

Gene expression levels of the insulin-like growth factor family in patients with AMD before and after ranibizumab intravitreal injections

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Purpose: The present study focused on the assessment of the mRNA levels of the insulin-like growth factor (IGF) family in patients with the exudative form of age-related macular degeneration (AMD) before and after ranibizumab intravitreal injections.

Patients and methods: An analysis of the expression profile of the IGF family of genes in patients with AMD was carried out using the oligonucleotide microarray and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) methods.

Results: In the peripheral blood mononuclear cells (PBMCs) obtained from AMD group receiving ranibizumab compared to the peripheral blood mononuclear cells from AMD group before ranibizumab treatment using oligonucleotide microarray technique, six statistically significant differentially expressed transcripts related to the IGF family were detected (unpaired *t*-test, $p < 0.05$, fold change > 1.5). Moreover, analysis using the real-time RT-qPCR technique revealed statistically significant differences in the *IGF2* and *IGF2R* mRNA levels (Mann-Whitney *U* test, $p < 0.05$) between the two groups that were studied. Statistical analyses of both oligonucleotide microarray and real-time RT-qPCR results demonstrated a significant decreased expression only for *IGF2* mRNA.

Conclusion: Our results revealed a changed expression of *IGF2* mRNA after ranibizumab treatment.

Keywords: age-related macular degeneration, IGF family, ranibizumab, mRNA

Introduction

Age-related macular degeneration (AMD) is a chronic, progressive disease that leads to permanent physiological and morphological changes in the retina and therefore leads to the irreparable impairment of the visual acuity. AMD is one of the most frequent causes of blindness in the population over 50 years of age in developed countries.¹⁻⁴ Moreover, a poor prognosis of visual outcomes in untreated cases was confirmed.⁵ Although it is believed that various genetic and environmental factors may have a significant impact on the development of AMD, the etiology of this complicated disease is still unknown.^{6,7}

Insulin-like growth factors (IGFs) are essential for the regulation of cellular processes, such as differentiation, apoptosis, and neovascularization.⁸ It is well known that IGFs can stimulate the proliferation of endothelial cells. Moreover, they can activate the vascular endothelial growth factor (VEGF) expression. Therefore, they can participate in the pathogenesis of various diseases that are associated with pathological angiogenesis.⁹ Locally produced IGFs seem to be especially important in the

development of ocular diseases such as AMD, diabetic retinopathy, or retinopathy of prematurity (ROP).¹⁰

The IGF family consists of two cell-surface receptors (IGF1R and IGF2R), two growth factor ligands (IGF1 and IGF2), six high-affinity IGF binding proteins (IGFBP1–6), and associated IGFBP degrading enzymes.^{11,12} Moreover, members of these growth factors include nine IGFBP-related proteins, which can bind to the IGFs but with a lower affinity than IGFBPs.¹³ IGFs and IGFs have amino acid sequences that are homologous to insulin or an insulin receptor, respectively.¹⁴

Ranibizumab (Lucentis; Novartis International AG, Basel, Switzerland) is an anti-VEGF agent that is commonly used in the form of intravitreal injections to treat the neovascular exudative type of AMD and choroidal neovascularization (CNV), which is secondary to many other reasons.^{15–17} The efficacy and safety of ranibizumab in the treatment of exudative AMD have been demonstrated in many previous studies including large samples studies and multicenter randomized clinical trials as well as in substantial clinical practice.^{15,18} However, systemic side effects of intraocular anti-VEGF therapy for AMD are posited.^{17,19} Moreover, gene expression or protein level changes in the blood samples of AMD patients after intravitreal anti-VEGF treatment are not well known.^{20–22} There are only a few data about the systemic expression profile of IGF-related genes in patients with ocular diseases or the influence of the anti-VEGF drugs that are used in the treatment of these diseases on its change.²³

Therefore, the present study focused on the assessment of the mRNA levels of the IGF genes in patients with the exudative form of AMD before and after ranibizumab intravitreal injection.

Patients and methods

Ethics statement

Permission to conduct the study was obtained from the Bioethics Committee of the Medical University of Silesia in Katowice (KNW/0022/KB1/61/10 and KNW/0022/KB1/61/II/10/12). All donors provided written informed consent in accordance with the Declaration of Helsinki. All of the patients were treated at the Department of Ophthalmology, University Hospital No 5, Medical University of Silesia, Katowice, Poland.

Subjects

Twenty-nine patients (15 women and 14 men, mean age 73 years, range 54–86 years) with diagnosed neovascular

AMD were enrolled into the study. All patients were subjected to the following ocular investigations: best-corrected visual acuity using Snellen charts, Goldmann applanation tonometry, indirect biomicroscopy in mydriasis (+78 D lens; Volk Optical, Mentor, OH, USA), optical coherence tomography (OCT, Cirrus HD-OCT 4000; Carl Zeiss Meditec AG, Jena, Germany), and fluorescein angiography (Fundus Camera FF 450 plus IR; Carl Zeiss Meditec AG).

The study group was homogenous in terms of age, race, and gender and consisted of highly selected patients who had no other diseases except for hypertension, had no ophthalmic surgery, and had no stroke or myocardial infarction during the previous 6 months. The main inclusion criteria for the study group included: CNV resulting from AMD, aged >50 years, best-corrected visual acuity 0.1–0.5 according to the Snellen chart, and no previous CNV therapies, such as anti-VEGF injections, photodynamic therapy, or laser therapies. The main exclusion criteria from the study group were as follows: uncontrolled, elevated intraocular pressure; glaucomatous optic neuropathy; chronic uveitis; retinal vein occlusion; other ocular neovascular diseases; and a previous history of myocardial infarction or stroke during the previous 6 months.

The time between first symptoms and ranibizumab injections was up to 3 months. Intravitreal injections were performed by an ophthalmologist who used an aseptic technique, using infiltration anesthesia at the injection site. Ranibizumab (0.5 mg) was administered intravitreally three times in the form of one injection every month during a period of 3 months. The reinjections were dependent on the CNV activity.

Tissues

Venous blood samples (5 mL) for molecular analysis were obtained from the all AMD patients included in the study before ranibizumab injections and 30 days after the application of the three injections of ranibizumab at around the same time of day and collected into tubes containing EDTA. Isolation of peripheral blood mononuclear cells (PBMCs) was performed using the Ficoll–Conray density gradient centrifugation for 30 minutes at 1,500 rpm at room temperature immediately after blood collection (specific gravity 1.077; Immunobiological Co., Gunma, Japan).

RNA extraction

Total RNA extraction was performed using TRIzol reagent (Invitrogen Life Technologies, CA, USA) according to the

producer's instructions. The purification, integrity, and quantitative assessment of the RNA extracts was performed as described previously.²⁴

Oligonucleotide microarray analysis

Analysis of the expression profile of the IGF-related genes was performed using commercially available oligonucleotide microarrays of HG-U133A (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's recommendations, as described previously.²⁴ The oligonucleotide microarray analysis was performed on eight samples out of 29 samples – three samples before and five samples after the ranibizumab intravitreal injections.

Real-time RT-qPCR

The mRNA level of *IGF1*, *IGF2*, *IGF1R*, *IGF2R*, and b-actin (*ACTB*) was determined using the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). The quantitative analysis was carried out using SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen, Valencia, CA, USA) and DNA Engine Opticon™ System (MJ Research Inc., MA, USA). The oligonucleotide primers and thermal profile have been previously described.^{25,26} The mRNA copy number was recalculated per 1 µg of the total RNA.

Statistical analyses

Analysis of the results obtained from oligonucleotide microarrays was performed using the GeneSpring 13.0 software (Agilent Technologies UK Ltd, South Queensferry, UK) and the PI-Grid platform. Normalization of the results was performed using the Robust Multi-Array Average

method. The differences in the expression levels of the IGF-related transcripts between the two groups were evaluated using the unpaired *t*-test with a cutoff at least a 1.5-fold change and $p < 0.05$. The real-time RT-qPCR results were subjected to statistical analysis using Statistica 12.0 software (StatSoft, Tulsa, OK, USA), and the Mann–Whitney *U* test was applied to compare the differences in the expression of *IGF1*, *IGF2*, *IGF1R*, and *IGF2R*. The values that were obtained using the real-time RT-qPCR were expressed as medians with the 25th and 75th quartiles and minimum and maximum.

Results

In the first step of the study, 26 ID mRNAs that are associated with the IGF family were selected from the NetAffx Analysis Center database of Affymetrix (<http://www.affymetrix.com/analysis/index.affx>). Among these, in the patient group receiving ranibizumab in comparison to the patient group before ranibizumab treatment, six statistically significant differentially expressed transcripts were detected (unpaired *t*-test, $p < 0.05$, fold change > 1.5) based on the oligonucleotide microarray data analysis (Table 1).

In the next step of the study, comparative analysis of the *IGF1*, *IGF2*, *IGF1R*, and *IGF2R* mRNA copies/µg of the total RNA was performed between the two groups that were studied using the real-time RT-qPCR technique. Our results revealed statistically significant differences in the *IGF2* and *IGF2R* mRNA levels (Mann–Whitney *U* test, $p = 0.017$, $p = 0.023$, respectively). However, there were no statistically significant differences in the *IGF1* and *IGF1R* mRNAs (Mann–Whitney *U* test, $p = 0.809$, $p = 0.422$, respectively) (Figure 1A and B).

Table 1 Characteristics of the IGF family transcripts that exhibit a differential expression in the PBMCs of patients with clinically diagnosed AMD before and after ranibizumab treatment

Fold change	Number of ID mRNAs	$p < 0.05$	$p < 0.02$	$p < 0.01$	$p < 0.005$	$p < 0.001$
FC all ID mRNAs	26	7	5	2	2	1
FC > 1.1	19	7	5	2	2	1
FC > 1.5	8	6	5	2	2	1
ID mRNA	Gene symbol	Gene name		FC after vs before		p-value
209541_at	IGF1	Insulin-like growth factor 1		2.32↓		0.0010
202409_at	IGF2	Insulin-like growth factor 2		1.81↓		0.0188
213910_at	IGFBP7	Insulin-like growth factor binding protein 7		2.54↓		0.0134
211959_at	IGFBP5	Insulin-like growth factor binding protein 5		2.31↓		0.0023
211958_at	IGFBP5	Insulin-like growth factor binding protein 5		1.60↓		0.0498
210095_s_at	IGFBP3	Insulin-like growth factor binding protein 3		2.18↓		0.0197

Notes: Unpaired *t*-test, ↓ – lower expression, differentially expressed genes with $p < 0.05$ and FC > 1.5 are indicated in bold.

Abbreviations: AMD, age-related macular degeneration; FC, fold change; IGF, insulin-like growth factor; PBMC, peripheral blood mononuclear cells.

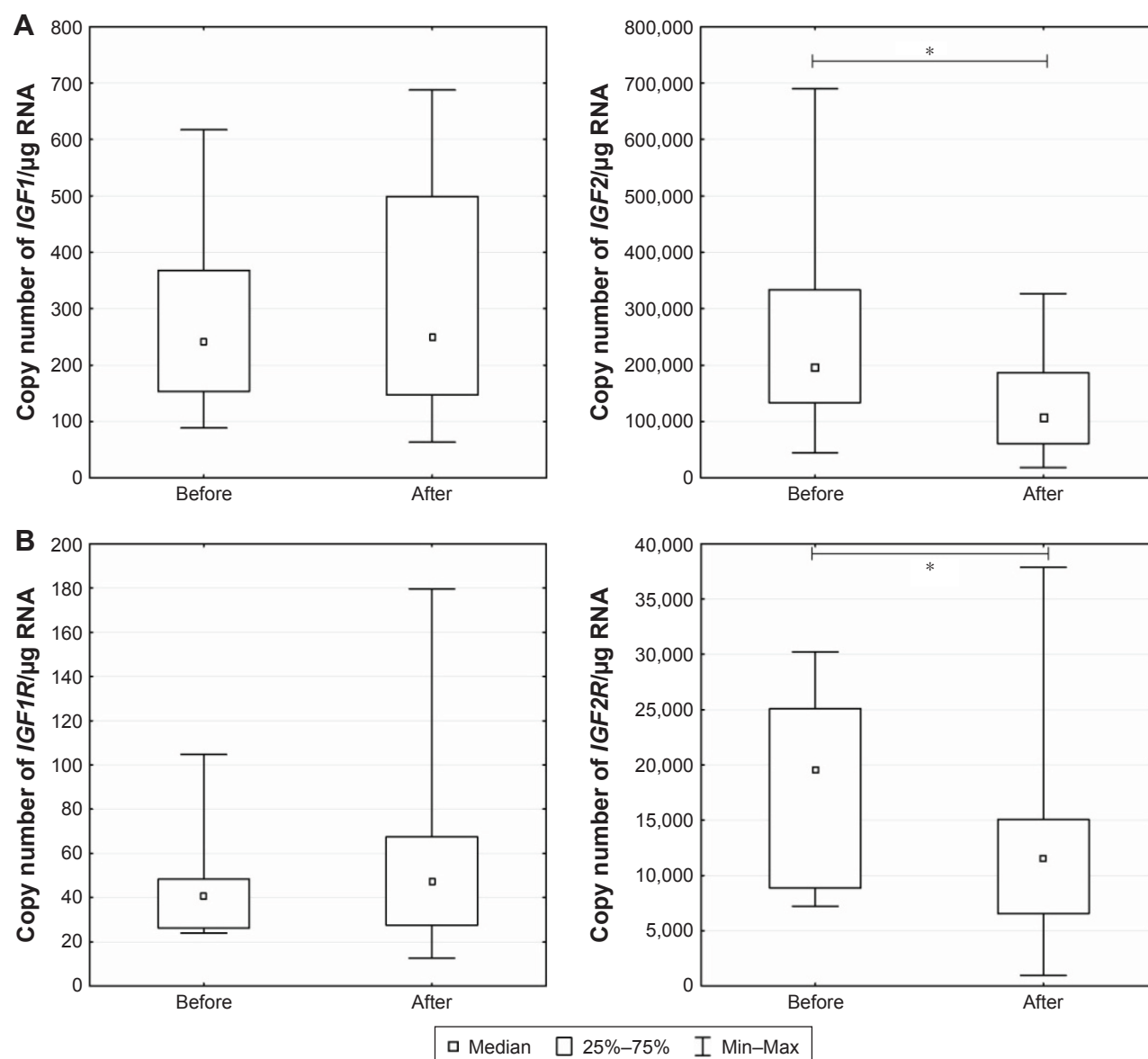


Figure 1 The mRNA levels of *IGF1*, *IGF2* (A) and *IGF1R*, *IGF2R* (B) in the PBMCs of patients with clinically diagnosed AMD before and after ranibizumab treatment.

Notes: Box and whisker plots present medians \pm quartiles and extreme values of copy numbers per 1 μ g of total RNA. Statistical significance: * $p < 0.05$, Mann–Whitney *U* test.

Abbreviations: AMD, age-related macular degeneration; IGF, insulin-like growth factor; PBMC, peripheral blood mononuclear cells.

Statistical analyses of both oligonucleotide microarray and real-time RT-qPCR results demonstrated a significant difference in expression only for *IGF2* mRNA.

Discussion

The IGF family plays an important role in the pathogenesis of many diseases, including eye diseases.²⁷ Lambooi et al²⁸ and Rosenthal et al²⁹ suggested its implication in the development of AMD. However, the exact role of IGFs in AMD still needs to be explained. In our study using oligonucleotide microarray, it was revealed that the *IGF1*, *IGF2*, *IGFBP1*, *IGFBP3*, and *IGFBP5* levels were significant downregulated

in the PBMCs from patients with AMD after ranibizumab treatment. Microarray analysis enables screening of gene expression profile. However, it is known that RT-qPCR has a higher sensitivity and specificity in comparison to the oligonucleotide microarray. Therefore, our study focused on the use of oligonucleotide microarray to investigate the expression profile of 26 ID mRNAs of IGF-related genes, and then the microarray data were verified using the real-time RT-qPCR technique. Validation of these results revealed statistically significant differences in the *IGF2* and *IGF2R* mRNA levels. The differences between microarray and RT-qPCR techniques may also result from carrying out

RT-qPCR assay on the larger study group. However, both analyses presented statistically significant differences only for *IGF2* mRNA. The mRNA level of the studied genes was determined in PBMC samples although, eg, vitreal samples may better reflect the retinal and choroidal conditions.³⁰ It is also known that human vitreous IGF2 level is several times higher than IGF1 level.³¹ However, vitreous samples are much harder to obtain than PBMC samples, and this was not possible with our patients.

Although both IGF1 and IGF2 can easily be detected in plasma,³² Haurigot et al¹⁰ showed that an increased intraocular but not systemic level of IGF1 can cause a disruption of blood–retinal barrier (BRB), which leads to various ocular diseases. Moreover, after treatment with IGF1, intravitreally injected, rats developed retinal vascular hyperpermeability that caused the degradation of BRB. These authors also observed an increased expression of VEGF in these rats.³³ On the other hand, Spranger et al³⁴ reported that an increased systemic expression of IGF1, IGF2, and IGFBP3 in proliferative diabetic retinopathy patients caused the disruption of BRB, which lead to the intravitreal accumulation of IGFs. These authors suggested that this is a more important source of these growth factors than local synthesis. Moreover, Hellstrom et al³⁵ suggested that the IGF1 level can be used to predict whether ROP will develop in premature infants. These authors showed that low serum levels of IGF1 could cause hypoxic retina, which can induce VEGF expression. Previous reports also disclosed that there is a correlation between systemic level of IGFs and the changes in eye structures.^{36,37} The abovementioned studies suggested that the source of elevated level of IGF family in ocular diseases still needs explanation. Moreover, there was revealed a significant correlation between serum and vitreous level of VEGF in proliferative diabetic retinopathy patients.³⁸ In turn, Romano et al³⁹ revealed altered expression of three microRNA both in rat retinas and in serum of AMD patients. However, only a few reports showed changed expression of various mRNAs or proteins in PBMCs obtained from AMD patients.^{40–42} Moreover, there are no published data regarding influence of intravitreally injected ranibizumab on IGF family expression level in the blood or vitreous of AMD patients. Previous research revealed only reduced concentration of VEGF after intravitreal ranibizumab treatment in the aqueous humor of AMD patients⁴³ or in the vitreous of diabetic proliferative retinopathy patients.⁴⁴ In turn, Manresa et al⁴⁵ showed no significant differences in homocysteine level after ranibizumab injections in the plasma and vitreous obtained from patients with AMD. Kong et al²³ measured the serum level of IGF1

and VEGF in infants with ROP, but after intravitreal bevacizumab injections. These authors revealed decreased serum levels of both of the growth factors 2 days after treatment. However, after this time, the IGF1 level was increased, but it was always lower than in infants who had been treated with laser. In this case, a lower level of IGF1 after bevacizumab treatment may cause slower vessel maturation than in the laser-treated infants. On the other hand, Hong et al⁴⁶ and Wu et al⁴⁷ revealed a decreased serum level of VEGF after bevacizumab treatment although other growth factors, including IGF1, were unchanged in infants with ROP. In our results, a decreased expression of IGF-related genes after anti-VEGF treatment was observed but in the PBMCs from the AMD patients who had been treated with ranibizumab. This is consistent with other results which showed that intravitreally injected anti-VEGF agents can be detected in serum/PBMCs and can therefore have a systemic influence.^{19,24} Moreover, some reports demonstrated the potential systemic effects of anti-VEGF therapy for AMD,^{20,24,48} eg, in our previous studies mean central retinal thickness was reduced in the treated eyes after intravitreal ranibuzumab injections. In addition, central retinal thickness was also decreased in the fellow eyes.²⁴ On the other hand, subfoveal choroidal thickness and foveal thickness were increased in patients with controlled acromegaly.⁴⁹ Moreover, Yazgan et al³⁷ showed positive correlation between macular choroidal thickness and serum IGF1 level. In turn, Polat et al⁵⁰ revealed no significant difference in mean central retinal thickness between the patients with acromegaly and the control group. However, retinal neovascularization was decreased after systemic inhibition of growth hormone or IGF1 in mice.³⁶

Neovascularization can also be promoted by IGF2.⁵¹ Kim et al⁵² reported that hypoxia induces *IGF2* expression and then retinal vascularization during ocular development. In turn, Spranger et al³⁴ detected IGF1, IGF2, and IGFBP3 in both the serum and vitreous in patients with proliferative diabetic retinopathy. These authors suggested that there is a possibility to treat this disease using inhibitors of the systemic IGF levels. These results are supported by Dransfield et al's⁵³ study which showed that the human monoclonal antibody against IGF2 can cause the inhibition of cancer cells both in vitro and in vivo. Maeng et al⁵⁴ also suggested that IGF2 could be a target for the treatment of diseases that are connected with neovascularization. It is also known that VEGF can decrease the expression of IGFBP3 and increase IGFBP5 at both the protein and mRNA levels in bovine aortic endothelial cells.⁵⁵ Moreover, in diabetic human retinal endothelial cells, IGF1R, IGFBP1, IGFBP2,

IGFBP3, and IGFBP5 were increased at the protein level and *IGFBP1*, *IGFBP2*, and *IGFBP5* also were increased at the mRNA level, while the *IGF1R* and *IGFBP3* expression was unchanged.⁵⁶

IGFBP1 can inhibit IGF1 and IGF2.⁵¹ In turn, IGFBP3 plays different roles. Some reports revealed that it can play an antiapoptotic role and that it can stimulate angiogenesis, but other reports showed that IGFBP3 could inhibit angiogenesis and counteract the effect of VEGF.²⁷ Although an increased level of IGF1 in vitreous proliferative diabetic retinopathy is seen, the IGFBP1 and IGFBP3 levels were unchanged.⁵⁷ In turn, infants with ROP had a lower plasma IGFBP3 level.⁵⁸ Similarly, it is known that IGFBP5 and IGFBP6 also have an inhibitory effect on IGFs.⁵¹ Some of these proteins can act through IGF-independent mechanisms.²⁷ Moreover, retinas in a hypoxia condition showed a decreased expression of IGFBP7 compared to retinas in a normal condition.¹³ Lofqvist et al⁵⁹ detected a greater expression of *IGF2* than *IGF1* in the photoreceptors and blood vessels of mouse retina. Moreover, these authors also observed an increased expression of *IGFBP3*, *IGFBP5*, and *IGFBP7* in the pathological vessels compared to normal vessels.

Jacobo et al⁶⁰ performed studies about the influence of the single-nucleotide polymorphisms of the *HTRA1* gene. The product of this changed gene cannot bind to IGF1 and therefore causes an amelioration of the effects of IGF1. These authors suggested that the polymorphisms of this gene can cause the development of AMD. In turn, Chiu et al¹² showed that a single-nucleotide polymorphism in *IGF1R* is connected with a greater risk of the development of AMD. Makarev et al⁶¹ performed an analysis of the pathway development and revealed a differential expression of 29 pathways in AMD, including the proangiogenic proliferation pathways, eg, those connected with IGF2. Moreover, Newman et al⁶² performed transcriptome analysis in choroid-RPE and retina tissue samples that had been obtained from AMD and control subjects. These authors suggested that IGF2 could be a target for AMD treatment. Cha et al,⁶³ who performed an analysis of the cytokine concentration in the aqueous humor that had been collected from patients with neovascular AMD, provided important information. These authors found a statistically significant higher level of IGF1, IGFBP2, IGFBP6, and IGFBP7 in the aqueous humor of patients who had been diagnosed with exudative AMD, thereby indicating their involvement in angiogenesis.

Dong et al⁶⁴ revealed that the polypyrimidine tract-binding protein-associated splicing factor can inhibit VEGF expression stimulated by IGF. Shaw et al⁶⁵ suggested that the IGF1

receptor ribozyme could reduce retinal neovascularization. In turn, Lee et al⁶⁶ showed that an extract of *Cinidium officinale* Makino and its bioactive compound, butylidenephthalide, could be used in the treatment of AMD because they inhibit IGFBP1, MCP1 and VEGF expression. Deissler et al⁶⁷ revealed that ranibizumab can inhibit the migration of the bovine retinal endothelial cells that are induced by VEGF, IGF, PGF, and FGF2. On the other hand, Shao et al⁶⁸ showed an upregulation of IGF1 both at the mRNA and protein level after bevacizumab treatment, but in xenografts that had been derived from ovarian cancer cell lines.

Conclusion

Our study indicated a differential gene expression of *IGF2* in PBMCs obtained from AMD patients before and after the intravitreal ranibizumab treatment. However, additional studies are needed to confirm our results conclusively, but this study may provide a valuable basis for further research that will be carried out on a larger group of patients.

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Disclosure

The authors report no conflicts of interest in this work.

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