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ORIGINAL PAPER

Impacts of intravitreal anti-VEGF therapy on retinal anatomy and neurophysiology in diabetic macular edema

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Abstract

Purpose To evaluate anatomical and neuroretinal functional aspects in patients with diabetic macular edema (DME) after intravitreal anti-vascular endothelial growth factor (VEGF) therapy, in particular aflibercept.

Materials and methods This prospective single-centered interventional study was performed at Afyonkarahisar Health Science University Faculty of Medicine, Department of Ophthalmology, where 32 eyes of 32 patients with DME were investigated. All patients received five intravitreal aflibercept injections on a monthly basis and were followed up for ≥ 6 months. After a comprehensive ophthalmological examination, including the measurements of visual acuity and intraocular pressure, and an antero-posterior segment slit-lamp biomicroscopy before and after full pupil dilation, fundus fluorescein angiography and optical coherence tomography were performed at baseline and during the third and sixth months post-therapy. Microperimetry and multifocal electroretinography were also performed at baseline and during the sixth months.

Results Mean visual acuity increased from 0.73 to 0.57 and 0.33 logarithm of the minimum angle of resolution (logMAR) during the third and sixth months, respectively ($p < 0.001$). Changes in intraocular pressure were not statistically significant ($p = 0.472$). There was statistically significantly decreased mean central macular thickness from 390.2 µm to 242.6 and 289.7 µm during the third and sixth months, respectively ($p < 0.001$). Significantly improved fixation patterns during the sixth month, along with significantly increased macular sensitivity from 8.2 to 14.2 dB ($p < 0.001$) and significantly decreased local deficit from − 10.3 to 5.5 dB ($p < 0.001$) were observed. Further, there was a significantly increased N1 amplitude in the first ring and significantly increased P1 amplitude in all rings (p for each parameter < 0.05). There was also significantly decreased N1 wave implicit time in all rings and significantly decreased P1 wave in the second, third, fourth and fifth rings (p for each parameter < 0.05).

Conclusions Patients with DME showed profound improvement in the retinal neurophysiological function, which was also accompanied by anatomical and ultrastructural integrity recovery after intravitreal aflibercept therapy. In the pathogenesis of DME, the influence of neurodegeneration has been increasingly gaining significant attention. Consequently, the need to assess neurophysiological effects of anti-VEGF

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therapy using a variety of diagnostic measures like electrophysiological studies and multimodal imaging technologies is undeniably growing.

Keywords Aflibercept · Diabetic macular edema · Fundus fluorescein angiography · Microperimetry · Multifocal electroretinography · Optical coherence tomography

Introduction

Diabetic retinopathy (DRP) is a common complication of diabetes mellitus (DM). In 2013, the International Diabetes Federation reported that 382 million people had DM and that number would rise to 592 million by 2035 [1, 2]. With growing DM epidemics worldwide, DRP, which is the most common microvascular complication, is anticipated to increase [3]. Diabetic macular edema (DME) is the most common manifestation of DRP which threatens vision leading to emergent deficits [4]. Epidemiological studies indicate that 26% of DRP patients are clinically present with DME [5].

Pathogenesis of DME is complex and multifactorial. Generally, DME develops as a result of fluid accumulation in the intraretinal layers, especially as a result of blood-retinal barrier damage. Vascular endothelial growth factors (VEGF) are thought to be more potent than histamine in altering vascular permeability [4]. In addition to causing retinal edema due to breakdown of the blood-retinal barrier and increased vascular permeability [6], prolonged release of these factors is also associated with neovascularization and endothelial cell proliferation. Levels of several growth factors, primarily VEGFs and cytokines, are therefore presumed to be high in patients with DM-related complications, including proliferative diabetic retinopathy and DME [7].

Microperimetry is a valuable tool used in the direct comparison of the retinal pathologies using psychophysical tests that allow for an objective evaluation of the fixation patterns. Evaluation of the central vision using microperimetry can provide additional useful information that is important to the management of the DME [8]. Multifocal electroretinography (mfERG), on the other hand, evaluates any localized pathologies in the outer retinal layer (ORL) and then reports responses from multiple retinal regions and can objectively provide a functional map of the retina [9].

In addition to reduced mean light sensitivity, DME is one of the major causes of visual loss in DM. However, vision assessment often offers insufficient details on severity of the retinal damage and its localization [10]. In view of this, the authors hypothesized that determining retinal improvements in terms of ultrastructural integrity, along with neuroretinal functional aspects, may therefore play an important role in optimizing management of DME patients. Therefore, the current study was intended to investigate the retinal anatomical and neuroretinal functional aspects in DME patients after intravitreal anti-VEGF therapy, in particular with aflibercept (EYLEA®, Bayer, Germany).

Materials and methods

Study design and patients

Thirty-two eyes of 32 naïve patients with DME who presented with visual symptoms at Afyonkarahisar Health Sciences University Faculty of Medicine, Department of Ophthalmology, were included in this prospective single-centered interventional study. The study method complied with the ethical principles set out in the Helsinki Declaration and received full approval from the Institutional Review Boards of the Afyonkarahisar Health Sciences University Ethics Committee. Eligible patients were informed of the course of the disease prior to the commencement of the anti-VEGF therapy, and informed consent was subsequently obtained from each patient.

Inclusion and exclusion requirements

Diabetic macular edema was characterized as a manifestation of retinal thickening caused by the accumulation of intraretinal fluid, predominantly in the inner and outer plexiform layers, which is considered to be the consequence of hyperpermeability of the retinal vasculature that may be present at any phase of diabetic retinopathy. Thus, requirements for inclusion to the study were absence of: prior posterior segment treatments including laser photocoagulation and pars plana vitrectomy; corneal and/or lens opacities that

could compromise optical coherence tomography (OCT) and multifocal electroretinography tests; retinal diseases other than DME, including age-related macular degeneration, hypertensive retinopathy or vascular occlusion; prior or active inflammatory ocular pathologies such as uveitis.

Exclusion requirements included failure to comply with the study visit schedule, insufficient cooperation in electrophysiological care, withdrawal of consent, any related complications from intravitreal injections, such as endophthalmitis, retinal detachment or intravitreal hemorrhage and any systemic complications associated with the treatment agent.

Ophthalmological examination and OCT analysis

A thorough ophthalmological examination was performed, including measurements of visual acuity in logarithm of the minimum angle of resolution (logMAR) and Goldmann applanation tonometry intraocular pressure (IOP) as well as antero-posterior segment slit-lamp biomicroscopy before and after maximum physiological pupil dilation.

Identification of fundus angiographic properties and classification of retinal lesions were performed using colored fundus and fundus fluorescein angiography images (Model, Zeiss, Visucam 5000, Germany). Optical coherence tomography scanning (Spectralis HRA + OCT, Heidelberg Engineering, Heidelberg, Germany) was used for measuring central macular thickness (CMT) and accompanying vitreoretinal interlaminar disorders. The measurement of CMT was taken by superimposing manual reference lines on the internal limiting membrane (ILM) and Bruch membrane (BM) for correction of segmentation errors. Width of an area between newly formed reference lines was determined using a device's automatic centralization software and subsequently used as data.

When errors were found during an automatic retinal segmentation analysis and a transition between segments in the automatic segmentation process, corrections were made by a manual software correction mode. Segmentation metrics were then measured again after the above corrections, and the results were calculated consecutively. The following parameters were measured consecutively in the central area of 1 mm diameter identified by Early Treatment Diabetic Retinopathy Study (ETDRS):

(a) Mean thickness of the retinal nerve fiber layer (RNFL)—a space extending from the ILM to the end of the RNFL;
(b) Mean thickness of the ganglion cell layer (GCL)—the space extending from the terminal margin of the RNFL to the end of the GCL;
(c) Mean thickness of the inner plexiform layer (IPL)—the space extending from the terminal margin of the GCL to the end of the IPL;
(d) Mean thickness of the inner nuclear layer (INL)—the space extending from the terminal margin of the IPL to the terminal margin of the INL;
(e) Mean thickness of the outer plexiform layer (OPL)—the space extending from the terminal margin of the INL to the end of the OPL;
(f) mean thickness of the outer nuclear layer (ONL)—the space extending from the terminal margin of the OPL to the end of the external limiting membrane (ELM);
(g) Mean thickness of the retinal pigment epithelium (RPE)—the space extending from the start of the RPE to the start of the BM;
(h) Mean thickness of the inner retinal layers (IRL)—the space extending from the ILM to the ELM (RNFL + GCL + IPL + INL + OPL + ONL); and
(i) Mean thickness of the outer retinal layers (ORL)—the space extending from the ELM to the BM (photoreceptor + RPE complex).

The mean values of superior, nasal, inferior and temporal zones of 1–3 mm inner ring (*pericentral area*) and 3–6 mm outer ring (*peripheral area*) determined by the ETDRS were calculated. Segmentation measurements were assessed by taking mean thickness values of the above-mentioned segment zones separately and by calculating means of the data obtained from these zones.

After initial 2.0 mg intravitreal aflibercept therapy, additional 5 consecutive doses were administered on a monthly basis for 5 months. Routine ophthalmological examination and OCT imaging were performed during each monthly visit. In addition, microperimetry and mfERG were performed at baseline and in the sixth month.

Electrophysiological studies

All microperimetry tests (MP 1, Nidek Instruments Inc., Padova, Italy) were performed by the same technician with the same device. This test provided a 45° non-mydriatic fundus view with an automatic ocular movement correction. Each patient had a pre-test preparation and prior visual adaptation for five minutes. During testing, a contralateral eye was kept closed. Goldmann III white stimulus and 4–2–1 ladder strategy were used. Sixty-eight randomized stimuli were applied to the retinal regions, including the central 10° area, using the grid system (Humphrey 10–2) (Fig. 1). A fixation target was set as a 2° diameter red cross. While a background illumination was 1.27 cd/m^2, the maximum stimulus illumination was 127 cd/m^2. The stimuli were projected onto a white background for 200 ms (ms). The stimulation attenuations ranged from 0 to 20 dB with the Goldmann-type magnitudes. The 4–2–1 ladder strategy was then established, and the last threshold value was accepted as the final one. Although the technician clarified the baseline threshold value, the actual threshold value of the examined eye was not stated. Also before progressing to the next magnitude of the illumination, the system conducted the same illumination intensity test for all test positions. The mean retinal sensitivity values were compared by measuring the mean value of the automatically preset measurement points in the polygon by using microperimetry-1 software.

Fixation assessment was conducted by tracking the fundus movement, while a patient was looking at the fixation target during testing. The automated tracking system calculated the horizontal and vertical shifts in the reference frame, mapping the patient's ocular movements. Recorded fixation points were classified into three categories for the fixation stability analysis. Under this situation, the fixation was defined as; "stable" when $> 75\%$ of fixations fell within 2° circle, provided its center was the center for all fixation points, "relatively unstable" when $< 75\%$ of fixations fell within 2° circle, but $> 75\%$ of them fell within 4° circle, and "unstable" when $< 75\%$ of fixations fell within 4° circle. In order to assess fixation localization, standard, circular and central fixation areas were determined by centering the fovea 2° with a diameter of approximately 700 μm. The fixation localizations were classified into three categories, i.e., when $> 50\%$ of the preferred fixation points fell within 2° circle, they were classified as "predominantly central," when $> 25\%$, but $< 50\%$ of the preferred fixation points fell within 2° circle, they were classified as "poorly central," and lastly, when $< 25\%$ of the preferred fixation points fell within 2° circle, they were classified as "predominantly eccentric." The fixation characteristics were automatically classified by microperimetry-1 software after placing a sign at the center of the foveal avascular zone.

As with microperimetry-1 test, all mfERG tests (Metrovision Monpack 3, Metrovision, France) were carried out by the same technician using the same device. Refraction error was corrected at a distance of 33 cm before testing. An ERG-jet electrode, which was placed on the cornea after topical anesthesia, was used as an active electrode during the procedure. After rubbing the skin with alcohol to eliminate the oily layer with low electrical conductivity, a ground electrode was placed slightly above the supraorbital rims on the forehead and a reference electrode was placed 1 cm ahead of an external canthus on the temporal region. The electrodes were then attached to a connection box in order to process the signal. The contralateral eye was closed, and the patient's chin was placed on the chinrest. The fixation was tracked with an infrared camera. The MERG61B test was performed in accordance with the International Society for Clinical Electrophysiology of Vision (ISCEV) criteria (Fig. 2). Preset visual patterns consisting of 61 hexagons with sizes adjusted to generate equivalent signals were used, and the recordings from 61 regions of the retina were made in five minutes. Screen resolution was set to 1024 × 768. Patients were seated 33 cm from the screen during the exposure and stimulated in the area of ± 30° horizontally and ± 24° vertically. The stimulus frequency was 17 Hz, the luminance was set to 100 candles/m^2, and the backlighting was set to 30 candles/m^2. Results of the noise level > 5 μV were discarded, and the procedure was continued until an acceptable noise level was achieved. Again, the results of attention loss and the number of rejected stimuli $> 20\%$ of the total stimuli were discarded.

During the concentric ring analysis the "first-row kernel wave" in each ring of N1, P1 and N2 wave amplitudes and implicit times was calculated. In this analysis, 0°–2° area was included within the first ring

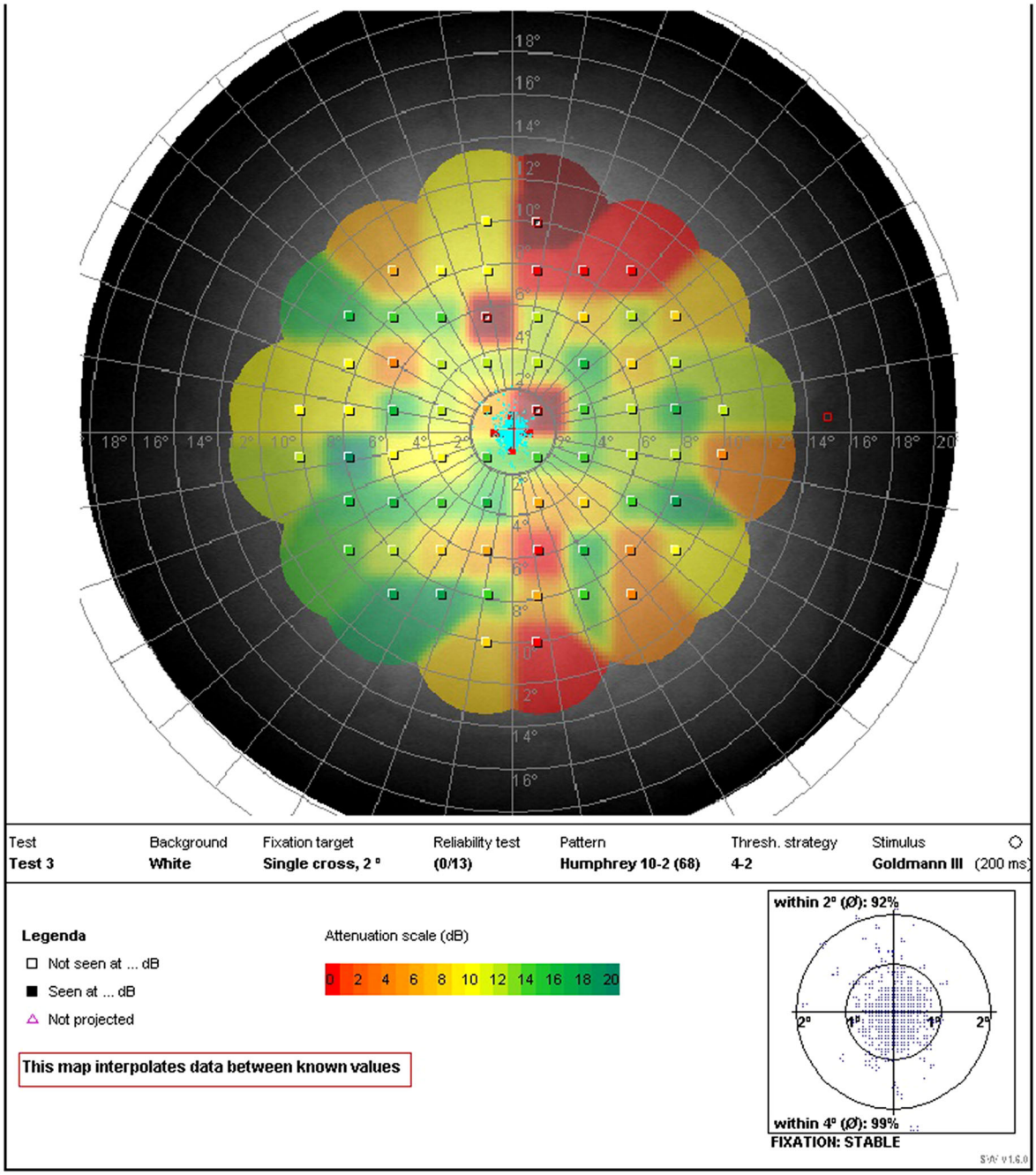

Fig. 1 Microperimetry-1 central 10° area using a grid system (Humphrey 10–2)

fixation; 2°–5° area within the second ring fixation; 5°–10° area within the third ring fixation; 10°–15° area within the fourth ring fixation; and > 15° area within the fifth ring fixation. The mean amplitude (nanovolt) and the implicit time (milliseconds) were recorded after all rings' analyses.

Statistics

Statistical package for social science (SPSS, 18.0 package Worldwide Headquarters SPSS Inc.) software was used for statistical analysis. General linear model and paired sample t test were used to compare visual

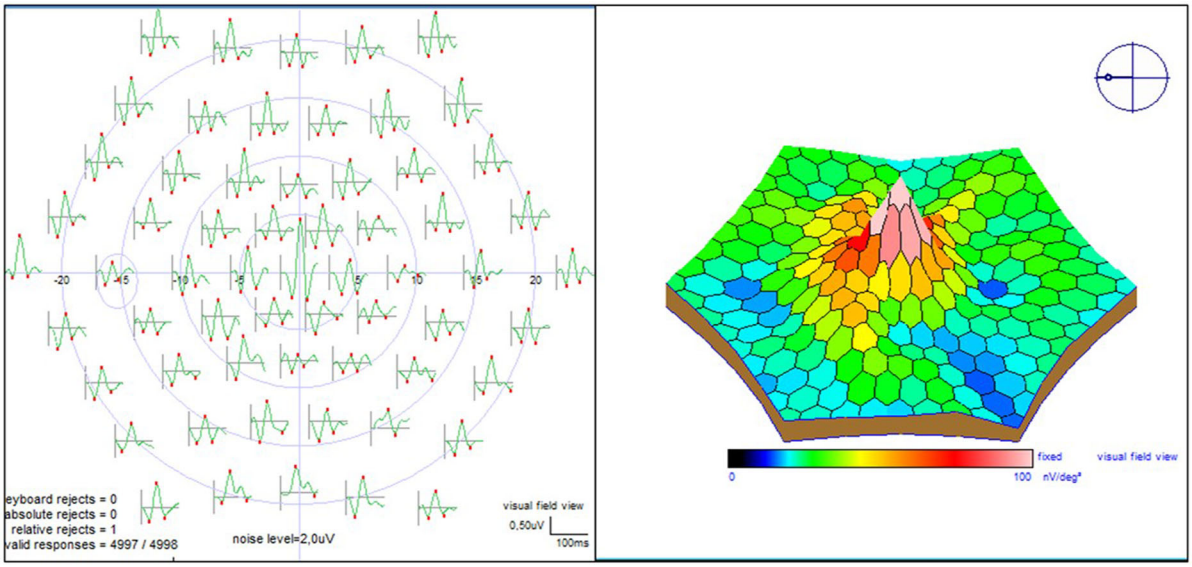

Fig. 2 MERG61B test in alignment with the International Society for Clinical Electrophysiology of Vision criteria

acuity, IOP and OCT data and microperimetry data to baseline data. Bonferroni corrections were applied as a result of the repeated test results. P values < 0.05 were considered to be statistically significant. Pearson bivariate correlation analysis was used to test the data correlations. One-way ANOVA test was used to compare data groups in various patient subgroups, such as the comparison of visual acuity data across different patient groups.

Results

Demographic characteristics of DME patients are demonstrated in Table 1. Three patients were excluded due to an emergent cerebrovascular disease, and three additional patients were excluded due to non-compliance with the visit schedules, other health issues and failure to adapt to microperimetry attraction, leaving only 32 patients who were followed up for ≥ 6 months. In addition to DM, nine patients were diagnosed with hypertension and one had cerebrovascular disease in the course of the study. Ocular complications such as retinal tears, retinal detachment, vitreous hemorrhage or endophthalmitis were not observed.

The mean visual acuity improved statistically significantly during the study ($p < 0.001$) (Table 2).

Table 1 Patients' demographic characteristics

		$\overline{X} \pm$ SD (Max–Min)
Age (Year)		61.4 ± 10.79 (42–79)
Diabetes duration (Year)		11.28 ± 3.46 (6–19)
HbA1c (%)		8.03 ± 0.71 (6.9–9.6)
		Number of patients/eyes
DRP severity	Moderate NPDR	2
	Severe NPDR	12
	Very severe NPDR	11
	Early PDR	6
	High-risk PDR	1
Type of DME	Focal	17
	Diffuse	15
Laterality	Right eye	22
	Left eye	10
Lens status	Pseudophakic	5
	Phakic	27

$\overline{X}$ Mean value; *SD* standard deviation; *DRP* diabetic retinopathy; *NPDR* non-proliferative DRP; *PDR* proliferative DRP; *DME* diabetic macular edema

There was a non-significantly decreased mean IOP from 16.19 to 15.97 mmHg in the sixth month ($p = 0.472$). However, three patients experienced high IOP during the study, which was administered with anti-glaucoma agents.

Table 2 Changes in best-corrected visual acuity, central macular thickness and central 1 mm macular thickness during the study (n = 32)

Period	Parameters	95% CI (confidence interval)		P value
	Best-corrected visual acuity (logMAR)	Lower limit	Upper limit	
Baseline	0.73 ± 0.33			
Third month	0.57 ± 0.20	0.10	0.23	< 0.001
Sixth month	0.33 ± 0.19	0.31	0.47	< 0.001
Central macular thickness (*μm*)				
Baseline	390.2 ± 134.8			
Third month	289.7 ± 56.6	62.9	138	< 0.001
Sixth month	242.6 ± 44.4	96.7	198.4	< 0.001
Central 1 mm macular thickness (*μm*)				
Baseline	425.4 ± 121.6			
Third month	332.8 ± 52.5	59.5	125.6	< 0.001
Sixth month	290.2 ± 41.6	89.4	181	< 0.001

Optical coherence tomography findings

Statistically significant decreases in mean CMT ($p < 0.001$) and mean central 1 mm macular thickness were observed ($p < 0001$) (Table 2). The segmentation data analysis findings that included baseline thickness of the ETDRS subfield values and changes during the study are demonstrated in Table 3.

Microperimetry findings

The changes in fixation stability as indicated in Table 4 revealed to be statistically significant by using marginal homogeneity test ($p = 0.003$). The changes in fixation localization ($p < 0.001$), retinal sensitivity ($p < 0.001$) and local deficit ($p < 0.001$) were also statistically significant (Tables 4 and 5).

Multifocal electroretinography findings

The N1 and P1 wave amplitudes and implicit times for each ring were statistically compared separately. Although increase in the N1 amplitude was observed in all rings, only the first ($p < 0.001$) and fifth ($p = 0.008$) rings increased statistically significantly. On the other hand, statistically significant decreases in N1 wave implicit time were observed in all rings (Table 6).

The mean P1 wave amplitude increased statistically significantly in all rings ($p = 0011$ for the first and third rings; $p = 0.003$ for the second ring; $p = 0.024$ for the fourth ring; and $p = 0.012$ for the fifth ring). With the exception of the first ring ($p = 0.251$), the mean P1 wave implicit time decreased statistically significantly in all other rings ($p < 0.001$ for the second, third and fifth rings; and $p = 0.022$ for the fourth ring) (Table 6).

Correlation analyses

Visual acuity improvement was positively correlated with the decreases in all OCT parameters, as seen in Fig. 3, for example, depicting the positive correlation between increased visual acuity and reduced CMT ($p < 0.001$; $r = 0.590$).

There was a statistically significant negative correlation between visual acuity and retinal sensitivity at baseline ($p < 0.001$; $r = -0.697$) and in the sixth month ($p < 0.001$; $r = -0.615$). However, visual acuity and retinal sensitivity changes in the course of the study were not significantly correlated. There was also statistically significant negative correlation between visual acuity and local deficit at baseline ($p < 0.001$; $r = -0.717$) and in the sixth month ($p = 0.002$; $r = -0.530$).

While there was statistically significant negative correlation between retinal sensitivity and CMT at

Table 3 Changes in the thickness of ETDRS subfields during the study ($n = 32$)

The ETDRS subfield	Baseline	Sixth month	*P* value
RNFL central	44.8	15.5	**0.002**
RNFL inner ring	46.3	29.6	**< 0.001**
RNFL outer ring	55.7	47.8	**< 0.001**
GCL central	34.3	16.7	**< 0.001**
GCL inner ring	54	51.9	0.245
GCL outer ring	40.7	38.4	**0.051**
IPL central	42.9	22.1	**0.027**
IPL inner ring	49.4	42.5	0.097
IPL outer ring	40.2	31.9	**< 0.001**
INL central	47.5	28.5	**0.002**
INL inner ring	51.1	43.7	**< 0.001**
INL outer ring	40.2	37.5	**< 0.001**
OPL central	36.8	28.3	**0.004**
OPL inner ring	37.9	36.4	0.332
OPL outer ring	31.9	31.5	0.674
ONL central	117.2	89.4	**0.001**
ONL inner ring	104.1	72.6	**< 0.001**
ONL outer ring	85.6	70.1	**0.001**
RPE central	16.3	16.6	0.877
RPE inner ring	14.9	13.9	0.244
RPE outer ring	12.9	12.5	0.331
IRL central	315.6	194.2	**< 0.001**
IRL inner ring	339.3	275.1	**< 0.001**
IRL outer ring	281.1	255.6	**< 0.001**
ORL central	113.2	104.5	0.240
ORL inner ring	101.8	93.7	0.137
ORL outer ring	95.6	88.4	0.245

Bold indicates $p < 0.05$

Table 4 Changes in fixation stability and localization during the study ($n = 32$)

	Baseline	Sixth month
Fixation stability		
Stable	9 (28.1%)	17 (53.1%)
Relatively unstable	21 (65.6%)	14 (43.8%)
Unstable	2 (6.3%)	1 (3.1%)
Fixation localization		
Predominantly central	14 (43.8%)	20 (62.5%)
Poor central	16 (50%)	12 (37.5%)
Predominantly eccentric	2 (6.3%)	0

baseline ($p < 0.001$; $r = -0.657$), no significant correlation was observed in the sixth month.

Though there was significant positive correlation between N1 wave amplitude of the first ring and retinal sensitivity at baseline ($p = 0.048$; $r = 0.353$), significant positive borderline correlation was observed in the sixth month ($p = 0.086$; $r = 0.308$). In addition, a borderline positive correlation of the N1 wave amplitude of the fourth ring and retinal sensitivity was observed in the sixth month ($p = 0.070$; $r = 0.324$). On the other hand, N1 wave implicit time of the first ring and CMT was significantly correlated in the sixth month ($p = 0.015$; $r = 0.426$).

The P1 wave amplitude of the first ring was significantly negatively correlated with CMT in the sixth month ($p = 0.024$; $r = -0.398$). But, a borderline negative correlation between P1 wave amplitude of the second ring and CMT was observed in the sixth month ($p = 0.071$; $r = -0.324$).

Discussion

Treatment of DME with intravitreal anti-VEGF agents, particularly aflibercept, as described in the current study, has been shown to be significantly associated with vastly improved retinal ultrastructural anatomy as well as neuroretinal functionality. Diabetic macular edema is the most common manifestation of DRP that commonly threatens vision [4]. With a growing incidence of DME worldwide, much attention has been paid to the prevention and treatment of this morbid complication [11].

Diabetic macular edema treatment currently consists of laser photocoagulation, pharmacological and surgical procedures. Diabetic macular edema and other microvascular complications can be prevented primarily by monitoring blood glucose levels as well as by managing other effective risk factors such as hypertension, hypercholesterolemia, microalbuminuria, proteinuria and pregnancy prior to complications requiring treatment.

Underlying pathophysiological mechanisms of neovascular ocular diseases are mostly believed to be mediated by the VEGFs. The VEGFs, which were discovered 20 years ago, are crucial mediator of endothelial cell proliferation and migration as well as progressive vasodilatation and vascular permeability [12]. In addition to playing a critical role in

Table 5 Changes in retinal sensitivity and local deficit during the study (n = 32)

Period	Parameters	95% CI (confidence interval)		P value
		Lower limit	Upper limit	
Retinal sensitivity (dB)				
Baseline	8.2 ± 2.9			
Sixth month	14.2 ± 2.5	− 6.6	− 5.3	< 0.001
Local deficit (dB)				
Baseline	− 10.3 ± 2.8			
Sixth month	− 5.5 ± 2.6	− 5.3	− 4.3	< 0.001

Table 6 Changes in the mean amplitude and implicit time of each ring in the N1 and P1 waves during the study (n = 32)

	Baseline (nv/deg^2)	Sixth month (nv/deg^2)	P value
Mean N1 wave amplitudes			
First ring	− 26.7 ± 8.8	− 30.1 ± 8.2	**< 0.001**
Second ring	− 21.5 ± 8.9	− 22.6 ± 7.7	0.355
Third ring	− 17.3 ± 5.8	− 22.5 ± 17.5	0.114
Fourth ring	− 14.7 ± 4.6	− 15.6 ± 5.4	0.150
Fifth ring	− 11.6 ± 3.6	− 12.8 ± 4.3	**0.008**
Mean N1 wave implicit time			
First ring	28.4 ± 4.5	26.9 ± 4.4	**< 0.001**
Second ring	28.8 ± 3.7	26.1 ± 3.1	**0.001**
Third ring	28.3 ± 2.8	26.8 ± 3.1	**< 0.001**
Fourth ring	28.2 ± 2.6	26.3 ± 2.8	**< 0.001**
Fifth ring	28.4 ± 2.4	26.8 ± 2.6	**< 0.001**
Mean P1 wave amplitude			
First ring	42.2 ± 13.6	48.3 ± 15.1	**0.011**
Second ring	40.2 ± 12.9	43.4 ± 12.4	**0.003**
Third ring	35.1 ± 10.9	37.1 ± 11.7	**0.011**
Fourth ring	29.1 ± 8.9	30.7 ± 9.8	**0.024**
Fifth ring	24.1 ± 7.8	25.5 ± 9.1	**0.012**
Mean P1 wave implicit time			
First ring	49.6 ± 7.4	47.7 ± 6.8	0.251
Second ring	49.1 ± 3.9	47.2 ± 3.6	**< 0.001**
Third ring	48.6 ± 3.1	47.6 ± 3.0	**< 0.001**
Fourth ring	48.1 ± 3.1	45.1 ± 7.5	**0.022**
Fifth ring	48.3 ± 3.2	46.8 ± 3.4	**< 0.001**

Bold indicates $p < 0.05$

vasculogenesis and angiogenesis, which involve several physiological processes such as wound healing, reproduction and organ development [13], the VEGFs have a major role in pathological neovascularization, especially in the development of solid tumors and certain vision-threatening diseases [14]. Diabetic retinal neovascularization has been shown to be particularly sensitive to anti-VEGF therapy. Neovascularization begins to regress as early as the first day of anti-VEGF therapy and the ultimate outcome is a complete regression [15]. In addition to other retinal vascular pathologies, anti-VEGFs have therefore emerged as the highly specific treatment paradigm for diabetic retinal disease.

Significant developments in the treatment of retinal vascular diseases have been made since last decade with the advent of the intravitreal anti-VEGF agents. Negative effects of PRP mainly on the visual field

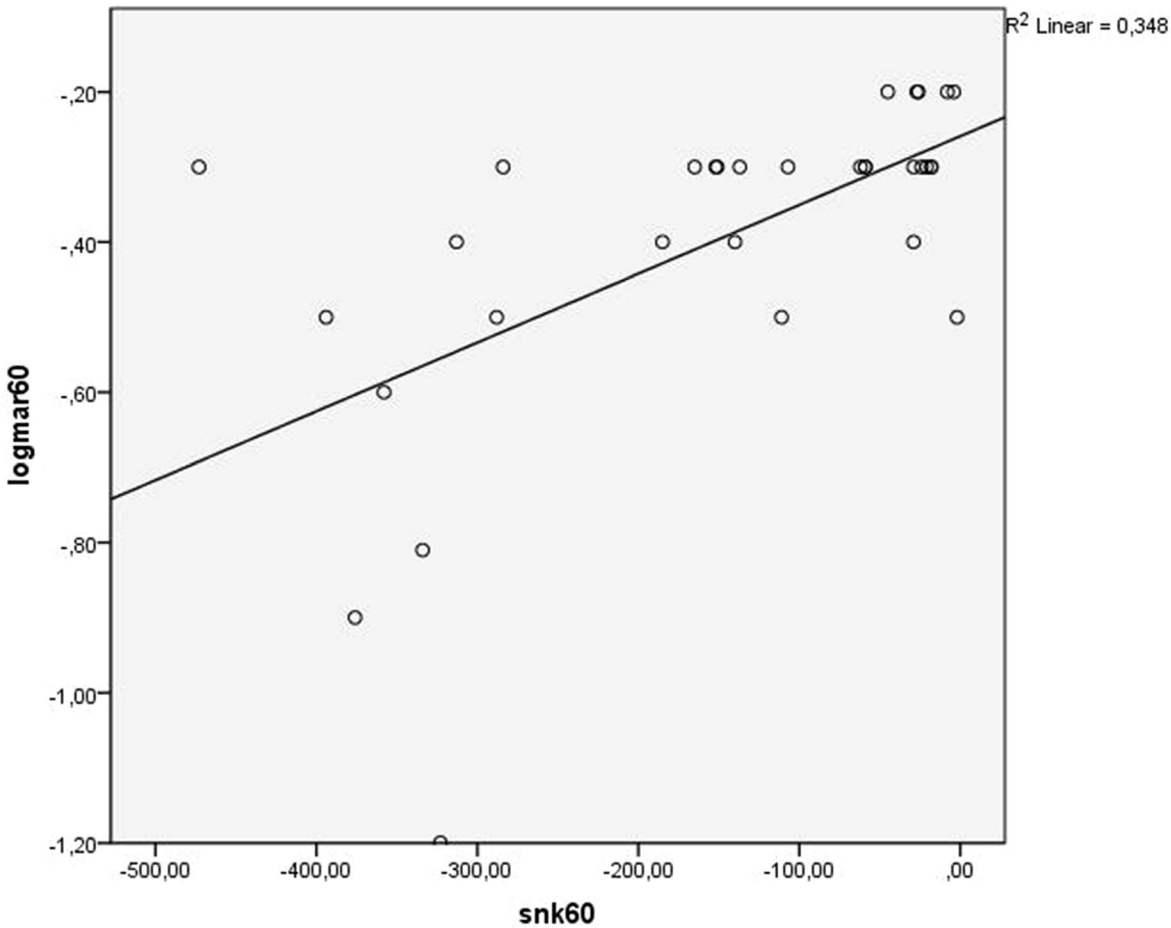

Fig. 3 Correlation between the best-corrected visual acuity improvement and central macular thickness reduction

have prompted researchers to consider alternative therapeutic strategies. Anti-VEGF agents decreased vascular permeability and proliferation based on early-phase studies with anti-VEGF monoclonal antibodies [15–17]. Further, they have been shown to reduce or at least stabilize non-proliferative diabetic retinopathy (NPDR) when used in DME therapy. However, these agents have not yet been recommended as first-line NPDR therapy without corresponding DME in the eyes concerned [15–17]. Aflibercept, an FDA-approved agent for DME therapy in 2014, is a recombinant fusion protein with a molecular weight of 115 kDa which inhibits the VEGF-A, VEGF-B and the placental growth factor [18]. It also binds the VEGF-A with an affinity 100 times higher than bevacizumab and ranibizumab [19].

In the current study, the treatment of naive DME patients with 2.0 mg aflibercept intravitreal injections on a monthly basis resulted in significantly improved visual acuity and OCT parameters beginning in the third month and progressed throughout the study, consistent with earlier literature studies including DaVinci, VISTA and VIVID trials [20–23]. The current study also revealed significant reductions in mean central 1 mm macular thickness and thicknesses of other retinal layers, including: central, inner and outer rings of the RNFL; central ring and outer ring of the GCL; central and outer ring of the IPL; central, inner and outer rings of the INL; outer ring of the OPL; central, inner and outer rings of the ONL; and central, inner and outer rings of the IRL. There was also a non-significant reduction in the ORL thickness. Although there are innumerable literature studies on segmentation analysis in the DME, no studies have yet evaluated the segmentation analysis results, particularly after aflibercept therapy, in DME patients. The

clinical results identified in the current study are therefore strongly considered to be indispensable with respect to the study area concerned.

Significantly decreased thickness of all retinal layers, except ONL, has been reported after OCT segmentation analysis of 33 naive DME patients treated with intravitreal ranibizumab [24]. In addition, the decrease in IRL thickness has been shown to be correlated with improved visual acuity, while the reverse was true for the outer retina after multiple linear regression analysis. Similarly, the current study found correlations between visual acuity improvement and changes in the thickness of quite more parameters relative to the above study, which included: CMT; central area and inner ring of the RNFL; central area of the GCL; central area, inner and outer rings of the IPL; central area and outer ring of the INL; and central area and inner ring of the IRL. No significant correlation between visual acuity improvement and outer ring thickness found. In this regard, it can essentially be inferred that decreased inner retinal thickness is accompanied by an increase in visual acuity. Furthermore, evaluation of changes in the retinal layers following treatment with ranibizumab and triamcinolone showed a correlation between decreased RNFL thickness and visual acuity improvement lasting > 12 months [25]. Although the thickness of all individual retinal layers decreased in either treatment arm, the highest reduction was observed in the ONL thickness. The current study also showed the highest degree of reduced thickness in the inner ring of the ONL (-31.5 µm), central ring of the RNFL (-29.4 µm) and central ring of the ONL (-27.8 µm) after intravitreal aflibercept therapy.

Diabetic retinopathy has recently been identified as a neurodegenerative ocular disease similar to retinitis pigmentosa and glaucoma [26, 27]. As a result, assessment of the RNFL thickness has been shown to be an effective tool for determining the development and progression of DRP as well as glaucoma-associated comorbidities [28]. Reproducibility of the peripapillary RNFL thickness assessments in glaucoma patients has been developed previously with the implementation of OCT in clinical practice [29]. The increased RNFL thickness has been reported in DRP patients with DME relative to DRP patients without DME and healthy individuals [30, 31]. Correspondingly, the current study revealed significantly decreased thickness in the central area and in the inner and outer RNFL rings, indicating that the RNFL thickness increases with DME. Moreover, prior to the onset of the first clinical signs of DRP, decreases in the thickness of IRLs in the macula area have been identified as a sign of early neurodegeneration [32]. Significant correlation between impaired visual acuity and decreased absolute GCL + IPL thickness after intravitreal anti-VEGF or dexamethasone therapy in DME has been demonstrated in a prior retrospective analytical study. In the current study, on the other hand, the decreases in GCL and IPL thicknesses after intravitreal aflibercept therapy correlated with the predicted visual acuity improvement. This discrepancy may be attributable to the fact that most of the patients in the above study, but not in the current study, had prior macular laser surgery. The laser therapy may have a detrimental effect on the final visual acuity, as this therapy not just decreases GCL and IPL thicknesses, but also has a destructive aspect [25].

The formation of cystoid spaces inside INL and OPL [33], and retinal swelling, which could induce intracytoplasmic swelling of the Müller cells and increase the OPL or Henle fiber layer thickness [34], has been reported after histological investigation in DM patients. Moreover, persistent retinal edema has been shown to cause necrosis of the Müller and surrounding nerve cells leading to the formation of cystoid spaces [35]. It has been also argued that the longer the duration of macular edema, the worse the effect on anatomical and functional outcomes [36]. Similarly, considerable decrease in the INL and OPL thicknesses after aflibercept therapy was identified in the current study and may therefore be seen as evidence of increased INL and OPL thickness in DM patients with DME.

In addition to DME, microperimetry can accurately measure the retinal sensitivity and fixation stability in various macular diseases and can easily and precisely identify the fixation characteristics [37]. Significantly improved macular sensitivity after intravitreal triamcinolone injections in DME patients has been reported in the past microperimetry studies [8, 38]. Another study reported significant improvements in 50% of the eyes with clinically significant DME after focal laser therapy [37]. Likewise, the current study revealed significant decreases in the mean light sensitivity, retinal sensitivity and fixation stability in DME patients after aflibercept therapy. The mean light sensitivity typically tended to decrease with both

severity of the macular change and its duration. This decrease is presumed to occur predominantly due to localized loss of light sensitivity and subsequent scattering in areas with severe tissue changes.

Quantification of the macular sensitivity and retinal fixation characteristics enables physicians to properly assess the effects of medical procedures. The fixation characteristics are important for many visual tasks, as many changes in size, shape and density have a drastic impact on visual performance. Certainly, patients' daily activities are positively influenced by microperimetry parameters and central visual field improvements. Therefore, sustainability of essential daily activities, including recognition, direction as well as reading surfaces and symbols, is highly dependent on the preservation of the central visual field [38]. Significantly decreased fixation stability control has been documented in DME patients compared to healthy subjects [10, 39, 40]. Also, the mean retinal sensitivity and fixation stability measured using liquid crystal display microperimetry have been found to be correlated with visual acuity in eyes with various macular pathologies [41]. Similarly, a correlation between fixation stability score and visual acuity, and between visual acuity and 4° central retinal sensitivity has been documented in eye with DME [42]. Conversely, a stable and central fixation, and a significant correlation between retinal sensitivity and retinal thickness, but not visual acuity, has been documented in patients with DME at different grades [43]. The current study, however, found a non-significant correlation between improved visual acuity and retinal sensitivity after intravitreal aflibercept therapy. Furthermore, microperimetry findings of the current study are similar to the earlier studies in the same sense that the macular sensitivity decreased in DME patients due to changes in the array and structure of the cone cells and/or the loss of photoreceptor cells. This decrease has also been assumed to arise from compression, degradation and disorientation of the cone cells as demonstrated in histological examinations [44]. Since thickness and integrity of the foveal photoreceptor layer are correlated with visual performance, eyes with persistent macular edema are therefore anticipated to demonstrate symptoms of visual disturbance [45, 46].

In addition to a highly significant correlation between scotoma size and structural damage, a significant correlation between visual acuity and foveal thickness and retinal sensitivity has been identified in DME patients [47]. In comparison, while there was a statistically significant negative correlation between CMT and retinal sensitivity at baseline, a non-significant correlation was found in the sixth month after intravitreal aflibercept therapy in the current study. Furthermore, in parallel to the current study, a study in which 30 eyes of 22 diabetic patients with clinically significant macular edema were compared with 30 eyes of 32 healthy subjects in terms of the stability of fixation and macular sensitivity reported significantly decreased retinal sensitivity in DME patients compared to healthy subjects [42]. Moreover, the fixation stability analysis of DME patients in the above study reported eight stable, 21 relatively unstable and one unstable fixation which were similar to the current study results. Corresponding findings, including predominantly central fixation in 18, poorly central fixation in 11 and predominantly eccentric fixation in 1 patient, were also reported.

Significant improvements in retinal sensitivity and fixation patterns and significant correlation between visual acuity and retinal sensitivity have been reported in 26 DME patients treated with intravitreal bevacizumab therapy. The unstable fixation in 20 eyes and relatively unstable fixation in six eyes at baseline has been reported, too. However, the unstable fixation decreased to 16 eyes and moderately unstable fixation increased to ten eyes during the follow-up. While predominantly eccentric fixation and poorly central fixation were detected in 20 and six eyes at baseline, during follow-up, poorly central fixation increased to ten eyes and predominantly eccentric fixation dropped to 16 eyes [48]. In addition to significantly decreased local deficit, the current study observed similar significantly increased mean retinal sensitivity, but with intravitreal aflibercept therapy. Significant improvements in fixation stability were also revealed using marginal homogeneity test. Further, there were predominantly central fixation localizations in 14 patients, poorly central fixation in 16 patients and predominantly eccentric fixation in two patients at baseline. However, predominantly central fixation decreased to 20 patients and poor central fixation localization to 12 patients in the sixth month.

The macular function has been previously investigated in DME patients. In this regard, similar to the current study in which DME patients were treated with intravitreal aflibercept, poorer fixation stability and fixation localization have been reported in DME

patients than in healthy individuals after intravitreal triamcinolone acetonide therapy [38]. Similar significant increase in the mean retinal sensitivity has been also recorded after intravitreal dexamethasone implant in 27 naïve DME patients [49]. Moreover, consistent findings have been reported following intravitreal ranibizumab therapy, including significantly increased macular sensitivity [50]. In fact, these studies were designed to determine neuroretinal function in DME patients after various intravitreal therapies. To the authors' knowledge, however, no literature studies have specifically investigated the effect of intravitreal aflibercept therapy on neuroretinal function in DME patients. As a result, it is fervently believed that clinical results of the current study may provide beneficial up-to-date knowledge on this treatment modality.

Differences between DM and control groups in all retinal ring zones have been reported following the analysis of changes in mfERG possible correlation between OCT and visual acuity. In addition, a significantly decreased width of both N1 and P1 waves, along with increased latencies, significant correlations between visual acuity and the width of P1 and N2 waves in the first ring, and a significant inverse relationship between visual acuity and central N1 latency have been identified in DME patients [34]. Similar baseline N1 and P1 wave amplitudes and implicit times were found in the current study. Furthermore, the N1 and P1 wave amplitudes decreased in stepwise pattern from the first to the fifth ring, which also corresponded to the ISCEV, in which mfERG responses correlated with greater amplitudes in fovea with the largest number of cone photoreceptors and bipolar cells, but gradually decreased to the periphery parallel to a sparse cone and bipolar cell population [51]. A comparative study reported no significant differences in the retinal thickness between type 1 and 2 DM patients and healthy subjects. However, there were significant differences in N1 and P1 wave amplitude and implicit time between groups, with type 2 DM patients having significantly lower amplitudes and prolonged implicit times relative to the other two groups, but not significant differences in mfERG between type 1 DM patients and healthy subjects [36]. Similar baseline mfERG results have been found in the current study, and as previously mentioned, mfERG may be a useful test for diagnosing subclinical retinopathy that may be insidious before signs and symptoms of retinopathy appear in DM patients.

The current study revealed negative correlation with borderline significance between baseline N1 amplitudes of the first and fourth ring and baseline visual acuity, which was inconsistent with previous micropathy studies such as best macular dystrophy, Stargardt disease and retinal venous occlusions in which significant correlations were identified [52, 53]. On the other hand, non-significant correlation between visual acuity and implicit time in the current study was consistent with earlier literature studies, which recorded a slight increase in latencies despite extreme visual losses and decreased amplitudes. Sometimes the data were found to drop beyond the normal limits, indicating that visual acuity was not always correlated with changes in the implicit time [52, 54].

Significant improvements in visual acuity with different retinal thicknesses have also been reported, despite modest correlations. Many eyes with thickened macula have also been shown to have excellent visual acuity, although visual acuity decreased in most eyes with normal CMT [55]. This indicates that OCT data alone might not be an adequate predictor of visual acuity as a primary outcome in DME studies. The current study, on the other hand, found significant correlations between CMT and N1 wave amplitudes of the first and fourth rings and between CMT and P1 wave amplitudes of the first, second, third and fourth rings. This also indicated the anatomical improvements which were accompanied by partial functional improvements. While there was no anticipation of consistency of the functional and anatomical tests at all times, it is obvious, after all, that structural and functional tests may never be completely understood.

Multifocal electroretinography has a propensity to lack local small abnormalities that may be identified in the OCT. And yet, OCT data may appear to fall within normal limits regardless of the obviously abnormal results of mfERG [56]. In addition, functional injuries can sometimes be identified by mfERG before detection of the structural changes in OCT. The N1 and P1 widths of the second ring and the N1 and P1 amplitudes of the first ring have been found to be predominantly stable until the fourth month of intravitreal dexamethasone implant therapy in DME patients [49]. Further, significantly increased mean P1 amplitudes in both central and peripheral rings ($p < 0.001$ for each) have been recorded in another mfERG study

after intravitreal triamcinolone acetonide therapy in DME patients. Although mean P1 latencies for the middle and peripheral rings were shortened, no major change occurred [57]. Furthermore, a comparison of the anatomical and functional effects of intravitreal ranibizumab with laser therapy in DME patients revealed moderate to severe central macular dysfunction in all cases treated with either therapy. Significantly increased amplitudes of the mfERG central responses in ranibizumab-treated eyes were also observed [50]. The current study, on the other hand, found significantly increased mean N1 wave amplitudes in the first and fifth rings, but non-significantly increased N1 amplitudes in the other rings. In addition, significantly increased P1 wave amplitudes in all rings and the N1 and P1 wave amplitudes of the first ring were observed after intravitreal aflibercept therapy.

While the anti-VEGF therapy is widely used in many retinal diseases, including DME, in some cases, due to numerous injections, concerns have emerged recently as to whether or not high levels of anti-VEGF administration could lead to toxic retinal effects, leading researchers to begin reviewing evidence in this regard. Significantly improved macular edema without any indication of retinal toxicity has been identified in patients with DME and retinal venous occlusion after intravitreal bevacizumab therapy [58]. The anti-VEGFs have been shown to target newly generated blood vessels, although there was no proof of the impact of these agents on healthy retinal tissue [59]. In order to determine the toxic effects of intravitreal aflibercept therapy on healthy retina, electrophysiological changes found in the fourth and fifth rings representing the neuroretinal activity of the peripheral macula were considered. The P1 or N1 wave amplitude impairments were not identified from any retinal rings. Most of the findings identified were neuroretinal functional improvement. Since all patients in the current study were naive cases with visual acuity $> 20/200$, and assuming either no or limited involvement in the fourth and fifth rings in most patients, it can be vehemently concluded that repeated intravitreal aflibercept injections do not lead to significant retinal toxicity.

The authors acknowledge the shortcomings of the current study. A smaller number of participants and a shorter follow-up duration of up to six months may have overshadowed more accurate results concerning anatomical and neuroretinal functional improvement of the retinal tissue. Therefore, a correspondingly greater study population with long-term follow-up would be worthwhile.

Conclusions

Major ultrastructural and neuroretinal functional improvements were observed in DME patients after intravitreal aflibercept therapy. The implications of neurodegeneration, which are increasingly at the frontline of DME pathogenesis presently, necessitate the neurophysiological effects of intravitreal anti-VEGF agents, including aflibercept, to be investigated using a combination of diagnostic measures.

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Compliance with ethical standards

Conflict of interest Author Mustafa Doğan received research grants from Afyon Kocatepe University. Authors Zübeyir Yozgat, Mehmet Cem Sabaner, Hamidu Hamisi Gobeka and Serpil Yazgan Akpolat declare that they have no conflict of interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants prior to the study commencement.

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