HYPERREFLECTIVE RETINAL SPOTS AND VISUAL FUNCTION AFTER ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR TREATMENT IN CENTER-INVOLVING DIABETIC MACULAR EDEMA

STELA VUJOSEVIC, MD, PHD,* MARIANNA BERTON, MD,* SILVIA BINI, MD,* MARGHERITA CASCIANO, MD,* FABIANO CAVARZERAN, SCD,* EDOARDO MIDENA, MD, PHD*†

Background: To assess and correlate early modifications in hyperreflective retinal spots (HRS), retinal sensitivity (RS), fixation stability, and best-corrected visual acuity (BCVA) after anti-vascular endothelial growth factor treatment in naive center-involving diabetic macular edema.

Methods: Cross-sectional comparative case–control series. Twenty diabetic patients underwent 3 consecutive intravitreal anti-vascular endothelial growth factor injections in the study eye (20 fellow eyes served as control), full ophthalmologic examination including spectral domain optical coherence tomography (Retinascan RS-3000; Nidek, Gamagori, Japan), and microperimetry (MP1; Nidek) at baseline (Visit-V1), 1 month after each injection (V2, V3, V4), and at 6 months (V5). Central retinal thickness, inner and outer retinal thickness, number of HRS, BCVA, RS, and bivariate contour ellipse area were evaluated by analysis of variance test with Bonferroni post hoc test. Correlation analyses were performed by Spearman correlation.

Results: In treated eyes, central retinal thickness and inner retinal thickness significantly decreased at V2, V3, V4 versus V1 ($P < 0.03$ at least for all); the mean number of HRS significantly decreased in both inner and outer retina at all follow-up visits versus V1 ($P < 0.008$ at least for all); mean RS and bivariate contour ellipse area remained statistically unchanged during the follow-up; BCVA significantly improved at V3, V4, and V5 versus V1 ($P = 0.009$ at least for all). In fellow eyes, central retinal thickness, HRS, RS, and BCVA did not change at any follow-up. The number of HRS correlated inversely with RS, directly with bivariate contour ellipse area, and not significantly with BCVA.

Conclusion: A significant decrease in HRS in the retina after anti-vascular endothelial growth factor treatment is documented. A decrease in HRS correlates with functional parameters, specifically RS. New parameters may be used for treatment evaluation in center-involving diabetic macular edema.

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Diabetic macular edema (DME) is the leading cause of impaired visual acuity in patients affected by diabetes mellitus.[1–3] The pathophysiology of DME involves many interconnected pathways with specific contributions, thus determining different DME phenotypes.[4] It is well known that diabetic retinopathy (DR), together with DME, is not only a vascular but also a neuroinflammatory disease.[5] Currently, intravitreal treatments, in particular anti-vascular endothelial growth factor (anti-VEGF) drugs, have been established as the first-line treatment in center-involving DME.[6] Slit-lamp biomicroscopy, fluorescein angiography, optical coherence tomography (OCT), fundus autofluorescence, microperimetry (MP), and retromode scanning laser ophthalmoscope have all been used for the diagnosis and follow-up of DME and also

for better identifying different DME characteristics.[7] Spectral domain OCT has become the new gold standard in the evaluation of DME.[8–10] Besides the evaluation of retinal thickness and volume, spectral domain OCT has been recently used for the evaluation of intraretinal hyperreflective spots (HRS, foci/dots), choroidal thickness, reflectivity of intra/subretinal fluid, and outer retina integrity.[10–20] The presence and characteristics of small intraretinal HRS have been recently described in diabetic patients, with different hypotheses regarding their origin and significance.[13–15,17,19] Although some authors hypothesized that HRS may represent precursors of hard exudates, others hypothesized an origin from degenerated photoreceptors and correlation to outer retina disruption.[13–15] On the contrary, other authors correlated the presence of HRS with inflammatory response in the retina, as HRS were detectable even in diabetic patients without any clinical sign of DR.[17,19] However, there are limited data in the literature on their changes and in particular correlation with visual function after anti-VEGF treatment in DME.[15]

The purpose of this study was to assess early changes in intraretinal HRS after anti-VEGF treatment in naive center-involving DME and correlate it with functional changes (retinal sensitivity and fixation determined with MP and visual acuity).

Material and Methods

Population

This is a prospective 6-month follow-up, observational consecutive case–control series, which included 20 treatment-naive diabetic patients (40 eyes) with center-involving DME treated with intravitreal anti-VEGF injections in 1 eye. All patients were recruited from Diabetic Retinopathy Clinic at the Department of Ophthalmology, University of Padova. Informed consent was obtained from each patient, and the research was carried out in accordance with the Declaration of Helsinki. Local Ethics Committee approval for the study was obtained.

From the *Department of Ophthalmology, University of Padova, Padova, Italy; and †Fondazione G.B. Bietti, IRCCS, Rome, Italy.

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Reprint requests: Edoardo Midena, MD, PhD, Department of Ophthalmology, University of Padova, Via Giustiniani 2, 35128 Padova, Italy; e-mail: edoardo.midena@unipd.it

The inclusion criteria were age >18 years, diagnosis of Type 2 diabetes mellitus, untreated center-involving DME needing anti-VEGF treatment (according to our standard clinical care), which included a loading phase of 3 consecutive intravitreal injections of 0.5 mg ranibizumab (Lucentis; Genentech, San Francisco, CA) and thereafter pro re nata regimen with monthly follow-up. Exclusion criteria were patients who needed anti-VEGF treatment in both eyes, any disease that affected the retina other than diabetes, any type of previous retinal treatment (macular or peripheral laser photocoagulation, vitrectomy, intravitreal steroids, and/or antiangiogenic drugs), any ocular surgery, ischemic maculopathy, proliferative DR, significant media opacities that precluded good quality fundus imaging, and history of glaucoma or ocular hypertension. The fellow (control) eye was not treated during the 6-month follow-up period. Each patient was evaluated at baseline (V1) and thereafter monthly. For the purpose of the study, 5 follow-up evaluations are reported at baseline (V1), 1 month after each injection during the loading phase (V2, V3, and V4), and at 6 months (V5). During each reported visit, all patients underwent a full ophthalmic examination (which included determination of best-corrected visual acuity [BCVA], anterior segment examination, Goldmann applanation tonometry, 90-D lens biomicroscopy, color fundus photograph, spectral domain OCT) and MP. Fluorescein angiography was performed at baseline in all patients to exclude ischemic changes in the macula and proliferative DR.

Study Procedures

Visual acuity. Best-corrected visual acuity for each eye was measured by a certified operator using the standard Early Treatment Diabetic Retinopathy Study (ETDRS) protocol at a distance of 4 meters with a modified ETDRS distance chart, transilluminated with a chart illuminator (Precision Vision, Bloomington, IL). Visual acuity was scored as the total number of letters read correctly and calculated according to the ETDRS score method.

Spectral domain optical coherence tomography. All eyes were examined with spectral domain OCT (Retinascan, RS-3000 advance; Nidek). After pupillary dilation, a single 0° linear scan, 6 mm in length, was centered onto the fovea. Moreover, a 12 × 9-mm raster scan map centered onto the fovea was also performed to obtain the central foveal thickness. The built-in real-time eye movement tracking system recognized eye structures in the simultaneous infrared confocal scanning laser ophthalmoscopy as blood vessels and the optic disk, minimizing the artifacts. After definition of a certain baseline scan as reference, the

tracking system further enabled image acquisition at the same retinal location throughout each follow-up examination for a precise evaluation of changes over time. To improve the definition, each 0° linear image consisted of 120 averaged B-scans in a single raster line scan. The linear scans consisted of 1,024 A-scan, whereas the map scans consisted of 512 A-scan, with high-definition (50 HD) frame enhancement software, using the ultrafine setting, with a light source of 880-nm wavelength. Increasing the luminosity and the contrast of the images, retinal details were better visualized.

Each linear retinal scan was analyzed, evaluating the presence of HRS. Two vertical lines were traced at 500 μm and 1,500 μm from the center of the fovea both in the nasal and the temporal sides, thus excluding the foveal avascular zone. The linear B-scan was also evaluated considering the direct fundus image given by the instrument to exclude HRS corresponding to vessels and hard exudates. A manual count of HRS, defined as small, punctiform discrete white lesions, was performed between the 2 markers.[17] The count was performed starting from the inner limiting membrane (ILM) to the outer nuclear layer (ONL), dividing the retina into three parts as follows: from ILM to inner plexiform layer (ILM–IPL), from inner nuclear layer (INL) to outer plexiform layer (INL–OPL), and ONL.

Central retinal thickness (CRT) corresponding to central subfield retinal thickness on OCT and mean macular volume were analyzed in this study. Each linear retinal scan was automatically divided in the inner (ILM–OPL) and outer retina (ONL). In case of error, by the automatic instrument tracing system, manual correction was performed by the grader. Central retinal thickness, mean macular volume, mean inner (ILM–OPL), and outer retinal (ONL) thickness (at 500 μm and 1,500 μm, mean value) at nasal and temporal sides were analyzed in this study.

All measurements were performed by 2 independent, masked experienced graders.[17] Each grader was blind to clinical data of all examined eyes.

Microperimetry. Microperimetry was performed on all subjects using the MP1 microperimeter (Nidek). For the purpose of this study, the following parameters were used: a fixation target consisting of a red ring, 1° in diameter; white monochromatic background at 4 asb, stimulus size Goldman III, with 200-millisecond projection time; a customized radial grid of 45 stimuli covering central 12° (centered onto the fovea), 1° apart (inner stimuli), and 2° apart (outer stimuli).[21] The starting stimulus light attenuation was set at 10 dB. A 4-2 double staircase strategy was used with an automatic eye tracker that compensates for eye movements. Pretest training was performed, and 5-minute mesopic visual adaptation was allowed before starting the test. All subjects underwent MP with dilated pupils. Mean retinal sensitivity was evaluated within central 4° and 12°, approximately covering 1-mm and 3-mm central retina area on the OCT map.[21,22] Moreover, mean retinal sensitivity between 2° and 6°, both nasally and temporally from the center of the fovea, was also evaluated (thus covering approximately an area between 500 μm and 1,500 μm on OCT nasally and temporally). Fixation stability was evaluated by the bivariate contour ellipse area (BCEA). The BCEA is the result of plotting the position of each fixation point on Cartesian axes and calculating the area of an ellipse that encompasses a given percentage of fixations. Each value represents the area of 3 ellipses on which the eye fixates the target for 68.2% (BCEA 68.2%), 95.4% (BCEA 95.4%), and 99.6% (BCEA 99.6%) of time, which correspond to 1, 2, and 3 standard deviations, respectively.[23]

Statistics

Sample measurements have been summarized with mean value and standard deviation. Time profile comparison between treated and fellow eyes was performed. Two-way analysis of variance (ANOVA) with repeated measures on both factors (eye and visit) has been applied. Complete model with eye, visit, and eye by visit interaction factors was considered.

In addition, 2 other analyses were performed: 1) one aimed to analyze time profiles of parameters in each eye and 2) the other aimed to compare treated versus fellow eye. For the first analysis, one-way ANOVA (visit factor) with repeated measures followed by Bonferroni test for multiple comparisons was applied. For the second, one-way ANOVA (eye factor) with repeated measures was applied to data from each of the 5 visits separately. In this analysis Bonferroni correction for multiple testing was applied to the significance level (alpha = 0.05/5).

Correlation at each follow-up visit between morphologic parameters (number of HRS in different retinal layers) and functional parameters (sensitivity within 4° and 12°, sensitivity nasally and temporally to the fovea, BCEA, and visual acuity) was evaluated by Spearman correlation coefficient. A correlation coefficient of at least 0.30 was interpreted as clinically significant.

All the analyses were made by SAS version.9.3 statistical software on a personal computer. $P < 0.05$ was interpreted as statistically significant, unless specified differently.

Results

Twenty eyes of 20 diabetic patients with center-involving DME were treated with 3 intravitreal

anti-VEGF injections in this study. Fellow eyes (20 eyes) were considered as control. Sixteen patients (80%) were men, and 4 (20%) were women. All patients had Type 2 DM, with a mean duration of 11.3 ± 10.0 years and a mean HbA1c of 56.4 ± 5.2 mmol/mol. The mean age of patients was 63.0 ± 8.2 years. All patients had nonproliferative DR.

Mean baseline CRT was 496.2 ± 119.1 μm in treated eyes and 365.3 ± 120.9 μm in fellow eyes ($P < 0.01$). Central retinal thickness significantly decreased in treated eyes at all visits versus the baseline visit V1, except the final visit (V5 at 6 months, $P = 1.0$) (Bonferroni post hoc test for multiple comparison versus baseline, $P < 0.03$ at least for all values from V2 to V4) (Table 1). Central retinal thickness did not significantly change in the fellow eye group at any follow-up visit (Table 1). Mean retinal volume values significantly decreased at V3 ($P = 0.0009$) and V4 ($P = 0.0006$) in treated eyes, and no changes were found in the fellow eye group.

Mean inner nasal retinal thickness significantly decreased at V2 ($P = 0.006$), V3 ($P < 0.001$), and V4 ($P < 0.001$) versus V1 in treated eyes (Bonferroni post hoc test for multiple comparison) (Table 1). Mean inner temporal retinal thickness significantly decreased at V2 ($P = 0.016$), V3 ($P = 0.005$), and V4 ($P = 0.001$) versus V1 in treated eyes (Bonferroni post hoc test for multiple comparison). No changes were found in fellow eyes in both inner nasal and inner temporal retinal thickness. Outer retinal thickness significantly decreased only in the temporal side at V3 ($P = 0.028$) and V4 ($P = 0.022$) versus baseline (Bonferroni post hoc test for multiple comparison) (Figure 1).

The mean number of HRS significantly decreased in all 3 measured layers (ILM–IPL, INL–OPL, and ONL) in treated eyes at all follow-up visits versus baseline (V1) (Bonferroni post hoc test for multiple comparison versus baseline, $P < 0.008$ at least for all) (Table 1, Figure 2). The major decrease in the HRS number was observed in the outer retina, specifically in the ONL, with a decrease varying between 40% and 65% (Table 1).

The mean number of HRS did not significantly change in the fellow eye group throughout the follow-up, except the increase in number in the INL–OPL layer versus baseline at V4 (14.8 ± 6.1 vs. 12.1 ± 4.2, $P = 0.02$) (Table 1, Figure 2).

Mean baseline retinal sensitivity within central 4° was 9.9 ± 5.6 dB in treated eyes and 13.1 ± 4.9 dB in fellow eyes ($P = 0.04$). Mean baseline retinal sensitivity within central 12° was 12.7 ± 5.0 dB in treated eyes and 14.5 ± 4.4 dB in fellow eyes ($P = 0.13$). Table 2 also shows mean values of retinal sensitivity in the nasal and temporal perifoveal area at each visit (Table 2).

In treated eyes, mean retinal sensitivity within central 4° and 12° increased at V3 and V4, without reaching statistical significance throughout the follow-up (ANOVA test for repeated measures, $P = 0.87$ and

Table 1. Mean Values and Standard Deviations of Morphologic Parameters Measured in Treated and Fellow Eyes at Follow-up Visits

Parameter	Eye	Visit				
		V1	V2	V3	V4	V5
CRT, μm	T	496.2^{A} ± 119.1	434.3^{c} ± 129.9	417.7^{d} ± 114.3	409.2^{e} ± 104.8	457.5 ± 137.8
	F	365.3 ± 120.9	377.5 ± 118.5	380.0 ± 127.2	348.9 ± 83.2	347.5 ± 92.6
Volume	T	12.1 ± 2.5	11.9^{B} ± 2.9	11.3^{e} ± 2.3	11.0^{e} ± 1.9	11.5 ± 2.3
	F	11.2 ± 3.0	11.3 ± 2.9	11.2 ± 2.8	10.7 ± 2.0	10.8 ± 2.3
HRS in ILM/IPL retinal layer	T	20.9^{B} ± 6.1	15.8^{e} ± 5.5 –24.6%*	15.2^{e} ± 5.7 –26.7%*	15.8^{e} ± 6.2 –25.1%*	18.4^{d} ± 8.7 –18.1%*
	F	12.7 ± 3.8	13.6 ± 2.0 +15.9%*	13.5 ± 3.0 +15.9%*	13.8 ± 2.5 +20.0%*	14.1 ± 3.1 +21.5%*
HRS in INL/OPL retinal layer	T	16.2^{A} ± 5.8	13.4^{d} ± 5.4 –17.3%*	12.5^{B,e} ± 5.3 –22.6%*	12.9^{e} ± 5.0 –18.8%*	13.8^{B,e} ± 5.8 –20.7%*
	F	12.1 ± 4.2	14.0 ± 5.4 +19.7%*	13.7 ± 5.0 +17.6%*	14.8^{c} ± 6.1 +25.3%*	15.0 ± 6.6 +22.2%*
HRS in ONL retinal layer	T	4.8^{B} ± 2.8	3.2^{d} ± 3.1 –39.9%*	2.2^{e} ± 2.7 –65.3%*	2.2^{e} ± 3.0 –60.0%*	2.5^{e} ± 3.2 –57.7%*
	F	1.8 ± 1.7	2.4 ± 2.1 +20.1%*	2.1 ± 1.5 +20.8%*	1.9 ± 1.7 +19.7%*	2.5 ± 1.8 +54.6%*

Comparison respect to fellow eye (one-way ANOVA with repeated measures, with significance P adjusted for multiple testing 0.05/5): $^{A}P < 0.01$; $^{B}P < 0.001$. Within eye comparison respect to V1 (Bonferroni post hoc test after one-way ANOVA with repeated measures): $^{c}P < 0.05$; $^{d}P < 0.01$; $^{e}P < 0.001$.

*Percentage change respect to V1.

F, fellow; T, treated; V1, baseline visit; V2, 1 month after the first anti-VEGF treatment; V3, 1 month after the second anti-VEGF treatment; V4, 1 month after the third anti-VEGF treatment; V5, at 6 months of follow-up.

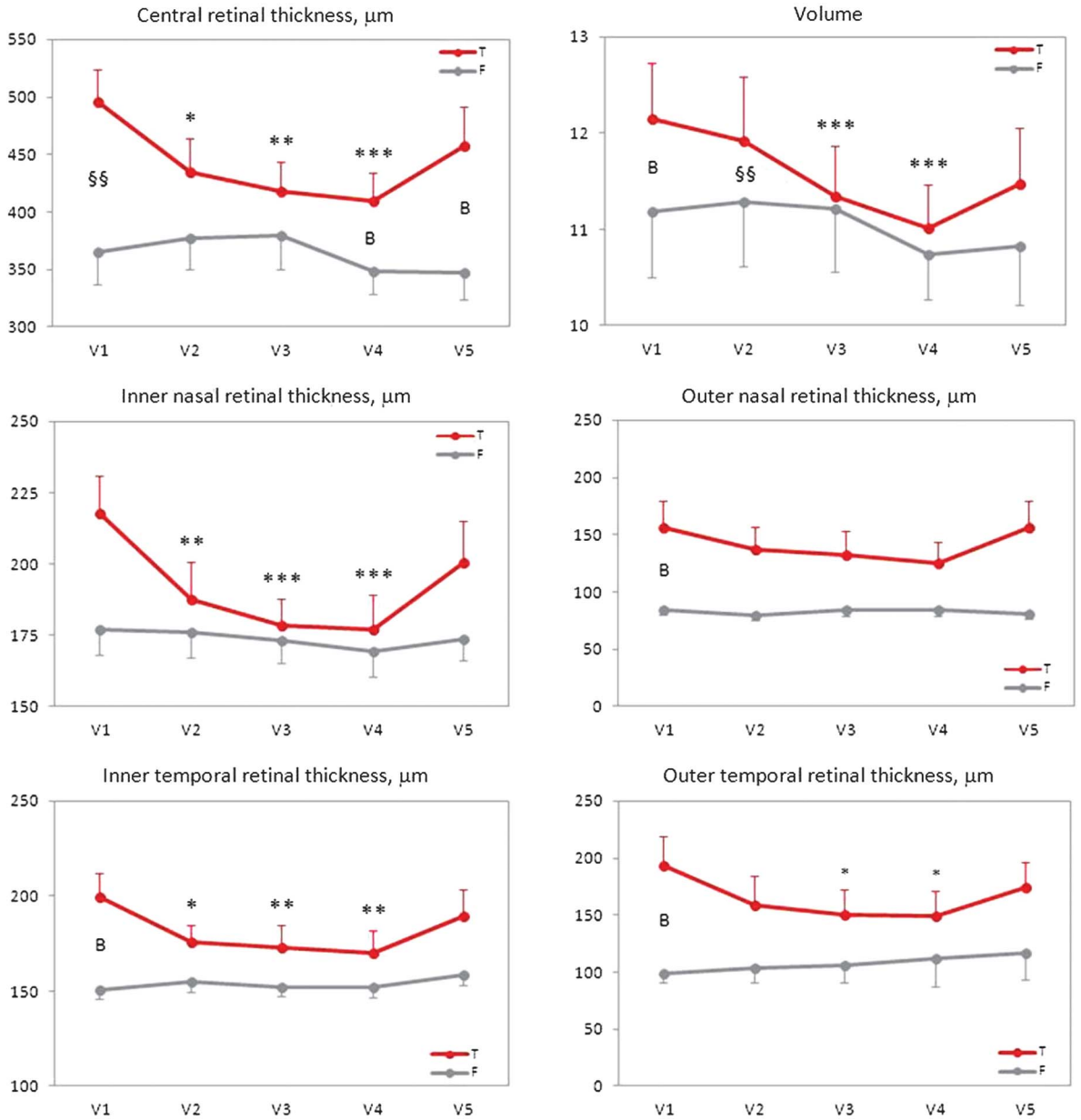

Fig. 1. Time profile of retinal thickness and volume in treated (T) and fellow (F) eyes. V1, baseline visit; V2, 1 month after the first anti-VEGF treatment; V3, 1 month after the second anti-VEGF treatment; V4, 1 month after the third anti-VEGF treatment; V5, at 6 months of follow-up. Retinal thickness values (CRT, inner and outer temporal and nasal retinal thickness) are expressed in micrometers. Retinal volume values are expressed in cubic millimeters. Statistical comparison respect to V1 mean value: Bonferroni post hoc test for multiple comparisons after one-way ANOVA with repeated measures, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical comparison between eyes (treated vs. fellow eye) at each visit separately: ANOVA for repeated measures with Bonferroni correction for multiple testing (Type I error alpha = 0.05/5), §§ $P < 0.01$, §§§ $P < 0.001$, B borderline P value (P between 0.01 and 0.02).

$P = 0.61$, respectively). In fellow eyes, mean retinal sensitivity within 4° and 12° did not significantly change throughout the follow-up (ANOVA test for repeated measures, $P = 0.61$ and $P = 0.81$, respectively). Mean retinal sensitivity within 4° and 12° was significantly different at V3, V4, and V5 in treated eyes versus fellow eyes (ANOVA test for repeated measures, $P < 0.009$ at least for all) (Table 2, Figure 2). In treated eyes, mean retinal sensitivity in the nasal and temporal perifoveal retina increased at V4, without reaching statistical significance (ANOVA test for repeated measures, $P = 0.18$ and $P = 0.17$, respectively). In fellow eyes, mean retinal sensitivity in the nasal and temporal perifoveal retina remained unchanged at all visits (ANOVA test for repeated measures, $P = 0.37$ and $P = 0.87$, respectively) (Table 2). As retinal

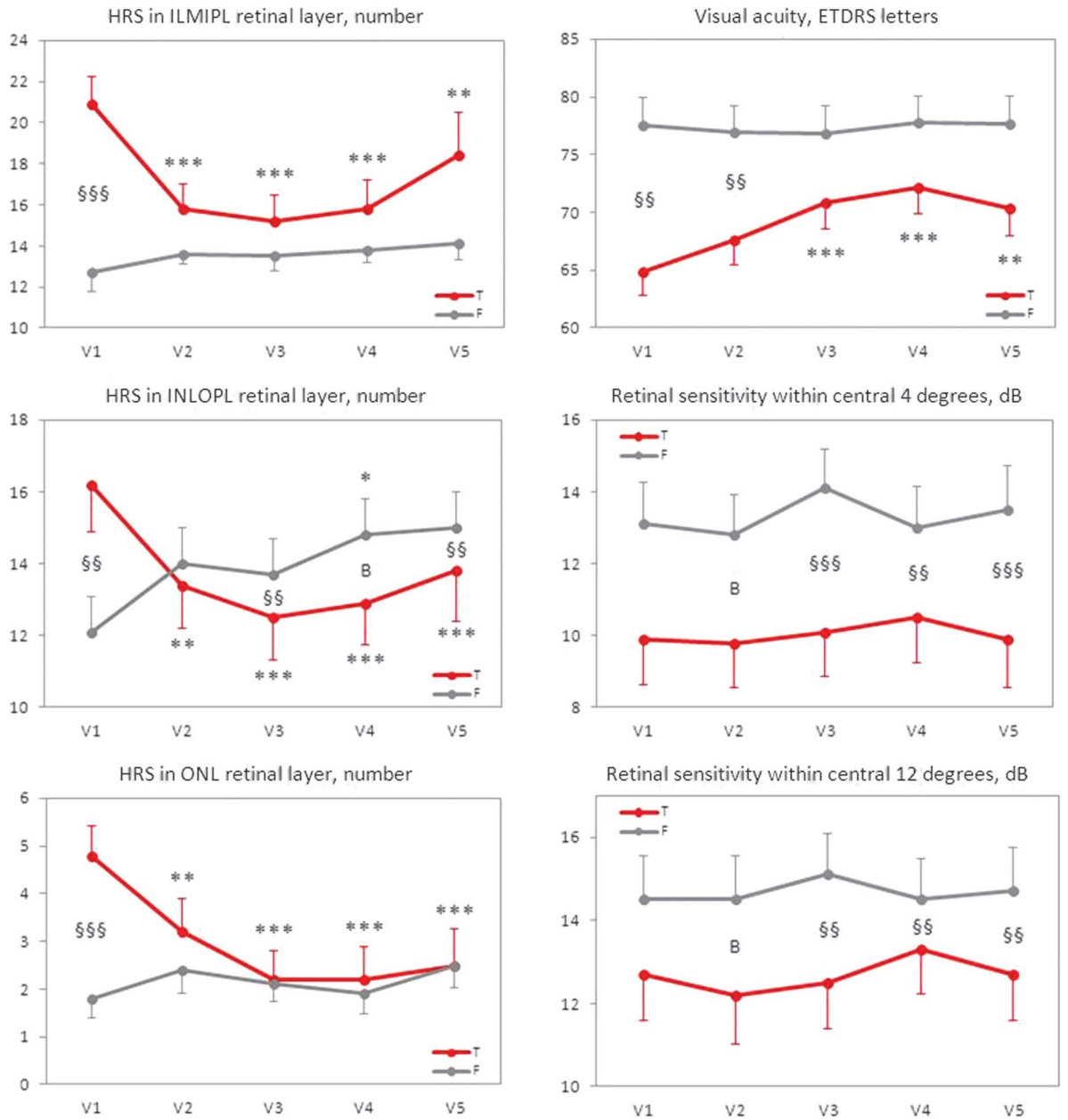

Fig. 2. Time profile of HRS, retinal sensitivity, and visual acuity in treated (T) and fellow (F) eyes. V1, baseline visit; V2, 1 month after the first anti-VEGF treatment; V3, 1 month after the second anti-VEGF treatment; V4, 1 month after the third anti-VEGF treatment; V5, at 6 months of follow-up. Statistical comparison respect to V1 mean value: Bonferroni post hoc test for multiple comparisons after one-way ANOVA with repeated measures, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical comparison between eyes (treated vs. fellow eye) at each visit separately: ANOVA for repeated measures with Bonferroni correction for multiple testing (Type I error alpha = 0.05/5), §§ $P < 0.01$, §§§ $P < 0.001$, B borderline P value (P between 0.01 and 0.02).

fixation is concerned, BCEA 68, BCEA 95, and BCEA 99 did not change in neither treated nor fellow eye group throughout the whole follow-up (ANOVA with repeated measures, $P > 0.236$, at least for all) (Table 2).

Mean baseline BCVA was 64.9 ± 9.1 ETDRS letters score in treated eyes and 77.6 ± 10.0 ETDRS letters score in fellow eyes ($P < 0.01$). In treated eyes, mean BCVA significantly improved at V3 (5.9 ETDRS letters increase, $P = 0.0002$), V4 (7.3 ETDRS letters increase, $P < 0.0001$), and V5 (5.5 ETDRS letters increase, $P = 0.0093$) versus baseline (Bonferroni post hoc test for multiple comparison). Mean BCVA did not change in fellow eyes throughout the whole follow-up (ANOVA with repeated measures, $P = 0.74$) (Table 2, Figure 2).

Table 2. Mean and Standard Deviation Values of Functional Parameters Measured in Treated and Fellow eyes at Follow-up Visits

Parameter	Eye	Visit				
		V1	V2	V3	V4	V5
Visual acuity (ETDRS letters)	T	64.9[A] ± 9.1	67.6[A] ± 9.4	70.8[e] ± 9.9	72.2[e] ± 10.0	70.4[d] ± 10.2
	F	77.6 ± 10.0	76.9 ± 10.1	76.8 ± 10.2	77.8 ± 9.2	77.7 ± 9.2
Retinal sensitivity within 4°	T	9.9 ± 5.6	9.8 ± 5.5	10.1[B] ± 5.6	10.5[A] ± 5.5	9.9[B] ± 5.6
	F	13.1 ± 4.9	12.8 ± 4.7	14.1 ± 4.5	13.0 ± 4.7	13.5 ± 4.7
Retinal sensitivity within 12°	T	12.7 ± 5.0	12.2 ± 5.3	12.5[A] ± 5.0	13.3[A] ± 4.7	12.7[A] ± 4.6
	F	14.5 ± 4.4	14.5 ± 4.4	15.1 ± 4.0	14.5 ± 4.1	14.7 ± 4.1
Nasal retinal sensitivity	T	13.1 ± 5.1	12.7 ± 5.4	13.3 ± 5.0	14.3 ± 4.9	13.4 ± 4.7
	F	15.1 ± 3.8	14.3 ± 4.4	15.5 ± 3.7	14.8 ± 4.1	14.9 ± 3.7
Temporal retinal sensitivity	T	12.7 ± 5.4	12.8 ± 5.2	13.0 ± 4.9	13.5 ± 5.0	12.8 ± 5.1
	F	14.7 ± 5.0	14.2 ± 4.4	14.9 ± 4.6	14.5 ± 4.6	14.1 ± 4.5
BCEA68	T	2.0 ± 1.2	2.3 ± 1.5	1.7 ± 1.3	1.8 ± 0.8	1.7 ± 1.0
	F	2.6 ± 2.1	2.5 ± 1.9	1.6 ± 0.6	2.2 ± 1.4	1.9 ± 0.9
BCEA95	T	5.4 ± 3.2	6.1 ± 4.1	4.5 ± 3.4	4.9 ± 2.1	4.6 ± 2.7
	F	5.1 ± 3.7	6.8 ± 5.0	4.3 ± 1.9	6.0 ± 3.9	5.0 ± 2.5
BCEA99	T	9.6 ± 5.6	10.9 ± 7.3	8.5 ± 6.8	8.8 ± 3.7	8.2 ± 4.8
	F	9.1 ± 6.5	12.3 ± 9.3	7.8 ± 3.4	10.8 ± 7.0	8.9 ± 4.5

Comparison respect to fellow eye (one-way ANOVA with repeated measures, with significance P adjusted for multiple testing 0.05/5): [A]$P < 0.01$; [B]$P < 0.001$. Within eye comparison respect to V1 (Bonferroni post hoc test after one-way ANOVA with repeated measures): [c]$P < 0.05$; [d]$P < 0.01$; [e]$P < 0.001$.

BCEA for evaluation of fixation stability; each value represents the area of 3 ellipses on which the eye fixates the target for 68.2% (BCEA 68.2%), 95.4% (BCEA 95.4%), and 99.6% (BCEA 99.6%) of time, which correspond to 1, 2, and 3 standard deviations, respectively.

F, fellow; T, treated; V1, baseline visit; V2, 1 month after the first anti-VEGF treatment; V3, 1 month after the second anti-VEGF treatment; V4, 1 month after the third anti-VEGF treatment; V5, at 6 months follow-up.

Spearman correlation coefficient showed significant and inverse correlation between the number of HRS and retinal sensitivity (within 4° and 12°, nasally and temporally to the fovea) (prevalently in the INL–OPL and ONL layers), $P = -0.31$ (at least for all); weak and not significant correlation of the number of HRS with BCVA (Table 3); direct and strong correlation of the number of HRS with BCEA 99 (mostly with HRS in the INL–OPL and ILM–IPL) (Table 3); weak and not significant correlation of the number of HRS with retinal thickness (*data not shown*).

Discussion

In this study, we report some detailed and specific early retinal changes in center-involving DME eyes treated with multiple anti-VEGF injections. Morphologic changes are compared with functional outcomes in terms of BCVA, retinal sensitivity, and BCEA determined with MP. The most relevant morphologic changes include reduction of CRT and HRS decrease. The reduction of CRT is well known after anti-VEGF treatment of DME.[24] A significant reduction in HRS was found in all retinal layers (ILM–IPL, INL–OPL, and ONL) in the perifoveal area after anti-VEGF treatment (Figure 3). This decrease was precocious, occurring immediately after the first anti-VEGF injection and persisted throughout the whole follow-up, until 6 months. In fellow eyes, the HRS number did not change. Framme et al[15] reported a significant reduction in HRS only in cases with complete resolution of DME and no correlation with BCVA improvement after anti-VEGF treatment. These authors concluded that HRS may indicate the integrity of retinal tissue, and being positively correlated with HbA1c values, a general severity of systemic diabetic disease. The significance of HRS (first described by Coscas et al[12] in age-related macular degeneration as small, punctiform hyperreflective elements, which were scattered throughout all retinal layers but mostly in the outer retina and interpreted as activated microglial cells during an inflammatory reaction) in diabetic eyes is still not uniform. Bolz et al[13] hypothesized that HRS may represent subclinical features of lipoprotein extravasation that act as precursors of hard exudates. Uji et al[14] reported that HRS in the outer retina were closely associated with disrupted external limiting membrane and inner segment and outer segment line and decreased visual acuity, suggesting an origin from degenerated photoreceptors or macrophages engulfing them. De Benedetto et al[19] described HRS in diabetic patients without macular edema or visual acuity impairment and correlated with poor glycometabolic control and hypertension. Vujosevic et al[17] reported an increase in HRS in diabetic patients versus normal

Table 3. Spearman Correlation Coefficient Between Functional and Morphologic Parameters in the Treated Eye at Different Visits

		Morphologic Parameters		
		HRS		
Functional Parameters	Visit	ILM–IPL	INL–OPL	ONL
Retinal sensitivity within 4°	V1	–0.04	–0.06	**–0.36**
	V2	–0.16	**–0.31**	**–0.44**
	V3	**–0.33**	**–0.40**	**–0.34**
	V4	–0.27	**–0.45**	–0.26
	V5	**–0.44**	**–0.53**	**–0.53**
Retinal sensitivity within 12°	V1	–0.09	–0.14	**–0.33**
	V2	–0.09	–0.27	**–0.36**
	V3	–0.25	**–0.38**	–0.28
	V4	**–0.39**	**–0.58**	**–0.41**
	V5	**–0.32**	**–0.44**	**–0.35**
Retinal sensitivity, nasal	V1	–0.07	–0.15	**–0.34**
	V2	–0.14	**–0.35**	**–0.48**
	V3	**–0.30**	**–0.46**	**–0.34**
	V4	**–0.33**	**–0.56**	**–0.37**
	V5	**–0.33**	**–0.46**	**–0.41**
Retinal sensitivity, temporal	V1	–0.14	–0.12	–0.26
	V2	–0.02	–0.14	–0.23
	V3	–0.19	**–0.31**	–0.23
	V4	**–0.36**	**–0.45**	**–0.31**
	V5	**–0.42**	**–0.43**	**–0.44**
Visual acuity (ETDRS letters)	V1	0.22	0.17	–0.23
	V2	0.16	–0.00	–0.15
	V3	–0.07	–0.05	–0.14
	V4	0.12	–0.01	–0.13
	V5	–0.10	–0.08	**–0.37**
BCEA99	V1	**0.40**	**0.42**	**0.31**
	V2	**0.44**	**0.65**	**0.41**
	V3	**0.51**	**0.61**	0.29
	V4	**0.46**	**0.70**	0.21
	V5	**0.58**	**0.65**	**0.48**

In bold are coefficients >0.30 that have been interpreted as clinically significant.
V1, baseline visit; V2, 1 month after the first anti-VEGF treatment; V3, 1 month after the second anti-VEGF treatment; V4, 1 month after the third anti-VEGF treatment; V5, at 6 months of follow-up.

subjects (even in diabetic eyes without any clinical sign of retinopathy), suggesting that HRS may represent aggregates of activated microglial cells which migrate, with more progressive disease (as showed in patients with nonproliferative DR vs. diabetic patients without DR), from the inner to outer retina. Moreover, as reported by the authors, the included patients neither had macular edema nor even subclinical (OCT) signs of DME nor hard exudates, thus excluding the possibility of HRS being lipid exudates or degenerated photoreceptors. Data from experimental studies in human diabetic retinas confirm the hypothesis that HRS may be secondary to microglial cell activation, showing that the resting microglia are physiologically located in the inner retinal layers and, when activated, microglial cells undergo significant changes in shape and size and aggregate among them to form the microglial aggregates.[25] The same authors reported the presence of "microglial perivasculitis," a local inflammatory response due to microglial activation, which secondarily affects ganglion cells (more inner retinal layers).[25] In fact, HRS were described in most inner retinal layers at early stages of diabetes mellitus. The release of inflammatory mediators (including VEGF) would provoke the extension of the inflammatory process through the entire retina, increasing both vascular permeability and neuronal damage (thus HRS migrating to the outer retinal layers), as occurring in DME.[13–15]

In this study, HRS correlated significantly and inversely with retinal sensitivity determined with MP (a decrease in the HRS number correlated with increased RS). Although retinal sensitivity improvement after anti-VEGF treatment did not reach statistical significance (probably because of high standard deviation and limited number of examined eyes),

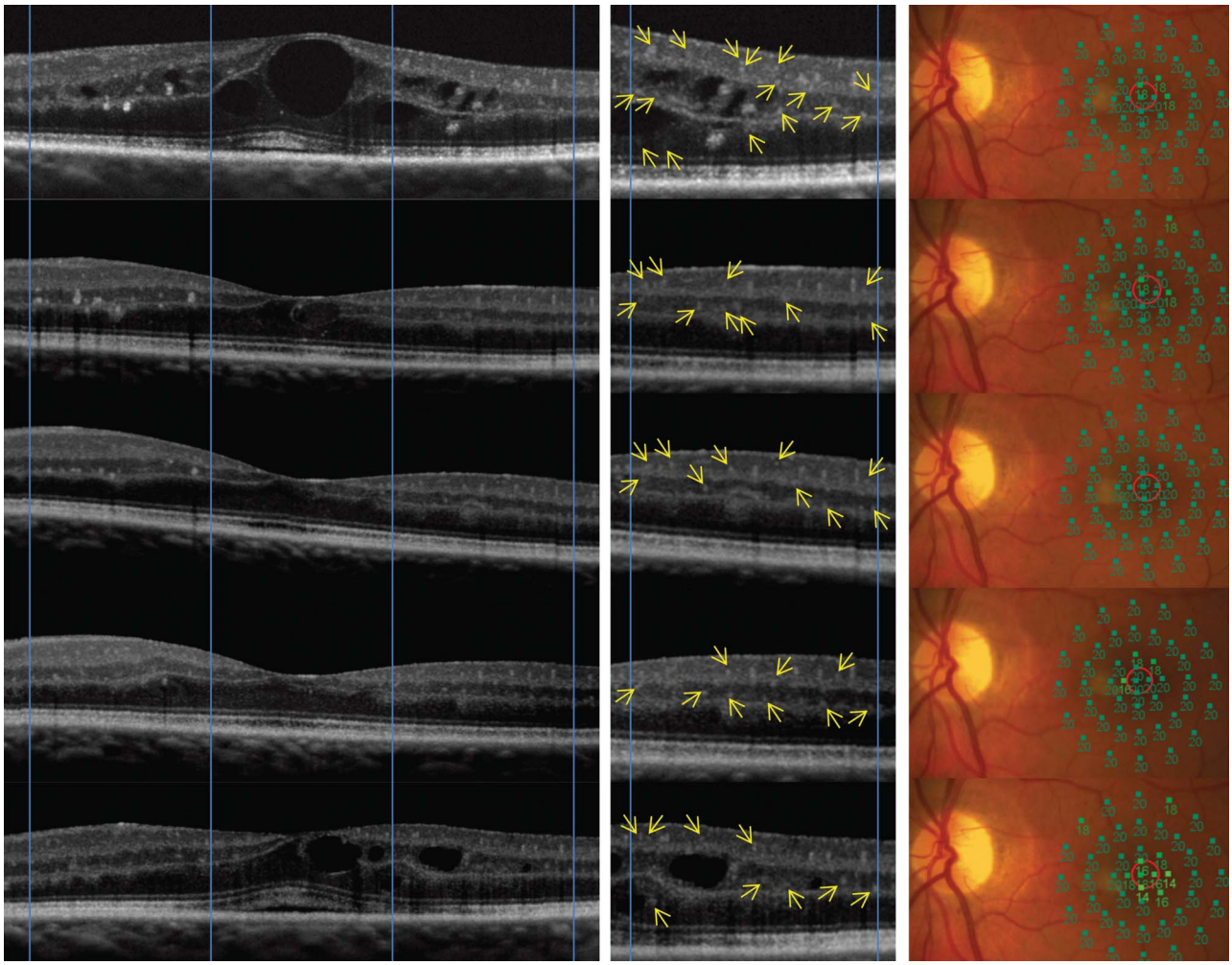

Fig. 3. Spectral domain OCT line scans with hyperreflective retinal spots and MP sensitivity maps in center-involving DME treated with anti-VEGF. Left eye of the patient treated with 3 consecutive ranibizumab intravitreal injections. Top row, at V1 (baseline visit); Row 2, at V2 (1 month after the first anti-VEGF treatment); Row 3, at V3 (1 month after the second anti-VEGF treatment); Row 4, at V4 (1 month after the third anti-VEGF treatment); Row 5, at V5 (at 6 months of follow-up). Vertical lines are traced at 500 μm and 1,500 μm from the center of the fovea both in the nasal and temporal sides and indicate the areas where the HRS were evaluated. Hyperreflective spots were considered as small, punctiform discrete white lesions evaluated from ILM to IPL; from INL to OPL; and ONL. To exclude HRS corresponding to vessels and hard exudates, the fundus image was evaluated and compared with OCT line scan. Images show (better seen in the zoomed temporal side of images and indicated with yellow arrows) progressive decrease in the HRS number, in all retinal layers, with anti-VEGF treatment versus baseline, and subsequent increase at final examination (6 months of follow-up).

correlation analysis performed at each visit showed that the HRS number is inversely related to macular function. This data might seem controversial. However, if HRS are considered as visible aggregates of activated microglial cells and therefore a marker of neuroinflammatory response in the retina, a decrease in the HRS number may lead to better functional outcome, thus clinically relevant. Therefore, the significance of HRS would merit further studies. Another functional parameter evaluated in this study is fixation stability, evaluated as BCEA and determined automatically by the microperimeter. Bivariate contour ellipse area represents the standard deviation of the horizontal and vertical eye movements during fixation.[26,27] Smaller BCEA means more stable fixation versus larger BCEA. This is a more recent and precise way of determining fixation stability than clinical evaluation previously proposed by Fuji et al[28] (stable, relatively unstable, and unstable fixation). In this study, we evaluated BCEA data obtained during the whole examination time of retinal sensitivity test (so called dynamic fixation). Dynamic fixation is considered more appropriate (versus static fixation, obtained during the initial 1-minute fixation test) when evaluating pathologic eyes because it better documents patient's fixation, as it may be influenced by patient attention.[23] Notwithstanding, we found small BCEA area in DME eyes and not significant changes during

the anti-VEGF treatment, as already stable fixation cannot improve reaching statistical significance (although absolute values were found decreased, Table 2). Therefore, center-involving DME eyes needing anti-VEGF treatment show preserved stability of fixation. This is in agreement with previous data reporting preserved fixation (mostly stable and central) in patients with DME, irrespective of the type of edema.[29] Moreover, a direct and strong correlation was found between the HRS number and BCEA, mostly HRS located in the inner retinal layers (ILM–IPL and INL–OPL). This means that at each single visit the higher number of HRS was associated with larger BCEA (although in this study corresponding mostly to stable fixation) and the smaller number of HRS with smaller BCEA. We have not found significant correlation between HRS decrease and BCVA increase. This may be due to the fact that BCVA increase reflects the morphologic changes of the very central retina, whereas we evaluated HRS spots between 500 and 1,500 μm from the fovea. We excluded the evaluation of HRS in the very central fovea, mostly because of its anatomical characteristics (lack of specific retinal layers), and therefore evaluation of HRS in the inner retinal layers may be underestimated in the fovea. Moreover, correlation between HRS and retinal thickness was only weak. In fact, HRS decreased in all retinal layers, whereas retinal thickness decreased mostly in the inner retinal layers (ILM–OPL). Therefore, it seems that HRS correlate better with functional data, rather than with morphologic changes. Thus, HRS may add information on functional outcome in DME eyes treated with anti-VEGF.

The major limitation of this short-term study is the limited number of examined eyes. However, the wide range of baseline data (e.g., CRT, RS) reflects the clinical heterogeneity of DME patients; thus, the information obtained from this study could be easily transferred in everyday clinical management of naive DME patients.

Data from this study show that anti-VEGF treatment preserves/ameliorates retinal sensitivity and preserves stability of fixation, adding more knowledge, to limited available information, on retinal sensitivity results in DME eyes treated with anti-VEGF.[30]

Indirectly, this study may confirm the necessity to treat patients with DME with multiple anti-VEGF injections, because at final visit almost all patients had recurrence of DME and worsening of both morphologic and functional parameters. However, this would need a longer-term study that was beyond the scope of this report.

In conclusion, in DME eyes, anti-VEGF treatment significantly reduces the number of perifoveal HRS, which significantly and inversely correlates with retinal sensitivity. Hyperreflective spots may become a new OCT parameter for evaluation of functional efficacy of treatments in center-involving DME.

Key words: diabetic macular edema, hyperreflective spots, anti-VEGF, OCT, microperimetry, retinal sensitivity, fixation.

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