PROTEOMICS OF PATIENTS WITH NEOVASCULAR AGE-RELATED MACULAR DEGENERATION RESISTANT TO ANTI-VEGF.

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RESUME

Age-related macular degeneration (AMD) is among the world's leading causes of blindness. The neovascular form presents deterioration of visual acuity and persistence of the disease in a quarter of the treated patients. Thus, there is a need for studies that elucidate the molecular pathways that act in choroidal neoangiogenesis beyond the vascular and endothelial growth factor (VEGF). The research is a cross-sectional study of a translational nature to assess the aqueous humor protein profiles of 25 patients divided into three groups: group 1 formed by patients with neovascular AMD treatment naive (called naive), who are accompanying posterori demonstrated good response to anti-VEGF injections; group 2 formed by patients with neovascular AMD resistant to anti-VEGF and group 3 formed by control patients, without systemic diseases or signs of retinopathy. We used mass spectrometry (label-freeLC-MS / MS) to make the proteomic characterization of the groups. A total of 2,336 proteins were identified, of which 185 were distinctly regulated and allowed differentiation of clinical conditions. Among these, thirty-nine proteins, including some new ones, were functionally analyzed as important effectors of lipid metabolism, oxidative stress, complement system and / or inflammatory pathways and angiogenesis. Thus, this study consolidates the understanding about the participation of other pathways in the pathophysiology of macular neovascularization and potential possible biomarkers, in addition to the known vascular and endothelial growth factor (VEGF).

INTRODUCTION

Age-related macular degeneration (AMD) will reach 288 million in 2040 (1) and is currently the leading cause of severe visual loss in patients over 50 in industrialized countries (1–5). The most aggressive form of AMD is neovascular, whose antiangiogenic (anti-VEGF) therapy is considered its gold standard treatment (6–8). However, a deterioration in visual acuity and persistence of the disease is observed in a quarter of the treated patients (9).

Therefore, there is a certain urgency in the molecular characterization of this disease that is able to provide more reliable information about the phenotype, its pathophysiology and resistance to standard therapy, corroborating with the identification of possible biomarkers of early diagnosis and new therapeutic targets, even if adjuvant (10).

PATIENTS AND METHODS

This is an experimental study whose aqueous humor samples stored at -80 ° C were analyzed by a DionexUltimate 3000 nano-UPLC chromatographic system (Thermo Fisher Scientific) coupled to an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific).

The initial sampling effort was 250 patients and the 25 subjects included in the research were separated into 3 groups: (i) patients with neovascular AMD who were naïve for treatment, called naive, who, in follow-up to posterori, demonstrated a good response to anti-VEGF; (ii) patients with neovascular AMD resistant to anti-VEGF (respecting AMOAKU criteria, 2015) (11) and (iii) control group composed of patients with cataracts, without systemic pathologies or underlying retinal disease.

In this study, for resistant individuals, we used criteria of poor response or lack of response to at least 06 monthly applications of anti-VEGF. Data analysis was performed with the programs ProgenesisQI, MetaboAnalyst and the String platform. The research was approved by Code of Ethics and Research / Platform Brazil, with CAAE 64921317.1.0000.5667.

RESULTS

A total of 2336 proteins were identified and of these, 185 allowed the distinction between the three groups analyzed in the research (Figure 1). 39 proteins correlate with visual function or with metabolic pathways directly or indirectly linked to choroidal neoangiogenesis, an essential condition for neovascular AMD (Table 1).

A posteriori statistical potency analysis showed that 5 patients per group have already demonstrated statistical robustness, reaching 86.4% of the samples with potency> 0.8.

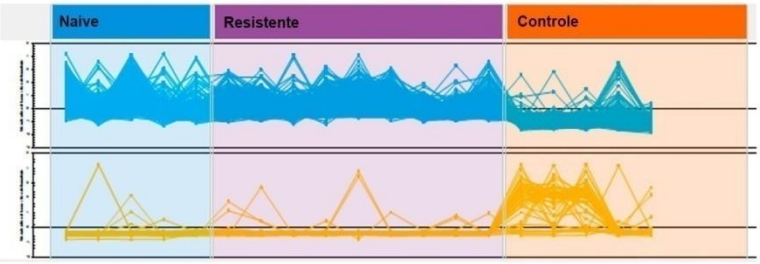

Figure 1: Profiles of relative abundance of grouped proteins. Each line represents a protein and each peak or valley, its abundance per patient. Proteins (A) have a predominance of positive regulation in cases of naive AMD and resistant AMD and a predominance of negative regulation in controls. The opposite in (B).

Table 1: Selected discriminant proteins by metabolic function related to the pathophysiology of AMD

	PROTEIN	FUNCTION	REGULATED POSITIVELY	REGULATED NEGATIVELY
VISUAL FUNCTION				
	Crystalline Alpha Chain B (P02511)	- visual perception - amyloid beta binder - response to hypoxia	Control	*Naive*
	Crystalline Beta A4 (P53673)	- visual perception	Control	*Naive*
	Crystalline Beta B1 (P53674)	- visual perception - lenticular development	Control	*Naive*
	Crystalline Beta 2	- visual perception	Control	Naive
	Crystalline Beta 3, IsoformCRA_a	- visual perception	Control	Naive
	Crystalline Gamma C (P0315)	- visual perception	Control	Resistant
	Matrix intercommotor of proteoglycan 1 (MIPG1)	- visual perception	*Naive*	Control
	Retinol Binding Protein 4 (RBP-4)	- eye development - response to retinoic acid	*Naive*	Control
LIPID METABOLISM				
	Apolipoprotein A-I, IsoformCRA_a	- lipid storage - biosynthetic lipoprotein process - negative regulation of lipase activity	Resistant	Control
	Apolipoprotein A-IV	- lipid homeostasis - lipid transport - removing superoxide radicals	Resistant	Control

	Phosphoinositide Phospholipase	- lipid catabolic process	Resistant	*Control*
	Retinol Binding Protein 3 (RBP-3)	- lipid metabolic process - visual perception	Resistant	*Control*
OXIDATIVE STRESS				
	Similar amyloid protein 11 (APLP1)	- oxidative stress - transition metal ion bond	*Naive*	*Control*
	Catalase (CAT)	- cellular response to oxidative stress - negative regulation of the apoptotic process	*Control*	*Naive*
	Enolase 1	- autoimmune stimulation	Control	Resistant
	Glutathione peroxidase (GPx)	- response to oxidative stress	Resistant	*Control*
	Lipocalin 1 (Tear prealbumin), IsoformCRA_a (LCN1)	- modulation of oxidative stress	Naive	Resistant
	Extracellular superoxide dismutase [Cu-Zn] (SOD)	- cellular response to oxidative stress - response to hypoxia	Resistant	*Control*
SYSTEM ACTIVATION COMPLEMENT				
	Heavy Chain MU Immunoglobulin	- adaptive immune response - B cell receptor signaling pathway - classic complement pathway activation - innate immune response - leukocyte migration - positive regulation of B cell activation	Naive	Resistant
	CFB	- complement activation	Control	Resistant
	Clusterine	- inflammatory response - Complement system regulation	Resistant	Control
	Complement C2	- complement activation	Resistant	Control
	Complement C3	- complement activation	Resistant	Control
	Complement C4-A	- complement activation -positive regulation of clearance (cleareance) of apoptotic cells	Resistant	Control
	Complement C7	- complement activation	Resistant	Control
	Complement C8 alpha chain	- complement activation	*Naive*	Control
	Complement Factor H protein related 1 (CFH1)	- complement activation - regulation of complement activation	*Naive*	Control
	Vitronectine	- regulation of complement activation - positive regulation of VEGF - constituent of the extracellular matrix - cell adhesion	Resistant	Control
INFLAMMATION				
	Monocytic differentiation CD14 antigen	- inflammatory response	Resistant	Control
	Heavy Chain MU Immunoglobulin	- adaptive immune response - B cell receptor signaling pathway - classic complement pathway activation - innate immune response - leukocyte migration - positive regulation of B cell activation	*Naive*	Resistant

	Plasma Kallikrein (KLKB1)	- inflammatory regulation - disassembly of the extracellular matrix - fibrinolysis - plasminogen activation	Resistant	Naive
	Clusterine	- inflammatory response - Complement system regulation	Resistant	Control
	Enolase 1	- autoimmune stimulation	Control	Resistant
	Pigment Epithelium Derived Factor (PEDF)	- negative regulation of angiogenesis - negative regulation of the inflammatory respon	*Naive*	*Control*
ANGIOGENESIS				
	Ectonucleotideopyrophospha tase / phosphodiesterase family - member 2 (ENPP2)	- regulation of angiogenesis	*Naive*	*Control*
	insulin-like growth factor protein (IGFBP-7)	- regulation of cell growth	*Naive*	*Control*
	insulin-like growth factor protein 7 (IGFBP-7), isoformCRA_a	- negative regulation of the insulin-like growth f signaling pathway	*Naive*	*Control*
	Pigment Epithelium Derived Factor (PEDF)	- negative regulation of angiogenesis -negative regulation of the inflammatory respons	*Naive*	*Control*
	Tissue Inhibitor Metalloproteinase 1 (TIMP1)	- regulation of angiogenesis	Resistant	*Control*
	Metallothionein - 1G	-cellular response to stimulation of vascular and growth factor -negative growth regulation	*Control*	*Naive*
	Vascular Endothelial Growth Factor Receiver 1 (VEGFR-1)	- endothelial vascular growth factor receptor sigı pathway - regulation of angiogenesis	Resistant	Naive
	Tyrosine kinase receptor, isofor (KDR)	- signaling pathway of the factor receptor endothelial vascular growth	Resistant	Naive
	Vitronectine	- complement regulation - positive regulation of VEGF - constituent of the extracellular matrix - cell adhesion	Resistant	Control
	Ubiquitin hydrolase carboxy-terminal	- response to ischemia	Control	*Naive*

* Thirty-nine different proteins correlated with visual function or with metabolic pathways directly or indirectly linked to choroidal neoangiogenesis. The repetition of some proteins is due to some having more than one of the selected cellular functions. Proteins with statistical significance (Anova p <0.005) comparing the naive, resistant and control groups with each other.

DISCUSSION

In addition to the VEGF pathway, some alternative pathogenic AMD pathways are being studied, with choroidal neoangiogenesis already correlated with oxidative stress, activation of the complement system, activation of the immune system and lipid metabolism (12–14).

Crystallines, the main components of the lens, also act in the regulation of astrocytes, remodeling of retinal vessels (15), regulation of apoptosis (16) and clearance of the outer segments of the photoreceptors by RPE (17). In neovascular AMD, numerous autoantibodies and immune complexes against crystalline alpha and beta have been demonstrated in the retina and choroid (18). Alpha-crystallines are expressed in cytosol and mitochondria of RPE cells, protecting them from oxidative stress. They also function as modulators of angiogenesis, VEGF and chaperone proteins derived from α-crystallines also function as inhibitors of oxidation-induced cell death (19). The crystalline beta proteins and the proteoglycan matrix matrix 1, also found in this research and which are involved in visual perception, allowed the grouping of replicates and the distinction between the 3 research groups. The literature lacks studies that consistently correlate these

proteins with the pathophysiology of AMD. Retinol-binding protein 4, in addition to annotations for Gene Ontology (GO term) involving visual perception and retinol metabolism, has also been described as a potential target in the treatment of atrophic AMD and Stargardt's disease (20).

Druses correspond to an accumulation of cellular debris including proteins, minerals and lipids between Bruch's membrane and the RPE (21). And the lipid accumulation at this site is considered an early event in the pathophysiology of AMD (22). Thus, in senile patients, oxidized lipoproteins and lipids are early triggers of AMD (23,24).

Thompson et al. (2015) propose a new mechanism for the formation and growth of druses. Such researchers confirmed the presence of calcium phosphate (PAH) in hollow spheres containing cholesterol in all sub-RPE deposits in the macula and periphery. By immunohistochemistry, they proved that innumerable proteins as a complement factor H, vitronectin and beta amyloid coated such spheres. The production and secretion of cholesterol-containing lipids and lipoproteins, secreted at least in part by the RPE (25), are recruited and retained in the aged Bruch's membrane (26,27) which sequentially, in a self-directed oligomerization process, facilitates depositions additional proteins leading, ultimately, to the formation and growth of druses (23). Studies also make an analogy between oxidation of lipoproteins in Bruch's membrane with atherosclerosis (23,24,28–30).

Druses act as a barrier between the choriocapillaries and the photoreceptors, limiting the oxygenation and nutritional support of the cones and rods, generating local hypoxia, a fundamental agent in the pathophysiology of AMD (31). The ubiquitin-proteasome system is one of the main components responsible for the degradation of proteins damaged or unnecessary to cellular metabolism produced by the heterophagy of the outer segments of the photoreceptors (32). Carboxy-terminal ubiquitin hydrolase was found to be positively regulated in controls and negatively regulated in the group with naïve treatment AMD. This finding is in agreement with researchers such as Gleen and collaborators (2012) (33).

The present research found numerous proteins frequently associated with lipoprotein particles, including complement factor H, amyloid-like protein 1 and vitronectin, which were highlighted by Thompson et al. (2015) in the pathophysiology of AMD. The GO descriptions also correlate vitronectin as a constituent of the extracellular matrix, with complement regulation, positive regulation of VEGF and cell adhesion. The AI and A-IV isoforms of apolipoprotein were also positively regulated in patients with resistant AMD, corroborating the findings of Levy et al. (2015), who demonstrated that apolipoprotein is a factor that promotes the survival of sub-retinal mononuclear phagocytes. and, therefore, a stimulator of chronic inflammation in AMD (34). Phosphoinositidephospholipase C and Protein 3 Retinol ligand (RBP-3) that are related to lipid metabolism were also found in the analyzed samples regulated positively in the resistant group and negatively in the control group. Morohoshi and collaborators (2012) identified anti-retinal antibodies against RBP-3 in patients with neovascular AMD (35).

The association between oxidative stress and age-related disorders has been extensively documented, such as Alzheimer's disease, Parkinson's disease, atherosclerosis, certain types of cancer and AMD (36–38). For comparative effect, the retina has the highest consumption of oxygen per gram of tissue in the human body, being together with the RPE extremely susceptible to damage caused by oxidative stress (39). When the anti-oxidant capacity of the eye is overcome, a large amount of oxidized proteins, lipids and factors related to inflammation are formed, which are constituents of druses (40). The photoreceptor

heterophagy by RPE cells is a constant source of polyunsaturated fatty acids, generating an environment rich in reactive oxygen species (ROS) (41) that can induce oxidative phospholipid modification. Exposure to ROS induces immune recognition with inflammatory damage, mainly through complement activation (39,42).

The present study found amyloid protein, catalase (CAT), enolase, glutathione peroxity (GPx), lipocalin and superoxide dismutase (SOD) regulated between the scenarios, which allowed the grouping of patients by their global protein profile in the three analyzed scenarios. A correlation was observed between the GO annotations of these proteins and changes in functionality caused by oxidative stress present in AMD. The retina contains a considerable number of antioxidant agents in the photoreceptors and RPE, mainly in the central portion of the retina, such as enzymes such as superoxide dismutase, glutathione peroxidase and catalase (43). The first two were found in increased concentration in cases of anti-VEGF-resistant AMD and the catalase was positively regulated in the control group, since, unlike the previous two, which are produced by oxidative injury, catalase is produced constitutively.

Amyloid protein, known in Alzheimer's disease (44), is also an important component of druses (45.46) and was found in the present study to be negatively regulated in the control group, without AMD. Lipocalin 1 (LCN 1), which had already been characterized as a protective agent against oxidative stress potentially caused by lipid peroxidation products (47.48), was found to be positively regulated in patients with naive AMD and negatively in anti-VEGF-resistant AMD in this study.

Key enzyme of the glycolytic pathway, also found in geographic atrophy, in neovascular atrophy of AMD (35,49,50) and with GO notes involved with oxidative stress, enolase was regulated positively in the control group and negatively in the resistant group. Autoimmune stimulation of enolase has been described by several authors. Adamus and colleagues evaluated anti-retinal autoantibodies against enolase at different stages of AMD (49).

Over the years, photoreceptors and retinal RPE cells are exposed to innumerable innate immune activators, in such a way that strict regulation of immunity is essential to prevent harmful inflammatory events. However, dysregulation of the complement system can potentially lead to chronic eye inflammation. One of the first indications that the complement system is involved in the progression of AMD is to find complement by-products (factor H) in druses (51). In the present study, there was an increase in C2, C3, C4, C7 found in patients with AMD resistant to anti-VEGF, thus corresponding to that described in the literature (12,52,53). Factor C8 was found to be positively regulated in the treatment-naïve neovascular AMD group. As reported by Lu at. al. (2018), complement factor B was found in greater concentration in control patients, functioning as a preventive factor for AMD (53).

Clusterin, according to the observed GO notes, is involved in inflammatory processes and the complement cascade. However, it can also play a protective role in the response to the redox environment (oxidative stress) of human RPE cells, which contributes to cell survival through the PI3K / Akt pathway, an important intracellular signaling pathway in the regulation of the cell cycle. Therefore, clusterin can be considered a preventive factor for AMD, which, when found in greater relative intensity in patients with resistant AMD in this study, denotes that this route remains active in an attempt to reestablish local homeostasis. Like amyloid protein, clusterin has also been described in the pathophysiology of Alzheimer's disease (54). The heavy chain of immunoglobulin Mu, in turn, despite its GO annotations correlating it with

immune response and complement activation, was a marker found in this research, where no association described with AMD was observed in the literature.

Plasma kallikrein and CD14 monocytic differentiation antigen were also found with increased relative intensity in the resistant AMD group in this research. However, the literature lacks studies demonstrating a correlation between such a system and AMD. Guo et al, (2004) and Ali et al (2005) correlated, even if indirectly, the CD14 antigen with apolipoprotein E (APOE), the main lipoprotein found in the retina (55–57) that plays a crucial role in local lipid transport (34). Thus, the CD14 antigen also participates in the pathway of lipid metabolism in the pathogenesis of AMD.

The impairment of RPE function is an early and crucial event that influences and drives choroidal neovascularization (58,59). The pigment epithelium derived factor (PEDF), well known for its participation in the suppression of angiogenesis and also with GO annotations showing negative regulation of the inflammatory response, was found with negative intensity in the control group of this research (cataract), an environment whose need for factors inflammation and angiogenesis inhibitors (such as PEDF) is small.

The growth factor insulin simile protein 7 and the pigment epithelium derived factor (PEDF) were found with lower concentration intensity in this research in the control group. In other words, it appears that these negative regulatory factors for angiogenesis had a positive production stimulus, in fact, predominantly in patients with AMD. The ectonucleotide pyrophosphatase was positively regulated in the neovascular AMD naive group in this experimental study, however, despite having a GO annotation in the regulation of neoangiogenesis, the literature is poor when correlating it with AMD.

The penetration of choroidal neovascularization through the Bruch's membrane is stimulated by the production of metalloproteinases (MMPs), which are enzymes that digest proteins in the extracellular matrix (60). MMP inhibitors called TIMPs act as inhibiting factors for angiogenesis (61). In this experimental study, TIMP-1 was found to be positively regulated in the persistent group, a poorly understood correlation in the literature.

Metallothionein - 1G was positively regulated in the control group in this study, which is consistent with the literature that correlates it with retinal neuroprotection (62).

Phosphorylation of protein kinases is an important mechanism in the proliferation and differentiation of endothelial cells, since the activity of tyrosine kinase receptors is a prerequisite for angiogenesis (63). The vascular and endothelial growth factor (VEGF) receptor, in turn, is certainly a key factor in the pathogenesis of choroidal neovascularization of neovascular AMD. A tyrosine kinase type III receptor and VGEF receptor 1 (VEGFR1) were found to be positively regulated in the resistant group and negatively regulated in the naive AMD group in the present study.

Specifically in the context of resistance, a cut can also be made to analyze only the AMD patients in this study. Two (2) proteins were positively regulated in the virgin treatment group (naive) and negatively in the group resistant to anti-VEGF. For 3 (three) other proteins, the opposite was verified.

In fact, the immunoglobulin Mu heavy chain is regulated positively in the naive group and negatively regulated in the resistant group. The same condition is repeated with lipocalin and, in relation to the previous one, it is a protein correlated with AMD more consistently in the literature. According to Ghosh et al. (2017), lipocalin functions as an initial AMD marker, particularly when lysosome-mediated clearance in RPE is compromised,

which contributes to the induction of a chronic inflammatory response. This hypothesis has also been demonstrated in an animal model of AMD (64).

Regarding the 3 (three) proteins regulated positively in the resistant group and negatively regulated in the naive group, the literature shows that one is directly linked to the inflammatory process (kallikreinplasmic) and two with VEGF (tyrosine kinase receptor, isoformMACRA_a and the receptor 1 of the Endothelial Vascular Growth Factor). When a slow loss of efficacy of the drug (anti-VEGF) is characterized over time, some authors introduced the new concept of resistance in the context of neovascular AMD. There is talk of medication tolerance when its effect can be improved if the dosage is increased or administered in a shorter period of time (65). This difference in the number of receptors in resistant patients, in addition to helping to understand the difficulty of controlling the subretinal neovascular membrane, may also corroborate with the explanation of the pathological tolerance that some patients develop to the gold standard treatment of neovascular AMD. That is, as the Vascular Endothelial Growth Factor Receptor 1 (VEGFR-1) is positively regulated in patients in the resistant group, such differentiation may explain, at least in part, the persistence of disease activity even with the use of anti-VEGF in patients with neovascular AMD.

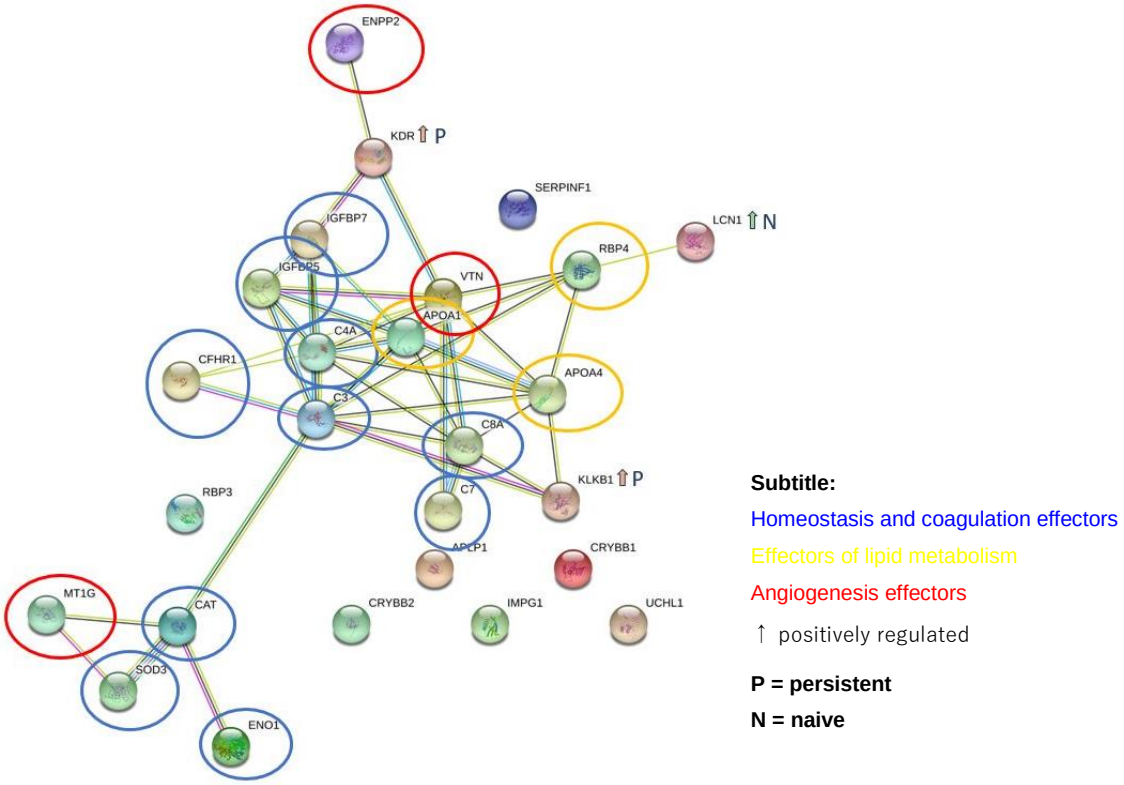

Figure 2: Grouping of selected proteins in molecular pathways / biological processes. The bonds between proteins represent interactions, according to the String program pattern.

When realizing that an important portion of the population with treated AMD remains with the disease in activity, the need to speed up the consolidation of alternative pathways that act in the final neoangiogenesis of AMD is understood. Recent research seeks therapeutic means using additional pathways in the pathophysiology of AMD and the proteins selected in this study can also contribute to this end. Expectations can be initially for those more strategic proteins, either by positive regulation and higher relative intensities (foldchange / VIP score), easier measurement in serum matrix (auto-antibodies) or by functional importance, in each of the additional pathways of choroidal neovascularization described in this research. Thus, the alpha

and beta or gamma crystalline proteins involved in visual function stand out, such as the crystalline alpha chain B, crystalline beta 2 and crystalline gamma C. In the path of lipid metabolism, important in the early diagnosis, the apolipoprotein AI isoformCRA_a is emphasized which has a correlation with the formation of druses, phosphoinositide phospholipase C with the highest foldchange and the retinol binding protein 3 for potential serum auto-antibody documentation. In the path of oxidative stress, catalase is the protein with the highest relative intensity (highest VIP score among all the discriminating proteins found). Along the way of complement activation, we had uniformity with the literature descriptions, with the group of patients with neovascular AMD general, showing a relative increase compared to the control group. All proteins found in this research and involved in this pathway can be potential candidates for biomarkers. In the inflammatory pathway, enolase 1 stands out, which is among the thirty proteins with the greatest discriminative capacity of the analyzed conditions (VIP score). And in the angiogenic pathway, finally, due to the need for further studies due to discrepancies in comparison with data in the literature, the metalloproteinase-TIMP tissue inhibitor stands out and, because it is at the heart of the current pathophysiology of choroidal neovascularization, the vascular and epithelial growth factor receptor 1 - VEGFR1.

More comprehensive prospective studies and validation studies in large cohorts are still needed to assess the diagnostic and prognostic capacity of selected protein classes.

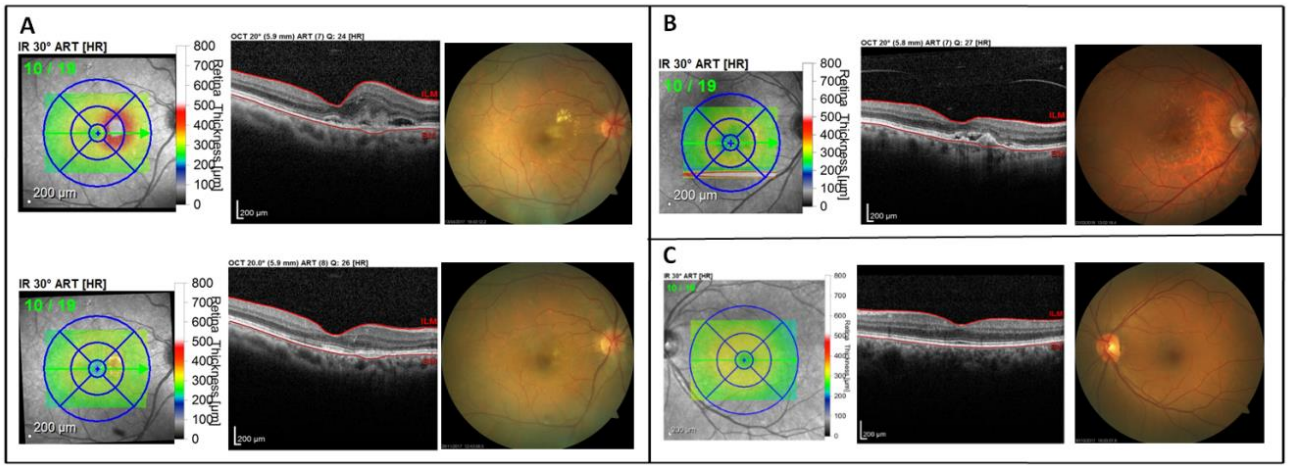

Figure 3: Panel of the groups analyzed: (A) patient from the naive group at the beginning of the research and in a posteriori follow-up demonstrate a good response to anti-VEGF. (B) patient in the resistant group, who shows disease activity despite 6 monthly applications of anti-VEGF. (C) control patient (with cataract), without systemic pathologies or underlying retinal disease.

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