Growth Factors

ISSN: 0897-7194 (Print) 1029-2292 (Online) Journal homepage: http://www.tandfonline.com/loi/igrf20

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To cite this article: Joana Mesquita, João Paulo Castro-de-Sousa, Sara Vaz-Pereira, Arminda Neves, Luís A. Passarinha & Cândida T. Tomaz (2018): Evaluation of the growth factors VEGF-a and VEGF-B in the vitreous and serum of patients with macular and retinal vascular diseases, Growth Factors, DOI: 10.1080/08977194.2018.1477140

To link to this article: https://doi.org/10.1080/08977194.2018.1477140

Published online: 03 Jul 2018.

GROWTH FACTORS
https://doi.org/10.1080/08977194.2018.1477140

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RESEARCH PAPER

Evaluation of the growth factors VEGF-a and VEGF-B in the vitreous and serum of patients with macular and retinal vascular diseases

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ABSTRACT

VEGF-A and VEGF-B are proangiogenic and key regulating factors for blood vessel growth. This study aims to compare VEGF-A and VEGF-B levels in the serum and vitreous of patients with neovascular pathology versus non-neovascular pathology. Our findings showed vitreous VEGF-A and VEGF-B levels increased in patients with neovascular disease, with higher levels of VEGF-A compared to VEGF-B ($p \leq .05$). In the diabetic retinopathy (DR) group, higher vitreous VEGF-A or VEGF-B were found in proliferative diabetic retinopathy (PDR) than in non-PDR. The strong correlation between VEGF-A and VEGF-B demonstrates a simultaneous pathological increase of cytokines ($p < .001$), suggesting besides VEGF-A, VEGF-B is another contributor to ocular pathologies involving angiogenesis. There was no correlation between vitreous and serum VEGF-A or VEGF-B; however, a correlation between vitreous (VEGF-A or VEGF-B) and macular volume ($p < .05$) in DR patients was found. Targeting VEGF-A and VEGF-B in macular and retinal vascular diseases, involving neovascularization, may improve treatment outcomes.

ARTICLE HISTORY
Received 3 February 2018
Revised 26 April 2018
Accepted 13 May 2018

KEYWORDS
ELISA; neovascular diseases; VEGF-A; VEGF-B; vitreous humor

Introduction

Angiogenesis is one of the mechanisms responsible for neovascularization, in which new blood vessels are contrived from the preexisting ones (Roy et al., 2006). Neovascularization is not only an essential physiological process like embryonic development but also induces several pathological processes, for instance, tumour growth, rheumatoid arthritis, psoriasis, and ocular diseases such as age-related macular degeneration (AMD), retinal vein occlusion (RVO) and diabetic retinopathy (DR) (Ferrara et al., 2003). The resulting proliferation often ends in visual impairment (Mesquita et al., 2018).

The vascular endothelial growth factor (VEGF) family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF) (Li et al., 2009). Alternative splicing results in several VEGF variants or isoforms (Penn et al., 2008).

VEGF-A is implicated in the angiogenesis of pathological eye conditions and, thus, is a highly significant therapeutic target (Witmer, 2003). Carmeliet et al. (1996) and Ferrara et al (1996) showed the essential role of VEGF-A in embryonic vasculogenesis and angiogenesis. VEGF-A induces an angiogenic response, essential in inflammation due to its capacity to increase vascular permeability and induce vascular leakage (Amadio et al., 2016). VEGF-A exists in four different isoforms created due to alternative exon splicing: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$; $VEGF_{165}$ is the most predominant isoform (Houck et al., 1991). VEGF-A binds to two receptor tyrosine kinases, that is, VEGF receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2), and also binds to neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). VEGFR-1 and VEGFR-2 regulate and signal physiological and pathological angiogenesis (Shibuya, 2006). Binding to VEGFR-1 promotes tumour growth, metastasis, and inflammation (Shibuya, 2006).

VEGF-B, a closely related molecule to VEGF-A, is one of the least studied members and the most controversial protein of the VEGF family (Li et al., 2009). The biological function of VEGF-B is enigmatic. Li and coworkers (2012) referred to VEGF-B as a multifunctional safeguarding molecule having a functional ambiguity role. VEGF-B has low angiogenic activity;

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however, it is a potent anti-apoptotic, rescuing vessels from death. It plays a specific role in angiogenesis during the repair process after brain injury and myocardium revascularization after myocardial infarction (Li et al., 2008; Nag et al., 2002). VEGF-B also plays an important role in the lipid metabolism, and its impairment causes obesity due to absorption and accumulation of fatty acids in the tissues. It was shown that lipid accumulation in the pathological tissue impairs insulin signalling, leading to insulin resistance and metabolic dysfunction and resulting in type-2 diabetes mellitus. Moreover, lipids in circulation are transported through vascular endothelium up to the tissue cells to help them perform their functions. VEGF-B regulates this process by controlling the expression of the endothelial fatty acids transporter protein, more specifically the regulation of endothelial cells long chain fatty acids uptake through VEGFR-1 and NRP-1 (Hagberg et al., 2010, 2013; Muhl et al., 2016). In vivo, VEGF-B causes choroidal and retinal neovascularization (Zhang et al., 2009). The 'angiogenic' activity of VEGF-B during ocular neovascularization is due to its vascular survival effect mediated by NPR-1 and VEGFR-1, which safeguards the neovessels from apoptosis (Zhang et al., 2009). Therefore, direct VEGF-B inhibition may be a potential therapy for the treatment of ocular neovascular diseases (Zhang et al., 2009). Alternative splicing of exon 6 resulted in two isoforms of VEGF-B: VEGF-B_{167} and VEGF-B_{186}. VEGF-B binds with high affinity to VEGFR-1 but not to VEGFR-2 or VEGF receptor 3 (VEGFR-3), competing with VEGF-A for VEGFR-1 binding (Olofsson et al., 1998). This selective binding is due to electrostatic surface potentials (Iyer et al., 2010).

Moreover, VEGF-A may also generate heterodimers with VEGF-B, affecting the formation of VEGF-A homodímers and bioavailability of VEGF-A to bind to its receptors and exert its activity (Olofsson et al., 1996).

Evidences show that patients with retinal diseases, such as DR, AMD, and RVO, have increased levels of vitreous VEGF-A (Aiello et al., 1994). Our research group has shown increased vitreous levels of VEGF-B in DR and its association with disease severity (Mesquita et al., 2017).

With the growing interest in the use of anti-angiogenics in the treatment of different diseases related to anomalous angiogenesis, inflammation and vessel hyperpermeability, more consideration must be paid to understand the accurate roles of the molecules of the VEGF family (Li et al., 2009).

In the present study, we investigated two cytokines levels (VEGF-A and VEGF-B) in vitreous and serum associated with neovascular eye pathologies: DR, AMD and RVO. Quantitative results and clinical characteristics of VEGF-A and B were compared between two groups of patients (neovascular and non-neovascular). For further exploration, the serum and vitreous concentration levels of VEGF-A or VEGF-B (in the neovascular group) were correlated with stage of DR to confirm if there was a growth of cytokines levels with disease progression, previous treatments, functional parameters, such as visual acuity and structural parameters, such as macular volume and central retinal thickness, assessed by optical coherence tomography (OCT).

A better knowledge of these cytokines and their correlation can lead us to better-targeted precise and specific therapies. This may also lead to disease prevention through the detection of ocular neovascularization before the appearance of clinical symptoms, subsequently leading to the prevention of blindness.

Materials and methods

Participants

This retrospective study involved 20 patients divided into two groups: (1) A neovascular group ($n = 17$) comprising RVO ($n = 2$), AMD ($n = 2$) and DR ($n = 13$) patients and (2) A non-neovascular group (or control group), which included patients with vitreomacular traction (VMT) syndrome ($n = 3$) of idiopathic aetiology but without any other ocular disorder (including neovascular disease).

The neovascular ocular disease was defined for this study as a retinal disease associated with abnormal blood vessel growth, which may lead to blindness; however, the location of neovascularization may differ.

The DR group was classified into patients with proliferative diabetic retinopathy (PDR) ($n = 8$) and non-proliferative diabetic retinopathy (NPDR) ($n = 5$).

For the DR group, the diabetes mellitus duration was defined as the duration from the first diagnosis of diabetes mellitus to the time of vitrectomy and sample collection. None of the patient samples selected for the study had undergone either pharmacological (intravitreal injections) or laser treatments within the 3 months of vitrectomy. Moreover, diabetic therapy remained unchanged for at least 3 months before vitrectomy.

A naïve patient was defined as a patient who has never been treated before for ocular conditions with any drug or non-drug therapy, including laser photocoagulation, bevacizumab, ranibizumab, aflibercept or triamcinolone.

Collection of samples and storage

Whole blood samples were collected from patients before the surgery. After allowing the blood to clot at room temperature for 30 minutes, the clot was removed by centrifugation at 3000 rpm for 10 minutes. After serum withdrawal, it was immediately frozen at −80 °C. Each patient underwent Pars Plana Vitrectomy (PPV) for treatment of the current disease. The undiluted vitreous samples were collected at the beginning of the PPV in sterilized tubes, placed immediately on ice and frozen at −80 °C until it was required for analysis.

The criteria for the samples were as follows:

1. Patients with a confirmed diagnosis of neovascular ocular disease (for the neovascular group) were included.
2. For the control group, non-diabetic patients with no PDR and no signs of neovascularization, retinal vascular occlusion or other ocular disorder, except VMT syndrome, were included.
3. For the diabetic population, all patients with diabetic treatment either stable or unchanged for at least 3 months before the vitrectomy procedure were included.
4. Patients with sample volume that would allow the confirmation of the results through repeated enzyme-linked immunosorbent assay (ELISA) for the determination of VEGF-A and VEGF-B levels simultaneously in both vitreous and serum.
5. Patient samples were excluded from the analysis if the patients performed any intravitreal treatment (with anti-VEGFs and/or corticosteroids) or laser therapy less than 3 months of vitrectomy.

Moreover, due to the limitation of sample size, restrictions due to controlled inclusion/exclusion criteria and the difficulty in the collection of proper large vitreous sample sizes, the conclusions in an overall population should be done drawn with caution.

Collection of retrospective data from patients

Clinical history of the patients was collected to confirm the diagnosis, concurrent diseases as well as baseline and clinical characteristics. Despite the exclusion of patients from the study, information on their previous concomitant eye treatments was collected.

Measurement of vitreous and serum VEGF-a and VEGF-B levels

ELISA was performed to measure the vitreous and serum levels of VEGF-B (VEGF-B ELISA Kit; Ref, E-EL-H2164; Elabscience; Wuhan, Hubei, China) and VEGF-A (VEGF-A ELISA Kit; Ref, E-EL-H0111; Elabscience; Wuhan, Hubei, China). Both analyses were conducted according to manufacturer instructions.

Measurement of structural parameters

OCT examinations were performed before PPV using the Heidelberg equipment (Heidelberg Engineering, Heidelberg, Germany). Thickness mapping of retinal layers was used to measure the central retinal thickness (CRT) and macular volume (MV) associated with the retinal pathologies.

Measurement of functional parameters

Best corrected visual acuity (BCVA) was measured with early treatment diabetic retinopathy study (ETDRS) charts (Precision Vision, La Salle, IL) at 4 m.

Statistical analysis

Statistical analysis was performed using IBM SPSS version 24.0 (IBM Corp., Armonk, NY) for Windows. A level of significance $(\alpha) \leq 0.05$ was set to reject the null hypothesis. Pearson test was used to correlate quantitative variables with a normal distribution while Spearman's correlation coefficient test was used to correlate quantitative variables without normal distribution. The Mann–Whitney was used to compare two groups on quantitative dependent variables, without normal distribution.

Ethical statement

The study was approved by the Institution Review Board, named Ethics Committee for Health of Centro Hospitalar de Leiria (CHL-15481) and Portuguese National Data Protection Commission. All patients included in this study, adhered to the tenets of the Declaration of Helsinki, gave their informed consent after appropriate explanation.

Results

Study population: baseline characteristics

In this retrospective study, samples from 20 patients (5 women and 15 men) with a mean age of 70.3 years were selected to be analysed based on specific inclusion and exclusion criteria.

Two of the patients with RVO submitted to PPV surgery had active neovascularization. Out of the two

AMD patients, one had active neovascularization and the other had reminiscent neovascularization at the time of surgery (confirmed by fluorescein angiography). The patient with reminiscent neovascularization was previously treated (more than 3 months before surgery) with 8 bevacizumab intravitreal injections (before approval of the license drug ranibizumab), 4 ranibizumab injections (after approval of ranibizumab by health authorities) and 5 aflibercept injections.

From the 13 diabetic patients analysed, eight had PDR and five had NPDR. The majority had DM type 2, with the exception of 1 patient who had DM type 1. This patient (with DM type 1) had PDR and was under insulin treatment. The other 12 patients with DM type 2 were under insulin therapy ($n = 2$), oral therapy ($n = 8$) or both insulin and oral therapy ($n = 2$).

Concerning the previous treatments undergone by the patients (3 months before the PPV), in the neovascular group, eight patients were naïve to treatment: 1 RVO, 1 AMD and 6 DR (2 patients had PDR and 4 had NPDR), and nine patients were non-naïve to the treatment. The non-naïve patients included one RVO, one AMD and seven DR patients (6 patients with PDR and 1 with NPDR). The control group did not have any ocular neovascular pathology besides VMT syndrome.

Characterization of the baseline demographic and clinical characteristics is summarized in Table 1.

After characterization, this pool of patients was re-grouped into the neovascular group and non-neovascular group as defined previously.

Comparison of vitreous and serum VEGF-a and VEGF-B levels between patients with neovascular and non-neovascular diseases

A statistically significant difference was observed between vitreous VEGF–A in the neovascular group [(343.30 ± 543.82 pg/mL), (mean ± standard deviation)] and vitreous VEGF-A in the non-neovascular group [(7.04 ± 1.35 pg/mL), ($Z = -1.958$, $p = .050$)]. Likewise, the mean difference of vitreous VEGF–B concentration was significantly higher in the neovascular group (208.83 ± 353.58 pg/mL) than the control group of non-neovascular disease [(1.94 ± 0.61 pg/mL) ($Z = -2.170$, $p = .030$)] (Figure 1).

Regarding the analysis of the serum, there were differences between the two groups (neovascular and non-neovascular), although these differences were not statistically significant (Figure 1).

Comparison of VEGF-a and VEGF-B levels in vitreous and serum of studied pathologies

The comparison between vitreous VEGF-A and vitreous VEGF-B levels showed an increase in both DR and RVO patients. AMD patients revealed lower vitreous levels for both cytokines (VEGF-A and VEGF-B), suggesting that this result is due to one patient who had reminiscent neovascularization (Figure 2).

Serum levels of VEGF-A and VEGF-B did not reveal any significant difference between neovascular pathologies and control group.

Table 1. Patient baseline clinical and demographic characteristics.

	Total ($n = 20$)	RVO ($n = 2$)	AMD ($n = 2$)	DR ($n = 13$)	Control group ($n = 3$)
Demography					
Age (yrs) (Mean, SD)	70.3 ± 10.7	78.0 ± 5.6	77.0 ± 7.0	68 ± 11.9	71.0 ± 6.2
Other variables					
BCVA (ETDRS letters) at baseline (Mean, SD)	–	65.0 ± 1.5	48.0 ± 3.5	62.1 ± 19.2	57.5 ± 3.5
CRT at baseline (Mean, SD)	–	328.5 ± 30.4	480.5 ± 140.7	413.8 ± 65.7	–
MV at baseline (Mean, SD)	–	8.07 ± 0.06	11.79 ± 3.08	9.59 ± 1.67	–
Diabetes mellitus type (n; %) in DR patients group					
DM 1 (*n*, %)	–	–	–	1, 8.3%	–
DM 2 (*n*, %)	–	–	–	12, 91.7%	–
Diabetes treatment (n; %) stable for at least 3 months before vitrectomy procedure					
Insulin therapy	–	–	–	3 27.3	–
Oral therapy	–	–	–	8 54.5	–
Insulin and oral therapy	–	–	–	2 18.2	–
Other DR characteristics					
Duration of DR (yrs) (Mean, SD)	–	–	–	1.6 ± 1.5	–
PDR (*n*; %)	–	–	–	8, 38.5	–
NPDR (*n*; %)	–	–	–	5, 61.5	–
Previous treatments for ocular condition (performed more than 3 months before vitrectomy)					
Laser photocoagulation (*n*)	–	1	0	7	–
Bevacizumab (*n*)	–	0	1	0	–
Ranibizumab (*n*)	–	0	1	3	–
Aflibercept (*n*)	–	0	1	0	–
Triamcinolone (*n*)	–	0	0	2	–

VEGF-A: vascular endothelial growth factor A; VEGF-B: vascular endothelial growth factor B; BCVA: best corrected visual acuity; CRT: central retinal thickness; MV: macular volume; RVO: retinal vein occlusion; AMD: age macular degeneration; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; NPDR: non-proliferative diabetic retinopathy; DM1: diabetes mellitus type 1; DM2: diabetes mellitus type 2; control group: patients with vitreomacular traction syndrome of idiopathic aetiology and without other ocular disorders; yrs: years; SD: standard deviation.

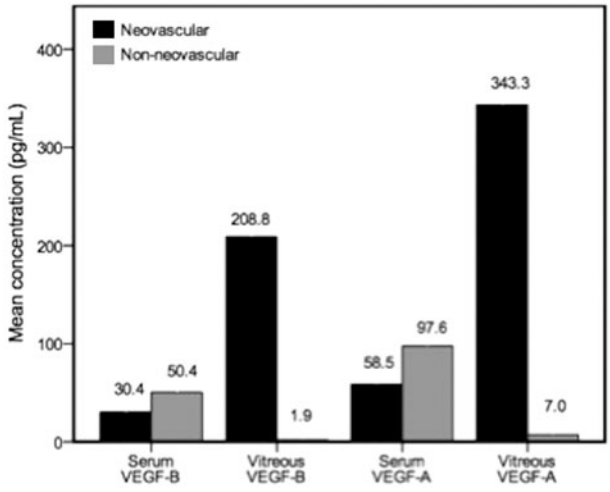

Figure 1. Mean serum and vitreous VEGF-A and VEGF-B concentrations between the neovascular ($n = 17$) and non-neovascular ($n = 3$) study arm, analysed with Mann–Whitney test ($p < .05$).

Comparison of vitreous and serum levels of VEGF-a and VEGF-B between PDR and NPDR patients

Analysis of the DR patients revealed statistical differences in VEGF-A and VEGF-B levels:

(a) Vitreous VEGF-A levels were higher in PDR (576.88 ± 681.15 pg/mL) than in NDPR (39.11 ± 71.13 pg/mL) ($Z = -2.342$, $p = .019$);
(b) Vitreous VEGF-B levels were also higher in PDR (357.55 ± 453.22 pg/mL) than in NDPR (17.75 ± 34.92 pg/mL) ($Z = -2.342$, $p = .019$);
(c) Serum VEGF-A levels were increased in NPDR (95.08 ± 47.37 pg/mL) compared to PDR (36.02 ± 28.05 pg/mL) ($Z = -2.196$, $p = .028$);
(d) Serum VEGF-B levels were higher in NPDR (46.38 ± 22.35 pg/mL) than in PDR (17.29 ± 14.74 pg/mL) ($Z = -2.049$, $p = .040$) (Figure 3).

Comparison of vitreous VEGF-a and VEGF-B levels between naïve and non-naïve patients with neovascular pathology

For this analysis, as defined previously, a non-naïve was a patient who never underwent any drug or non-drug therapy to treat his/her ocular disease, including laser photocoagulation, bevacizumab, ranibizumab, aflibercept or triamcinolone.

Although the analysed patients had performed intravitreal treatments and/or laser therapy 3 months before vitrectomy, a comparison of the mean values between naïve ($n = 8$) and non-naïve ($n = 9$) patients in the neovascular group ($n = 17$) demonstrated a statistically significant difference ($p < .05$) between vitreous VEGF-A and VEGF-B. Higher levels of vitreous

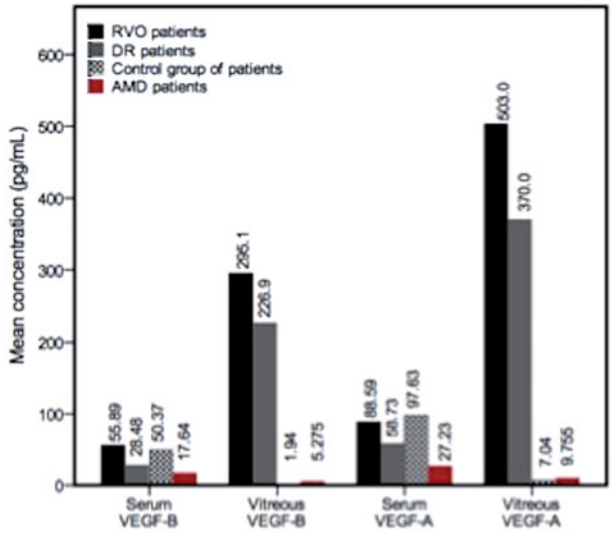

Figure 2. Mean vitreous and serum levels of VEGF-A and VEGF-B among different studied pathologies and control group of patients, analysed with Mann–Whitney test ($p > .05$). VEGF-A: vascular endothelial growth factor; VEGF-B: vascular endothelial growth factor B; RVO: retinal vein occlusion ($n = 2$); DR –diabetic retinopathy ($n = 13$); AMD: age macular degeneration ($n = 2$). The control group included patients with VMT syndrome with no other ocular disorders ($n = 3$).

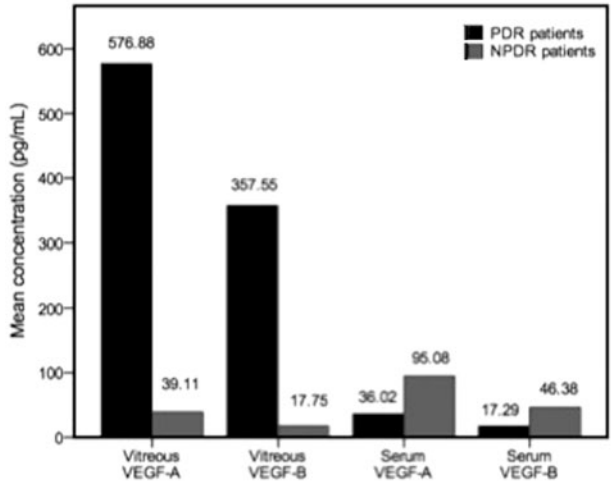

Figure 3. Comparison of the mean vitreous and serum levels of VEGF-A and VEGF-B between NPDR ($n = 5$) and PDR ($n = 8$), analysed with Mann–Whitney test ($p < .05$). PDR: proliferative diabetic retinopathy, NPDR – non-proliferative diabetic retinopathy.

VEGF-A and VEGF-B in the group of non-naïve patients was observed (Table 2).

Analysis of vitreous VEGF-a and VEGF-B levels in the group of patients with neovascular disease per treatment group

For this analysis, the non-naïve neovascular patients ($n = 9$) were divided into five groups, describing the

Table 2. Comparison of the mean concentration levels of vitreous VEGF-A and vitreous VEGF-B in the neovascular group between naïve and non-naïve patients ($p < .05$).

Patients in the neovascular group ($n = 17$)						
	Non-naïve ($n = 9$)		Naïve ($n = 8$)			
	Mean	SD	Mean	SD	*Z*	*p*
Vitreous VEGF-B	321.48	441.11	82.09	169.42	−1.925	.054
Vitreous VEGF-A	516.26	665.50	148.71	296.79	−2.021	.043

VEGF-A: vascular endothelial growth factor A, VEGF-B: vascular endothelial growth factor B, SD: Standard deviation.

treatments performed since they were diagnosed until vitrectomy (note that the selected samples for the neovascular group did not undergo any treatment 3 months before the surgery):

Group A: patients who were administered only laser;

Group B: patients who were administered bevacizumab, ranibizumab and aflibercept;

Group C: patients who were administered ranibizumab and laser;

Group D: patients who were administered laser and triamcinolone;

Group E: patients who were administered laser, triamcinolone and ranibizumab.

Overall, the results revealed high vitreous levels of both growth factors as seen in Figure. 4.

Correlation between vitreous VEGF-a and vitreous VEGF-B, serum VEGF-a and serum VEGF-B in the neovascular group of patients

There was a statistically significant, positive and strong correlation between vitreous VEGF-A and vitreous VEGF-B in patients with neovascular pathology ($n = 17$), that is, $r_{sp} = 0.983$, $p < .001$. Likewise, there was a positive and strong correlation between serum VEGF-A and serum VEGF-B ($r = 0.970$, $p < .001$) in the neovascular group (Figure 5).

Similarly, the correlation coefficient between VEGF-A and VEGF-B in vitreous and serum for DR patients ($n = 13$) was statistically significant, positive and strong; $r_{sp} = 0.984$, $p < .001$ and $r_{sp} = 0.973$, $p < .001$, respectively (Figure 6).

Correlation between vitreous and serum VEGF-a and between vitreous with serum VEGF-B in the neovascular group of patients

The correlation coefficient between vitreous VEGF-A and serum VEGF-A for the neovascular group was not statistically significant ($n = 17$; $r_{sp} = -0.051$; $p = .844$). Also, there was no correlation between vitreous VEGF-A and serum VEGF-A in the DR patients group ($n = 13$; $r_{sp} = -0.209$; $p = .494$).

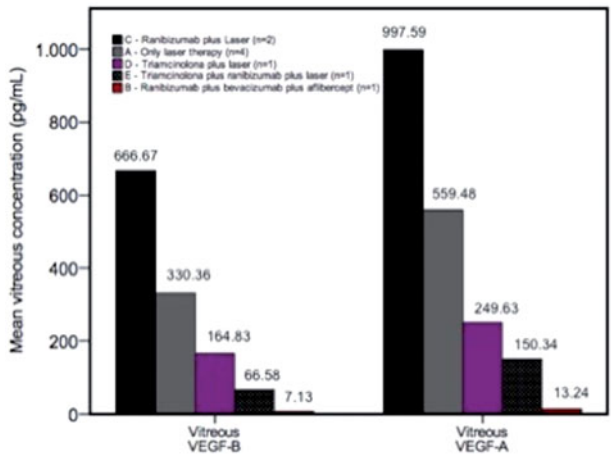

Figure 4. Mean vitreous concentration levels of VEGF-A and VEGF-B among the 5 treatment groups in the neovascular patients group (non-naïve patients, $n = 9$), using descriptive analysis. VEGF-A: vascular endothelial growth factor; VEGF-B: vascular endothelial growth factor B; Group A: Patients that only performed laser treatment; Group B: Patients that performed either or, ranibizumab, bevacizumab, aflibercept; Group C: Patients treated with ranibizumab plus laser; Group D: Patients treated with triamcinolone and laser; Group E: patients treated with ranibizumab plus triamcinolone plus laser; SD: standard deviation.

Similarly, the correlation coefficient between vitreous VEGF-B and serum VEGF-B levels was not statistically significant for neovascular patients' population ($n = 17$; $r_{sp} = 0.027$; $p = .918$). Relatively to DR patients, there was no correlation between vitreous VEGF-B and serum VEGF-B ($n = 13$; $r_{sp} = -0.110$; $p = .721$).

Correlations of vitreous VEGF-a or vitreous VEGF-B with structural and functional parameters in DR patients

In the DR patients group, a statistically significant, positive and moderate correlation was observed between vitreous VEGF-A and MV ($r_{sp} = 0.560$, $p = .046$) and between vitreous VEGF-B and MV ($r_{sp} = 0.588$, $p = .035$). No statistically significant correlation was found either for the visual acuity or CRT (Table 3).

Discussion

VEGF-A is not only the prototype angiogenic factor but also a mediator of angiogenesis and vascular permeability in inflammatory disorders (Amadio et al., 2016). VEGF-B, unlike VEGF-A, is insignificant in angiogenesis or vascular permeability (Li et al., 2012). Its role is to be a survival factor in pathological

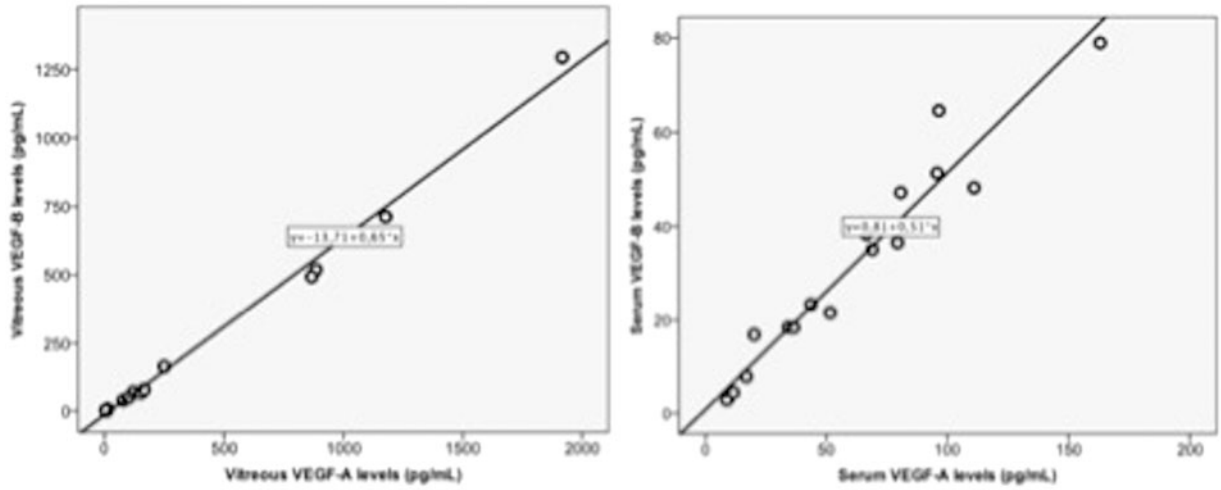

Figure 5. Correlation between (a) vitreous VEGF-A versus vitreous VEGF-B and (b) serum VEGF-A versus serum VEGF-B (in patients with neovascular pathology, $n = 17$), analysed by Spearman's correlation (in vitreous samples) and by Pearson correlation coefficient (in serum samples) ($p < .001$).

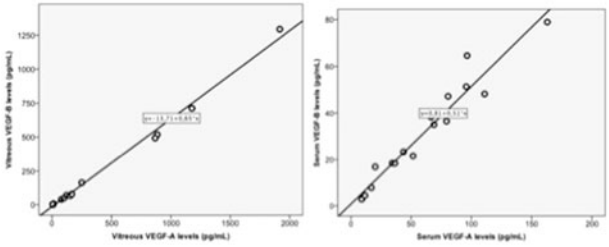

Figure 6. Correlation between: (a) vitreous VEGF-A versus vitreous VEGF-B and (b) serum VEGF-A versus serum VEGF-B, in DR patients, ($n = 13$), analysed by Spearman's correlation coefficient ($p < .001$).

Table 3. Correlations between structural (CRT and MV), functional parameters (VA) and concentration levels of vitreous VEGF A and B in a group of DR patients ($n = 13$), analyzed by Spearman's correlation coefficient ($p < .05$).

	CRT	MV	BCVA
Vitreous VEGF-B levels (pg/mL)	0.008	0.588*	−0.103
Vitreous VEGF-A levels (pg/mL)	−0.041	0.560*	−0.118

*$p \leq .05$.
VEGF-A: vascular endothelial growth factor A; VEGF-B: vascular endothelial growth factor; CRT: central retinal thickness; MV: macular volume; BCVA: best corrected visual acuity.

processes in several body systems such as the eye, brain or heart (Zhang et al., 2009).

Our results demonstrated that vitreous VEGF-A and VEGF-B are increased in patients with neovascular diseases as compared to the control group of patients with non-neovascular diseases. The pathologies which had higher concentrations of vitreous VEGF-A and VEGF-B were RVO and DR, supporting the assumptions that these cytokines are important contributors in the pathogenesis of neovascular eye diseases (Li et al., 2009). Enhanced concentrations of both factors were also expected in AMD patients, but such results were not observed due to the fact that one AMD patient was in a reminiscent phase of the disease, despite being treated more than 3 months before the PPV. Moreover, increased levels of VEGF-A than VEGF-B demonstrated that the development of new drugs is in the right direction by inhibiting the growth factor A.

A combination action of drugs causing VEGF-A inhibition while targeting VEGF-B (apoptotic proprieties) should be beneficial (Li et al., 2012).

Concerning the analysis of DR patients, an investigative analysis demonstrated statistically significant and high values of vitreous VEGF-A and VEGF-B in PDR patients, suggesting the increase in VEGF-A and VEGF-B levels with the progression of the disease and, thus, confirming the benefit of inhibiting those two cytokines in the treatment of DR. Research reports from Funatsu et al. (2002), Gao et al. (2016) and Watanabe et al. (2005) also demonstrated increased levels of vitreous VEGF-A in PDR patients as compared to their control group. In addition, evidences are provided that vitreous VEGF-B levels are increased in diabetics and depend on disease severity from NPDR to PDR (Mesquita et al., 2017). In contrast, the results obtained with serum samples in the DR patients showed higher VEGF-A and VEGF-B levels in NPDR. Higher serum VEGF-A levels have been determined by some investigators in NPDR patients as compared to PDR (Burgos et al., 1997; Hernandez et al., 2001; Krizova et al., 2015; Lee et al., 2005; Praidou et al., 2009; Simó et al., 2002), whereas others described no serum level differences among distinctive groups of diabetics (Aiello et al., 1994; Burgos et al., 1997; Hernandez et al., 2001, 2002; Krizova et al., 2015; Praidou et al., 2009; Simó et al., 2002).

A hypothesis mentions that the high VEGF-A and VEGF-B serum concentration levels in the NPDR are due to undiagnosed concurrent systemic diseases, such as cancer and rheumatoid arthritis, that may alter serum VEGF-A values, especially in our analyzed aged population (Ferrara et al., 2003; Mesquita et al., 2018). Another hypothesis suggests that the stage of DR and diabetic therapy may alter VEGF results as demonstrated by Baharivand and coworkers (2012), which showed that serum VEGF-A levels are lower in diabetics on oral therapy, well-controlled diabetes cases, and in early stages of DR. One last hypothesis to explain our results is through the fact that the production of VEGF-A or VEGF-B by the retina may not be related with their systemic levels. In this case, while Lee, Chae and Kim (2005) attributed the progression of DR to the circulating systemic VEGF-A, Burgos et al. (1997) and Funatsu et al. (2002) suggested that not the serum diffusion, but the intraocular synthesis of VEGF was the principal contributing factor for the high VEGF-A levels observed in the vitreous humor of PDR patients. To confirm this hypothesis, we showed in our study no correlation between serum and vitreous levels of either VEGF-A and VEGF-B, proposing a dissociation between the eye and other organs and an intraocular synthesis of vitreous VEGF-A and VEGF-B. Other researchers measured the levels of VEGF-A in vitreous and serum of patients and tried to find a correlation between serum and vitreous (Abdel et al., 2008; Aiello et al., 1994; Ambati et al., 1997; Baharivand et al., 2012; Burgos et al., 1997; Celik et al., 2005; Deng et al., 1999; Funatsu et al., 2002; Gao et al., 2016; Hernandez et al., 2001, 2002; Hogeboom et al., 2002; Krizova et al., 2015; Lee et al., 2005; Malik et al., 2005; Ozturk et al., 2009; Praidou et al., 2009; Simó et al., 2002; Watanabe et al., 2005; Zhou & Zhang, 1997). However, most of the researchers did not find any correlation with the exception of Baharivand and colleagues (2012) who found a positive correlation between serum and vitreous VEGF-A.

The comparison of vitreous VEGF-A and vitreous VEGF-B (in non-naïve patients) with the five treatment groups in the neovascular population suggested the existence of disease recurrence. Anti-VEGFs are the gold standard treatment for neovascular and potentially blinding diseases, although there are limitations to the use of such therapies. One limitation is the unknown duration of anti-neovascular effects. Anti-angiogenics reduce progression of neovascularization after the treatment, but the duration of the effect is for a limited time. Some patients require several intravitreal injections to achieve successful treatment or stabilized condition while others respond insufficiently even with several antiangiogenic treatments (Singer et al., 2016). Another limitation is the detectable antiangiogenic drug levels in the systemic circulation that may cause an increase in thromboembolic and cardiovascular events. Although the death rates do not seem to be increased due to the use of antiangiogenic drugs, their long-term consequences are still unknown (Singer et al., 2016). Lastly, VEGF-A and VEGF-B play an important role in neuroprotection, including the retinal neurons, and cell survival. Therefore, VEGF-A and VEGF-B blockage may also cause significant adverse events if there is a significant systemic distribution of the drugs (Singer et al., 2016).

An interesting finding was the significant correlation between VEGF-A and VEGF-B either in serum or vitreous, demonstrating that they can increase simultaneously. This may support the statement that VEGF-B, besides VEGF-A, is another important contributor to the pathogenesis of angiogenic diseases.

A statistically significant and positive correlation was found between vitreous VEGF-A and MV or between vitreous VEGF-B and MV in the diabetic population, demonstrating their effects in the establishment of oedema.

Overall, the results suggest an overexpression of vitreous VEGF-A and VEGF-B, with higher levels of VEGF-A and a simultaneous increase of those cytokines in neovascular eye pathologies. It was also confirmed that vitreous VEGF-A and VEGF-B increase with stages of DR, being more enhanced in PDR patients. Both are correlated with the increase in MV, demonstrating the importance of those cytokines in the establishment of macular oedema.

There are several pharmaceutical agents and non-drug procedures available to battle these multifactorial neovascular diseases. Despite lasers and anti-angiogenics being the leading treatments for neovascular diseases, about 50% of patients have an insufficient response to angiogenic therapy. Some would benefit from an early therapy switch as shown in the EARLY studies (Gonzalez et al., 2016). The combination therapy may be the rational approach to fight neovascular diseases by targeting VEGF factors while combating multiple and complex factors in the inflammatory and angiogenic cascade.

Only with a profound knowledge of pathophysiology, vascular, inflammatory and biochemical mechanisms of the targeted molecules, it will be possible to develop a new treatment approach. Thus, targeting VEGF-A or/and VEGF-B as an additional treatment options for neovascular ocular diseases might provide

better outcomes, sustained duration of action, and increased efficiency.

Acknowledgments

We thank Nurse Cristina Matias for her support in all aspects of collection, preparation and samples storage, José Pereira for statistical analysis, and the orthoptist team from the Centro Hospitalar de Leiria for assistance in data collection.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by FEDER funds through the POCI – COMPETE 2020 – Operational Programme Competitiveness and Internationalisation in Axis I – Strengthening research, technological development and innovation Project [POCI-01-0145-FEDER-007491] and National Funds by FCT – Foundation for Science and Technology Project [UID/Multi/00709/2013].

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