Retinal Vessel Functionality Is Linked With *ARMS2* A69S and *CFH* Y402H Polymorphisms and Choroidal Status in AMD Patients

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Received: January 12, 2021
Accepted: March 27, 2021
Published: April 26, 2021

Citation: Krytkowska E, Ulańczyk Z, Grabowicz A, et al. Retinal vessel functionality is linked with *ARMS2* A69S and *CFH* Y402H polymorphisms and choroidal status in AMD patients. *Invest Ophthalmol Vis Sci.* 2021;62(4):30.
https://doi.org/10.1167/iovs.62.4.30

Purpose. We aimed to investigate the reactivity of retinal vessels to a flickering stimulus in patients with age-related macular degeneration (AMD) and healthy participants. We also assessed whether the parameters of retinal vessels are dependent on genetic predisposition.

Methods. A total of 354 patients with AMD and 121 controls were recruited for the study. All participants underwent thorough ophthalmologic examination and static and dynamic retinal vessel analysis. AMD risk polymorphisms were genotyped in the *CFH* and *ARMS2* genes.

Results. We found no differences between the AMD group and controls in central retinal arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE), arteriovenous ratio (AVR), dynamic analysis of arteries (DAAs), or dynamic analysis of veins (DAVs). Eyes with early AMD presented with significantly higher AVR values than eyes with late AMD. In the AMD group, DAA correlated positively with both choroidal thickness (Rs = 0.14, $P = 0.00096$) and choroidal volume (Rs = 0.23, $P < 0.0001$), and no such associations were observed in the controls. We found significantly lower DAA (1.47 ± 1.50) in TT homozygotes for the *ARMS2* A69S polymorphism in comparison with GG homozygotes (2.38 ± 1.79) and patients with GG + GT genotypes (2.28 ± 1.84). We also observed less prominent DAV (3.24 ± 1.71) in patients with TC + CC genotypes in the *CFH* Y402H polymorphism compared with TT homozygotes (3.83 ± 1.68).

Conclusions. Our findings suggest that retinal microcirculation appears to be associated with the genetic background, choroidal parameters, and clinical features of the patients with AMD.

Keywords: age-related macular degeneration (AMD), CFH, ARMS2, retinal vasculature, choroid

Age-related macular degeneration (AMD) is a disease that affects the macular region of the retina, causing progressive decrease in central vision. Clinically relevant disease is most prevalent in the elderly population, but early signs of AMD can also be found in individuals below 50 years of age.[1] Early clinical manifestations of the disease are the formation of lipid-rich extracellular deposits called drusen and abnormalities of the retinal pigment epithelium (RPE) seen on fundoscopy as hyper- or hypopigmentation.[2] Symptoms of the late phase of disease are geographic atrophy (GA) or choroidal neovascularization (CNV), both leading to severe and permanent visual impairment and legal blindness that affect quality of life and functional independence.[3]

AMD is considered a disease with a complex pathogenesis, and metabolic, functional, genetic, and environmental factors seem to contribute to it.[4] Age is one of the most constant unmodifiable factors; 60 to 80-year-old people are at a threefold greater risk of developing advanced AMD than persons younger than 60 years.[5,6] A positive family history is also considered an important risk factor for AMD because siblings of patients have a three to sixfold higher risk than the general population.[7] To date, using genomewide screening approaches, 34 genetic loci, including 52 gene variants that have been linked to AMD, have been identified.[8] All these genes can be clustered into five physiologically relevant pathways: immune

response and complement (*CFH*, *C9*, *C2/CFB*, *CFI*, and *C3*), extracellular matrix remodeling (*COL8A1*, *COL10A1*, *TGFBR1*, *TIMP3*, *ADAMTS9*, *HTRA1*, and *B3GALTL*), lipid transport (*APOE*, *LIPC*, *CETP*, *BAIAP2*, and *L2*), angiogenesis (*VEGFA*, *TGFBR1*, and *ADAMTS9*), and cell survival (*ARMS2*, *RAD51B*, and *TNFRSF10*).[9,10] The significance of genetic predisposition extends beyond the high susceptibility of a person to develop AMD and may also affect the response to treatment.[11] Some researchers have reported a preferential association of *CFH* and *ARMS2/HTRA1* risk variants with different forms of advanced AMD.[12,13] Specifically, *CFH* and *ARMS2* risk variants have been associated with progression toward GA and CNV, respectively.[13,14] Even though this may imply a different influence of the two genes, their variants substantially increase the likelihood of both types of late AMD, indicating their contribution to biological processes before the onset of advanced disease.

Furthermore, cardiovascular risk factors, including smoking, hypertension, and obesity, have been suggested to intensify microvascular aging processes.[15,16] It has been suggested that vascular factors play an important role in AMD pathogenesis,[17] and that choroidal and retinal blood flows are disturbed in AMD, which may result in the initiation or progression of AMD.[18,19] As we have shown in our previous study, the state of the retinal microvasculature may also reflect generalized disturbances in cardiovascular disorders (CVDs), such as stroke, coronary heart disease, and hypertension.[20] Retinal vascular caliber changes, assessed quantitatively on fundus photographs, have been shown to predict CVD independently of traditional risk factors, suggesting that, compared to other risk measures of CVD, retinal vascular changes may carry additional prognostic information.[21]

It has been suggested by McClintic et al. that functional assessments of the retinal microcirculation may be more useful in CVD risk prediction than static analysis alone.[22] Thus, a dynamic vessel analyzer (DVA) estimates retinal vessel functionality at baseline and after flicker stimulation. It is based on the premise that in accordance with the neurovascular coupling theory, flicker light increases retinal neural activity, provoking retinal arterial and venous dilation via the release of nitric oxide (NO).[23] Endothelial cells play an important role in modulating microvascular tone and autoregulation because NO is an endothelial-derived vasodilator, whereas endothelin is a potent vasoconstrictor.[24] Importantly, diminished arterial and venous dilative responses have been linked to different vascular diseases.[25] Because AMD has been previously linked to cardiovascular diseases, such a relationship may be confirmed with altered vessel reactivity in the DVA test. Indeed, dynamic vessel analysis in eyes with drusen and RPD showed diminished retinal arterial dilation in response to flicker light stimulation.[26] However, the literature describing the changes in DVA in the course of AMD is scarce,[27,28] and studies have been conducted on relatively small groups of patients, which makes inference impossible.

The aim of the present study was to compare the differences in the reactivity of the retinal vessels to a flickering stimulus in patients with AMD and healthy participants and to assess whether the status of retinal vessels is dependent on concomitant vascular diseases. Subsequently, we searched for the correlation between flicker-induced vasodilatation and morphological status of the retina and choroid on the basis of spectral-domain optical coherence tomography (SD-OCT) images. We also assessed the relationship between high-risk AMD polymorphisms, including *CFH* Y402H and *ARMS2* A69S, and both static and dynamic retinal vascular function. We hypothesize that static and dynamic retinal vascular function is associated with high-risk AMD polymorphisms, choroidal status, and concomitant vascular diseases.

Materials and Methods

Subjects and Initial Management

We recruited 354 patients with AMD and 121 age-matched controls with no symptoms or signs of macular degeneration from the outpatient population of the First Department of Ophthalmology of Pomeranian Medical University in Szczecin, Poland. All patients with AMD were enrolled in the study at the moment of diagnosis, prior to the introduction of anti-VEGF treatment. The exclusion criteria were significant chronic systemic conditions (diabetes mellitus, renal failure, neoplastic disease, hepatic dysfunction, etc.) or ongoing retinal disease except AMD (in the AMD group). All patients enrolled in the study signed a consent form before enrollment, in accordance with the tenets of the Declaration of Helsinki.

Data regarding medical history and current drug use were collected with a particular focus on the history of cardiovascular diseases, including physician-diagnosed heart and vascular diseases. Prior to ophthalmic examination, the actual arterial blood pressure (BP) was directly measured in all subjects using a noninvasive BP system with a manual aneroid manometer. The mean result from 3 measurements obtained with 5-minute resting intervals was calculated. The systemic mean arterial pressure (MAP) was calculated as follows: $\text{MAP} = \text{diastolic BP} + 1/3\ (\text{systolic BP} - \text{diastolic BP})$ mm Hg. The following medical parameters were also assessed: waist circumference (cm), waist/hip ratio (WHR), and body mass index (BMI; weight [kg]/height [m]2). Using the reported average number of cigarettes smoked per day and the number of years of smoking, we calculated cumulative pack-years.

Ophthalmologic Examination

All participants underwent complete ophthalmologic examination, including best corrected distance visual acuity with Snellen charts, slit lamp biomicroscopy, and detailed fundus examination after pupil mydriasis with 1% tropicamide solution, to determine the ocular health status of the fundus. OCT images were obtained using a Spectralis OCTA (Heidelberg Engineering, Heidelberg, Germany). IOP measurement with the Goldmann applanation tonometer, axial length, and anterior chamber depth calculations were performed to eliminate known factors that interfere with reliable OCT image analysis. If the presence of a neovascular membrane could not be clearly excluded or confirmed, a fluorescence angiography test was performed. The results were analyzed for the presence of factors that met the exclusion criteria, which included any posterior eye disease that could potentially affect the choroidal or retinal vasculature, including but not limited to glaucoma, retinal vessel occlusion, retinopathy of any type, high myopia (≥ 6 D), uveitis, and ocular tumor. Eyes were excluded if they had cataract surgery in the previous 3 months, and any previous laser therapy or vitreoretinal surgery. Additionally, fundus autofluorescence examination was performed for more accurate drusen classification.

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The severity of AMD was classified according to Ferris et al.[29]: (1) early AMD: medium drusen (63–125 µm) and no pigmentary abnormalities, (2) intermediate AMD: large drusen or pigmentary abnormalities associated with at least medium drusen, and (3) late AMD: lesions associated with neovascular AMD or geographic atrophy. The examinations were carried out in a blinded manner.

Retinal Vessel Analysis

For static retinal vessel analysis (SVA), the FF450 plus fundus camera (Zeiss AG, Jena, Germany) was used, and 30 degrees retinal photographs of each subject were collected and analyzed using VISUALIS and VesselMap Software (IMEDOS Systems, Ltd., Jena, Germany). Examination was conducted in a half-lit room. The participant focused on the fixation bar within the retinal camera, whereas the fundus was examined under green light; thus, preserved central vision is crucial for precise test conduction. The standard parameters for this evaluation were as follows: central retinal arteriolar equivalent (CRAE), which relates to the diameter of the central retinal artery; central retinal venular equivalent (CRVE), which relates to the diameter of the central retinal vein; and arteriovenous ratio (AVR), which represents the CRAE/CRVE ratio. After 20 minutes, DVA was conducted. The major temporal arterial and venous segments approximately 1.5 mm long were evaluated in each eye. The measurements were located 1- to 2-disc diameters from the optic disc. The selection criteria for the arterial and venous segment locations were as follows: no crossing or bifurcation in the measured segment, a curvature not exceeding 30 degrees, a distance from the neighboring vessels of at least one vessel diameter, and sufficient contrast with the surrounding fundus. The measurement of the baseline vessel diameter for 50 seconds was followed by 3 cycles of 20-second flicker provocation and 80-second steady illumination, during which the vessel diameter returned to baseline. The total duration of the measurement, including baseline and observation between flicker provocations, was 352 seconds. The response was measured as the difference between the mean vascular diameter for the last 10 seconds of flicker stimulation and the mean vascular diameter for the 30 seconds immediately preceding this flicker stimulation, divided by the latter value. The response was expressed as the mean of the calculations for the three flicker cycles. Only one artery and one vein were measured in each eye.

Genotyping

Venous blood samples (approximately 7.5 mL) collected in EDTA tubes were centrifuged (2000 rpm, 4°C, 10 minutes). Then, red blood cells were lysed using ammonium chloride-based lysing buffer (BD Biosciences, Franklin Lakes, NJ, USA). Nucleated cells were then counted, and DNA isolation was subsequently performed with a total DNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. AMD risk polymorphisms were genotyped as previously described.[30] In *CFH*, rs1061170, encoding a Y402H interchange, was genotyped by restriction analysis with EagI, HhaI, and Hsp92II enzymes. In *ARMS2*, LOC387715 rs10490924, encoding an A69S interchange, was determined by direct DNA sequence analysis using an Applied Biosystems 3130 XL instrument for DNA sequencing. Molecular analysis was performed by an outward cooperating laboratory according to generally approved standards.

Statistical Analysis

Because most of the analyzed retinal vessel parameters showed distributions significantly different from normal distribution (Shapiro-Wilk test, $P < 0.05$), nonparametric tests were applied. The Kruskal-Wallis and Mann-Whitney U tests were used to compare quantitative and rank variables between groups. The strength of associations between quantitative and rank variables was measured with Spearman rank correlation coefficients (Rs). Fisher's exact test was used to compare qualitative variables between groups. We considered any P values < 0.05 as statistically significant. Because simultaneous testing of multiple comparisons within a family of hypotheses carries the risk of increasing rejection error, the conservative Bonferroni's correction was calculated to decrease the risk of indication of a significant difference when it resulted only from random variation. Consequently, the nominal significance level of each test was lowered by a specific correction for multiple comparisons. To take into account the issue of multiple testing, we corrected the P value threshold by dividing the standard 0.05 level byproduct of the number of retinal vessel parameters (5), the number of ophthalmological, clinical and genetic parameters correlated with retinal vessel parameters (30), and the number of study groups (2). Thus, associations of retinal vessel parameters with the other variables at $P < 0.05/300 = 0.00016$ was considered statistically significant after Bonferroni correction for multiple testing.

Results

Clinical Characteristics of the Study Groups

In this study, we enrolled 354 patients with AMD (175 dry AMD and 179 wet AMD) and 121 healthy controls. Table 1 lists the clinical features of the patients and controls. In the study groups, we examined vascular-related risk factors because epidemiological evidence collected so far suggests that atherosclerosis is involved in AMD. However, no significant differences in age or well-known atherosclerotic risk factors, including hypertension, history of ischemic heart disease, or cardiac infarction, were detected between the AMD and control groups. The proportion of past smokers and the number of smoking pack-years were significantly higher in the AMD group than in the controls ($P = 0.0004$ and $P < 0.0001$, respectively).

Retinal Vessel Parameters

Next, we analyzed retinal vessel parameters in the study groups (Table 2). The eyes of the patients with AMD and controls did not differ significantly in parameters evaluated by SVA and DVA. Both CRAE, relating to the diameter of the central retinal artery, and CRVE, relating to the diameter of the central retinal vein, did not show statistically significant differences between AMD and control eyes ($P = 0.55$ and $P = 0.88$, respectively). Accordingly, we observed no differences between the groups in AVRs ($P = 0.31$). Regarding the dynamic retinal vessel analysis, no significant differences in either arterial or venous responses to flicker stimulation were identified between eyes from patients with AMD and controls ($P = 0.31$ and $P = 0.22$, respectively).

Table 1. Characteristics of the Study Groups

Parameter	AMD Group	Control Group	*P* Value*
Number of subjects	354	121	—
Sex M/F	135/219	32/89	0.02
Patient's age, y, mean ± SD	73.4 ± 8.0	73.1 ± 6.0	0.41
BMI, kg/m^2, mean ± SD	26.9 ± 4.2	26.6 ± 3.7	0.43
WHR, arbitrary units, mean ± SD	0.90 ± 0.09	0.88 ± 0.09	0.13
Waist circumference, cm, mean ± SD	103.3 ± 9.1	102.1 ± 7.3	0.33
MAP, mm Hg, mean ± SD	98.30 ± 11.10	98.72 ± 9.66	0.86
Current smokers, *N*	44 (14%)	6 (6%)	0.0503
Former smokers, *N*	166 (51%)	30 (31%)	**0.0004**
Smoking pack-years, mean ± SD	13.6 ± 18.9	6.0 ± 13.1	**0.00007**
Period without smoking, y, mean ± SD	6.8 ± 10.9	5.3 ± 10.2	0.055
Hypertension, *N*	209 (65%)	69 (71%)	0.27
Duration of hypertension, y, mean ± SD	8.2 ± 9.5	9.2 ± 9.9	0.27
History of ischemic heart disease, *N*	52 (16%)	11 (11%)	0.33
Duration of ischemic heart disease, y, mean ± SD	1.2 ± 4.2	0.8 ± 3.3	0.26
History of myocardial infarction, *N*	20 (6%)	6 (6%)	1.00
History of cerebral stroke, *N*	9 (3%)	3 (3%)	1.00
History of peripheral artery disease, *N*	16 (5%)	6 (6%)	0.61
History of aortic aneurysm, *N*	5 (2%)	0	0.59
Hypotensive drugs/vasodilators, *N*	210 (65%)	68 (70%)	0.39
Hormonal drugs, *N*	55 (17%)	20 (21%)	0.45
Thyroxine, *N*	44 (14%)	20 (21%)	0.11
Steroids, *N*	6 (2%)	1 (1%)	1.00
Other hormonal drugs, *N*	4 (1%)	0	0.58
Statins, *N*	86 (27%)	35 (36%)	0.07
NSAIDs, *N*	65 (20%)	19 (20%)	1.00
Cardiac medications/anti-arrhythmic drugs, *N*	45 (14%)	14 (14%)	0.87
Anti-asthmatic drugs, *N*	24 (7%)	3 (3%)	0.16
Antidepressants, *N*	15 (5%)	5 (5%)	0.79

N, number of subjects.
* Mann–Whitney test/Fisher's exact test. In bold, a *P* value < 0.05 was considered statistically significant.

Table 2. Retinal Vessel Parameters in the Study Groups

Parameter	AMD Group Median (IQR)	Control Group Median (IQR)	*P* Value*
AVR	0.85 (0.09)	0.85 (0.09)	0.31
CRAE	181.4 (23.1)	182.8 (21.18)	0.55
CRVE	215.2 (27.1)	213.6 (26.43)	0.88
DAA (%)	1.6 (2.9)	1.8 (2.9)	0.31
DAV (%)	3.0 (2.5)	3.3 (2.4)	0.22

IQR, interquartile range; AVR, arteriovenous ratio; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; DAA, dynamic analysis of arteries; DAV, dynamic analysis of veins.
* Mann–Whitney *U* test.

Interestingly, when we analyzed the same parameters in groups of patients with different stages of AMD (Table 3), we observed significantly higher AVR values in eyes with early AMD than in eyes with late AMD (median = 0.87 and median = 0.85, respectively, $P = 0.03$). This may indicate that AMD progression is linked with structural changes in the retinal vasculature.

Representative static vessel analyses of the patients with early and late stage of AMD are shown in the Figure.

Correlations Between Retinal Vessel Parameters and Retinal and Choroidal Characteristics

Next, we investigated possible correlations between retinal vessel parameters and ophthalmological characteristics of our patients with AMD and controls. In both patients with AMD and controls, choroidal thickness correlated positively with CRAE (Rs = 0.21, $P < 0.0001$ in AMD eyes and Rs = 0.20, $P = 0.007$ in controls, respectively) and CRVE (Rs = 0.14, $P = 0.0007$ in AMD eyes and Rs = 0.28, $P = 0.0002$ in controls, respectively). Similarly, choroidal volume correlated positively with CRAE (Rs = 0.24, $P < 0.0001$ in AMD eyes and Rs = 0.30, $P < 0.0001$ in controls, respectively) and CRVE (Rs = 0.25, $P < 0.0001$ in AMD eyes and Rs = 0.38, $P < 0.0001$ in controls, respectively). This finding indicates that the thicker the choroid is, the larger the diameter of both the central retinal vein and central retinal artery. Interestingly, visual acuity correlated positively with CRAE in AMD eyes (Rs = 0.14, $P = 0.001$), whereas no such association was observed for controls. Associations of choroidal thickness with CRAE in the AMD group and associations of choroidal volume with CRAE and CRVE for both patients with AMD and controls remained significant after correction for multiple tests ($P < 0.00016$).

Then, we investigated whether flicker-induced vasodilatation is associated with morphological changes in the retina and choroid obtained from OCT images. In the AMD group, arterial response to flicker stimulation correlated positively with both choroidal thickness (Rs = 0.14, $P = 0.00096$) and choroidal volume (Rs = 0.23, $P < 0.0001$), and no such associations were observed in the controls. This indicates that the function of the vascular endothelium in the arterial vessels of the retina is linked with parameters of choroidal vessels: the higher the flicker-induced arterial vasodilatation values are, the thicker and larger the volume of the choroid.

Table 3. Retinal Vessel Parameters in Patients With Different Stages of AMD

Parameter	*P* Value*	Early Median (IQR)	Intermediate Median (IQR)	Late Median (IQR)	*P* Value† E vs. I	*P* Value† I vs. L	*P* Value† E vs. L
AVR	0.05	0.87 (0.08)	0.85 (0.09)	0.85 (0.08)	0.33	0.11	0.03
CRAE	0.21	184.50 (21.0)	185.2 (16.85)	180.18 (21.25)	0.68	0.08	0.43
CRVE	0.31	210.55 (25.7)	216.5 (25.4)	214.18 (24.8)	0.17	0.94	0.14
DAA (%)	0.74	1.65 (2.5)	1.98 (2.65)	1.5 (2.65)	0.49	0.55	0.78
DAV (%)	0.95	3.35 (1.95)	3.03 (1.65)	3.1 (2.2)	0.70	0.94	0.87

* Kruskal–Wallis test.
† Mann–Whitney *U* test.
E, early, I, intermediate; L, late; AVR, arteriovenous ratio; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; DAA, dynamic analysis of arteries; DAV, dynamic analysis of veins.
In bold, a *P* value < 0.05 was considered statistically significant.

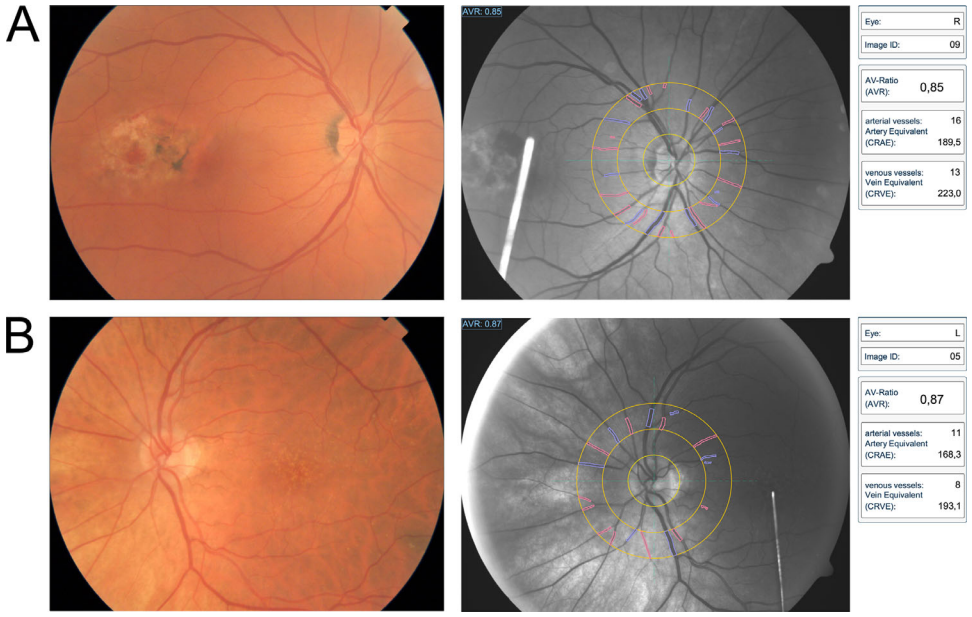

Figure. Static vessel analysis of a patient with late (**A**) and early (**B**) AMD, with AVR, CRAE, and CRVE values presented.

Interestingly, in the AMD group, we found a positive correlation between arterial response to flicker stimulation and total retinal volume (Rs = 0.16, $P = 0.0003$). This indicates that better vascular endothelial function implies less advanced retinal atrophy. Moreover, we also found strong positive correlations between arterial and venous responses to flicker stimulation in both patients with AMD (Rs = 0.28, $P < 0.0001$) and controls (Rs = 0.33, $P < 0.0001$). Associations of arterial response to flicker stimulation with choroidal volume in AMD group and associations of arterial with venous responses to flicker stimulation for both patients with AMD and controls remained significant after correction for multiple tests ($P < 0.00016$).

Correlations Between Retinal Vessel Parameters and Clinical Characteristics of the Patients

Because narrowing of vessels in the course of atherosclerosis is tightly linked with the development and progression of AMD, we focused on associations between analyzed retinal vessel parameters and general characteristics of our patients with AMD and controls, including medical history and current drug use. We observed a negative correlation between flicker-induced arterial vasodilation and age in both the AMD and control groups (Rs = −.16, $P = 0.00018$ and Rs = −0.21, $P = 0.005$, respectively). In the AMD group, we also observed a negative correlation between WHR values and AVRs (Rs = −0.12, $P = 0.003$). This indicates that overweight and obesity are associated with a prominent narrowing of the ocular microvasculature. Accordingly, higher MAP values were accompanied by lower AVRs (Rs = −0.19, $P < 0.0001$), further supporting the notion of narrowing the arteries in the eyes of patients who present with increased BP. Association of MAP with AVR remained significant after correction for multiple tests ($P < 0.00016$).

Our next aim was to further assess whether static and dynamic retinal vessel characteristics differ in the eyes of patients with known atherosclerotic risk factors and a history of cardiovascular disease. We observed significantly lower DAA values in patients with AMD with ischemic heart disease and aortic aneurysm than in individuals without such diseases (ischemic heart disease: median = 0.85, no ischemic heart disease: median = 2.25, $P = 0.002$; aortic aneurysm: median = 0.6, no aortic aneurysm: median = 1.95, $P = 0.04$). No such differences were observed in controls. In patients with AMD with hypertension, we also observed significantly lower CRAE (hypertension: median

Table 4. Retinal Vessel Parameters in Patients With Different High Risk AMD Genotypes

		CFH Y402H Genotype Groups						
Parameter	***P* Value***	**TT (*N* = 61) Mean ± SD Median (IQR)**	**TC + CC (*N* = 234) Mean ± SD Median (IQR)**	***P* Value†**	**TT + TC (*N* = 194) Mean ± SD Median (IQR)**	**CC (*N* = 101) Mean ± SD Median (IQR)**	***P* Value†**	***P* Value† (TT vs. CC)**
AVR	0.58	0.84 ± 0.06 0.84 (0.09)	0.85 ± 0.06 0.86 (0.08)	0.33	0.85 ± 0.07 0.85 (0.09)	0.85 ± 0.06 0.86 (0.07)	0.98	0.48
CRAE	0.48	183.74 ± 14.66 185.75 (23.36)	181.44 ± 15.12 180.75 (19.55)	0.26	181.96 ± 15.06 182.73 (20.01)	181.91 ± 15.03 181.5 (20.6)	1.0	0.49
CRVE	0.20	219.73 ± 19.59 217.05 (29.79)	214.25 ± 18.10 212.65 (27.8)	0.08	215.81 ± 18.91 213.93 (28.6)	214.77 ± 17.88 212.6 (27.68)	0.76	0.16
DAA, %	0.35	2.50 ± 2.09 2.0 (3.5)	2.06 ± 1.73 1.63 (2.5)	0.29	2.08 ± 1.7 1.8 (2.7)	2.30 ± 2.0 1.5 (2.7)	0.62	0.69
DAV, %	0.08	3.83 ± 1.68 3.4 (2.6)	3.24 ± 1.71 3.0 (1.9)	**0.03**	3.36 ± 1.71 3.05 (2.0)	3.38 ± 1.74 3.1 (2.1)	0.82	0.10
		***ARMS* A69S Genotype Groups**						
Parameter	***P* Value***	**GG (*N* = 95) Mean ± SD Median (IQR)**	**GT + TT (*N* = 201) Mean ± SD Median (IQR)**	***P* Value†**	**GG + GT (*N* = 251) Mean ± SD Median (IQR)**	**TT (*N* = 45) Mean ± SD Median (IQR)**	***P* Value†**	***P* Value† (GG vs. TT)**
AVR	0.07	0.86 ± 0.07 0.86 (0.07)	0.84 ± 0.06 0.85 (0.09)	0.12	0.85 ± 0.06 0.85 (0.08)	0.86 ± 0.07 0.87 (0.09)	0.27	0.92
CRAE	0.61	183.52 ± 15.62 182.95 (21.35)	181.15 ± 14.63 180.75 (19.8)	0.43	181.92 ± 14.98 182.28 (19.84)	182.0 ± 15.22 183.0 (24.18)	0.74	0.86
CRVE	0.53	215.06 ± 19.22 214.1 (30.6)	215.60 ± 18.19 212.9 (27.3)	0.85	215.86 ± 17.85 214.1 (26.9)	212.99 ± 21.86 210.5 (35.15)	0.32	0.54
DAA, %	**0.04**	2.38 ± 1.79 1.95 (2.6)	2.03 ± 1.82 1.5 (2.5)	0.11	2.28 ± 1.84 1.95 (2.5)	1.47 ± 1.50 0.93 (1.3)	**0.01**	**0.008**
DAV, %	0.31	3.44 ± 1.51 3.2 (1.8)	3.31 ± 1.81 3.05 (2.2)	0.43	3.42 ± 1.68 3.2 (2.1)	3.01 ± 1.89 2.5 (1.4)	0.14	0.11

* Kruskal–Wallis test for comparison among three genotypes of indicated polymorphisms.

† Mann–Whitney *U* test.

AVR, arteriovenous ratio; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; DAA, dynamic analysis of arteries; DAV, dynamic analysis of veins; *N*, number of subjects.

In bold, a *P* value < 0.05 was considered statistically significant.

= 179.93, no hypertension: median = 186.88, $P = 0.001$) and CRVE (hypertension: median = 212.45, no hypertension: median = 218.4, $P = 0.006$).

Finally, we investigated whether specific drugs could also affect retinal vessel parameters. In the AMD group, taking hypotensive medication was associated with lower values of the same parameters as those observed for hypertensive subjects: CRVE (hypotensive drug use: median = 212.88, no hypotensive drug use: median = 217.7, $P = 0.02$) and CRAE (hypotensive drug use: median = 180.55, no hypotensive drug use: median = 186.6, $P = 0.002$). Accordingly, lower CRAEs were observed in the eyes of the patients taking anticoagulants (anticoagulant users: median = 178.7, no anticoagulant users: median = 183.4, $P = 0.04$). No such differences were found in the controls. Interestingly, DAV was reduced in patients with AMD taking nonsteroidal anti-inflammatory drugs (NSAID use: median = 2.6, no NSAID use: median = 3.25, $P = 0.04$) and taking cardiac medications (cardiac drug use: median = 1.0, no cardiac drug use: median = 1.95, $P = 0.03$).

Associations Between *CFH* Y402H and *ARMS2* A69S Polymorphisms and Retinal Vessel Parameters

In order to explore whether SNPs associated with an increased risk of AMD (*CFH* Y402H and *ARMS2* A69S) influence retinal vessel characteristics in our patients, we correlated these parameters with different genotypes in these two SNPs (Table 4). We found that the TT genotype in *CFH* Y402H was associated with an evidently higher venous response to flicker stimulation than the TC + CC genotypes (median = 3.4, median = 3.0, respectively, $P = 0.03$). However, we should be rather cautious in interpreting the results of the Mann–Whitney test because the Kruskal–Wallis test showed only borderline differences between the TT and TC + CC genotype groups ($P = 0.08$). Importantly, in the case of *ARMS2* A69S, we observed significant differences in arterial vasodilatation values between the tested genotypes ($P = 0.04$, Kruskal–Wallis test). In detail, GG homozygotes presented the highest, whereas TT homozygotes presented the lowest DAA responses. Moreover, patients with GG + GT genotypes presented with a significantly higher arterial response to flicker stimulation than TT homozygotes ($P = 0.01$).

Discussion

AMD remains the leading cause of blindness in the industrialized world. However, the exact pathogenesis of this disease remains largely unknown. Recently, disturbances in choroidal and retinal blood flows leading to chronic hypoxia have been implicated in the initiation or progression of AMD. Because the state of the retinal microvasculature may also reflect generalized processes occurring in the course of various CVDs and a number of CVD risk factors (age, obesity, cigarette smoking, hypertension, and high cholesterol) have also been associated with an increased risk of AMD,[31] the link between CVDs and AMD is receiving more attention. Thus, in this study, we aimed to assess the reactivity of retinal vessels to flickering stimuli in patients with AMD and to investigate whether retinal vascular function is associated with disease grading, choroidal and retinal characteristics, genetic background, and clinical and demographic data, with a particular focus on vascular-related risk factors.

First, we evaluated static and dynamic retinal vessel parameters in our study groups. We did not find any differences in retinal vessel diameters between AMD and control eyes at baseline or after flicker light application. Functional assessments of the retinal microcirculation in patients with AMD have not been extensively investigated. In 2019, Rabiolo et al. found no significant difference between eyes with drusen, reticular pseudodrusen and controls for CRAE, CRVE, and AVR, which is in agreement with our results.[26] In the same study, DVA revealed significantly reduced arterial dilation in eyes with drusen compared with controls, contradicting our results, as we observed no significant alterations in this parameter. However, it is worth noting that the study by Rabiolo et al. was conducted on a very small group of 23 patients with drusen. In another study by Lanzl et al., no significant differences in mean arterial dilation were found in patients with AMD in comparison with controls,[27] further supporting our results. The 10 patients with exudative AMD enrolled in this study were evaluated pre- and post-treatment with a single intravitreal application of bevacizumab, and, interestingly, arterial constriction after stimulation occurred more slowly in the post-treatment group than in the control group. However, the scarce literature on retinal vessel analysis in AMD and the small sample size in those studies makes it difficult to draw valuable conclusions.

Our results are in concordance with several studies on large cohorts, including the Blue Mountains Eye Study, Beijing Eye Study, and Beaver Dam and Rotterdam Eye Study (BDES), in which the authors found no association between the diameter of retinal vessels and AMD risk.[32–34] Accordingly, we found no statistically significant differences between the AMD and control groups in terms of AVR. However, when we investigated the particular AMD stages, we observed lower AVR values in eyes with late AMD. This may support the hypothesis that the structural changes in the retinal vasculature might be accompanied by progression of AMD. In fact, retinal changes depending on the AMD stage have previously been described by Yang et al., as dilated retinal arteriolar caliber was shown to be associated with early AMD.[35] Similarly, Jeganathan et al. identified wider venular caliber to be associated independently with early AMD, whereas there was no significant association between retinal arteriolar caliber and early AMD or between arteriolar or venular caliber and late AMD.[36] The authors attributed this association to some cardiovascular risk factors, such as inflammatory processes, oxidative stress, or nitric oxide (NO) production dysregulation. Interestingly, a smaller AVR was also reported in the context of increased carotid artery stiffness, which is considered an early marker of atherosclerosis.[37]

Next, we focused on the correlations between retinal vessel parameters and choroidal characteristics of the patients. Several previous studies noted that abnormal choroidal circulation may be involved in the development of AMD.[38–40] Choriocapillaris dropout has been observed from early stages of AMD, and vascular dropout appears to be linearly related to the progression of AMD based on histopathological studies.[18,41,42] Several previous studies have proposed that AMD affects both the quantity and morphology of inner retinal and choroidal vasculature.[17,43,44] In early patients with AMD with reticular pseudodrusen, retinal thinning was accompanied by choroidal and retinal vascular loss, which further suggests a possible link

between retinal atrophy and choroidal vasculature alterations.[45] The results of our study are in agreement with these previously published works and show that with the loss of choroidal tissue, a reduction in the diameter of retinal arterioles and venules occurs. It is probable that chronic choroidal ischemia may contribute to retinal atrophy and decreased metabolic demands that may induce a secondary decrease in retinal vascular flow.[45] The positive correlation between the arterial response to flicker stimulation and choroidal thickness and volume may in part support the hemodynamic theory of AMD proposed by Friedman.[46] According to this theory, lipids accumulate in Bruch's membrane and sclera, similar to atherosclerotic plaques in the arteries, resulting in the higher stiffness of the tissues. This leads to increased choroidal vascular resistance, which further leads to either an increase in choriocapillaris hydrostatic pressure or a decrease in choroidal perfusion, depending on the relative resistances of the ophthalmic artery and the cerebral artery.[47] This further supports the notion that AMD is the eye localization of the systemic disease of atherosclerosis.

In fact, several studies have linked atherosclerotic changes and a markedly increased risk of CVD with the incidence of AMD. Taniguchi et al. reported that neovascular AMD is associated with atherosclerosis, whereas Klein et al. similarly reported the presence of carotid plaques and carotid artery intima-media thickness to be weakly associated with the incidence of late AMD.[48,49] The Age-Related Eye Disease Study (AREDS) found advanced AMD to be associated with increased cardiovascular mortality,[50] whereas the Atherosclerosis Risk in Communities Study (ARCS) observed a significant association between late AMD and incident coronary heart disease (CHD) in a population at high risk for CHD.[51] Therefore, to better understand possible associations between AMD and atherosclerosis risk factors, we investigated the relationship between analyzed retinal vessel parameters and general characteristics of our patients with AMD and controls, including medical history and current drug use. We found significant associations between both static and dynamic retinal vessel parameters and ischemic heart disease, aortic aneurysm, and hypertension. Our results confirm the previously described relationship between arterial hypertension and the reduced diameter of the retinal vessels, where it might take the form of generalized or focal arteriolar narrowing as a result of local autoregulatory mechanisms in response to elevated intraluminal pressure.[52,53] Either remodeling or rarefaction of retinal/choroidal vessels may contribute to an increase in vascular resistance, resulting in a decrease in blood flow and ischemia, and these effects may be reflected by changes in retinal vessel static and dynamic tests. Indeed, the history of ischemic heart disease, aortic aneurysm, or hypertension in our patients with AMD was associated with a significantly decreased reactivity of arterial vessels to flicker stimulation. All these diseases are characterized by complex etiopathogenesis related to both environmental and genetic factors, but atherosclerosis remains the common point of these entities,[54] once again suggesting its vital role in AMD pathogenesis.

In patients with AMD, both antihypertensive and anticoagulant therapies were associated with significantly reduced CRAE and CRVE parameters. According to our previous study, conducting effective BP-lowering therapy does not go hand in hand with the normalization of the functions of the vascular endothelium.[20] This may indicate sources of microcirculation disturbances other than elevated BP in patients with hypertension. In contrast, it was postulated that statin use might potentially contribute to reduced progression of AMD due to lipid-lowering, anti-inflammatory, and anti-angiogenic effects.[55] We also observed a reduced venous response to flicker stimulation in patients with AMD using NSAIDs or cardiac medications. Rubio-Ruiz et al. proposed that in chronic, low-grade inflammatory conditions, such as aging, COX-2 contributes greatly to vasoconstriction; thus, NSAIDs directly affect vascular responses.[56] On the other hand, Modjtahedi et al. recently described a lower risk of exudative AMD in longer-term NSAID users, suggesting its protective effect, although the group concluded a generally low impact of NSAIDs on AMD incidence.[57]

Finally, we found a significantly lower arterial response to flicker stimulation in TT homozygotes for the *ARMS* A69S polymorphism and a less prominent venous response to flicker stimulation in patients with TC + CC genotypes in the *CFH* Y402H polymorphism. Carriers of at least one high-risk C allele in the Y402H variant are two to three times more likely to develop AMD than homozygous individuals with T alleles,[58] whereas individuals harboring TT alleles in the *ARMS* A69S polymorphism have up to a 10-fold increase in the risk of late AMD. Our results suggest that high-risk alleles in these polymorphisms are associated with decreased arterial or venous responses to flicker stimulation, however this does not necessarily mean a causal relationship. In population-based trials, the pathways surrounding venous retinal microcirculation maladaptation have demonstrated strong correlations with indicators of systemic inflammation,[59,60] which corresponds to the function of *CFH* and *ARMS2*, as *CFH* is a crucial regulator of the complement system, whereas *ARMS2* might also play a role in the pro-inflammatory pathway.[61,62] Although there have been several studies conducted in murine models on the role of *CFH* in retinal development and function,[63,64] there is less evidence of the influence of *CFH* on retinal vessel parameters in humans. Only one study investigated retinal vessel dilation in healthy young carriers of the C risk allele at the Y402H polymorphism and found no abnormal flicker-induced retinal vessel dilation[28]; however, the study included only 18 homozygous C carriers. We cannot exclude the possibility that the vascular response could alter over the lifespan of these individuals and be poorer when they develop AMD. To the best of our knowledge, our study provides the first evidence that high-risk alleles in the *CFH* Y402H and *ARMS2* A69S polymorphisms are associated with decreased arterial or venous responses to flicker stimulation in a population of patients with AMD.

Conclusions

Overall, we analyzed static and dynamic retinal vessel parameters in patients with AMD with a particular focus on morphological changes in the choroid, underlying vascular disorders, and high-risk AMD polymorphisms. Our findings suggest that retinal microcirculation appears to be associated with the genetic background, choroidal parameters, and clinical features of the patients with AMD.

Acknowledgments

Supported by the Polish National Centre for Research and Development (grant number: STRATEGMED1/234261/2NCBR/2014) and European Union funds from the European Union Regional Development Fund, Interreg Cooperation Program

V A Mecklenburg-Western Pomerania/Brandenburg/Poland for 2014-2020: "Consolidating cross-border cooperation through exchange of knowledge and skills in the field of modern diagnostic imaging methods in ophthalmology."

Disclosure: **E. Krytkowska**, None; **Z. Ulańczyk**, None; **A. Grabowicz**, None; **K. Mozolewska-Piotrowska**, None; **K. Safranow**, None; **A. Pałucha**, None; **M. Krawczyk**, None; **P. Sikora**, None; **E. Matczyńska**, None; **A. Stahl**, None; **B. Machaliński**, None; **A. Machalińska**, None

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