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Effect of intravitreal ranibizumab injections on aqueous humour concentrations of vascular endothelial growth factor and pigment epithelium-derived factor in patients with myopic choroidal neovascularisation

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ABSTRACT

Aims To investigate aqueous humour changes in vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) levels in patients with choroidal neovascularisation (CNV) secondary to pathological myopia (mCNV) before and after intravitreal ranibizumab injection (IVR).

Methods This was a prospective, case–control study investigating aqueous levels of VEGF and PEDF in eyes with mCNV treated with IVR.

Results Mean VEGF and PEDF levels in the aqueous humour of control patients were 25.7±4.9 pg/mL and 12.6±3.5 ng/mL, respectively. Lower levels of both VEGF (19.5±5.4 pg/mL) and PEDF (4.7±2.2 ng/mL) were found in patients with mCNV before IVR. After IVR, aqueous VEGF levels significantly reduced to 6.5 ±2.7 pg/mL, while PEDF levels significantly increased to 35.8±11.4 ng/mL. VEGF and PEDF levels significantly correlated with each other, and with best-corrected visual acuity and central retinal thickness.

Conclusions The VEGF and PEDF levels in aqueous humour were significantly lower in the myopic group than in controls. Moreover, IVR resulted in reduced VEGF and increased PEDF levels in patients with mCNV. In mCNV, neovascularisation is associated with inappropriate VEGF and PEDF expression. A balance between VEGF and PEDF is crucial to prevent CNV development.

Trial registration number NCT02175940.

INTRODUCTION

Pathological myopia (PM), one of the main causes of legal blindness worldwide, is characterised by myopic maculopathy, which can cause progressive macular degeneration, choroidal neovascularisation (CNV) formation, along with macular hole, posterior staphyloma and retinoschisis.[1 2] The most frequent sight-threatening complication of PM is CNV, and nearly 10% of patients with degenerative retinal findings develop CNV.[2 3] Different therapeutic approaches have been evaluated for this condition, such as thermal laser photocoagulation, macular translocation, surgical removal, photodynamic therapy with verteporfin (PDT-V) and vascular endothelial growth factor (VEGF) inhibitors.[1 4] Previous studies have demonstrated that intravitreal anti-VEGF compounds for CNV secondary to PM provide both functional and anatomic improvement.[5] More recently, the REPAIR[i] study showed that ranibizumab was effective in preventing vision loss and improving vision during a 12-month follow-up in CNV secondary to PM,[6] whereas the RADIANCE[ii] study demonstrated that individualised ranibizumab treatment was effective in improving and sustaining visual acuity (VA) and was generally well tolerated in patients with myopic CNV.[7] Lastly, in a 4-year follow-up of highly myopic patients with CNV treated with anti-VEGF drugs, Ruiz-Moreno *et al*[8] reported that intravitreal bevacizumab and ranibizumab are effective therapies and show similar clinical effects. However, VEGF concentrations in the aqueous humour of patients with myopic CNV (mCNV) are lower than that in normal controls[9] and in patients with age-related macular degeneration (AMD).[10]

Pigment epithelium-derived factor (PEDF) is a multifunctional protein involved in neuronal survival and differentiation, which is ubiquitously expressed and distributed in the human body.[11] Decreased PEDF levels have been linked to several retinal diseases, such as AMD, diabetic retinopathy and neuroretinal dystrophies. PEDF levels in myopic patients are not significantly different than in controls, and intravitreal bevacizumab injections do not affect intraocular PEDF levels.[12] Therefore, the role of VEGF in the pathogenesis of mCNV is controversial. This study was undertaken to investigate aqueous humour changes in VEGF and PEDF levels in patients with CNV secondary to PM (mCNV) after intravitreal ranibizumab injection (IVR).

PATIENTS AND METHODS

This was a prospective, case–control study investigating aqueous VEGF and PEDF levels in eyes with mCNV treated with IVR at the Medical Retina Department, University of Molise, Campobasso, Italy, between July 2013 and January 2014.

The primary goals of this study were to establish whether mCNV is associated with different concentrations of VEGF and PEDF and to compare

[i]REPAIR: Ranibizumab for the Treatment of Choroidal Neovascularisation (CNV) Secondary to Pathological Myopia (PM): an Individualized Regimen.
[ii]RADIANCE: Ranibizumab And PDT [verteporfIn] evAluation iN myopic Choroidal nEovascularization.

changes after IVR. Secondary goals include comparing central macular thickness (CMT) and best-corrected visual acuity (BCVA) before and after treatment, and exploring the correlation among CMT, BCVA, VEGF and PEDF levels.

Inclusion criteria were as follows: PM, defined as spherical equivalent >6 D and axial length >26 mm (Carl Zeiss IOLMaster V.4.07; Carl Zeiss Meditec, Dublin, California, USA); posterior pole myopic retinal changes (posterior staphyloma, chorioretinal atrophy, papillary crescent); fluorescein angiography (FA), indocyanine green angiography (ICGA) and optical coherence tomography (OCT) detection of the subfoveal or juxtafoveal CNV (CNV was classified as juxtafoveal if the lesion was closer than 200 mm, but not under the geometric centre of the foveal avascular zone), as recently pointed out by Mitry and Zambarakji;[14] clear ocular media; and duration of symptoms no longer than 4 weeks before enrolment. Exclusion criteria were as follows: previous treatment for CNV, including previous intravitreal drug injection or PDT-V, presence of other maculopathies, such as diabetic retinopathy or retinal vascular occlusion, history of recent myocardial infarction or other thromboembolic events, ongoing uncontrolled hypertension or glaucoma, refractive media opacities and eye surgery. Eligible patients were treated with an off-label IVR, regardless of the location of the lesion.

At baseline, all patients underwent BCVA measurement using an early treatment diabetic retinopathy study chart at 4 m, fundus biomicroscopy, FA, ICGA and spectral domain (SD)-OCT. All examinations, with the exception of angiographic tests, were repeated at all follow-up appointments. Angiographic tests and OCT scans were recorded using Spectralis SD-OCT (Heidelberg Engineering, Heidelberg, Germany). CMT was calculated after acquiring a sequence of 193 horizontal sections recorded in the high-resolution mode (1024 A-scans/30°) and covering an area of 20° (horizontal)×20° (vertical) with a distance of approximately 30 µm between individual sections. On follow-up examinations, the image processing software allowed re-evaluation at exactly the same location. The images were then processed by the 'Thickness Map' analysis program. Field 1 of the map analysis protocol (central 1 mm) was used for central retinal thickness (CRT) calculations. All patients with mCNV received two IVRs of 0.5 mg in 0.05 mL (Genentech, South San Francisco, California, USA), at baseline and about 1 month later. Immediately before each intravitreal injection aqueous sampling was performed by aspirating 0.05–0.1 mL of aqueous using a 30-gauge needle connected to a tuberculin syringe at the temporal limbus. The undiluted aqueous samples were then transferred into sterile containers and immediately stored in a –80°C freezer until analysis.

Reference samples were obtained from 20 age-matched patients undergoing cataract surgery. Exclusion criteria for the control group were any type of retinal disease, glaucoma, previous vitrectomy, laser coagulation, diabetes mellitus, use of immunosuppressive drugs, malignant tumours at any location and participation in any study using investigational drugs within three months preceding inclusion. Aqueous humour samples were obtained and stored in the same fashion described above for eyes with mCNV.

VEGF levels were determined in 50 µL of diluted sample with a human VEGF ELISA kit (EHVEGF, Pierce Biotechnology, Rockford, Illinois, USA) according to the manufacturer's instructions and using an extended standard curve including concentrations at 16, 8 and 4 pg/mL. This VEGF kit permits the detection of VEGF165 and VEGF121 isoforms. All assays were performed in duplicate. The minimum detectable VEGF concentrations were 3.5 pg/mL. Values <3.5 pg/mL were considered equal to 1 for statistical analysis. PEDF levels were determined in 50 µL of diluted sample using a human PEDF ELISA kit (HUMAN PEDF ELISA, BioVendor, Laboratornì medicina a.s. Karasek, Czech Republic) according to the manufacturer's instructions and using an extended standard curve. Assay range: 0.15–6 ng/mL; detection limit: 0.045 ng/mL. Values <0.045 ng/mL were considered equal to 1 for statistical analysis. All assays were performed in duplicate.

The sample size calculation indicated that at least 20 patients were necessary to have an alpha of 0.05 and a beta of 0.80. Data were recorded in a spreadsheet and analysed by SPSS statistical software (SPSS Version 22, Chicago, Illinois, USA). All data are expressed as means±SD. To analyse statistical differences, we used the Wilcoxon signed-rank test to evaluate intragroup significance between preinjection and postinjection data. The Mann–Whitney U test was used to compare the control and myopic groups. The relationship between clinical and laboratory variables was analysed using non-parametric methods (Spearman's rho correlations). A p value <0.05 was considered statistically significant.

RESULTS

A total of 20 eyes with mCNV in 20 patients met the study criteria and were enrolled for aqueous assessment. Table 1 shows the demographic and clinical characteristics of the participants. Twenty aqueous samples obtained from 20 age-matched patients undergoing cataract surgery served as controls.

Mean VEGF and PEDF levels in aqueous humour of control patients were 25.7±4.9 pg/mL and 12.6±3.5 ng/mL, respectively. When compared with control samples, statistically significant lower levels of both VEGF (19.5±5.4 pg/mL) and PEDF (4.7±2.2 ng/mL) were found in patients with mCNV before IVR (p<0.05 and <0.01, respectively). After IVR, aqueous VEGF levels significantly reduced to 6.5±2.7 pg/mL (p<0.001) and PEDF levels significantly increased to 35.8±11.4 ng/mL (p<0.0001). These data are shown in figure 1.

A significant improvement in BCVA was noticed after IVR (38.2±8.71 letters, p<0.05). Similarly, a reduction in CRT was recorded following intravitreal treatments (226.1±15.3, p<0.01). These data are shown in figure 2.

In the aqueous of eyes with mCNV, VEGF and PEDF levels significantly correlated with each other and with BCVA and CMT. This correlation became more significant after IVR (table 2).

No serious adverse effects were observed during the study period. Adverse ocular and non-ocular events are reported in table 3.

Table 1 Demographic and clinical characteristics of patients with myopic choroidal neovascularisation at study entry

Characteristics	Patients	Controls	p Value
Number	20	20	–
Gender (male, female)	10, 10	10, 10	–
Age, years (mean±SD)	58.6±6.5	65.6±3.3	0.07
Median spherical equivalent, D (mean±SD)	−9.4±1.8	2.5±1.4	<0.01
Median axial length, mm (mean±SD)	30.1±2.3	23.8±1.2	<0.01
Mean BCVA, letters (letters±SD)	24.5±7.9	75±8.3	<0.01
Central macular thickness, µm (mean±SD)	263.4±17.9	289.9±15.3	0.02

BCVA, best-corrected visual acuity.

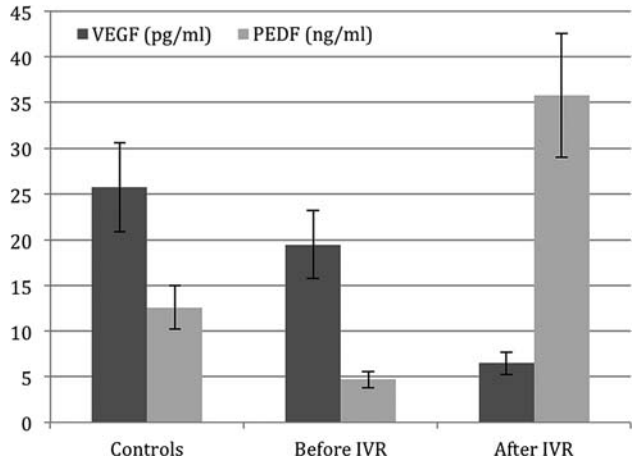

Figure 1 Mean vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF) levels in the aqueous humour of controls and patients with myopic choroidal neovascularisation before and after intravitreal ranibizumab. Dark grey columns indicate VEGF (pg/mL) and light grey columns indicate PEDF (ng/mL). IVR, intravitreal ranibizumab.

DISCUSSION

VEGF and PEDF levels in the aqueous humour of myopic patients were significantly lower than in the control group. These findings are in agreement with those reported by Shin *et al*,[13 14] who found significantly lower VEGF levels in the aqueous humour of highly myopic patients compared with non-myopic subjects, whereas PEDF concentrations did not differ significantly between groups. Chen *et al*[15] recently demonstrated that aqueous VEGF levels in highly myopic patients were significantly lower than controls. Moreover, as ascertained by Chan *et al*,[16] in patients with mCNV, intravitreal anti-VEGF injections increase PEDF levels about 6–7 times from baseline. Although the increase we found is of the same order of magnitude recorded by Chan *et al*,[16] the absolute values are quite different; this may be a result of the different methods used for determining PEDF levels (ChemiKine, Chemicon International, Temecula, California, USA vs HUMAN PEDF ELISA, BioVendor, Laboratornì medicina a.s. Karasek, Czech Republic, respectively). However, our values are in accordance with those recently obtained by Wang *et al*,[17] who used the same methods.

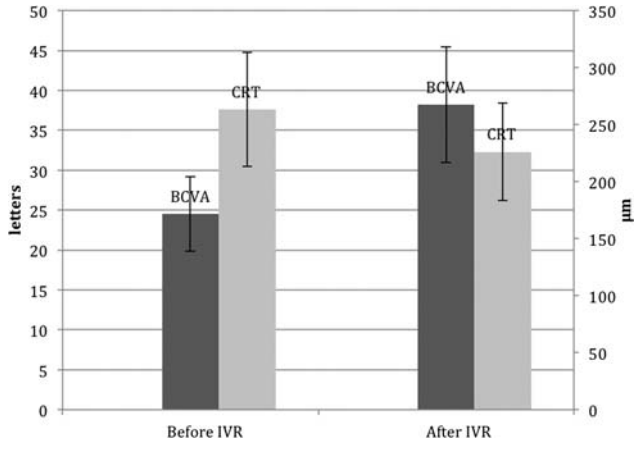

Figure 2 Best-corrected visual acuity (BCVA) and central retinal thickness (CRT) changes 1 month after intravitreal ranibizumab (IVR). Dark grey columns indicate BCVA and light grey columns indicate CRT.

Table 2 Correlation (Spearman's rho) between laboratory and clinical variables in patients with mCNV before and after IVR

	Before IVR		After IVR	
	Rho	p Value	Rho	p Value
VEGF—BCVA	−0.41	0.04	−0.49	0.008
VEGF—CMT	0.46	0.03	0.51	0.007
PEDF—BCVA	0.44	0.04	0.56	0.005
PEDF—CMT	−0.42	0.04	−0.57	0.007
VEGF—PEDF	−0.51	0.03	−0.58	0.005

BCVA, best-corrected visual acuity; CMT, central macular thickness; IVR, intravitreal ranibizumab; mCNV, myopic choroidal neovascularisation; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor.

Patients with mCNV have aqueous humour VEGF levels lower than control subjects. The most plausible explanation for the lower VEGF concentration found in the aqueous humour of patients with mCNV is due to the dilution effect in the anterior chamber and vitreous cavity of myopic patients since the axial length is longer and therefore the intraocular volume is larger in patients with high myopia.[9] However, in patients with PM, a significant correlation between axial length and macular choroidal thickness and VEGF levels has also been demonstrated.[15] Moreover, diminished VEGF production in high myopia could be due to generalised retinal pigment epithelium (RPE) cell degenerative changes, which lead to photoreceptor degeneration.[18] The significantly lower concentration of PEDF in eyes with chorioretinal atrophy-associated high myopia also results from the degeneration of RPE cells and/or retinal ganglion cells, which are the main sources of PEDF in the eye.[19]

VEGF and PEDF levels in the aqueous humour of patients with PM are quite different than the levels recorded in patients with diabetes or with AMD.[10] Despite differences in AMD, diabetes and PM pathogenesis, there is evidence that VEGF may play a role in the development of CNV in PM, as well as in other neovascular eye diseases.[20] In fact, accumulating evidence confirms that anti-VEGF injections are indicated for the treatment of mCNV, and that their use is effective in improving and sustaining both morphological (CRT) and functional (BCVA) parameters in patients with mCNV.[4–8] Nevertheless, the long-term safety and efficacy of anti-VEGF agents remains yet unknown since the risk of developing chorioretinal atrophy secondary to successful treatment with VEGF inhibitors represents a key factor in determining final visual outcome.[4] For this reason, the mean number of injections/year in patients with PM is far less than what is performed in AMD. Yet, in myopic eyes, there is an increased likelihood of developing retinal tears and retinal detachments.[21]

PEDF levels are altered in diseases characterised by retinopathy, such as AMD, diabetic retinopathy and PM. PEDF is deficient in the vitreous of patients with CNV and its concentration decreases with age; however, the effects of PEDF on CNV are complex, and its inhibitory effect on neovascularisation in animal models and in patients with neovascular AMD has been well ascertained.[22] Angiogenesis is controlled by a balance of the expression of angiogenic and antiangiogenic factors: VEGF is a potent inducer of vascular endothelial cell growth derived from blood vessels, whereas PEDF is a powerful angiogenesis inhibitor with high neuroprotective effects. In choroidal diseases involving angiogenesis, such as active CNV, alterations in the concentration of biomolecules that stimulate or inhibit growth of new blood vessels actively contribute to the development of

Table 3 Incidence of ocular and non-ocular adverse events (AEs) in treated patients with myopic choroidal neovascularisation

	Number of patients (%)
Ocular AEs	
Eye pain	40
Ocular hyperaemia	50
Conjunctival haemorrhage	15
Retinal haemorrhage	5
Increased intraocular pressure	5
Corneal erosion	5
Non-ocular AEs	
Arterial hypertension	5
Nausea/vomiting	5
Orthostatic hypotension	5

diseases.[23] VEGF and PEDF both possess multiple biological activities and functions that affect a wide variety of cells. Inappropriate expression levels are associated with diseases that involve neovascularisation: a critical balance between VEGF and PEDF is crucial to prevent the development of CNV.[24] CNV occurs when damage to retinal vessels causes areas of vessel closure and retinal ischaemia that result in elevated levels of hypoxia-inducible factor 1 (HIF-1). HIF-1 stimulates expression of a group of hypoxia-regulated genes that together stimulate the growth of new vessels. Although hypoxia has not been demonstrated as a player in mCNV pathogenesis, HIF-1 levels are elevated in this type of choroidal neovascularisation, and thus the same group of hypoxia-regulated gene products may play a role. While there are substantial differences in the pathogenesis of retinal and subretinal neovascularisation, there is considerable overlap in the vasoactive mediators that participate in these events; for example, increased VEGF expression in mCNV typifies the rationale for anti-VEGF therapy for this pathology.[25]

Interestingly, baseline CMT was higher in control patients due to macular atrophy in high myopia. Our results show that a single IVR is effective in treating patients with mCNV as BCVA improvement associated with a CMT reduction was observed in all treated subjects. IVR resulted in reduced aqueous VEGF and increased PEDF levels in patients with mCNV. Moreover, VEGF upregulates PEDF expression via VEGFR-1 through autocrine signalling.[26] Therefore, a regulatory interaction between the two counterbalancing systems of angiogenic stimulators and inhibitors, VEGF and PEDF, occurs. In mCNV, PEDF is expressed in significantly lower levels than VEGF in RPE cells, RPE basal lamina, Bruch's membrane and choroidal stroma. Balancing these factors may play a key role in maintaining human retina homeostasis and avoiding CNV formation. VEGF and PEDF expression levels vary with the severity of CNV. When CNV was active, VEGF and PEDF are both strongly expressed in CNV lesions. However, after CNV has developed, the expression of both VEGF and PEDF decreases. VEGF-A exerts antiapoptotic, chemotactic, mitogenic and proinflammatory activities, and emerging evidence suggests that it also displays marked neurotrophic and neuroprotective properties.[27] Concomitantly, PEDF release from retinal cells is downregulated, providing a permissive condition for retinal neovascularisation.[22] In experimental animal models, intravitreal PEDF inhibits retinal neovascularisation, counteracting angiogenesis through normalisation of intraocular levels of VEGF and other proangiogenic molecules. These findings suggest that PEDF treatment may represent an effective therapy for ocular diseases characterised by neovascularisation.[28] The significant correlation between VEGF and PEDF levels found in our patients with mCNV further confirms the close relationship between these two compounds, suggesting that VEGF-induced PEDF gene upregulation and a feedback mechanism may be present in CNV.[23]

In summary, our results show that the mechanism by which anti-VEGF counteracts the neovascularisation process in mCNV is also mediated by an increase in PEDF levels. In animal models, intravitreal bevacizumab decreases the transcriptional expression of VEGF-A and increases the expression of PEDF in the pigment epithelium, modulating the expression of angiogenesis-related factors. Together, these findings suggest that PEDF constitutes a novel target for the treatment of ocular diseases characterised by neovascularisation.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted according to the ethical principles of the Declaration of Helsinki and was approved by the ethics committee of University of Molise.

Provenance and peer review Not commissioned; externally peer reviewed.

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