

Association between VEGF gene polymorphisms and the therapeutic response to bevacizumab in Diabetic Macular Edema

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Research Article

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Abstract

Introduction. Diabetic macular edema (DME) is the most frequent and disabling manifestation of diabetic retinopathy (DR). The polymorphisms of VEGFA gene has been associated with the development of DME. To investigate the relationship between the VEGFA polymorphism gene and the response to antiangiogenic treatment in DME.

Methods. 82 treatment-naïve patients with type 2 diabetes mellitus (T2DM) and clinically significant DME were studied. Intravitreal bevacizumab was administered and patients were evaluated at the first and third month. DNA was obtained to identify the polymorphisms rs2010963, rs699947 and rs35569394. The direct sequencing technique was used. Friedman test were used for comparison. Distribution of genotypes and alleles were analyzed by the Chi-square test and Fisher's exact test. $P < 0.05$ was considered as significant.

Results. The rs2010963 polymorphism in the responders the most frequent genotype was the CG (59%) ($p = 0.0036$, OR 3.2), with G being the most frequent allele ($p < 0.0001$, OR 6.6). This indicates that G allele have a higher probability of responding to treatment with bevacizumab. No significant differences were found for the polymorphisms rs699947 and rs35569394.

Conclusion. There is a relationship between genetic variability and treatment response, including intronic and promoter-region polymorphisms. We hypothesized that the allele G in the VEGF rs2010963 polymorphism is related to variability in the response to bevacizumab treatment in patients with DME.

Introduction

Diabetes mellitus is a public health problem; affects approximately 530 million people globally of which 95% have type 2 diabetes mellitus (T2DM) [1]. In Mexico, the prevalence has shown an increase in recent decades, it is estimated that 17 to 18.3% of the population, aged 20 years or older, have T2DM with an estimated 14 to 14.6 million affected adults [2]. Diabetes mellitus is characterized by a chronic hyperglycemic state that triggers increased oxidative stress, activates of the polyol pathway, and promotes the accumulation of advanced glycation end products (AGEs). These processes cause damage to endothelial cells of blood vessels, affecting the micro and macrovasculature [3].

Diabetic macular edema (DME) is the most frequent and disabling manifestation of diabetic retinopathy (DR); It is defined as the thickening or inflammation of the macula, which is the central region of the retina and is responsible for the most precise vision. This process is the result of the abnormal accumulation of extracellular fluid and displacement of the intraretinal or sub foveal space, caused by alterations in the permeability of the blood vessels present in the peripheral retina near the fovea [4]. The clinical relevance of this complication is when clinically significant macular edema (CSME) occurs and therapy is required to stop or delay its progression to avoid vision loss as a result of retinal layer disorder [5].

During the progression of DR, increased capillary permeability leads to a loss of vascular volume, capillary perfusion is inhibited in some areas, and tissue hypoxia and ischemia occur [6]. Angiogenesis-inducing pathways are activated, including hypoxia-inducible factor type 1 (HIF-1 α) in retinal cells, which translocate to the nucleus and positively regulates the transcription of genes involved in adaptation mechanisms, most notably vascular endothelial growth factor A (VEGFA) [6]. In the development of EMD, VEGFA is recognized as the main mediator of pathological progression because its overexpression constitutes the molecular activator of vascular permeability [6, 7].

In the degenerative process of diabetic microangiopathy that culminates in DME and the continuous presence of macular vascular leakage, the overexpression of VEGF is intrinsically linked as a potent mediator of vascular permeability and angiogenesis [8]. Genetic variability may be related to VEGFA expression and susceptibility to developing DME and probably defines the severity of the disease [9]. The -2578 C/A polymorphism (rs699947) has been investigated in pathologies associated with hypervascularization [10]. At the ocular level, it is proposed that the adenine (A) allele in this position is associated with an increased concentration of VEGFA in the vitreous humor, which allows for greater ligand-receptor binding, promoting neovascularization, blood retinal barrier (BRB) rupture, and macular leakage, increasing the risk of severe forms of DR [10, 11]. It has been found that two VEGF gene polymorphisms, the -634 C/G (rs2010963) occurring in 5'-UTR and an 18 base pair (bp) insertion/deletion (I/D) polymorphism at position -2549 of the VEGF promoter region (rs35569394) has also been described. There is evidence that these functional polymorphisms are associated with immune-mediated conditions, particularly microvascular diseases [12]. However, the -2549 insertion/deletion (I/D) polymorphism influences the basal transcription rate of the gene, with the deletion (D) variant negatively modifying the binding affinity to the transcription factor, which reduces VEGFA expression, indicating that the homozygous D/D genotype is probably a protective factor due to the decrease in VEGFA concentration [13].

The therapeutics for DME focus on neutralizing the overexpression of VEGFA with the intention of stopping or delaying the progression of blindness. Currently, the clinical guideline for the control of DME establishes therapy with anti-angiogenics such as aflibercept, ranibizumab or bevacizumab administered intravitreally, which results in a decrease in central macular thickness and improvement of visual acuity [14]. In addition, adjuvant therapies such as reticular or focal laser photocoagulation and intravitreal corticosteroids are used; these are mainly used in cases where the use of antiangiogenics did not yield considerable results [15, 16].

Bevacizumab is a humanized monoclonal antibody initially approved as an oncological treatment; however, it was found to have ophthalmological efficacy for the treatment of neovascular and edematous pathologies in the retina [17]. Studies showed that administration of bevacizumab produces a significant reduction in macular thickness in patients with DME compared to therapies such as laser or laser and triamcinolone [18]. The effectiveness of bevacizumab is similar to other anti-angiogenic treatments such as ranibizumab or aflibercept; but the use of bevacizumab is beneficial due to the cost-benefit ratio [19].

Although there is evidence of the efficacy of bevacizumab and other antiangiogenics in the treatment of DME, the therapeutic response is variable among patients. This suggests the existence of intrinsic factors involved in the variability of the response. It has been shown that, after the application of anti-angiogenic therapy, some patients show an anatomical and functional improvement, while others do not have a significant response regardless of the dosage [20, 21].

It is suggested that differences in therapeutic response are linked to individual-specific factors, including genetic factors such as single nucleotide polymorphisms (SNPs) [22]. In this context, the VEGFA gene polymorphism emerges as a determining factor for the therapeutic effect of bevacizumab. Studies have evaluated the relationship between these polymorphisms and the safety of bevacizumab use [23]. The rs2010963 polymorphism has been identified as being associated with adverse events such as hypertension and proteinuria. Since VEGF polymorphism represent the primary risk contributors for DME, we aimed to investigate if there is a relationship between the polymorphism of the VEGFA gene promoter region and the response to antiangiogenic treatment in diabetic macular edema [23, 24].

Materials and methods

This is a report about observational, comparative, prospective, and longitudinal study was conducted in accordance with the Declaration of Helsinki and approved by the institution's ethics and research committee with the register number 2019R23BI.

Eligibility, Search, and Selection Criteria

Participants were adults, diagnosed with type 2 diabetes mellitus (T2DM) of more than five years duration and clinically significant macular edema, who were treatment-naïve. All participants provided written informed consent. A complete ophthalmological evaluation was performed including Snellen visual acuity test, Goldmann contact tonometry, slit-lamp examination, and an optical coherence tomography (OCT) examination of the fundus. All imaging modalities were carried out by an experienced ophthalmic photographer with the Heidelberg SPECTRALIS OCT, Heidelberg Engineering, Heidelberg, Germany. The diagnosis of clinically significant DME was made using the parameters of the Early Treatment Diabetic Retinopathy Study (ETDRS). Patients included in the study had at least one eye with center-involving DME defined as central subfield thickness (CST) of more than 300 μm on SD-OCT. Intravitreal bevacizumab was administered and patients were evaluated at the first and third month of drug administration.

Patients were considered non-responsive to bevacizumab if they had persistent intraretinal and/or subretinal fluid on OCT (CST $>300 \mu\text{m}$ and $\leq 10\%$ CST decrease after bevacizumab injection). Patients were excluded from the study if they had any history of intraocular inflammation, or postsurgical macular edema. Patients were also excluded if any intraocular surgery such as cataract surgery or vitrectomy was performed or if they had any other treatment such as focal laser or intravitreal dexamethasone. Other disease conditions that may affect best-corrected visual acuity (BCVA) or central retinal thickness

measurements, such as AMD, retinal vein occlusion, epiretinal membrane and glaucoma, were also excluded.

DNA extraction and genotyping

Whole blood samples were collected using EDTA as an anticoagulant and DNA extraction was performed using the QIAamp mini kit (QIAGEN, Germany), subsequently amplification was performed by polymerase chain reaction (PCR). Each 25µL PCR reaction contained 1X Buffer (20 µM Tris-Cl, 100 µM KCl, 0.1 µM EDTA and 0.5% (v/v) Tween 20), 200 ng of genomic DNA, 0.2 mM of each dNTP, 2 U of Taq polymerase, 1 mM of oligonucleotides (Table 1) and 1.5 mM of MgCl₂. An Axygen/Maxygene PCR System 2400 thermocycler (Perkin Elmer Co. Norwalk, Connecticut) was used. Amplification conditions were: initial denaturation at 95°C for 10 minutes, followed by 35 cycles with denaturation at 95°C for 1 minute, annealing at specific temperature for each fragment to be amplified for 1 minute (Table 1), extension at 72°C for 1 minute and final extension at 72°C for 10 minutes.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel (90V/ 40 minutes), only for the rs35569394 polymorphism was a 3.0% gel used (90V/ 90 minutes), all amplified products were observed with the UVP BioLite™ MultiSpectralSource photodocumentation system using the LaunchVision Works LS program. To analyze the rs2010963 and rs699947 polymorphisms, the direct sequencing technique using fluorescent terminators (Big Dye Terminator, Thermo Fisher Scientific, Vilnius, Lithuania) was used.

Statistical Analysis

We performed the statistical analyses using GraphPad Prism 8 (GraphPad Software, CA, USA). The baseline characteristics of all participants not were distributed normally according to the Kolmogorov–Smirnov test. Therefore, the Mann Whitney test for independent means, Friedman test were used for comparison. Distribution of genotypes and alleles was analyzed by the Chi-square test and Fisher's exact test, and the data were expressed as the median (minimum and maximum) and frequencies. $P < 0.05$ was considered as significant.

Results

A total of 102 patients with type 2 diabetes mellitus and diabetic macular edema (DME) were included in the study. All participants underwent a comprehensive ophthalmological examination and provided blood samples prior to the intravitreal administration of bevacizumab, and were scheduled for follow-up review at one and three months after application. Demographic data are shown in Table 2.

In the table 3 presents the macular thickness and volume results obtained via OCT and the visual acuity in Snellen and LogMar scale. The figure 1 illustrates a representative case showing the reduction of macular thickness and volume. The reduction in macular thickness was evaluated, basal and 3 months after and participants were classified as responders and non-responders. 63 patients (62%) were

responders (79 eyes) and 39 (38%) were non-responders (33 eyes). In patients with bilateral DME, the response was similar in both eyes. When performing the analysis of the decrease in macular thickness and volume in non-responders, the change was not significant ($p = 0.1031$ and 0.1778 respectively). Figure 2

The responder patients there was a significant reduction three months after the application of bevacizumab 23% to macular thickness and 12% to macular volume with a significantly difference ($p < 0.0001$ for both). Figure 3

The distribution of genotypes rs2010963, rs699947, and rs35569394 was found to be in Hardy-Weinberg equilibrium. The results of the allele and genotype frequency analysis are shown in Table 4. There was no statistically significant difference between the rs699947 or rs35569394 and the response to treatment with bevacizumab. The genotype distribution of the rs2010963 VEGF-A gene polymorphism was statistically different between the study groups ($p = 0.0003$).

Discussion

Diabetic macular edema (DME) remains the main cause of vision impairment in diabetic patients and the vascular endothelial growth factor (VEGF) plays an important role in the pathogenesis of DME. Anti-VEGF agents remains as the first-line therapy in DME treatment with outstanding results anatomic and functional results. However, some patients do not respond satisfactorily to anti-VEGF treatment, more than 30% present persistent DME even after intravitreal injection, suggesting other pathogenic factors might contribute to the pathogenesis of DME. Recent studies have shown that the genetic factors may affect the results of the intravitreal anti-VEGF injections in patients with DME. However, to our knowledge, this is the first study to determine whether the variants of VEGF gene affect the response anti-VEGF therapy.

Although anti-VEGF therapy has had a significant benefit for most of the patients, inadequate response to treatment and treatment failures to treatment. A theory proposed to explain treatment failure imply that suboptimal response to anti-VEGF agents, might be explained due to a short-term response that stays undetected at monthly follow-up visits. The response to anti-VEGF injection is likely influenced by a combination of numerous clinical, genetic, and environmental factors. Despite the fact that genetic studies have been highly successful in identifying genes and pathways driving DME risk, our understanding of how these genetic variants contribute to DME progression is still limited. A large number of studies have investigated the associations of genetic polymorphisms with the development of DME, but not with the response to anti-VEGF treatment for DME.

The present study was conducted to investigate the relationship between the rs2010963, rs699947, rs35569394 polymorphisms of the VEGFA gene and the response to treatment with bevacizumab. In our findings, we found that 65% of patients responded to treatment, while only 35% were non-responders. Regarding the study of the rs2010963 polymorphism, in the responders the most frequent genotype was the CG with a 59% and GG with 32% while CC only a 9%. The G allele was more frequent in responders

than C. These results indicate that carriers of the G allele have a higher probability of responding to treatment with bevacizumab and the odds ratio indicates that carriers of the G allele are twice as likely to respond to the treatment compared with carriers of the C allele. No significant differences were found for the polymorphisms rs699947 and rs35569394.

Polat O and colleagues described that the rs2010963 polymorphism, and particularly the C allele, is associated with increased VEGF gene expression in the retina [25]. El-Shazly also reported that patients with diabetic retinopathy and diabetic macular edema carrying the C allele had elevated levels of VEGFA compared to patients without retinal involvement; however, visual improvement was shown in patients carrying the CC genotype [26]. On the other hand, the study by Hagstrom SA, in patients with age-related macular degeneration, and the study by Chionh F, in metastatic colorectal cancer, found that the rs2010963 polymorphism is not associated with the therapeutic effect of bevacizumab. This contrast of results suggests a possible relevance of the ethnic origin of the studied populations, which highlights the importance of studies in the Mexican population to establish the relationship of the polymorphism with intravitreal therapy with bevacizumab [26, 27, 28]. However, the SNP rs2010963 VEGF-A promoter region's 5'-UTR was identified for Awata et al. that the serum VEGF levels were higher in healthy individuals with C/C genotype [29]. Vailati et al. showed that VEGFA gene expression in the human retina was higher in subjects with the C allele [30].

Considering our results on clinically significant visual improvement directly associated with a reduction in macular thickness greater than 50 μm , the rs2010963 polymorphism was identified as being related to the efficacy of bevacizumab compared to the rs699947 and rs35569394 polymorphisms.

The strengths of our study were that it was prospective and used the same anti-VEGF agent for all patients. This study has some limitations, the sample size was relatively small, and the genetic analysis was limited to three genetic variants of VEGF gene. Research on a larger group of patients treated also with other VEGF inhibitors (e.g. aflibercept, faricimab, ranibizumab and brolucizumab) is warranted to validate our observations.

Undoubtedly, further studies are needed to confirm the association of the VEGF rs2010963 polymorphism with treatment response in patients with DME. Our findings are important because of the contrast with other populations. We hypothesized that the VEGF rs2010963 polymorphism is related to variability in the response to bevacizumab treatment in patients with EMD.

Conclusion

DME is the main cause of vision impairment in diabetic patients. However, not all patients respond satisfactorily to anti-VEGF treatment. The poor response to treatment indicates the presence of other factors might be involved in the pathogenesis of DME. There is a relationship between genetic variability and treatment response, including intronic and promoter-region polymorphisms. We hypothesized that the allele G in the VEGF rs2010963 polymorphism is related to variability in the response to bevacizumab treatment in patients with DME.

Declarations

Authors' Contributions

O.M.L. and H.J.P.C.: Writing—original draft, writing—review and editing, visualization. H.J.P.C., P.L.S., R.M.L.M., M.G.V., S.V.R.: Writing—review and editing, supervision. O.M.L., H.J.P.C., G.J.R.N., A.R.E., H.G.F.C. AND T.S.F.P.: Conceptualization, methodology, formal analysis, writing—review and editing, supervision.

Author Disclosure Statement

There is no conflict of interest within this article.

Ethics declarations

This study was performed in accordance with Declaration of Helsinki. Informed consent was obtained from the participants.

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Data availability

Data are available upon request to the corresponding author.

Consent to publish

The authors certify that this work has not been published or is being considered for publication in another journal. Also, if accepted for publication, the authors transfer the property rights (copyright) of this work to Graefe's Archive for Clinical and Experimental Ophthalmology.

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Tables

Table 1. Oligonucleotides used for amplification of the 5' untranslated region of the VEGFA gene

Polymorphism	Oligonucleotides	Size	Tm
-634C>G (rs2010963)	5'-CGACGGCTTGGGGAGATTGC -3'	274pb	56.7°C
	5'-GGGCGGTGTCTGTCTGTCTG-3'		
-2578C>A (rs699947)	5'-CATACCGATGGAAGTGG -3'	297pb	57.2°C
	5'-GTTTCTGACCTGGCTATTT -3'		
-2549 I/D (rs35569394)	5'-AAGATCTGGGTGGATAATCAGACT-3'	188pb (II)	61.0°C
	5'-AACTCTCCACATCTTCCCTAAGTG-3'	170pb (DD)	

Table 2. Demographic characteristics of the subjects who participated in the study.

Variable	Results	P
Male	50 (49%)	0.3785*
Female	52 (51%)	
Age		
Male	67 ($\pm$ 6.6) years	0.067*
Female	71 ($\pm$ 4.3) years	
Body mass index (BMI)		
Male	24.1 ($\pm$ 1.2) Kg/m ²	0.056*
Female	23 ($\pm$ 2.4) Kg/m ²	
Time of evolution of DM2		
Male	20 ($\pm$ 6.7) years	0.459*
Female	22 ($\pm$ 8.7) years	
Glucose		
Initial	220 ($\pm$ 34) mg/dL	0.068*
3 Months	216 ($\pm$ 42) mg/dL	
HbA1c		
Initial	9.15 ($\pm$ 1.3) %	0.069*
3 Months	8.45 ($\pm$ 2.3) %	
DME		
Unilateral	73 (72%)	-----
Bilateral	29 (28%)	

*Mann Whitney Test

Table 3. Macular volume and thickness obtained by OCT and visual acuity of the subjects included in the study.

	Initial (Basal)	3 months	p
Macular Thickness by OCT (µm)			
Non-Responders	354 (233 to 604)	376 (224 to 576)	0.3454*
Responders	396 (207 to 722)	300 (159 to 580)	<0.0001*
Macular Volume by OCT (mm3)			
Non-Responders	9.56 (7.56 to 12.62)	9.9 (7.89 to 12.11)	0.3176*
Responders	10.29 (7.4 to 16.27)	9.42 (6.49 to 13.59)	0.0007*
Visual acuity Snellen (LogMar)			
Non-Responders			
Snellen	20/200 (20/40 to HM*)	20/200 (20/40 to HM*)	
LogMar	1 (0.5 to 2.6)	1 (0.5 to 2.6)	0.0285**
Responders			
Snellen	20/200 (20/60 to HM*)	20/200 (20/40 to HM*)	
LogMar	1 (0.5 to 2.6)	1 (0.5 to 2.1)	0.0321**

*HM = Hand movement

**Wilcoxon test

Table 4. Analysis of genotypic and allelic frequencies of the polymorphisms rs2010963, rs699947 and rs35569394.

Responder			No responder				
rs2010963							
Genotypic frequency							
	n	%	n	%		p	OR
CC	6	9	11	28			
CG	37	59	22	57	CC vs CG	0.0036*	3.2
GG	20	32	6	15	CC vs GG	<0.001*	6.6
Rs2010963							
Allele frequency							
C	43	36	44	56			
G	77	64	34	44	C vs G	0.008*	2.07
rs699947							
Genotypic frequency							
CC	36	57	23	59			
CA	24	38	15	38	CC vs CA	0.5097*	-
AA	3	5	1	3	CC vs AA	0.2619*	-
rs699947							
Allele frequency							
C	96	76	61	78		0.4334*	-
A	30	24	17	22			
rs35569394							
Genotypic frequency							
DD	6	10	2	5			
ID	55	87	35	90	DD vs ID	0.4235*	-
II	2	3	2	5	DD vs II	0.2671*	-
rs35569394							
Allele frequency							
D	67	53	39	50			
I	59	47	39	50	D vs I	0.3886*	-

*Fisher's exact test

Figures

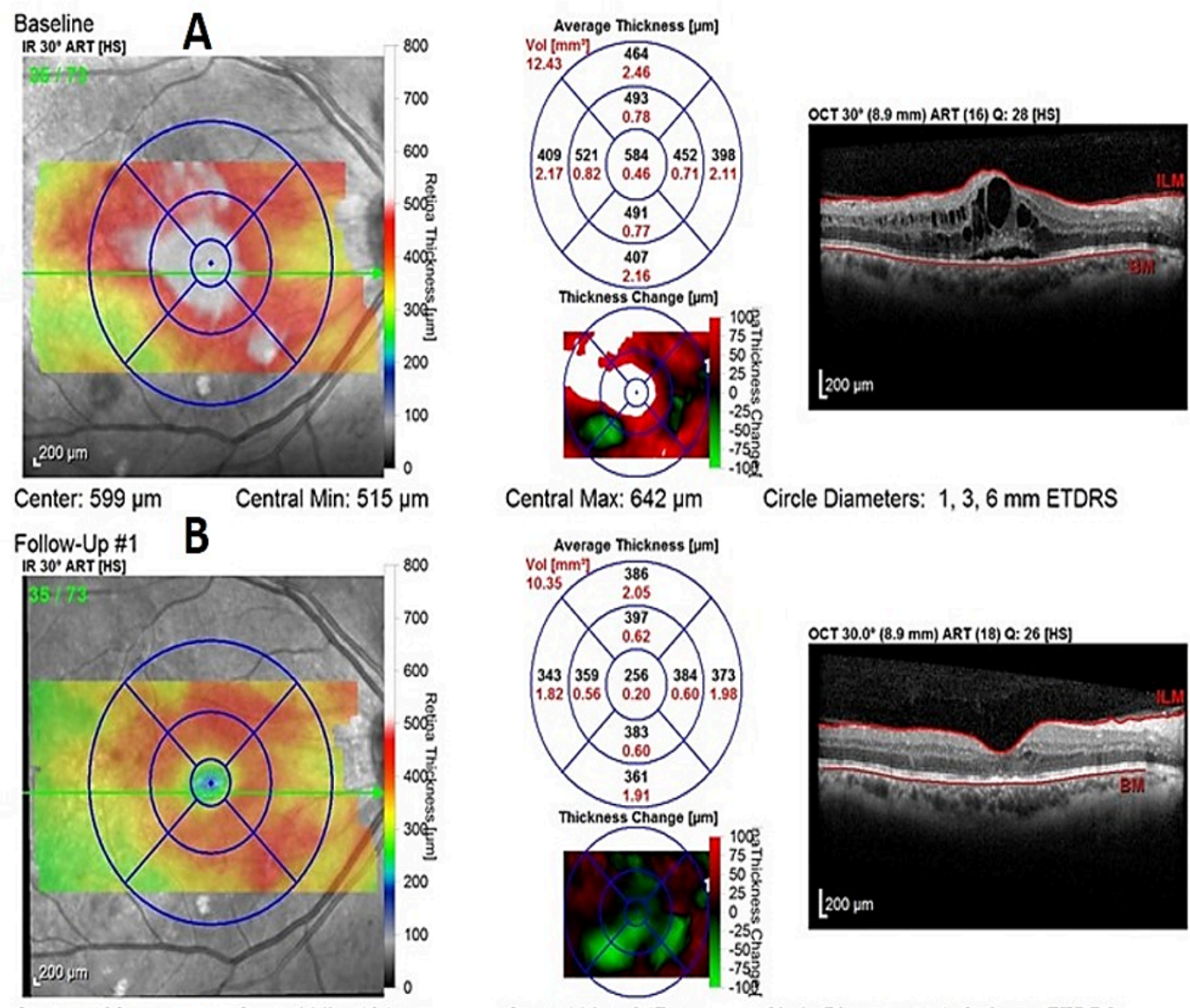


Figure 1

Representation of the result obtained from optical coherence tomography (OCT). (A) Macular edema is observed with an increase in macular thickness and volume, (B) 3 months after the application of Bevacizumab, a decrease in macular edema is observed.

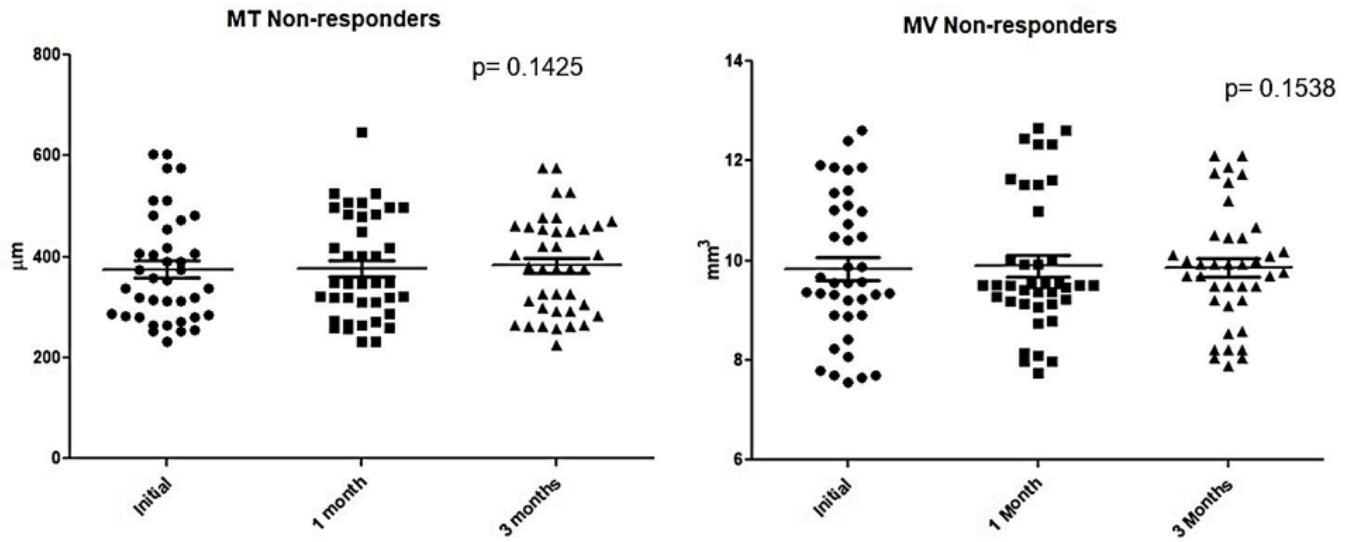


Figure 2

Macular thickness (MT) and macular volume (MV) of non-responder patients showing the changes three months after the application of bevacizumab, there is no significant difference.

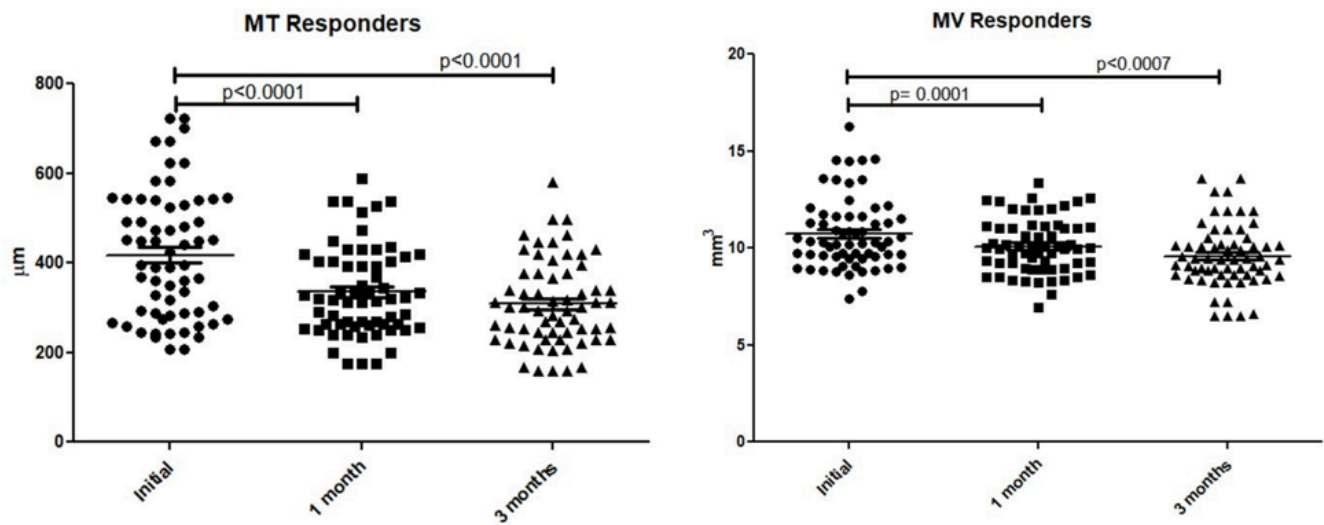


Figure 3

Macular thickness (MT) and macular volume (MT) of responder patients showing the changes three months after the application of bevacizumab, showing a significant difference.