coherence tomography (OCT), and optical coherence tomography angiography (OCT-A) was performed to confirm the late AMD after the OCT examination.

2.4. AMD group

AMD group consisted of subjects who underwent ophthalmological evaluation and were diagnosed with early, exudative, or atrophic AMD (Fig. 2.4.1).

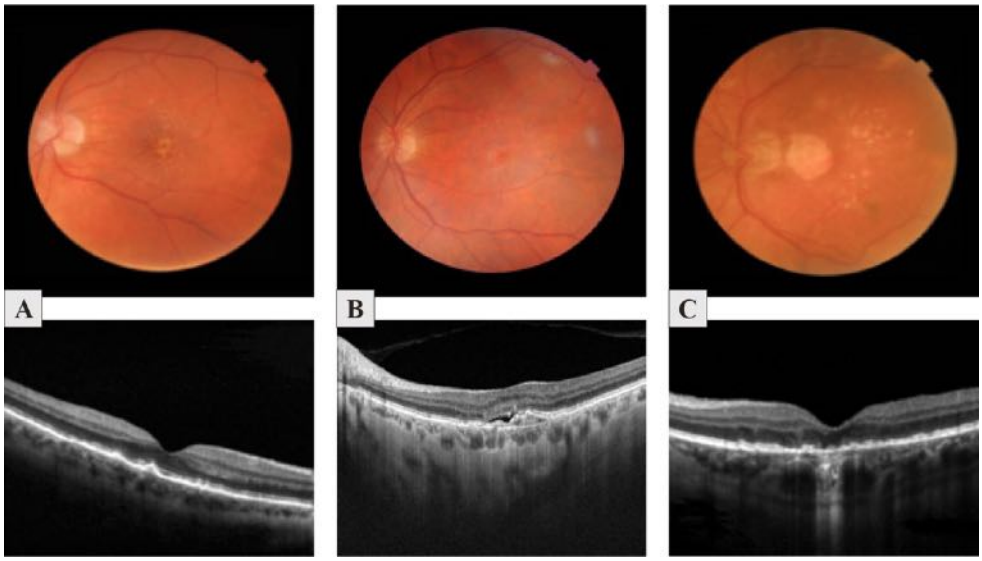

Fig. 2.4.1. *Macular changes in early, exudative, and atrophic AMD*

A – Early AMD; B – Exudative AMD; C – Atrophic AMD.

2.4.1. AMD exclusion criteria

1. Unrelated eye disorders, e.g., high refractive error, cloudy cornea, lens opacity (nuclear, cortical, or posterior subcapsular cataract) except minor opacities, keratitis, acute or chronic uveitis, glaucoma, or diseases of the optic nerve.
2. Systemic illnesses, e.g., diabetes mellitus, malignant tumours, systemic connective tissue disorders, chronic infectious and non-infectious

diseases, hypertension, coronary artery disease, stroke or conditions following organ or tissue transplantation.
3. Ungraded colour fundus photographs resulting from obscuring the ocular optic system or because of fundus photograph quality.
4. Use of antiepileptic or sedative drugs.

2.4.2. Exudative AMD response to anti-VEGF injection treatment

The anti-VEGF treatment efficacy was evaluated for exudative AMD patients presenting exudative or haemorrhagic features in the macula but who had no previous intravitreal anti-VEGF injections or any other treatment and were followed-up at least six months after the first injection of anti-VEGF. Central macular thickness (CMT) and best-corrected visual acuity (BCVA) measurements were performed before therapy, at three months, and at six months after the first anti-VEGF intravitreal injection. Insufficient effect in structural changes was considered when according to OCT there was an increase in CMT of 100 μM after six months from the first injection, or persistent macular oedema. CMT changes were calculated accordingly: CMT before treatment minus CMT after three or six months.

BCVA was evaluated before therapy, at three months and at six months after the first anti-VEGF intravitreal injection. BCVA deterioration was considered when patients loss one or more signs from the chart. BCVA changes during the treatment period were calculated according to the formula: BCVA after three or six months minus BCVA before treatment.

Based on OCT and BCVA clinical data, patients with exudative AMD were divided into two groups: responders and non-responders. The non-responders' group consisted of patients with at least two signs: reduced BCVA, increased CMT of 100 μM after six months from the first injection compared to the CMT before treatment, and persistent macular oedema. The responders' group included patients who had less than two of those signs.

2.5. Control group

Subjects who underwent ophthalmological evaluation were involved in the control group.

2.5.1. Control group inclusion criteria

1. Older than 18 years.
2. Patients after senile cataract surgeries (without any other ocular comorbidities).
3. Signed informed consent form.

2.5.2. Control group exclusion criteria:

1. Unrelated eye disorders, e. g., high refractive error, cloudy cornea, lens opacity (nuclear, cortical, or posterior subcapsular cataract) except minor opacities, keratitis, acute or chronic uveitis, glaucoma, or diseases of the optic nerve.
2. Systemic illnesses, e. g., diabetes mellitus, malignant tumours, systemic connective tissue disorders, chronic infectious and non-infectious diseases, hypertension, coronary artery disease, stroke or conditions following organ or tissue transplantation.
3. Ungraded colour fundus photographs resulting from obscuring the ocular optic system or because of fundus photograph quality.
4. Use of antiepileptic or sedative drugs.

2.6. Sample size

According to the power calculator [168] using the minor allele frequencies of all the selected SNPs [169], which were ≥ 10 %, and the global AMD prevalence (8.7 %) [2], we determined that the sample size should be at least about 100 subjects in a group. We confirmed that our collected sample sizes for early and exudative AMD and control groups were sufficient to reach 80 % or higher power for the selected SNPs analysis. Unfortunately, the atrophic AMD group is too small to reach at least the 80 % power, but the atrophic AMD is rarer condition than the early and exudative AMD in Lithuanian population to collect enough samples.

2.7. Deoxyribonucleic acid extraction from peripheral venous blood

Deoxyribonucleic acid **(**DNA) extraction was carried out at the Laboratory of Ophthalmology, Neuroscience Institute, LSMU. The DNA was extracted from 200 μL peripheral venous blood samples collected in ethylenediaminetetraacetic acid (EDTA) – containing vacutainer tubes and stored at –80 °C until the DNA extraction procedure. Genomic DNA extraction was performed utilizing a genomic DNA extraction kit based on silica-based membrane technology (*Thermo Fisher Scientific*, Lithuania), based on the manufacturer's recommendations.

2.8. Single nucleotide polymorphism selection

2.8.1. *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), *COL10A1* (rs1064583) genetic variants

Based on the multifactorial AMD pathogenesis, the SNPs selected for this study were:

- an intronic variant in the *COL8A1* gene (rs13095226), which was found to have associations with advanced AMD [33, 79–83].
- variant near *FRK/COL10A1* (rs1999930) that has also been associated with advanced AMD development [80], suggesting *COL10A1* as a potential genetic locus for future association studies.
- two variants in *RAD51B* gene (rs8017304 and rs2588809) were selected regarding the oxidative stress, which has been associated with the various types of DNA damage that play an important role in ageing and age-related diseases [90, 91], including AMD [92, 93] and previously published associations between genetic variations in the *RAD51B* gene and AMD [22, 81, 82, 94].
- It has also been variant in the *TRIB1* gene (rs6987702) has been selected based on the previous studies suggesting that TRIB1 expression is regulated by inflammatory stimulation [98]. Also, the variant has been found to be associated with AMD in African-American and Mexican-American populations [100], suggesting a significant role for TRIB1 in the development of AMD.

However, association studies of these SNPs have not been performed in both forms-early and exudative AMD. Therefore, considering previously studied associations between genetic variations and AMD, we have selected three previously described SNPs in *RAD51B* (rs8017304), *TRIB1* (rs6987702), *COL8A1* (rs13095226), and four novel genetic loci in *RAD51B* (rs2588809), *TRIB1* (rs4351376; rs4351379), and *COL10A1* (rs1064583) as potential biomarkers for early and exudative AMD.

2.8.2. *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) genetic variants

Based on the database the interactions between the IL-9 and IL-10 have been shown [170], so five *IL-9* gene SNPs (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and three SNPs in the *IL-10* gene (rs1800871, rs1800872, and rs1800896) with minor allele frequencies greater than 0.1 in the Europe population were selected from the SNP database [169].

- rs2069885: a missense variant which results in a threonine to methionine amino acid substitution;
- rs1859430 intronic variant, located in the coding region of *IL-9*;
- rs2069884 intronic variant, located in the coding region of *IL-9*;
- rs11741137: a downstream, gene variants without known functions;
- rs2069870: an upstream gene variant without known functions.

In addition, *IL-9* rs1859430, rs11741137, rs2069884 and rs2069885 have already been identified as potential biomarkers for other diseases like asthma or alergic rihnitis [116–121,171].

The widely studied *IL-10* rs1800871, rs1800872 and rs1800896 SNPs were selected as potential biomarkers for AMD based on their location in the upstream *IL-10* promoter region and their associations with IL-10 mRNA transcription and IL-10 protein expression [135].

For the first time, all these eight SNPs were selected for an AMD association study, suggesting their and IL-9 and IL-10 roles in the interleukin signaling pathway in AMD development.

2.8.3. *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genetic variants

In the present study, two tag SNPs (intron variants rs3025033 and rs2146323) covering two haploblocks (Fig. 2.8.3.1) (rs833068 have already been analyzed in our previous research study) [172] were selected from the Utah Residents with Northern and Western European ancestry (CEU) population using the public HapMap genotyping database. The pairwise option of the online LD Tag SNP selection tool [173] was used with the following settings: with $r^2 = 0.8$ set, and a minimum number of SNPs tagged by each tag SNP was 2.

Two other *VEGFA* promoter polymorphisms: -2578C/A (rs699947) and -1154G/A (rs1570360), were selected additionally based on the previous inconsistent results [150] and potential multiple SNP associations with the AMD [155].

Finally, 4 SNPs in *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) were selected for genotyping and further analysis.

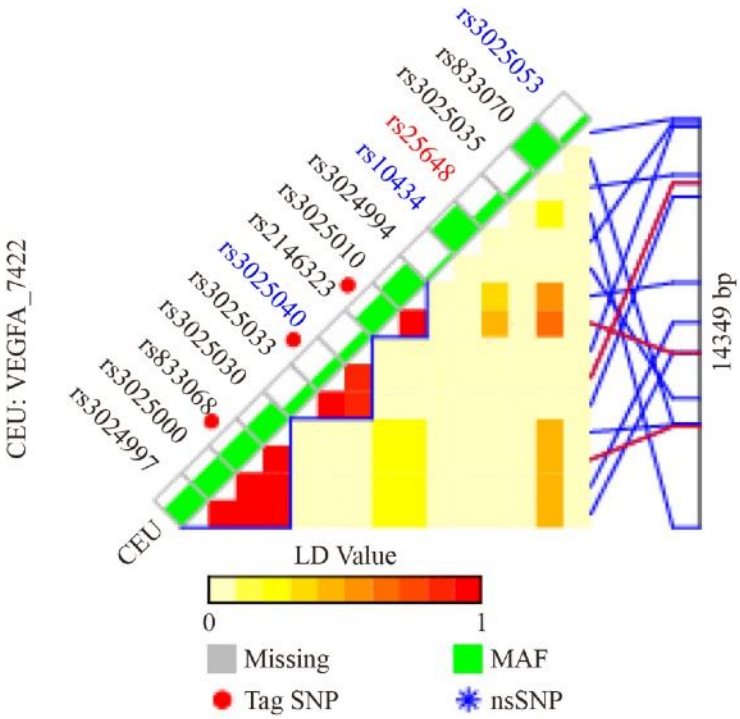

Fig. 2.8.3.1. *Tag SNPs with haploblocks in CEU population, HapMap data*

LD Tag SNP selection tool [173] was used with the following settings: selected *VEGFA* genetic variants genotyping data in CEU population from HapMap database with the LD threshold $r^2 = 0.8$ and a minimum number of SNPs tagged by each tag SNP = 2.

2.9. Genotyping

The genotyping of nineteen SNPs: *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), *COL10A1* (rs1064583), *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) and *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) was carried out at the Laboratory of Ophthalmology, Neuroscience Institute, LUHS, using predesigned TaqMan™ Genotyping assays (Thermo Fisher Scientific, Pleasanton, USA) according to the manufacturer's recommendations.

TaqMan™ SNP Genotyping Assay is a fluorescence-based genotyping assay for real-time polymerase chain reaction (RT-PCR), which includes two differentially labelled, allele-specific TaqMan minor groove binder (MGB) probe and PCR primer pairs that uniquely amplify and provide high specificity for the allele of interest.

TaqMan™ MGB Probes incorporate a 5' fluorescent reporter dye (FAM™- 6-carboxyfluorescein or VIC®) and a 3' nonfluorescent quencher (NFQ) – TAMRA™ (6-carboxytetramethylrhodamine, succinimidyl ester). As with the standard TaqMan™ assay, when the probe is intact, the proximity of the reporter dye to the quencher suppresses reporter fluorescence.

During polymerization, strand synthesis begins to displace any TaqMan™ probes that have hybridized to the target sequence and the 5'>3' exonuclease activity of the specific Taq DNA polymerase cleaves the reporter dye from the probe. Since the reporter is no longer subjected to quencher, it starts to fluorescense. This fluorescence is measured, and the level is directly proportional to the amount of target DNA accumulating during the PCR reaction.

Genotyping assay preparation protocol is presented in Table 2.9.1. Prepared mixture was poured into 96-well plates. 95 well were filled with different DNA samples and the last well was used as a no template control (NTC) were 1 µl of nuclease-free water was used instead of DNA. Then plate was covered with the adhesive film, centrifuged, and run on the RT- PCR system "Step One Plus" according to the following conditions: 95 °C for 10 min (Taq polymerase activation), 92 °C for 15 sec for denaturation, and 60 °C for 1 min for annealing and extension. The program was set to monitor the complete amplification for 45 cycles.

Table 2.9.1. *Genotyping assay preparation*

Reagents	**1X, µl**	**96X, µl**
TaqMan™ Universal Master Mix II, no UNG (*Applied Biosystems by Thermo Fisher Scientific*, Lithuania)	5 µl	480 µl
TaqMan™ SNP Genotyping Assay Assay (20×) (*Applied Biosystems by Thermo Fisher Scientific*, UK)	0,5 µl	48 µl
Nuclease-free water (*Applied Biosystems by Thermo Fisher Scientific*, Lithuania)	3,5 µl	350 µl
Total genotyping mixture volume	9 µl	878 µl
Genomic DNA	1 µl	
Total volume per well	10 µl	

The identification of all single-nucleotide polymorphisms was performed on a "StepOnePlus" real-time PCR quantification system (Thermo Fisher Scientific, Singapore). Genotyping results were obtained using Genotyping program on the StepOne V.2.3 software (Fig. 2.9.2).

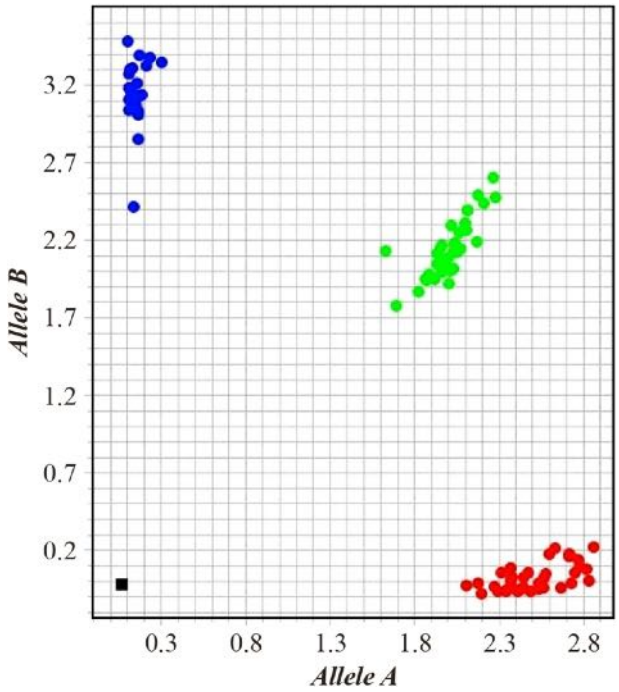

Fig. 2.9.2. *Example genotypes obtained using TaqMan™ chemistry and an "Step-One Plus" RT-PCR machine*

Different colours define three different genotypes for tartgeted SNP: A fluorescent signal from only the VIC dye indicates homozygosity for Allele "A"; the presence of only FAM dye fluorescence indicates homozygosity for Allele "B," and the presence of both fluorescent signals indicates Allele "A"/Allele "B" heterozygosity.

2.10. Quality control of genotyping

5 % randomly chosen samples were repetitively genotyped for all four SNPs to confirm the same rate of genotypes from initial and repetitive genotyping.

2.11. Serum protein concentration measurement

Obtained serum was portioned into 200 µl aliquots to Eppendorf tubes and frozen at –80 °C.

Human IL-9, IL-10, VEGF-A and VEG-FR2/KDR assays were performed using the Invitrogen ELISA Kit (Cat. No. BMS2081) for human IL-9, assay range: 3.1–200 pg/mL, sensitivity 0.5 pg/mL; Invitrogen ELISA Kit (Cat. No. BMS215-2) for human IL-10, assay range: 3.15–200 pg/mL, sensitivity 1 pg/mL; Invitrogen ELISA kit (Cat. No. BMS277-2) for VEGF-A,

assay range: 15.6 – 1000 pg/mL, sensitivity 7.9 pg/mL; Invitrogen ELISA kit (Cat. No. BMS2019) for VEGF-R2/KDR, assay range: 78–5000 pg/mL, sensitivity 7 pg/mL, following the manufacturer's instructions. Protein concentrations were analysed and calculated using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. Samples were excluded if serum cytokine concentrations were below the detection range.

2.12. Statistical analysis

Statistical analysis was performed using the SPSS/W 27.0 software (Statistical Package for the Social Sciences for Windows, Inc., Chicago, Illinois, USA). Continuous data (age, protein serum concentrations, BCVA, and CMT) were evaluated for normality by the Kolmogorov-Smirnov test. Continuous variables presented median with interquartile range (IQR) based on non-normal data distributions. The Mann-Whitney test was used to compare two groups for non-normally distributed data.

Categorical data (gender, genotype, and allele distributions) are presented as absolute numbers with percentages in brackets and compared between the early, exudative, atrophic AMD and control groups using the Chi-square (χ^2) test. Hardy-Weinberg equilibrium (HWE) was evaluated to compare the observed and expected frequencies of *RAD51B* (rs8017304; rs2588809), *TRIB1* (rs6987702; rs4351379, rs4351376), *COL8A1* (rs13095226), *COL10A1* (rs1064583), *VEGFA* (rs1570360, rs699947, rs3025033 and rs2146323), *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) using χ^2 test in the control group as well. Fisher's exact test was used only to compare the allele distributions between exudative AMD subgroups: 'responders' and 'non-responders', when the number of subjects was less than 50 in one subgroup.

The impact of SNPs on early, exudative, and atrophic AMD was evaluated using binomial logistic regression analysis. Results presented as odds ratios (ORs) with 95 % confidence interval (CI) and adjusted by covariate effect for age in the exudative AMD groups. The logistic regression results were expressed as statistical genetic models (codominant: heterozygotes *vs.* major allele homozygotes and minor allele homozygotes *vs.* major allele homozygotes; dominant: minor allele homozygotes and heterozygotes *vs.* major allele homozygotes; recessive: minor allele homozygotes *vs.* major allele homozygotes and heterozygotes; overdominant: heterozygotes *vs.* homozygotes with major allele and minor allele homozygotes; the additive

model was used to evaluate the impact of each minor allele of genotype on AMD: minor allele homozygotes *vs.* heterozygotes *vs.* major allele homozygotes). The best genetic model was selected based on the Akaike information criterion (AIC); therefore, the best genetic models had the lowest AIC values.

Haplotype analysis was performed in the early AMD and control groups, and exudative AMD and control groups separately, using online SNPStats software (*https://www.snpstats.net/snpstats/*) [174]. Linkage disequilibrium (LD) was measured and presented as D' and r^2 values. The haplotype associations with AMD were calculated by logistic regression and presented as ORs and 95 % CI and values adjusted for age in the exudative AMD analysis. Haplotypes with less than 1 % frequencies were pooled into one group and described as "rare".

A two-sided test with a value less than 0.05 was considered statistically significant. Also, we introduced an adjusted significance threshold, Bonferroni correction, for multiple comparisons alpha = 0.05/n (where n-the number of analysed SNPs in one study). Graphs were performed using GraphPad Prism version 9.0.0 for Mac, GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Study I. *RAD51B*, *TRIB1*, *COL8A1* and *COL10A1* genetic variants with a predisposition to age-related macular degeneration

Study I involved 498 patients: 254 patients diagnosed with early AMD, 244 patients with exudative AMD and 942 healthy controls. The Control group was formed of 942 subjects that matched gender classification in the early and exudative AMD group structure; however, subjects of the control group were younger than AMD patients ($p<0.001$), so the further analysis was performed adjusted by age (Table 3.3.1).

Table 3.3.1. *Demographic data of the Study I*

Characteristic	Early AMD, n = 254	Exudative AMD, n = 244	Control, n = 942	p value
Gender				
Male, n (%)	83 (32.7)	87 (35.7)	350 (37.2)	0.188*
Female, n (%)	171 (67.3)	157 (64.3)	592 (62.8)	0.665**
Age years; median (IQR)	73 (12)	76 (11)	53 (0)	**<0.001*** **<0.001****

IQR – interquartile range; p – significance level, significance when $p<0.05$; p values marked with bold indicate statistically significant p values; *Early AMD *vs.* Control group; **Exudative AMD *vs.* Control group.

Analysis of *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), and *COL10A1* (rs1064583) genetic variants with a predisposition to AMD

Hardy-Weinberg equilibrium analysis

The study included seven SNPs, but genotyping results showed that G allele at *TRIB1* rs4351376 was observed in all study subjects (n = 1440), so it was not included in further analysis.

Quality assessment based on HWE analysis showed that distribution of genotypes of *TRIB1* rs6987702 and *COL10A1* rs1064583 deviated from HWE in the control group ($p<0.05$), and those two SNPs were excluded from further statistical analysis as well (Supplementary material 2, Table 1).

Statistical analysis of four SNPs (rs8017304; rs2588809; rs4351379; rs13095226) in early and exudative AMD and control groups showed statistically significant results only in the exudative AMD group (Table 3.1.2). No statistically significant differences were observed in early AMD analysis (Table 3.1.3). Genotype (TT, TC and CC) distributions of *COL8A1* rs13095226 were significantly different between exudative AMD and control groups (60.2 %, 33.6 % and 6.1 % *vs.* 64.9 %, 32.3 % and 2.9 %, respectively, p = 0.036) but the result did not remain significant after Bonferroni correction (p>0.05/4) (Table 3.1.2).

Table 3.1.2. *RAD51B (rs8017304, rs2588809), TRIB1 (rs4351379), COL8A1 (rs13095226) genotype and allele frequencies in exudative AMD and control groups*

Gene/ marker	Genotype/ allele	Exudative AMD, n (%) n = 244	Control group, n (%) n = 942	p value
RAD51B rs8017304	AA AG GG	117 (48.0) 110 (45.1) 17 (7.0)	451 (47.9) 401 (42.6) 90 (9.6)	0.423
	A G	544 (70.5) 144 (29.5)	1303 (69.2) 581 (30.8)	0.570
RAD51B rs2588809	CC CT TT	188 (77.0) 53 (21.7) 3 (1.2)	740 (78.6) 185 (19.6) 17 (1.8)	0.652
	C T	429 (87.9) 59 (12.1)	1665 (88.4) 219 (11.6)	0.775
TRIB1 rs4351379	GG GC CC	217 (88.9) 26 (10.7) 1 (0.4)	826 (87.7) 109 (11.6) 7 (0.7)	0.780
	G C	460 (94.3) 28 (5.7)	1761 (93.5) 123 (6.5)	0.524
COL8A1 rs13095226	TT TC CC	147 (60.2) 82 (33.6) 15 (6.1)	611 (64.9) 304 (32.3) 27 (2.9)	0.036
	T C	376 (77.0) 112 (23.0)	1526 (81.0) 358 (19.0)	0.051

p – significance level and Bonferroni corrected significance level when p<0.05/4.

Table 3.1.3. *RAD51B (rs8017304; rs2588809), TRIB1 (rs4351379), COL8A1 (rs13095226) genotype and allele frequencies in early AMD and control groups*

Gene/ marker	Genotype/ allele	Early AMD, n (%) n = 254	Control group, n (%) n = 942	p value
RAD51B rs8017304	AA AG GG	125 (49.2) 102 (40.2) 27 (10.6)	451 (47.9) 401 (42.6) 90 (9.6)	0.745
	A G	352 (69.3) 156 (30.7)	1303 (69.2) 581 (30.8)	0.955
RAD51B rs2588809	CC CT TT	196 (77.2) 50 (19.7) 8 (3.1)	740 (78.6) 185 (19.6) 17 (1.8)	0.410
	C T	442 (87.0) 66 (13.0)	1665 (88.4) 219 (11.6)	0.398
TRIB1 rs4351379	GG GC CC	214 (84.3) 39 (15.4) 1 (0.4)	826 (87.7) 109 (11.6) 7 (0.7)	0.229
	G C	467 (91.9) 41 (8.1)	1761 (93.5) 123 (6.5)	0.222
COL8A1 rs13095226	TT TC CC	166 (65.4) 74 (29.1) 14 (5.5)	611 (64.9) 304 (32.3) 27 (2.9)	0.095
	T C	406 (79.9) 102 (20.1)	1526 (81.0) 358 (19.0)	0.585

p – significance level and Bonferroni corrected significance level when p<0.05/4.

Binomial logistic regression showed that rs13095226 CC genotype is associated with increased odds of exudative AMD development under the codominant (OR = 3.426; 95 % CI: 1.355–8.667; p = 0.009) and recessive (OR = 3.540; 95 % CI: 1.415–8.856; p = 0.007) genetic models (Table 3.1.4). These results remained significant after Bonferroni correction (p<0.05/4).

Table 3.1.4. *COL8A1 rs13095226 associations with exudative AMD*

Genetic model	Genotype/allele	OR* (95 % CI)	p value	AIC
Codominant	TC *vs.* TT CC *vs.* TT	0.906 (0.589–1.394) 3.426 (1.355–8.667)	0.653 **0.009**	640.511
Recessive	CC *vs.* TC + TT	3.540 (1.415–8.856)	**0.007**	638.713

*OR adjusted for age in logistic regression models; p-significance level, statistically significant when p<0.05/4; p values marked with bold indicate statistically significant p values OR: odds ratio; 95 % CI: 95 % confidence interval; AIC – Akaike information criterion.

It has been suggested that AMD development differs between males and females [175], so we performed the SNP analysis in males and females separately and found that genotype (CC, CT and TT) distributions of *RAD51B* rs2588809 were significantly different between females with early AMD and control group females (78.4 %, 17.5 % and 4.1 % *vs.* 78 %, 20.9 % and 1 %, respectively, p = 0.017), but the result did not remain significant after Bonferroni correction (p>0.05/4) (Table 3.1.5). No associations were found in genotype distribution analysis between females with exudative AMD and control females.

Table 3.1.5. *RAD51B (rs8017304; rs2588809), TRIB1 (rs4351379), COL8A1 (rs13095226) genotype and allele frequencies in females with early AMD and control females*

Gene/ marker	Genotype/ allele	Early AMD, n (%) n = 171	Control group, n (%) n = 592	p value
RAD51B rs8017304	AA AG GG	91 (53.2) 62 (36.3) 18 (10.5)	291 (49.2) 243 (41) 58 (9.8)	0.530
	A G	244 (71.3) 98 (28.7)	825 (69.7) 359 (30.3)	0.554
RAD51B rs2588809	CC CT TT	134 (78.4) 30 (17.5) 7 (4.1)	462 (78) 124 (20.9) 6 (1)	0.017
	C T	298 (87.1) 44 (12.9)	1048 (88.5) 136 (11.5)	0.486
TRIB1 rs4351379	GG GC CC	141 (82.5) 29 (17) 1 (0.6)	516 (87.2) 71 (12) 5 (0.8)	0.229
	G C	311 (90.9) 31 (9.1)	1103 (93.2) 81 (6.8)	0.165
COL8A1 rs13095226	TT TC CC	107 (62.6) 56 (32.7) 8 (4.7)	386 (65.2) 189 (31.9) 17 (2.9)	0.474
	T C	270 (78.9) 72 (21.1)	961 (81.2) 223 (18.8)	0.360

p – significance level and Bonferroni corrected significance level when p<0.05/4.

Also, binomial logistic regression after adjustment for age, showed that rs2588809 TT genotype is associated with increased odds of early AMD development in females under the codominant (OR = 6.977; 95 % CI: 1.887–25.796; p = 0.009) model (Table 3.1.6). These results remained significant after Bonferroni correction (p<0.05/4).

Table 3.1.6. *RAD51B rs2588809 associations with early AMD in females*

Genetic model	Genotype/allele	OR* (95 % CI)	p value	AIC
Codominant	CT *vs.* CC TT *vs.* CC	0.723 (0.415–1.262) 6.977 (1.887–25.796)	0.254 **0.004**	550.809

*OR adjusted for age in logistic regression models; p-significance level, statistically significant when p<0.05/4; p values marked with bold indicate statistically significant p-values OR: odds ratio; 95 % CI: 95 % confidence interval; AIC – Akaike information criterion.

In male groups, we found that genotype (TT, TC and CC) distributions of *COL8A1* rs13095226 were significantly different between males with early AMD and control group males (71.1 %, 21.7 % and 7.2 % *vs.* 64.3 %, 32.9 % and 2.9%, respectively, p = 0.036), but the result did not remain significant after Bonferroni correction (p>0.05/4) (Table 3.1.7). No statistically significant differences between genotype distributions in males with exudative AMD and control males were found.

Table 3.1.7. *RAD51B (rs8017304; rs2588809), TRIB1 (rs4351379), COL8A1 (rs13095226) genotype and allele frequencies in males with early AMD and control males*

Gene/ marker	Genotype/ allele	Early AMD, n (%) n = 83	Control group, n (%) n = 350	p value
RAD51B rs8017304	AA AG GG	34 (41) 40 (48.2) 9 (10.8)	160 (45.7) 158 (45.1) 32 (9.1)	0.712
	A G	108 (65.1) 58 (34.9)	478 (68.3) 222 (31.7)	0.424
RAD51B rs2588809	CC CT TT	62 (74.7) 20 (24.1) 1 (1.2)	278 (79.4) 61 (17.4) 11 (3.1)	0.260
	C T	144 (86.7) 22 (13.3)	617 (88.1) 83 (11.9)	0.620
TRIB1 rs4351379	GG GC CC	73 (88) 10 (12) 0 (0)	310 (88.6) 38 (10.9) 2 (0.6)	0.755
	G C	156 (94) 10 (6)	658 (94) 42 (6)	0.991
COL8A1 rs13095226	TT TC CC	59 (71.1) 18 (21.7) 6 (7.2)	225 (64.3) 115 (32.9) 10 (2.9)	0.036
	T C	136 (81.9) 30 (18.1)	565 (80.7) 135 (19.3)	0.720

p – significance level and Bonferroni corrected significance level when p<0.05/4.

Binomial regression analysis showed that rs8017304 GG genotype is associated with decreased odds of exudative AMD development in males under the recessive (OR = 0.165; 95 % CI: 0.028–0.962; p = 0.045) genetic model (Table 3.1.8). Also, rs13095226 CC genotype is associated with increased odds of exudative AMD development in males under the codominant (OR = 5.893; 95 % CI: 1.220–28.462; p = 0.027) and recessive (OR = 5.129; 95 % CI: 1.093–24.060; p = 0.038) genetic models (Table 3.1.8).

On the other hand, these results did not remain significant after Bonferroni correction (p>0.05/4) (Table 3.1.8).

Table 3.1.8. *RAD51B rs8017304 and COL8A1 rs13095226 associations with exudative AMD in males*

Genetic model	Genotype/allele	OR* (95 % CI)	p value	AIC
RAD51B rs8017304				
Recessive	GG *vs.* AG + AA	0.165 (0.028–0.962)	0.045	212.773
COL8A1 rs13095226				
Codominant	TC *vs.* TT CC *vs.* TT	1.427 (0.415–1.262) 5.893 (1.220–28.462)	0.343 0.027	214.642
Recessive	CC *vs.* TC + TT	5.129 (1.093–24.060)	0.038	213.541

*OR adjusted for age in logistic regression models; p-significance level, statistically significant when p<0.05/4; p values marked with bold indicate statistically significant p-values OR: odds ratio; 95 % CI: 95 % confidence interval; AIC – Akaike information criterion.

RAD51B haplotype associations with AMD

RAD51B haplotypes were constructed of two SNPs: rs8017304 and rs2588809. Analysis was performed to evaluate the haplotype impact on early and exudative AMD development. Linkage disequilibrium (LD) was measured and assessed by both D′ and r2 measures. The r^2 and D' values were 0.055 and 0.425 in early AMD and 0.425 and 0.457 and 0.063 in exudative AMD analysis.

Unfortunately, haplotype analysis did not reveal any statistically significant associations with early or exudative AMD development after adjustment for age.

3.2. Study II. *IL-9* and *IL-10* genetic variants and IL-9 and IL-10 serum levels in age-related macular degeneration

Study II enrolled 1209 participants: 343 subjects in early AMD, 422 in exudative AMD, and 61 in atrophic AMD groups. Also, 383 persons were involved as healthy controls (Table 3.2.1).

Table 3.2.1. *Demographic data of the Study II*

Characteristic	Early AMD group, n = 343	Exudative AMD group, n = 422	Atrophic AMD group, n = 61	Control group, n = 383	p value
Gender					**0.019***
Males, n (%)	105 (30.6)	149 (35.3)	22 (36.1)	149 (38.9)	0.291**
Females, n (%)	238 (69.4)	273 (64.7)	39 (63.9)	234 (61.1)	0.672***
Age, median (IQR)	73 (13)	77 (10)	80 (9)	72 (11)	0.266* **<0.001**** **<0.001****

IQR – interquartile range; p-significance level, statistically significant when p<0.05; p values marked with bold indicate statistically significant p-values; *early AMD *vs.* control group; **exudative AMD *vs.* control group; ***atrophic AMD *vs.* control group.

Hardy-Weinberg equilibrium

We evaluated the distributions of rs1859430, rs2069870, rs11741137, rs2069885, rs2069884, rs1800871, rs1800872 and rs1800896 genotypes in the control group using HWE. Seven SNPs were in HWE (p>0.05), but rs2069870 did not fulfil the HWE requirements because there were observed only two genotypes (Supplementary material 2, Table 2).

IL-9 (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872 and rs1800896) genotype and allele frequencies in early, exudative, and atrophic AMD

We analyzed 8 SNPS (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884, rs1800871, rs1800872 and rs1800896) and their genotype and allele distributions between the early, exudative and atrophic AMD, and control groups. Our statistical analysis revealed that genotype distributions of *IL-9* rs1859430 (GG, GA and AA) differs between early AMD and control groups (65.6 %, 27.7 % and 6.7 % *vs.* 60.8 %, 35.5 % and 3.7 %, p = 0.024). No statistically significant differences between study groups and *IL-9* (rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872 and rs1800896) were found (Table 3.2.2).

Table 3.2.2. *Distributions of IL-9 and IL-10 SNPs genotype and allele frequencies in early, exudative, atrophic AMD, and control groups*

Gene/ marker	Genotype/ allele	Group				p value*	p value**	p value***
		Early AMD, n (%) n = 343	**Exudative AMD, n (%) n = 422**	**Atrophic AMD, n (%) n = 61**	**Control, n (%) n = 383**			
IL-9 rs1859430	GG GA AA	225 (65.6) 95 (27.7) 23 (6.7)	262 (62.1) 143 (33.9) 17 (4.0)	33 (54.1) 24 (39.3) 4 (6.6)	233 (60.8) 136 (35.5) 14 (3.7)	**0.024**	0.871	0.426
	G A	545 (79.4) 141 (20.6)	667 (79.0) 177 (21.0)	90 (73.8) 32 (26.2)	602 (78.6) 146 (21.4)	0.625	0.472	0.088
IL-9 rs2069870	AA AG GG	222 (64.7) 121 (35.3) 0 (0)	262 (62.1) 160 (37.9) 0 (0)	33 (54.1) 28 (45.9) 0 (0)	235 (61.4) 148 (38.6) 0 (0)	0.349	0.832	0.282
	A G	565 (82.4) 121 (17.6)	684 (81.0) 160 (19.0)	94 (77.0) 28 (23.0)	618 (80.7) 148 (19.3)	0.410	0.853	0.350
IL-9 rs11741137	CC CT TT	238 (69.4) 91 (26.5) 14 (4.1)	290 (68.7) 120 (28.6) 12 (2.8)	41 (67.2) 18 (29.5) 2 (3.3)	247 (64.5) 126 (32.9) 10 (2.6)	0.117	0.389	0.846
	C T	657 (82.7) 119 (17.3)	700 (82.9) 144 (17.1)	100 (82.0) 22 (18.0)	620 (80.9) 146 (19.1)	0.053	0.297	0.788
IL-9 rs2069885	GG GA AA	240 (70.0) 90 (26.2) 13 (3.8)	293 (69.4) 119 (28.2) 10 (2.4)	41 (67.2) 18 (29.5) 2 (3.3)	250 (65.3) 123 (32.1) 10 (2.6)	0.172	0.453	0.892
	G A	570 (83.1) 116 (16.9)	705 (83.5) 139 (16.5)	100 (82.0) 22 (18.0)	623 (81.3) 143 (18.7)	0.382	0.246	0.867

Table 3.2.2. *Continued*

Gene/ marker	Genotype/ allele	Group				p value*	p value**	p value***
		Early AMD, n (%) n = 343	**Exudative AMD, n (%) n = 422**	**Atrophic AMD, n (%) n = 61**	**Control, n (%) n = 383**			
IL-9 rs2069884	GG GT TT	239 (69.7) 91 (26.5) 13 (3.8)	292 (69.2) 120 (28.4) 10 (2.4)	41 (67.2) 18 (29.5) 2 (3.3)	250 (65.3) 123 (32.1) 10 (2.6)	0.199	0.495	0.892
	G T	569 (82.9) 117 (17.1)	704 (83.4) 140 (16.6)	100 (82.0) 22 (18.0)	623 (81.3) 143 (18.7)	0.424	0.273	0.867
IL-10 rs1800871	GG GA AA	208 (60.6) 123 (35.9) 12 (3.5)	252 (59.7) 152 (36.0) 18 (4.3)	38 (62.3) 20 (32.8) 3 (4.9)	232 (60.6) 133 (34.7) 18 (4.7)	0.705	0.903	0.957
	G A	539 (78.6) 147 (21.4)	656 (77.7) 188 (22.3)	96 (78.7) 26 (21.3)	597 (77.9) 169 (22.1)	0.770	0.918	0.852
IL-10 rs1800872	GG GT TT	208 (60.6) 123 (35.9) 12 (3.5)	252 (59.7) 152 (36.0) 18 (4.3)	38 (62.3) 20 (32.8) 3 (4.9)	232 (60.6) 133 (34.7) 18 (4.7)	0.705	0.903	0.957
	G T	539 (78.6) 147 (21.4)	656 (77.7) 188 (22.3)	96 (78.7) 26 (21.3)	597 (77.9) 169 (22.1)	0.770	0.918	0.852
IL-10 rs1800896	TT TC CC	103 (30.0) 175 (51.0) 65 (19.0)	112 (26.5) 207 (49.1) 103 (24.4)	14 (23.0) 27 (44.3) 20 (32.8)	103 (26.9) 203 (53.0) 77 (20.1)	0.642	0.319	0.084
	T C	381 (55.5) 305 (44.5)	431 (51.1) 413 (48.9)	55 (45.1) 67 (54.9)	409 (53.4) 357 (46.6)	0.413	0.350	0.088

p-significance level, Bonferroni corrected significance level $p<0.05/8$; p-values marked with bold indicate statistically significant p values; * Early AMD *vs.* control group; ** exudative AMD *vs.* control group; ***atrophic AMD *vs.* control group.

Binary logistic regression analysis was performed to evaluate the impact of SNPs on early, exudative, and atrophic AMD. It showed that *IL-9* rs1859430 GA genotype was associated with decreased odds of early AMD under the codominant (OR = 0.700; 95 % CI: 0.507–0.966; p = 0.030), and overdominant (OR = 0.673; 95 % CI: 0.490–0.925; p = 0.015) genetic models after adjustment for gender. *IL-9* rs11741137 CT genotype was associated with decreased odds of early AMD under the overdominant model after adjustment for gender (OR = 0.720; 95 % CI: 0.522–0.994; p = 0.046). Moreover, we found that *IL-10* rs1800896 CC genotype was associated with increased odds of atrophic AMD (OR = 2.013; 95 % CI: 1.078–3.759; p = 0.028) under the recessive model after adjustment for age (Table 3.2.3), but these results did not remain significant after Bonferroni correction (p<0.05/8). No statistically significant associations were found in the exudative AMD group.

Table 3.2.3. *Associations between IL-9 rs185943 and rs11741137 on early AMD and IL-10 rs1800896 on atrophic AMD*

Genetic model	Genotype/allele	OR* (95 % CI)	p value	AIC
Early AMD				
IL-9 rs1859430				
Codominant	GA *vs.* GG AA *vs.* GG	0.700 (0.507–0.966) 1.713 (0.857–3.424)	**0.030** 0.128	1000.541
Overdominant	GA *vs.* GG+AA	0.673 (0.490–0.925)	**0.015**	994.759
IL-9 rs11741137				
Overdominant	CT *vs.* CC+TT	0.720 (0.522–0.994)	**0.046**	996.749
Atrophic AMD				
IL-10 rs1800896				
Recessive	CC *vs.* TC+TT	2.013 (1.078–3.759)	**0.028**	319.973

*OR adjusted for gender in early AMD and adjusted for age in atrophic AMD group p-significance level, Bonferroni corrected significance level p<0.05/8; p values marked with bold indicate statistically significant p values; OR – odds ratio; CI – confidence interval; AIC – Akaike information criteria.

We performed the SNP analysis in males and females separately and found that *IL-9* rs11741137 (CC, CT and TT), *IL-9* rs2069885 (GG, GA and AA) and *IL-9* rs2069884 (GG, GT and TT) genotypes were distributed statistically significantly differently comparing males with early AMD and control males: 73.3 %, 20 % and 6.7 % *vs.* 66.4 %, 31.5 % and 2 %, p = 0.032; 73.3 %, 20 % and 6.7 % *vs.* 67.1 %, 30.9 % and 2 %, p = 0.039; 73.3 %, 20 % and 6.7 % *vs.* 67.1 %, 30.9 % and 2 %, p = 0.039, respectively (Table 3.2.4).

No statistically significant associations were determined in other AMD groups or females.

Table 3.2.4. *IL-9 rs11741137, rs2069885, and rs2069884 genotypes and alleles frequencies in early AMD and control males*

Gene/marker	Genotypes/ alleles	Group		p value
		Early AMD, n (%) n = 105	**Control, n (%) n = 149**	
IL-9 rs11741137	CC CT TT	77 (73.3) 21 (20.0) 7 (6.7)	99 (66.4) 47 (31.5) 3 (2.0)	**0.032**
	C T	175 (83.3) 35 (13.3)	245 (82.2) 53 (17.8)	0.743
IL-9 rs2069885	GG GA AA	77 (73.3) 21 (20.0) 7 (6.7)	100 (67.1) 46 (30.9) 3 (2.0)	**0.039**
	G A	175 (83.3) 35 (13.3)	246 (82.6) 52 (17.4)	0.818
IL-9 rs2069884	GG GT TT	77 (73.3) 21 (20.0) 7 (6.7)	100 (67.1) 46 (30.9) 3 (2.0)	**0.039**
	G T	175 (83.3) 35 (13.3)	246 (82.6) 52 (17.4)	0.818

p-significance level, Bonferroni corrected significance level $p<0.05/8$; p values marked with bold indicate statistically significant p values.

Binomial logistic regression analysis showed that *IL-9* rs1859430 GA genotype was associated with decreased odds of early AMD in males under the codominant (OR = 0.547; 95 % CI: 0.302–0.991; p = 0.047) and overdominant (OR = 0.526; 95 % CI: 0.292–0.948; p = 0.033) genetic models, respectively (Table 3.2.5). *IL-9* rs11741137 CT genotype was associated with decreased odds of early AMD in males under the codominant model (OR = 0.543; 95 % CI: 0.301–0.979; p = 0.042) (Table 3.2.5). Unfortunately, these results did not remain significant after Bonferroni correction ($p>0.05/8$).

Table 3.2.5. *Associations of IL-9 rs1859430 and rs11741137 with early AMD in males*

Genetic model	Genotype/allele	OR (95 % CI)	p value	AIC
IL-9 rs1859430				
Codominant	GA *vs.* GG AA *vs.* GG	0.547 (0.302–0.991) 1.667 (0.54–5.010)	**0.047** 0.363	342.864
Overdominant	GA *vs.* GG+AA	0.526 (0.292–0.948)	**0.033**	341.702
IL-9 rs11741137				
Overdominant	CT *vs.* CC+TT	0.543 (0.301–0.979)	**0.042**	342.175

p-significance level, Bonferroni corrected significance level p<0.05/8; p values marked with bold indicate statistically significant p values; OR – odds ratio; CI – confidence interval; AIC-Akaike information criteria.

IL-9 and IL-10 haplotype associations with AMD

Strong pairwise LD between studied polymorphisms was observed (Table 3.2.6).

Table 3.2.6. *Linkage disequilibrium between IL-9 and IL-10 SNPs*

SNPs	AMD *vs.* Controls	
	D’	r^2
rs1859430-rs2069870	0.9828	0.8339
rs1859430-rs11741137	0.9167	0.6752
rs1859430-rs2069885	0.9397	0.6877
rs1859430-rs2069884	0.9401	0.6922
rs2069870-rs11741137	0.8271	0.6366
rs2069870-rs2069885	0.8436	0.6419
rs2069870-rs2069884	0.8445	0.6471
rs11741137-rs2069885	0.9942	0.9579
rs11741137-rs2069884	0.9942	0.9633
rs2069885-rs2069884	0.9995	0.9932
rs1800871-rs1800872	0.9997	0.9994
rs1800871-rs1800896	0.9883	0.2454
rs1800872-rs1800896	0.9883	0.2454

SNPs – single nucleotide polymorphisms; D' is the deviation between the expected haplotype frequency and the observed frequency [D’ scale: 0,1]. R^2 is the squared correlation coefficient of the haplotype frequencies [r^2 scale: 0,1]; p-significance level, statistically significant when p<0.05.

Table 3.2.7. *IL-9 haplotype association with AMD*

Haplotype	rs1859430	rs2069870	rs11741137	rs2069885	rs2069884	Frequency	OR (95 % CI)	p value
Haplotype associations with early AMD								
1	G	A	C	G	G	0.7715	1	–
2	A	G	T	A	T	0.1468	0.76 (0.54–1.08)	0.13
3	A	G	C	G	G	0.0335	0.49 (0.25–0.95)	**0.035**
4	A	A	T	A	T	0.018	1.74 (0.72–4.22)	0.22
5	G	A	T	A	T	0.0108	0.08 (0.01–0.61)	**0.015**
6	A	A	C	G	G	0.0103	2.25 (0.66–7.66)	0.19
7 (rare)	*	*	*	*	*	0.009	1.29 (0.32–5.21)	0.72
Haplotype associations with exudative AMD								
1	G	A	C	G	G	0.7713	1	–
2	A	G	T	A	T	0.1487	0.96 (0.69–1.35)	0.83
3	A	G	C	G	G	0.0408	0.99 (0.57–1.73)	0.98
4	A	A	T	A	T	0.0165	1.11 (0.46–2.66)	0.82
5 (rare)	*	*	*	*	*	0.0227	0.37 (0.15–0.92)	**0.033**
Haplotype associations with atrophic AMD								
1	G	A	C	G	G	0.7558	1	–
2	A	G	T	A	T	0.1518	1.25 (0.65–2.40)	0.5
3	A	G	C	G	G	0.0453	2.34 (0.93–5.88)	0.071
4	G	A	T	A	T	0.0167	<0.00 (–)	1
5	A	A	T	A	T	0.0162	1.05 (0.23–4.68)	0.95
6 (rare)	*	*	*	*	*	0.0142	0.55 (0.07–4.43)	0.57

p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p-values; rare – pooled rare haplotypes; OR – odds ratio; CI – confidence interval.

We identified *IL-9* and *IL-10* haplotypes and analyzed their frequencies between the early, exudative, atrophic AMD, and control. The results of frequencies of haplotypes have shown that haplotypes of *IL-9* SNPs (rs1859430A-rs2069870G-rs11741137C-rs2069885G-rs2069884G and rs1859430G-rs2069870A-rs11741137T-rs2069885A-rs2069884T) are associated with the decreased odds of early AMD (OR = 0.49; 95 % CI: 0.025–0.95; p = 0.035 and OR = 0.08; 95 % CI: 0.01–0.61; p = 0.015, respectively). The rare haplotype set showed the association with decreased odds of exudative AMD (OR = 0.37; 95 % CI: 0.015–092; p = 0.033), and no associations were observed with atrophic AMD (Table 3.2.7). Analysis of *IL-10* haplotypes did not show any associations with early, exudative, or atrophic AMD (Supplementary material 2, Table 3).

IL-9 and IL-10 serum concentrations in AMD and control groups

IL-9 and IL-10 serum levels were measured in patients with early AMD (n = 20), exudative AMD (n = 26), atrophic AMD (n = 20) and controls (n = 19). Interleukin serum concentration measurements were performed in groups considering the age and gender distributions between patients and controls. IL-9 concentrations in most samples were below the detection range, and it was not analyzed. IL-10 serums concentration differences were observed between study groups (Fig. 3.2.1). IL-10 serum concentrations were lower in exudative AMD than in controls (8.0 (2.7) pg/mL *vs.* 8.8 (2.4) pg/mL, p = 0.049), patients with early AMD (8.0 (2.7) pg/mL *vs.* 9.2 (1.7) pg/mL, p = 0.017), and atrophic AMD (8.0 pg/mL *vs.* 9.4 (1.5) pg/mL, p = 0.008), respectively.

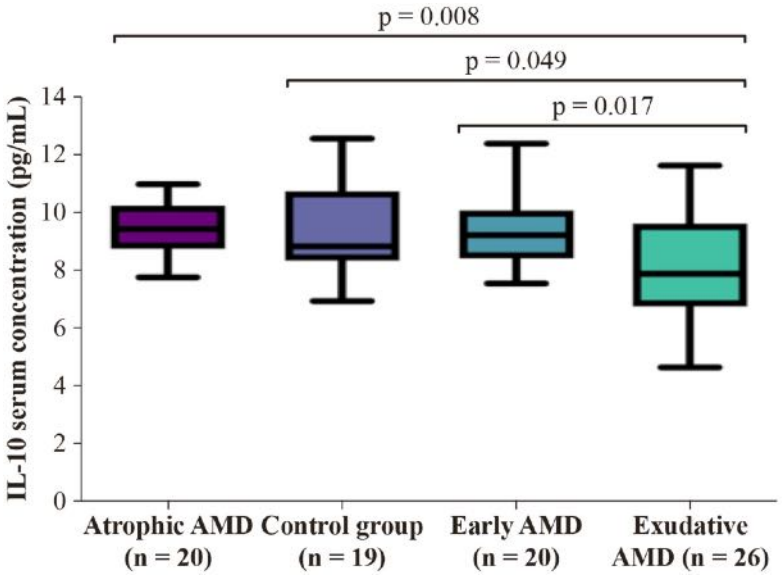

Fig. 3.2.1. *IL-10 concentrations in study groups*

IL-10 serum levels in control group (8.8 pg/mL), early AMD (9.2 pg/mL), exudative AMD (8.0 pg/mL) and atrophic AMD (9.4 pg/mL) groups. The bars represent the median with interquartile variation (1st and 3ed quartile).

Further analysis was performed to evaluate the associations between the IL-10 serum concentrations and *IL-10* SNPs. IL-10 concentrations were evaluated based on two genetic models: dominant and recessive. We found that exudative AMD patients with minor allele T at *IL-10* rs1800896 had lower IL-10 serum concentrations than those with wild type genotype CC: 7.2 (2.3) *vs.* 9.3 (1.4); p = 0.048 (Table 3.2.8).

Table 3.2.8. *Associations between IL-10 concentrations and IL-10 genotypes*

Genetic model	Genotype/allele	Early AMD (pg/mL), median (IQR)	p value	Exudative AMD (pg/mL), median (IQR)	p value	Atrophic AMD (pg/mL), median (IQR)	p value	Control group (pg/mL), median (IQR)	p value
IL-10 rs1800871									
Dominant	GA+AA *vs.* GG	9.2 (15.8) *vs.* 9.1 (1.5)	0.571	8.6 (3.9) *vs.* 7.7 (2.6)	0.312	9.3 (1.1) *vs.* 9.5 (2.2)	0.230	8.7 (2.6) vs. 8.8 (3.9)	0.442
Recessive	AA *vs.* GA+GG	(–)* *vs.* 9.2 (1.4)	–	(–)**vs.* 7.9 (2.9)	-	(–)* *vs.* 9.4 (1.6)	–	(–)* vs. 8.8 (2.9)	–
IL-10 rs1800872									
Dominant	GT+TT *vs.* GG	9.2 (15.8) *vs.* 9.1 (1.5)	0.571	8.6 (3.9) *vs.* 7.7 (2.6)	0.312	9.3 (1.1) *vs.* 9.5 (2.2)	0.230	8.7 (2.6) *vs.* 8.8 (3.9)	0.442
Recessive	TT *vs.* GT+GG	(–)* *vs.* 9.2 (1.4)	–	(–)**vs.* 7.9 (2.9)	–	(–)* *vs.* 9.4 (1.6)	–	(–)* *vs.* 8.8 (2.9)	–
IL-10 rs1800896									
Dominant	TC+CC *vs.* TT	9.1 (1.8) *vs.* 9.3 (7.8)	0.800	7.2 (2.3) *vs.* 9.3 (1.4)	**0.048**	9.4 (2.2) *vs.* 9.3 (0.7)	0.800	8.8 (3.4) *vs.* 8.7 (–)	0.573
Recessive	CC *vs.* TC+TT	9.2 (–) *vs.* 9.1 (2.7)	0.546	6.7 (2.2) *vs.* 8.5 (2.5)	0.054	8.7 (1.5) *vs.* 9.5 (1.2)	0.153	8.7 (–) *vs.* 8.8 (2)	0.958

IQR – interquartile range; * indicates that certain genotype was not determined; p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values. IL-10 concentrations were evaluated based on two genetic models: dominant, when IL-10 concentrations compared between at least one IL-10 rs1800871, rs1800872 and rs1800896 minor allele carriers (homozygous+heterozygous) and subjects with wild type genotypes; and recessive, when IL-10 concentrations compared between IL-10 rs1800871, rs1800872 and rs1800896 minor allele carriers (homozygous) and subjects with wild type allele carriers (homozygous+heterozygous).

3.3. Study III. *VEGFA* genetic variants and VEGF-A and VEGF-R2/KDR serum levels in age-related macular degeneration

Study III consisted of 1132 participants. Three hundred and thirty-nine subjects in early AMD and four hundred nineteen in the exudative AMD group have been enrolled. The Control group involved 374 healthy subjects (Table 3.3.1).

Table 3.3.1. *Demographic data of the Study III*

Characteristic	Early AMD, N = 339	Exudative AMD, N = 419	Control group, N = 374	p value
Gender				
Males, n (%)	104 (30.7)	149 (35.6)	139 (37.2)	0.068*
Females, n (%)	235 (69.3)	270 (64.4)	235 (62.8)	0.639**
Age, median (IQR)	73 (13)	77 (10)	72 (10)	0.382* **<0.001****

IQR – interquartile range; p – significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p-values; *Early AMD *vs.* Control group; **Exudative AMD *vs.* Control group.

VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) genotype and allele associations with early and exudative AMD

Genotype distributions of rs1570360, rs699947, rs3025033, and rs2146323 were evaluated in the control group using HWE. Three SNPs were in HWE ($p>0.05$), but rs1570360 did not conform the HWE requirements ($p<0.001$) (Table 3.3.2).

Statistical analysis showed that rs3025033 genotypes (AA, AG, and GG) were distributed significantly different between exudative AMD and control groups (69.2 %, 27.7 % and 3.1 % *vs.* 61 %, 33.4 %, and 5.6 %, respectively, $p = 0.029$) (Table 3.3.2). Still, these results did not remain significant after Bonferroni correction. G allele at rs3025033 was statistically significantly less frequent in the exudative AMD group than controls (16.9 % *vs.* 22.3 %, respectively, $p = 0.007$) (Table 3.3.2).

Binomial logistic regression revealed that G allele at rs3025033 was significantly associated with about 30 % lower odds of exudative AMD under the dominant model (OR = 0.67; 95 % CI: 0.49–0.80; $p = 0.0088$) and additive (OR = 0.7; 95 % CI: 0.54–0.90; $p = 0.0058$) genetic models, even after Bonferroni correction (Table 3.3.3).

Table 3.3.2. *Frequencies of VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) genotypes and alleles in early AMD, exudative AMD, and control groups*

Gene/marker	Genotype/ allele	Early AMD, n (%) N = 339	Exudative AMD, n (%) N = 419	Control group, n (%) N = 374	HWE p value	p value*	p value**	p value***
VEGFA rs1570360	GG AG AA	162 (47.8) 128 (37.8) 49 (14.5)	183 (43.7) 168 (40.1) 68 (16.2)	177 (47.3) 125 (33.4) 72 (19.3)	**<0.001**	0.186	0.136	0.512
	G A	452 (66.7) 226 (33.3)	534 (63.7) 304 (36.3)	479 (64) 269 (36)		0.298	0.897	0.234
VEGFA rs699947	AA AC CC	86 (25.4) 174 (51.3) 79 (23.3)	119 (28.4) 187 (44.6) 113 (27)	112 (29.9) 173 (46.30 89 (23.8)	0.168	0.317	0.589	0.183
	A C	346 (51) 332 (49)	425 (50.7) 413 (49.3)	397 (53.1) 351 (46.9)		0.441	0.348	0.902
VEGFA rs3025033	AA AG GG	215 (63.4) 112 (33) 12 (3.5)	290 (69.2) 116 (27.7) 13 (3.1)	228 (61) 125 (33.4) 21 (5.6)	0.482	0.399	**0.029**	0.242
	A G	542 (80) 136 (20)	696 (83.1) 142 (16.9)	581 (77.7) 167 (22.3)		0.296	**0.007**	0.119
VEGFA rs2146323	CC AC AA	142 (41.9) 157 (46.3) 40 (11.8)	191 (45.6) 169 (40.3) 59 (14.1)	158 (42.2) 157 (42) 59 (15.8)	0.06	0.248	0.603	0.236
	C A	441 (65) 237 (35)	551 (65.8) 287 (34.2)	473 (63.2) 275 (36.8)		0.477	0.296	0.773

HWE p value – Hardy Weinberg equilibrium significance level p = 0.05; p – significance level and Bonferroni corrected significance level when p<0.05/4; p values marked with bold indicate statistically significant p values. *Early AMD *vs.* Control group; **Exudative AMD *vs.* Control group; ***Early AMD *vs.* Exudative AMD.

Table 3.3.3. *Binomial logistic regression analysis of VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) in early and exudative AMD and control groups*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	p value	AIC	OR (95 % CI)*	p value	AIC
VEGFA rs1570360							
Codominant	AG *vs.* GG	1.12 (0.81–1.55)	0.499	989.3	1.25 (0.91–1.73)	0.171	1045.7
	AA *vs.* GG	0.74 (0.49–1.13)	0.168		0.94 (0.63–1.41)	0.763	
Dominant	AG+AA *vs.* GG	0.98 (0.73–1.32)	0.9	990.7	1.14 (0.85–1.52)	0.38	1045.5
Recessive	AA *vs.* GG+AG	0.71 (0.48–1.05)	0.087	987.8	0.85 (0.58–1.24)	0.4	1045.6
Overdominant	AG *vs.* GG+AA	1.21 (0.89–1.64)	0.23	989.2	1.27 (0.94–1.72)	0.11	1043.8
Additive	A	0.91 (0.75–1.11)	0.34	989.8	1.02 (0.84–1.23)	0.88	1046.2
VEGFA rs699947							
Codominant	AC *vs.* AA	1.31 (0.92–1.86)	0.132	990.4	0.97 (0.69–1.37)	0.870	1045.9
	CC *vs.* AA	1.16 (0.76–1.75)	0.492		1.28 (0.86–1.89)	0.226	
Dominant	AC+CC *vs.* AA	1.26 (0.90–1.75)	0.17	988.8	1.07 (0.78–1.47)	0.67	1046.1
Recessive	CC *vs.* AA+AC	0.97 (0.69–1.38)	0.88	990.7	1.30 (0.93–1.81)	0.13	1043.9
Overdominant	AC *vs.* AA+CC	1.23 (0.91–1.64)	0.18	988.9	0.87 (0.65–1.16)	0.34	1045.4
Additive	C	1.08 (0.88–1.33)	0.45	990.1	1.12 (0.92–1.37)	0.25	1044.9

Table 3.3.3. *Continued*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	**p value**	**AIC**	**OR (95 % CI)***	**p value**	**AIC**
VEGFA rs3025033							
Codominant	AG *vs.* AA	0.95 (0.69–1.30)	0.751	990.8	0.69 (0.51–0.95)	**0.025**	1040.7
	GG *vs.* AA	0.61 (0.29–1.26)	0.181		0.50 (0.24–1.03)	0.061	
Dominant	AG+GG *vs.* AA	0.90 (0.67–1.22)	0.5	990.3	0.67 (0.49–0.90)	**0.0088**	1039.4
Recessive	GG *vs.* AA+AG	0.62 (0.30–1.27)	0.18	988.9	0.56 (0.27–1.15)	0.11	1043.7
Overdominant	AG *vs.* AA+GG	0.98 (0.72–1.34)	0.91	990.7	0.73 (0.53–0.99)	**0.045**	1042.3
Additive	G	0.87 (0.68–1.13)	0.3	989.6	0.70 (0.54–0.90)	**0.0058**	1038.7
VEGFA rs2146323							
Codominant	AC *vs.* CC	1.11 (0.81–1.53)	0.509	989.9	0.84 (0.61–1.15)	0.280	1046.5
	AA *vs.* CC	0.75 (0.48–1.20)	0.231		0.79 (0.51–1.21)	0.280	
Dominant	AC+AA *vs.* CC	1.01 (0.75–1.37)	0.92	990.7	0.83 (0.62–1.11)	0.2	1044.6
Recessive	AA *vs.* CC+AC	0.71 (0.46–1.10)	0.12	988.3	0.86 (0.57–1.28)	0.45	1045.7
Overdominant	AC *vs.* CC+AA	1.19 (0.89–1.60)	0.24	989.4	0.89 (0.67–1.20)	0.45	1045.7
Additive	A	0.93 (0.75–1.15)	0.49	990.2	0.88 (0.72–1.07)	0.2	1044.7

*OR adjusted for age in exudative AMD analysis; p – significance level and Bonferroni corrected significance level when $p<0.05/4$; p values marked with bold indicate statistically significant p values; OR – odds ratio; CI – confident interval AIC – Akaike information criteria.

VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) genotype and allele associations with early and exudative AMD by gender

We analyzed *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genotype and allele associations with early and exudative AMD by gender. We found that rs3025033 association with exudative AMD remained only in females (Supplementary material 2, Tables 4 and 5), but not in males (Supplementary material 2, Tables 6 and 7). After the strict Bonferroni correction, three significant results remained. We found association between rs3025033 AG genotype and exudative AMD under the codominant model (OR = 0.57; 95 % CI: 0.37–0.87; p = 0.009); also, we revealed that G allele is associated with lower odds of exudative AMD for females under the dominant (OR = 0.55; 95 % CI: 0.37–0.82; p = 0.0032) and additive models (OR = 0.60; 95 % CI: 0.42–0.84; p = 0.0028) (Supplementary material 2, Table 5). AIC value shows that the additive model was best for revealing rs3025033 association with exudative AMD in females (Supplementary material 2, Table 5).

The logistic regression analysis showed significant associations between rs2146323 and exudative AMD in females, but these results did not survive Bonferroni correction (Supplementary material 2, Table 5). Moreover, no associations between rs2146323 with early AMD were found nor in females (Supplementary material 2, Tables 4 and 5) nor males (Supplementary material 2, Tables 6 and 7).

Also, we compared the genotype and allele frequencies between females with early AMD and exudative AMD and males with early AMD and exudative AMD. We found that allele G at rs3025033 was less frequent in females with exudative AMD than in females with early AMD (14.4 % *vs.* 20 %, respectively p = 0.019). Still, the result did not survive Bonferroni correction (Supplementary material 2, Table 4).

Haplotype analysis

VEGFA rs1570360, rs699947, rs3025033, and rs2146323 haplotype analysis was performed in separate early and exudative AMD groups.

Pairwise linkage disequilibrium (LD) between studied polymorphisms was observed (Table 3.3.4).

Table 3.3.4. *Linkage disequilibrium between VEGFA SNPs*

SNPs	Early AMD *vs.* Controls		Exudative AMD *vs.* Controls	
	D'	r^2	D'	r^2
rs1570360-rs699947	0.07182	0.2521	0.7087	0.2641
rs1570360-rs3025033	0.3771	0.0204	0.3919	0.0210
rs1570360-rs2146323	0.0898	0.0077	0.1191	0.0138
rs699947-rs3025033	0.1503	0.0066	0.0389	0.0004
rs699947-rs2146323	0.9904	0.5051	0.9871	0.4970
rs3025033-rs2146323	0.0866	0.0036	0.1844	0.0150

SNP – single nucleotide polymorphism; D' is the deviation between the expected haplotype frequency and the observed frequency [D' scale: 0,1]. R^2 is the squared correlation coefficient of the haplotype frequencies [r^2 scale: 0,1]; p – significance level when $p<0.05$.

Haplotype analysis revealed a protective role of rs1570360A-rs699947A-rs3025033G-rs2146323A haplotype in exudative AMD (OR = 0.46, 95 % CI: 0.23–0.90; p = 0.023) (Table 3.3.5). Any statistical haplotype associations were found in early AMD (Supplementary material 2, Table 8).

Table 3.3.5. *VEGFA haplotype associations with exudative AMD*

Haplotype	rs1570360	rs699947	rs302503	rs214632	Frequency (%)			OR (95% CI)	p value
					Exudative AMD	Controls	Total		
1	G	C	A	C	35.1	31.71	33.52	1.00	–
2	A	A	A	C	16.2	15.55	15.89	0.93 (0.68–1.27)	0.65
3	G	A	A	A	14.65	15.22	14.88	0.86 (0.61–1.22)	0.4
4	A	A	A	A	11.4	10.01	10.76	1.07 (0.73–1.57)	0.73
5	G	C	G	C	8.62	10.35	9.38	0.81 (0.53–1.23)	0.32
6	G	A	G	A	5.08	6.14	5.58	0.69 (0.40–1.20)	0.19
7	A	C	A	C	5.44	4.56	5.04	1.17 (0.69–1.98)	0.56
8	A	A	G	A	2.99	5.09	3.98	0.46 (0.23–0.90)	**0.023**
rare	*	*	*	*	NA	NA	0.95	0.36 (0.09–1.46)	0.15

p-significance level $p<0.05$; p values marked with bold indicate statistically significant p values; OR – odds ratio; CI – confident interval; AIC – Akaike information criteria; rare – haplotypes with frequencies <1 %.

VEGF-A and VEGF-R2/KDR serum concentration analysis

Serum protein concentrations were measured in 20 patients with exudative AMD before treatment and 21 control group samples. The control group for VEGF-A and VEGF-R2/KDR serum concentration measurement consisted of subjects considering the age and gender distributions based on the exudative AMD group.

We compared the VEGF-A serum concentrations between exudative AMD and control groups but did not find a significant difference (422.674 (677.02) *vs.* 615.489 (425.49), respectively, p = 0.424) (Fig. 3.3.1).

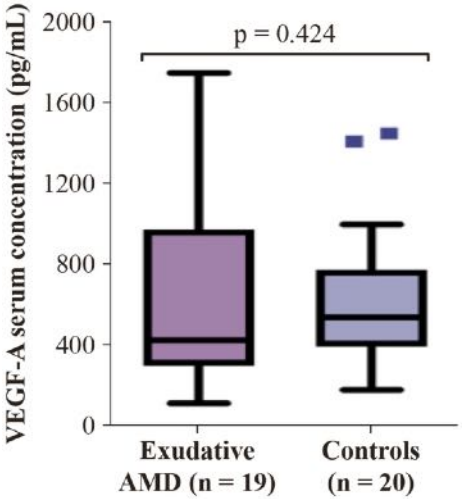

Fig. 3.3.1. *VEGF-A serum concentrations in study groups*

The bars represent the median with interquartile range (1st quartile and 3rd quartile). VEGF-A serum concentration in exudative AMD group: 422.674 (677.02) pg/mL and control group: 615.489 (425.49) pg/mL. Mann-Whitney U test, p = 0.424.

Also, we compared the VEGF-R2/KDR serum concentrations between exudative AMD and control groups, but there was no statistical difference as well (12759.2 (5358.85) *vs.* 15428.35 (6698.03), respectively, p = 0.183) (Fig. 3.3.2).

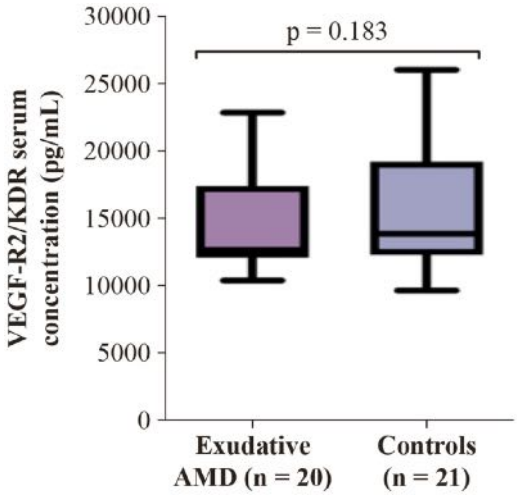

Fig. 3.3.2. *VEGF-R2/KDR serum concentrations in study groups*

The bars represent the median with interquartile range (1st quartile and 3rd quartile). VEGF-R2/KDR serum concentration in exudative AMD group: (12759.2 (5358.85) pg/mL and control group: 15428.35 (6698.03) pg/mL. Mann-Whitney U test, p = 0.183.

VEGF-A and VEGF-R2/KDR serum concentrations by *VEGFA* genotypes

We also performed VEGF-A and VEGF-R2/KDR serum concentration and *VEGFA* rs1570360, rs699947, rs302503, and rs2146323 genotype association analysis. We found that patients with exudative AMD carrying at least one C allele at rs699947 have statistically significantly higher VEGF-A serum concentrations compared to wild-type allele A homozygous genotypes carriers (485.95 (945.93) *vs.* 194.97 (–), respectively, p = 0.046) (Table 3.3.7).

Table 3.3.7. *VEGF-A and VEGF-R2/KDR serum concentration associations with VEGFA polymorphisms in exudative AMD and controls*

Genetic model	VEGF-A				VEGF-R2/KDR			
	Exudative AMD (pg/mL), median (IQR)	**p value**	**Control group (pg/mL), median (IQR)**	**p value**	**Exudative AMD (pg/mL), median (IQR)**	**p value**	**Control group (pg/mL), median (IQR)**	**p value**
VEGFA rs1570360								
Dominant AG+AA *vs.* GG	383.55 (427.3) *vs.* 640.36 (1234.32)	0.142	711.25 (472.66) *vs.* 526.14 (391.91)	0.181	12588.6 (6294.67) *vs.* 13402.08 (5875.31)	0.569	14435.95 (7035.44) *vs.* 16211.6 (7547.35)	0.916
Recessive AA *vs.* AG+GG	726.99 (–) *vs.* 421.42 (646.25)	0.737	727.89 (–) *vs.* 581.23 (518.70)	0.615	17876.6 (–) *vs.* 12673.9 (4531.86)	0.560	15428.35 (–) *vs.* 15473.4 (7038.14)	0.451
VEGFA rs699947								
Dominant AC+CC *vs.* AA	485.95 (945.93) *vs.* 194.97 (–)	**0.046**	546.82 (553.1) *vs.* 711.25 (272.64)	0.531	13357.85 (5451.18) *vs.* 11403.18 (–)	0.130	14735.2 (7207.88) *vs.* 16872.8 (10171.29)	0.347
Recessive CC *vs.* AC+AA	422.67 (1231.86) *vs.* 434.75 (678.21)	0.447	526.14 (9396.71) *vs.* 711.254 (479.99)	0.156	14044.95 (7679.2) *vs.* 12418.33 (4638.02)	0.231	16211.6 (6731.85) *vs.* 14435.95 (7038.14)	0.654
VEGFA rs3025033								
Dominant AG+GG *vs.* AA	401.86 (734.59) *vs.* 517.55 (1022.04)	0.935	629.32 (336.84) *vs.* 526.14 (621.82)	0.944	12588.6 (1324.06) *vs.* 17349.15 (7709.45)	0.623	14089.38 (6374.95) *vs.* 17524.8 (8218.45)	0.307
Recessive GG *vs.* AG+AA	– (–) *vs.* 454.31 (819.36)	–	– (–) *vs.* 581.23 (377.25)		– (–) *vs.* 12673.9 (5468.76)	–	– (–) *vs.* 15819.96 (6356.65)	
VEGFA rs2146323								
Dominant AC+AA *vs.* CC	383.55 (1282.99) *vs.* 454.311 (527.24)	0.866	735.12 (743.12) *vs.* 536.48 (318.29)	0.227	13357.85 (5271.05) *vs.* 12673.9 (5624.84)	0.874	13443.55 (7708) *vs.* 15819.98 (7312.38)	0.270
Recessive AA *vs.* AC+CC	– (–) *vs.* 42.67 (677.02)	–	739.89 (–) *vs.* 546.82 (484.33)	0.402	– (–) *vs.* 12759.2 (5358.85)	–	15880.4 (–) *vs.* 15428.35 (6868.4)	0.952

IQR – interquartile range; p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values.

3.4. Study IV. Immunogenetic marker associations with exudative age-related macular degeneration treatment efficacy

3.4.1. Central macular thickness and best-corrected visual acuity associations with treatment response

We evaluated the treatment response of exudative AMD and differentiated patients into responders (n = 22) and non-responders (n = 97). BCVA and the CMT before treatment and after 3 and 6 months and BCVA and CMT changes after 3 and 6 months were compared between responders and non-responders (Table 3.4.1.1).

We found that after 6 months of treatment with anti-VEGF injections, responders had significantly lower CMT than non-responders (median (IQR): 271 (96) *vs.* 319.5 (101), respectively, p = 0.034) and better BCVA than non-responders (median (IQR): 0.40 (0.35) *vs.* 0.30 (0.29), respectively, p = 0.028). Also, CMT and BCVA changes after 3 and 6 months of treatment were significantly different between responders and non-responders CMT significantly decreased in responders than non-responders after 3 and 6 months (p = 0.005 and p = 0.001, respectively) (Table 3.4.1.1). BCVA increased in responders compared to non-responders after 3 months (p = 0.003), but after 6 months of treatment, the BCVA was better in non-responders than in responders (p<0.001) (Table 3.4.1.1).

Table 3.4.1.1. *Central macular thickness and best-corrected visual acuity associations with the response to anti-VEGF treatment in exudative AMD patients*

Parameter	Measurement point	Responders Median (IQR), n=97	Non-responders Median (IQR), n=22	p value
CMT (μM)	Before treatment	338 (100.0)	284 (108.5)	0.269
	After 3 months	270 (100)	291 (107.5)	0.315
	After 6 months	271 (96)	319.5 (101)	**0.034**
CMT changes (μM)	After 3 months	45 (98)	10 (51)	**0.005**
	After 6 months	51 (105)	–10 (144)	**0.001**
BCVA	Before treatment	0.30 (0.24)	0.40 (0.42)	0.306
	After 3 months	0.40 (0.30)	0.30 (0.45)	0.607
	After 6 months	0.40 (0.35)	0.30 (0.29)	**0.028**
BCVA changes	After 3 months	0.025 (0.10)	–0.1 (0.09)	**0.003**
	After 6 months	0.05 (0.13)	0.30 (0.29)	**<0.001**

BCVA – best-corrected visual acuity; CMT – central macular thickness; IQR – interquartile range; p-significance level, statistically significant when p<0.05; p values marked with bold indicate statistically significant p values.

3.4.2. *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702 and rs4351379), *COL8A1* (rs13095226), *COL10A1* (rs1064583) genetic variant associations with exudative AMD treatment efficacy

In this part of our study, we compared the distributions of *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702 and rs4351379), *COL8A1* (rs13095226), *COL10A1* (rs1064583) genotypes and alleles between responders and non-responders' groups. Still, the analysis did not show any statistically significant differences (Table 3.4.2.1).

Table 3.4.2.1. *Distribution of RAD51B (rs8017304; rs2588809), TRIB1 (rs6987702; rs4351379), COL8A1 (rs13095226), COL10A1 (rs1064583) genotypes and alleles in responders and non-responders*

Gene/marker	***Genotype/ allele***	***Responders, n (%) N = 45***	***Non-responders, n (%) N = 12***	***p value****
RAD51B rs8017304	AA AG GG	26 (57.8) 17 (37.8) 2 (4.2)	4 (33.3) 6 (50) 2 (16.7)	0.179
	A G	69 (76.7) 21 (23.3)	14 (58.3) 10 (41.7)	0.119
RAD51B rs2588809	CC CT TT	36 (80) 9 (20) –	8 (66.7) 4 (33.3) –	0.269
	C T	81 (90) 9 (10)	20 (83.3) 4 (16.7)	0.468
TRIB1 rs6987702	TT TC CC	27 (60) 16 (35.6) 2 (4.4)	8 (66.7) 3 (25) 1 (8.3)	0.720
	T C	70 (77.8) 20 (22.2)	19 (79.2) 5 (20.8)	1
TRIB1 rs4351379	GG GC CC	39 (86.7) 6 (13.3) –	11 (91.7) 1 (8.3) –	0.541
	G C	84 (93.3) 6 (6.7)	23 (95.8) 1 (4.5)	1

Table 3.4.2.1. *Continued*

Gene/marker	***Genotype/ allele***	***Responders, n (%) N = 45***	***Non-responders, n (%) N = 12***	***p value****
COL8A1 rs13095226	TT TC CC	27 (60) 17 (37.8) 1 (2.2)	6 (50) 5 (41.7) 1 (8.3)	0.546
	T C	71 (78.9) 19 (21.1)	17 (70.8) 7 (29.2)	0.419
COL10A1 rs1064583	AA AG GG	19 (42.2) 22 (48.9) 4 (8.9)	6 (50) 5 (41.7) 1 (8.3)	0.888
	A G	60 (66.7) 30 (33.3)	17 (70.8) 7 (29.2)	0.809

p-significance level, p is statistically significant when <0.05; *Fisher Exact Test was used to compare the allele distributions.

Moreover, we wanted to find if there are associations between *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702 and rs4351379), *COL8A1* (rs13095226), *COL10A1* (rs1064583) and CMT or BCVA before anti-VEGF treatment and during the treatment. Statistical analysis showed that rs8017304 heterozygous and homozygous minor allele carriers had increased CMT before treatment than wild type genotype carriers ($p = 0.004$) (Table 3.4.2.2). Also, we revealed that CMT decreased more for rs4351379 heterozygous and homozygous minor allele carriers than for wild type genotype carriers after 6 months of treatment ($p = 0.03$). Any associations between these SNPs and BCVA were determined (Table 3.4.2.2). Overall, these results show that *RAD51B* rs8017304 is associated with increased CMT in exudative AMD, but it is not associated with treatment efficacy. On the other hand, *TRIB1* rs4351379 is not associated with the CMT in exudative AMD but with better anti-VEGF treatment response after 6 months (Table 3.4.2.2).

Table 3.4.2.2. *Associations between the distribution of RAD51B (rs8017304 and rs2588809), TRIB1 (rs6987702 and rs4351379), COL8A1 (rs13095226), COL10A1 (rs1064583) and central macular thickness and best-corrected visual acuity*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***RAD51B* rs8017304**		**AG+GG**	**AA**	
CMT (μM)	Before treatment	353 (57)	303.5 (97.75)	**0.004**
	After 3 months	320 (114.5)	278 (97.25)	0.051
	After 6 months	297 (113)	281 (83.5)	0.205
CMT changes (μM)	After 3 months	21.5 (77.75)	19 (62.5)	0.831
	After 6 months	26 (100.5)	25 (80)	0.488
BCVA	Before treatment	0.3 (0.3)	0.2 (0.25)	0.624
	After 3 months	0.3 (0.47)	0.275 (0.32)	0.580
	After 6 months	0.3 (0.38)	0.3 (0.25)	0.630
BCVA changes	After 3 months	0 (0.1)	0.045 (0.11)	0.911
	After 6 months	0 (0.14)	0.045 (0.1)	0.464
***RAD51B* rs2588809**		**CT+TT**	**CC**	
CMT (μM)	Before treatment	350 (112)	329 (73)	0.217
	After 3 months	270 (86)	307 (114)	0.951
	After 6 months	295 (76)	283 (108)	0.872
CMT changes (μM)	After 3 months	25 (86)	19 (61)	0.217
	After 6 months	30 (91)	39 (98)	0.576
BCVA	Before treatment	0.25 (0.24)	0.25 (0.24)	0.093
	After 3 months	0.25 (0.3)	0.32 (0.44)	0.175
	After 6 months	0.2 (0.32)	0.3 (0.34)	0.201
BCVA changes	After 3 months	0 (0.1)	0.03 (0.1)	0.841
	After 6 months	0 (0.16)	0.02 (0.1)	0.880
***TRIB1* rs6987702**		**TC+CC**	**TT**	
CMT (μM)	Before treatment	349.5 (69.25)	326 (93.5)	0.090
	After 3 months	326.5 (124.5)	278 (183.25)	0.092
	After 6 months	306 (128.5)	282 (68.75)	0.457
CMT changes (μM)	After 3 months	15 (69.75)	20.5 (69.75)	0.562
	After 6 months	47 (154)	28.5 (70.75)	0.471
BCVA	Before treatment	0.275 (0.27)	0.25 (0.27)	0.645
	After 3 months	0.35 (0.47)	0.275 (0.35)	0.792
	After 6 months	0.31 (0.37)	0.3 (0.32)	0.510
BCVA changes	After 3 months	0.05 (0.21)	0 (0.1)	0.839
	After 6 months	0.005 (0.21)	0.025 (0.1)	0.641

Table 3.4.2.2. *Continued*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***TRIB1* rs4351379**		**GC+CC**	**GG**	
CMT (μM)	Before treatment	357 (138)	337 (76.5)	0.444
	After 3 months	253.5 (136.5)	307 (92.5)	0.294
	After 6 months	265.5 (86.25)	297 (101)	0.133
CMT changes (μM)	After 3 months	36.5 (144)	19 (62)	0.170
	After 6 months	87.5 (88.25)	26.5 (93.5)	**0.030**
BCVA	Before treatment	0.325 (0.21)	0.25 (0.25)	0.592
	After 3 months	0.45 (0.53)	0.275 (0.32)	0.497
	After 6 months	0.41 (0.37)	0.3 (0.32)	0.332
BCVA changes	After 3 months	0.125 (0.32)	0 (0.1)	0.591
	After 6 months	0.085 (0.16)	0 (0.16)	0.178
***COL8A1* rs13095226**		**TC+CC**	**TT**	
CMT (μM)	Before treatment	326 (80)	347 (100)	0.795
	After 3 months	280 (64)	308 (127)	0.786
	After 6 months	282 (65)	288 (116)	0.762
CMT changes (μM)	After 3 months	19 (54)	22 (82)	0.879
	After 6 months	30 (72)	39 (126)	0.696
BCVA	Before treatment	0.2 (0.25)	0.3 (0.24)	0.733
	After 3 months	0.3 (0.35)	0.3 (0.34)	0.697
	After 6 months	0.3 (0.25)	0.32 (0.35)	0.495
BCVA changes	After 3 months	0 (0.2)	0.03 (0.1)	0.524
	After 6 months	0 (0.14)	0.04 (0.1)	0.170
***COL10A1* rs1064583**		**AG+GG**	**AA**	
CMT (μM)	Before treatment	342 (114)	330.6 (61)	0.949
	After 3 months	307 (124.5)	285 (75.5)	0.578
	After 6 months	295 (130)	283 (86)	0.872
CMT changes (μM)	After 3 months	21 (71)	19 (71)	0.872
	After 6 months	30 (86)	39 (116)	0.921
BCVA	Before treatment	0.25 (0.25)	0.25 (0.25)	0.942
	After 3 months	0.3 (0.42)	0.3 (0.29)	0.698
	After 6 months	0.32 (0.35)	0.3 (0.23)	0.669
BCVA changes	After 3 months	0.04 (0.15)	0 (0.1)	0.455
	After 6 months	0.02 (0.13)	0 (0.1)	0.541

BCVA – best-corrected visual acuity; CMT – central macular thickness; IQR – interquartile range; p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values.

3.4.3. *IL-9* and *IL-10* genetic variant and IL-9 and IL-10 serum level associations with exudative AMD treatment efficacy

In this stage of a study, we compared the distributions of *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and *IL-10* (rs1800871, rs1800872 and rs1800896) genotypes and alleles between responders and non-responders' groups, but the analysis did not show any statistically significant differences (Table 3.4.3.1).

Table 3.4.3.1. *Distribution of IL-9 (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and IL-10 (rs1800871, rs1800872 and rs1800896) genotypes and alleles in responders and non-responders*

Gene/ marker	Genotype/ allele	Responders, n (%) N = 97	Non-responders, n (%) N = 22	p value*
IL-9 rs1859430	GG GA AA	50 (51.5) 42 (43.3) 5 (5.2)	13 (59.1) 8 (36.4) 1 (4.5)	0.814
	G A	142 (73.2) 52 (26.3)	34 (77.3) 10 (22.7)	0.704
IL-9 rs2069870	AA AG GG	49 (50.5) 48 (49.5) –	13 (59.1) 9 (40.9) –	0.313
	A G	146 (75.3) 48 (24.7)	35 (79.5) 9 (20.5)	0.696
IL-9 rs11741137	CC CT TT	56 (57.7) 39 (40.2) 2 (2.1)	15 (68.2) 6 (27.3) 1 (4.5)	0.458
	C T	151 (77.8) 43 (22.2)	36 (81.8) 8 (18.2)	0.686
IL-9 rs2069885	GG GA AA	58 (59.8) 37 (38.1) 2 (2.1)	15 (68.2) 6 (27.3) 1 (4.5)	0.540
	G A	153 (78.9) 41 (21.1)	36 (81.8) 8 (18.2)	0.837
IL-9 rs2069884	GG GT TT	58 (59.8) 37 (38.1) 2 (2.1)	15 (68.2) 6 (27.3) 1 (4.5)	0.540
	G T	153 (78.9) 41 (21.1)	36 (81.8) 8 (18.2)	0.837

Table 3.4.3.1. *Continued*

Gene/ marker	Genotype/ allele	Responders, n (%) N = 97	Non-responders, n (%) N = 22	p value*
IL-10 rs1800871	GG GA AA	55 (56.7) 36 (37.1) 6 (6.2)	11 (50) 8 (36.4) 3 (13.6)	0.481
	G A	146 (75.3) 48 (24.7)	30 (68.2) 14 (31.8)	0.345
IL-10 rs1800872	GG GT TT	55 (56.7) 36 (37.1) 6 (6.2)	11 (50) 8 (36.4) 3 (13.6)	0.481
	G T	146 (75.3) 48 (24.7)	30 (68.2) 14 (31.8)	0.345
IL-10 rs1800896	TT TC CC	29 (29.9) 46 (47.4) 22 (22.7)	7 (31.8) 10 (45.5) 5 (22.7)	0.982
	T C	104 (53.6) 90 (46.4)	24 (54.5) 20 (45.5)	1

p-significance level, p is statistically significant when <0.05; *Fisher Exact Test was used to compare the allele distributions.

Also, we evaluated *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and *IL-10* (rs1800871, rs1800872 and rs1800896) associations with CMT and BCVA before the treatment and during the treatment. Statistical analysis showed that rs1859430, rs2069870 and rs2069884 heterozygous and homozygous minor allele carriers had worse BCVA before treatment than wild type genotype carriers ($p = 0.018$; $p = 0.012$; $p = 0.041$, respectively) (Table 3.4.3.2). Moreover, we found that rs2069885 heterozygous and homozygous minor allele carriers had more improved BCVA after 6 months than wild type genotype carriers ($p = 0.032$) (Table 3.4.3.2). These associations show that *IL-9* rs1859430, rs2069870 and rs2069884 SNPs are associated with worse BCVA before anti-VEGF treatment, while rs2069885 might be associated with the improved BCVA after 6 months of anti-VEGF treatment (Table 3.4.3.2).

Table 3.4.3.2. *Associations between distribution of IL-9 (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and IL-10 (rs1800871, rs1800872 and rs1800896) and central macular thickness and best corrected visual acuity*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***IL-9* rs1859430**		**GA+AA**	**GG**	
CMT (μM)	Before treatment	325 (104.5)	334.12 (99)	0.697
	After 3 months	264 (83.5)	287.63 (122.5)	0.729
	After 6 months	271 (87)	284.5 (110.75)	0.266
CMT changes (μM)	After 3 months	31 (73.5)	23.5 (89.5)	0.599
	After 6 months	26 (100.5)	30 (106.25)	0.845
BCVA	Before treatment	0.25 (0.25)	0.32 (0.3)	**0.018**
	After 3 months	0.3 (0.25)	0.4 (0.43)	0.124
	After 6 months	0.3 (0.26)	0.31 (0.45)	0.714
BCVA changes	After 3 months	0 (0.1)	0.035 (0.11)	0.750
	After 6 months	0.02 (0.1)	0 (0.2)	0.136
***IL-9* rs2069870**		**AG+GG**	**AA**	
CMT (μM)	Before treatment	327.5 (102.75)	336 (104)	0.744
	After 3 months	265 (91)	276 (114.5)	0.911
	After 6 months	272 (89.5)	283 (105)	0.422
CMT changes (μM)	After 3 months	28 (74.75)	24 (88.5)	0.774
	After 6 months	25 (101)	30 (103)	0.623
BCVA	Before treatment	0.25 (0.25)	0.32 (0.3)	**0.012**
	After 3 months	0.3 (0.24)	0.4 (0.43)	0.097
	After 6 months	0.3 (0.25)	0.32 (0.45)	0.639
BCVA changes	After 3 months	0 (0.1)	0.03 (0.12)	0.703
	After 6 months	0.016 (0.1)	0 (0.2)	0.122
***IL-9* rs11741137**		**CT+TT**	**CC**	
CMT (μM)	Before treatment	330 (103)	332.5 (119)	0.752
	After 3 months	264 (93)	276.5 (109.25)	0.977
	After 6 months	271 (55)	284.5 (116.5)	0.291
CMT changes (μM)	After 3 months	42 (86)	24 (84.75)	0.544
	After 6 months	33 (103)	28.5 (102.5)	0.564
BCVA	Before treatment	0.3 (0.25)	0.31 (0.3)	0.070
	After 3 months	0.4 (0.24)	0.335 (0.42)	0.324
	After 6 months	0.32 (0.3)	0.3 (0.42)	0.761
BCVA changes	After 3 months	0 (0.1)	0 (0.1)	0.313
	After 6 months	0.02 (0.1)	0 (0.2)	0.057

Table 3.4.3.2. *Continued*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***IL-9* rs2069885**		**GA+AA**	**GG**	
CMT (μM)	Before treatment	332 (109.5)	327.5 (113)	0.405
	After 3 months	264 (94.5)	276.5 (107.5)	0.657
	After 6 months	271 (66.5)	284.5 (116.25)	0.510
CMT changes (μM)	After 3 months	25 (83)	26 (86.25)	0.640
	After 6 months	24 (104)	30 (103.75)	0.547
BCVA	Before treatment	0.3 (0.25)	0.31 (0.3)	0.053
	After 3 months	0.4 (0.29)	0.35 (0.41)	0.261
	After 6 months	0.30 (0.32)	0.3 (0.38)	0.761
BCVA changes	After 3 months	0 (0.11)	0 (0.1)	0.348
	After 6 months	0.02 (0.11)	0 (0.2)	**0.032**
***IL-9* rs2069884**		**GT+TT**	**GG**	
CMT (μM)	Before treatment	332.5 (109.5)	327.5 (113)	0.623
	After 3 months	264 (94.5)	276.5 (107.5)	0.824
	After 6 months	271 (66.5)	284.5 (116.25)	0.416
CMT changes (μM)	After 3 months	25 (83)	26 (86.25)	0.703
	After 6 months	24 (104)	30 (103.75)	0.669
BCVA	Before treatment	0.3 (0.25)	0.31 (0.3)	**0.041**
	After 3 months	0.4 (0.29)	0.35 (0.41)	0.258
	After 6 months	0.3 (0.32)	0.3 (0.38)	0.895
BCVA changes	After 3 months	0 (0.11)	0 (0.1)	0.266
	After 6 months	0.02 (0.11)	0 (0.2)	0.050
***IL-10* rs1800871**		**GA+AA**	**GG**	
CMT (μM)	Before treatment	351.5 (108.5)	309 (83.5)	0.288
	After 3 months	276 (96)	262 (107.5)	0.451
	After 6 months	281 (92.5)	271 (106.5)	0.760
CMT changes (μM)	After 3 months	26.5 (100.25)	22 (75)	0.499
	After 6 months	36.5 (121)	25 (90)	0.393
BCVA	Before treatment	0.3 (0.27)	0.3 (0.2)	0.662
	After 3 months	0.375 (0.41)	0.4 (0.3)	0.466
	After 6 months	0.29 (0.38)	0.4 (0.28)	0.719
BCVA changes	After 3 months	0 (0.1)	0 (0.11)	0.776
	After 6 months	0.005 (0.18)	0 (0.12)	0.821

Table 3.4.3.2. *Continued*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***IL-10* rs1800872**		**GT+TT**	**GG**	
CMT (μM)	Before treatment	351.5 (108.5)	309 (83.5)	0.288
	After 3 months	276 (96)	262 (107.5)	0.451
	After 6 months	281 (92.5)	271 (106.5)	0.760
CMT changes (μM)	After 3 months	26.5 (100.25)	22 (75)	0.499
	After 6 months	36.5 (121)	25 (95)	0.393
BCVA	Before treatment	0.3 (0.27)	0.3 (0.2)	0.662
	After 3 months	0.375 (0.41)	0.4 (0.3)	0.466
	After 6 months	0.29 (0.38)	0.4 (0.28)	0.719
BCVA changes	After 3 months	0 (0.1)	0 (0.11)	0.776
	After 6 months	0.005 (0.18)	0 (0.12)	0.821
***IL-10* rs1800896**		**TC+CC**	**TT**	
CMT (μM)	Before treatment	323 (117)	344 (79.5)	0.981
	After 3 months	276 (109)	270 (91)	0.947
	After 6 months	282 (106)	268 (90.25)	0.865
CMT changes (μM)	After 3 months	28 (87)	24 (80)	0.769
	After 6 months	27 (100)	34.5 (107.25)	0.936
BCVA	Before treatment	0.3 (0.34)	0.3 (0.23)	0.458
	After 3 months	0.32 (0.3)	0.4 (0.41)	0.107
	After 6 months	0.3 (0.35)	0.335 (0.35)	0.060
BCVA changes	After 3 months	0 (0.1)	0.04 (0.2)	0.103
	After 6 months	0 (0.17)	0.015 (0.17)	0.184

BCVA – best-corrected visual acuity; CMT – central macular thickness; IQR – interquartile range; p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values.

IL-10 serum protein concentrations were compared between responders and non-responders, but the analysis did not reveal significant differences.

IL-10 serum concentrations in responders *vs.* non-responders: 7.08 (2.64) *vs.* 7.67 (4.04), respectively p = 1 (Fig. 3.4.3.1).

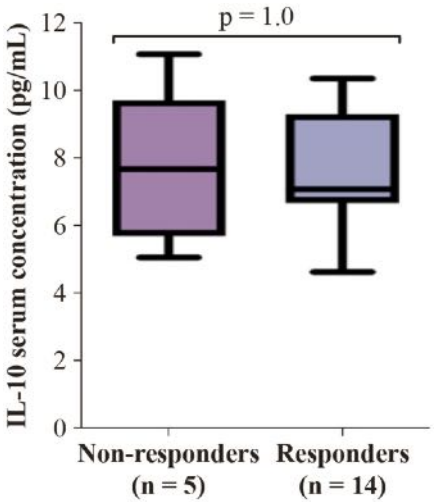

Fig. 3.4.3.1. *IL-10 serum concentrations in non-responders and responders*

The bars represent the median with interquartile range (1st quartile and 3rd quartile). VEGF-A serum concentration in non-responders' group: 7.67 (4.04) pg/mL and responders' group: 7.08 (2.64) pg/mL. Mann-Whitney U test, p = 1.

3.4.4. *VEGFA* genetic variant and VEGF-A and VEGF-R2/KDR serum level associations with exudative AMD treatment efficacy

In further analysis, we compared the distributions of *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 genotypes and alleles between responders and non-responders' groups, but the analysis did not show any statistically significant differences (Table 3.4.4.1).

Table 3.4.4.1. *Distribution of VEGFA rs1570360, rs699947, rs3025033, rs2146323 genotypes and alleles in responders and non-responders*

Gene/ marker	Genotype/ allele	Responders, n (%) N = 97	Non-responders, n (%) N = 22	p value*
VEGFA rs1570360	GG GA AA	48 (48.5) 36 (37.1) 13 (13.4)	9 (40.9) 11 (50.0) 2 (9.1)	0.524
	G A	132 (68.0) 62 (32.0)	29 (65.9) 15 (34.1)	0.859
VEGFA rs699947	AA AC CC	27 (27.8) 36 (37.1) 34 (35.1)	4 (18.2) 14 (63.6) 4 (18.2)	0.073
	A C	90 (46.4) 104 (53.6)	22 (50.0) 22 (50.0)	0.739

Table 3.4.4.1. *Continued*

Gene/ marker	Genotype/ allele	Responders, n (%) N = 97	Non-responders, n (%) N = 22	p value*
VEGFA rs3025033	AA AG GG	71 (73.2) 24 (24.7) 2 (2.1)	14 (63.6) 6 (27.3) 2 (9.1)	0.233
	A G	166 (85.6) 28 (14.4)	34 (77.4) 10 (22.7)	0.177
VEGFA rs2146323	CC CA AA	49 (50.5) 37 (38.1) 11 (11.3)	10 (45.5) 10 (45.5) 2 (9.1)	0.811
	C A	135 (69.6) 59 (30.4)	30 (68.2) 14 (31.8)	0.858

p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values; *Fisher Exact Test was used for allele comparison.

In *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 and anti-VEGF treatment response analysis, we found that rs699947 heterozygous and homozygous minor allele carriers had worse BCVA before treatment and after 3 and 6 months than wild type genotype carriers (median (IQR): 0.40 (0.21) *vs.* 0.30 (0.25), $p = 0.027$; 0.40 (0.30) *vs.* 0.30 (0.34), $p = 0.003$: 0.40 (0.30) *vs.* 0.30 (0.35), $p = 0.022$, respectively) (Table 3.4.4.2). Also, we found that rs699947 heterozygous and homozygous minor allele carriers had higher CMT after 6 months of anti-VEGF treatment than wild type genotype carriers (median (IQR): 293 (104) *vs.* 257 (50), $p = 0.032$) (Table 3.4.4.2).

Table 3.4.4.2. *Associations between VEGFA rs1570360, rs699947, rs3025033, rs2146323 and central macular thickness and best-corrected visual acuity*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***VEGFA* rs1570360**		**GA+AA**	**GG**	
CMT (μM)	Before treatment	353.5 (64.75)	325 (107)	0.547
	After 3 months	318 (120.25)	270 (98)	0.732
	After 6 months	268 (86)	293 (123)	0.282
CMT changes (μM)	After 3 months	24 (89)	42 (109.5)	0.458
	After 6 months	55 (109)	27 (113)	0.347
BCVA	Before treatment	0.25 (0.35)	0.30 (0.24)	0.312
	After 3 months	0.375 (0.27)	0.32 (0.35)	0.570
	After 6 months	0.325 (0.37)	0.30 (0.32)	0.736
BCVA changes	After 3 months	0.035 (0.11)	0.00 (0.10)	0.364
	After 6 months	0.05 (0.27)	0.01 (0.10)	0.603

Table 3.4.4.2. *Continued*

Parameter	Measurement point	Median (IQR)	Median (IQR)	p value
***VEGFA* rs699947**		**AC+CC**	**AA**	
CMT (μM)	Before treatment	308.5 (122.5)	341 (95)	0.650
	After 3 months	273.5 (89.25)	276 (101)	0.358
	After 6 months	293 (104)	257 (50)	**0.032**
CMT changes (μM)	After 3 months	24 (108)	32 (73)	0.476
	After 6 months	27 (123.5)	58 (96)	0.227
BCVA	Before treatment	0.40 (0.21)	0.30 (0.25)	**0.027**
	After 3 months	0.40 (0.30)	0.30 (0.34)	**0.003**
	After 6 months	0.40 (0.30)	0.30 (0.35)	**0.022**
BCVA changes	After 3 months	0.00 (0.18)	0.00 (0.10)	0.508
	After 6 months	0.025 (0.18)	0.02 (0.10)	0.621
***VEGFA* rs3025033**		**AG+GG**	**AA**	
CMT (μM)	Before treatment	266.5 (–)	332 (102.5)	0.562
	After 3 months	233 (–)	276 (102.5)	0.263
	After 6 months	282 (107)	282 (102)	0.763
CMT changes (μM)	After 3 months	15 (102)	39 (87)	0.502
	After 6 months	14 (123)	48 (98)	0.267
BCVA	Before treatment	0.16 (–)	0.30 (0.24)	0.510
	After 3 months	0.30 (–)	0.35 (0.35)	0.773
	After 6 months	0.275 (–)	0.32 (0.30)	0.700
BCVA changes	After 3 months	0.14 (–)	0.00 (0.10)	0.427
	After 6 months	0.115 (–)	0.02 (0.10)	0.195
***VEGFA* rs2146323**		**CA+AA**	**CC**	
CMT (μM)	Before treatment	285 (73)	341.5 (100)	0.068
	After 3 months	262 (53)	277.5 (100.75)	0.367
	After 6 months	281 (85.5)	283 (119)	0.779
CMT changes (μM)	After 3 months	26.5 (79.25)	46.5 (130.5)	0.957
	After 6 months	34.5 (93.25)	40.5 (158.75)	0.709
BCVA	Before treatment	0.25 (0.35)	0.30 (0.24)	0.963
	After 3 months	0.40 (0.30)	0.335 (0.37)	0.835
	After 6 months	0.30 (0.40)	0.31 (0.30)	0.992
BCVA changes	After 3 months	0.05 (0.10)	0.00 (0.10)	0.496
	After 6 months	0.05 (0.15)	0.005 (0.10)	0.705

BCVA – best-corrected visual acuity; CMT – central macular thickness; IQR – interquartile range; p-significance level, statistically significant when $p<0.05$; p values marked with bold indicate statistically significant p values.

VEGF-A serum concentrations were compared between responders and non-responders, but analysis did not reveal significant differences.

VEGF-A serum concentrations in responders *vs.* non-responders: 422.674 (896.94) *vs.* 450.552 (1393.43), respectively p = 0.966 (Fig. 3.4.4.1).

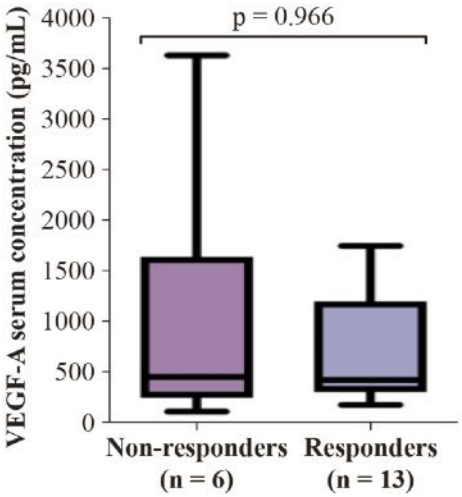

Fig. 3.4.4.1. *VEGF-A serum concentrations in non-responders and responders*

The bars represent the median with interquartile range (1st and 3rd quartile). VEGF-A serum concentration in non-responders' group: 450.552 (1393.43) pg/mL and responders' group: 422.674 (896.94) pg/mL. Mann-Whitney U test, p=0.966.

VEGF-R2/KDR serum concentrations did not differ between responders and non-responders statistically significantly as well: 12759.200 (6864.58) *vs.* 12845.475 (3602.83), respectively, p = 0.547 (Fig. 3.4.4.2).

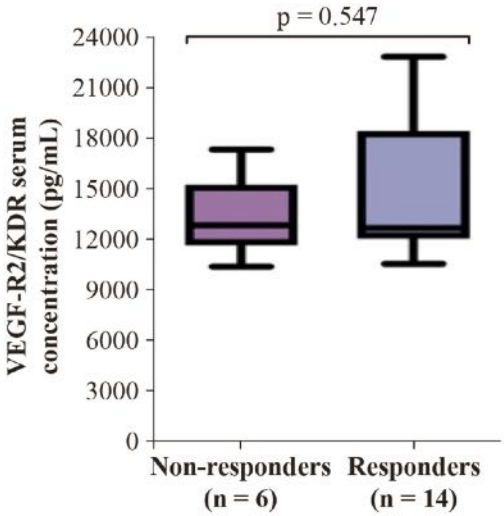

Fig. 3.4.4.2. *VEGF-R2/KDR serum concentrations in non-responders and responders*

The bars represent the median with interquartile range (1st quartile and 3rd quartile). VEGF-R2/KDR serum concentration in non-responders' group: 12845.475 (3602.8) pg/mL and responders' group: 12759.200 (6864.58) pg/mL. Mann-Whitney U test, p = 0.547.

4. DISCUSSION

RAD51B, *TRIB1*, *COL8A1* and *COL10A1* genetic variants with a predisposition to age-related macular degeneration

Study I included 7 SNPs, 5 of which were newly selected as risk factors for the development of AMD: *RAD51B* (rs2588809), *TRIB1* (rs6987702; rs4351376; rs4351379), *COL10A1* (rs1064583), and two others have already been found to be associated with AMD development: *RAD51B* (rs8017304) and *COL8A1* (rs13095226). Our study showed that one SNP (rs13095226), increased odds of developing exudative AMD in the Lithuanian population. Also, we found that rs2588809 TT genotype is associated with increased odds of early AMD development in females, but not in males.

Lipoprotein metabolism was affected by an SNP near the *TRIB1* locus called rs17321515. *TRIB1* rs17321515 was associated with the alterations of triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) and even higher risk of coronary heart disease (CHD), but the results remain controversial among studies [176–178]. The other SNP (rs2954021), located in the *TRIB1* gene, was associated with increased plasma levels TG and risk of coronary heart disease (CAD) [179,180] as were the other three variants: rs2001945, rs2001845 and rs2001844 [181]. In addition, rs2001844 was associated with increased plasma levels TG and reduced HDL-C [181]. Several association studies also reported a significant association between HDL-C and the development of AMD [182–186], while other studies did not show a significant association between HDL-C and the development of AMD [20, 187–192]. Though the association between HDL-C and AMD is controversial, the variant rs6987702 in the lipid trait-associated gene *TRIB1* showed a significant association with AMD development; unfortunately, these results did not survive rigorous correction for multiple testing [100]. None of the SNPs in *TRIB1* achieved a statistically significant association with early or exudative AMD development in the present study.

Studies of variants in *RAD51B* found statistically significant associations of SNPs in *RAD51B* with breast cancer incidence [193–196]. In addition, *RAD51B* rs911263 has been associated with rheumatoid arthritis [197, 198]; two SNPs (rs11158728 and rs927220) have been associated with the development of nasopharyngeal carcinoma [199], and one (rs34094401) has been associated with Parkinson's disease [200]. In addition, the literature review explains that risk alleles in *RAD51B* rs8017304, *COL8A1* rs13081855 and *COL10A1* rs3812111 are associated with a greater risk of developing advanced AMD [33]. The other three GWAS also demonstrated an associa-

tion of the *RAD51B* gene with AMD [22,82,94]. Seddon et al. performed a 12-year follow-up study including 2765 individuals and found that *RAD51B* was significantly associated with AMD progression (HR: 0.8; 95 % CI: 0.60–0.97, p = 0.03) [82]. In another study, Seddon and co-authors (2015) analysed AMD progression and found 834 of 2951 subjects who had progressed from no AMD, early AMD or intermediate AMD to advanced AMD. Also, AMD progression was confirmed by the associations with the *RAD51B* [94]. Fritsche et al. also demonstrated a strong association between *RAD51B* ($p<5 \times 10^{(-8)}$) and advanced AMD in Europeans and Asians [81]. Chu and colleagues also analyzed the influence of *RAD51B* on AMD using two cohorts from Caucasian and Han Chinese populations. They identified two novel SNPs in *RAD51B* (rs17105278 and rs4902566) and confirmed the association between rs8017304 and the development of AMD in Caucasians [22], suggesting a significant role for RAD51B-associated DNA damage/DNA repair mechanisms in AMD pathogenesis.

Recent studies indicated that variants in the *COL8A1* gene are associated with AMD development [80–82, 94]. One of the GWAS studies was conducted on 979 patients with advanced AMD and 1,709 controls and found a significant association between rs13095226 and AMD (p = 2.5e-06) in the European population [79]. In this study, the authors also found the frequency of rs13095226 C allele in patients with polypoidal choroidal vasculopathy (PCV), neovascular AMD (nAMD), and control subjects was similar to the frequency in Americans, while differences between Americans and Asians were found [79]. A meta-analysis of advanced AMD confirmed the previously found associations with advanced AMD [80]. In contrast, Yang's study, which included 300 cases with nAMD, 300 cases with PCV and 300 control subjects, showed no significant associations between *COL8A1* rs13095226 and nAMD or PCV development denied its risk factor role for nAMD or PCV in Chinese individuals [73]. Another variant of the same gene, the *COL8A1* rs13081855 polymorphism, has also been associated with the development of exudative AMD [83]. As in non-AMD patients, one of the common variants in the *COL8A1* gene (rs669676) was associated with myopic choroidal neovascularisation (OR: 1.88; 95 % CI: 1.18–2.98, p = 0.0076) but did not survive multiple testing [200]. Our study in the Lithuanian population discovered a strong association between a variant in *COL8A1* (rs13095226) and the development of exudative AMD; the rs13095226 genotype CC was associated with 3.5-fold increased odds of developing exudative AMD.

We also found a study showing the contribution of the extracellular collagen matrix (*FRK/COL10A1*) to the development of advanced AMD. A coding variant rs3812111 in *COL10A1*, a collagen protein that may maintain the structure and function of the extracellular matrix [80]. Ferrington

et al. analyzed the *COL10A1* rs3812111 A/T (non-risk allele/risk allele) ratios (0.69/0.65) in controls/patients with age-related macular degeneration and found it similar [201]. To the best of our knowledge, there are no studies analyzing the *COL10A1* rs1064583 polymorphism in patients with AMD.

IL-9 and *IL-10* genetic variants and IL-9 and IL-10 serum levels in age-related macular degeneration

Study II aimed to analyze the relationships between the immunogenetic markers *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and *IL-10* (rs1800871, rs1800872, rs1800896) polymorphisms and their haplotypes, serum levels IL -9 and IL -10 and the different forms of AMD.

IL-9 is encoded by the *IL-9* gene, located on chromosome 5q31.1 [115]. Several *IL-9* gene variants (rs31563, rs1859430, rs11741137, rs2069885) have already been identified as potential biomarkers for atopic dermatitis, asthma or its severity, also respiratory syncytial virus (RSV) infection and even clinical features of pituitary adenoma [116–121].

On the other hand, Elyaman et al. pointed out the multifaceted role of IL-9, which includes a regulator of both pathogenic and protective mechanisms of the immune response [202].

Two other authors emphasized gender differences in the development of asthma and RSV infections, considering gender-dependent mechanisms. Schuurhof et al. found that the major allele at rs2069885 was associated with increased susceptibility to severe RSV infection in boys and with an opposite association in girls. Aschard et al. emphasized the importance of complex mechanisms such as sex heterogeneity and pleiotropy in revealing the genes involved in asthma phenotypes [119].

Controversial results were also observed in several studies that reported no significant associations between *IL-9* promoter polymorphism A-345G and RSV bronchiolitis [203], or T113M in *IL-9* and atopic bronchial asthma [204], rs1859430 and rs2069868 and Graves' disease [54] or *IL-9* rs2069885 and allergic rhinitis in Iranian women [205]. Schürks et al. attempted to demonstrate associations between *IL-9* rs2069885 and inflammatory pathways in women with migraine, but the results did not survive correction for multiple testing [206]. Also, *IL-9* rs31563 C > T and rs31564 G > T were not associated with gastric cardiac adenocarcinoma and oesophageal cancer in a Chinese population [136, 207].

According to the databases, no *IL-9* SNPs were analyzed in AMD. Our study shows that the *IL-9* rs1859430 GA genotype and the *IL-9* rs11741137 CT genotype were associated with lower odds of early AMD. Unfortunately,

we applied Bonferroni correction in our study because of multiple comparisons, and these results did not survive this stringent correction.

Although these results did not survive Bonferroni correction, differences were observed between the male and female groups, as in previous studies [118, 119, 175]. The same tendencies remained only in the male group in further analysis. Potentially different inflammatory processes in men and women should be investigated, considering the role of hormones on the molecular mechanisms of inflammation and the response to inflammation, which may lead to different disease outcomes [208].

We also performed a haplotype analysis and identified the *IL-9* haplotypes rs1859430A-rs2069870G-rs11741137C-rs2069885G-rs2069884G and rs1859430G-rs2069870A-rs11741137T-rs2069885A-rs2069884T, which were associated with the lower odds of early AMD ($p = 0.035$ and $p = 0.015$, respectively). The rare haplotype carriers were associated with the lower odds of exudative AMD ($p = 0.033$), which may play a role in IL-9 dependent inflammatory process in AMD.

In our study, serum levels of IL-9 were not determined due to low detection rates. On the other hand, IL-9 has been previously investigated in several AMD studies. IL-9 cytokine in aqueous humour was measured, but no differences were found between neovascular AMD and control groups [111]. IL-9 was also measured in aqueous humour samples from neovascular AMD before treatment by intravitreal drug injection and afterwards and compared with the control group, but statistical analysis showed no differences between the study groups [112]. In another study, the concentrations of IL-9 in plasma and aqueous humour were not reported between the exudative AMD and control groups due to the low detection rate [113]. Shallow detection limits of IL-9 in the aqueous humour of patients with neovascular AMD have also been reported [114].

In contrast, Lin et al. showed that the cytokine IL-9 was overexpressed in stimulated RPE cells, which may be associated with AMD development [19]. Unfortunately, no other studies analyzing IL-9 protein concentrations in patients with AMD were found. Otherwise, only a few diseases/conditions were reported. Elevated IL-9 levels have been found in asthma patients, patients with allergic rhinitis and peanut allergy, and in patients with some autoimmune diseases [109].

IL-10 cytokine and genetic variants of the *IL-10* gene were also included in our study. IL-10 protein is encoded by the *IL-10* gene located on chromosome 1q32.1 [134], and the three most studied point mutations in the *IL-10* promoter -592 A/C (rs1800872), -819 C/T (rs1800871), and -1082 G/A (rs1800896) have been described as leading genetic variants for genetic association studies [135]. Our study also showed that the *IL-10* rs1800896

CC genotype was associated with 2-fold increased odds of atrophic AMD (p=0.028), but even these results did not survive strict Bonferroni correction.

Shevchenko et al. reported similar results. They found a higher *IL-10* – 1082 GG genotype frequency in AMD patients than in controls [138]. In another study, the associations between the *IL-10* -592 A/C, -819 C/T or -1082 G/A polymorphism and advanced AMD were not found [137]. Furthermore, no other such studies were found. However, these SNPs were associated with other diseases, e. g. RSV bronchiolitis, oesophageal cancer in a Chinese population [136] and no associations with cardiac adenocarcinoma of the stomach in the same population [207], suggesting further investigation for the associations of the IL-10 pathway with cancer development. Previous studies have also shown that increased IL-10 levels trigger alternative macrophage activation in the eye that may be associated with the development of choroidal neovascularization [129, 130]. We also analysed serum levels of IL-10 and found that protein serum levels were lower in exudative AMD than in controls ($p = 0.049$), as well as in patients with early AMD ($p = 0.017$) and atrophic AMD ($p = 0.008$). Similar results were found by the other group of researchers, who found lower levels of the IL-10 in wet and dry AMD groups than in controls ($p<0.05$ and $p<0.05$, respectively). They also found IL-10 levels were higher in wet AMD than in the dry AMD group ($p = 0.009$) [133]. Subhi Y et al. reached the opposite conclusion when they found that patients with nAMD had increased plasma levels of IL-10 compared to healthy controls ($p<0.0001$), and the statistically significant results remained after multivariate analysis (after adjustment for demographic data, comorbidities and lifestyle factors) IL-10 ($p<0.001$) [131]. Statistically significantly increased IL-10 serum levels in AMD patients were also found by Nassar et al. [132]. Opposite results were shown when the expression of IL-10 did not differ significantly between T follicular helper cells from AMD patients and non-AMD controls in another study [209]. Interestingly, IL-10 levels in aqueous humour did not differ between neovascular AMD and control groups [112–114]. Few other studies did not even determine IL-10 in AMD patients' plasma or aqueous humour samples [112–114]. In contrast, others showed that IL-10 levels did not differ between aqueous humour and serum samples [210].

Furthermore, we found that IL-10 levels in the exudative AMD group are associated with the minor allele T and that patients with exudative AMD carrying the *IL-10* rs1800896 CT or TT genotype have lower IL-10 serum concentrations than patients with wild-type (CC) genotype. These results confirm the role of the *IL-10* promoter polymorphism (rs1800896) in the changes in IL-10 levels [37], which may be responsible for the immune response in the development of exudative AMD. It is important to highlight

that the gene variants *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884) and *IL-10* (rs1800871, rs1800872, rs1800896), as well as the IL-9 and IL-10 serum levels, have never been investigated in AMD in the Lithuanian population and our study was the first of its kind, suggesting further research on AMD development.

VEGFA genetic variants and VEGF-A and VEGF-R2/KDR serum levels in age-related macular degeneration

Study II looked at four SNPs in the *VEGFA* gene and their association with early and exudative AMD. The study results showed that the G allele at rs3025033 was significantly associated with a lower likelihood of exudative AMD. We also found that these associations with exudative AMD persisted only in women but not in men suggesting gender role in AMD pathogenesis. In addition, differences in the frequency of rs3025033 allele G were observed between women with exudative AMD and women with early AMD, but the result did not survive Bonferroni correction. In addition, associations were found between rs2146323 and exudative AMD in women, but these outcomes did not survive Bonferroni correction.

Haplotype analysis of *VEGFA* SNPs revealed that individuals carrying the rs1570360A-rs699947A-rs3025033G-rs2146323A haplotype had a lower risk of exudative AMD, suggesting a protective role for this haplotype.

It is interesting to note that haplotypes of these four SNPs were not analysed previously.

Mori et al. (2010) investigated the association of *VEGFA* (rs1570360) with AMD but failed to obtain significant results [150]. The other three studies investigated rs1570360 associations with efficacy in the treatment of exudative AMD, but not with the occurrence of AMD [159, 160, 163].

The *VEGFA* promoter polymorphism rs699947 has been extensively studied in a variety of angiogenesis-related diseases, including many different cancers [211–213] and their response to treatment with anti-VEGF agents [214].

This SNP has also been studied in patients with AMD, but no statistically significant differences in the genotypic distribution of *VEGFA* rs699947 were found, nor were any significant associations found [16, 150–152]. Researchers have also tried to find differences between AMD subtypes by comparing neovascular and atrophic AMD groups but found no significant results [215].

The previous results were found in much smaller study groups. However, our study confirmed these results in larger sample groups, including 339 subjects in the early AMD group and 419 in the exudative AMD group, and 374 subjects in the control group.

Other researchers studied rs699947 associations with anti-VEGF treatment [157–161] or response to photodynamic therapy (PDT) [162–164], but conflicting results suggest the role of other risk factors [53] or SNP combinations in response to AMD treatment [216, 217]. Our current analysis of SNPs and VEGF-A and VEGF-R2 serum concentrations revealed that carriers of at least one C allele in rs699947 had statistically significantly higher VEGF-A serum concentrations compared with carriers of the homozygous genotype with wild-type A allele (485.95 (945.93) *vs.* 194.97 (–), $p = 0.046$).

Bulgu et al. (2014) included an intronic *VEGFA* variant rs3025033 in their study and genotyped it in 82 AMD patients and 80 controls. Unfortunately, about 98 % of the genotypes were determined to be AA, so no further statistical analysis was performed [153].

Li et al. (2021) evaluated the effect of rs3025033 on VEGF165b protein production but did not obtain significant results; on the other hand, and they showed that this SNP promotes cell proliferation in human retinal vascular endothelial cells [218].

Another study by Immonen et al. (2010) included the association analysis of rs3025033 and response to PDT, but no associations were found [164].

In contrast, the frequencies of *VEGFA* rs2146323 differed significantly between non-responders and responders to PDT [164]. However, similar frequencies of this SNP were found between exudative AMD patients and controls [151] or all AMD patients and controls [153]. Study showed that the rs2146323 C allele was protective against dry AMD, while the A allele was associated with the disease. Furthermore, there was a statistically significant difference in genotype frequencies of this SNP between wet and dry AMD types [153]. Our study showed associations between rs2146323 and exudative AMD only in women, but these results did not persist when we applied strict Bonferroni correction. Furthermore, we found no associations between this SNP and serum concentrations of VEGF-A or VEGF-R2.

Other widely studied SNPs show significant associations with AMD, indicating the possible contribution of the *VEGFA* rs1413711 and rs833061 polymorphisms to AMD susceptibility [154].

Bulgu et al. (2014) also found that the ancestral allele G in rs1413711 was protective for all AMD patients, and the AA genotype was a risk factor for AMD and especially a strongly increased risk factor for dry AMD [153]. In another study, opposite results were shown: rs1413711 CC (GG) genotype was significantly associated with a higher risk of exudative AMD [155]. The controversial results of these studies may be explained by differences in the size of the study groups and populations (Turks and Caucasians of Northern European origin).

Habibi et al. (2014) showed that the frequencies of *VEGFA* rs2010963 CC and *VEGFA* rs3025039 TT genotypes were higher in Tunisian AMD patients than in controls [145].

These results confirmed previous associations between *VEGFA* rs3025039, and wet AMD in the Japanese population [156]. A more in-depth analysis showed that genotype TT in rs3025039 was associated with increased serum VEGF-A protein levels [16].

In addition, a comprehensive analysis of *VEGFA* promoter and gene polymorphisms showed that SNPs +674, +4618, +5092, +9162 and +9512 and their haplotypes, CTCCT and TCACC, were associated with a 15-fold increased risk of exudative AMD and the haplotype of *VEGFA* promoter SNPs -460T, -417T, -172C, -165C, -160C, -152G, -141A, -116A, +405C was associated with an approximately 18-fold increased risk [155]. Two of these polymorphisms were included in our study and, together with two other SNPs, resulting in a protective haplotype for exudative AMD. It is important to elucidate the SNP combinations and their role in AMD to understand the pathogenesis and potential treatment strategies better.

VEGF-A serum levels, as well as *VEGFA* SNPs, are widely studied in AMD patients. While we found no statistical differences in serum VEGF-A levels between exudative AMD patients and controls, we confirmed the results of several other studies that included all AMD patients or only exudative AMD patients and consisted of 27 to 71 samples per group in different populations [219–226]. Other studies showed significantly increased VEGF-A levels in exudative AMD patients compared to control subjects [112, 143]. A few studies also found significantly increased VEGF-A levels in patients with total AMD than controls [16, 144, 145].

Associations with VEGF-R2 protein have not been studied as extensively as those with VEGF-A, but conflicting results have also been found. Ornek et al. (2016) found that decreased serum VEGF-R2 levels were associated with both dry and wet AMD. Furthermore, negative correlations between VEGFR-2 and foveal retinal thickness in AMD patients and a significant positive correlation with subfoveal choroidal thickness indicated the possible role of VEGF-R2 in AMD development [146]. The other two studies showed opposite associations and found increased VEGF-R2 protein levels in patients with exudative AMD than in controls [147,148], suggesting different roles of VEGF-R2 in the pathogenesis of AMD.

Immunogenetic marker associations with exudative age-related macular degeneration treatment efficacy

Study 4 was performed only in the exudative AMD group. We evaluated the exudative AMD treatment response and divided patients into responders (n = 22) and non-responders (n = 97) based on the ophthalmological parameters. Associations between SNPs from Study 1, 2 and 3 with treatment response and best-corrected visual acuity and the CMT before treatment and after 3 and 6 months and BCVA and CMT changes after 3 and 6 months were evaluated.

Firstly, we compared the distributions of *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702 and rs4351379), *COL8A1* (rs13095226), *COL10A1* (rs1064583) genotypes and alleles between responders and non-responders, but it did not show any statistically significant differences. Otherwise, we found that *RAD51B* rs8017304 is associated with increased CMT in exudative AMD, but it is not associated with treatment efficacy. On the other hand, *TRIB1* rs4351379 is not associated with the CMT in exudative AMD but with better anti-VEGF treatment response after 6 months.

Further analysis revealed that *IL-9* rs1859430, rs2069870 and rs2069884 SNPs are associated with worse BCVA before anti-VEGF treatment. In contrast, rs2069885 might be associated with the improved BCVA after 6 months of anti-VEGF treatment.

VEGFA SNP analysis showed that rs699947 C allele was associated with worse BCVA before and after 3 and 6 months of treatment. Also, this SNP was associated with higher CMT after 6 months of anti-VEGF therapy, so the results show that the *VEGFA* rs699947 C allele is associated with worse treatment efficacy.

Other scientists also seek to find the associations between genetic markers and response to anti-VEGF treatment. They focus on genetic variants previously associated with the exudative AMD development because their associations with the AMD pathogenesis can be related to the response to treatment with anti-VEGF agents. Widely studied *HTRA1* (rs11200638), A69S at *LOC387715/ARMS2*, rs10490924 in *ARMS2/HTRA1* or CFH Y402H were associated with a poorer visual outcome for anti-VEGF treatment [227–229]. These findings could lead to the individualized treatment regimens based on patients' genotypes to achieve optimal treatment response in AMD.

While none of the *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702 and rs4351379), *COL8A1* (rs13095226), *COL10A1* (rs1064583), *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884), and *IL-10* (rs1800871, rs1800872, rs1800896) polymorphisms has been analysed with the response of exudative AMD treatment previously, the *VEGFA* SNPs have been involved into several studies as potential biomarkers which could help

to achieve the optimal treatment response and develop individualised therapeutic approaches for exudative AMD.

Researchers analyzed rs699947 associations with anti-VEGF treatment and found similar results showing that rs699947 AA genotype had a higher chance of increasing best-corrected visual acuity or better overall response when intravitreal ranibizumab [157, 158] or bevacizumab [159] treatment applied for AMD. *VEGFA* rs699947 was also associated with the better response to the photodynamic therapy not only with intravitreal injections [164]. Moreover, few studies found associations between *VEGFA* rs699947 and treatment efficacy: AA genotype carriers had higher chances of good response compared to other genotypes [161] or demonstrated an association with retinal thickness [230] but statistical significance did not remain after corrections for multiple testing. Of course, there was a study which showed no associations between *VEGFA* rs699947 and anti-VEGF response as well [151]. Opposite results were shown in one study where ranibizumab treatment was significantly more effective as measured by BCVA in patients harbouring the C allele at rs699947, and the genotype AA was associated with the absence of early functional response to ranibizumab [231].

VEGFA rs1570360 and rs3025033 with two others *VEGFA* SNPs (rs699947 and rs2010963) were analyzed with the photodynamic therapy response, but no associations were found [163, 164].

While Yildiz et al. study confirmed that *VEGFA* rs2146323 has no relationship to any effect on anti-VEGF response [151], differences in rs2146323 genotype distributions were found between PDT non-responders and PDT responders [164]. Hagstrom et al. demonstrated an association between *VEGFA* rs2146323 and retinal thickness; however, the adjusted p value was not statistically significant showing no associations with the response to anti-VEGF therapy (ranibizumab or bevacizumab) for exudative AMD [230].

Other *VEGFA* gene SNPs (rs699946 and rs3025000) were also related with better visual outcomes after anti-VEGF treatment [232, 233].

However, VEGF-A serum levels have not been studied so widely with the response to intravitreal anti-VEGF treatment. It was only shown that aflibercept significantly decreased serum and plasma VEGF-A concentrations one month after injection [234] and conbercept significantly reduced serum VEGF-A level one day and one week after injection only [235]. In contrast, ranibizumab had no significant effect on serum or plasma VEGF-A concentration changes [234, 235].

Our study compared VEGF-A and VEGF-R2/KDR serum protein concentrations between responders and non-responders, but the analysis did not reveal significant differences. VEGF-R2/KDR serum levels were not analysed in previous studies with exudative AMD treatment response.

CONCLUSIONS

1. *COL8A1* rs13095226 CC genotype, compared to TT and TC genotypes, is associated with increased odds of exudative AMD development. *RAD51B* rs2588809 TT genotype compared to CC genotype, is associated with increased odds of early AMD development in females.
2. *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotypes A-G-C-G-G and G-A-T-A-T are associated with the decreased odds of early AMD. Also, minor allele T in *IL-10* rs1800896 is associated with lower IL-10 serum levels in exudative AMD.
3. *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotype A-A-G-A are associated with decreased odds of exudative AMD. Also, *VEGFA* rs3025033 G allele is associated with decreased odds of exudative AMD only in females, and the C allele in *VEGFA r*s699947 is associated with elevated VEGF-A serum concentrations in patients with exudative AMD.
4. Significant associations between immunogenetic markers and exudative AMD treatment efficacy were determined:
   4.1. *RAD51B* rs8017304 minor allele is associated with increased central macular thickness in exudative AMD before anti-VEGF treatment. Also, *TRIB1* rs4351379 minor allele is associated with more decreased central macular thickness after 6 months of treatment.
   4.2. *IL-9* rs1859430, rs2069870 and rs2069884 minor alleles are associated with worse best-corrected visual acuity before anti-VEGF treatment. On the other hand, *IL-9* rs2069885 minor allele is associated with better improvement of best-corrected visual acuity after 6 months of anti-VEGF treatment.
   4.3. *VEGFA* rs699947 allele C is associated with worse best-corrected visual acuity before and after 3 and 6 months of anti-VEGF treatment, and with higher central macular thickness after 6 months of anti-VEGF treatment.

PRACTICAL RECOMMENDATIONS

Our study results suggested the new AMD biomarker complexes: *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotypes A-G-C-G-G and G-A-T-A-T, *VEGFA* rs3025033 G allele as well as *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotype A-A-G-A, *IL-10* rs1800896 T allele with lower IL-10 serum levels, and the *VEGFA r*s699947 C allele with elevated VEGF-A serum levels could help better understand the AMD pathogenesis differences between early and late AMD forms. Also, the exudative AMD treatment efficacy associated biomarkers (*RAD51B* rs8017304, *IL-9* rs1859430, rs2069870 and rs2069884 and *VEGFA* rs699947) should be included in future studies looking for the best exudative treatment response or treatment for the other AMD forms.

SANTRAUKA

Įvadas

Amžinė geltonosios dėmės degeneracija (AGDD) yra progresuojanti neurodegeneracinė centrinės makulos liga, sukelianti regos sutrikimus ir aklumą. Makula yra tinklainės dalis, kurioje yra išsidėstę fotoreceptoriai atsakingi už regos aštrumą ir spalvų suvokimą [1]. Dažniausiai liga yra diagnozuojama apie 60-uosius gyvenimo metus arba vėliau [2], bet pirmieji ligos požymiai gali būti stebimi ir žymiai anksčiau – apie 40-uosius gyvenimo metus [3]. AGDD sukelia regos aštrumo sumažėjimą, taip prastindama gyvenimo kokybę, kadangi darosi sunku atpažinti veidus ar atskirti spalvas [4]. Pagal su amžiumi susijusių ligų studija (*angl* Age-Related Eye Disease Study (AREDS)), AGDD yra klasifikuojama į pradinę, vidutinę ir vėlyvąją AGDD formas [5]. Nors pradinė AGDD yra daug dažnesnė ir diagnozuojama anksčiau, eksudacinė AGDD gali sukelti visišką apakimą net 80 proc. pacientų [6]. AGDD patogenezė nėra iki galo aiški, todėl amžius ir senėjimo procesai yra stipriai susiję su AGDD pasireiškimu, ypač išsivysčiusiose šalyse, kur didėjantis išgyvenamumas siejamas ir su su amžiumi susijusių lygų vystymusi [7]. Ankstesni tyrimai parodė, kad Europoje pradine AGDD sergančiųjų skaičiai išaugs iki 21,5 milijono, o vėlyvaja AGDD sergančiųjų skaičius pasieks 4,8 milijono. Taip sparčiai didėjantis sergančiųjų AGDD skaičius iki 2040-ųjų metų pasieks 288 millijonus pasaulyje [8].

Senėjimo proecesai akyje apima drūzogenezę ir oksidacinį stresą, kurie skatina AGDD vystymąsi [9]. Drūzos arba lipidų, baltymų ir kolageno dalelės, taip pat aktyviosios deguonies formų sankaupos tarp tinklainės pigmentinio epitelio (TPE) ir Brucho membranos [10], sukelia lėtinį uždegimą tinklainėje, kuris sukelia negrįžtamus TPE ir fotoreceptorių [11, 12] bei kraujo-akies barjero pakitimus sutrikdydamos normalias akie funkcijas [13]. Taip pat, susikaupusios medžiagos skatina TPE išskirti didelius kiekius įvairių uždegimo veiksnių. Jų ilgalaikis veikimas sukelia fotoreceptorių ir TPE ląstelių degeneraciją bei atrofiją tinklainėje [14].

Atlikti mokslinai tyrimai parodė, jog sutrikęs uždegiminis atsakas skatina išsiskirti didelius proangiogeninių mediatorių, tokių kaip kraujagyslių endotelio augimo faktoriaus A (KEAF-A), kiekius, skatinančius angiogenezę [15–17]. Kita vertus, naujų, tačiau silpnų ir pralaidžių kraujagyslių gyslainėje augimas sukelia vietinę edemą, skatinančią eksudacinės AGDD vystymąsi [17]. Kol kas yra rastas tik eksudacinės AGDD gydymas anti-KEAF injekcijomis, todėl daug dėmesio skiriama į KEAF signalinio kelio sąsajas su gydymo efektyvumu [18], įskaitant ir kitas šiame kelyje dalyvaujančias molekules[15]. Buvo nustatyta, kad uždegimo veiksniai, tokie kaip IL-10 ir IL-9, yra susiję su

AGDD patogeneze per specifinį NF-kB ir JAK/STAT signalinį kelią, kuris galėtų būti potencialus taikinys eksudacinės AGDD gydymui [19].

Nors itin svarbūs AGDD rizikos veiksniai yra gyvenimo būdo įpročiai, tokie kaip rūkymas, alkoholio vartojimas ir nesubalansuota mityba [20], genetiniai veiksniai išlieka pačiais svarbiausiais AGDD patogenezėje. Dar daugiau, besivystantys genetiniai tyrimai ir inovatyvūs tyrimų metodai skatina mus ieškoti potencialių biožymenų, kurie galėtų prisidėti prie ankstyvos AGDD diagnostikos ir gydymo sukūrimo.

Genetikai užimant itin svarbų vaidmenį AGDD vystymesi, daugelis mokslininkų ieško vieno nukleotido polimorfizmų (VNP) sąsajų su AGDD pasireiškimu. Mes taip pat pasirinkome anustatyti ir įvertinti *IL-9* ir *IL-10,* kolegeną koduojančių genų *COL8A1* ir *COL10A1*, su lipidų transportu susijusio geno *TRIB1* [21], su oksidaciniu stresu ir DNR pažaidų taisymu siejamo geno *RAD51B* [22] ir su kraujagyslių augimu susijusio *VEGFA* geno vieno nukleotido polimorfizmų sąsajas su AGDD pasireiškimu bei eksudacinės AGDD gydymo anti-KEAF injekcijomis efektyvumu. Manome, jog pasirinkti VNP ir IL-9, IL-10, VEGF-A ir VEGF-R2/KDR baltymai gali būti reikšmingai susiję su AGDD pasireiškimu bei eksudacinės AGDD gydymo efektyvumu.

Tikslas ir uždaviniai

Tyrimo tikslas:

Tyrimo tikslas buvo nustatyti naujus imunogenetinius molekulinius žymenis pacientų sergančių amžine geltonosios dėmės degeneracija kraujyje bei įvertinti jų sąsajas su eksudacinės amžinės geltonosios dėmės degeneracijos gydymo efektyvumu.

Tyrimo uždaviniai:

1. Nustatyti *RAD51B* (rs8017304 ir rs2588809), *TRIB1* (rs6987702, rs4351379 ir rs4351376), *COL8A1* (rs13095226) ir *COL10A1* (rs1064583) genų polimorfizmų sąsajas su pradinės ir eksudacinės amžinės geltonosios dėmės degeneracijos pasireiškimu.
2. Nustatyti *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885 ir rs2069884), *IL-10* (rs1800871, rs1800872 ir rs1800896) genų polimorfizmų bei IL-9 ir IL-10 koncentracijų kraujo serume sąsajas su pradinės, eksudacinės ir atrofinės amžinės geltonosios dėmės degeneracijos pasireiškimu.
3. Nustatyti *VEGFA* (rs1570360, rs699947, rs3025033 ir rs2146323A) geno polimorfizmų bei VEGF-A ir VEGF-R2/KDR koncentracijų

kraujo serumo sąsajas su pradinės ir eksudacinės amžinės geltonosios dėmės degeneracijos pasireiškimu.

4. Nustatyti *RAD51B* (rs8017304 ir rs2588809), *TRIB1* (rs6987702, rs4351379 ir rs4351376), *COL8A1* (rs13095226), *COL10A1* (rs1064583), *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885 ir rs2069884), *IL-10* (rs1800871, rs1800872 ir rs1800896), *VEGFA* (rs1570360, rs699947, rs3025033 ir rs2146323A) genų polimorfizmų bei IL-10, VEGF-A ir VEGF-R2/KDR kraujo serumo koncentracijų sąsajas su eksudacinės amžinės geltonosios dėmės degeneracijos gydymo anti-KEAF injekcijomis efektyvumu.

Naujumas

Mūsų atliktas tyrimas leido identifikuoti naujus prognostinius su uždegimu susijusius AGDD biožymenis:

1. *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotipas A-G-C-G-G susijęs su 2 kartais, o G-A-T-A-T susiję 12 kartų mažesne pradinės AGDD pasireiškimo galimybe.
2. *VEGFA* rs3025033 G alelis ir *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotipas A-A-G-A susiję su 2 kartais mažesne eksudacinės AGDD pasireiškimo galimybe. Kita vertus, *VEGFA* rs3025033 G alelis susijęs su mažesne eksudacinės AGDD pasireiškimo galimybe tik moterims.
3. *RAD51B* rs2588809 TT genotipas, lyginant su CC genotipu, yra susijęs su 7 kartais didesne pradinės AGDD pasireiškimo galimybe taip pat tik moterims.

Remiantis mums prieinamomis duomenų bazėmis, mes pirmą kartą nustatėme, jog *IL-10* rs1800896 T alelis yra susijęs su mažesne IL-10 koncentracija kraujo serume, o *VEGFA r*s699947 C alelis su didesne VEGF-A koncentracija kraujo serume sergantiems eksudacine AGDD.

Taip pat, mes pirmieji nustatėme šių imunogenetinių žymenų sąsajas su eksudacinės AGDD gydymo anti-KEAF injekcijomis efektyvumu:

1. *RAD51B* rs8017304 retesnysis alelis susijęs su didesniu centriniu tinklainės storiu prieš gydymą anti-KEAF; *TRIB1* rs4351379 retesnysis alelis susijęs su didesniu centrinio tinklainės storio sumažėjimu po šešių mėnesių gydymo anti-KEAF injekcijomis.
2. *IL-9* rs1859430, rs2069870 ir rs2069884 retesnieji aleliai yra susiję su blogesniu regos aštrumu prieš anti-KEAF gydymą; *IL-9* rs2069885 retasis alelis yra susijęs su didesniu geriausio koreguoto regos aštrumo pagerėjimu praėjus šešiems mėnesiams nuo gydymo anti-KEAF injekcijomis pradžios.

3. *VEGFA* rs699947 C alelis yra susijęs su blogesniu geriausiu koreguotu regos aštrumu prieš gydymą, po trijų mėnesių ir šešių mėnesių nuo gydymo anti-KEAF injekcijomis pradžios. Taip pat, jis susijęs su didesniu centriniu tinklainės storiu praėjus šešiems mėnesiams nuo gydymo anti-KEAF injekcijomis pradžios.

Nustatyti imunogenetiniai žymenys ir jų kompleksai ateityje galėtų prisidėti prie efektyviausio AGDD gydymo užtikrinimo.

Tyrimo planavimas ir metodai:

Tyrimai buvo atliekami Lietuvos sveiktos mokslų universiteto, Neuromokslų instituto Oftalmologijos laboratorijoje, gavus Kauno regioninio biomedicininių tyrimų etikos komiteto (BE-2-/48) leidimą.

Visas mokslinis tyrimas buvo padalintas į keturis atskirus tyrimus (Tyrimas 1–4) (1 lentelė). Visiems tiriamiesiems atliktas išsamus oftalmologinis ištyrimas ir iriamieji suskirstyti į keturias grupes: sergantys pradine AGDD, eksudacine arba atrofine AGDD bei kontrolinės grupės asmenys. Taip pat, sergantys eksudacine AGDD buvo suskirstyti į atsakančius į gydymą ir neatsakančius į gydymą anti-KEAF injekcijomis, remiantis gautais oftalmologinių tyrimų rezultatais.

1 lentelė. *Tyrimo struktūra*

Tyrimo Nr.	Tyrimo pavadinimas	Tiriamųjų skaičius				
		Pradinė AGDD	Eksudacinė AGDD	Atrofinė AGDD	Kontrolinė grupė	Iš viso
1	*RAD51B, TRIB1, COL8A1* ir *COL10A1* polimorfizmų sąsajos su amžine geltonosios dėmės degeneracija	254	244	–	942	1440
2	*IL-9* ir *IL-10* polimorfizmų bei IL-10 koncentracijos kraujo serume sąsajos su amžine geltonosios dėmės degeneracija	343	422	61	383	1209
3	*VEGFA* polimorfizmų ir VEGF-A ir VEGF-R2/KDR koncentracijos kraujo serume sąsajos su amžine geltonosios dėmės degeneracija	339	419	–	374	1132
4	Imunogenetinių žymenų sąsajos su eksudacinės amžinės geltonosios dėmės degeneracijos gydymo efektyvymu	–	119	–	–	119

Deoksiribonukleininės rūgšties (DNR) išskyrimui kraujas buvo surinktas į vakuuminius mėgintuvėlius su antikoaguliantu EDTA (etilendiamintetraacto rūgštis), kad nesusidarytų mikrokrešuliai ir DNR būtų apsaugota nuo degradacijos. Tyrime naudojama DNR buvo išskirta iš periferinio kraujo baltųjų kraujo ląstelių – leukocitų. DNR skyrimas atliktas iš 200 µl kraujo, naudojant silikagelio kolonėlių genominės DNR skyrimo rinkinį (Thermo Fisher Scientific, Lietuva).

Devyniolikos VNP: *RAD51B* (rs8017304 ir rs2588809), *TRIB1* (rs6987702, rs4351379 ir rs4351376), *COL8A1* (rs13095226), *COL10A1* (rs1064583), *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885 ir rs2069884) and *IL-10* (rs1800871, rs1800872 ir rs1800896) ir *VEGFA* (rs1570360, rs699947, rs3025033 ir rs2146323) genotipavimas buvo atliktas naudojant molekulinių pradmenų ir zondų rinkinius „TaqMan™ Genotyping assay“ (Thermo Fisher Scientific, JAV). Genotipavimas atliktas tikro laiko polimerazės grandinės reakcijos (TL-PGR) metodu, TL-PGR gausintuvu „StepOne Plus“ (Applied Biosystems by Thermo Fisher Scientific, Singapūras) pagal gamintojo rekomendacijas.

IL-9, IL-10, VEGF-A ir VEGFR2/KDR baltymų koncentracijos tiriamųjų kraujo serume buvo nustatytos imunofermentiniu ELISA metodu, naudojant komercinius IL-9 (Kat. Nr. BMS2081), IL-10, (Kat. Nr. BMS215-2), VEGF-A (Kat. Nr. BMS277-2) ir VEGF-R2/KDR (Kat. Nr. BMS2019) ELISA rinkinius. Baltymų koncentracija nustatyta ir suskaičiuota „Multiskan FC“ mikroplokštelių fotometru (Thermo Fisher Scientific, JAV) ties 450 nm bangos ilgiu. Mėginiai, kurie nepasiekė minimalios koncentracijos ribos, nebuvo įtraukti į skaičiavimus.

Statistinė duomenų analizė buvo atlikta naudojant kompiuterinę programą „IBM SPSS Statistics 27.0“ (Statistical Package for the Social Sciences for Windows, Inc., IL, Čikaga, JAV). Duomenys pateikiami realiaisiais skaičiais (proc.) arba mediana, tarpkvartiliniu pločio (IQR) vertėmis. Duomenų, kurie nėra normaliai pasiskirstę, palyginimui tarp dviejų grupių buvo naudotas Mano-Vitnio U testas.

Genų polimorfizmų genotipų pasiskirstymas kontrolinėje grupėse vertintas pagal Hardžio-Veinbergo dėsnį (*http://www.oege.org/software/hwe-mr-calc.shtml*). Polimorfizmų genotipų pasiskirstymo homogeniškumo tarp sergančiųjų pradine, eksudacine ar atrofine AGDD ir kontrolinės grupės palyginimui taikytas Chi kvadrato testas (χ^2). Fisher vienpusio ir dvipusio kriterijų skaičiavimas atliktas lyginant genotipų ir alelių pasiskirstymus į gydymą atsakančių ir neatsakančių pacientų grupes. Atlikus dvinarę logistinę regresinę analizę, įvertintas galimybių santykis (GS) AGDD pasireikšti, atsižvelgiant į paveldėjimo modelius. GS buvo koreguotas pagal amžių arba lytį. Ši analizė atlikta atskiroms grupėms nurodant GS su 95 proc. pasikliautinuoju

intervalu (PI). Pasirenkant geriausią paveldėjimo modelį buvo vertinamas Akaike informacinis kriterijus (AIK), kurio mažiausia vertė nurodo tinkamiausią modelį. Haplotipų analizė atlikta naudojant elektroninę laisvai prieinamą statistinės analizės programą SNPStats software (*https://www.snpstats.net/snpstats/*) [174].

Skirtumai vertinti kaip statistiškai reikšmingi, kai $p<0,05$. Genotipų analizėje taikyta daugkartinių lyginimų Bonferoni korekcija.

Rezultatai

Pirmiausia buvo įvertintos *RAD51B* (rs8017304 ir rs2588809), *TRIB1* (rs6987702 ir rs4351379) bei *COL8A1* (rs13095226) VNP sąsajos su pradinės ir eksudacinės AGDD pasireiškimu. Rezultatai parodė, jog *COL8A1* rs13095226 CC genotipas yra susijęs su padidėjusia eksudacinės AGDD pasireiškimo galimybe pagal kodominantinį (GS = 3,426; 95 proc. PI: 1,355–8,667; p = 0,009) ir recesyvinį (GS = 3,540; 95 proc. PI: 1,415–8,856; p=0,007) genetinius modelius. Vertinant kitų VNP sąsajas su AGDD, statistiškai reikšmingų rezultatų nustatėme.

Tęsiant tyrimą buvo atlikta *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885 ir rs2069884) ir *IL-10* (rs1800871, rs1800872 ir rs1800896) genotipavimas bei IL-9 ir IL-10 koncentracijos kraujo serume nustatymas sergantiems AGDD ir kontrolinės grupės asmenims. Dvinarė logistinė regresija parodė, jog *IL-9* rs1859430 GA genotipas susijęs su mažesne pradinės AGDD pasireiškimo galimybe pagal kodominantinį (GS = 0,700; PI: 0,507–0,966; p = 0,030) ir viršdominantį (GS = 0,673; 95 proc. PI: 0,490–0,925; p = 0,015) genetinius modelius, koreguojant pagal lytį. *IL-9* rs11741137 CT genotipas susijęs su mažesne pradinės AGDD pasireiškimo galimybe pagal viršdominantinį modelį, koreguojant pagal lytį (GS = 0,720; 95 proc. PI: 0,522–0,994; p = 0,046). Taip pat, mes nustatėme, jog *IL-10* rs1800896 CC genotipas susijęs su didesne atrofinės AGDD pasireiškimo galimybe pagal recesyvinį modelį, koreguojant pagal amžių (GS = 2,013; 95 proc. PI: 1,078–3,759; p = 0,028). Statistiškai reikšmingų rezultatų eksudacinės AGDD grupėje nenustatėme. Atlikta dvinarė logistinė regresija taip pat parodė, jog *IL-9* rs1859430 GA genotipas susijęs su mažesne pradinės AGDD pasireiškimo galimybe vyrams pagal kodominantinį (GS = 0,547; 95 proc. PI: 0,302–0,991; p = 0,047) ir viršdominantinį (GS = 0,26; 95 proc. PI: 0,292–0,948; p = 0,033) genetinius modelius. *IL-9* rs11741137 CT genotipas susijęs su mažesne pradinės AGDD pasireiškimo galimybe pagal kodominantinį modelį (GS = 0,543; 95 proc. PI: 0,301–0,979; p = 0,042). Kita vertus, šie rezultatai neišliko statistiškai reikšmingi pritaikius Bonferoni korekciją ($p>0.05/8$).

IL-9 ir *IL-10* haplotipų analizė parodė, jog *IL-9* rs1859430A-rs2069870G-rs11741137C-rs2069885G-rs2069884G ir rs1859430G-rs2069870A-rs11741137T-rs2069885A-rs2069884T haplotipai yra susiję su mažesne pradinės AGDD pasireiškimo galimybe (GS = 0,49; 95 proc. PI: 0,025–0,95; p = 0,035 ir GS = 0,08; 95 proc. PI: 0,01–0,61; p = 0,015, atitinkamai). Retųjų haplotipų rinkinys buvo susijęs su mažesne eksudacinės AGDD pasireiškimo galimybe (GS = 0,37; 95 proc. PI: 0,015–0,092; p = 0,033). *IL-10* haplotipų analizė neparodė statistiškai reikšmingų rezultatų.

Pamatavome IL-9 ir IL-10 koncentracijas tiriamųjų kraujo serume: sergančių pradine AGDD (n = 20), eksudacine AGDD (n = 26), atrofine AGDD (n = 20) ir kontrolinės grupės asmenis (n = 19). Nustatėme, jog IL-10 koncentracija buvo mažesnė sergančių eksudacine AGDD kraujo serume nei kontrolinės grupės asmenų (8,0 (2,7) pg/ml *vs.* 8,8 (2,4) pg/ml, p = 0,049), taip pat mažesnė nei pradine AGDD sergančių asmenų (8,0 (2,7) pg/ml *vs.* 9,2 (1,7) pg/ml, p = 0,017) bei mažesnė nei atrofine AGDD sergančių asmenų (8,0 (2,7) pg/ml *vs.* 9,4 (1,5) pg/ml, p = 0,008), atitinkamai. Detalesnė analizė parodė, kad asmenys turintys bent vieną *IL-10* rs1800896 T alelį, turi mažesnę IL-10 koncentraciją kraujo serume, lyginant su asmenimis turinčiais CC genotipą: 7,2 (2,3) *vs.* 9,3 (1,4); p = 0,048. IL-9 nebuvo nustatytas tiriamųjų kraujo serume.

Kitoje tyrimo dalyje analizavome *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) ir VEGF-A bei VEGF-R2/KDR baltymų sąsajas su AGDD. Statistinė analizė parodė, jog rs3025033 G alelis susijęs su mažesne eksudacinės AGDD pasireiškimo galimybe pagal dominantinį (GS = 0,67; 95 proc. PI: 0,49–0,80; p = 0,0088) ir adityvinį (GS = 0,7; 95 proc. PI: 0,54–0,90; p = 0,0058) genetinius modelius. Atlikus analizę vyrų ir moterų gupėse atskirai, nustatėme, kad rs3025033 AG genotipas susijęs su mažesne ekdudacinės AGDD pasireiškimo galimybe moterims pagal kodominantinį modelį (GS = 0,57; 95 proc. PI: 0,37–0,87; p = 0,009). Taip pat, nustatėme, kad G alelis susijęs su mažesne eksudacinės AGDD pasireiškimo galimybe moterims pagal dominantinį (GS = 0,55; 95 proc. PI: 0,37–0,82; p = 0,0032) ir adityvinį (GS = 0,60; 95 proc. PI: 0,42–0,84; p = 0,0028) modelius.

VEGFA rs1570360, rs699947, rs3025033 ir rs2146323 haplotipų analizė parodė, jog rs1570360A-rs699947A-rs3025033G-rs2146323A haplotipas yra susijęs su mažesne eksudacinės AGDD pasireiškimo galimybe (GS = 0,46, 95 proc. PI: 0,23–0,90; p = 0,023).

Nors išmatavę VEGF-A ir VEGF-R2/KDR koncentracijas sergančių eksudacine AGDD kraujo serume prieš gydymą anti-KEAF injekcijomis skirtumų nenustatėme, tačiau nustatėme, kad *VEGFA* rs699947 C alelis, lyginant su AA genotipu, statistiškai reikšmingai susijęs su didesne VEGF-A koncent-

racija sergančių eksudacine AGDD kraujo serume: 485.95 (945.93) *vs.* 194.97 (–), atitinkamai, p = 0.046.

Nustatę šių žymenų sąsajas su pradinės, eksudacinės ar atrofinės AGDD pasireiškimu, įvertinome šių žymenų sąsajas su eksudacinės AGDD gydymo anti-KEAF efektyvumu. Statistinė analizė parodė, jog *RAD51B* rs8017304 retesnysis alelis susijęs su didesniu centriniu tinklainės storiu prieš gydymą anti-KEAF injekcijomis (p = 0,004). *TRIB1* rs4351379 retesnysis alelis susijęs su didesniu centrinio tinklainės storio sumažėjimu po šešių mėnesių gydymo anti-KEAF injekcijomis (p = 0,03).

IL-9 rs1859430, rs2069870 ir rs2069884 retesnieji aleliai yra susiję su blogesniu regos aštrumu prieš anti-KEAF gydymą (p = 0,018; p = 0,012; p = 0,041, atitinkamai). Kita vertus, *IL-9* rs2069885 retasis alelis yra susijęs su didesniu geriausio koreguoto regos aštrumo pagerėjimu praėjus šešiems mėnesiams nuo gydymo anti-KEAF pradžios (p = 0,032). IL-10 koncentracija kraujo serume nebuvo susijusi su eksudacinės AGDD gydymo efektyvumu.

Galiausiai, *VEGFA* VNP ir VEGF-A bei VEGF-R2/KDR baltymų koncentracijos kraujo serume buvo įvertintos kartu su eksudacinės AGDD gydymo anti-KEAF injekcijomis efektyvumu. Nustatėme, jog *VEGFA* rs699947 C alelis yra susijęs su blogesniu geriausiu koreguotu regos aštrumu prieš gydyma (p = 0,027), po trijų mėnesių (p = 0,003) ir šešių mėnesių (p = 0,022) nuo gydymo anti-KEAF pradžios. Taip pat, jis susijęs su didesniu centriniu tinklainės storiu praėjus šešiems mėnesiams nuo gydymo anti-KEAF pradžios (p = 0,032). VEGF-A bei VEGF-R2/KDR baltymų koncentracijos nebuvo susijusios su eksudacinės AGDD gydymo anti-KEAF efektyvumu.

Išvados

1. *COL8A1* rs13095226 CC genotipas, lyginant su TT ir TC genotipais, yra susijęs su didesne eksudacinės amžinės geltonosios dėmės degeneracijos pasireiškimo galimybe, o *RAD51B* TT genotipas, lyginant su CC genotipu, yra susijęs su didesne pradinės AGDD pasireiškimo galimybe taip pat tik moterims.
2. *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotipai A-G-C-G-G ir G-A-T-A-T yra susiję su mažesne pradinės amžinės geltonosios dėmės degeneracijos pasireiškimo galimybe. Taip pat, *IL-10* rs1800896 T alelis yra susijęs su mažesne IL-10 koncentracija kraujo serume sergantiems eksudacine amžine geltonosios dėmės degeneracija.
3. *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotipas A-A-G-A yra susijęs su mažesne eksudacinės amžinės geltonosios

dėmės degeneracijos pasireiškimo galimybe. Taip pat, *VEGFA* rs3025033 G alelis yra susijęs su mažesne eksudacinės amžinės geltonosios dėmės degeneracijos pasireiškimo galimybe moterims, o *VEGFA r*s699947 C alelis yra susijęs su didesne VEGF-A koncentracija kraujo serume sergantiems eksudacine amžine geltonosios dėmės degeneracija.

4. Statistiškai reikšmingos genetinių žymenų sąsajos nustatytos su eksudacinės amžinės geltonosios dėmės degeneracijos gydymo anti-KEAF injekcijomis efektyvumu:
   4.1. *RAD51B* rs8017304 retesnysis alelis susijęs su didesniu centriniu tinklainės storiu prieš gydymą anti-KEAF. *TRIB1* rs4351379 retesnysis alelis susijęs su didesniu centrinio tinklainės storio sumažėjimu po šešių mėnesių gydymo anti-KEAF injekcijomis.
   4.2. *IL-9* rs1859430, rs2069870 and rs2069884 retesnieji aleliai yra susiję su blogesniu regos aštrumu prieš anti-KEAF gydymą. Kita vertus, *IL-9* rs2069885 retasis alelis yra susijęs su didesniu geriausio koreguoto regos aštrumo pagerėjimu praėjus šešiems mėnesiams nuo gydymo anti-KEAF pradžios.
   4.3. *VEGFA* rs699947 C alelis yra susijęs su blogesniu geriausiu koreguotu regos aštrumu prieš gydyma, po trijų mėnesių ir šešių mėnesių nuo gydymo anti-KEAF pradžios. Taip pat, jis susijęs su didesniu centriniu tinklainės storiu praėjus šešiems mėnesiams nuo gydymo anti-KEAF pradžios.

Praktinės rekomendacijos

Mūsų tyrimo rezultatai atskleidė naujus AGDD biožymenų kompleksus: *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, rs2069884 haplotipai A-G-C-G-G ir G-A-T-A-T, *VEGFA* rs3025033 G alelis ir *VEGFA* rs1570360, rs699947, rs3025033, rs2146323 haplotipas A-A-G-A, *IL-10* rs1800896 T alelis kartu su sumažėjusia IL-10 koncentracija kraujo serume bei *VEGFA r*s699947 C alelis su padidėjusia VEGF-A koncentracija kraujo serume serum, kurie gali padėti geriau suprasti AGDD patogenezę bei pradinės ir vėlyvosios AGDD patogenezinius skirtumus. Taip pat, su eksudacinės AGDD gydymo efektyvumu susiję biožymenys (*RAD51B* rs8017304, *IL-9* rs1859430, rs2069870, rs2069884 ir *VEGFA* rs699947) turėtų būti įtraukiami į būsimus AGDD tyrimus, analizuojančius atsaką į eksudacinės AGDD gydymą ar kuriant kitų AGDD formų gydymo strategijas.

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PUBLICATIONS

Thesis publications:

1. **Vilkevičiūtė Alvita;** Čebatorienė, Džastina; Kriaučiūnienė, Loresa; Liutkevičienė, Rasa. *VEGFA* Haplotype and VEGF-A and VEGF-R2 Protein Associations with Exudative Age-Related Macular Degeneration // Cells Cells. Basel : MDPI. ISSN 2073-4409, 2022, vol. 11, no. 6, p. 996. DOI: 10.3390/CELLS11060996. [Impact factor: 6.600, Aggregate impact factor.: 7.567, Quartile: Q2 (2020. InCites JCR SCIE)]
2. **Vilkevičiūtė, Alvita;** Čebatorienė, Džastina; Kriaučiūnienė, Loresa; Žemaitienė, Reda; Liutkevičienė, Rasa. *IL-9* and *IL-10* Single-Nucleotide Variants and Serum Levels in Age-Related Macular Degeneration in the Caucasian Population // Mediators of inflammation. London : Hindawi LTD. ISSN 0962-9351, 2021, vol. 2021, p. 1-13. DOI: 10.1155/2021/6622934. [Impact factor: 4.711, Aggregate impact factor: 6.652, Quartile: Q2 (2020. InCites JCR SCIE)]
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Hindawi
Disease Markers
Volume 2019, Article ID 5631083, 8 pages
https://doi.org/10.1155/2019/5631083

Hindawi

Research Article

RAD51B (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), and *COL10A1* (rs1064583) Gene Variants with Predisposition to Age-Related Macular Degeneration

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Received 14 August 2018; Accepted 27 March 2019; Published 2 May 2019

Academic Editor: Giuseppe Murdaca

Background. Age-related macular degeneration (AMD) is a progressive neurodegenerative disease of a central part of the neural retina (macula) and a leading cause of blindness in elderly people. While it is known that the AMD is a multifactorial disease, genetic factors involved in lipid metabolism, inflammation, and neovascularization are currently being widely studied in genome-wide association studies (GWAS). The aim of our study was to evaluate the impact of new single nucleotide polymorphisms (SNPs) in *RAD51B*, *TRIB1*, *COL8A1*, and *COL10A1* genes on AMD development. *Methods.* Case-control study involved 254 patients diagnosed with early AMD, 244 patients with exudative AMD, and 942 control subjects. The genotyping of *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs6987702, rs4351379, and rs4351376), *COL8A1* (rs13095226), and *COL10A1* (rs1064583) was carried out using TaqMan assays by a real-time polymerase chain reaction (RT-PCR) method. *Results.* Statistically significant difference was found in genotype (TT, TC, and CC) distribution of *COL8A1* rs13095226 between exudative AMD and control groups (60.2%, 33.6%, and 6.1% vs. 64.9%, 32.3%, and 2.9%, respectively, $p = 0.036$). Also, comparing with TT+TC, rs13095226 CC genotype was associated with 3.5-fold increased odds of exudative AMD development (OR = 3.540; 95% CI: 1.415-8.856; $p = 0.007$). *Conclusion.* Our study revealed a strong association between a variant in *COL8A1* (rs13095226) and exudative AMD development.

1. Introduction

Age-related macular degeneration (AMD) is a progressive neurodegenerative disease of a central part of neural retina (macula) [1]. AMD is a leading cause of central vision loss, and while it is diagnosed in elderly people (in those aged 60 and over) [1], the first signs of the disease can occur in people aged 40 [2]. One of the main processes involved in AMD pathogenesis is called drusogenesis [3]. Drusen are described as small particles of lipid, protein, and collagen detachments accumulated between the retinal pigment epithelium (RPE) and the Bruch's membrane (BrM) in the retina [3]. RPE controls fluid transportation between the choriocapillaris and the retina including lipid transportation and metabolism as well as oxygen transportation [4]. The BrM is a five-layer extracellular matrix located between the RPE and the choroid. It regulates metabolic exchange between RPE cells and blood flow from the choroid through a semipermeable filtration barrier [5]. Any alteration in the structure of BrM might impact dysfunction of the RPE and the outer retina [6]. One

of the most complicated biological processes which may affect BrM is ageing: age-related processes urge the accumulation of incompletely digested phospholipids [7], combining with oxidative stress results of lipid peroxidation [8] and lysosomal defects in photoreceptor outer segments [9]. Oxidative stress-induced lipid and protein accumulation leads to RPE injury or early RPE cell death with changes in BrM [10, 11]. Damaged RPE cells release huge amounts of different cytokines and chemokines which could result in chronic inflammation over time [12–14]. One of the cytokines is a vascular endothelial growth factor A (VEGF-A) which can also induce angiogenesis [15–17]. This process leads to a growth of new fragile and leaky vessels resulting in exudative hemorrhage and acute vision loss [18]. According to the Age-Related Eye Disease Study (AREDS), AMD can be classified into early, intermediate, and late stages [19]. Early AMD is described as the appearance of drusen and retinal pigment abnormalities; when at least one large druse, numerous medium-sized drusen, or geographic atrophy (GA) without extension to the center of the macula occurs, the intermediate stage of AMD is diagnosed; late AMD is divided into dry AMD with the GA of the RPE and neovascular AMD which is characterized by choroidal neovascularization with detachments of the RPE, hemorrhages, and/or scars [20]. While it is known that the AMD is a multifactorial disease, genetic factors involved in lipid metabolism, inflammation, and neovascularization are currently being widely studied in genome-wide association studies (GWAS). Products of a collagen gene family are composed of types I, II, III, and V of fibril-forming interstitial and type IV basement membrane collagens. Collagens keep the architecture and function of normal tissues as well as the structure and function of the extracellular matrix [21, 22]. Recent studies detected an intronic variant in *COL8A1* gene (rs13095226 T/C), suggesting an association with advanced AMD [23–27]. The *COL8A1* gene is located on human chromosome 3 and encodes one of the two alpha chains of type VIII collagen which is a central component of multiple basement membranes of corneal endothelial cells, including BrM, choroidal stroma, and endothelia of blood vessels, playing a role in the maintenance of vascular integrity and structure [28, 29]. BrM and new vessel formation play a key role in pathogenesis of AMD [30]. Another collagen gene family member is *COL10A1* gene, located on human chromosome 6, which encodes the alpha chain of type X collagen. This short-chain collagen is expressed by hypertrophic chondrocytes during endochondral ossification, and it was shown that the expression of *COL10A1* was significantly downregulated in patients with osteoarthritis [31], but its expression was higher in diverse solid tumors and correlated with tumor vasculature [32]. The new variant near *FRK/COL10A1* (rs1999930) was found to be associated with advanced AMD development [24] and suggested the *COL10A1* as a potential locus for future association studies. While the oxidative stress has been linked to the various types of DNA damage playing a significant role in ageing and age-related disorders [33, 34], few studies [35, 36] revealed a significant association between oxidative stress-linked DNA damage and AMD development. Recently, GWAS pinpointed new genetic variations in the *RAD51B* gene associated with AMD [25, 26, 37, 38]. RAD51B is involved in homologous recombinational repair of DNA double-strand breaks by promoting the activity of the central recombinase [39]. Absence of RAD51B protein is thought to disrupt the formation of the RAD51 nucleoprotein filament, which is the initial stage of homologous recombinational repair [40]. G-protein-coupled receptor-induced proteins, playing a role in the mitogen-activated protein kinases- (MAPK-) related signaling cascade [41, 42] which mediates cell proliferation, differentiation, and apoptosis [43] and can regulate lipid metabolism through this pathway [44], are encoded by tribbles pseudokinase 1 (*TRIB1*) gene located in human chromosome 8. It has also been suggested that the *TRIB1* expression is regulated by inflammatory stimulation [45]. There was found one SNP in *TRIB1* gene (rs6987702) to be associated with AMD in African American and Mexican American population [46], suggesting a significant role of *TRIB1* in AMD development.

Association studies of these SNPs, however, have not been conducted in both (early and exudative) AMD forms. Genetic differences between developments of early and exudative AMD still need to be researched and understood better which might lead to future studies with focus on certain molecular pathways involved in the pathogenesis of AMD. Also, genetic variations combined with other risk factors or molecular changes can benefit physicians in creating personalized genetic therapies and/or lifestyle programs for increased risk individuals.

Considering previously studied links between genetic variations and AMD, we chose three previously described SNPs in RAD51B (rs8017304), TRIB1 (rs6987702), and COL8A1 (rs13095226) and four new genetic loci in RAD51B (rs2588809), TRIB1 (rs4351376 and rs4351379), and COL10A1 (rs1064583) genes as potential biomarkers for early and exudative AMD. The aim of our study was to determine and evaluate their impact on the development of early and exudative AMD in the Lithuanian population.

2. Materials and Methods

The study was conducted in the Department of Ophthalmology, Hospital of Lithuanian University of Health Sciences and Laboratory of Ophthalmology, Neuroscience Institute, Lithuanian University of Health Sciences. Ethical approval was obtained from the Ethics Committee for Biomedical Research (number: BE-2-/13).

Ophthalmological evaluation, study group formation, DNA extraction and genotyping methods, and statistical analysis were described in detail in our previous studies [47, 48].

3. Results

3.1. Demographic Characteristics of the Study Groups. Our study involved 498 patients (254 patients with a diagnosis of early AMD and 244 patients with exudative AMD) and 942 healthy controls. The control group was formed of 942 subjects classified into different genders, which matched gender classification in the early and exudative AMD group

Table 1: Demographic characteristics.

Characteristic	Group			p value
	Early AMD ($n = 254$)	Exudative AMD ($n = 244$)	Control ($n = 942$)	
Gender				
Male, n (%)	83 (32.7)	87 (35.7)	350 (37.2)	0.188*
Female, n (%)	171 (67.3)	157 (64.3)	592 (62.8)	0.665**
Age (years), median (IQR)	73 (12)	76 (11)	53 (0)	<0.001* <0.001**

*Early AMD group vs. the control group. **Exudative AMD group vs. the control group.

Table 2: Analysis of Hardy-Weinberg equilibrium in control group subjects.

SNP	Allele frequencies	Genotype distribution in the control group	p value
RAD51B rs8017304	G (0.31) A (0.69)	90/401/451	0.950
RAD51B rs2588809	T (0.12) C (0.88)	17/185/740	0.175
TRIB1 rs6987702	C (0.27) T (0.73)	83/343/516	**0.019**
TRIB1 rs4351379	C (0.07) G (0.94)	7/109/826	0.111
COL8A1 rs13095226	C (0.19) T (0.81)	27/304/611	0.138
COL10A1 rs1064583	G (0.31) A (0.69)	52/482/408	**<0.001**

structure; however, subjects of the control group were younger than AMD patients ($p < 0.001$) (Table 1).

3.2. Hardy-Weinberg Equilibrium. When genotyping results showed that G allele at *TRIB1* rs4351376 was observed in all study subjects (100%), it was not included in further analysis. Hardy-Weinberg equilibrium (HWE) analysis showed that the distribution of genotypes of rs6987702 and rs1064583 deviated from the HWE in the control group and those two SNPs were excluded from further statistical analysis as well (Table 2).

3.3. Analysis of Single Nucleotide Polymorphisms. Analysis of four SNPs (rs8017304, rs2588809, rs4351379, and rs13095226) in the early and exudative AMD and control groups showed a statistically significant difference only in genotype (TT, TC, and CC) distribution of *COL8A1* rs13095226, when compared to the exudative AMD and control groups (60.2%, 33.6%, and 6.1% vs. 64.9%, 32.3%, and 2.9%, respectively, $p = 0.036$) (Table 3).

Binomial logistic regression revealed that the rs13095226 CC genotype is associated with 3.5-fold increased odds of exudative AMD development under the recessive model (OR = 3.540; 95% CI: 1.415-8.856, $p = 0.007$) as well as under the codominant model (OR = 3.426; 95% CI: 1.355-8.667, $p = 0.009$) (Table 4). Results remained statistically significant even after applying Bonferroni correction ($p < 0.05/4$) (Table 4).

3.4. Haplotype Associations with AMD. In this section, we performed haplotype association analysis and evaluated the impact of haplotype constructed of two SNPs: rs8017304 and rs2588809 on early and exudative AMD development. Linkage disequilibrium (LD) analysis was assessed by both D' and r^2 measures. The r^2 values were 0.055 and 0.063 in early AMD and exudative AMD analysis, respectively; for the haplotype block, $|D'|$ values were 0.425 and 0.457 in early AMD and exudative AMD analysis, respectively.

Unfortunately, haplotype analysis did not reveal any significant associations with early or exudative AMD development after adjustment for age.

4. Discussion

Our study involved 498 AMD patients (254 patients with the diagnosis of early AMD and 244 patients with exudative AMD) and 942 healthy control subjects. Since we chose 7 SNPs, 5 of those were newly selected as risk factors for AMD development, *RAD51B* (rs2588809), *TRIB1* (rs6987702, rs4351376, and rs4351379), and *COL10A1* (rs1064583), and 2 were already associated with AMD development, *RAD51B* (rs8017304) and *COL8A1* (rs13095226). Unfortunately, our study revealed only one SNP (rs13095226) which was associated with 3.5-fold increased odds for exudative AMD development in the population of Lithuania ($p = 0.007$).

One of the SNPs near the *TRIB1* locus called rs17321515 was found affecting lipoprotein metabolism. It was strongly associated with triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) and even with increased risk of coronary heart disease (CHD), but results remain controversial among the studies [49–51]. Another SNP (rs2954021) located in *TRIB1* gene was associated with increasing plasma TG levels and coronary artery disease (CAD) risk [52, 53] as well as the other three variants: rs2001945, rs2001845, and rs2001844 [54]. Furthermore, rs2001844 was associated with increased plasma TG and reduced HDL-C [54]. Several association studies reported a significant association between HDL-C and AMD development as well [55–59]; unfortunately, others did not reveal any significant associations between HDL-C and AMD development [60–66]. On the other hand, while the association of HDL-C and AMD is controversial, variant in lipid trait-associated gene *TRIB1* rs6987702 showed a significant association with AMD development, but those results did not survive strict corrections for multiple testing [46]. In our study, none of the SNPs in *TRIB1* reached a statistically significant association with early or exudative AMD development.

Table 3: *RAD51B* (rs8017304 and rs2588809), *TRIB1* (rs4351379), and *COL8A1* (rs13095226) SNPs in patients with early and exudative AMD and control groups.

Gene/marker	Genotype/allele	Early AMD, *n* (%)	Control group, *n* (%)	*p* value	Exudative AMD, *n* (%)	Control group, *n* (%)	*p* value
RAD51B rs8017304	AA	125 (49.2)	451 (47.9)		117 (48.0)	451 (47.9)	
	AG	102 (40.2)	401 (42.6)	0.745	110 (45.1)	401 (42.6)	0.423
	GG	27 (10.6)	90 (9.6)		17 (7.0)	90 (9.6)	
	A	352 (69.3)	1303 (69.2)	0.955	544 (70.5)	1303 (69.2)	
	G	156 (30.7)	581 (30.8)		144 (29.5)	581 (30.8)	0.570
RAD51B rs2588809	CC	196 (77.2)	740 (78.6)		188 (77.0)	740 (78.6)	
	CT	50 (19.7)	185 (19.6)	0.410	53 (21.7)	185 (19.6)	0.652
	TT	8 (3.1)	17 (1.8)		3 (1.2)	17 (1.8)	
	C	442 (87.0)	1665 (88.4)	0.398	429 (87.9)	1665 (88.4)	
	T	66 (13.0)	219 (11.6)		59 (12.1)	219 (11.6)	0.775
TRIB1 rs4351379	GG	214 (84.3)	826 (87.7)		217 (88.9)	826 (87.7)	
	GC	39 (15.4)	109 (11.6)	0.229	26 (10.7)	109 (11.6)	0.780
	CC	1 (0.4)	7 (0.7)		1 (0.4)	7 (0.7)	
	G	467 (91.9)	1761 (93.5)	0.222	460 (94.3)	1761 (93.5)	
	C	41 (8.1)	123 (6.5)		28 (5.7)	123 (6.5)	0.524
COL8A1 rs13095226	TT	166 (65.4)	611 (64.9)		147 (60.2)	611 (64.9)	
	TC	74 (29.1)	304 (32.3)	0.095	82 (33.6)	304 (32.3)	**0.036**
	CC	14 (5.5)	27 (2.9)		15 (6.1)	27 (2.9)	
	T	406 (79.9)	1526 (81.0)	0.585	376 (77.0)	1526 (81.0)	
	C	102 (20.1)	358 (19.0)		112 (23.0)	358 (19.0)	0.051

AMD: age-related macular degeneration. p value < 0.05 indicated in bold is statistically significant.

Table 4: Binomial logistic regression analysis of the *COL8A1* (rs13095226) in patients with exudative AMD and controls.

SNP	Exudative AMD Model/genotype	OR; 95% CI; p^*	AIC value
	Codominant		
rs13095226	TC vs. TT	0.906; 0.589-1.394; 0.653	640.511
	CC vs. TT	3.426; 1.355-8.667; **0.009**	
	Recessive		
rs13095226	CC vs. TT+TC	3.540; 1.415-8.856; **0.007**	638.713

$^*p$ was adjusted for age in logistic regression models. OR: odds ratio; 95% CI: 95% confidence interval; AIC: akaike information criterion.

Studies of variants in *RAD51B* revealed statistically significant associations of SNPs in *RAD51B* with breast cancer occurrence [67–70]. Also, *RAD51B* rs911263 was associated with rheumatoid arthritis (RA) [71, 72]; two SNPs (rs11158728 and rs927220) were associated with nasopharyngeal carcinoma development [73], and one (rs34094401) was associated with Parkinson's disease [74]. Also, literature review explains that risk alleles at *RAD51B* rs8017304, rs13081855 near *COL8A1/FILIP1L* locus, and rs3812111 in *COL10A1* are associated with a greater risk for advanced AMD development [25]. Other three GWAS proved *RAD51B* gene association with AMD as well [26, 37, 38]. Seddon et al. conducted a 12-year follow-up study of 2765 individuals and revealed that *RAD51B* (HR: 0.8; 95% CI: 0.60-0.97, $p = 0.03$) was significantly related to AMD progression [26]. In another study, Seddon and coauthors [38] analyzed the progression of AMD and found 834 from 2951 subjects, who progressed from no AMD, early AMD, or intermediate AMD to an advanced AMD form, and also confirmed that *RAD51B* was associated with AMD progression. Fritsche et al. [25] also proved a strong *RAD51B* association ($p < 5 \times 10^{-8}$) with advanced AMD in Europeans and Asians. Chu et al. analyzed *RAD51B* influence on AMD using two cohorts from Caucasian and Han Chinese populations, as well. Scientists identified two new SNPs in *RAD51B* (rs17105278 and rs4902566) and confirmed the association between rs8017304 and AMD development in Caucasians [37], suggesting a significant role of *RAD51B* associated with DNA damage/DNA repair mechanism in AMD pathogenesis.

Recent studies indicated that variants in *COL8A1* gene were associated with AMD development [24–26, 38]. One of the GWAS studies was performed on 979 patients with advanced AMD and 1709 control subjects and revealed a significant association between rs13095226 and AMD ($p = 2.5e-06$) in the European population [23]. Also, in this study, authors found that the frequencies of minor allele C at rs13095226 in patients with polypoidal choroidal vasculopathy (PCV), neovascular AMD (nAMD), and controls were similar to the frequencies in Americans, while differences were found between Americans and Asians [23]. Meta-analysis of advanced AMD confirmed previously found associations to advanced AMD [24], while in contrast, Yang et al.'s study including 300 cases with nAMD, 300 cases with PCV, and 300 control subjects did not show the association of *COL8A1* rs13095226 with nAMD or PCV, suggesting that

this variant may not be a risk factor for nAMD or PCV in Chinese subjects [75]. Also, another variant of the same gene, polymorphism of *COL8A1* rs13081855, was linked to exudative AMD development [27]. As in non-AMD patients, one of the common variants in *COL8A1* gene (rs669676) was found to be associated with myopic choroidal neovascularization (OR: 1.88; 95% CI: 1.18-2.98, $p = 0.0076$) but did not survive multiple testing [74]. Our study of the Lithuanian population discovered a strong association between a variant in *COL8A1* (rs13095226) and exudative AMD development; genotype rs13095226 CC was determined to be associated with 3.5-fold increased odds of exudative AMD development.

We also found a study, which showed the contribution of the extracellular collagen matrix (FRK/COL10A1) pathways to the development of advanced AMD. A coding variant rs3812111 in *COL10A1*, a collagen protein, could be involved in maintaining the structure and function of the extracellular matrix [24]. Ferrington et al. analyzed that the *COL10A1* rs3812111 A/T (nonrisk allele/risk allele) ratio was 0.69/0.65 in controls/age-related macular degeneration [76]. To our knowledge, there are no studies analyzing *COL10A1* (rs1064583) polymorphism in patients with AMD.

The following limitations of this study have to be declared:

(i) Patients with atrophic AMD have not been analyzed

(ii) Sample size of AMD and healthy controls was relatively small

(iii) Possibility of other risk factors was not included in the study

A thorough medical examination of the study objects can be acknowledged as one of the main strengths of our study. All patients were examined for chronic infectious and noninfectious diseases by a general practitioner. It is also important to highlight that RAD51B (rs8017304 and rs2588809), TRIB1 (rs6987702, rs4351379, and rs4351376), COL8A1 (rs13095226), and COL10A1 (rs1064583) gene variants have never been studied in the Baltic states, namely, in the Lithuanian population, and our study was the first of its type.

5. Conclusion

Our study revealed a strong association between variant in *COL8A1* (rs13095226) and exudative AMD development.

Data Availability

The genotyping data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

None of the authors have any proprietary interests or conflicts of interest related to this submission.

Acknowledgments

This work was funded by a grant (No. SEN-11/2015) from the Research Council of Lithuania.

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Hindawi
Mediators of Inflammation
Volume 2021, Article ID 6622934, 13 pages
https://doi.org/10.1155/2021/6622934

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Research Article

IL-9 and *IL-10* Single-Nucleotide Variants and Serum Levels in Age-Related Macular Degeneration in the Caucasian Population

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Received 25 November 2020; Revised 1 March 2021; Accepted 5 April 2021; Published 13 April 2021

Academic Editor: Ayumi Ouchi

Considering the immunological impairment in age-related macular degeneration (AMD), we aimed to determine the associations of *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884 and *IL-10* rs1800871, rs1800872, and rs1800896 polymorphisms and their haplotypes, as well as the serum levels of IL-9 and IL-10 with AMD. 1209 participants were enrolled in our study. SNPs were genotyped using TaqMan SNP genotyping assays by real-time PCR method. IL-9 and IL-10 serum levels were evaluated using ELISA kits. Our study results have shown that haplotypes A-G-C-G-G and G-A-T-A-T of *IL-9* SNPs are associated with the decreased odds of early AMD occurrence ($p = 0.035$ and $p = 0.015$, respectively). A set of rare haplotypes was associated with the decreased odds of exudative AMD occurrence ($p = 0.033$). Also, IL-10 serum levels were lower in exudative AMD than in controls ($p = 0.049$), patients with early AMD ($p = 0.017$), and atrophic AMD ($p = 0.008$). Furthermore, exudative AMD patients with *IL-10* rs1800896 CT and TT genotypes had lower IL-10 serum concentrations than those with wild-type (CC) genotype ($p = 0.048$). In conclusion, our study suggests that IL-10 serum levels can be associated with a minor allele at *IL-10* rs1800896 and exudative AMD. The haplotypes of IL-9 SNPs were also associated with the decreased odds of early and exudative AMD.

1. Introduction

Inflammation is a typical process involved in the pathogenesis of many diseases. While the inflammation is characterized as a signal transfer cascade which helps to identify and eliminate foreign materials and induce tissue recovery [1], the long-term inflammation and excessive proinflammatory molecule excretion can cause chronic conditions, such as cancer [2], type 2 diabetes mellitus [3], and neurodegenerative disorders [4], including age-related macular degeneration (AMD) [5]. AMD is a worldwide leading cause of progressive and irreversible blindness affecting 1 out of 4 people older than 75 years in developed countries [6]. Still, early signs of the disease can appear when people are in their 50s [7]. The particular pathophysiology of AMD is not clear, so this ocular impairment is described as a multifactorial disease because of its associations with environmental [8, 9] and genetic factors [10], metabolite profile [11], and even microbiome [12] changes. Increasing age, female gender, and ethnicity with the highest prevalence in Europeans at 12.3–30% have also been pinpointed as relevant risk factors [13, 14].

According to the Age-Related Eye Disease Study (AREDS), AMD is divided into early, intermediate, and late stages [15]. Early AMD is usually asymptomatic with defined lipid, protein, and collagen detachments between retinal pigment epithelium (RPE) and Bruch's membrane (BrM) in the retina [16] called drusen and retinal pigment abnormalities. The intermediate stage is described as a presence of at least one large drusen, numerous medium-sized drusen, or geographic atrophy (GA) without extension to the center of the macula. The late AMD is divided into dry or atrophic AMD with the GA of the RPE, and neovascular or exudative

AMD is diagnosed when choroidal neovascularization with detachments in the RPE, hemorrhages, and/or scars appears and causes central vision impairments [17].

Drusogenesis or accumulation of lipids and other metabolites remains a significant AMD process, resulting in chronic inflammation that directly affects RPE, choroidal capillaries, and BrM [18]. The oxidative stress caused by reactive oxygen species (ROS), nitric oxide (NO), oxidized lipoproteins, advanced glycosylation end products (AGER), and apoptotic cells is the leading cause of ocular inflammation [19–21]. These accumulated substances also promote the RPE to release large amounts of different inflammatory factors. These factors' long-term exposure leads to the degeneration and atrophy of photoreceptors and RPE cells in the retina [22]. During the inflammation, complement system components are activated whose persistent accumulation impairs RPE and promotes inflammatory cells' (leukocytes, microglial cells, and macrophages) activation [18]; when macrophage recruitment is impaired at the site of inflammation, accumulating metabolites stimulate the release of proangiogenic mediators, such as vascular endothelial growth factor (VEGF), which induce progressive angiogenesis [19, 23–25]. New, however weak, permeable, and leaking blood vessels in the choroid cause local edema leading to acute vision loss with hemorrhages and fibrotic scars [26]. These pathological processes are responsible for the degradation of BrM and the extracellular matrix and lead to exudative AMD development [24]. Lin et al. found inflammation factors (IL-10, IL-1ra, IL-9, and IL-13) that may be associated with AMD's pathogenesis and revealed their function and regulation via specific NF-jB and JAK-STAT pathways, encouraging for the new exudative AMD treatment [23].

Genetic variations in cytokine coding genes can also cause cytokine expression changes that affect the balance between pro- and anti-inflammatory cytokines and disturb the appropriate immune response, leading to disease development. IL-10 level changes were linked to the genetic alterations mostly known as three *IL-10* -1082 (rs1800896), -819 (rs1800871), and -592 (rs1800872) promoter site single-nucleotide polymorphisms (SNPs) [27]. Otherwise, only one study reported SNP in the IL-9 gene associated with the IL-9 expression. It showed that individuals carrying the A allele of the -351 polymorphism in IL-9 promoter were linked to the increased synthesis of IL-9 [28].

Considering the immunological impairment in AMD development, we aimed to determine the possible associations of *IL-9* rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884 and *IL-10* rs1800871, rs1800872, and rs1800896 polymorphisms and their haplotypes, as well as the serum levels of IL-9 and IL-10 with the early, exudative, and atrophic AMD. We also aimed to evaluate the associations between these polymorphisms and IL-9 and IL-10 concentrations.

2. Materials and Methods

2.1. Study Subjects. The study was approved by the Ethics Committee for Biomedical Research, Lithuanian University of Health Sciences (No. BE-2-/48).

The study groups were made of subjects who were admitted to the Hospital of Lithuanian University of Health Sciences Ophthalmology Department for preventive ophthalmological evaluation. In our study, 1209 participants were enrolled: 343 subjects in early AMD, 422 in exudative AMD, and 61 in the atrophic AMD groups. Also, 383 persons were involved as healthy controls (Table 1). Using the global AMD prevalence (8.7%) [13] and the minor allele frequencies from https://www.ncbi.nlm.nih.gov/snp/, we calculated that our collected sample sizes for the early and exudative AMD and control groups were sufficient to reach 80% or higher power for the selected SNP analysis. Unfortunately, the atrophic AMD group is too small to reach at least 80% power, and according to the power calculator (http://csg.sph.umich.edu/abecasis/cats/gas_power_calculator/), the sample size should be about 100 cases, but the atrophic AMD is a rarer condition than the early and exudative AMD in Lithuanian population to collect enough samples.

Study subjects underwent ophthalmological evaluation and general examination [29]. Participants were enrolled in our study, according to the previously published criteria [29].

2.2. Ophthalmological Evaluation. All the study subjects were evaluated by slit-lamp biomicroscopy to assess corneal and lenticular transparency. Classification and grading of lens opacities were performed according to the Lens Opacities Classification System III. On each examination, intraocular pressure was measured. Pupils were dilated with tropicamide 1%. Fundoscopy, using a direct monocular ophthalmoscope, and slit-lamp biomicroscopy with a double aspheric lens +78 diopters were performed. Results of eye examinations were recorded on most standardized forms. For a detailed analysis of the macula, stereoscopic color fundus photographs of the macula, centered at 45° and 30° to the fovea, were obtained with a Visucam NM Digital camera (Carl Zeiss Meditec AG, Germany).

All the AMD patients underwent optical coherence tomography (OCT), and fluorescence angiography was performed in patients suspected of having late AMD after the OCT examination.

The classification system of AMD formulated by the Age-Related Eye Disease Study was used: early mild AMD consisted of a combination of multiple small drusen and several intermediate (63–124 μm in diameter) drusen, or retinal pigment epithelial abnormalities and the presence of extensive intermediate drusen characterized early intermediate AMD and at least one large (≥125 μm in diameter) drusen, or geographic atrophy not involving the center of the fovea. Advanced AMD was characterized by geographic atrophy involving the fovea and/or any of the neovascular AMD features [15].

2.3. Control Group Formation. The control group consisted of subjects who had no ophthalmologic pathology on examination and agreed to participate in this study. After senile cataract surgeries, the patients were also included in the control group, while they have no other ocular comorbidity. The exclusion criteria were (i) unrelated eye disorders, e.g., high refractive error, cloudy cornea, lens opacity (nuclear, cortical,

or posterior subcapsular cataract) except minor opacities, keratitis, acute or chronic uveitis, glaucoma, or diseases of the optic nerve; (ii) systemic illnesses, e.g., diabetes mellitus, malignant tumors, systemic connective tissue disorders, chronic infectious diseases, or conditions following organ or tissue transplantation; and (iii) ungraded color fundus photographs resulting from obscuration of the ocular optic system or because of fundus photograph quality.

2.4. General Medical Examination. Data on hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease, and stroke were obtained during an examination by a family doctor and gathered from medical records for all study subjects.

2.5. Polymorphism Selection. As the IL-9 and IL-10 have been shown having interactions (https://version-11-0b.string-db.org/cgi/network?networkId=bMDXv9BDi65x), five SNPs (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) in the IL-9 gene and three SNPs (rs1800871, rs1800872, and rs1800896) in the *IL-10* gene whose minor allele frequencies in the Europe population are more than 0.1 were selected from the dbSNP database https://www.ncbi.nlm.nih.gov/snp/. The rs2069885 is a missense variant (resulting in a threonine to methionine amino acid substitution), the rs1859430 and rs2069884 intronic variants are located in the coding region of *IL-9*, and rs11741137 is a downstream, and rs2069870 is an upstream gene variant with no known function. Moreover, rs1859430, rs11741137, rs2069884, and rs2069885 have already been published and identified as potential biomarkers for the other diseases [30–35]; also, rs2069870 along with the SNP in *IL-26* demonstrated influenced susceptibility to develop allergic rhinitis [36].

The rs1800871, rs1800872, and rs1800896 SNPs are located in the upstream *IL-10* promoter region and associated with transcription of IL-10 mRNA and IL-10 protein expression in vitro [37].

All these SNPs were selected for the association study in the AMD group for the first time, suggesting their role in IL-9 and IL-10 signaling pathway and AMD development.

2.6. The DNA Extraction and Genotyping. After collecting the venous blood samples, the DNA salting-out method was used for preparing genomic DNA from the white blood cells. Eight SNPs were genotyped on the *Step One Plus* real-time PCR system (Applied Biosystems, Foster City, USA). The TaqMan SNP genotyping assays for all eight chosen SNPs were performed according to the manufacturer's protocol.

For quality control, 5% of randomly chosen samples for each of the 8 SNPs were selected for repetitive analysis.

2.7. Quantification of IL-10 and IL-9 Serum Levels. IL-9 and IL-10 serum levels were measured in 19 control subjects, 20 patients with early AMD, 20 atrophic AMD, and 26 exudative AMD. These two assays were performed using the Invitrogen ELISA Kit (Cat. No. BMS2081) for human IL-9, standard curve sensibility range: 3.1–200 pg/mL, sensitivity 0.5 pg/mL; Invitrogen ELISA Kit (Cat. No. KHC0101) for human IL-10, standard curve sensibility range: 0–500 pg/mL, sensitivity <1 pg/mL, following the manufacturer's instructions, and they were analyzed on the Multiskan FC Microplate Photometer (Thermo Scientific, Waltham, MA) at 450 nm. The samples were excluded if the levels of serum cytokines were below the detection range.

2.8. Statistical Analysis. Statistical analysis was performed using the SPSS/W 20.0 software (Statistical Package for the Social Sciences for Windows, Inc., Chicago, Illinois, USA). Age and interleukin serum level data distributions were evaluated for normality by the Kolmogorov-Smirnov test. Continuous variables presented median with interquartile range (IQR) based on data distribution. For nonnormally distributed data, Mann-Whitney U test was used to compare two groups, and the Kruskal-Wallis test for the three groups (statistically significant differences observed when $p < 0.05$).

Categorical data (gender, genotype, and allele distributions) are presented as absolute numbers with percentages in brackets and compared between the early, exudative, and atrophic AMD and control groups using the χ^2 test.

The impact of gene polymorphisms on early, exudative, and atrophic AMD was evaluated using binomial logistic regression analysis and presented as odds ratios (ORs) with 95% confidence interval (CI) after gender adjustment in early AMD and age in the exudative and atrophic AMD groups. Logistic regression analysis results were expressed as genetic models (codominant: heterozygotes versus wild-type homozygotes and minor allele homozygotes versus wild-type homozygotes; dominant: minor allele homozygotes and heterozygotes versus wild-type homozygotes; recessive: minor allele homozygotes versus wild-type homozygotes and heterozygotes; overdominant: heterozygotes versus wild-type homozygotes and minor allele homozygotes; the additive model was used to evaluate the impact of each minor allele on AMD). The best genetic model selection was based on the Akaike information criterion (AIC); therefore, the best genetic models were those with the lowest AIC values. We introduced an adjusted significance threshold for multiple comparisons $\alpha = 0.00625$ (0.05/8, since we analyzed eight different SNPs) due to multiple association calculations [38].

Haplotype analysis was performed between the early AMD and control groups, exudative AMD and control groups, and atrophic AMD and control groups using online SNPStats software (https://www.snpstats.net/snpstats/) [39]. Two haplotype blocks were constructed based on different chromosomes where the SNPs were located. Linkage disequilibrium (LD) analysis was assessed by D' and r^2 measures. The associations between the haplotypes with frequencies of at least 1% and different AMD forms were calculated by logistic regression and presented as ORs and 95% CI and p values adjusted for gender in early AMD and age in exudative and atrophic AMD analysis. Statistically significant differences observed when $p < 0.05$.

3. Results

3.1. Hardy-Weinberg Equilibrium. We evaluated the distributions of rs1859430, rs2069870, rs11741137, rs2069885, rs2069884, rs1800871, rs1800872, and rs1800896 genotypes in the control group using the Hardy-Weinberg equilibrium (HWE). Seven SNPs were in HWE ($p > 0.05$), but

Table 1: Demographic data of the studied groups.

	Early AMD group ($n = 343$)	Exudative AMD group ($n = 422$)	Atrophic AMD group ($n = 61$)	Control group ($n = 383$)	p value
Males, n (%)	105 (30.6)	149 (35.3)	22 (36.1)	149 (38.9)	0.019*
Females, n (%)	238 (69.4)	273 (64.7)	39 (63.9)	234 (61.1)	0.291** 0.672***
Age, median (IQR)	73 (13)	77 (10)	80 (9)	72 (11)	0.266* <0.001** <0.001***

*Early AMD vs. control group. **Exudative AMD vs. control group. ***Atrophic AMD vs. control group. IQR: interquartile range; p: significance level, statistically significant differences observed when $p < 0.05$.

rs2069870 did not fulfill the HWE requirements because there were observed only two genotypes (Supplementary Materials, Table S1).

3.2. IL-9 (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and IL-10 (rs1800871, rs1800872, and rs1800896) Analysis in Early, Exudative, and Atrophic AMD. We analyzed 8 SNPS (rs1859430, rs2069870, rs11741137, rs2069885, rs2069884, rs1800871, rs1800872, and rs1800896) and their genotype and allele distributions between the early, exudative, and atrophic AMD and control groups. Our statistical analysis revealed that genotype distributions of *IL-9* rs1859430 (GG, GA, and AA) differ between the early AMD and control groups (65.6%, 27.7%, and 6.7% vs. 60.8%, 35.5%, and 3.7%, $p = 0.024$). Any differences between study groups and *IL-9* (rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) were found (Table 2).

We performed the binary logistic regression analysis to evaluate these SNPs' impacts on early, exudative, and atrophic AMD. The analysis showed that *IL-9* rs1859430 GA genotype was associated with 30% decreased odds of early AMD (OR = 0.700; CI: 0.507-0.966; $p = 0.030$) under the codominant model, and about 33% decreased under the overdominant model after adjustment for gender (OR = 0.673; CI: 0.490-0.925; $p = 0.015$). *IL-9* rs11741137 CT genotype was associated with 28% decreased odds of early AMD under the overdominant model after adjustment for gender (OR = 0.720; CI: 0.522-0.994; $p = 0.046$). Also, we found that *IL-10* rs1800896 CC genotype was associated with 2-fold increased odds of atrophic AMD (OR = 2.013; CI: 1.078-3.759; $p = 0.028$) under the recessive model after adjustment for age (Table 3). Since we analyzed 8 SNPs in our study, we applied the Bonferroni correction (significance threshold, $p = 0.05/8$), and the results did not survive this strict correction. No statistically significant associations were found in the exudative AMD group (data not shown).

While it has been suggested that AMD pathogenesis can be differentiated by gender [14], we performed the SNP analysis in males and females separately and found that *IL-9* rs11741137 (CC, CT, and TT), *IL-9* rs2069885 (GG, GA, and AA), and *IL-9* rs2069884 (GG, GT, and TT) genotypes were distributed statistically significantly between males with early AMD and control males: 73.3%, 20%, and 6.7 vs. 66.4%, 31.5%, and 2%, $p = 0.032$; 73.3%, 20%, and 6.7 vs. 67.1%, 30.9%, and 2%, $p = 0.039$; 73.3%, 20%, and 6.7 vs. 67.1%, 30.9%, and 2%, $p = 0.039$, respectively (Table 4). No statistically significant associations were found in the exudative or atrophic AMD groups and female group analysis (data not shown).

Binomial logistic regression analysis revealed that *IL-9* rs1859430 GA genotype was associated with 46 and 48% decreased odds of early AMD in males under the codominant and overdominant genetic models (OR = 0.547; CI: 0.302-0.991; $p = 0.047$ and OR = 0.526; CI: 0.292-0.948; $p = 0.033$, respectively). *IL-9* rs11741137 CT genotype was associated with 46% decreased odds of early AMD in males (OR = 0.543; CI: 0.301-0.979; $p = 0.042$) under the codominant model (Table 5).

None of the results survived strict Bonferroni correction (significance threshold, $p = 0.05/8$).

3.3. Haplotype Association with the Predisposition to AMD Occurrence. Strong linkage disequilibrium between studied polymorphisms was observed (Table 6).

While the haplotype analyses identified many of their sets, any differences in the haplotype frequencies between the atrophic AMD and control groups were observed (Table 7). The results of the frequencies of haplotypes among patients with early AMD and controls have shown that haplotypes A-G-C-G-G and G-A-T-A-T of *IL-9* SNPs (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) are associated with the decreased odds of early AMD occurrence (OR = 0.49; 95% CI: 0.025-0.95; $p = 0.035$ and OR = 0.08; 95% CI: 0.01-0.61; $p = 0.015$, respectively). The set of rare haplotypes was associated with the decreased odds of exudative AMD occurrence (OR = 0.37; 95% CI: 0.015-092; $p = 0.033$) (Table 7).

3.4. IL-9 and IL-10 Serum Levels in the AMD and Control Groups. IL-9 and IL-10 serum levels were measured in patients with early AMD ($n = 20$), exudative AMD ($n = 26$), atrophic AMD ($n = 20$), and controls ($n = 19$). Subgroups for interleukin serum level measurements consisted of study subjects considering the age and gender distributions in subgroups. IL-9 levels did not reach the detection range, and it was not analyzed. IL-10 serum levels differed between study groups ($p = 0.02$) (Figure 1). When comparing the IL-10 serum levels between every two groups, we found that IL-10 serum levels were lower in exudative AMD than in

Table 2: Distributions of IL-9 and IL-10 SNP genotypes and alleles in the early, exudative, and atrophic AMD and control groups.

SNP	Genotypes/alleles	Group						
		Early AMD ($n = 343$), n (%)	Exudative AMD ($n = 422$), n (%)	Atrophic AMD ($n = 61$), n (%)	Control ($n = 383$), n (%)	p value*	p value**	p value***
IL-9 rs1859430	GG	225 (65.6)	262 (62.1)	33 (54.1)	233 (60.8)	0.024	0.871	0.426
	GA	95 (27.7)	143 (33.9)	24 (39.3)	136 (35.5)			
	AA	23 (6.7)	17 (4.0)	4 (6.6)	14 (3.7)			
	G	545 (79.4)	667 (79.0)	90 (73.8)	602 (78.6)	0.625	0.472	0.088
	A	141 (20.6)	177 (21.0)	32 (26.2)	146 (21.4)			
IL-9 rs2069870	AA	222 (64.7)	262 (62.1)	33 (54.1)	235 (61.4)	0.349	0.832	0.282
	AG	121 (35.3)	160 (37.9)	28 (45.9)	148 (38.6)			
	GG	0 (0)	0 (0)	0 (0)	0 (0)			
	A	565 (82.4)	684 (81.0)	94 (77.0)	618 (80.7)	0.410	0.853	0.350
	G	121 (17.6)	160 (19.0)	28 (23.0)	148 (19.3)			
IL-9 rs11741137	CC	238 (69.4)	290 (68.7)	41 (67.2)	247 (64.5)	0.117	0.389	0.846
	CT	91 (26.5)	120 (28.6)	18 (29.5)	126 (32.9)			
	TT	14 (4.1)	12 (2.8)	2 (3.3)	10 (2.6)			
	C	657 (82.7)	700 (82.9)	100 (82.0)	620 (80.9)	0.053	0.297	0.788
	T	119 (17.3)	144 (17.1)	22 (18.0)	146 (19.1)			
IL-9 rs2069885	GG	240 (70.0)	293 (69.4)	41 (67.2)	250 (65.3)	0.172	0.453	0.892
	GA	90 (26.2)	119 (28.2)	18 (29.5)	123 (32.1)			
	AA	13 (3.8)	10 (2.4)	2 (3.3)	10 (2.6)			
	G	570 (83.1)	705 (83.5)	100 (82.0)	623 (81.3)	0.382	0.24	60.867
	A	116 (16.9)	139 (16.5)	22 (18.0)	143 (18.7)			
IL-9 rs2069884	GG	239 (69.7)	292 (69.2)	41 (67.2)	250 (65.3)	0.199	0.495	0.892
	GT	91 (26.5)	120 (28.4)	18 (29.5)	123 (32.1)			
	TT	13 (3.8)	10 (2.4)	2 (3.3)	10 (2.6)			
	G	569 (82.9)	704 (83.4)	100 (82.0)	623 (81.3)	0.424	0.273	0.867
	T	117 (17.1)	140 (16.6)	22 (18.0)	143 (18.7)			
IL-10 rs1800871	GG	208 (60.6)	252 (59.7)	38 (62.3)	232 (60.6)	0.705	0.903	0.957
	GA	123 (35.9)	152 (36.0)	20 (32.8)	133 (34.7)			
	AA	12 (3.5)	18 (4.3)	3 (4.9)	18 (4.7)			
	G	539 (78.6)	656 (77.7)	96 (78.7)	597 (77.9)	0.770	0.918	0.852
	A	147 (21.4)	188 (22.3)	26 (21.3)	169 (22.1)			
IL-10 rs1800872	GG	208 (60.6)	252 (59.7)	38 (62.3)	232 (60.6)	0.705	0.903	0.957
	GT	123 (35.9)	152 (36.0)	20 (32.8)	133 (34.7)			
	TT	12 (3.5)	18 (4.3)	3 (4.9)	18 (4.7)			
	G	539 (78.6)	656 (77.7)	96 (78.7)	597 (77.9)	0.770	0.918	0.852
	T	147 (21.4)	188 (22.3)	26 (21.3)	169 (22.1)			
IL-10 rs1800896	TT	103 (30.0)	112 (26.5)	14 (23.0)	103 (26.9)	0.642	0.319	0.084
	TC	175 (51.0)	207 (49.1)	27 (44.3)	203 (53.0)			
	CC	65 (19.0)	103 (24.4)	20 (32.8)	77 (20.1)			
	T	381 (55.5)	431 (51.1)	55 (45.1)	409 (53.4)			
	C	305 (44.5)	413 (48.9)	67 (54.9)	357 (46.6)	0.413	0.350	0.088

*Early AMD vs. control group. **Exudative AMD vs. control group. ***Atrophic AMD vs. control group. p: significance level and Bonferroni corrected significance level when $p = 0.05/8$.

Table 3: The impact of IL-9 rs185943 and rs11741137 on early AMD and IL-10 rs1800896 on atrophic AMD.

Model	Genotype/allele	OR* (95% CI)	p value	AIC
		Early AMD		
		IL-9 rs1859430		
Codominant	GA vs. GG	0.700 (0.507-0.966)	**0.030**	1000.541
	AA vs. GG	1.713 (0.857-3.424)	0.128	
Dominant	GA+AA vs. GG	0.794 (0.585-1.077)	0.137	998.541
Recessive	AA vs. GA+GG	1.926 (0.972-3.816)	0.060	997.109
Overdominant	GA vs. GG+AA	0.673 (0.490-0.925)	**0.015**	994.759
Additive	A	0.938 (0.731-1.203)	0.613	1000.499
		IL-9 rs11741137		
Codominant	CT vs. CC	0.734 (0.530-1.015)	0.062	997.863
	TT vs. CC	1.489 (0.646-3.431)	0.350	
Dominant	CT+TT vs. CC	0.788 (0.577-1.077)	0.135	998.512
Recessive	TT vs. CT+CC	1.635 (0.714-3.745)	0.245	999.381
Overdominant	CT vs. CC+TT	0.720 (0.522-0.994)	**0.046**	996.749
Additive	T	0.883 (0.675-1.156)	0.365	999.931
		Atrophic AMD		
		IL-10 rs1800896		
Codominant	TC vs. TT	1.023 (0.503-2.079)	0.951	321.969
	CC vs. TT	2.043 (0.938-4.450)	0.072	
Dominant	TC+CC vs. TT	1.296 (0.670-2.505)	0.441	323.962
Recessive	CC vs. TC+TT	2.013 (1.078-3.759)	**0.028**	319.973
Overdominant	TC vs. TT+CC	0.719 (0.408-1.266)	0.253	323.257
Additive	C	1.454 (0.967-2.187)	0.072	321.302

*Adjusted for gender in early AMD and adjusted for age in atrophic AMD group. OR: odds ratio; CI: confidence interval; p: significance level and Bonferroni corrected significance level when $p = 0.05/8$; AIC: Akaike information criterion.

controls (8.0 (2.7) pg/ml vs. 8.8 (2.4) pg/ml, $p = 0.049$) and also in patients with early AMD (8.0 (2.7) pg/ml vs. 9.2 (1.7) pg/ml, $p = 0.017$) and atrophic AMD (8.0 pg/ml vs. 9.4 (1.5) pg/ml, $p = 0.008$).

We also performed the IL-10 serum level and SNP association analysis and found that exudative AMD patients with *IL-10* rs1800896 CT and TT genotypes had lower IL-10 serum concentrations than those with wild-type (CC) genotype: 7.2 (2.3) *vs.* 9.3 (1.4); $p = 0.048$ (Table 8).

4. Discussion

Our study is aimed at analyzing the associations between the immunogenetic markers *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) polymorphisms and their haplotypes, serum IL-9 and IL-10 levels and the different AMD forms.

IL-9 belongs to the IL-2Ryc chain family and works as a pleiotropic cytokine in inflammatory processes [40]. T lymphocytes, or more specific Th2, were described as the primary source for the IL-9 production [41]. Nevertheless, further studies identified other cell types, including Th9, mast cells, innate lymphoid cells (ILCs), NK cells, and even Foxp3+ Tregs, as well as mucin-producing cells, and eosinophils could also produce IL-9 [42–44]. Moreover, Dardalhon et al. have identified unique T cells that produce both IL-9

Table 4: Distributions of IL-9 rs11741137, rs2069885, and rs2069884 genotypes and alleles in the early AMD and control male groups.

SNP	Genotypes/alleles	Group: Early AMD ($n = 105$), n (%)	Group: Control ($n = 149$), n (%)	p value
rs11741137	CC	77 (73.3)	99 (66.4)	**0.032**
	CT	21 (20.0)	47 (31.5)	
	TT	7 (6.7)	3 (2.0)	
	C	175 (83.3)	245 (82.2)	0.743
	T	35 (13.3)	53 (17.8)	
rs2069885	GG	77 (73.3)	100 (67.1)	**0.039**
	GA	21 (20.0)	46 (30.9)	
	AA	7 (6.7)	3 (2.0)	
	G	175 (83.3)	246 (82.6)	0.818
	A	35 (13.3)	52 (17.4)	
rs2069884	GG	77 (73.3)	100 (67.1)	**0.039**
	GT	21 (20.0)	46 (30.9)	
	TT	7 (6.7)	3 (2.0)	
	G	175 (83.3)	246 (82.6)	0.818
	T	35 (13.3)	52 (17.4)	

p: significance level and Bonferroni corrected significance level when $p = 0.05/8$.

Table 5: The impact of IL-9 rs1859430 and rs11741137 on early AMD in males.

Model	Genotype/allele	OR (95% CI)	p value	AIC
		IL-9 rs1859430		
Codominant	GA vs. GG	0.547 (0.302-0.991)	**0.047**	342.864
	AA vs. GG	1.667 (0.54-5.010)	0.363	
Dominant	GA+AA vs. GG	0.671 (0.390-1.155)	0.150	344.355
Recessive	AA vs. GA+GG	1.966 (0.661-5.843)	0.224	342.961
Overdominant	GA vs. GG+AA	0.526 (0.292-0.948)	**0.033**	341.702
Additive	A	0.863 (0.562-1.327)	1.327	346.005
		IL-9 rs11741137		
Codominant	CT vs. CC	0.574 (0.317-1.041)	0.068	341.517
	TT vs. CC	3.000 (0.751-11.983)	0.120	
Dominant	CT+TT vs. CC	0.720 (0.415-1.248)	0.242	345.071
Recessive	TT vs. CT+CC	3.476 (0.878-13.769)	0.076	342.969
Overdominant	CT vs. CC+TT	0.543 (0.301-0.979)	**0.042**	342.175
Additive	T	0.929 (0.590-1.464)	0.751	346.357

*Only two genotypes were determined. OR: odds ratio; CI: confidence interval; p: significance level and Bonferroni corrected significance level when $p = 0.05/8$; AIC: Akaike information criterion.

Table 6: Linkage disequilibrium between studied polymorphisms.

	SNP1 (D'; r^2)	SNP2 (D'; r^2)	SNP3 (D'; r^2)	SNP4 (D'; r^2)	SNP5 (D'; r^2)	SNP6 (D'; r^2)	SNP7 (D'; r^2)	SNP8 (D'; r^2)
SNP1 (D'; r^2)		0.9828; 0.8339	0.9167; 0.6752	0.9397; 0.6877	0.9401; 0.6922			
SNP2 (D'; r^2)			0.8271; 0.6366	0.8436; 0.6419	0.8445; 0.6471			
SNP3 (D'; r^2)				0.9942; 0.9579	0.9942; 0.9633			
SNP4 (D'; r^2)					0.9995; 0.9932			
SNP5 (D'; r^2)								
SNP6 (D'; r^2)							0.9997; 0.9994	0.9883; 0.2454
SNP7 (D'; r^2)								0.9883; 0.2454
SNP8 (D'; r^2)								

D' is the deviation between the expected haplotype frequency and the observed frequency [D' scale: 0,1]. r^2 is squared correlation coefficient of the haplotype frequencies [r^2 scale: 0,1]. SNP1: rs1859430; SNP2: rs2069870; SNP3: rs11741137; SNP4: rs2069885; SNP5: rs2069884; SNP6: rs1800871; SNP7: rs1800872; SNP8: rs1800896.

and IL-10, leading to tissue inflammation [45]. Previously, IL-9 was described as a growth factor for T cells and mast cells [46, 47]. It is known that IL-9 can promote the growth and function of the erythroid progenitor, fetal thymocyte, myeloid precursor cells, and human megakaryoblastic leukemic cell lines [48]. IL-9 behavior is regulated through the specific IL-9 receptor (IL9R, which is contained of two subunits: the alpha chain (IL-9Rα) and the common gamma chain receptor). IL-9 binds the IL-9Rα subunit and forms the IL-9R heterocomplex. Because of the lack of specific enzymatic activity, the JAK/STAT pathway needs to be activated, and JAK is the initiator of the following phosphorylation cascades [49]. Previous inflammation-associated studies were reviewed and showed the pathogenic role of IL-9 in inflammatory disease development [48]. On the other hand, Elyaman et al. have been suggested the diverse role of IL-9, including both a regulator of pathogenic and protective mechanisms of immune responses [50].

IL-9 is encoded by the *IL-9* gene located on chromosome 5q31.1 [51]. Several *IL-9* variants (rs31563, rs1859430, rs11741137, and rs2069885) have already been published and identified as potential biomarkers for atopic dermatitis, asthma and its severity, respiratory syncytial virus (RSV) infection, and pituitary adenoma [30–35].

Namkung et al. reported that rs31563 (−4091G/A) at the *IL-9* gene was associated with increased susceptibility to atopic dermatitis [34]. Another study revealed that *IL-9* rs1859430 genotype frequencies were lower in asthma

Table 7: Haplotype association with the predisposition to AMD occurrence.

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	Frequency	OR (95% CI)	p value
Haplotype associations with early AMD											
1	G	A	C	G	G	—	—	—	0.7715	1	—
2	A	G	T	A	T	—	—	—	0.1468	0.76 (0.54-1.08)	0.13
3	A	G	C	G	G	—	—	—	0.0335	0.49 (0.25-0.95)	**0.035**
4	A	A	T	A	T	—	—	—	0.018	1.74 (0.72-4.22)	0.22
5	G	A	T	A	T	—	—	—	0.0108	0.08 (0.01-0.61)	**0.015**
6	A	A	C	G	G	—	—	—	0.0103	2.25 (0.66-7.66)	0.19
7 (rare)	*	*	*	*	*	—	—	—	0.009	1.29 (0.32-5.21)	0.72
8	—	—	—	—	—	G	G	C	0.4549	1	—
9	—	—	—	—	—	G	G	T	0.3275	1.13 (0.89-1.44)	0.31
10	—	—	—	—	—	A	T	T	0.2166	1.00 (0.75-1.32)	0.98
11 (rare)	—	—	—	—	—	*	*	*	0.001	<0.001 (-)	1
Haplotype associations with exudative AMD											
1	G	A	C	G	G	—	—	—	0.7713	1	—
2	A	G	T	A	T	—	—	—	0.1487	0.96 (0.69-1.35)	0.83
3	A	G	C	G	G	—	—	—	0.0408	0.99 (0.57-1.73)	0.98
4	A	A	T	A	T	—	—	—	0.0165	1.11 (0.46-2.66)	0.82
5 (rare)	*	*	*	*	*	—	—	—	0.0227	0.37 (0.15-0.92)	**0.033**
6	—	—	—	—	—	G	G	C	0.4751	1	—
7	—	—	—	—	—	G	G	T	0.3026	0.87 (0.68-1.10)	0.24
8	—	—	—	—	—	A	T	T	0.2205	0.94 (0.72-1.22)	0.63
9 (rare)	—	—	—	—	—	*	*	*	0.0018	0.34 (0.02-5.66)	0.45
Haplotype associations with atrophic AMD											
1	G	A	C	G	G	—	—	—	0.7558	1	—
2	A	G	T	A	T	—	—	—	0.1518	1.25 (0.65-2.40)	0.5
3	A	G	C	G	G	—	—	—	0.0453	2.34 (0.93-5.88)	0.071
4	G	A	T	A	T	—	—	—	0.0167	<0.00 (-)	1
5	A	A	T	A	T	—	—	—	0.0162	1.05 (0.23-4.68)	0.95
6 (rare)	*	*	*	*	*	—	—	—	0.0142	0.55 (0.07-4.43)	0.57
7	—	—	—	—	—	G	G	C	0.4758	1	—
8	—	—	—	—	—	G	G	T	0.3046	0.62 (0.38-1.02)	0.06
9	—	—	—	—	—	A	T	T	0.218	0.78 (0.47-1.29)	0.33
10 (rare)	—	—	—	—	—	*	*	*	0.0016	<0.00 (-)	1

Rare: pooled rare haplotypes; OR: odds ratio; CI: confidence interval; p: significance level when $p < 0.05$; SNP1: rs1859430; SNP2: rs2069870; SNP3: rs11741137; SNP4: rs2069885; SNP5: rs2069884; SNP6: rs1800871; SNP7: rs1800872; SNP8: rs1800896.

patients than in controls under the recessive GA+AA ($p = 0.021$) and heterozygous GA ($p = 0.031$) models. Also, those patients had significantly lower A-T (rs1859430-rs2066758) haplotype frequency ($p = 0.006$) and higher G-T (rs1859430-rs2066758) haplotype frequency ($p \leq 0.001$). Moreover, they showed that rs1859430 AG genotype was associated with the higher IL-9 serum levels compared with other genotypes in the disease group ($p < 0.05$), and the rs2066758 CC genotype was linked to the partial pressure of carbon dioxide ($PaCO_2$) ($p = 0.041$) [35]. Two authors underlined the differences between gender groups in asthma and RSV infection development, considering the sex-dependent mechanisms. Aschard et al. showed that polysensitization (SPTQ) and forced expiratory volume in one second divided by height square (FEV(1)/H(2)) were associated with two *IL-9* variants rs2069885 and rs2069882 ($p = 0.02$ and $p = 0.002$, respectively, after Bonferroni's correction). This study underlines the importance of complex mechanisms, such as heterogeneity, according to sex and pleiotropy, to reveal the genes involved in asthma phenotypes [33]. Schuurhof et al. revealed that the major allele at rs2069885 was associated with increased susceptibility to severe RSV infection in boys' and girls' opposite associations. Furthermore, the haplotype T-T rs2069885 and rs1799962 was a risk marker for severe RSV bronchiolitis in girls [32]. One more study found that study subjects with the dominant genotype for these *IL-9* polymorphisms (rs11741137, rs2069885, and rs1859430) were associated with a severe

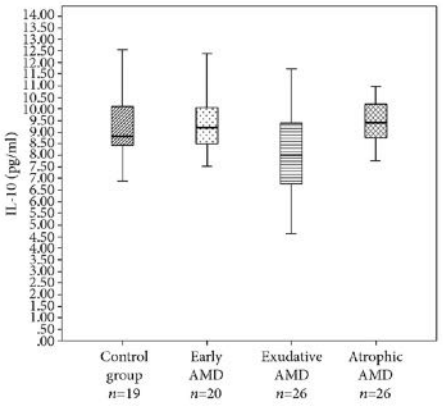

FIGURE 1: Interleukin 10 concentrations between the groups. IL-10 serum levels in the control (8.8 pg/ml), early AMD (9.2 pg/ml), exudative AMD (8.0 pg/ml), and atrophic AMD (9.4 pg/ml) groups. Kruskal-Wallis test ($p = 0.020$). The bars represent the median with interquartile variation (1st quartile and 3rd quartile).

asthma exacerbation if exposed to increased dust mite levels ($p = 0.02$ to 0.03). It was even replicated it in another study ($p = 0.04$) [52]. *IL-9* rs1859430 G/A and A/A genotypes were also found to be associated with increased odds of having recurrent PA under the codominant ($p = 0.003$ and $p = 0.006$, respectively), dominant ($p = 0.011$), and recessive ($p = 0.021$) genetic models [30].

Controversial results were observed in several studies as well, and they reported no significant associations between *IL-9* promoter polymorphism A−345G and RSV bronchiolitis [52], T113M in *IL-9* and atopic bronchial asthma [53], rs1859430 and rs2069868 and Graves' disease [54], or *IL-9* rs2069885 and allergic rhinitis in Iranian women [55]. Schürks et al. tried to show associations between *IL-9* rs2069885 and inflammatory pathways among women with migraine, but results did not survive the corrections for multiple testing [56]. Also, *IL-9* rs31563 C>T and rs31564 G>T were not associated with gastric cardiac adenocarcinoma and esophageal cancer in a Chinese population [57, 58].

According to the databases, any *IL-9* SNPs were analyzed in AMD. Our study shows that *IL-9* rs1859430 GA genotype and *IL-9* rs11741137 CT genotypes were associated with decreased odds of early AMD. Unfortunately, in our study, we applied Bonferroni's correction because of the multiple comparisons, and associations between study groups and SNPs in *IL-9* did not survive this strict correction.

In further analysis, the same tendencies remained only in the male group. Even if these results did not survive Bonferroni's correction, differences between male and female groups were observed as in previous studies [14, 32, 33]. Potentially different inflammation processes in men and women should be studied, considering the hormone role on molecular mechanisms in inflammation and response to inflammation, which may lead to opposite disease outcomes [59].

Also, we performed the haplotype analysis and identified the haplotypes A-G-C-G-G and G-A-T-A-T of *IL-9* SNPs (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884), which were associated with the decreased odds of early AMD ($p = 0.035$ and $p = 0.015$, respectively). The set of rare haplotypes was associated with the decreased odds of exudative AMD ($p = 0.033$), which may have a potential role in the IL-9-dependent inflammation process involved in AMD development.

In our study, the serum IL-9 levels were not determined because of the low detection rates. On the other hand, IL-9 was previously involved in several AMD studies. IL-9 cytokine in the aqueous humor was measured, but differences between neovascular AMD and the control groups were not found [60]. IL-9 was also measured in aqueous humor samples of neovascular AMD before the treatment by intravitreal drug injection and after, and compared with the control group, but the statistical analysis did not show any differences between study groups [61]. In another study, plasma and aqueous humor IL-9 levels between exudative AMD and the control groups were not reported because of the low detection rate [62]. Extremely low limits of detection of IL-9 in the aqueous fluid of patients with neovascular AMD were reported as well [63].

Contrarily, Lin et al. revealed that cytokine IL-9 was overexpressed in stimulated RPE cells, with a potential association with AMD development [23]. Unfortunately, no other studies, including IL-9 and AMD, were found. Otherwise, few diseases/conditions were reported, and elevated IL-9 levels were found in asthmatic patients, patients with allergic rhinitis and a peanut allergy, and those suffering from some autoimmune diseases [48].

IL-10 belongs to the IL-10 family, and as an anti-inflammatory cytokine, the IL-10 takes part in the regulation of the inflammatory response [64]. Macrophages are the primary source of IL-10. Still, other immune cells (monocytes, dendritic cells, B lymphocytes, T helper 1 (Th1) and Th2 lymphocytes, mast cells, NK cells, cytotoxic T cells, and granulocytes like neutrophils and eosinophils) can secrete this interleukin [65, 66]. As a pleiotropic cytokine, IL-10 inhibits the antigen-presenting cells via the inhibition of expression of major histocompatibility complex (MHC) class II molecules. It also suppresses the expression of IL-1, IL-6, IL-8, IL-12, and tumor necrosis factor-alpha (TNF-α). Furthermore, IL-10 promotes proliferation, activation, and differentiation and helps prevent cell apoptosis in B cells [27, 65]. The immunosuppressive IL-10 activity is mediated by the heterodimeric IL-10 receptors (IL-10R1 and IL-10R2). IL-10R1 primary ligates to IL-10 and dimerizes with IL10R2 leading to the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. Phosphorylated STAT3 molecules that enter the cell's nucleus induce changes in the expression of immunomodulatory genes leading to the inhibition of the secreted proinflammatory cytokines and downstream immune response, and regulate the activity of growth factors, such as VEGF [67, 68]. The IL-10 level changes were reported as a

Table 8: Serum IL-10 levels in relation to the genotypes.

Model		Early AMD (pg/ml), median (IQR)	p value	Exudative AMD (pg/ml), median (IQR)	p value	Atrophic AMD (pg/ml), median (IQR)	p value	Control group (pg/ml), median (IQR)	p value
				IL-10 rs1800871					
Dominant	GA+AA vs. GG	9.2 (15.8) vs. 9.1 (1.5)	0.571	8.6 (3.9) vs. 7.7 (2.6)	0.312	9.3 (1.1) vs. 9.5 (2.2)	0.230	8.7 (2.6) vs. 8.8 (3.9)	0.442
Recessive	AA vs. GA+GG	(-) vs. 9.2 (1.4)	—	(-) vs. 7.9 (2.9)	—	(-) vs. 9.4 (1.6)	—	(-) vs. 8.8 (2.9)	—
				IL-10 rs1800872					
Dominant	GT+TT vs. GG	9.2 (15.8) vs. 9.1 (1.5)	0.571	8.6 (3.9) vs. 7.7 (2.6)	0.312	9.3 (1.1) vs. 9.5 (2.2)	0.230	8.7 (2.6) vs. 8.8 (3.9)	0.442
Recessive	TT vs. GT+GG	(-) vs. 9.2 (1.4)	—	(-) vs. 7.9 (2.9)	—	(-) vs. 9.4 (1.6)	—	(-) vs. 8.8 (2.9)	—
				IL-10 rs1800896					
Dominant	TC+CC vs. TT	9.1 (1.8) vs. 9.3 (7.8)	0.800	7.2 (2.3) vs. 9.3 (1.4)	**0.048**	9.4 (2.2) vs. 9.3 (0.7)	0.800	8.8 (3.4) vs. 8.7 (-)	0.573
Recessive	CC vs. TC+TT	9.2 (-) vs. 9.1 (2.7)	0.546	6.7 (2.2) vs. 8.5 (2.5)	0.054	8.7 (1.5) vs. 9.5 (1.2)	0.153	8.7 (-) vs. 8.8 (2)	0.958

*Only two genotypes were determined.

significant pathophysiological modulator in many diseases and reviewed previously [27].

IL-10 and genetic variants of *IL-10* were also included in our study. IL-10 is encoded by the *IL-10* gene located on chromosome 1q32.1, and three most studied point mutations in *IL-10* promoter -592 A/C (rs1800872), -819 C/T (rs1800871), and -1082G/A (rs1800896) were described as leading genetic variations for SNP association studies [37]. Our present study showed that the *IL-10* rs1800896 CC genotype was associated with 2-fold increased odds of atrophic AMD ($p = 0.028$), but these results did not survive strict Bonferroni's correction as well.

Shevchenko et al. reported similar results. They found a higher *IL10*-1082 GG genotype frequency in AMD patients than in the controls [69], while in another study, the associations of *IL-10* -592 A/C, -819 C/T, and -1082G/A polymorphisms and late AMD were not determined [70]. Moreover, no more such studies were found, but these SNPs were associated with other conditions. For example, children with *IL-10* -592 CC or -592 AA genotypes had a higher risk of hospitalization for RSV bronchiolitis than those with heterozygous genotype [52]. Also, *IL-10* rs1800872 T>G polymorphism was associated with an increased risk of esophageal cancer in a Chinese population [58]. It was still not associated with gastric cardiac adenocarcinoma in the same population [57], suggesting further investigations for the IL-10 signaling pathway associations with cancer development.

Previous studies have also shown that the elevated IL-10 levels in the eye induce the alternative macrophage activation, which can be associated with choroidal neovascularization development [71, 72]. We also analyzed serum IL-10 levels and found that IL-10 serum levels were lower in exudative AMD than in controls ($p = 0.049$), and also in patients with early AMD ($p = 0.017$) and atrophic AMD ($p = 0.008$). Similar results were found by the other researcher group, which revealed lower concentrations of IL-10 cytokine in the wet and dry AMD groups than in controls ($p < 0.05$ and $p < 0.05$). Also, they determined that IL-10 levels were higher in wet AMD than in the dry AMD group ($p = 0.009$) [73]. Opposite results were found when Subhi et al. revealed that patients with neovascular AMD had higher plasma levels of IL-10 compared to healthy controls ($p < 0.0001$), and statistically significant results remained even after multivariate analysis (after adjusting for demographics, comorbidities, and lifestyle factors) IL-10 ($p < 0.001$) [74]. Statistically, significantly elevated IL-10 serum levels in AMD patients were determined by Nassar et al. as well [75]. On the other hand, the expression of IL-10 did not differ between Tfh cells from AMD patients and non-AMD controls [76]. It is interesting that IL-10 levels in the aqueous humor did not differ between the neovascular AMD and control groups [61–63]. Few other studies have not even determined IL-10 in plasma or aqueous humor samples of AMD patients [61–63]. In contrast, the others showed that IL-10 levels do not differ between intraocular fluid and serum samples [77].

Moreover, we found that IL-10 levels in the exudative AMD group are associated with the minor allele T, and patients with exudative AMD carrying *IL-10* rs1800896 CT and TT genotypes have lower IL-10 serum concentrations than those with wild-type (CC) genotype. These findings confirm the *IL-10* promoter polymorphism (rs1800896) role on IL-10 level changes [37], which can be responsible for the immune response in exudative AMD development. It is important to highlight that *IL-9* (rs1859430, rs2069870, rs11741137, rs2069885, and rs2069884) and *IL-10* (rs1800871, rs1800872, and rs1800896) gene variants, as well as serum IL-9 and IL-10 levels, have never been studied in AMD, in the Lithuanian population, and our study was the first of its type. While a thorough medical examination of the study objects can be acknowledged as one of our study's

main strengths, we should declare that the relatively small sample size and the other risk factors that were not involved in our study fall into this study's limitations.

5. Conclusions

In conclusion, inflammation is an underlying mechanism in AMD development. We have found lower IL-10 serum levels in patients with exudative AMD than healthy controls and early or atrophic AMD patients. A minor allele at *IL-10* rs1800896 was associated with the lower IL-10 serum levels in the exudative AMD group. The haplotypes of IL-9 SNPs were also associated with the decreased odds of early and exudative AMD occurrence. Further studies will be needed to elucidate this regulatory pathway's underlying mechanism and its association with AMD clinical symptoms.

Data Availability

Data will be provided in case a request is made by editors, reviewers, or scientists.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

A.V. and L.K. are responsible for the conceptualization. A.V. and D.C. are responsible for the data curation. A.V. is responsible for the writing of the original draft preparation. A.V., D.C., R.Z., and R.L. are responsible for the methodology. A.V., D.C., and L.K. are responsible for the investigation. A.V. is responsible for the formal analysis. L.K. and R.Z. are responsible for the validation. R.L. is responsible for the supervision. R.L. is responsible for the writing—reviewing and editing.

Supplementary Materials

Table S1: genotype distribution in the control group using Hardy-Weinberg equilibrium. Seven SNPs were in HWE ($p > 0.05$), but rs2069870 did not fulfill the HWE requirements because there were observed only two genotypes. *(Supplementary Materials)*

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Article

VEGFA Haplotype and VEGF-A and VEGF-R2 Protein Associations with Exudative Age-Related Macular Degeneration

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Abstract: Our study aimed to reveal the associations between *VEGFA* SNPs (rs1570360, rs699947, rs3025033, and rs2146323), their haplotypes, VEGF-A and VEGF-R2 serum concentrations, and early and exudative AMD. A total of 339 subjects with early AMD and 419 with exudative AMD groups, and 374 healthy subjects, were genotyped for four *VEGFA* SNPs (rs1570360, rs699947, rs3025033, and rs2146323). VEGF-A and VEGFR-2 serum concentrations were measured in exudative AMD and controls. The results revealed that rs3025033 G allele was significantly associated with lower odds of exudative AMD under the dominant model (OR = 0.67; 95% CI: 0.49–0.80; p = 0.0088) and additive (OR = 0.7; 95% CI: 0.54–0.90; p = 0.0058) models after Bonferroni correction. In the female group, rs3025033 AG genotype was associated with exudative AMD under the codominant model (OR = 0.57; 95% CI: 0.37–0.87; p = 0.009) and G allele under the dominant (OR = 0.55; 95% CI: 0.37–0.82; p = 0.0032) and additive models (OR = 0.60; 95% CI: 0.42–0.84; p = 0.0028). Haplotype analysis revealed that individuals carrying rs1570360, rs699947, rs3025033, and rs2146323 haplotype A-A-G-A had decreased risk of exudative AMD (OR = 0.46, 95% CI: 0.23–0.90; p = 0.023). The VEGF-A and VEGF-R2 serum concentrations did not differ between study groups; we found that patients with exudative AMD carrying at least one C allele at rs699947 have statistically significantly higher VEGF-A serum concentrations compared to AA genotype carriers (485.95 (945.93) vs. 194.97 (-), respectively, p = 0.046). In conclusion, we found that *VEGFA* rs3025033 and haplotype rs1570360A-rs699947A-rs3025033G- rs2146323A play a protective role for exudative AMD in the Caucasian population. Furthermore, rs699947 is associated with elevated VEGF-A serum concentrations in exudative AMD.

Keywords: age-related macular degeneration; *VEGFA*; haplotype; serum concentration; rs1570360; rs699947; rs3025033; rs2146323

Citation: Vilkeviciute, A.; Cebatoriene, D.; Kriauciuniene, L.; Liutkeviciene, R. *VEGFA* Haplotype and VEGF-A and VEGF-R2 Protein Associations with Exudative Age-Related Macular Degeneration. *Cells* **2022**, *11*, 996. https://doi.org/10.3390/cells11060996

Academic Editors: Sarah Xin Zhang, Joshua Jianxin Wang and Michael E. Boulton

Received: 17 January 2022
Accepted: 11 March 2022
Published: 15 March 2022

1. Introduction

Age-related macular degeneration (AMD) is widely described as a multifactorial, progressive, neurodegenerative disease of the macula, causing loss of visual functions leading to blindness. The macula is a part of the retina where the photoreceptors, responsible for visual acuity and colour perception, are concentrated [1]. Most eye function impairments are associated with age-related alterations and, together with the other risk factors such as environmental and genetic factors, lead to severe eye conditions [2]. Exponential population ageing is a recent worldwide problem affecting human health, including eye diseases. It is known that AMD affects about 10% of people older than 65 years and more than 25% of people older than 75 years in developed countries [2]. The previous study's projection shows that the number of people with early AMD will increase up to 21.5 million and late AMD up to 4.8 million in Europe [3], while worldwide the numbers are expected to increase dramatically, even up to 288 million in 2040 [3].

AMD can be classified based on histopathological changes [4]. Early and intermediate AMD often may not cause any symptoms. Early AMD signs include small drusen (protein

and lipid deposit) formation between the retinal pigment epithelium (RPE) and Bruch's membrane [2]. The intermediate form is described as a presence of at least one large drusen, numerous medium-sized drusen, or geographic atrophy (GA) without extension to the center of the macula with mild symptoms, such as mild blurriness, in their central vision or trouble seeing in low lighting. The advanced AMD is divided into dry or atrophic AMD with the GA of the RPE, and neovascular or exudative AMD is diagnosed when choroidal neovascularization with detachments in the RPE haemorrhages and/or scars appear and cause progressive blurring or other central vision impairments [5].

Considering the multifactorial AMD pathogenesis, drusogenesis and oxidative stress remain the essential processes [6]. Accumulation of molecular debris, including lipid and protein particles as well as reactive oxygen species (ROS) leading to chronic inflammation, cause the irreversible damage of RPE cells and photoreceptors in the macula [7,8], and also the breakdown of the blood–ocular barrier, which is a normal function to protect the eye [9].

The above-mentioned pathological alterations cause ocular damage, resulting in cytokine release and pro-angiogenic and anti-angiogenic factor disbalance, including increased vascular endothelial growth factor A (VEGFA) production, which plays a crucial role in angiogenesis and vascular permeability. Moreover, vascular leakage and inflammation caused by excessive VEGFA release were found to play a critical role for choroidal neovascularisation and exudative AMD development [7,10].

VEGFA can regulate angiogenesis in the vascular endothelium binding two types of VEGF family receptors: VEGF-R1, encoded by FMS-related tyrosine kinase 1; *FLT1* gene (OMIM* 165070) and VEGF-R2, encoded by kinase insert domain receptor; *KDR* gene (OMIM* 191306) [11]. One of these high-affinity receptor tyrosine kinases, VEGF-R2, is a primary angiogenic receptor associated with VEGFA-stimulated vascular permeability [12,13], and the VEGF-R1 focuses on endogenous VEGFA inhibition [12,14].

While only the exudative AMD is already treated with anti-VEGFA inhibitors, in recent years, the VEGFA and its signaling pathway have been targeted for the most effective therapy development [15], including the other molecules based on the pathogenic processes in which they are involved [16].

Although AMD involves many risk factors, a substantial genetic contribution reveals the genetic marker associations with AMD susceptibility [17], including a relationship between the *VEGFA* and *VEGFR2* gene variants and AMD as well [18–21]. Regarding the significant associations and conflicting results between genetic markers and AMD [22] in previous studies, we aimed to investigate the associations between four single nucleotide polymorphisms (SNPs) in *VEGFA* (OMIM* 192240), and VEGF-A and VEGFR-2/KDR protein roles in AMD in the Caucasian population to focus on a potential tool for early diagnosis.

2. Materials and Methods

2.1. Study Groups

This study was conducted after the approval of the Ethics Committee for Biomedical Research, Lithuanian University of Health Sciences (No. BE-2-/48; 8 October 2018).

Study groups consisted of subjects admitted to the Hospital of Lithuanian University of Health Sciences Ophthalmology Department for ophthalmological evaluation. In our study, 1132 participants were enrolled: 339 subjects in early AMD and 419 in the exudative AMD group. The Control group was formed of 374 healthy subjects (Table 1). Using the global AMD prevalence (8.7%) [23] and the minor allele frequencies from [24], we calculated that our collected sample sizes for the early, exudative AMD and control groups were sufficient to reach 80% or higher statistical power for the selected *VEGFA* SNP analysis.

An ophthalmological evaluation was performed for all the study subjects, and data about general health and other diseases were obtained from study subjects. All study subjects agreed to participate and signed an informed consent form.

Table 1. Demographic characteristics of study groups.

Characteristic	Early AMD N = 339	Exudative AMD N = 419	Control Group N = 374	*p* Value
Gender				
Males, n (%)	104 (30.7)	149 (35.6)	139 (37.2)	0.068 *
Females, n (%)	235 (69.3)	270 (64.4)	235 (62.8)	0.639 **
Age, median (IQR)	73 (13)	77 (10)	72 (10)	0.382 *
				<0.001 **

IQR—interquartile range; *p*—significance level when $p = 0.05$; * Early AMD vs. Control group; ** Exudative AMD vs. Control group.

2.2. AMD Groups

All the AMD patients underwent optical coherence tomography (OCT), and optical coherence tomography angiography (OCT-A) was performed in patients suspected of having late AMD after the OCT examination. Exudative AMD was diagnosed in one eye of the study subjects.

Age-Related Eye Disease Study (AREDS) classification was used for AMD diagnosis and has been described previously [25].

Early AMD was defined as a combination of multiple small drusen and several intermediate drusen (63–124 µm in diameter) or retinal pigment epithelial abnormalities.

Intermediate AMD was characterized by the presence of extensive intermediate drusen and at least one large (giant) druse (≥125 µm in diameter) or geographic atrophy (GA) not involving the center of the fovea.

Advanced AMD was characterized by GA involving the fovea and/or any of the features of neovascular AMD.

2.3. The Control Group

Subjects without ophthalmologic pathologies and patients after senile cataract surgeries (without any other ocular comorbidities) were included in the control group. The following exclusion criteria were also described in previous publications [26]:

(1) unrelated eye disorders, e.g., high refractive error, cloudy cornea, lens opacity (nuclear, cortical, or posterior subcapsular cataract) except minor opacities, keratitis, acute or chronic uveitis, glaucoma, or diseases of the optic nerve;
(2) systemic illnesses, e.g., diabetes mellitus, malignant tumors, systemic connective tissue disorders, chronic infectious diseases, hypertension, coronary artery disease, stroke or conditions following organ or tissue transplantation;
(3) ungraded colour fundus photographs resulting from obscuring the ocular optic system or because of fundus photograph quality.

2.4. DNA Extraction

DNA extraction was carried out at the Laboratory of Ophthalmology, Neuroscience Institute, LUHS. The DNA was extracted from 200 µL peripheral venous blood samples utilizing silica-based membrane technology, using a genomic DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, Vilnius, Lithuania), based on the manufacturer's recommendations.

2.5. SNP Selection

In the present study, two tag SNPs (intronic variants rs3025033A/G and rs2146323A/C) covering two haploblocks (Figure 1) (rs833068 have already been analyzed in our previous research study) [26] were selected from the CEU population using the public HapMap database. The pairwise option of the online Tag SNP tool was used with the following settings: $r^2 = 0.8$ set and the minimum number of SNPs tagged by each tag SNP was 2.

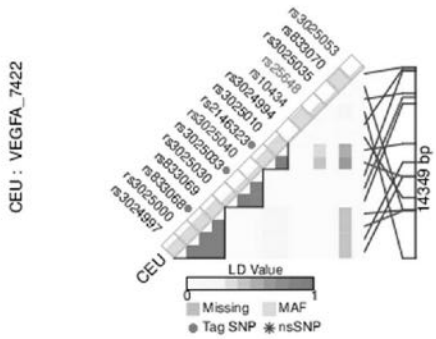

Figure 1. Haploblocks in CEU population, HapMap data. $r^2 = 0.8$ set; a minimum number of SNPs tagged by each tag SNP = 2.

Two other *VEGFA* promoter polymorphisms, −2578C/A (rs699947) and −1154G/A (rs1570360), were selected additionally based on the previous inconsistent results [27] and potential multiple SNP associations with the AMD [19].

Finally, 4 SNPs in *VEGFA* gene were selected for genotyping and further analysis: rs1570360 (chromosome6:43770093 (GRCh38)), rs699947 (chromosome6:43768652 (GRCh38)), rs3025033 (chromosome6:43783338 (GRCh38)), rs2146323 (chromosome6:43777358 (GRCh38)).

2.6. Genotyping

The genotyping of four *VEGFA* gene polymorphisms, rs1570360, rs699947, rs3025033, and rs2146323, was carried out at the Laboratory of Ophthalmology, Neuroscience Institute, LUHS. The identification of all single-nucleotide polymorphisms was performed on a "StepOnePlus" real-time PCR quantification system (Thermo Fisher Scientific, Singapore) using predesigned TaqMan® Genotyping assays (Thermo Fisher Scientific, Pleasanton, CA, USA) according to the manufacturer's recommendations. Genotyping results were obtained using Genotyping program on the StepOne software.

2.7. Quality Control of Genotyping

A total of 5% randomly chosen samples were repetitively genotyped for all four SNPs to confirm the same rate of genotypes from initial and repetitive genotyping.

2.8. Serum VEGF-A and VEGF-R2 Concentration Measurement

Obtained serum was partitioned into 200 µL aliquots to Eppendorf tubes and frozen at −80 °C. Serum VEGF-A and VEGF-R2 concentrations were determined by sandwich enzyme-linked immunosorbent assays, using the Human VEGF-A ELISA (Cat. No. BMS277-2) and VEGF-R2/KDR ELISA (Cat. No. BMS2019) kits following the manufacturer's instructions. Results were observed using a Multiskan FC microplate photometer (Thermo Scientific, Waltham, MA, USA) at 450 nm. VEGF-A assay sensitivity was 7.9 pg/mL, VEGF-R2/KDR—7 pg/mL.

2.9. Statistical Analysis

Statistical analysis was performed using the SPSS/W 27.0 software (Statistical Package for the Social Sciences for Windows, Inc., Chicago, IL, USA). Continuous data (age, protein serum level data distributions were evaluated for normality by the Kolmogorov–Smirnov test. Continuous variables presented as median with interquartile range (IQR) based on data distribution. The Mann–Whitney test was used to compare two groups for non-normally distributed data.

Categorical data (gender, genotype, and allele distributions) are presented as absolute numbers with percentages in brackets and compared between the early, exudative, AMD, and control groups using the *chi-square* (χ^2) test. Hardy–Weinberg equilibrium was evaluated to compare the observed and expected frequencies of *VEGFA* rs1570360, rs699947, rs3025033, and rs2146323 using χ^2 test in the control group as well.

The impact of SNPs on early and exudative AMD was evaluated using binomial logistic regression analysis. Results are presented as odds ratios (OR) with 95% confidence interval (CI) and adjusted by covariate effect for age in the exudative AMD groups. Logistic regression analysis results were expressed as genetic models (codominant: heterozygotes vs. major allele homozygotes and minor allele homozygotes vs. major allele homozygotes; dominant: minor allele homozygotes and heterozygotes vs. major allele homozygotes; recessive: minor allele homozygotes vs. major allele homozygotes and heterozygotes; overdominant: heterozygotes vs. major allele homozygotes and minor allele homozygotes); the additive model was used to evaluate the impact of each minor allele on AMD: major allele homozygotes vs. heterozygotes *vs.* minor allele homozygotes. The best genetic model selection was based on the Akaike information criterion (AIC); therefore, the best genetic models had the lowest AIC values. We introduced an adjusted significance threshold for multiple comparisons alpha = 0.0125 (0.05/4, since we analyzed four SNPs in the *VEGF-A* gene) [28].

Haplotype analysis was performed in the early AMD and control groups, and exudative AMD and control groups separately, using online SNPStats software (https://www.snpstats.net/snpstats/ (accessed on 15 December 2021)) [29]. Linkage disequilibrium (LD) analysis was assessed by D′ and r^2 measures. The associations between the haplotypes and different AMD forms were calculated by logistic regression and presented as ORs and 95% CI and values adjusted for age in exudative AMD analysis. Haplotypes with less than 1% frequencies were pooled into one group and described as "rare". A two-sided test with a value less than 0.05 was considered statistically significant. Graphs were performed using GraphPad Prism version 9.0.0 for Mac, GraphPad Software, San Diego, CA, USA, www.graphpad.com (accessed on 21 February 2022).

3. Results

3.1. VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) Genotype and Allele Associations with Early and Exudative AMD

VEGFA SNPs' genotype distributions were evaluated in the control group using Hardy–Weinberg equilibrium (HWE). Three SNPs were in HWE ($p > 0.05$), but rs1570360 did not conform the HWE requirements ($p < 0.001$) (Table 2).

Analysis of genotype and allele distributions showed statistically significant differences of rs3025033 genotypes (AA, AG, and GG) between exudative AMD and control groups (69.2%, 27.7% and 3.1% vs. 61%, 33.4%, and 5.6%, respectively, $p = 0.029$) (Table 2). Still, these results did not survive Bonferroni correction.

Allele frequency analysis showed that G allele at rs3025033 was statistically significantly less frequent in the exudative AMD group than controls (16.9% vs. 22.3%, respectively, $p = 0.007$) (Table 2).

Binomial logistic regression revealed that G allele at rs3025033 was significantly associated with lower odds of exudative AMD under the dominant model (OR = 0.67; 95% CI: 0.49–0.80; $p = 0.0088$) and additive (OR = 0.7; 95% CI: 0.54–0.90; $p = 0.0058$) model as best according to AIC value, even after Bonferroni correction (Table 3).

Table 2. Frequencies of *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genotypes and alleles in early AMD, exudative AMD, and control groups.

SNP Genotype/Allele	Early AMD N = 339 n (%)	Exudative AMD N = 419 n (%)	Control Group N = 374 n (%)	HWE *p* Value	*p* Value *	*p* Value **	*p* Value ***
rs1570360							
GG	162 (47.8)	183 (43.7)	177 (47.3)	<0.001	0.186	0.136	0.512
AG	128 (37.8)	168 (40.1)	125 (33.4)				
AA	49 (14.5)	68 (16.2)	72 (19.3)				
G	452 (66.7)	534 (63.7)	479 (64)		0.298	0.897	0.234
A	226 (33.3)	304 (36.3)	269 (36)				
rs699947							
AA	86 (25.4)	119 (28.4)	112 (29.9)	0.168	0.317	0.589	0.183
AC	174 (51.3)	187 (44.6)	173 (46.30				
CC	79 (23.3)	113 (27)	89 (23.8)				
A	346 (51)	425 (50.7)	397 (53.1)		0.441	0.348	0.902
C	332 (49)	413 (49.3)	351 (46.9)				
rs3025033							
AA	215 (63.4)	290 (69.2)	228 (61)	0.482	0.399	**0.029**	0.242
AG	112 (33)	116 (27.7)	125 (33.4)				
GG	12 (3.5)	13 (3.1)	21 (5.6)				
A	542 (80)	696 (83.1)	581 (77.7)		0.296	**0.007**	0.119
G	136 (20)	142 (16.9)	167 (22.3)				
rs2146323							
CC	142 (41.9)	191 (45.6)	158 (42.2)	0.06	0.248	0.603	0.236
AC	157 (46.3)	169 (40.3)	157 (42)				
AA	40 (11.8)	59 (14.1)	59 (15.8)				
C	441 (65)	551 (65.8)	473 (63.2)		0.477	0.296	0.773
A	237 (35)	287 (34.2)	275 (36.8)				

HWE *p* value—Hardy–Weinberg equilibrium significance level $p = 0.05$; *p*—significance level and Bonferroni corrected significance level when $p < 0.05/4$; *p*-values marked with bold indicate statistically significant *p*-values. * Early AMD vs. Control group; ** Exudative AMD vs. Control group; *** Early AMD vs. Exudative AMD.

Table 3. Binomial logistic regression analysis of *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) in early and exudative AMD and control groups.

				rs1570360			
	Early AMD vs. Control Group				Exudative AMD vs. Control Group		
Model	Genotype/Allele	OR (95% CI)	*p* Value	AIC	OR (95% CI) *	*p* Value	AIC
				rs1570360			
Codominant	GG	1		989.3	1		1045.7
	AG	1.12 (0.81–1.55)	0.499		1.25 (0.91–1.73)	0.171	
	AA	0.74 (0.49–1.13)	0.168		0.94 (0.63–1.41)	0.763	
Dominant	GG	1		990.7	1	0.38	1045.5
	AG + AA	0.98 (0.73–1.32)	0.9		1.14 (0.85–1.52)		
Recessive	GG + AG	1		987.8	1	0.4	1045.6
	AA	0.71 (0.48–1.05)	0.087		0.85 (0.58–1.24)		
Overdominant	GG + AA	1		989.2	1	0.11	1043.8
	AG	1.21 (0.89–1.64)	0.23		1.27 (0.94–1.72)		
Additive	A	0.91 (0.75–1.11)	0.34	989.8	1.02 (0.84–1.23)	0.88	1046.2
				rs699947			
Codominant	AA	1		990.4	1		1045.9
	AC	1.31 (0.92–1.86)	0.132		0.97 (0.69–1.37)	0.87	
	CC	1.16 (0.76–1.75)	0.492		1.28 (0.86–1.89)	0.226	
Dominant	AA	1		988.8	1		1046.1
	AC + CC	1.26 (0.90–1.75)	0.17		1.07 (0.78–1.47)	0.67	

Table 3. *Cont.*

				rs1570360			
		Early AMD vs. Control Group			**Exudative AMD vs. Control Group**		
Model	**Genotype/Allele**	**OR (95% CI)**	***p* Value**	**AIC**	**OR (95% CI) ***	***p* Value**	**AIC**
Recessive	AA + AC	1			1		
	CC	0.97 (0.69–1.38)	0.88	990.7	1.30 (0.93–1.81)	0.13	1043.9
Overdominant	AA-CC	1			1		
	AC	1.23 (0.91–1.64)	0.18	988.9	0.87 (0.65–1.16)	0.34	1045.4
Additive	C	1.08 (0.88–1.33)	0.45	990.1	1.12 (0.92–1.37)	0.25	1044.9
				rs3025033			
Codominant	AA	1			1		
	AG	0.95 (0.69–1.30)	0.751	990.8	0.69 (0.51–0.95)	**0.025**	1040.7
	GG	0.61 (0.29–1.26)	0.181		0.50 (0.24–1.03)	0.061	
Dominant	AA	1			1		
	AG + GG	0.90 (0.67–1.22)	0.5	990.3	0.67 (0.49–0.90)	**0.0088**	1039.4
Recessive	AA + AG	1			1		
	GG	0.62 (0.30–1.27)	0.18	988.9	0.56 (0.27–1.15)	0.11	1043.7
Overdominant	AA + GG	1			1		
	AG	0.98 (0.72–1.34	0.91	990.7	0.73 (0.53–0.99)	**0.045**	1042.3
Additive	G	0.87 (0.68–1.13)	0.3	989.6	0.70 (0.54–0.90)	**0.0058**	1038.7
				rs2146323			
Codominant	CC	1			1		
	AC	1.11 (0.81–1.53)	0.509	989.9	0.84 (0.61–1.15)	0.28	1046.5
	AA	0.75 (0.48–1.20)	0.231		0.79 (0.51–1.21)	0.28	
Dominant	CC	1			1		
	AC + AA	1.01 (0.75–1.37	0.92	990.7	0.83 (0.62–1.11)	0.2	1044.6
Recessive	CC + AC	1			1		
	AA	0.71 (0.46–1.10)	0.12	988.3	0.86 (0.57–1.28)	0.45	1045.7
Overdominant	CC + AA	1			1		
	AC	1.19 (0.89–1.60)	0.24	989.4	0.89 (0.67–1.20)	0.45	1045.7
Additive	A	0.93 (0.75–1.15)	0.49	990.2	0.88 (0.72–1.07)	0.2	1044.7

OR—odds ratio; CI—confident interval; *p*—significance level and Bonferroni corrected significance level when $p < 0.05/4$; *p*-values marked with bold indicate statistically significant *p*-values; AIC—Akaike information criteria; * Ods adjusted for age in exudative AMD analysis.

3.2. VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) Genotype and Allele Associations with Early and Exudative AMD by Gender

We analyzed *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genotype and allele associations with early and exudative AMD by gender and found that rs3025033 association with exudative AMD remained only in females (Supplementary Material, Tables S1 and S2), but not in males (Supplementary Material S1, Tables S3 and S4). After the strict Bonferroni correction, three significant results remained. We found association between rs3025033 AG genotype and exudative AMD under the codominant model (OR = 0.57; 95% CI: 0.37–0.87; $p = 0.009$); we also revealed that G allele is associated with lower odds of exudative AMD for females under the dominant (OR = 0.55; 95% CI: 0.37–0.82; $p = 0.0032$) and additive models (OR = 0.60; 95% CI: 0.42–0.84; $p = 0.0028$) (Supplementary Material, Table S2). AIC value shows that the additive model was best for revealing rs3025033 association with exudative AMD in females (Supplementary Material, Table S2).

While the logistic regression analysis showed significant associations between rs2146323 and exudative AMD in females, the results did not survive Bonferroni correction (Supplementary Material, Table S2). No associations were found between rs2146323 and early AMD in females (Supplementary Material S1, Tables S1 and S2) nor males (Supplementary Material, Tables S3 and S4).

We also compared the genotype and allele frequencies between females with early AMD and exudative AMD and males with early AMD and exudative AMD. We found that

allele G at rs3025033 was less frequent in females with exudative AMD than in females with early AMD (14.4% vs. 20%, respectively $p = 0.019$). Still, the result did not survive Bonferroni correction (Supplementary Material, Table S1).

3.3. Haplotype Analysis

Haplotype analysis was performed in separate groups of AMD. Pairwise linkage disequilibrium (LD) between studied polymorphisms was observed (Table 4).

Table 4. Linkage disequilibrium between the four *VEGFA* SNPs.

SNPs	Early AMD vs. Controls			Exudative AMD vs. Controls		
	D′	r^2	*p* Value	D′	r^2	*p* Value
rs1570360–rs699947	0.07182	0.2521	0.0	0.7087	0.2641	0.0
rs1570360–rs3025033	0.3771	0.0204	0.0	0.3919	0.0210	0.0
rs1570360–rs2146323	0.0898	0.0077	0.001	0.1191	0.0138	0.0
rs699947–rs3025033	0.1503	0.0066	0.0021	0.0389	0.0004	0.4298
rs699947–rs2146323	0.9904	0.5051	0.0	0.9871	0.4970	0.0
rs3025033–rs2146323	0.0866	0.0036	0.0232	0.1844	0.0150	0.0

SNP—single nucleotide polymorphism; D′ is the deviation between the expected haplotype frequency and the observed frequency (D′ scale: 0,1). r^2 is the squared correlation coefficient of the haplotype frequencies (r^2 scale: 0,1); *p*—significance level; significant when $p < 0.05$.

Haplotype analysis revealed that there is no association between *VEGFA* haplotypes and early AMD (Table 5), but individuals carrying rs1570360, rs699947, rs3025033, and rs2146323 haplotype A-A-G-A had decreased risks of exudative AMD (OR = 0.46, 95% CI: 0.23–0.90; $p = 0.023$) (Table 6).

Table 5. Associations between *VEGFA* haplotypes and risk of early AMD.

Haplotype	SNP1	SNP2	SNP3	SNP4	Frequency (%)			OR (95% CI)	*p* Value
					Early AMD	Controls	Total		
1	G	C	A	C	31.13	31.71	31.43	1.00	-
2	G	A	A	A	18.87	15.22	16.98	1.30 (0.92–1.84)	0.14
3	A	A	A	C	16.08	15.55	15.8	1.08 (0.78–1.50)	0.64
4	G	C	G	C	13.14	10.35	11.68	1.31 (0.87–1.97)	0.19
5	A	A	A	A	9.17	10.01	9.59	0.96 (0.63–1.46)	0.86
6	G	A	G	A	3.53	6.14	4.88	0.61 (0.33–1.13)	0.12
7	A	C	A	C	4.7	4.56	4.63	1.14 (0.64–2.01)	0.66
8	A	A	G	A	3.39	5.09	4.29	0.63 (0.34–1.17)	0.14
rare	*	*	*	*	NA	NA	0.72	0.00 (-)	1

OR—odds ratio; CI—confident interval; *p*—significance level; significant when $p < 0.05$; *p*-values marked with bold indicate statistically significant *p*-values; AIC—Akaike information criteria; SNP1 rs1570360; SNP2 rs699947; SNP3 rs3025033; SNP4 rs2146323; rare—pooled haplotypes with frequencies < 1 %. *—allele of rare haplotype. Global haplotype association *p* value: 0.025.

Table 6. Associations between *VEGFA* haplotypes and risk of exudative AMD.

Haplotype	SNP1	SNP2	SNP3	SNP4	Frequency (%)			OR (95% CI)	*p* Value
					Exudative AMD	Controls	Total		
1	G	C	A	C	35.1	31.71	33.52	1.00	-
2	A	A	A	C	16.2	15.55	15.89	0.93 (0.68–1.27)	0.65
3	G	A	A	A	14.65	15.22	14.88	0.86 (0.61–1.22)	0.4
4	A	A	A	A	11.4	10.01	10.76	1.07 (0.73–1.57)	0.73
5	G	C	G	C	8.62	10.35	9.38	0.81 (0.53–1.23)	0.32
6	G	A	G	A	5.08	6.14	5.58	0.69 (0.40–1.20)	0.19
7	A	C	A	C	5.44	4.56	5.04	1.17 (0.69–1.98)	0.56
8	A	A	G	A	2.99	5.09	3.98	0.46 (0.23–0.90)	**0.023**
rare	*	*	*	*	NA	NA	0.95	0.36 (0.09–1.46)	0.15

OR—odds ratio; CI—confident interval; *p*—significance level; significant when $p < 0.05$; *p*-values marked with bold indicate statistically significant *p*-values; AIC—Akaike information criteria; SNP1 rs1570360; SNP2 rs699947;

SNP3 rs3025033; SNP4 rs2146323; rare—haplotypes with frequencies < 1 %;. *—allele of rare haplotype. Global haplotype association p value: 0.13.

3.4. VEGF-A and VEGF-R2/KDR Serum Concentration Analysis

Serum protein concentrations were measured in 20 patients with exudative AMD before treatment and 21 control group samples. The Control group for VEGF-A and VEGF-R2/KDR serum concentration measurement consisted of subjects considering the age and gender distributions based on the exudative AMD group.

We compared the VEGF-A serum concentrations between exudative AMD and control groups but did not find a significant difference (422.674 (677.02) vs. 615.489 (425.49), respectively, $p = 0.424$) (Figure 2).

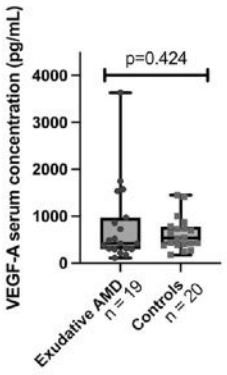

Figure 2. VEGF-A serum concentrations between groups. The bars represent the median with interquartile range (first quartile and third quartile) and whiskers from min to maximum values. VEGF-A serum concentration in exudative AMD group: 422.674 (677.02) pg/mL and control group: 615.489 (425.49) pg/mL. Mann–Whitney-U test, $p = 0.424$.

We also compared the VEGF-R2/KDR serum concentrations between exudative AMD and control groups, but there was also no statistical difference (12,759.2 (5358.85) vs. 15,428.35 (6698.03), respectively, $p = 0.183$) (Figure 3).

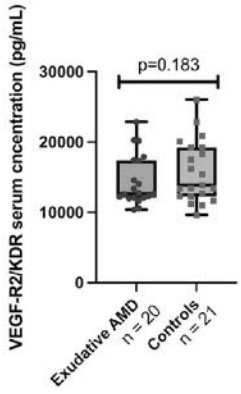

Figure 3. VEGF-R2/KDR serum concentrations between groups. The bars represent the median with interquartile range (first quartile and third quartile) and whiskers from minimum to maximum. VEGF-R2/KDR serum concentration in exudative AMD group: (12,759.2 (5358.85) pg/mL and control group: 15,428.35 (6698.03) pg/mL. Mann–Whitney-U test, $p = 0.183$.

3.5. VEGF-A and VEGF-R2/KDR Concentrations by VEGFA Genotypes

We also performed VEGF-A and VEGF-R2/KDR serum concentration and *VEGFA* rs1570360, rs699947, rs302503, and rs2146323 genotype association analysis and found that patients with exudative AMD carrying at least one C allele at rs699947 have statistically significantly higher VEGF-A serum concentrations compared to wild-type allele A homozygous genotypes carriers (485.95 (945.93) vs. 194.97 (-), respectively, $p = 0.046$) (Table 7). However, there were only two subjects with the AA genotype.

Table 7. VEGF-A and VEGF-R2/KDR concentration associations with *VEGFA* polymorphisms in exudative AMD and controls.

Model	VEGF-A				VEGF-R2/KDR			
	Exudative AMD (pg/mL), Median (IQR)	*p* Value	Control Group (pg/mL), Median (IQR)	*p* Value	Exudative AMD (pg/mL), Median (IQR)	*p* Value	Control Group (pg/mL), Median (IQR)	*p* Value
rs1570360								
Dominant AG + AA vs. GG	383.55 (427.3) vs. 640.36 (1234.32)	0.142	711.25 (472.66) vs. 526.14 (391.91)	0.181	12,588.6 (6294.67) vs. 13,402.08 (5875.31)	0.569	14,435.95 (7035.44) vs. 16,211.6 (7547.35)	0.916
Recessive AA vs. AG + GG	726.99 (-) vs. 421.42 (646.25)	0.737	727.89 (-) vs. 581.23 (518.70)	0.615	17,876.6 (-) vs. 12,673.9 (4531.86)	0.560	15,428.35 (-) vs. 15,473.4 (7038.14)	0.451
rs699947								
Dominant AC + CC vs. AA	485.95 (945.93) vs. 194.97 (-)	**0.046**	546.82 (553.1) vs. 711.25 (272.64)	0.531	13,357.85 (5451.18) vs. 11,403.18 (-)	0.130	14,735.2 (7207.88) vs. 16,872.8 (10,171.29)	0.347
Recessive CC vs. AC + AA	422.67 (1231.86) vs. 434.75 (678.21)	0.447	526.14 (9396.71) vs. 711.254 (479.99)	0.156	14,044.95 (7679.2) vs. 12,418.33 (4638.02)	0.231	16,211.6 (6731.85) vs. 14,435.95 (7038.14)	0.654
rs3025033								
Dominant AG + GG vs. AA	401.86 (734.59) vs. 517.55 (1022.04)	0.935	629.32 (336.84) vs. 526.14 (621.82)	0.944	12,588.6 (1324.06) vs. 17,349.15 (7709.45)	0.623	14,089.38 (6374.95) vs. 17,524.8 (8218.45)	0.307
Recessive GG vs. AG + AA	- (-) vs. 454.31 (819.36)	-	- (-) vs. 581.23 (377.25)	-	- (-) vs. 12,673.9 (5468.76)	-	- (-) vs. 15,819.96 (6356.65)	-
rs2146323								
Dominant AC + AA vs. CC	383.55 (1282.99) vs. 454.311 (527.24)	0.866	735.12 (743.12) vs. 536.48 (318.29)	0.227	13,357.85 (5271.05) vs. 12,673.9 (5624.84)	0.874	13,443.55 (7708) vs. 15,819.98 (7312.38)	0.270
Recessive AA vs. AC + CC	- (-) vs. 42.67 (677.02)	-	739.89 (-) vs. 546.82 (484.33)	0.402	- (-) vs. 12,759.2 (5358.85)	-	15,880.4 (-) vs. 15,428.35 (6868.4)	0.952

IQR—interquartile range; any missing IQR value is due to the small sample size in the study subgroup; *p*—significance level; significant when $p < 0.05$; *p*-values marked with bold indicate statistically significant *p*-values.

4. Discussion

This study analyzed four SNPs in the *VEGFA* gene and their associations with early and exudative AMD. Study results revealed that the G allele at rs3025033 was significantly associated with lower odds of exudative AMD. We also found that these associations

with exudative AMD remained only in females but not in males, suggesting the potential gender role in AMD development. Furthermore, the differences of rs3025033 allele G frequencies were observed between females with exudative AMD and females with early AMD, but the results did not survive Bonferroni correction. Moreover, associations between rs2146323 and exudative AMD in females were found, but the results did also not survive Bonferroni correction.

A haplotype of *VEGFA* SNPs analysis revealed that individuals carrying rs1570360, rs699947, rs3025033, and rs2146323 haplotype A-A-G-A had decreased risk of exudative AMD, showing a protective role of this haplotype. No studies have previously included all these SNPs in AMD analysis. Only Mori et al. (2010) analyzed *VEGFA* −116A, rs1570360 associations with AMD, but did not reveal significant results [27]. Another three studies examined rs1570360 associations with exudative AMD treatment efficacy but not with the AMD occurrence [30–32].

VEGFA promoter polymorphism −2578C/A (rs699947) is widely studied in a large number of angiogenesis-associated diseases, including many different types of cancers [33–35] and their response to anti-VEGFA agent treatment [36].

This SNP was also studied in patients with AMD, but statistically significant differences in the genotypic distribution of *VEGFA* rs699947 were not found, and any significant associations were revealed [20,27,37,38]. Scientists also tried to find the differences between the AMD subtypes comparing neovascular and atrophic AMD groups but did not reveal significant results [39].

Previous results were found in much smaller study groups. Still, our study confirmed those findings in bigger samples groups, including 339 subjects in early AMD and 419 in the exudative AMD groups, and 374 subjects in the control group.

Other research studies have analyzed rs699947 associations with anti-VEGFA treatment [30,31,40–42] or photodynamic therapy response [32,43,44] but conflicting results suggest that other risk factors [45] or SNP combinations may be associated with the AMD treatment as well [46,47]. Our analysis of SNPs and VEGF-A and VEGF-R2 serum concentrations revealed that carriers of at least one C allele at rs699947 have statistically significantly higher VEGF-A serum concentrations compared to wild-type allele A homozygous genotype carriers (485.95 (945.93) vs. 194.97 (-), respectively, $p = 0.046$).

Bulgu et al. (2014) included in their study an intronic VEGFA variant rs3025033 and genotyped it for 82 AMD patients and 80 controls. Unfortunately, about 98% of genotypes were determined as AA, so no further statistical analysis was performed [48].

Li et al. (2021) analyzed the rs3025033 effect on VEGF165b protein production but did not reveal significant results; on the other hand, they showed that this SNP promoted cell proliferation in human retinal vascular endothelial cells (hRVECs) [49].

Another study performed by Immonen et al. (2010) analyzed the associations between rs3025033 and photodynamic therapy response, but no associations were revealed [44].

In contrast, the frequencies of the *VEGFA* +5092, rs2146323 were significantly different in photodynamic therapy non-responders and responders [44]. However, similar frequencies of this SNP were found between the exudative AMD patients and controls [37] and any AMD patients and controls [48]. Furthermore, it was shown that rs2146323 C allele was protective against dry AMD, and the allele A was associated with the disease. Moreover, there was a significant difference in the genotype frequencies of this SNP between the wet type of AMD and dry type AMD [48]. Our study results showed associations between rs2146323 and exudative AMD only in females, but these results did not survive when we applied strict Bonferroni correction. Furthermore, we did not find any associations between this SNP and VEGF-A or VEGF-R2 serum concentrations.

Other widely studied SNPs show significant associations with the AMD and highlight the possible *VEGFA* rs1413711 and rs833061 polymorphisms contributions to AMD susceptibility [50].

Bulgu et al. (2014) found that the presence of ancestral allele (G) in rs1413711 was protective for all AMD patients, and the AA genotype was a risk factor for AMD and

even a highly increased risk factor for dry AMD [48]. Opposite results were shown in another study: SNP +674, rs1413711 CC genotype was significantly associated with a higher risk of exudative AMD [19]. The conflicting results of these studies may be explained by differences in the size of study groups and populations (Turkish and Caucasian of Northern European origin).

Habibi et al. (2014) showed that *VEGFA* +405, rs2010963 CC and *VEGFA* +936, rs3025039 TT genotype frequencies were higher in Tunisian AMD patients than in controls [51].

These results confirmed the previous associations between *VEGFA* +936, rs3025039, and wet AMD in the Japanese population [52]. Deeper analysis showed that genotype TT for rs3025039 was associated with elevated VEGF-A protein serum levels [20].

Moreover, an extensive analysis of *VEGFA* promoter and gene polymorphisms showed that SNPs +674, +4618, +5092, +9162, and +9512 their haplotypes, CTCCT and TCACC, were associated with a 15-fold increased risk of exudative AMD and the promoter SNPs −460T, −417T, −172C, −165C, −160C, −152G, −141A, −116A, +405C haplotype was associated with about an 18-fold greater risk [19]. Two of these polymorphisms were included in our study and, together with another two SNPs, revealed a protective haplotype for exudative AMD. It is important to elucidate the SNP combinations and their role in AMD to understand the pathogenesis and possible treatment strategies better.

VEGF-A serum levels, as well as VEGFA SNPs, are widely studied in AMD patients. While we did not find any statistical differences in VEGF-A serum levels between the exudative AMD patients and controls, we confirmed results from several other studies which included total AMD patients or only exudative AMD patients, consisting of 27 to 71 samples per group in different populations [53–60]. Further studies revealed significantly elevated VEGF-A levels in exudative AMD patients compared to controls [61,62]. Significantly elevated VEGF-A levels in total AMD patients compared to controls were also found in a few studies [20,51,63].

VEGF-R2 protein associations are not so widely analyzed as VEGF-A, but conflicting results were also found. Örnek et al. (2016) found that decreased VEGFR-2 serum levels were associated with both dry and wet type AMD. Furthermore, negative correlations between VEGFR-2 with foveal retinal thickness in AMD patients and a significant positive correlation with subfoveal choroidal thickness revealed the possible VEGF-R2 role in AMD development [64]. Another two studies showed opposite associations and found elevated VEGFR2 protein levels in patients with exudative AMD group than in the controls [65,66], suggesting the role of VEGFR-2 in the pathogenesis of AMD.

5. Conclusions

In conclusion, we found that rs3025033 polymorphism of *VEGFA* and the haplotype rs1570360A-rs699947A-rs3025033G-rs2146323A can play a protective role for exudative AMD in our population. Furthermore, the C allele at rs699947 is associated with elevated VEGF-A serum concentrations in exudative AMD patients. Since the genetic variations were associated with exudative AMD, our findings need to be replicated in additional studies. While the main strength of our study was a large number of early and exudative AMD patients, further studies are still needed to investigate the pharmacologic role of these angiogenesis-related markers in AMD therapy.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells11060996/s1, Table S1: Frequencies of *VEGFA* (rs1570360, rs699947, rs3025033 and rs2146323) genotypes and alleles in females with early AMD and exudative AMD, and controls, Table S2: Binomial logistic regression analysis of *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) in females with early and exudative AMD and controls, Table S3: Frequencies of *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) genotypes and alleles in males with early AMD and exudative AMD, and controls, Table S4: Binomial logistic regression analysis of *VEGFA* (rs1570360, rs699947, rs3025033, and rs2146323) in males with early and exudative AMD and controls.

Author Contributions: Conceptualization, R.L. and A.V.; methodology, A.V., D.C., L.K. and R.L.; validation, A.V., D.C. and R.L.; formal analysis, D.C., L.K. and R.L.; investigation, A.V. and D.C.; resources, L.K. and R.L.; data curation, A.V., R.L. and D.C.; writing—original draft preparation, A.V.; writing—review and editing, A.V. and R.L.; supervision, R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee for Biomedical Research, Lithuanian University of Health Sciences (No. BE-2-/48; 8 October 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data relevant to the study are included in the article or uploaded as supplementary information.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTS

Supplementary material 1

KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS

Lietuvos sveikatos mokslų universitetas, A. Mickevičiaus g. 9, LT-44307 Kaunas, tel. (+370) 37 32 72 29; el. paštas: kaunorbtek@lsmuni.lt

LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2018-10-08 Nr. BE-2-48

Biomedicininio tyrimo pavadinimas: **„Naujų amžinės geltonosios dėmės degeneracijos (AGDD) ir diabetinės retinopatijos (DR) žymenų paieška diagnozei ir gydymui naudojant integruotą imunogenetinių žymenų ir metabolomikos analizę“**	
Protokolo Nr.:	**1**
Data:	**2018-06-15**
Versija:	**2**
Asmens informavimo forma	**Versija Nr. 2, data 2018-06-15 (pagrindiniam tyrimui)** **Versija Nr. 3, data 2018-09-10 (genetiniam tyrimui)**
Pagrindinis tyrėjas:	**Doc. dr. Rasa Liutkevičienė** **Gyd. Rasa Čiumbraitė**
Biomedicininio tyrimo vieta: Įstaigos pavadinimas: Adresas:	**Lietuvos sveikatos mokslų universiteto ligoninė Kauno klinikos, Akių ligų klinika** Eivenių g. 2, LT-50161 Kaunas **Klaipėdos universitetinė ligoninė** Liepojos g. 49, LT-92288 Klaipėda

Išvada:
Kauno regioninio biomedicininių tyrimų etikos komiteto posėdžio, įvykusio **2018 m. spalio mėn. 2 d.** (protokolo Nr. BE-10-13) sprendimu pritarta biomedicininio tyrimo vykdymui.

Mokslinio eksperimento vykdytojai įsipareigoja: (1) nedelsiant informuoti Kauno regioninį biomedicininių tyrimų etikos komitetą apie visus nenumatytus atvejus, susijusius su studijos vykdymu, (2) iki sausio 15 dienos – pateikti metinį studijos vykdymo apibendrinimą bei, (3) per mėnesį po studijos užbaigimo, pateikti galutinį pranešimą apie eksperimentą.

Kauno regioninio biomedicininių tyrimų etikos komiteto nariai			
Nr.	Vardas, Pavardė	Veiklos sritis	Dalyvavo posėdyje
1.	Prof. Edgaras Stankevičius	Fiziologija, farmakologija	Ne
2.	Prof. Skaidrius Miliauskas	Pulmunologija, vidaus ligos	Taip
3.	Med. dr. Jonas Andriuškevičius	Chirurgija	Taip
4.	Doc. Gintautas Gumbrevičius	Klinikinė farmakologija	Taip
5.	Prof. Kęstutis Petrikonis	Neurologija	Taip
6.	Dr. Ramunė Kasperavičienė	Filologija	Taip
7.	Dr. Žydrūnė Luneckaitė	Visuomenės sveikata	Taip
8.	Aušra Degutytė	Visuomenės sveikata	Ne
9.	Jurgita Laurinaitytė	Teisė	Ne

Kauno regioninis biomedicininių tyrimų etikos komitetas dirba vadovaudamasis etikos principais nustatytais biomedicininių tyrimų Etikos įstatyme, Helsinkio deklaracijoje, vaistų tyrinėjimo Geros klinikinės praktikos taisyklėmis.

Kauno RBTEK pirmininkas Prof. Edgaras Stankevičius

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS * KAUNO REGIONINIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS

Supplementary material 2

Table 1. *RAD51B, TRIB1, COL8A1 and COL10A1 genotype distributions in the control group using Hardy-Weinberg equilibrium*

Gene	SNP	Allele frequencies		Genotype distribution in control group	HWE p value
RAD51B	rs8017304	G (0.31)	A (0.69)	90/401/451	0.950
RAD51B	rs2588809	T (0.12)	C (0.88)	17/185/740	0.175
TRIB1	rs6987702	C (0.27)	T (0.73)	83/343/516	**0.019**
TRIB1	rs4351379	C (0.07)	G (0.94)	7/109/826	0.111
COL8A1	rs13095226	C (0.19)	T (0.81)	27/304/611	0.138
COL10A1	rs1064583	G (0.31)	A (0.69)	52/482/408	**<0.001**

p-significance level, p is statistically significant when <0.05.

Table 2. *IL-9 and IL-10 genotype distributions in the control group using Hardy-Weinberg equilibrium*

Gene	SNP	Allele frequencies		Genotype distributions in control group	HWE p value
IL-9	rs1859430	A (0.21)	G (0.79)	14/136/233	0.280
IL-9	rs2069870	G (0.16)	A (0.84)	0/121/160	**<0.001**
IL-9	rs11741137	T (0.19)	C (0.81)	10/126/247	0.195
IL-9	rs2069885	A (0.19)	G (0.81)	10/123/250	0.260
IL-9	rs2069884	T (0.19)	G (0.81)	10/123/250	0.260
IL-10	rs1800871	A (0.22)	G (0.78)	18/133/232	0.848
IL-10	rs1800872	T (0.22)	G (0.78)	18/133/232	0.848
IL-10	rs1800896	C (0.47)	T (0.53)	77/203/103	0.204

p-significance level, p is statistically significant when <0.05.

Table 3. *IL-10 haplotype associations with early, exudative, and atrophic AMD*

Haplotype	rs1800871	rs1800872	rs1800896	Frequency	OR (95 % CI)	p value
Haplotype associations with early AMD						
1	G	G	C	0.4549	1	–
2	G	G	T	0.3275	1.13 (0.89–1.44)	0.31
3	A	T	T	0.2166	1.00 (0.75–1.32)	0.98
4 (rare)	*	*	*	0.001	<0.001 (–)	1

Table 3. *Continued*

Haplotype	rs1800871	rs1800872	rs1800896	Frequency	OR (95 % CI)	p value
Haplotype associations with exudative AMD						
1	G	G	C	0.4751	1	–
2	G	G	T	0.3026	0.87 (0.68–1.10)	0.24
3	A	T	T	0.2205	0.94 (0.72–1.22)	0.63
4 (rare)	*	*	*	0.0018	0.34 (0.02–5.66)	0.45
Haplotype associations with atrophic AMD						
1	G	G	C	0.4758	1	–
2	G	G	T	0.3046	0.62 (0.38–1.02)	0.06
3	A	T	T	0.218	0.78 (0.47–1.29)	0.33
4 (rare)	*	*	*	0.0016	<0.00 (–)	1

Rare – pooled rare haplotypes; OR – odds ratio; CI – confidence interval; p-significance level $p<0.05$.

Table 4. *Frequencies of VEGFA (rs1570360, rs699947, rs3025033 and rs2146323) genotypes and alleles in females with early AMD and exudative AMD, and controls*

Gene/ marker	Genotype/ allele	Early AMD, n (%) N = 235	Exudative AMD, n (%) N = 270	Control group, n (%) N = 235	p value*	p value**	p value***
VEGFA rs1570360	GG AG AA	115 (48.9) 88 (37.4) 32 (13.6)	119 (44.1) 107 (39.6) 44 (16.3)	108 (46) 79 (33.6) 48 (20.4)	0.142	0.285	0.498
	G A	318 (67.7) 152 (32.3)	345 (63.9) 195 (36.1)	295 (62.8) 177 (37.2)	0.097	0.646	0.208
VEGFA rs699947	AA AC CC	61 (26) 122 (51.9) 52 (22.1)	81 (30) 118 (43.7) 71 (26.3)	70 (29.8) 113 (48.1) 52 (22.1)	0.618	0.490	0.182
	A C	244 (51.9) 226 (48.1)	280 (51.8) 260 (48.1)	253 (53.8) 217 (46.2)	0.556	0.530	0.984
VEGFA rs3025033	AA AG GG	148 (63) 80 (34) 7 (3)	199 (73.3) 64 (23.7) 7 (2.6)	147 (62.6) 75 (31.9) 13 (5.5)	0.374	**0.017**	**0.032**
	A G	376 (80) 94 (20)	462 (85.6) 78 (14.4)	369 (78.5) 101 (21.5)	0.573	**0.003**	**0.019**
VEGFA rs2146323	CC AC AA	96 (40.9) 111 (47.2) 28 (11.9)	124 (45.9) 109 (40.4) 37 (13.7)	92 (39.1) 107 (45.5) 36 (15.3)	0.560	0.308	0.299
	C A	303 (64.5) 167 (35.5)	357 (66.1) 183 (33.9)	291 (61.9) 179 (38.1)	0.417	0.165	0.584

p – significance level and Bonferroni corrected significance level when p = 0.05/4; *Early AMD *vs.* Control group; **Exudative AMD *vs.* Control group; ***Early AMD *vs.* Exudative AMD.

Table 5. *Binomial logistic regression analysis of VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) in females with early and exudative AMD and controls*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	p value	AIC	OR (95 % CI)*	p value	AIC
VEGFA rs1570360							
Codominant	AG *vs.* GG	1.05 (0.70–1.56)	0.826	653.6	1.10 (0.73–1.67)	0.648	647.1
	AA *vs.* GG	0.63 (0.37–1.05)	0.077		0.89 (0.53–1.48)	0.640	
Dominant	AG+AA *vs.* GG	0.89 (0.62–1.27)	0.52	655.1	1.02 (0.70–1.48)	0.91	645.8
Recessive	AA *vs.* GG+AG	0.61 (0.38–1.00)	**0.049**	651.7	0.85 (0.53–1.37)	0.5	645.3
Overdominant	AG *vs.* GG+AA	1.18 (0.81–1.73)	0.39	654.8	1.14 (0.78–1.68)	0.5	645.4
Additive	A	0.84 (0.66–1.07)	0.15	653.5	0.97 (0.76–1.24)	0.79	645.7
VEGFA rs699947							
Codominant	AC *vs.* AA	1.24 (0.81–1.90)	0.327	656.6	0.82 (0.53–1.28)	0.387	643.6
	CC *vs.* AA	1.15 (0.69–1.92)	0.601		1.35 (0.81–2.24)	0.256	
Dominant	AC+CC *vs.* AA	1.21 (0.81–1.81)	0.35	654.7	0.98 (0.65–1.46)	0.91	645.8
Recessive	CC *vs.* AA+AC	1.00 (0.65–1.55)	1	655.6	1.51 (0.97–2.34)	0.064	642.4
Overdominant	AC *vs.* AA+CC	1.17 (0.81–1.67)	0.41	654.9	0.72 (0.50–1.05)	0.09	642.9
Additive	C	1.08 (0.84–1.40)	0.56	655.2	1.14 (0.88–1.46)	0.32	644.8
VEGFA rs3025033							
Codominant	AG *vs.* AA	1.06 (0.72–1.56)	0.771	655.6	0.57 (0.37–0.87)	**0.009**	638.7
	GG *vs.* AA	0.53 (0.21–1.38)	0.195		0.42 (0.15–1.12)	0.083	
Dominant	AG+GG *vs.* AA	0.98 (0.68–1.43)	0.92	655.5	0.55 (0.37–0.82)	**0.0032**	637.1
Recessive	GG vs. AA+AG	0.52 (0.21–1.34)	0.17	653.7	0.49 (0.18–1.31)	0.15	643.7
Overdominant	AG *vs.* AA+GG	1.10 (0.75–1.62)	0.62	655.3	0.60 (0.39–0.91)	**0.015**	639.9
Additive	G	0.91 (0.67–1.25)	0.57	655.2	0.60 (0.42–0.84)	**0.0028**	636.9

Table 5. *Continued*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	**p value**	**AIC**	**OR (95 % CI)***	**p value**	**AIC**
VEGFA rs2146323							
Codominant	AC *vs.* CC	0.99 (0.67–1.47)	0.977	656.4	0.65 (0.44–0.98)	**0.040**	642.9
	AA *vs.* CC	0.75 (0.42–1.32)	0.313		0.65 (0.37–1.14)	0.135	
Dominant	AC+AA *vs.* CC	0.93 (0.64–1.35)	0.71	655.4	0.65 (0.45–0.95)	**0.026**	640.9
Recessive	AA *vs.* CC+AC	0.75 (0.44–1.27)	0.28	654.4	0.80 (0.47–1.36)	0.42	645.1
Overdominant	AC *vs.* CC+AA	1.07 (0.75–1.54)	0.71	655.4	0.73 (0.50–1.06)	0.1	643.1
Additive	A	0.90 (0.69–1.17)	0.42	654.9	0.77 (0.59–1.00)	0.049	641.9

OR – odds ratio; CI – confident interval; p – significance level and Bonferroni corrected significance level when p = 0.05/4; AIC – Akaike information criteria; *ORs adjusted for age in exudative AMD analysis.

Table 6. *Frequencies of VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) genotypes and alleles in males with early AMD and exudative AMD, and controls*

Gene/ marker	Genotype/ allele	Early AMD, n (%) N = 104	Exudative AMD, n (%) N = 149	Control group, n (%) N = 139	p value*	p value**	p value***
VEGFA rs1570360	GG AG AA	47 (45.2) 40 (38.5) 17 (16.3)	64 (43) 61 (40.9) 24 (16.1)	69 (49.6) 46 (33.1) 24 (17.3)	0.684	0.378	0.920
	G A	134 (64.4) 74 (35.6)	189 (63.4) 109 (36.6)	184 (66.2) 94 (33.8)	0.686	0.488	0.818
VEGFA rs699947	AA AC CC	25 (24) 52 (50) 27 (26)	38 (25.5) 69 (46.3) 42 (28.2)	42 (30.2) 60 (43.2) 37 (26.6)	0.488	0.671	0.844
	A C	102 (49) 106 (51)	145 (48.7) 153 (51.3)	144 (51.8) 134 (48.2)	0.512	0.451	0.933
VEGFA rs3025033	AA AG GG	67 (64.4) 32 (30.8) 5 (4.8)	91 (61.1) 52 (34.9) 6 (4)	81 (58.3) 50 (36) 8 (5.8)	0.623	0.756	0.775
	A G	166 (79.8) 42 (20.2)	234 (78.5) 64 (21.5)	212 (76.3) 66 (23.7)	0.352	0.516	0.727
VEGFA rs2146323	CC AC AA	46 (44.2) 46 (44.2) 12 (11.5)	67 (45) 60 (40.3) 22 (14.8)	66 (47.5) 50 (36) 23 (16.5)	0.333	0.744	0.701
	C A	138 (66.3) 70 (33.7)	194 (65.1) 104 (34.9)	182 (65.5) 96 (34.5)	0.840	0.926	0.831

p – significance level and Bonferroni corrected significance level when p = 0.05/4; *Early AMD *vs.* Control group; **Exudative AMD *vs.* Control group; ***Early AMD *vs.* Exudative AMD.

Table 7. *Binomial logistic regression analysis of VEGFA (rs1570360, rs699947, rs3025033, and rs2146323) in males with early and exudative AMD and controls*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	p value	AIC	OR (95 % CI)*	p value	AIC
VEGFA rs1570360							
Codominant	AG *vs.* GG	1.28 (0.73–2.24)	0.395	337.1	1.49 (0.89–2.49)	0.132	400.3
	AA *vs.* GG	1.04 (0.50–2.14)	0.916		1.08 (0.56–2.10)	0.815	
Dominant	AG+AA *vs.* GG	1.20 (0.72–1.99)	0.49	335.3	1.35 (0.84–2.15)	0.21	399.1
Recessive	AA vs. GG+AG	0.94 (0.47–1.85)	0.85	335.8	0.91 (0.49–1.69)	0.76	400.6
Overdominant	AG vs. GG+AA	1.26 (0.74–2.15)	0.39	335.1	1.46 (0.90–2.36)	0.13	398.3
Additive	A	1.07 (0.76–1.50)	0.71	335.7	1.12 (0.81–1.54)	0.49	400.2
VEGFA rs699947							
Codominant	AC *vs.* AA	1.46 (0.78–2.70)	0.234	336.1	1.25 (0.72–2.20)	0.428	402.1
	CC *vs.* AA	1.23 (0.61–2.47)	0.569		1.24 (0.66–2.32)	0.505	
Dominant	AC+CC *vs.* AA	1.37 (0.77–2.44)	0.28	334.7	1.25 (0.74–2.10)	0.4	400
Recessive	CC vs. AA+AC	0.97 (0.54–1.72)	0.91	335.8	1.08 (0.64–1.82)	0.78	400.6
Overdominant	AC vs. AA+CC	1.32 (0.79–2.19)	0.29	334.7	1.13 (0.71–1.80)	0.61	400.4
Additive	C	1.11 (0.78–1.57)	0.56	335.5	1.11 (0.81–1.52)	0.5	400.2
VEGFA rs3025033							
Codominant	AG *vs.* AA	0.77 (0.45–1.34)	0.360	336.9	0.90 (0.55–1.48)	0.690	402.1
	GG *vs.* AA	0.76 (0.24–2.42)	0.637		0.67 (0.22–2.00)	0.468	
Dominant	AG+GG *vs.* AA	0.77 (0.46–1.30)	0.33	334.9	0.87 (0.54–1.40)	0.57	400.3
Recessive	GG vs. AA+AG	0.83 (0.26–2.61)	0.74	335.7	0.69 (0.23–2.05)	0.5	400.2
Overdominant	AG vs. AA+GG	0.79 (0.46–1.36)	0.4	335.1	0.93 (0.57–1.52)	0.78	400.6
Additive	G	0.82 (0.53–1.26)	0.36	335	0.86 (0.58–1.28)	0.47	400.1

Table 7. *Continued*

Genetic model	Genotype/allele	Early AMD *vs.* Control group			Exudative AMD *vs.* Control group		
		OR (95 % CI)	p value	AIC	OR (95 % CI)*	p value	AIC
VEGFA rs2146323							
Codominant	AC *vs.* CC	1.32 (0.76–2.29)	0.322	335.6	1.19 (0.72–1.98)	0.502	402.1
	AA *vs.* CC	0.75 (0.34–1.65)	0.474		0.98 (0.50–1.93)	0.949	
Dominant	AC+AA vs. CC	1.14 (0.68–1.90)	0.61	335.6	1.12 (0.70–1.79)	0.62	400.4
Recessive	AA vs. CC+AC	0.66 (0.31–1.39)	0.27	334.6	0.90 (0.48–1.71)	0.76	400.6
Overdominant	AC *vs.* AA+AA	1.41 (0.84–2.37)	0.19	334.1	1.20 (0.74–1.93)	0.46	400.1
Additive	A	0.97 (0.68–1.38)	0.85	335.8	1.03 (0.75–1.42)	0.86	400.6

OR – odds ratio; CI – confident interval; p – significance level and Bonferroni corrected significance level when p=0.05/4; AIC – Akaike information criteria; *ORs adjusted for age in exudative AMD analysis.

Table 8. *VEGFA haplotype associations with early AMD*

Haplotype	rs1570360	rs699947	rs302503	rs214632	Frequency (%)			OR (95 % CI)	p value
					Early AMD	Controls	Total		
1	G	C	A	C	31.13	31.71	31.43	1.00	–
2	G	A	A	A	18.87	15.22	16.98	1.30 (0.92–1.84)	0.14
3	A	A	A	C	16.08	15.55	15.8	1.08 (0.78–1.50)	0.64
4	G	C	G	C	13.14	10.35	11.68	1.31 (0.87–1.97)	0.19
5	A	A	A	A	9.17	10.01	9.59	0.96 (0.63–1.46)	0.86
6	G	A	G	A	3.53	6.14	4.88	0.61 (0.33–1.13)	0.12
7	A	C	A	C	4.7	4.56	4.63	1.14 (0.64–2.01)	0.66
8	A	A	G	A	3.39	5.09	4.29	0.63 (0.34–1.17)	0.14
rare	*	*	*	*	NA	NA	0.72	0.00 (–)	1

OR – odds ratio; CI – confident interval; p – significance level when p = 0.05; AIC – Akaike information criteria; rare – pooled haplotypes with frequencies <1 %.

CURRICULUM VITAE

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Education:

2017–2021	Lithuanian University of Health Sciences, PhD studies in Biology
2015–2017	Lithuanian University of Health Sciences, Master's degree in medical biology
2011–2015	Lithuanian University of Health Sciences, Bachelor's degree in medical and veterinary genetics

Work Experience:

2019-02–2019-06	assistant lecturer, Lithuanian University of Health Sciences, Institute of Biological Systems and Genetics Research
from 2017	junior researcher, Lithuanian University of Health Sciences, Neuroscience Institute, Laboratory of Ophthalmology
2015–2017	laboratory assistant, Lithuanian University of Health Sciences, Neuroscience Institute, Laboratory of Ophthalmology

Scientific Projects:

2019-07-01–2019-08-30	Lithuanian Research Council students' scientific summer internship project "Molecular markers for throat cancer" (student' supervisor). No. 09.3.3-LMT-K-712-15-0210
2018–2020	Lithuanian Business Support Agency project "The invention of a new methodology and genetic testing kit for early depression and bipolar disorder diagnosis and medication treatment" (junior researcher). No. J05-LVPA-K-03-0042
2015–2018	Lithuanian Research Council project "Healthy and patients with age-related macular degeneration new genetic and metabolomic and biomarkers search" (laboratory assistant). No. SEN-11/2015

Professional Memberships:

From 2016	Lithuanian genetics association
From 2018	LSMU PhD students council

Honours and Awards:

2018	Awarded by Lithuanian science academy for the best master thesis "Association of genetic markers and age-related macular degeneration". Supervisor prof. Dr. Rasa Liutkevičienė
2019	3rd place winner in the contest LSMU "Best PhD Student 2019"

PADĖKA

Labiausiai dėkinga šio mokslinio darbo vadovei prof. dr. Rasai Liutkevičienei už nuolatinį pozityvumą ir motyvaciją, pasitikėjimą ir palaikymą bei nuoseklų ir rūpestingą vadovavimą visam moksliniam darbui.

Dėkoju Lietuvos sveikatos mokslų universiteto ligoninės Kauno klinikų Akių ligų klinikų gydytojams prisidėjusiems prie šio mokslinio darbo atlikimo. Ypač dėkoju savo draugams ir kolegoms, gydytojams Rūtai Mockutei, Džastinai Čebatorienei ir Mantui Banevičiui bei Oftalmologijos laboratorijos jaunesniosioms mokslo darbuotojoms Gretai Gedvilaitei ir Kristei Kaikarytei už pagalbą atliekant mokslinį darbą ir palaikymą.

Esu ypač dėkinga savo mylimai šeimai ir mylimiems draugams už supratingumą bei nuolatinį palaikymą. Be Jūsų, mano pasiekimai būtų nieko verti.