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PEDF and VEGF plasma level alterations in patients with dry form of age-related macular degeneration – a possible link to the development of the disease

Zaburzenie osoczowego poziomu PEDF i VEGF u pacjentów z suchą postacią zwyrodnienia plamki związanego z wiekiem – potencjalny czynnik patogenetyczny w rozwoju choroby

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Streszczenie: **Cel:** celem pracy jest ocena poziomu i wzajemnych interakcji czynnika wywodzącego się z nabłonka barwnikowego (PEDF) oraz czynnika wzrostu śródbłonka naczyniowego (VEGF) we krwi obwodowej pacjentów w przebiegu suchej i wysiękowej postaci zwyrodnienia plamki związanego z wiekiem (AMD).
Materiał i metody: badaniem objęto 31 pacjentów z suchą postacią AMD oraz 46 pacjentów z wysiękową postacią AMD. Grupę kontrolną stanowiło 46 zdrowych ochotników bez zmian na dnie oka, pacjenci z obu grup byli w podobnym wieku. Poziom PEDF oraz VEGF w osoczu oznaczano metodą ELISA.
Wyniki: poziom osoczowego PEDF okazał się istotnie niższy w grupie pacjentów z suchą postacią AMD – według porównania tego parametru w grupach kontrolnej i z wysiękową postacią schorzenia. Na podstawie analizy wieloczynnikowej wykazano, że sucha postać AMD stanowi niezależny czynnik związany z niższym stężeniem PEDF w osoczu (ß = -0,34; p = 0,026). W grupie z wysiękową postacią schorzenia stężenie VEGF we krwi obwodowej istotnie korelowało ze stężeniem PEDF (Rs = +0,63; p = 0,002). Ponadto wyższe stężenia zarówno PEDF, jak i VEGF obserwowano w osoczu pacjentów manifestujących obustronną neowaskularyzację naczyniówkową.
Wnioski: zaburzenia wzajemnych interakcji między czynnikami hamującymi proces angiogenezy w przebiegu AMD a stymulującymi go mogą stanowić element istotnie przyczyniający się do rozwoju obu postaci schorzenia – i suchej, i wysiękowej.

Słowa kluczowe: zwyrodnienie plamki związane z wiekiem, neowaskularyzacja naczyniówkowa, czynnik wywodzący się z nabłonka barwnikowego, czynnik wzrostu śródbłonka naczyniowego.

Summary: **Purpose:** The aim of the study is to explore the interaction between stimulators and inhibitors of angiogenesis by measuring pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) plasma levels in patients with the wet and dry forms of age-related macular degeneration (AMD).
Material and methods: Forty-six subjects with the wet form, 31 with the dry form of AMD as well as 47 non-AMD healthy controls were enrolled in the study. Plasma concentrations of VEGF and PEDF were measured using ELISA test.
Results: A significant decrease in the PEDF plasma level in patients with the dry form of AMD was found. Multivariate analyses of patients and controls adjusted for age, sex, smoking, and concomitant vascular diseases as independent variables revealed that the dry form of AMD was the only independent factor associated with lower plasma PEDF levels (ß = -0.34; p = 0.026). On the contrary, in the wet AMD group, a strong positive correlation between VEGF and PEDF concentrations was observed (Rs = +0.63; p = 0.002), and significantly higher PEDF and VEGF plasma levels in patients with bilateral manifestations of the disease were also found.
Conclusions: These findings suggest that different manifestations of AMD, i.e. the dry and wet forms, may be associated with various altered concentrations of counterbalancing stimulators and inhibitors of the angiogenesis process.

Key words: age-related macular degeneration, choroidal neovascularization, pigment epithelium-derived factor, vascular endothelial growth factor.

Introduction

Age-related macular degeneration (AMD) is one of the leading causes of irreversible vision loss among people 65 years of age and older in the western world (1). It represents a degenerative and progressive condition involving the retinal pigment epithelium (RPE), Bruch's membrane and the choriocapillaris. The dise-

ase has been broadly classified into two clinical states: a wet, exudative form and a dry, atrophic form. The most common dry type is characterized by drusen formation together with the presence of RPE defects and local retinal atrophy in the macular region. In the wet form of AMD, which is more severe, blood vessels grow out from the choroid behind the retina, leading to irreversible vision loss in a very short time. The resulting choroidal neovascularization (CNV) is accompanied by signs of subretinal leakage, local haemorrhaging and the proliferation of a number of retinal cell types (2).

Despite the fact that our understanding of molecular events presaging AMD has grown over the last decade, the pathogenesis of AMD has not yet been completely defined. Numerous pathogenic theories, as well as multiple factors contributing to disease progression, e.g. genetic predispositions, oxidative stress and choroidal hypoperfusion in the macular region, suggest that AMD is probably a disease of multifactor origin (3). There is evidence to suggest that the precise balance between stimulators and inhibitors of angiogenesis, such as vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), respectively, is essential for maintaining the homeostasis in the human retina. The presence of ocular angiogenesis is believed to be controlled by this regulatory interaction between two counterbalancing systems that stimulate or inhibit new vessels formation. The balance between these two factors may play a key role in the formation of CNV. In order to initiate angiogenesis, the equilibrium between the positive and negative regulators is likely to be shifted in such a way that mitogenic factors become enhanced or inhibitory factors become decreased (4).

In this study, we sought to explore this issue by measuring PEDF and VEGF plasma levels in patients with wet and dry forms of AMD.

Material and methods

Characteristics and selection of the study groups

The study group included 77 patients with AMD who were diagnosed and treated at the Ophthalmology Department of Pomeranian Medical University of Szczecin. 46 age- and sex-matched volunteers without AMD (defined as the absence of drusen, pigmentary abnormalities and neovascularization), were enrolled as the control group.

All of the enrolled subjects underwent a complete ophthalmic examination, i.e. visual acuity assessment, intraocular pressure measurements, and dilated fundus examinations using slit-lamp biomicroscopy. In all cases, 30° colour stereo fundus photographs of the macular region of both eyes were taken. The AMD phenotypes were characterized by fluorescein angiography as well as by optical coherence tomography imaging. According to AMD type, the patients were divided into two subgroups: 1) 'exudative, wet AMD' (46 subjects) characterized by serous or haemorrhagic retinal pigment epithelium detachment, choroidal neovascularization, subretinal haemorrhaging and a fibrous scar in at least one eye; 2) 'dry, atrophic AMD' (31 subjects) defined as the presence of changes in the retinal pigment epithelium including hypopigmentation, depigmentation or the absence of the RPE with visible choroidal vessels with soft or reticular drusen. The wet form of AMD was considered to be advanced when the entire lesion diameter exceeded 3 disc diameter (together with retinal oedema, fibrous scars or haemorrhaging areas). In patients with atrophic AMD the early stages were defined as the presence of single small drusen and local extrafoveal areas of RPE and/or retinal atrophy. Advanced stage of atrophic AMD was diagnosed in patients who presented large, confluent soft drusen or/and geographic atrophy affecting the foveal centre.

Patients who had previously undergone laser or intravitreal treatment were excluded from the study. In cases where different stages of the disease were diagnosed in both eyes the patient was categorized according to the severity of lesions in the worse eye. The data regarding medical history and smoking were collected and special attention was paid to arterial hypertension and pre-existing cardiovascular and cerebrovascular conditions.

Exclusion criteria included significant chronic systemic conditions, for example collagen or neoplastic disease, diabetes mellitus, renal failure and hepatic dysfunction, as well as any evidence of retinal disease with the exception of AMD (in AMD groups), i.e. glaucoma, intraocular inflammatory diseases or recent (within 3 months) ocular surgery.

The study adhered to the tenets of the Declaration of Helsinki and approval was obtained from the Local Research Ethics Committee. Moreover, each patient gave written informed consent for the involvement in the study.

Laboratory assays

Venous blood was collected from all subjects in tubes containing EDTA as an anticoagulant. The plasma was separated by centrifugation (15 min, 2000 g, 20° C), within 2 hours after collection, divided into aliquots and frozen at -70° C until the assays were performed. Plasma concentrations of human VEGF and PEDF were measured using commercially available enzyme-linked immunosorbent assays (ELISA), Quantikine human immunoassays (R&D Systems, Minneapolis, MN, USA) for VEGF and the Chemikine PEDF ELISA Kit (Millipore, Temecula, CA, USA) for PEDF, according to the manufacturer's protocol. The results were analysed using a log-log quadratic curve fit.

Statistical analysis

Non-parametric tests were used because the distributions of the analysed parameters were significantly different from normal distributions (Shapiro-Wilk test, $p<0.05$). Differences between the analysed groups of patients were tested by the Kruskal-Wallis test followed by the Mann-Whitney test for quantitative variables and a chi-square test for qualitative variables. Associations between the level of plasma proteins and the selected parameters (age, sex, smoking, coexisting hypertension, ischaemic heart disease and history of stroke), were evaluated using Spearman's rank correlation coefficient for age and a Mann-Whitney test for the other variables. Multiple linear regression was used for the multivariate analysis. The VEGF and PEDF concentrations were normalized by logarithmic transformation before their inclusion in the multivariate models as dependent variables. A p-value of $p<0.05$ was considered statistically significant.

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Results

Characteristics of study subjects

The characteristics of the patients and the controls are summarized in table I. The AMD and control groups were matched for age and gender, as well as the selected well-known AMD risk factors, including hypertension, history of ischaemic heart disease and stroke. The rate of former smokers was significantly higher in both atrophic and wet AMD groups compared to the control group ($p = 0.03$); however no difference in current smokers between the AMD and control groups was observed ($p = 0.13$). In the wet AMD group a positive association between age and lesion diameter as well as between age and bilateral CNV was noted. Patients with bilateral CNV and a larger diameter of the macular lesion were significantly older compared to those with monocular manifestation of AMD (median: 78 *vs* 71 years; $p = 0.020$) and with a smaller CNV area (median: 76 *vs* 69 years; $p = 0.014$).

VEGF plasma concentrations

There were no significant differences in VEGF plasma levels between the study and control groups (fig. 1a). However, significantly higher VEGF plasma levels were found in patients with a bilateral manifestation of CNV (median: 173 *vs* 83 pg/ml; $p = 0.050$). The plasma levels of VEGF were higher in females in all of the groups with the differences being significant in the control group (median: 185 *vs* 85 pg/ml; $p = 0.022$) and borderline significant in the wet AMD group (median: 133 *vs* 67 pg/ml; $p = 0.052$). Interestingly, in the control group, lower levels of VEGF were observed in patients with arterial hypertension (median: 96 *vs* 191 pg/ml; $p = 0.036$). Similarly, lower VEGF plas-

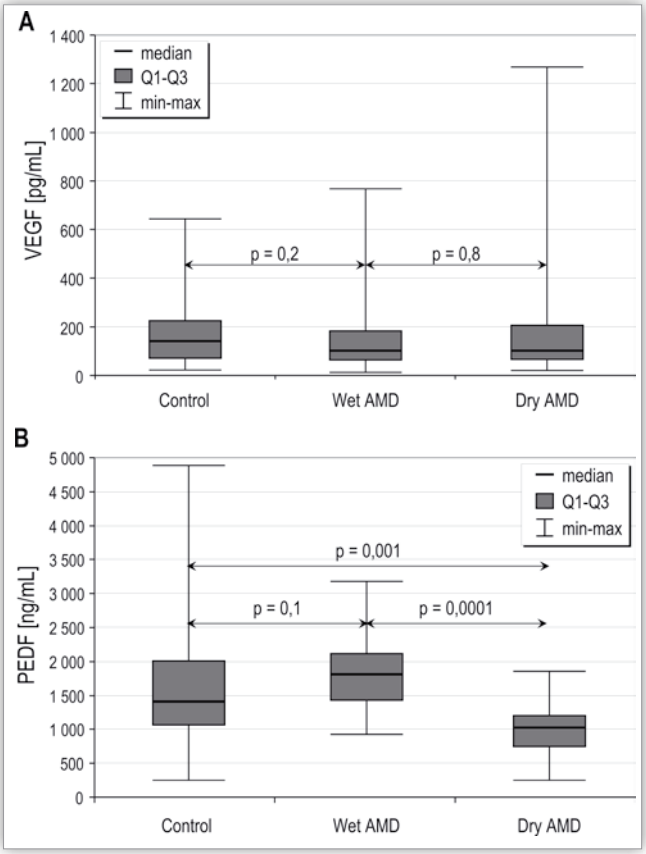

Fig. 1. VEGF (panel A) and PEDF (panel B) plasma concentrations in the study groups. Q1-Q3 – upper and lower quartiles.

Ryc. 1. Poziom VEGF (panel A) i PEDF (panel B) w osoczu u pacjentów z poszczególnych badanych grup. Q1-Q3 – kwartyl górny i dolny.

	Exudative AMD/ Postać wysiękowa AMD	Dry AMD/ Postać sucha AMD	Control group/ Grupa kontrolna	p*
Number of subjects/ Liczebność	46	31	46	
Sex (male/ female)/ Płeć (mężczyźni/ kobiety)	23/ 23	15/ 16	13/ 33	0.07
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Patient's age (years)/ Wiek pacjenta (lata)	71.98 ± 8.76	69.06 ± 8.5	72.7 ± 8.29	0.19
	%	**%**	**%**	
Bilateral CNV/ Obustronna CNV	30			
Advanced stages/ Zmiany zaawansowane	54	58		0.82
Smoking status/ Palenie tytoniu				
– current/ aktualnie	11	19	4.5	0.13
– past/ w przeszłości	62	68	39	**0.03**
Hypertension/ Nadciśnienie tętnicze	51	61	64	0.41
History of ischemic heart disease/ Choroba niedokrwienna serca w wywiadzie	16	35	33	0.11
History of stroke/ Udar mózgu w wywiadzie	2	6	4	0.7

Tab. I. Clinical characteristics of the study groups.

Tab. I. Charakterystyka kliniczna badanych grup.

* test $\chi 2$ for qualitative variables and Kruskal-Wallis ANOVA for quantitative variables were used/
*test $\chi 2$ dla zmiennych nominalnych i ANOVA Kruskala-Wallisa dla zmiennych mierzalnych

ma concentrations were detected in patients with a history of smoking (median: 122 pg/ml for past smokers *vs* 227 pg/ml for non-smokers, p = 0.008). No correlation was found between VEGF levels and age or history of angina and stroke. Multiple linear regression analysis adjusted for age, gender, presence of coronary artery disease, arterial hypertension, smoking (current or past) and presence of the dry form of AMD (or its absence in the control group) as independent variables showed that hypertension and smoking were independent determinants of lower VEGF plasma levels (β = -0.24; p = 0.043 for smoking and β = -0.31; p = 0.012 for hypertension). Interestingly, an older age predicted higher VEGF plasma levels (β = +0.27; p = 0.024). A similar model adjusted for the wet form of the disease and controls only revealed smoking as a predictor of lower VEGF plasma levels (β = -0.22; p = 0.067). Neither the dry nor the wet form of AMD was found to be independent predictor of plasma VEGF concentration.

PEDF plasma concentrations

Analysis of plasma PEDF levels showed significantly lower levels in the atrophic AMD group when compared to the control group (p = 0.0018) and to the wet AMD group (p<0.0001), and no significant difference was found between the wet AMD group and the controls (fig. 1b). Multivariate analyses of patients and controls adjusted for age, sex, arterial hypertension and current or previous smoking as independent variables revealed that the atrophic type of AMD was the only independent factor associated with lower plasma PEDF concentrations (β = -0.34; p = 0.026). As with VEGF, higher levels of PEDF were found in patients with a bilateral manifestation of CNV (median: 2643 ng/ml *vs* 1756 ng/ml; p = 0.040). No association between PEDF level and age, gender, arterial hypertension, history of angina or stroke was found in any of the study groups.

Correlations between VEGF and PEDF plasma levels

Further analysis showed a strong positive correlation between VEGF and PEDF concentrations in the wet AMD group (Rs = +0,64; p = 0.0026) (fig. 2). A similar positive correlation, although borderline significant, was observed in the control group (Rs = +0.32; p = 0.085). No correlation between the two analysed parameters was found in the dry AMD group.

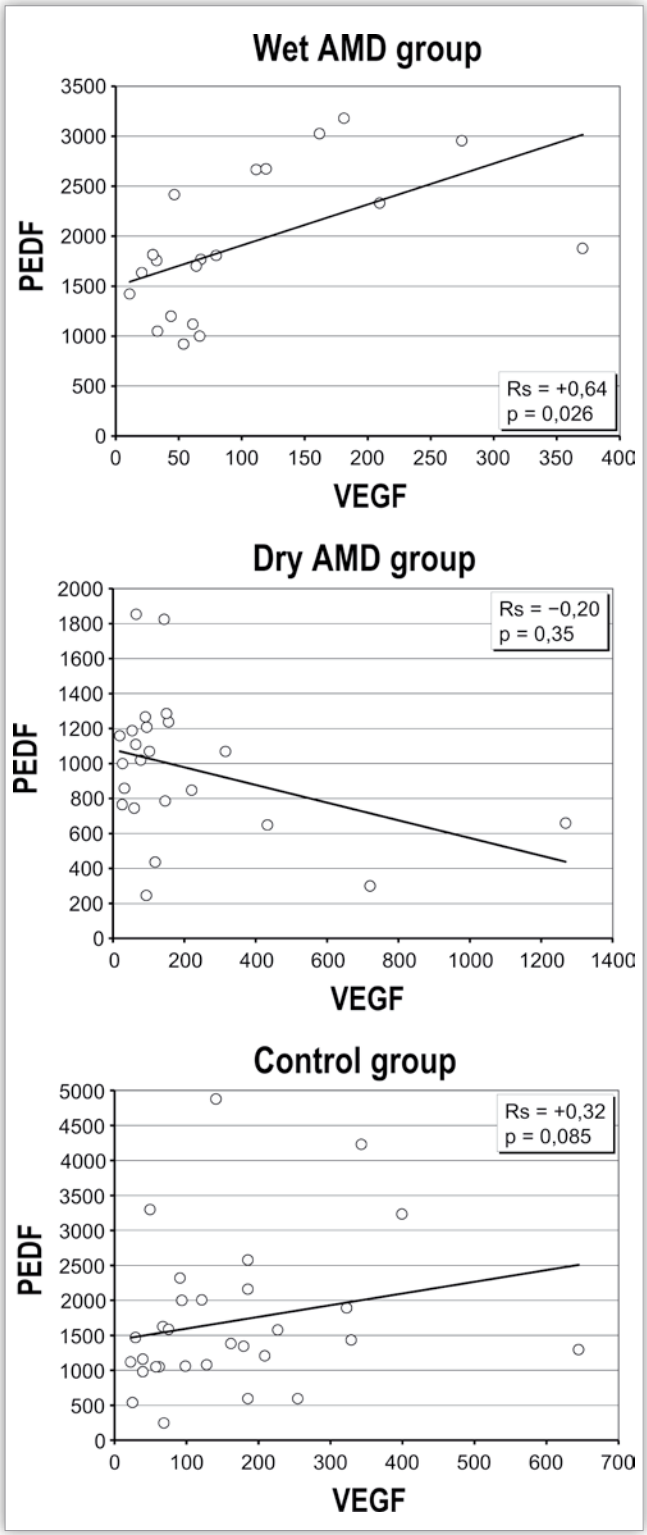

Fig. 2. Correlation between VEGF and PEDF plasma concentrations in the study groups. R – Spearman's rank correlation coefficient.
Ryc. 2. Analiza korelacji zmian stężeń VEGF i PEDF w poszczególnych badanych grupach. R – współczynnik korelacji rang Spearmana.

Discussion

VEGF is an endothelial cell mitogen and vaso-permeability factor, and its expression is induced by hypoxia in retinal cells (5). Increased VEGF levels were detected in the retina and vitreous of patients with ischaemic ocular neovascular disorders, as well as in animal models of ischaemia-induced retinopathy and retinal vein occlusion (6). As reported by previous investigations, VEGF concentrations in peripheral blood were found to be elevated in patients with exudative AMD (7,8). The results of different studies on VEGF expression in AMD patients are conflicting, however. Both our results and studies by Thill et al. showed no significant differences between plasma VEGF levels in the AMD and healthy control groups (9). Furthermore, the results of the study by Duh et al. yielded evidence to show that VEGF vitreous levels were at or below the level of detectability in the choroidal neovascularization group (10). This may indicate that the local secretion of VEGF in hypoxic retinas may not influence its systemic concentration. Moreover, the possibility that other causative factors could determine or influence the elevated plasma VEGF levels observed by other authors cannot be excluded. According to our multivariate analyses, the VEGF

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plasma level depends on a variety of systemic interactions, i.e. vascular hypertension, cigarette smoking and age.

On the other hand, the pigment epithelium-derived factor displays a potent neuroprotective, neurotrophic and antiangiogenic activity. The generally held belief is that RPE cells are the main producers of PEDF, which is released towards the neural retina into the interphotoreceptor matrix, as a diffusible factor (11). Although PEDF levels in the aqueous humour of eyes with chorioretinal atrophy associated with high levels of myopia have been reported to be low, PEDF levels in patients with the dry form of age-related maculopathy have not been determined (12). In the present study, we addressed this issue and revealed the plasma PEDF concentration to be significantly decreased in the peripheral blood of patients with the dry form of AMD compared to patients with the wet form of the disease and the controls. Thus, it is possible that the reduced levels of PEDF in these patients resulted from the degeneration of retinal pigment epithelium cells, which are the main sources of this factor in the eye. Indeed, it is widely recognized and generally accepted that the wet form of AMD follows and arises from its dry form. Since PEDF is the most potent natural inhibitor of angiogenesis, it seems reasonable to suppose that the loss of PEDF creates a permissive environment for CNV development in patients with dry AMD.

Nevertheless, the status of intraocular VEGF and PEDF in ocular angiogenesis and CNV formation is still elusive. It has been proposed that accumulated oxidative stress inhibits the synthesis and secretion of PEDF but not VEGF in adult RPE cells, leading to an equilibrium shift between the molecules and giving a selective advantage to the angiogenic-promoting activity of VEGF (13). However, experimental evidence to date does not support the contention that the ratio of PEDF and VEGF is a permissive event in the generation of ocular neovascularization. While some studies have shown decreased levels of PEDF in ocular tissues accompanying various types of neovascularization (14-16), many studies have shown a simultaneous increase in VEGF and PEDF during ocular blood vessel formation (17,18). It was recently demonstrated that VEGF expression in choroidal neovascular membranes positively correlated with the expression of PEDF, and that the expression of both factors was dependent on the activity of neovascularization. The positive correlation (expression-expression) between these two factors, as observed in our study, may indicate their synergistic effect on endothelial proliferation. Our results echoed the findings of Tong et al., who detected an analogous association between both growth factors and found that both PEDF and VEGF were in parallel secreted to aqueous humour of patients with active CNV (16). Furthermore, the results of our study show that the severity of retinal lesions affects the total expression of factors since the levels of VEGF and PEDF were found to be significantly increased in the peripheral blood of patients with a bilateral manifestation of CNV.

There may be possible explanations for these observations. The amount of PEDF secreted by retinal cells positively correlated with oxygen concentrations, which means that its production is augmented by hyperoxia and decreased by hypoxia (19). Consequently, its increase in CNV lesions may be a response to increased neovascularization, providing higher oxygen concentrations. Moreover, VEGF appears to upregulate the secretion of PEDF in an autocrine manner. It has been shown that VEGF secreted by retinal pigment epithelium cells stimulates PEDF expression via the VEGFR-1 receptor, and that PEDF regulates angiogenesis and cell proliferation through a negative feedback mechanism (20). The function of PEDF in cell survival may promote the growth of CNV stroma cells and thus stabilize the CNV membrane to inhibit its further expansion (21). It has been shown that PEDF immunoreactivity in the eyes of AMD patients was the most intense in disciform scars. This observation strongly supports the notion that the equilibrium between PEDF and VEGF could be shifted towards PEDF in late CNV to prevent further expansion of CNV vasculature (22). According to other theories, PEDF was found to cause opposing effects on CNV and endothelial cell function: low doses are inhibitory, but high doses can augment the development of neovascularization. Such opposing effects may be explained by differential PEDF binding and dissociations with its multiple receptors with different affinities (23). Nevertheless, the complete elucidation of all regulatory interactions between stimulators and inhibitors of angiogenesis in the course of AMD requires further studies.

In conclusion, we found a significant decrease in PEDF plasma levels in patients with the dry type of AMD, which supports the speculation that local RPE disturbances are reflected in systemic oscillations of PEDF plasma levels. Furthermore, the strong positive correlation between the concentrations of VEGF and PEDF in the wet form of the disease and their parallel relationship with disease severity suggest their synergistic interaction in CNV formation. Our findings indicate that different manifestations of AMD, i.e. the dry and wet forms, may be associated with various altered concentrations of the counterbalancing stimulators and inhibitors of angiogenesis. Since the effectiveness of therapeutic approaches is still not satisfactory, the complete elucidation of all pathophysiological mechanisms responsible for the development of AMD seems to be of great importance.

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