Circulating Anti-endothelial Cell Antibodies in Patients with Geographic Atrophy Related to Dry Age-Related Macular Degeneration

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Article

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Abstract: *Background and Objectives*: Age-related macular degeneration (AMD) is one of the leading causes of central vision loss among elderly patients and its dry form accounts for the majority of cases. Although several causes and mechanisms for the development and progression of AMD have previously been identified, the pathogenesis of this complex disease is still not entirely understood. As the inflammation and immune system involvement are strongly suggested to play a central role to promote the degenerative process and to stimulate the onset of complications, we aimed to analyze the frequency of serum anti-retinal (ARA) and anti-endothelial cell antibodies (AECA) in patients with dry AMD and to determine their relationship with the clinical features of the disease, notably area of geographic atrophy (GA). *Materials and Methods*: The study included 41 patients with advanced stage of dry AMD and 50 healthy controls without AMD, matched for gender and age. ARA were detected by indirect immunofluorescence using monkey retina as an antigen substrate, and the presence of AECA was determined using cultivated human umbilical vein endothelial cells and primate skeletal muscle. *Results:* ARA were detected 36 (87.8%) AMD patients (titers range from 1:20 to 1:320) and in 16 (39.0%) (titers range from 1:10 to 1:40) of controls (p=0.0000). Twenty of the 41 patients (48.8%) were positive for AECA, while in a control group AECA were present only in 5 sera (10.0%). The titers of AECA in AMD patients ranged from 1:100 to 1:1000 and in the control group the AECA titers were 1:100 (p=0.0001). There were no significant correlations between the presence of AECA and disease activity. *Conclusions*: The study represents evidence for the potential involvement of autoimmune processes against retina and retinal vessel antigens in the pathogenesis of geographic atrophy associated with dry AMD.

Keywords: anti-retinal antibodies; anti-endothelial cell antibodies; age-related macular degeneration; geographic atrophy; indirect immunofluorescence

1. Introduction

Age-related macular degeneration (AMD) is one of the most common causes of central blindness in people over 50 years of age in well-developed countries [1]. The loss of central vision is the result of geographical atrophy (GA) in a course of dry (atrophic, non-exudative) AMD or macular neovascularization (MNV) related to wet (exudative) AMD. Dry AMD accounts for 85-90% of all AMD cases [2]. Advances form of dry AMD is geographic atrophy (GA), defined as round or oval area of atrophy outer retinal layers, retinal pigment epithelium (RPE) and choroidal vessels with a diameter of at least 175 μm. GA typically starts in the perifoveal region and gradually progress to involve the fovea with time, leading to central scotomas and permanent loss of visual acuity. According to statistical data newly recognized GA affects over 5 millions of people around the world

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and is significant cause of irreversible central vision loss [3]. Median time from diagnosis to central vision loss ranges from 1.4 to 2.5 years, which is related to fovea involvement by GA [4].

AMD appears to be complex and involve numerous processes and mechanisms involved in its development and progression. Both non-modifiable risk factors, such as age, female gender, white race, genetic background influence the development and progression of this condition [5–7]. There are over 30 identified genes linked to the risk of AMD development, however the polymorphism of complement factor H gene (Y402H) is thought to be the strongest genetic risk variant for AMD [8–10]. The modifiable risk factors include: cigarette smoking, cardiovascular diseases, high lipid levels, abdominal obesity, diet with low intake of antioxidants, exposure to ultraviolet radiation, as well as local factors such as cataract surgery and blue irises [5,6,11–14].

There is growing evidence that autoimmunity against retinal antigens and inflammatory reactions dependent on complement system activation might be involved in the pathogenesis and progression of AMD [15–18]. With aging chronic inflammation, called para-inflammation or inflammaging, is thought to be an adaptive response of the immune system due to increased oxidative stress and noxious insults to maintain local tissue homeostasis. In patients with AMD, this chronic inflammatory reaction become dysregulated and contributes to macular damage [19]. There are publications demonstrated that various ocular diseases in a course of which autoimmune and inflammatory components play a crucial role may be associated with the presence of serum ARA [20–30]. However, the detailed role of these autoantibodies in the AMD pathogenesis remains unclear. There are speculations that their occurrence may be an epiphenomenon developing in response to macular damage or alternatively ARA may be directly involved in the development and progression of AMD [31–36].

Although the prevalence of serum AECA has been reported previously by us in patients with wet AMD [37], on reviewing the literature, no publication regarding the presence of circulating AECA in a course of dry AMD is currently available. To our best knowledge this is the first research on the occurrence of circulating AECA in patients with dry AMD.

The purpose of our study was to determine the prevalence of serum AECA in patents with dry AMD in order to determine their relationship with the clinical features of the disease, notably area of GA.

2. Materials and Methods

2.1. Patients and Controls

Forty one patients with unilateral or bilateral geographic atrophy in a course of dry AMD were enrolled in the study. Exclusion criteria included the following: other retinal diseases (MNV in the contralateral eye, macular teleangiectasia, degenerative myopia), infectious or non-infectious uveitis, glaucoma, paraneoplastic retinopathies, diabetic retinopathy, systemic autoimmune comorbidities, systemic steroid and immunosuppressive therapy. Fifty sex- and age-matched healthy subjects, planned for moderate senile cataract surgery with no clinical signs of AMD or any chronic eye disease served as a control group. The determination of absence of AMD in controls was based on ophthalmological examination and supported by a classification system of AMD prepared by Ferris et al.[2]. This system is based on fundus lesions observed within 2 disc diameters of the fovea. Individuals with no drusen or pigmentary changes or with small drusen (<63 μm) in macula were considered to have no features of AMD.

Baseline ophthalmic examination in patients and controls included best corrected visual acuity (BCVA) assessment with Snellen charts, anterior segment and fundus examination. The diagnosis of dry AMD was based on the presence of characteristic clinical features on fundoscopy and on the results of optical coherence tomography (OCT) (Topcon 3D OCT 2000, Japan) and fundus autofluorescence (FAF) (Heidelberg Engineering, Spectralis HRA-OCT, Germany) which was used to measure the area of geographic atrophy. In each patient the color fundus picture was also obtained.

 doi:10.20944/preprints202404.1565.v1

2.2. Autoantibody Assays

Each subject of the study was collected 5 ml of peripheral blood from peripheral vein, next clotted and centrifuged at 3500 rpm (1970g) for 10 minutes to recover serum. Aliquoted serum samples were stored at -80° until analysis. Collected material was studied for ARA by indirect immunofluorescence (IIF) method using commercially available frozen sections of normal monkey retina. For detection of antigen bound autoantibodies a secondary goat's anti-human IgA, G, M polyclonal antibody labelled by fluorescein isothiocyanate was used (Euroimmun AG, Lubeck, Germany). A serial dilution of patients' or controls' serum: 1:10, 1:20, 1:40, 1:80 etc. was used to titer positive samples against ARA, whereas starting serum 1:100 dilution was used for screening against AECA, in which human umbilical vein endothelial cells (HUVEC) and iliopsoas muscle sections were antigen substrates (Euroimmun AG, Lubeck, Germany). All details of IIF processing, including tittering, incubation and washing protocol were according to the manufacturer's recommendations. In brief, incubation time for the serum or the secondary antibody was 30 minutes. After each incubation with diluted serum and next the secondary antibody, biochips were rinsed with phosphate buffered saline with Tween 20. Samples with positive immunological reaction to retinal vessels were screened for AECA using IIF on monkey iliopsoas muscle and HUVECs. All immunohistochemical slides were evaluated using fluorescence microscope EUROSTAR-Bluelight (Carl Zeiss, Euroimmun, Germany). Scoring of IIF results was done by two independent investigators, unaware of the diagnosis or the stage of disease. Results were recorded as the presence of fluorescence signal in the layers of the retina, iliopsoas muscle and HUVECs and the highest serum dilution. A commercial negative tissue control was included on each slide to eliminate the misinterpretation causes by autofluorescence of the tissue.

This study complied with the Declaration of Helsinki. Approval by the Jagiellonian University Bioethical Committee (approval no. 1072.6120.37.2019) was obtained. All subjects provided written informed consent to participate in the study.

2.3. Statistical Analysis

Correlation between the nominal variables and ordinal variables was determined as the association between the variables and the significance level was evaluated with chi-square test for two-way table. A nonparametric Spearman's rank correlation coefficient was used to assess the statistical dependence between discrete and continuous variables. The Student's-t distribution or non-parametric Mann-Whitney U tests were used to compare the means in two groups. Type I statistic error p-value <0.05 was taken to be significant.

3. Results

3.1. Epidemiology and Characteristics of Patients with Dry AMD and a Control Group

The study group included 41 patients (82 eyes) with dry AMD; 15 males (36.6%) and 26 females (63.4%) aged 61 - 90 years (mean: 76.3 years). The control group consisted of 20 males (40%) and 30 females (60%) aged 59 – 86 years (mean: 71.9 years). No statistical differences of age, gender distribution were noted between AMD cases and controls. There were no significant statistical differences between the study and control groups of prevalence to cardiovascular diseases and hyperlipidaemia, either. Table 1. shows the clinical characteristics of patients with dry AMD and a control group.

 doi:10.20944/preprints202404.1565.v1

Table 1. Epidemiologic and clinical characteristic of patients with dry age-related macular degeneration (AMD) and a control group.

Characteristics	AMD group n = 41	Control group n = 50	P value
Sex			
Females	26 (63.4%)	31 (62.0%)	0.7390
Males	15 (36.6%)	19 (38.0%)	
Age	61-90 yrs (mean: 76.3 yrs)	59-86 yrs (mean: 71.9 yrs)	0.0770
Arterial hypertension	20 (48.8%)	11 (22.4%)	0.5324
Ischaemic heart disease	6 (14.6%)	3 (6.1%)	0.1931
Artherosclerosis	5 (12.2%)	1 (2.0%)	0.0678
Hyperlipidaemia	4 (9.8%)	3 (6.1%)	0.6109

The baseline BCVA ranged from counting fingers to 0.8 by Snellen charts. On fundoscopy characteristic changes for dry AMD were observed including: areas of chorioretinal atrophy in 80 eyes and large drusen with changes at the level of retinal pigment epithelium (RPE) in 2 eyes. OCT scans revealed typical findings for dry AMD; large drusen or the atrophy of the outer retinal layers involving photoreceptors, Bruch's membrane and choriocapillaris.

Based on the FAF examination the mean area of the geographic atrophy ranged from 0.24 mm^2 to 29.12 mm^2 (mean: 9.81 mm^2).

3.2. Serum Antiretinal Antibodies in Dry AMD Patients and in a Control Group

IIF test performed on monkey retina showed ARA in 36 (87.8%) of the 41 AMD patients (range from 1:20 to 1:320) and in 16 from 50 (39.0%) (range from 1:10 to 1:40) of controls (p=0.0000). The comparison of distribution and titers of serum ARA in AMD patients and in a control group are presented in Figure 1.

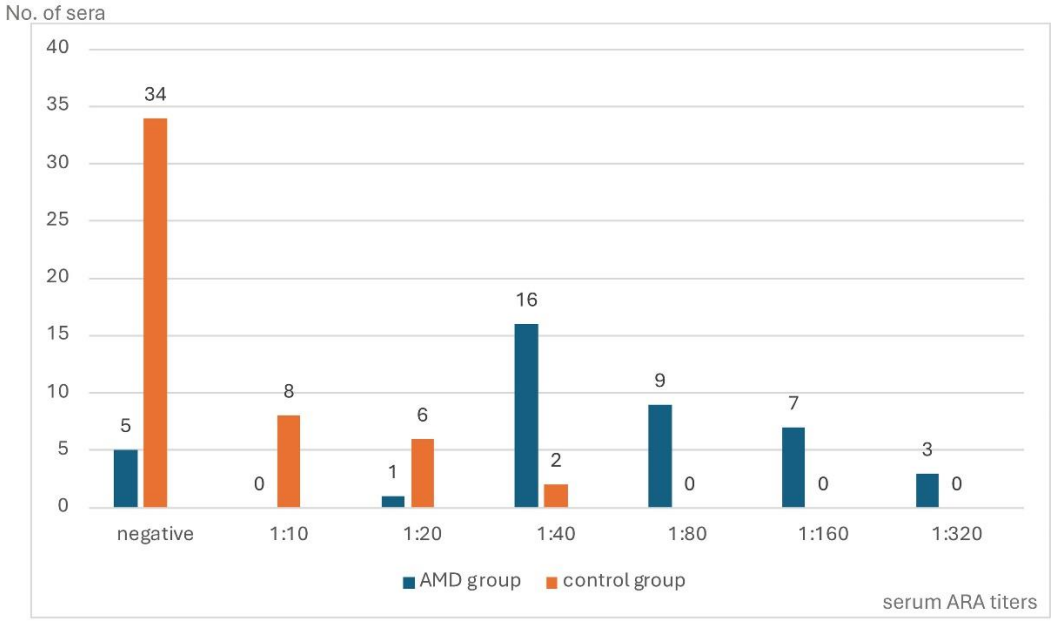

Figure 1. The distribution of circulating antiretinal antibodies (ARA) titers in patients with dry age-related macular degeneration (AMD) and in a control group.

Interestingly, patients with dry AMD demonstrated the presence of three types of retinal staining on monkey retina in IIF test, while control sera showed two staining patterns of reactivity

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with retinal tissue. Differences in ARA types in AMD and controls were statistically significant in cases with positive reaction against cones and rods, p=0.0000 and p=0.0001, respectively, while immunofluorescence within the cytoplasmic components of retinal nuclear layer cells showed no statistical differences between two analyzed groups. Table 2 shows distribution of ARA in serum of patients with dry AMD and controls according to the staining pattern and titer.

Table 2. Distribution of seruum anti-retinal antibodies in patients with dry age-related macular degeneration (AMD) and controls according to the titer and immunofluorescence staining pattern on retinal tissue.

Type of immunofluorescence staining pattern	Study group	Titre							P value
		Negative	1:10	1:20	1:40	1:80	1:160	1:320	
Cones, n (%)	AMD n=41	17 (41.5)	0 (0)	1 (2.4)	13 (31.7)	5 (12.2)	3 (7.3)	2 (4.9)	0.0000
	Control n=50	50 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Rodes, n (%)	AMD n=41	20 (48.8)	0 (0)	0 (0)	2 (4.8)	10 (24.4)	7 (17.1)	2 (4.8)	0.0005
	Control n=50	34 (68.0)	7 (14.0)	7 (14.0)	2 (4.0)	0 (0)	0 (0)	0 (0)	
Cytoplasmic components of retinal nuclear layer cells, n (%)	AMD n=41	34 (82.9)	0 (0)	0 (0)	5 (12.2)	1 (2.4)	1 (2.4)	0 (0)	0.3230
	Control n=50	34 (96)	0 (0)	1 (2)	1 (2)	0 (0)	0 (0)	0 (0)	
Retinal vessels, n (%)	AMD n=41	21 (51.2)	0 (0)	0 (0)	9 (22)	5 (12.2)	4 (9.8)	2 (4.9)	0.0001
	Control n=50	45 (90)	3 (6)	2 (4)	0 (0)	0 (0)	0 (0)	0 (0)	

The sera of 31 (75,6%) patients with dry AMD showed more than one immunofluorescence type, while in a control group the complexity of ARA types was observed only in 4 (8%) cases (p=0.0000). Figure 2 presents various immunofluorescence pattern reactions of AMD patients sera with monkey retina.

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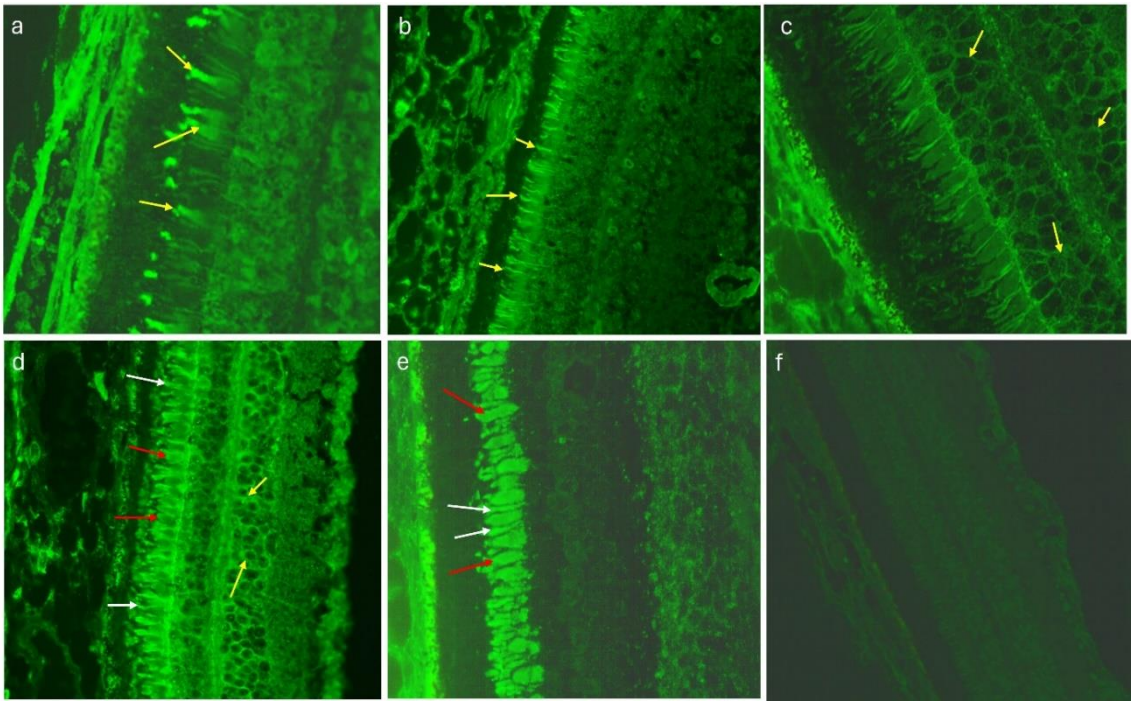

Figure 2. Monkey retina – indirect immunofluorescence test (IIF): **(a)** a positive staining of cones (yellow arrows), magnification 400x; **(b)** a positive staining of rods (yellow arrows), magnification 200x; **(c)** a positive staining of cytoplasmic components of both retinal nuclear layers (yellow arrows), magnification 400x; **(d)** a combined positive reaction within cones (red arrows), rods (white arrows) and cytoplasmic components of both retinal nuclear layers (yellow arrows), magnification 200x; **(e)** a positive reaction within photoreceptors: cones (red arrows) and rods (white arrows), magnification 200x; **(f)** a negative control, magnification 200x.

3.3. Anti-Endothelial Cells Antibodies in Patients with Dry AMD and in a Control Group

Sera of 20 (48.8%) AMD patients showed a highly distinctive pattern of tubular arrangement of the fluorescence signal on monkey retina sections within the retinal vessels (Figure 3a,b). In a control group this type of immunofluorescence was observed only in 5 (10%) sera. This difference was significant statistically (p=0.0001).

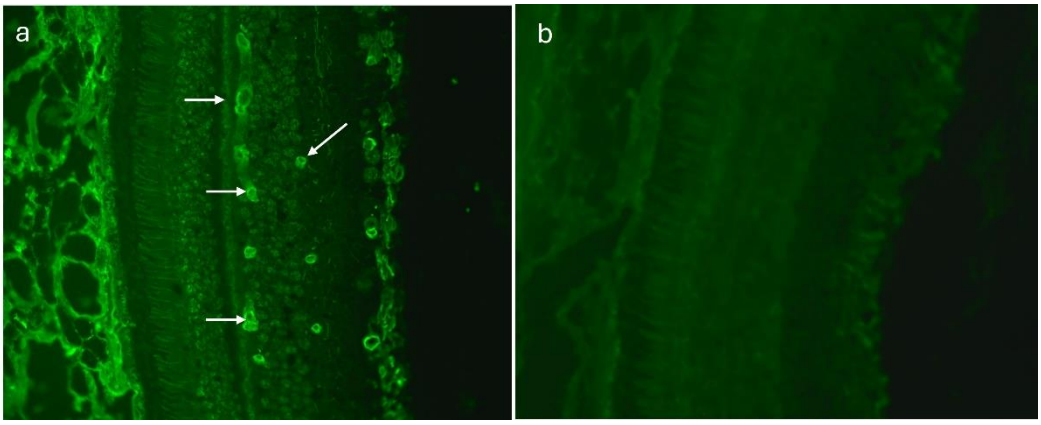

Figure 3. (a) Indirect immunofluorescence test (IIF) performed on monkey retina revealed a positive retinal vessels pattern staining (white arrows), magnification 200x; **(b)** Monkey retina - a negative control, magnification 200x.

Since this type of immunofluorescence was suggesting anti-endothelial specificity of autoantibodies, we repeated the assay with a primary human cell line from umbilical vein (HUVEC)

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and skeletal iliopsoas muscle sections of monkey and as the substrates. The fluorescence signal located between the fibers of transversal sections of the muscle had a characteristic pattern corresponding to the striated muscle vessels. The HUVEC cellular substrate has no other epitopes than endothelial cells. Verification of auto-antibodies in IIF tests performed on monkey iliopsoas muscle and HUVEC cells confirmed the presence of AECA in 20 (48.8%) patients with dry AMD in titers ranging from 1:100 to 1:1000. All 20 (48.8%) sera of AMD patients showed the positive reaction with iliopsoas muscle and 13 (31.7%) of them with HUVEC demonstrating an immunofluorescence pattern typical for AECA (Figures 4 and 5) .

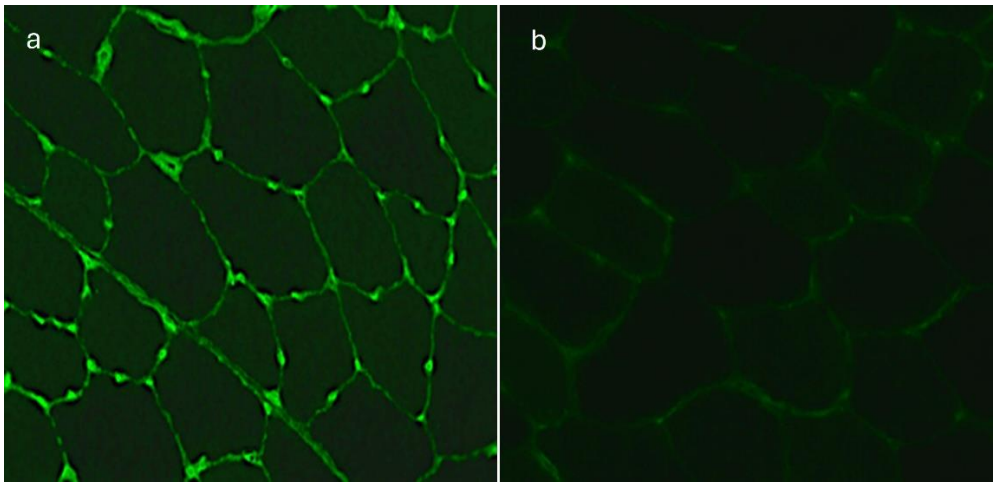

Figure 4. (a) Iliopsoas muscle - indirect immunofluorescence (IIF) test demonstrates a positive reaction with serum of AMD patient, immunofluorescence of endothelial cells of muscle vessels is observed, magnification 400x; (b) Iliopsoas muscle - a negative reaction with a control serum, magnification 400x.

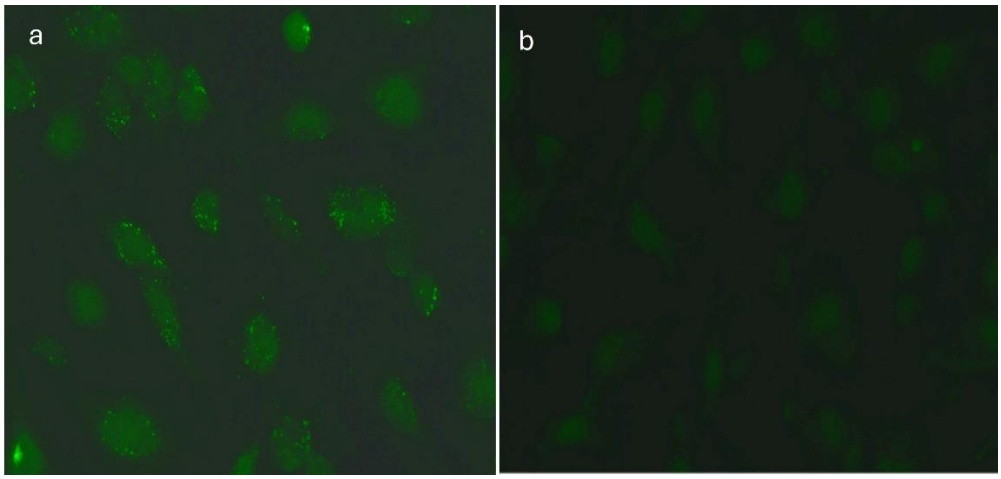

Figure 5. (a) Human umbilical vein endothelial cells (HUVEC) – indirect immunofluorescence (IIF) test shows a positive reaction with AMD patient serum, immunofluorescence of endothelial cells is present, magnification 400x; (b) Human umbilical vein endothelial cells (HUVEC) - a negative reaction with a control serum, magnification 400x.

Only five (10.0%) of control sera showed the positive immunofluorescence pattern reaction to the retinal vessels in IIF test on normal monkey retina at titers ranging from 1:10 to 1:80. The verification carried out on monkey iliopsoas muscle and HUVEC, confirmed the presence of circulating AECA in all five (10.0%) control sera at titer 1:100.

Table 3 presents the distribution of AECA in the sera of patients with dry AMD and controls according to the immunofluorescence staining pattern and titer.

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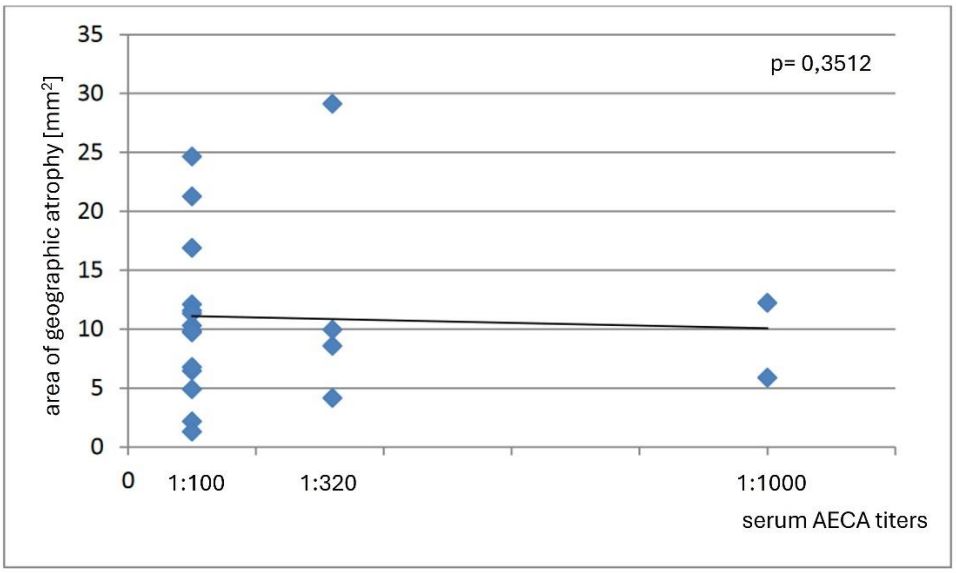
Table 3. Distribution of anti-endothelial cell antibodies (AECA) in the sera of patients with dry age-related macular degeneration (AMD) and controls according to the titer and immunofluorescence staining pattern on retinal tissue.

Type of immunofluorescence staining pattern	Study group	Titer				P value
		Negative	1:100	1:320	1:000	
Iliopsoas muscle, n (%)	AMD n=41	21 (51.2)	14 (34.1)	4 (9.8)	2 (4.9)	0.0000
	Control n=50	45 (90)	5 (10)	0 (0)	0 (0)	
HUVEC, n (%)	AMD n=41	28 (68.3)	8 (19.5)	3 (7.3)	2 (4.9)	0.0001
	Control n=50	45 (90)	5 (10)	0 (0)	0 (0)	

HUVEC –Human Umbilical Vein Endothelial Cells.

3.4. Circulating Anti-Retinal and Anti-Endothelial Cells Antibodies and Clinical Features of Dry AMD

In analyzed group of patients no correlation was found between immunofluorescence staining patterns of reactivity against retinal tissue, complexity and serum levels of circulating ARA and clinical features of dry AMD. Our observations revealed no association between serum levels of circulating AECA and area of geographic atrophy, either (p = 0,3512) (Figure 6).



AECA – anti-endothelial cell antibody

Figure 6. The graph shows no association between the area of geographic atrophy and serum anti-endothelial cell antibodies (AECA) titers in patients with dry age-relate macular degeneration.

The simultaneous occurrence of circulating ARA and AECA showed no differences with cases that sera showed to be positive only for ARA or AECA in terms of area of geographic atrophy.

4. Discussion

GA is a multifactorial disease in which intrinsic and extrinsic factors lead to RPE dysfunction. With age, RPE dysfunction progresses, leading to drusen and lipofuscin deposition [10,38]. Drusen and lipofuscin components, as well as other oxidative stress products, such as advanced glycation end products, were reported to induce inflammation through multiple pathways, including the complement cascade [38,39]. When regulatory components in these pathways are compromised, especially by genetic risk factors, initiation of chronic inflammation (para-inflammation) may lead to

 doi:10.20944/preprints202404.1565.v1

damage and ultimately retinal cell death, which constitutes a typical feature of GA [10]. The mechanism of the late stage of dry AMD is not fully understood, but inflammation and immune dysregulation are believed to play a significant role in its pathogenesis [40]. Pathological mechanisms initiating excessive apoptosis of photoreceptors in the course of AMD have also been implicated as one of the potential pathogenetic factors in this multifactorial condition [41].

In the current study circulating ARA were detected at significantly higher frequencies and at higher titers in patients with GA as compared with controls. There are few data in the literature on the prevalence of serum ARA in patients with dry AMD; however, most of those studies reported autoantibody analysis for both wet and dry AMD [32,42]. Recently, there have been more studies investigating the presence of ARA in the serum of patients with exudative AMD. The results of these studies revealed the presence of ARA in 46% to 95.9% of patients with exudative AMD [35,43–46]. This discrepancy in the occurrence of circulating ARA may result from the use of different diagnostic methods, different dilutions of the tested sera and may also be related to the heterogeneity of the studied groups, and differences in the type and the stage of the disease. The previous studies revealed the associations between the serum ARA levels and the stage of the disease and moreover treatment with intravitreal VEGF inhibitors led to decrease in the titers of these autoantibodies [35]. Considering that exudative and dry AMD are two forms of the same condition, it seems that the effect of ARA is still insufficiently understood in patients with dry AMD. Korb et al. [36] described ARA in patients with dry AMD and found that they occurred more frequently as compared to healthy population, however the study did not analyzed the association with the stage of the disease, thus it is difficult to compare those results with our observations. In our study serum ARA were detected more frequently in individuals with GA than in controls without AMD, suggesting their involvement in the development of the disease. Our results may support the hypothesis that circulating ARA may play some role in GA pathogenesis. Since it has been shown that circulating ARA may appear 3 to15 years prior to clinical manifestation of the disease, it seems to be reasonable for further research and to investigate their role in the pathogenesis and the progression of GA in patients with dry AMD [47,48].

To the best of our knowledge, the current study is the first to report the presence of circulating AECA in patients with dry AMD. We found significantly elevated serum expression of AECA in patients with GA. Studies on the association between circulating AECA with dry AMD appear to be limited. Machalinska et al. [49] reported circulating endothelial cells (CECs) in patients with wet and dry AMD, but serum AECA levels were not assessed. Their study indicated that AMD is accompanied by endothelial dysfunction. Increased serum CECs counts in patients with AMD reflect severe vascular abnormalities and may be involved in the pathogenesis of the disease. The authors also emphasized the need to search for a common pathological mechanism for AMD and systemic vascular diseases.

AECA were first described by Lindqvist and Osterland in 1971 [50]. These autoantibodies were reported in several autoimmune diseases associated with vasculitis, such as systemic lupus erythematosus (SLE), systemic scleroderma (SSc), rheumatoid arthritis, Kawasaki disease, granulomatosis with polyangiitis (GPA), multiple sclerosis and diabetes mellitus [22,51,52]. However, the presence of AECA is not a disease-specific marker but can play an important role in monitoring disease activity, and can also be used to assess the risk of recurrence or complications [52]. In patients with SSc there was no correlation with AECA and SSc activity, but the authors suggested that the presence of AECA might indicate vascular complications in SSc [51]. In some reports, it has been suggested that AECA may be a good predictor of relapses in patients with small vessel vasculitis [53].

It has been hypothesized that AECA occur in response to endothelial cell injury. However, it is also possible that they themselves have cytotoxic activity, and their presence may be inherent to the destructive inflammatory process [47,52,54]. The cytotoxic activity of AECA can affect endothelial cells through complement-dependent mechanisms or as antibody-dependent cell-mediated response [54]. Some publications suggested that AECA, by inducing the overexpression of adhesion molecules (including selectins, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1) and

 doi:10.20944/preprints202404.1565.v1

the production of cytokines and chemokines, may lead to endothelial damage through adhesion to endothelial cells. An autoimmune basis of vasculitis is therefore suggested [52,55,56]. AECA were shown to induce apoptosis of progenitors of bone marrow endothelial cells, which leads to disturbances in endothelial regeneration and healing of vascular lesions [57]. Some studies also suggested that AECA may exert pathogenic effects by activating endothelial cells to induce a procoagulant phenotype and subsequent thrombosis [56,58–60].

The results of our study revealed significant differences in the occurrence of circulating AECA in patients with dry AMD and controls; these autoantibodies were detected more frequently and at higher titers in patients than in controls. Our previous study in patients with wet AMD also showed significant differences in the prevalence of AECA between the study group and healthy subjects, while there was no association between the disease advancement and average serum AECA titers [37]. These observations are in accordance with the results revealed in a current research that showed no association between AECA titers and the clinical activity of the disease assessed as an area of GA.

The described mechanisms of initiation and progression of tissue damage related to the presence of circulating AECA cannot be excluded in the course of dry AMD. However, further studies are needed to determine whether AECA are involved in the pathogenesis of dry AMD or whether they occur secondary to endothelial cell damage in the course of the disease.

5. Conclusions

Our findings are important in light of previous research suggesting that AMD is an immune-mediated inflammatory disease. While AECA are probably involved in the pathogenesis and progression of AMD, it cannot be excluded that their presence is secondary to preexisting retinal damage. Further research is needed to clarify the clinical and pathological significance of AECA in dry AMD, which may have important implications for future treatment of this sight-threatening disease.

Author Contributions: Conceptualization, A.K.-T.; Data curation, K.Ż.-Ł., and W.P.-M.; Formal analysis, .K.Ż.-Ł., I.K.-B., B.R.-D., and A.K.-T.; Investigation, J.W., I.K.-B.,W.P-M., and M.S.; Methodology, J.W., M.S., and A.K.-T.; Project administration, K.Ż-Ł.; Resources, B.R.-D.; Software, K.Ż.-Ł.; Supervision, M.S., and A.K.-T.; Validation, W.P.-M., and B.R.-D.; Visualization, K.Ż-Ł.; Writing – original draft, K.Ż.-Ł, A.K.-T., and I.K.-B.; Writing – review & editing, A.K.-T., and B.R.-D.; Funding acquisition, K.Ż.-Ł. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Jagiellonian University (protocol code 1072.6120.37.2019, dated from 31st January 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The patients signed an institutional informed consent for the use of medical records and the publication of this information for research purposes.

Data Availability Statement: Data supporting the findings of this study are available upon request from the corresponding author.

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

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