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Research Article

Study of nonperfusion area changes after ranibizumab intravitreal injection for diabetic macular edema by means of widefield OCT angiography

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Short Title: Retinal Ischemia after ranibizumab

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Abstract

Introduction: To evaluate changes of retinal capillary non-perfusion areas (RCNPA) and the retinal capillary vessel density (RCVD) of the superficial capillary plexus (SCP) and deep capillary plexus (DCP) using widefield optical coherence tomography angiography (WFOCTA) in patients with diabetic retinopathy (DR) and diabetic macular edema (DME) treated with intravitreal ranibizumab injection (IRI).

Materials and Methods: 24 eyes of 24 patients with DR and DME candidates to a loading dose of IRI were enrolled. All patients underwent WFOCTA with the PLEX Elite 9000 device with 15 × 9 mm scans centered on the foveal center at baseline (T0) and 1 month after each intravitreal injection at 30 days (T1), 60 days (T2), and 90 days (T3). In all patients, the variation of RCNPA and the RCVD of the of the SCP and DCP were calculated using automatic software written in Matlab (MathWorks, Natick, MA).

Results: The SCP showed a significant longitudinal variation of RCNPA ($p = 0.04$). Post-hoc analysis revealed a statistically significant reduction of RCNPA at T1 ($p = 0.04$) and a not significant reduction at T2 ($p=0.18$) and T3 ($p=0.96$). The DCP showed longitudinal changes of the RCNPA that tended to statistical significance ($p = 0.09$). Post-hoc analysis revealed a trend towards a statistically significant reduction of RCNPA at T3 ($p = 0.09$) not statistically significant, at T1 ($p=0.17$) and T2 ($p=0.75$). The RCVD of SCP and DCP showed no significant changes in any of the time points.

Conclusions: Widefield OCT angiography showed a decrease of RCNPA after IRI, probably related to the reperfusion of retinal capillaries.

Introduction

Diabetic retinopathy (DR) is still a cause of blindness in the working-age patients in developed countries [1]. DR is a microvascular disease characterized by increased vascular permeability, macular oedema, ischemia and retinal neovascularization (NV) [2]. The hypoxia resulting from the nonperfusion areas increases the expression of vascular endothelial growth factor (VEGF), that promotes retinal NV, edema and vessel abnormalities, leading to advanced stages of DR [3-9]. Conventional fluorescein angiography (FA) and widefield fluorescein angiography (WFFA) have shown high sensitivity and specificity for DR assessment, particularly for retinal ischemia detection, being the latter more accurate for the study of the far peripheral retina; nevertheless, both imaging techniques are invasive requiring dye injection [10]. The introduction of conventional optical coherence tomography angiography (OCTA) without dye injection, was very helpful for the diagnosis and monitoring of DR but it was limited to a small central retinal field [11,12]. Recently, the last generation of OCTA devices known as widefield OCTA (WFOCTA), allowed to explore also the mid-peripheral retina in a safe and not invasive modality, thus WFOCTA is now considered an important tool to investigate retinal vascular diseases such as DR in clinical settings [13]. Some authors reported the use of WFOCTA in DR for the investigation of retinal ischemia showing a higher detection rate of WFOCTA compared to ultrawidefield FA (UWF FA). A regression of retinal ischemia assessed by means of WFOCTA has also been demonstrated after intravitreal steroid implant for DME, whilst no reperfusion of retinal ischemia has been detected after anti-VEGF treatment [13]. The aim of this study was to evaluate changes in retinal capillary non-perfusion areas (RCNPA) and retinal capillary vessels density (RCVD) of the superficial capillary plexus (SCP) and deep capillary plexus (DCP) in patients with diabetic macular edema (DME) undergoing a loading dose of intravitreal ranibizumab injection (IRI).

Materials and Methods

Study participants

Twenty-four eyes of 24 patients with diabetic retinopathy classified according to the simplified version of the Early Treatment Diabetic Retinopathy Study classification of the American Academy of Ophthalmology Guidelines Committee, complicated by DME candidates to a loading dose of IRI were enrolled. The diagnosis of DR was made by means of fundus examination. In addition, FA and spectral OCT (SD-OCT) were performed in all cases. Criteria for inclusion were: (1) age >18 years old; (2) presence of treatment naïve center-involved DME; (3) central macular thickness (CMT) > 300 µm as

measured using SD-OCT at the baseline examination. The exclusion criteria were: (1) any previous ocular surgery (included intravitreal injections); (2) laser treatments; (3) history of glaucoma and ocular hypertension; (4) other retinal vascular diseases; (5) significant media opacities. The study adhered to the tenets of the Declaration of Helsinki and was approved by our Institutional Review Board (n. WRD/2020). Written informed consent was obtained from all participants of the study.

Study protocol

All patients underwent a complete ophthalmologic examination, including best-corrected visual acuity assessment, intraocular pressure measurement, slit-lamp biomicroscopy evaluation, and dilated fundus examination. Moreover, in all cases WFOCTA with the PLEX Elite 9000 device (Carl Zeiss Meditec Inc., Dublin, CA) with 15 × 9 mm scans centered on the foveal center was performed at baseline (T0) and 1 month after each intravitreal injection at 30 days (T1), 60 days (T2), and 90 days (T3). For each eye, WFOCTA volumes covering a 15x9 mm retinal area and centered at the fovea were acquired at each time point using a FastTrack motion correction software. Poor quality images showing a signal strength index lower than 8 and with relevant motion or tilt artifacts were excluded from the analysis and repeated. All participants' eyes were imaged three times each, and the best quality image was chosen to be investigated in the study. Severe tilt artifacts were excluded directly from the study by the two retinal specialists (LT and RDA) adjusting the system's working distance until good signal strength and good alignment between the beam pivot and pupil plane were observed throughout the B-scan. All selected images were carefully visualized by two retinal specialists in consensus to ascertain the correctness of segmentation and in case of erroneous recognition by the software of the position of the boundaries of the inner limiting membrane (ILM) and retinal pigment epithelium (RPE) manual correction was executed using the segmentation and propagation editing tool from the device. To identify and quantify in detail the main outcome measures all WFOCTA images (field of view of 9 mm × 15 mm, pixel resolution of 0.015 mm) were segmented at the SCP and DCP levels using automatic segmentation by PLEX Elite 9000 device to define the two capillary plexuses.

Semiautomated nonperfusion analysis

A custom-made semi-automatic software, written in Matlab (MathWorks, Natick, MA), was used to identify vessels within the WFOCTA images and to infer regions of ischemia. The algorithm proceeded in five steps as previously described [14]. In the first step each raw WFOCTA image was low-pass filtered based on a 2-D gaussian smoothing kernel with standard deviation of 5 mm (350 pixels) [15]. In the second step, the smoothed image was subtracted from the raw image to obtain a high-pass filtered WFOCTA where slow changing intensity, plausibly associated to the different sensitivity of the imaging technology within the field of view of the image, was automatically corrected [16]. In the third step, the processed image was normalized (between 0 and 1) and thresholded (above 0.5) to create a binary image that highlighted vessels (vessel image). In order to identify regions of ischemia, the variability in the contrast of the image where vessels are present was exploited [17]. In the fourth step a texture-based approach was utilized by computing the metric of entropy within small regions of 5 pixel x 5 pixel, i.e., 0.075 x 0.075 mm2. In the fifth and last step, the regions of retinal capillary non perfusion were identified using the entropy image, after being masked based on the vessel image, through a thresholding approach where pixels with entropy below 0.3 were deemed as possibly ischemic. In fact, the entropy metric, which measured the variability of the image intensity within the region, tended to be low in regions were a smooth intensity values were present because of vessels absence.

Clusters of pixels with low entropy (below 0.3) were finally identified as ischemic regions only if they covered an area above 20 pixel x 20 pixel, i.e. 0.3 x 0.3 mm2 [18]. The final outcome of the algorithm was carefully visualized and corrected (if needed) by the two independent and highly expert ophthalmologists.

Statistical analysis

Shapiro-Wilks test was performed to evaluate the departure from normal distribution. Variables were summarized as mean and standard deviation (SD). One-way ANOVA for repeated measures was performed to evaluate the effect of time and treatment during follow-up. Post-hoc analysis was performed using paired t-tests and multiple comparison correction using the False Discovery Rate (FDR) [19]. For all analyses, a $p<0.05$ was considered as statistically significant. Statistical analysis was performed using IBM SPSS Statistics v20.0 software (SPSS Inc. Chicago, Illinois, USA).

Main outcome measures

The main outcome measures examined in the study were the following:
1) RCNPA 1 month after each IRI at SCP and DCP level from WFOCTA scans;
2) RCVD changes 1 month after each IRI of SCP and DCP from WFOCTA scans.

Results

Among all scans acquired, 8 scans showed segmentation errors that were manually modified by the two independent retinal specialists as previously described in methods section. After segmentation correction they were used in step one. In contrast, 2 additional scans could not be used in step one because of the presence of significant image artifacts.
In all eyes retinal RCNPA at SCP and DCP and RCVD of SCP and DCP were calculated.
Data examined from SCP and DCP at the different times did not show significant departure from gaussianity (all p's>0.05).
The SCP showed a significant temporal variation of RCNPA ($p =0.04$). Post-hoc analysis revealed a statistically significant reduction in RCNPA at T1 ($p = 0.04$). The reduction of RCNPA compared to T0 was still present, although not statistically significant, at T2 ($p=0.18$) and T3 ($p=0.96$). In the SCP the average RCNPA was 14.82 ± 3.24 mm2 at T0, 11.27 ± 2.40 mm2 at T1, 11.87 ± 1.92 mm2 at T2 and 11.51 ± 2.58 mm2 at T3 (shown in Fig. 1–2). The DCP showed longitudinal changes of RCNPA that tended to statistical significance ($p = 0.09$). Post-hoc analysis revealed a trend towards a statistically significant reduction of RCNPA at T3 ($p = 0.09$). The reduction was also present, although not statistically significant, at T1 ($p=0.17$) and T2 ($p=0.75$). In DCP the mean and SD RCNPA was 15.80 ± 3.48 mm2 at T0, 11.38 ± 2.63 mm2 at T1, 11.82 ± 2.0 mm2 at T2 and 9.7 ± 2.27 mm2 at T3 (shown in Fig. 1–2). The RCVD of SCP and DCP showed no significant changes in any of the time points (shown in Fig. 3). The density of SCP and DCP showed no significant changes in any of the time points.

Discussion

In this study we investigated RCNPA changes and RCVD at SCP and DCP after IRI in patients with DR complicated by DME using a WFOCTA scan of 15 × 9 mm. The RCVD of SCP and DCP showed no significant changes in any of the time points. A decrease of RCNPA in SCP at T1, with a loss of effect at T2 and T3, was observed. We did not find significant changes of nonperfusion areas at DCP, although a decreasing trend of ischemic areas was detected, with changes up to 3% at T3. Previous studies investigated changes in retinal capillary perfusion density and retinal non perfusion areas after intravitreal therapy for DME both using intravitreal injection of anti-VEGF and dexamethasone implant [13,14,20,21]. In a study with data obtained using FA, Campochiaro et al. [20] showed that monthly injections of ranibizumab can slow, but not completely prevent, retinal capillary closure in patients with DME. Couturier et al. [13] reported no significant arterioles and venules reperfusion and no modification of nonperfusion retinal areas after 3 anti VEGF injections for DME using swept source (SS)-WF OCTA scans and UWF FA. The different outcomes concerning retinal reperfusion was probably related the image analysis modality being subjective in the Couturier et al. study and based on semiautomated algorithm in our study.
Querques et al. [21], in a pilot study, analyzed the modifications of retinal peripheral ischemic areas after dexamethasone intravitreal implant in patients affected by DME using ultra-widefield FA. Dexamethasone implant was effective in improving ischemic index in all patients and in decreasing breakdown of the blood–retinal barrier.

In a study on patients with diabetes complicated with diabetic macular edema, Toto et al. [14] evaluated the changes of retinal capillary nonperfusion areas and retinal capillary vessel density of the SCP and DCP treated with an intravitreal dexamethasone implant. They found a significant decrease in retinal nonperfusion capillary areas at 1 month after dexamethasone implant in the SCP, although no significant modification was found in the DCP. In the current study after a loading dose of IRI, a reduction of ischemic area in SCP was reported at T1, with a loss of effect at T2 and T3. No statistically significant reduction of ischemic areas in DCP after treatment was detected although a trend towards a statistically significant reduction was observed. It has been demonstrated that high levels of VEGF in the retina recruit leukocytes into the retinal vasculature, in part, through stimulation of VEGFR1 and promote endothelial cell adhesion and vessel plugging through activation of NF-κB and increased expression of adhesion molecules such as VCAM-1. Thus, high levels of VEGF cause closure of retinal vessels, which can be reopened by VEGF suppression with intraocular injections of a VEGF-neutralizing protein. Lack of intervention can cause permanent vessel closure with retinal nonperfusion [22-23]. The reduction of effect found at T2 and T3 could be correlated to two hypotheses one of them relate to the possibility that only some vessels of SCP and DCP have the potential to reperfuse after the first injection. Consequentially, the following injections do not obtain the same effect due to reduced amount of 'responsive' vessels. This mechanism could prevail over VEGF-suppression, which was observed to rise at an increasing number of injections. Mastropasqua et al. demonstrated a reduction of humor aqueous VEGF levels during a loading dose of five aflibercept intravitreal injections [24]. The second hypothesis is that a delayed intervention when permanent closure of vessels has occurred cannot reverse the process. Several previous studies reported that, in patients with DR, DCP is more compromised than SCP and is also the early target of vascular changes [23,25-28]. Thus we can hypothesize that loss of reperfusion is valid particularly for the DCP. In the current work we did not find significant modifications of vessel density both at the SCP and DCP. We can argue that the semiautomated algorithm is sensible in identifying ischemic areas that are of a certain dimension i.e. equal or greater than 0.075 x 0.075 mm2 and can fail in identifying reperfusion of small vessels. The study strength is the implementation of a semiautomated algorithm that quantifies nonperfusion areas. The application of the algorithm is indeed time saving compared to manual approaches [21]. Moreover, the algorithm makes a step toward the objectivation of OCTA image analysis allowing for robust comparison among different clinical studies. A limitation of the semiautomated approach is that large vessels (with a section >5 pixels) may provide regions of low entropy; however, the regions of large vessels can be easily masked based on the thresholded vessel image. The study promotes further trials to understand the relation between intravitreal treatment and modifications on retinal ischemic areas, with the aim to limit vascular damage in patients with DR.

Statement of Ethics

Study approval statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Ophthalmology Clinic, Department of Medicine and Science of Ageing, approval number DME/2020) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent to participate statement: Written informed consent was obtained from participants to partecipate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

The following statements should be used “Conceptualization, Lisa Toto and Rossella D’Aloisio; methodology, software and formal analysis, Antonello Maria Chiarelli; investigation, Daniele Libertini, Giada D’Onofrio, Chiara De Nicola, Emanuele Doronzo.; writing—original draft preparation, Lisa Toto, Antonello Maria Chiarelli, Rossella D’Aloisio; writing—review and editing, Rodolfo Mastropasqua; All authors have read and agreed to the published version of the manuscript.”

Data Availability Statement

Data supporting reported results can be provided upon request to: l.toto@unich.it

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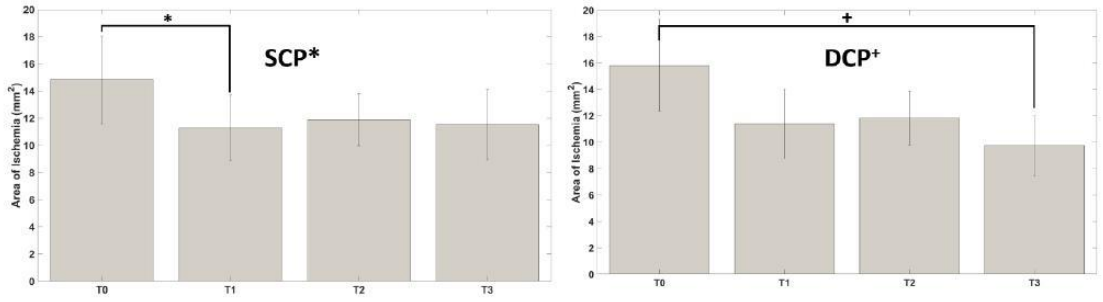
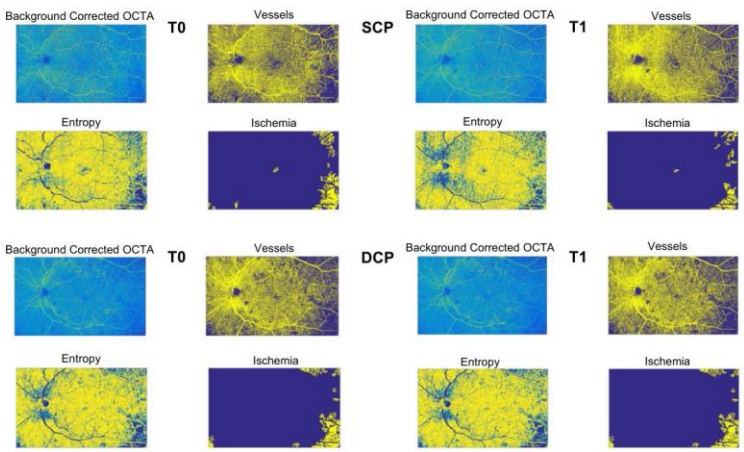
Figure Legends

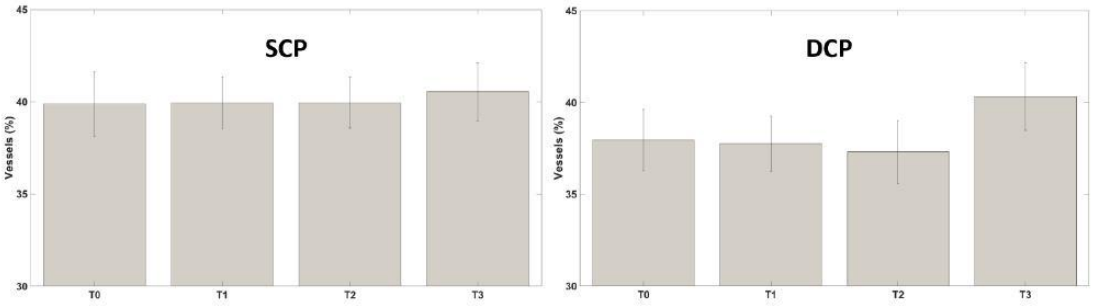
Fig. 1. Graphs showing retinal nonperfusion capillary areas (mm2) modification during follow-up of superficial capillary plexus (SCP) and of deep capillary plexus (DCP) (right and left). + $p<0.10$, *$p<0.05$ (longitudinal effects).

Fig. 2. Retinal nonperfusion capillary areas (mm^2) modification during follow-up of SCP and of DCP (right and left).

Fig. 3. Graphs showing retinal capillary vessel density (%) modification during follow-up of SCP and of DCP (right and left).


SCP*
Area of ischemia (mm²)
T0
T1
T2
T3
DCP+
Area of Ischemia (mm²)
T0
T1
T2
T3




SCP
DCP
Vessels (%)
45
40
35
30
T0
T1
T2
T3