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Imaging retinal inflammatory biomarkers after intravitreal steroid and anti-VEGF treatment in diabetic macular oedema

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ABSTRACT.

Purpose: **To evaluate changes of specific retinal imaging biomarkers [intraretinal hyper-reflective retinal spots: HRS ; subfoveal neuroretinal detachment: SND; and increased foveal autofluorescence: IFAF after intravitreal steroid or anti-vascular endothelial growth factor treatment in diabetic macular oedema (DME)] as possible indicators of retinal inflammatory condition.**

Methods: **Retrospective analysis of images and clinical charts of 49 eyes (49 patients) with DME treated with intravitreal dexamethasone (dexamethasone, 23 eyes) or intravitreal ranibizumab (ranibizumab, 26 eyes). All patients had fundus colour photograph, spectral domain optical coherence tomography (SD OCT) and fundus autofluorescence (FAF), best-corrected visual acuity (BCVA) and microperimetry recorded before and 1 month after the end of treatment. Central macular thickness (CMT), number of HRS and presence of SND were evaluated by SD OCT. Fundus autofluorescence images were evaluated for area of (IFAF). Retinal sensitivity within 4° and 12° from fovea was quantified by microperimetry. Changes in morphologic and functional parameters were assessed, and correlation was performed by Pearson's correlation.**

Results: **Best-corrected visual acuity and CMT improved in all patients, ($p < 0.05$, for both groups). Mean number of HRS decreased after both treatments ($p < 0.0001$). Subfoveal neuroretinal detachment resolved in 85.7% dexamethasone-treated eyes ($p = 0.014$) and in 50% ranibizumab-treated eyes ($p = 0.025$). Mean IFAF area decreased in both groups, ($p < 0.0001$, for both). A significantly higher decrease in CMT was observed in dexamethasone- versus ranibizumab-treated eyes, ($p = 0.032$). In dexamethasone group, higher number of HRS at baseline and larger IFAF were correlated with higher increase in retinal sensitivity; eyes with SND at baseline had major decrease in CMT versus those without SND, ($p = 0.003$).**

Conclusion: **Higher number of HRS, larger area of IFAF and presence of SND may indicate a prevalent inflammatory condition in DME with specific response to targeted treatment.**

Key words: diabetic macular oedema – fundus autofluorescence – hyper-reflective retinal spots – microperimetry – OCT – subfoveal neuroretinal detachment

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Introduction

Diabetic macular oedema (DME) is the major cause of vision loss in diabetic patients (Das et al. 2015). Although hyperglycaemia is the most important risk factor contributing to the pathogenesis of DME, the exact mechanism it acts through is still unclear. However, hyperglycaemia determines modifications in at least four major biochemical pathways including diacylglycerol (DAG)–protein kinase C (PKC), advanced glycation end products/receptor for advanced glycation end products, polyol (sorbitol) and hexosamine pathways (Zhang et al. 2014). The activation of these metabolic pathways leads to increased oxidative stress, inflammation and vascular dysfunction (Das et al. 2015). Oxidative stress and inflammation result in upregulation of cytokines and growth factors such as interleukins (ILs), angiopoietins, tumour necrosis factor, matrix metalloproteinases and vascular endothelial growth factor (VEGF), which contribute to breakdown of blood–retinal barrier and development of DME (Das et al. 2015; Simo' & Hernandez 2015). Therefore, the important role played by inflammation in the pathogenesis of DME represents the rationale for its treatment by intravitreal steroids and/or anti-VEGF drugs (Funk et al. 2010; Sohn et al. 2011; Simo' & Hernandez 2015).

Recently, there has been an increased interest in clinical evaluation

of specific retinal parameters as non-invasive biomarkers of retinal inflammation in diabetic retinopathy (DR) and DME. These include presence of hyper-reflective retinal spots (HRS), subfoveal neuroretinal detachment (SND) and increased foveal autofluorescence (IFAF) (Vujosevic et al. 2011, 2013, 2016a; Sonoda et al. 2014; Madeira et al. 2015). In fact, small HRS (with specific characteristics) may be considered aggregates of activated microglial cells, present in both inner and outer retina in diabetic patients (Vujosevic et al. 2013, 2016a). Recently, it has been shown that HRS decrease after anti-VEGF treatment in DME patients (Framme et al. 2012; Vujosevic et al. 2016a).

Subfoveal neuroretinal detachment is an accumulation of subretinal fluid, with a distinct upper edge determined by the detached retina, visible as hyper-reflective area on spectral domain optical coherence tomography (SD OCT) (Otani et al. 1999). Different pathophysiologic mechanisms have been hypothesized for SND in DME eyes including outer limiting membrane disruption causing microglial cell attraction or impaired choroidal blood flow (Aloisi 2001; Nagaoka et al. 2004; Gaucher et al. 2008; Sonoda et al. 2014; Seo et al. 2016). Subfoveal neuroretinal detachment in DME has prevalence of 15–30%, and it is correlated with higher concentration of inflammatory cytokines in the vitreous and poorer visual prognosis after treatment (Otani et al. 1999; Framme et al. 2012; Sonoda et al. 2014).

Modifications of fundus autofluorescence (FAF) have been recently described in DME, and different patterns of increased foveal autofluorescence have been described (Pece et al. 2009; Vujosevic et al. 2011). Fundus autofluorescence signals are thought to derive from lipofuscin present in RPE cells in degenerative acquired and inherited macular diseases, but in DME, it is thought that areas of IFAF are caused by accumulation of oxidative products induced by activated microglial cells (Schmitz-Valckenberg et al. 2008; Xu et al. 2008; Pece et al. 2009; Vujosevic et al. 2011).

The purpose of this study was to evaluate and compare, non-invasively, changes in specific retinal imaging biomarkers (HRS, SND and IFAF) after intravitreal steroid or anti-VEGF treatment in DME as possible indicators of retinal inflammatory condition.

Materials and Methods

Population

This study is a retrospective comparative case evaluation of images and clinical charts of 49 eyes of 49 naïve patients with centre involving DME, treated with either intravitreal implant of dexamethasone 0.7 mg (Ozurdex, Allergan, Inc., Irvine, California, USA), or with 3 monthly intravitreal injections of ranibizumab 0.5 mg (Lucentis, Genentech, San Francisco, USA) Novartis and evaluated 1 month after the end of their treatment (Figs. 1 and 2). All patients had complete ophthalmologic examination with best-corrected visual acuity determination (BCVA), slit-lamp biomicroscopy examination, fundus colour photography, SD OCT, FAF and fluorescein angiography. All exams (except fluorescein angiography) were performed at baseline and 1 month after the end of their treatment. Fluorescein angiography was performed only before treatment. Inclusion criteria were previously untreated centre involving DME with central macular thickness (CMT) on SD OCT ≥300 μm; BCVA between Early Treatment Diabetic Retinopathy Study (ETDRS) score 35 (corresponding to 0.1 Snellen decimal) and ETDRS score 80, (corresponding to 0.8 Snellen decimal). Moreover, in order to be eligible for this study, patients had to have all imaging modalities of reasonably good quality obtained on the same day. Exclusion criteria were HbA1c >86 mmol/mol; a positive history of ocular surgery or any other macular/retinal treatment (laser, intravitreal injections); a history of uncontrolled glaucoma or ocular hypertension; iris neovascularization; and ischaemic maculopathy. 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.

Visual acuity

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

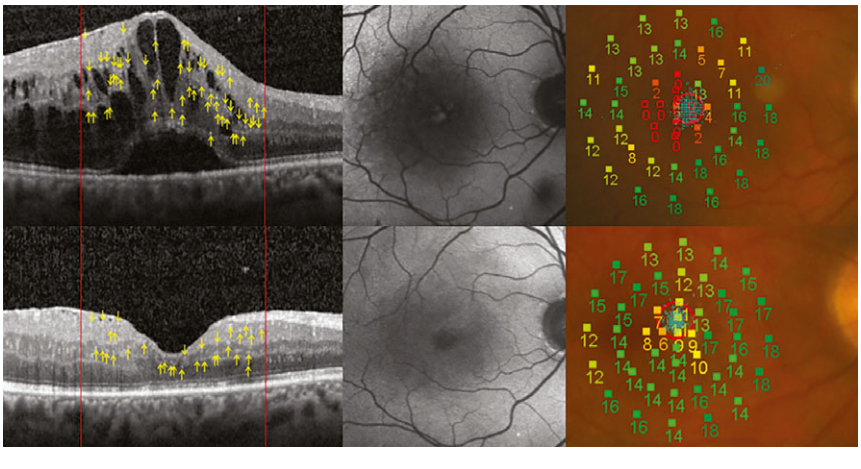

Fig. 1. Right eye of a patient with diabetic macular oedema and subfoveal neuroretinal detachment treated with intravitreal injection of dexamethasone. Upper row: spectral domain optical coherence tomography (SD OCT) image, fundus autofluorescence (FAF) and microperimetry before treatment; Lower row: SD OCT image, FAF and microperimetry 1 month after treatment. Red vertical lines on OCT image delimitate area within 3000 μm centred on the fovea where the count of total retinal hyper-reflective spots (HRS) was performed (yellow arrows). There is a decrease in HRS number before and after treatment. Fundus autofluorescence images show reduction in increased autofluorescence area in the fovea before and after treatment. Microperimetry maps show retinal sensitivity and fixation before and after treatment.

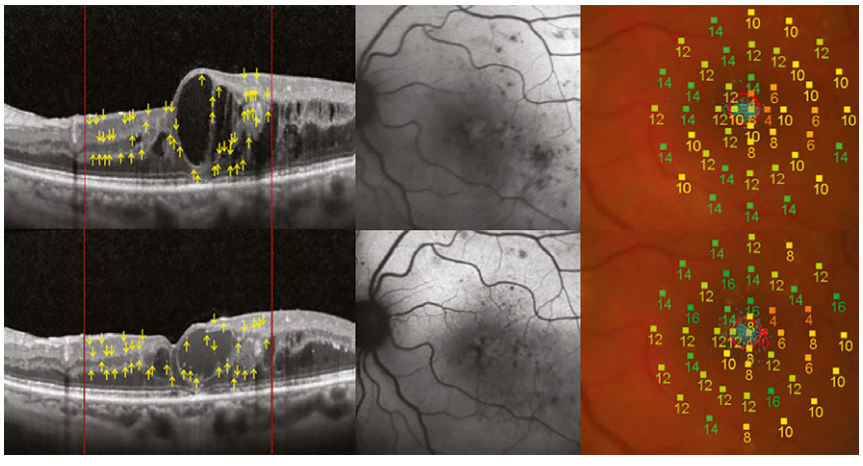

Fig. 2. Left eye of a patient with cystoid diabetic macular oedema treated with 3 monthly intravitreal injections of ranibizumab. Upper row: spectral domain optical coherence tomography SD OCT image, fundus autofluorescence (FAF) and microperimetry before treatment; Lower row: SD OCT image, FAF and microperimetry 1 month after treatment. Red vertical lines on OCT image delimitate area within 3000 μm centred on the fovea where the count of total retinal hyper-reflective spots (HRS) was performed (yellow arrows). There is a decrease in HRS number before and after treatment. Fundus autofluorescence images show reduction in increased autofluorescence area in the fovea before and after treatment. Microperimetry maps show retinal sensitivity and fixation before and after treatment.

Microperimetry

Microperimetry was performed on all subjects using the MP1 Microperimeter (Nidek, Gamagori, Japan). Standard parameters were used for DME patients: a fixation target consisting of red ring, 1° in diameter; white, monochromatic background at four asb, stimulus size Goldman III, with 200 ms projection time; customized radial grid of 45 stimuli covering central 12° (centred onto the fovea), 1° apart (inner stimuli) and 2° apart (outer stimuli) (Vujosevic et al. 2016a). 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 min mesopic visual adaptation was allowed before starting the test. All subjects underwent microperimetry with dilated pupils. Mean retinal sensitivity was evaluated within central 4° and 12°, approximately covering 1-mm and 3-mm central retina area on OCT map (Vujosevic et al. 2006, 2011).

Spectral domain optical coherence tomography

SD OCT was performed with SPECTRALIS (Software 5.3.0.15; Heidelberg Engineering, Heidelberg, Germany) OCT, with following scan patterns: one linear scan of 8.8 mm at 0° centred on the fovea in High Speed mode with a resolution of 100 automatic real-time (ART) module and macular map 6 × 6 mm with resolution of 50 ART centred on the fovea. Pre- and posttreatment measurements were made through HEIDELBERG EYE EXPLORER software (HEYEX™ Heidelberg Engineering), considering CMT, count of total retinal HRS (calculated in the area of 3000 μm centred on the fovea) and presence of SND.

Fundus autofluorescence

Fundus autofluorescence of the macula was recorded with a confocal scanning laser ophthalmoscope (Heidelberg Retinal Angiograph 2; Heidelberg Engineering), using blue wavelength (solid-pumped laser; 488 nm) for excitation, whereas emitted light was detected above 500 nm because of a barrier filter. Decreased foveal autofluorescence (due to the blockage caused by luteal pigment) was considered normal. The area of increased autofluorescence in the fovea (IFAF) was evaluated before and after treatment. The area with IFAF was manually outlined with the mouse-driven arrow on the computer screen, using the scanning laser ophthalmoscope software (HEIDELBERG EYE EXPLORER software (HEYEX™; HRA 2; Heidelberg Engineering)). All values were automatically calculated and reported in square millimetres.

Fluorescein angiography

Fluorescein angiography was performed at baseline (before treatment) in all patients using the same instrument as for FAF (Heidelberg Retinal Angiograph 2; Heidelberg Engineering). Fluorescein angiography images were evaluated for the presence of significant retinal capillary dropout in the macula.

Statistical analysis

Comparison of characteristics between two treatment groups (intravitreal dexamethasone or intravitreal ranibizumab) at baseline was performed by Student's *t*-test. The comparison of changes between two treatment groups was performed by Wilcoxon Mann–Whitney test. The comparison of changes within a single treatment group was performed by Wilcoxon signed-rank test (for quantitative variables: BCVA, CMT, number of HRS and dimension of IFAF area). The percentage of presence of SND (qualitative parameter) before treatment in two treatment groups was compared by Fisher's exact test. The resolution of SND after treatment was compared by Fisher's (between treatment group)/McNemar exact test (for within treatment group). Comparison between BCVA, retinal sensitivity (RS) (either within 4 and 12 central degrees) and CMT variation in relation to presence of SND in two treatment groups was evaluated by Student's *t*-test. Pearson's correlation coefficient was used to evaluate correlation between change in some functional and morphological parameters (e.g. BCVA, RS and CMT) and specific baseline parameters (HRS and IFAF). Correlation coefficient >0.2 was considered as clinically significant. All analyses were performed using SAS 9.3 statistical software (SAS Institute, Cary, NC, USA) on a personal computer. The results of the statistical tests were considered significant when $p < 0.05$.

Results

A total of 49 eyes of 49 patients were evaluated: 23 patients were treated

Table 1. Descriptive characteristics of patients in two treatment groups.

Characteristic	All patients	Dexamethasone	Ranibizumab	Difference (significance), p
Gender, M/F	30/19 (61.2%/38.8%)	15/8 (65.2%/34.8%)	15/11 (57.7%/42.3%)	0.589
Age, yrs	66.0 ± 7.0 (43–77)	66.9 ± 6.5 (55–76)	65.2 ± 7.6 (43–77)	0.410
Diabetes type, DM2/DM1	42/7 (85.7%/14.3%)	20/3 (87.0%/13.0%)	22/4 (84.6%/15.4%)	0.815
Diabetes duration, yrs	14.9 ± 10.4 (1–47)	15.7 ± 9.7 (2–40)	14.1 ± 11.1 (1–47)	0.601
HbA1c, mmol/mol	57.3 ± 9.2 (38–82)	56.4 ± 7.4 (42–68)	58.2 ± 10.6 (38–82)	0.508
Arterial hypertension, presence	32 (69.6%)	17 (73.9%)	15 (57.7%)	0.225

M = men, F = female; yrs = years, DM2 = diabetes mellitus type 2, DM1 = diabetes mellitus type 1, HbA1c = glycated haemoglobin, IVDEX = intravitreal dexamethasone treatment group, IVR = intravitreal ranibizumab treatment group.

with intravitreal dexamethasone (dexamethasone) and 26 with intravitreal ranibizumab (ranibizumab). Before treatment, no significant difference was observed in mean age, duration and type of diabetes, level of haemoglobin A1c and presence of associated arterial hypertension between the two treatment groups (Table 1).

Mean BCVA before treatment was 50.8 ± 14.9 letters in dexamethasone group and 57.6 ± 10.2 letters in ranibizumab group, ($p = 0.1558$). Best-corrected visual acuity significantly increased after treatment in both groups: 6.61 ± 8.53 letters, ($p < 0.0001$) in dexamethasone group, and 6.4 ± 7.4 letters, ($p < 0.0001$) in ranibizumab group. There was no significant difference between the two treatment groups as BCVA is concerned, ($p = 0.6224$; Table 2). Before treatment, mean retinal sensitivity within 4° was 5.9 ± 4.1 dB in dexamethasone group and 7.1 ± 3.0 dB in ranibizumab group, ($p = 0.4867$). Mean retinal sensitivity within 12° was 9.4 ± 3.9 dB in dexamethasone group and

Table 2. Morphologic and functional parameters at baseline and after treatment.

	Ranibizumab (N = 26)			Dexamethasone (N = 23)			
	Mean	SD	Range	Mean	SD	Range	p-value†
ETDRS							
Baseline	57.6	10.2	35–72	50.8	14.9	35–78	0.1558
Post-treatment	64.0	8.7	41–85	57.4	15.7	30–80	
Absolute change	6.4	7.4	−3 to 28	6.6	8.5	−5 to 41	0.6224
p-value	<0.0001*			<0.0001*			
S4 (dB)							
Baseline	7.08	2.98	2.90–12.20	5.94	4.12	0.90–13.20	0.4867
Post-treatment	7.35	3.79	1.80–14.00	6.08	3.74	0.40–11.20	
Absolute change	0.27	2.63	−2.90 to 5.80	0.15	3.14	−2.00 to 9.20	0.5819
p-value	1.0*			0.2266*			
S12 (dB)							
Baseline	10.99	2.85	7.20–14.80	9.41	3.95	2.80–15.00	0.5050
Post-treatment	10.42	3.51	3.80–16.70	9.64	4.38	1.30–13.80	
Absolute change	−0.58	2.00	−3.60 to 2.40	0.23	1.90	−2.70–3.50	0.4868
p-value	1.0*			1.0*			
CMT (μm)							
Baseline	518.0	113.0	321–745	594.6	182.5	292–979	0.1549
Post-treatment	354.8	135.7	201–586	301.0	87.1	160–468	
Absolute change	−163.1	113.4	−438 to 69	−293.6	206.5	−777 to −56	0.0320
p-value	<0.0001*			<0.0001*			
HRS (number)							
Baseline	80.6	18.2	34–125	101.3	16.4	77–134	0.0003
Post-treatment	52.5	14.1	31–100	68.8	10.4	45–91	
Absolute change	−28.0	16.4	−72 to 1	−32.4	12.1	−52 to −6	0.1353
p-value	<0.0001*			<0.0001*			
IFAF (mm^2)							
Baseline	0.42	0.19	0.09–0.82	0.54	0.41	0.06–1.79	0.5544
Post-treatment	0.22	0.16	0.00–0.60	0.27	0.27	0.00–0.98	
Absolute change	−0.20	0.11	−0.44 to −0.03	−0.27	0.23	−0.81 to −0.05	0.6094
p-value	<0.0001*			<0.0001*			

N = number, ETDRS = Early Treatment Diabetic Retinopathy Study letter score, S4 = retinal sensitivity within central 4° and expressed in decibel, S12 = sensitivity within central 12° and expressed in decibel, CMT = central macular thickness, HRS = hyper-reflective spots, IFAF = increased foveal autofluorescence expressed in mm2; SD = standard deviation.
*Wilcoxon matched-pairs signed-rank test, †Wilcoxon Mann–Whitney test.

Table 3. Serous neuroretinal detachment in diabetic macular oedema eyes: presence and resolution in treatment groups.

Treatment group	SND presence before treatment	SND presence after treatment			SND resolution after treatment
		SND present before treatment	SND absent before treatment	p-value†	
Dexamethasone	7/23 (30.4%)	1/7 (14.3%)	0/16 (0.0%)	0.0143	6/7 (85.7%)
Ranibizumab	10/26 (38.5%)	5/10 (50.0%)	0/16 (0.0%)	0.0253	5/10 (50.0%)
p-value*	0.7643				0.3043

SND = serous neuroretinal detachment.
*Fisher's exact test, †McNemar's test.

11.0 ± 2.9 dB in ranibizumab group, (p = 0.5050). After treatment, there was no significant difference in retinal sensitivity change between the two treatment groups, (p = 0.5819 for retinal sensitivity change within 4° and p = 0.4868 for retinal sensitivity change within 12°; Table 2). Mean CMT before treatment was 594.6 ± 182.5 μm in dexamethasone group and 518.0 ± 113.0 μm in ranibizumab group, (p = 0.1549). Central macular thickness significantly decreased after both treatments. Mean change was −293.6 ± 206.5 μm, (p < 0.0001) in dexamethasone eyes and −163.1 ± 113.4 μm, (p < 0.0001) in ranibizumab eyes. Decrease in CMT was significantly greater in eyes treated with dexamethasone versus ranibizumab, (p = 0.0320; Table 2). Mean total HRS number before treatment was 101.3 ± 16.4 in dexamethasone group and 80.6 ± 18.2 in ranibizumab group, (p = 0.0003). Hyper-reflective retinal spots number significantly decreased after both treatments: −32.4 ± 12.1 HRS in dexamethasone and −28.0 ± 16.4 HRS in ranibizumab group, (p < 0.0001 for both groups). Hyper-reflective retinal spots decrease was not different between the two treatment groups (p = 0.1353; Table 2). At baseline, mean IFAF area was 0.54 ± 0.41 mm^2 in dexamethasone, and 0.42 ± 0.19 mm^2 in ranibizumab group, (p = 0.5544). After both treatments, mean IFAF area significantly decreased, −0.27 ± 0.23 mm^2 in dexamethasone eyes and −0.20 ± 0.11 mm^2 in ranibizumab eyes, (p < 0.0001 for both groups). Mean IFAF decrease was not different between the two treatment groups, (p = 0.6094; Table 2). Fluorescein angiography showed no ischaemic DME in all cases at baseline.

Subfoveal neuroretinal detachment was present in 17 of 49 eyes (34.7%): seven (30.4%) in dexamethasone group and 10 (38.5%) in ranibizumab group, (p = 0.76). Subfoveal neuroretinal detachment resolved in 85.7% of eyes treated with dexamethasone, (p = 0.014, McNemar's test) and in 50% of eyes treated with ranibizumab, (p = 0.025, McNemar's test). Fisher's exact test (p = 0.3) showed no significant difference in SND resolution in two treatment groups, although there was a trend towards higher response in dexamethasone group (Table 3).

In eyes treated with dexamethasone, there was a clinically significant and positive correlation between HRS number at baseline and 4° RS (ρ = 0.46, Pearson's correlation) and 12° RS (ρ = 0.69, Pearson's correlation) variation; IFAF area at baseline and 4° RS change (ρ = 0.27, Pearson's correlation); a significant inverse correlation between HRS number at baseline and CMT change (ρ = −0.28); and a significant inverse correlation (p = 0.003) between presence of SND at baseline and CMT change (Fig. 3, Table 4). In eyes treated with ranibizumab, there was a significant inverse correlation between IFAF area at baseline and BCVA changes (ρ = −0.21) and SND presence/absence at baseline and increase in 4° RS (2.60 ± 2.25 dB versus −1.19 ± 1.63 dB, p = 0.005) and 12° RS (1.38 ± 0.87 dB versus −1.80 ± 1.41 dB, p < 0.001; Fig. 3, Table 4). Intragrader agreement showed excellent repeatability with intraclass correlation coefficient (ICC) at least 0.99 (95% Confidence Interval ranging from 0.98 to 0.99) for all evaluations.

Discussion

In this study, some early specific retinal morphologic and functional changes after intravitreal steroid and anti-VEGF treatment in naïve centre involving DME are reported. More specifically, HRS, area of IFAF, presence of SND and CMT as morphologic parameters, and BCVA and retinal sensitivity (determined by microperimetry) as functional parameters were evaluated. The goal was to analyse, non-invasively, in a clinical setting, the changes of specific parameters (biomarkers) *as possible indicators* of retinal inflammation in DME-treated eyes. Recent studies in humans, analysing samples of aqueous humour or vitreous, have shown that inflammation plays a crucial role in DR and DME (Funatsu et al. 2009; Funk et al. 2010; Sohn et al. 2011; Sonoda et al. 2014; Dong et al. 2015; Vujosevic et al. 2016b;). Higher levels of inflammatory factors (including ICAM-1, MCP-1, IL-6, IL-8, IP-10, IL-1β and VEGF) were detected in vitreous and aqueous humour of patients with DME, compared to non-diabetic patients and diabetics without DR, but also compared to patients with different degrees of DME (positive relationship between concentration of these cytokines and macular thickness) (Funatsu et al. 2009; Dong et al. 2015).

Moreover, specific inflammatory patterns (increased cytokines and chemokines) secreted by activated microglial cells were documented in early stages of DR (Madeira et al. 2015; Vujosevic et al. 2016b). Madeira et al. reported microglial activation in different retinal degenerative diseases including DR, contributing to chronic neuroinflammation, with release of proinflammatory mediators, increased oxidative and nitrosative stress (Madeira et al. 2015). Omri et al. demonstrated, in diabetic rats, an accumulation of activated microglial cells in the subretinal space due to the decrease in RPE pores. This is a consequence of blockage of normal process of cell trafficking between retina and choroid, with consequent further damage to the retina (Omri et al. 2011). Currently, there is an

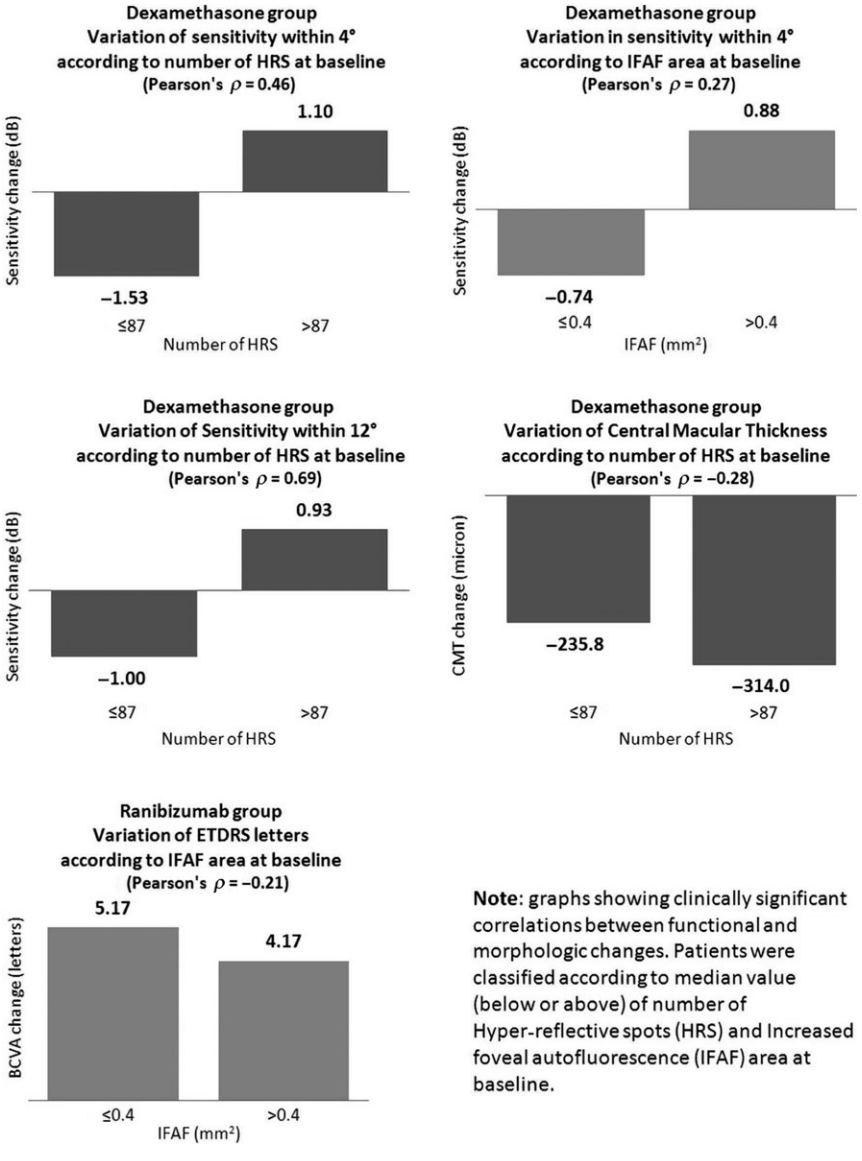

Fig. 3. Correlation between changes in functional and morphologic variables and the number of hyper-reflective spots, extension of IFAF area at baseline. HRS = hyper-reflective spots, IFAF = increased foveal autofluorescence, BCVA change = change in best-corrected visual acuity, dB = decibel, CMT = central macular thickness (μm), ρ = Pearson's correlation coefficient.

increasing interest in the evaluation of specific retinal parameters such as number of HRS, area of IFAF and presence of SND as possible, non-invasive, biomarkers of neuroretinal inflammation (Coscas et al. 2009, 2013; Vujosevic et al. 2011, 2013, 2016a; Framme et al. 2012; Sonoda et al. 2014; Madeira et al. 2015). In fact, Coscas et al. were the first to report the presence of HRS on SD OCT, as small in size, punctiform hyper-reflective elements, suggesting that they may represent aggregates of activated microglia cells (Coscas et al. 2009, 2013). Vujosevic et al. described HRS in the early stages of DR and also in diabetics without any clinical sign of DR, and hypothesized that HRS represent aggregates of microglial activated cells. They also highlighted how these spots were, at early stages, mainly located in the inner retinal layers (physiological location of resting resident retinal microglia) and then migrate to outer retina (Vujosevic et al. 2013). Moreover, HRS were evaluated in patients with DME, and specifically, their modification after anti-VEGF treatment (Framme et al. 2012; Vujosevic et al. 2016a). Framme et al. reported a significant reduction in HRS after anti-VEGF treatment, but only in cases with complete resolution of DME and no correlation to visual acuity outcome (Framme et al. 2012). Vujosevic et al. evaluated HRS changes in eyes treated with ranibizumab, reporting a significant and precocious reduction in HRS in all retinal layers (inner limiting membrane–inner plexiform layer, inner nuclear layer–outer plexiform layer and outer nuclear layer) in the perifoveal area (Vujosevic et al. 2016a). The number of counted HRS was inferior in the previous study compared to present data. This is due to evaluation of a smaller retinal area and also to the area located between 500 and 1500 μm and not involving the centre of the fovea. Notwithstanding, the decrease of HRS after anti-VEGF treatment is very similar in both studies (Vujosevic et al. 2016a). Moreover, in the present study, we report a significant reduction in HRS after both dexamethasone and ranibizumab treatment, thus an effect of both drugs on activated microglial cells. Whereas it is well known that steroids (and in particular intravitreal dexamethasone) are anti-inflammatory by definition (suppress leukostasis and BRB breakdown by inhibiting leucocyte recruitment in DR *in vivo*; block phospholipase A2; inhibit ICAM-1, IL-6, MCP-1, VEGF, SDF-1, CCR3 and TLRs3; stimulate adenosine and activate A1-receptor; decrease paracellular permeability; increase tight junction integrity) (Tamura et al. 2005; Uckermann et al. 2005; Nehmé & Edelman 2008; Augustin et al. 2010), even anti-VEGF agents have an anti-activated microglia action, in addition to anti-angiogenic one (Forstreuter et al. 2002; Ishida et al. 2003) ranibizumab blocks all active forms of VEGF-A, the most abundant and including more than one angiogenic potential in humans is VEGF165 form (Bouïs et al. 2006). It had been proved that VEGF164 (mouse isoform of human VEGF165) induces retinal ICAM-1 expression and leukostasis (Ishida et al. 2003). Moreover, it has also been demonstrated that VEGF induces chemotaxis and proliferation of microglial cells (Forstreuter et al. 2002). Therefore, both drugs significantly decrease HRS number, reducing microglia related retinal 'inflammatory' condition.

Fundus autofluorescence has been recently used for the evaluation of

Table 4. Correlation between changes in functional and morphologic variables and presence of SND at baseline.

	BCVA change	S4 change	S12 change	CMT change
Dexamethasone group				
SND				
No	6.56 ± 9.95	−0.96 ± 0.83	−0.34 ± 1.26	**−215.4 ± 150.8**
Yes	6.71 ± 4.39	3.10 ± 5.39	1.73 ± 2.80	**−472.4 ± 214.2**
p	0.970	0.321	0.110	**0.003**
Ranibizumab group				
SND				
No	4.00 ± 3.16	**−1.19 ± 1.63**	**−1.80 ± 1.41**	−183.8 ± 117.1
Yes	5.60 ± 5.44	**2.60 ± 2.25**	**1.38 ± 0.87**	−130.1 ± 104.3
p	0.372	**0.005**	**< 0.001**	0.248

SND = serous neuroretinal detachment, BCVA change = change in best-corrected visual acuity (Early Treatment Diabetic Retinopathy Study letter score), S4 change = change in retinal sensitivity within central 4° (decibel), S12 change = change in retinal sensitivity within central 12° (decibel), CMT change = change in central macular thickness (μm), clinically significant correlation has been reported in bold character. p = Student's *t*-test p-value.

DME (Pece et al. 2009; Vujosevic et al. 2011; Reznicek et al. 2013; Yoshitake et al. 2015). Vujosevic et al. previously documented IFAF in 77% of diabetic patients with clinically significant macular oedema. Areas of IFAF were associated with decreased RS determined with microperimetry indicating that DME with IFAF is, at least functionally, more severe than DME without IFAF (Vujosevic et al. 2011). It was hypothesized that IFAF in DME may relate to activated retinal microglial cells (Xu et al. 2008). Diabetes induces activation of microglial cells with oxidation of proteins and lipids, and thus, accumulation of oxidative products in the retina might be responsible for the increased FAF signal (Xu et al. 2008; Vujosevic et al. 2011). Thus, IFAF may be considered as an imaging biomarker of microglial activation in DME. In this study, areas of IFAF significantly decreased after both steroid and anti-VEGF treatment.

The pathophysiology of SND has been correlated to the condition of external limiting membrane (ELM), as the integrity of ELM seems to be a key factor to prevent fluid accumulation in the outer retina (Gaucher et al. 2008). A disruption of the ELM in eyes with DR can be accompanied by cell damage, and these damaged cells and debris are strong attractors of scavenger cells to the retina. These cells could be the source of IL-6 (Aloisi 2001; Sonoda et al. 2014). In fact, SND has been correlated with higher intravitreal levels of IL-6 (Sonoda et al. 2014). Seo et al. reported that ranibizumab is effective in the treatment of different types of DME (diffuse, cystoid and SND), but with poorer visual acuity outcome in SND type, closely related to ELM and ellipsoid zone integrity (Seo et al. 2016). We have documented a significant decrease in CMT and resolution of SND after both steroid and anti-VEGF treatment. Central macular thickness decrease was greater after steroid versus anti-VEGF treatment. This finding may merit further evaluation especially in cases with huge increase in retinal thickness, as a single treatment with intravitreal dexamethasone implant may give more rapid and significant decrease in retinal thickness than three ranibizumab injections. There was also a trend towards greater resolution of SND after steroid treatment versus anti-VEGF (86% versus 50%). This difference was not statistically significant. It may be due to low incidence (approximately 34%) of SND cases. No data are currently available on direct evaluation and comparison of SND resolution after steroid and anti-VEGF treatment. We compared a single injection of dexamethasone implant with a loading dose (three injections of ranibizumab), as the first-line treatment. When starting anti-VEGF treatment, at least a loading dose should be performed before evaluating any stable effect. Therefore, it is reasonable that these dosing regimens may be clinically comparable.

Functional outcomes (visual acuity and retinal sensitivity) were quite similar shortly after treatment in both groups. However, the correlation analysis showed that dexamethasone better improves retinal sensitivity if HRS are more numerous at baseline and the area of IFAF is larger and better decreases CMT if SND is present. On the contrary, ranibizumab increases visual acuity and retinal sensitivity in a more limited outcome in case of larger IFAF area at baseline. Moreover, ranibizumab better increases RS when SND is present, (even if both dexamethasone and ranibizumab increase RS in case of SND presence).

The major limits of this study are its retrospective nature (that might have biased the selection of the treatment eventually influencing the results) and the limited number of examined eyes, which could have influenced the lack of statistical significance of some results. A prospective, randomized study, with an increased sample size, could better define differences in specific morphological and functional response to the two types of treatment.

In conclusion, this study suggests that DME with high number of HRS and large area of IFAF shows better morphologic and functional results (in terms of retinal sensitivity) if, at least initially, treated with steroids versus anti-VEGF; DME with SND shows better morphologic results when treated with steroids versus anti-VEGF, whereas functional results are quite similar with both treatments. Moreover, the number of HRS decreased significantly after both treatments and correlate to functional data. Thus, the number of HRS may become a functional surrogate marker of DME. These data may be helpful to improve the characterization of imaging biomarkers in DME and to promote a more personalized treatment.

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