FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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Peripapillary Neuro-Vascular Coupling in the Early Stages of Diabetic Retinopathy

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE OFTALMOLOGIA

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NOVEMBRO 2017

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Peripapillary Neuro-Vascular Coupling in the Early Stages of Diabetic Retinopathy

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Abstract

Purpose: To study how retinal nerve fiber layer (RNFL) thickness correlates with changes in radial peripapillary capillary (RPC) density in the early stages of Diabetic Retinopathy (DR), using optical coherence tomography angiography (OCT-A).

Methods and Materials: A cross-sectional evaluation of RNFL thickness and RPC density was performed with OCT-A (Avanti RTVue-XR 100, Optovue Inc, Fremont, CA). Parameters were calculated using the in-built software and a custom-developed macro. Both diabetic and control patients were included. Seven-field photographs of the fundus were taken for DR staging. Diabetic eyes were divided into two groups: diabetic patient without DR (No DR) and nonproliferative DR (NPDR). Univariate and multivariate linear regression models were used for analysis; multilevel mixed-effects models allowed to account for correlated outcomes.

Results: We included 136 eyes (n=43 control; n=25 noRD; n=68 NPDR) from 74 subjects (mean[SD] age 63.35[9.66] years; 53.68% female). When compared to controls, we found a significant decrease in RNFL thickness in the No DR (β=–8.01, P=0.003) and in the NPDR group (β=–6.29, P=0.008). We also observed a significant decrease in RPC density in both groups, even after adjusting for potential confounders (No DR: β=–1.97, P=0.049; NPDR: β=–2.55, P=0.048). Furthermore, in diabetic eyes, time since the diagnosis of diabetes was found to correlate negatively with RPC density (β=–0.07, P=0.037).

Conclusions: Our results suggest that peripapillary vascular changes occur prematurely in the course of DR. Whether the reduction in RPC density is a significant pathophysiological hallmark of DR progression warrants further research.

Keywords: Diabetic Retinopathy; Optic Disc; Radial Peripapillary Capillaries; Optical Coherence Tomography Angiography.

Resumo

Objetivo: Estudar a correlação entre espessura da camada de fibras nervosas (CFN) da retina e densidade capilar peripapilar radiária (CPR) nos estadios iniciais da Retinopatia Diabética (RD), utilizando a angiografia por tomografia de coerência ótica (OCT-A).

Métodos e materiais: Realizou-se uma avaliação transversal da espessura da CFN da retina e da densidade CPR com recurso a OCT-A (Avanti RTVue-XR 100, Optovue Inc, Fremont, CA). Os parâmetros foram calculados usando o software incluído no aparelho e uma macro customizada. Tanto doentes diabéticos como controlos saudáveis foram incluídos. As fotografias do fundo ocular de sete campos foram utilizadas para o estadiamento da RD. Os olhos dos indivíduos diabéticos foram divididos em dois grupos: diabéticos sem RD (sem RD) e diabéticos com RD não-proliferativa (RDNP). Para a análise estatística, usaram-se modelos de regressão linear univariada e multivariada, bem com modelos de efeitos mistos multinível.

Resultados: Foram incluídos 136 olhos (n = 43 controlos; n = 25 sem RD; n = 68 RDNP) de 74 indivíduos (idade média [DP] 63,35 [9,66] anos, 53,68% do sexo feminino). Comparativamente ao grupo controlo, verificou-se uma diminuição significativa na espessura da CFN, tanto no grupo "sem RD" (β = -8.01; P = 0,003) como no grupo "RDNP" (β = -6,29; P = 0,008). Também se observou uma diminuição significativa da densidade CPR em ambos os grupos, mesmo após o ajuste para possíveis variáveis confundidoras (sem DR: β = -1,97; P = 0,049 e NPDR: β = -2,55; P = 0,048). Além disso, nos olhos dos doentes diabéticos, o tempo após o diagnóstico da diabetes foi correlacionou-se negativamente com a densidade RPC (β = -0,07; P = 0,037).

Conclusões: Os nossos resultados sugerem que as alterações vasculares peripapilares ocorrem numa fase prematura do curso da RD. São necessários mais estudos para confirmar se a redução da densidade CPR constitui um biomarcador fisiopatológico significativo da progressão da RD.

Palavras-chave: Retinopatia diabética; Disco ótico; Capilares peripapilares radiários; Angiografia de Tomografia de Coerência Óptica.

Introduction

Data from the 2015 International Diabetes Federation Atlas report that diabetes mellitus (DM) affects 415 million people worldwide. [1] Fueled by the progressive ageing of the population, increased caloric consumption and sedentary lifestyle, [2] this number is expected to rise to 640 million by 2040, [1] turning diabetes in one of the largest global health issues of the 21st century.

Diabetic retinopathy (DR) is the most common microvascular complication of DM [3], causing blindness or severe visual impairment to approximately 9 million people worldwide [4]. With the rising number of diabetics and global ageing, the prevalence of vision-threatening DR is expected to double in the next decade [5].

The chronopathology of DR is slow and can be separated in four overlapping clinical stages: 1) retinal damage without any visible microvascular abnormalities in the fundus; 2) non-proliferative retinal microvascular changes (mild and moderate non-proliferative DR with or without diabetic macular edema (DME); 3) more advanced pre-proliferative changes (severe non-proliferative DR); and 4) Proliferative diabetic retinopathy (PDR) and advanced stages of DR. Clinically, the abnormalities seen in DR can conceptually be split into three categories: those resulting from leaking microvasculature (hemorrhages, lipid exudates, retinal edema); those resulting from structural damage to the microvasculature wall (microaneurysms); and those resulting from ischemia with subsequent overproduction of vascular growth factors (cotton-wool patches, intraretinal microvascular abnormalities, preretinal neovascularization, fibrous proliferation and vitreous hemorrhage). [6] These clinical signs of vascular dysfunction are paramount for the diagnosis, staging and management of DR. In fact, the most commonly used grading system in clinical and epidemiological studies of DR is the Early Treatment of Diabetic Retinopathy Study (ETDRS) scale [7], which relies upon a number of photographically detectable microvascular lesions as indicators of disease progression. For a long time, this limited the diagnostic and therapeutic focus to the vascular system. However, it is now widely accepted that DR involves both the neuronal and the vascular compartments[8] and a growing body of evidence suggests that the neuroretinal structure is compromised early in the course of the disease, perhaps even before overt vessel involvement (i.e., microaneurysms, hemorrhages and exudates) becomes apparent [9].

The axons from all the ganglion cells, which relay retinal information to the brain, travel in the retinal nerve fiber layer (RNFL) and converge to the optic disc. Therefore, the neurovascular coupling (i.e., the relationship between vascular and neuronal components) of the peripapillary region is particularly interesting in DR, as it may unveil changes early in disease progression. A recent large meta-analysis demonstrated that peripapillary RFNL thickness was significantly decreased in diabetic patients with no clinical signs of DR, when compared with healthy control patients [10]. Previous studies suggested that RNFL and ganglion cell layer (GLC) changes might correlate with the stage of diabetic polyneuropathy, rather than with the stage of DR [11, 12]. However, the putative contribution of early capillary compromise to these neuroretinal changes has not been addressed. Limitations of conventional clinical imaging tools, such as fluorescein angiography, have hampered the study of the capillaries, as these do not allow a clear visualization of small capillary networks. Since the recent advent of the optical coherence tomography angiography (OCT-A), precise measurements of the radial peripapillary capillary (RPC) density have become possible [13].

We hypothesize that peripapillary capillaries are compromised in the early stages of DR, most likely affecting the nourishment of the RNFL and, thus, its gross thickness. In the present study, we aim to assess how RNFL thickness correlates with the density of the superficial RPCs, across the early clinical stages of DR.

Methods and Materials

Study Design and Participants

This hospital-based, cross-sectional, prospective, observational cohort study was conducted at the Department of Ophthalmology of Centro Hospitalar e Universitário de Coimbra (CHUC). The research study adhered to the tenets of the Declaration of Helsinki. All participants provided oral and written informed consent.

From April 2017 to October 2017, consecutive patients visiting the Retina Clinic of CHUC were invited to participate. Included patients were older than 18 years old and diagnosed with either type 1 or type 2 DM, with or without DR in the early stages (up to ETDRS Level 53). Patients presenting with proliferative DR (ETDRS Level 61 or higher) or with clinically significant macular edema were excluded. Other exclusion criteria included refractive error ≥6 diopters of spherical equivalent; diagnosis of ocular hypertension or glaucoma; any previous intravitreal injection or eye surgery (except phacoemulsification for cataract extraction); any previous laser retina treatment; any other retinal or choroidal diseases (e.g., age-related macular degeneration, vitreoretinal traction, epiretinal membrane, macular hole or uveitis); any media opacity likely to jeopardize image acquisition.

Concomitantly, subjects without diabetes mellitus and with no diagnosed vitreoretinal disease were also invited to participate, and server as the control group. The same exclusion criteria applied.

Clinical Evaluation

Through a structured questionnaire and review of medical records, the following data were collected: age; gender; systemic and ophthalmologic comorbidities and treatments; current medication (including eye drops); and, for patients with diabetes mellitus, time since the diagnosis, type of diabetes and current medication for diabetes (including use or no use of insulin) were also collected. When a recent (< 3 months) glycosylated hemoglobin analysis was available, the value was also collected. Otherwise, blood was drawn for the glycosylated hemoglobin assay on the day of the clinical evaluation.

All included patients were submitted to a complete bilateral ophthalmologic examination, with an experienced ophthalmologist. Distance BCVA of study patients was determined using a modified ETDRS chart with Sloan Letters (Cat. No. L220, Lighthouse Enterprises, NY, USA). Current refraction was determined, intraocular pressure (IOP) was

measured with a Goldmann tonometer and anterior segment biomicroscopy along with dilated fundus examination were performed.

Diabetic Retinopathy Staging

For DR staging, seven standard 45º-field photographs of the eye fundus of diabetic patients were taken with a Nikon Digital SLR Camera D7000 (Nikon Corporation, Japan) mounted on a TRC-NW7SF Mark II Retinal Camera (TopCon Corporation, Japan) by orthoptic technicians certified for this technique. DR staging was performed according to the Modified Airlie-House Classification [14], by a certified grader by the Coimbra Ophthalmology Reading Center (CORC). The grader was blinded to every study variable.

Optical Coherence Tomography (OCT) Circumpapillary Retinal Nerve Fiber Layer Analysis

Optical coherence tomography RNFL thickness analysis (Avanti RTVue-XR 100, Optovue Inc, Fremont, CA, USA) was performed prior to OCT-A. Average RFNL thickness was used for comparison with OCT-A data. Images with inadequate signal strength (<6/10) were excluded from the analysis.

Optical Coherence Tomography Angiography (OCT-A) Image Acquisition

Optical coherence tomography angiography (OCT-A) images were obtained with a commercial spectral-domain OCT system (Avanti RTVue-XR 100, Optovue Inc, Fremont, CA, USA), as previously described [15]. Each patient underwent a single imaging session consisting of 4.5 x 4.5-mm-diameter peripapillary scans (4.5-mm scan). The Motion Correction Technology (MCT) of Optovue software was used to compensate for motion artifacts [16]. Images with inadequate signal strength due to significant motion artifact or low signal strength (<6/10) were excluded from the analysis.

A split-spectrum amplitude-decorrelation angiography (SSADA) algorithm was used to identify perfused vessels, including larger retinal vessels and the surrounding capillary network, for each scan. The specifics of similar algorithms have been previously published [17]. Briefly, the algorithm distinguishes the movement of red blood cells within the lumen of retinal and choroidal vessels between cross-sectional scans. The decorrelation algorithm identifies perfused retinal vessels from surrounding static tissue on the basis of signal amplitude variation differences in non-static versus static tissue. Vessels with slow or no flow cannot be visualized with this technique.

OCT-A Image Analysis

In this study, we analyzed the perfused peripapillary vasculature, which includes the larger blood vessels as well as the radial peripapillary capillaries (RPC). For RPC analysis, the Optovue in-built software (AngioVue version 2017.1.0.116, Optovue Inc, Fremont, CA, USA) automatically segments the internal limiting membrane (ILM) and the posterior boundary of the RNFL and performs a z-projection of the maximum decorrelation value to generate an en face OCT-A image (henceforth, referred to as "RNFL slab") (Figure 1B). The OCT-A images of all patients were reviewed to ensure proper segmentation of OCT scans. Furthermore, a superficial OCT-A slab (50 μm deep from ILM) was customized on AngioVue in order to isolate only the most superficial layer of RPCs (henceforth, referred to as "superficial slab") (Figure 1A).

The in-built software (IBS) provides a quantification of vessel density in the pre-specified z-projection, both in the circumpapillary region and in the whole scan. However, this analysis does not exclude the larger blood vessels and, thus, any detected differences cannot be specifically ascribed to differences in RPC density. Thus, we developed a custom macro on Fiji (SciJava Consortium) [18] for this purpose, based on previously described methodologies [19]. Briefly, the capillaries are first blurred out, which allows to create a mask for the larger blood vessels (Figure 1C). This mask is then subtracted from the original OCT-A acquisition (Figure 1D) and, by default isomodes, a threshold for capillary detection is set (Figure 1E). To ensure that the same region of interest (ROI) was included in all OCT-A images, a fixed annular ROI defined by two concentric circles with 1.95-mm (inner) and 3.45-mm (outer) diameters (Figure 1F) was manually centered at the optic nerve head using the choroid slab and then transposed to both the RNFL and the superficial slabs. This 3.45-mm outer circle diameter represents the standard circumpapillary scan dimension for RNFL thickness measurement currently employed by the majority of commercially available OCT systems.

The annular RPC density for both slabs was computed as the number of pixels associated with perfused capillaries over the number of pixels in the annular ROI after removal of major blood vessels. The global RPC density was calculated as the percentage of pixels associated with perfused capillaries in the entire scan over the number of pixels in the entire image after removal of the inner 1-95-mm circular area and of the major blood vessels.

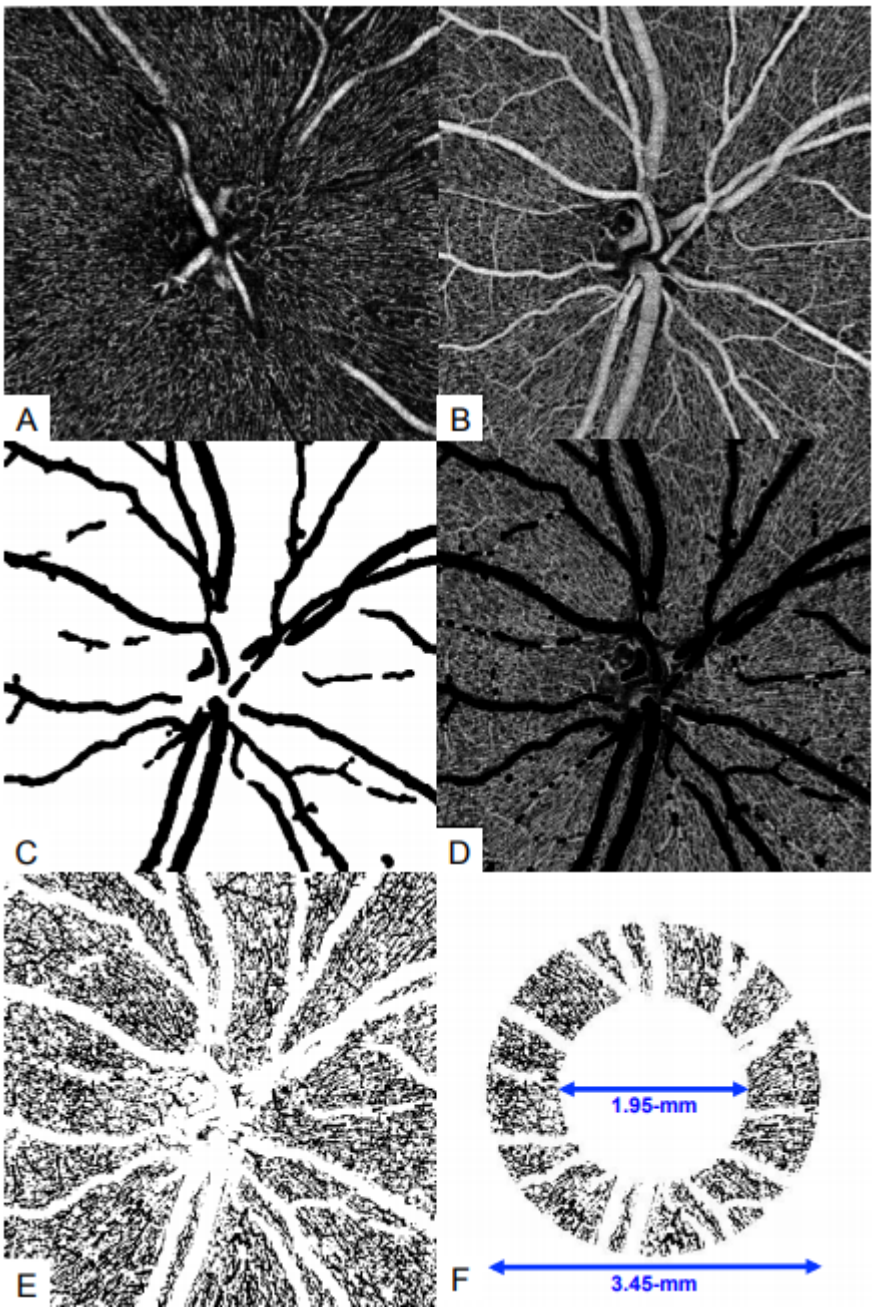

Figure 1. OCT-A image processing steps. (A) Custom superficial OCT-A slab (50 μm deep from ILM) to isolate only the most superficial later of RPCs. (B) Pre-specified OCT-A slab (from the ILM to the posterior boundary of the RNFL) segmented by the in-built software for RPC analysis. (C) Larger blood vessels mask created after the small capillaries are blurred out. (D) The mask in (C) was subtracted from the original OCT-A slab in (B). (E) By default isomodes, the threshold for capillary (in black) detection is set. (F) A fixed annular ROI defined by two concentric circles with 1.95-mm (inner) and 3.45-mm (outer) diameters is manually centered at the optic nerve head.

Statistical Analysis

The study population demographics, clinical and structural characteristics were summarized using traditional descriptive methods.

To evaluate the fitness of the custom-developed analysis (CDA) for RPC density, we assessed how well its output values correlated with the output from the Optovue in-built software (IBS), using univariate linear regression models.

Since we included both eyes of the same patient (unless one of the eyes met the exclusion criteria), we used multilevel mixed-effect linear models to evaluate the influence of DR on RPC density. These models are appropriate for research designs with nested data. In this context, the units of analysis considered were the eyes (at a lower level), which are nested within patients (at a higher level). Given the aims of the current work, the analysis was centered in two outcomes – RPC density and RNFL thickness. First, univariate analyses were performed for all potential confounders (i.e., age and refractive error) and then all variables with a $P<0.05$ were included in the multivariate models. Furthermore, using the same statistical approach, we evaluated the influence of diabetes-specific predictors (i.e., HbA1c level and time since diagnosis) on RPC density in eyes of diabetic patients alone (excluding the control group).

All statistics were performed on STATA (version 14.2, StataCorp LCC, College Station, TX, USA). $P<0.05$ were considered statistically significant.

Results

Patients' Demographic and Clinical Characteristics

We analyzed and imaged a total of 148 eyes of 74 patients. Figure 2 presents the flowchart of the study and details the reasons for exclusion. A total of 136 eyes were considered for the analysis. Henceforth, all results presented pertain only to the eyes included in the final study sample (43 control eyes from 24 subjects and 93 diabetic eyes from 60 patients).

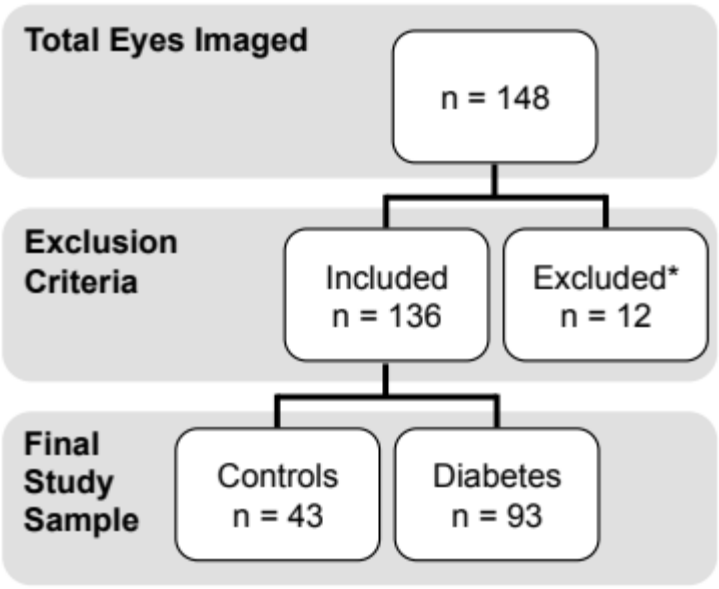

Figure 2. Flowchart of the study. *Exclusion reasons were epiretinal membrane (n=2), branch retinal artery occlusion (n=1), central retinal vein occlusion (n=1), branch retinal vein occlusion (n=1), diabetic macular edema (n=2), proliferative diabetic retinopathy (n=2) and poor quality scan (n=3).

Of the 93 eyes from diabetic patients, 25 had no signs of DR (No DR), while the remaining 68 eyes were classified as nonproliferative DR (NPDR), in various stages. The distribution of the included diabetic eyes according to ETDRS staging is depicted in Figure 3.

For the remainder of the analysis, eyes from diabetic patients were divided into two groups (No DR and NPDR). Demographic and clinical characteristics from these two groups and from the control group are summarized in Table 1.

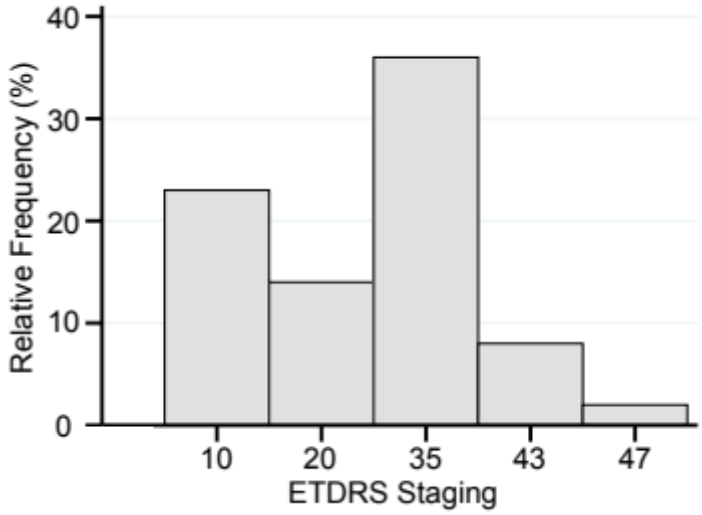

Figure 3. ETDRS staging of diabetic study eyes. Histogram of relative frequency depicting the distribution of the diabetic study eyes (n=93) by DR severity, according to the modified Airlie-House ETDRS grading scheme. For simplicity, final grades (i.e., 35A, 35B, 35C, etc) are represented within the respective umbrella grade (i.e., 35).

Table 1. Demographic and Clinical Characteristics of the Eyes Included for Analysis (n = 136) by Study Group.

	Control (n=43)	No DR (n=25)	NPDR (n=68)	Total (n=136)
Age, years (mean ± SD)	59.42 ± 8.36	67.48 ± 11.11	64.31 ± 9.10	63.35 ± 9.66
Gender, male (n, %)	8 (18.60)	11 (44.00)	44 (64.71)	63 (46.32)
Hypertension, prevalence (%)	60.00	72.00	97.06	86.11
Time with DM, years (mean ± SD)	NA	17.04 ± 8.38	19.79 ± 9.15	19.95 ± 8.99
HbA1C, % (mean ± SD)	NA	6.34 ± 0.48	7.52 ± 1.42	7.20 ± 1.35
Insulin, prevalence (%)	NA	12.00	55.88	44.09
BCVA, logMAR (mean ± SD)	0.10 ± 0.17	0.06 ± 0.10	0.06 ± 0.11	0.06 ± 0.12
IOP, mmHg (mean ± SD)	14.69 ± 1.93	14.68 ± 2.08	14.80 ± 2.88	14.75 ± 2.59
SE, Diopters (mean ± SD)	0.53 ± 0.89	– 0.28 ± 2.34	0.53 ± 1.08	0.33 ± 1.49

BCVA. Best-Corrected Visual Acuity. DM. Diabetes Mellitus. DR. Diabetic Retinopathy. HbA1c. glycosylated hemoglobin. IOP. Intraocular Pressure. logMAR. Logarithm of the Minimum Angle of Resolution. NA. Not Applicable. NPDR. Non-Proliferative Diabetic Retinopathy. SD. Standard Deviation. SE. Spherical Equivalent.

Annular and Global RPC Density Measurements

The mean values of RNFL thickness and of both annular and global RPC densities, calculated for both the RNFL and the superficial slabs, are presented in Table 2. For the RNFL slab, RPC density values from the IBS are also presented.

Crude analysis of the values in Table 2 suggests that diabetic eyes (both in the No DR and NPDR groups) present lower RNFL thickness and RPC density values (when measured in the RNFL slab) when compared to controls. The difference in RPC density on the superficial slab is less stark.

Table 2. Demographic and Clinical Characteristics of the Eyes Included for Analysis (n = 136), Organized by Study Group.

	RNFL Slab				Superficial Slab		
	Annular ROI Density (%)		Global ROI Density (%)		Annular ROI Density (%)	Global ROI Density (%)	RNFL Thickness (μm)
	CDA	BIS	CDA	BIS			
Control	32.26 ± 2.43	53.17 ± 2.61	30.01 ± 1.92	50.38 ± 2.14	26.81 ± 1.81	25.67 ± 1.44	99.60 ± 9.05
No DR	28.79 ± 3.20	48.88 ± 6.13	27.35 ± 2.35	46.72 ± 4.88	26.09 ± 3.04	25.31 ± 2.54	90.00 ± 12.96
NPDR	29.77 ± 2.72	49.73 ± 3.54	27.70 ± 2.31	47.45 ± 3.01	25.73 ± 2.34	24.69 ± 2.31	94.01 ± 8.37

CDA. Custom-Developed Analysis. BIS. Built-In Software. DR. Diabetic Retinopathy. RNFL. Retinal Nerve Fiber Layer. ROI. Region of Interest. NPDR. Non-Proliferative Diabetic Retinopathy

Interestingly, the output values from the IBS on RPC density at the RNFL slab suggest the same trends, although these values are much higher than those ascertained by the custom-developed analysis (CDA). This was expected, since the latter excludes the high intensity pixels pertaining the larger blood vessels. RPC density measurements from the IBS and the CDA correlated well, both for the annular ($\beta = 0.56$, $P < 0.001$) and the global ROI ($\beta = 0.56$, $P < 0.001$) (Figure 4). However, there is relevant dispersion of individual data points around the predicted lines, for the analysis of both the annular ($R^2 = 0.61$) and global ROI ($R^2 = 0.63$). The reason why the values are not exactly equivalent probably relates to the fact that

the area occupied by the larger blood vessels varies between patients. Since the CDA computes the RPC density over the total number of pixels after exclusion of the larger blood vessels, it is able to overcome this limitation inherent to the IBS analysis. Furthermore, as stated before, our aim is to study capillary networks. Consequently, henceforth, only RPC values calculated by the CDA will be considered.

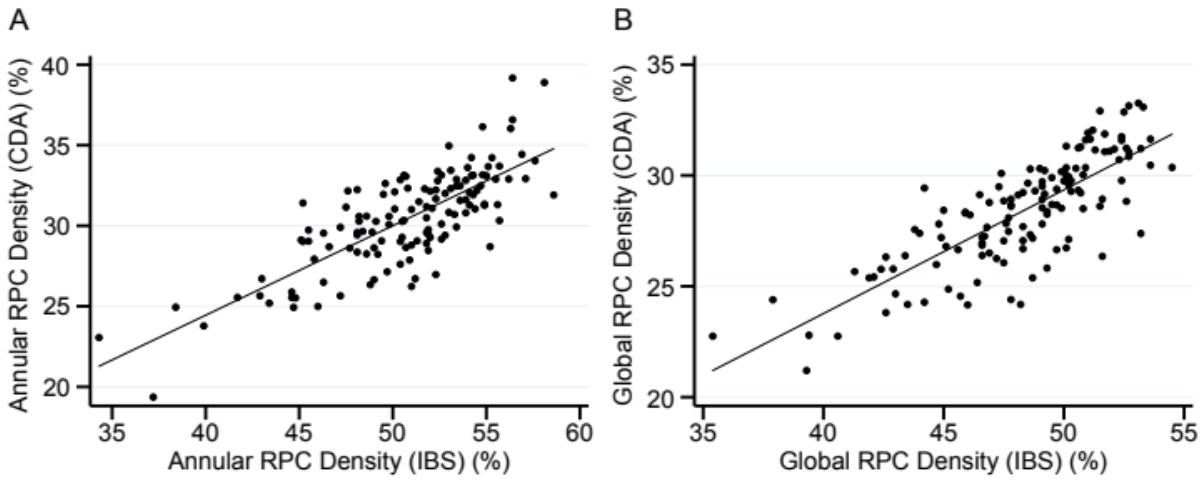

Figure 4. Agreement between the in-built software (IBS) and the custom-developed analysis (CDA) outputs on RPC density. Scatter plots with fitted lines demonstrate that the values from the CDA largely agree with the output values of the IBS, both for the annular (A) and global (B) RPC densities.

RNFL Thickness and RPC Density are Decreased in Diabetic Patients

On univariate analysis, advancing age ($\beta = 0.56$, $P < 0.001$) and diabetes, both in the absence ($\beta = -8.01$, $P = 0.003$) and in the presence ($\beta = -6.29$, $P = 0.008$) of clinical signs of DR, correlated with a significantly thinner RNFL, while hyperopic eyes showed a significantly thicker RNFL ($\beta = 1.41$, $P = 0.026$) (Table 3). In order to control for potential confounding between these factors, they were all included in the multivariate model, and NPDR eyes, compared with controls, still presented a statistically significant thinner RNFL, demonstrating that NPDR is an independent risk factor.

Table 3. Univariate and Multivariate Mixed-Effects Multilevel Assessment of Factors Associated with RNFL Thickness.

	Univariate Analysis[1]		Multivariate Analysis[2]	
	β (95% CI)	P	β (95% CI)	P
Age	–0.35 (–0.57, –0.13)	0.002	–0.09 (–0.15, –0.03)	0.003
Female Gender	0.92 (–3.53, 5.37)	0.685		
Hypertension	0.45 (–6.50, 7.40)	0.899		
Study Group				
No DR	–8.01 (–13.34, –2.67)	0.003	–0.80 (–2.61, 1.01)	0.386
NPDR	–6.29 (–10.92, –1.67)	0.008	–1.70 (–3.33, –0.06)	0.042
BCVA	–9.32 (–22.84, 4.19)	0.176		
IOP	–0.31 (–0.89, 0.27)	0.298		
SE	1.41 (0.17, 2.65)	0.026	0.24 (–0.10, 0.59)	0.171

β. Regression Coefficient. BCVA. Best-Corrected Visual Acuity. DR. Diabetic Retinopathy. IOP. Intraocular Pressure. NPDR. Non-Proliferative Diabetic Retinopathy. SE. Spherical Equivalent.

[1] In the mixed-effects multilevel univariate linear model, the demographic/clinical variables were analyzed, including one covariate at a time.

[2] In the mixed-effects multilevel multivariate linear model, only the significant ($P<0.05$) variables from the univariate model were included.

The annular RPC density in the RNFL slab should correlate best with findings on RNFL thickness. Accordingly, the factors previously shown to associate with RNFL thickness were also found to be significantly associated with changes in annular RPC density (Table 4). Namely, both No DR and NPDR eyes, compared to controls, demonstrated significantly reduced annular RPC densities (No DR: $\beta = –3.33$, $P < 0.001$; NPDR: $\beta = –2.55$, $P < 0.001$), even after adjustment for potential confounders (No DR: $\beta = –1.97$, $P = 0.049$; NPDR: $\beta = –2.55$, $P = 0.048$).

Table 4. Univariate and Multivariate Mixed-Effects Multilevel Assessment of Factors Associated with Annular RPC Density.

	Univariate Analysis[1]		Multivariate Analysis[2]		
	β (95% CI)	P	β (95% CI)		P
Age	–0.10 (–0.16, –0.04)	0.001	–0.08	(–0.15, –0.02)	0.012
Female Gender	0.92 (–0.27, 2.11)	0.129			
Hypertension	–0.76 (–2.52, 0.99)	0.394			
Study Group					
No DR	–3.33 (–4.80, –1.86)	<0.001	–1.97	(–3.93, –0.01)	0.049
NPDR	–2.55 (–3.71, –1.39)	<0.001	–2.55	(–3.38, 0.12)	0.048
BCVA	–0.54 (–5.50, 4.41)	0.830			
IOP	–0.12 (–0.35, 0.11)	0.301			
SE	0.43 (0.02, 0.84)	0.037	0.39	(0.01, 0.77)	0.045

β. Regression Coefficient. BCVA. Best-Corrected Visual Acuity. DR. Diabetic Retinopathy. IOP. Intraocular Pressure. NPDR. Non-Proliferative Diabetic Retinopathy. SE. Spherical Equivalent.

[1] In the mixed-effects multilevel univariate linear model, the demographic/clinical variables were analyzed, including one covariate at a time.

[2] In the mixed-effects multilevel multivariate linear model, only the significant (P<0.05) variables from the univariate model were included.

Regarding annular RPC density in the superficial slab, although a significant reduction was found on univariate analysis in the NPDR group (β = –1.15, P = 0.037), the significance level did not hold after multivariate adjustment (β = –0.24, P = 0.678).

Data presented above strongly suggests that both RNFL thickness and respective RPC density within the annular ROI are decreased in diabetic eyes, when compared to control eyes. However, it is unclear whether the profile of association between RNFL thickness and annular RPC density is changed in diabetic eyes. When we performed subgroup analyses of this association, we found that RNFL thickness was strongly associated with RPC density in No DR (β = 1.46, P = 0.002) and in NPDR eyes (β = 0.58, P = 0.013), but not in the control group (β = –0.31, P = 0.352) (Table 5). The significance level held for the NPDR group (β = 0.57, P = 0.016) and it was marginally significant for the No DR group (β = 0.99, P = 0.054), after adjusting for the previously identified potential confounders (age and spherical equivalent) (Table 5). This finding suggests that, in diabetic eyes, RNFL is more prone to thinning,

should the RPC density be decreased, while control eyes are able to preserve RNFL thickness, even for lower RPC density values (Figure 5).

Table 5. Univariate and Multivariate Mixed-Effects Multilevel Assessment of Association between RNFL thickness and Annular RPC density, by Study Group.

	Univariate Analysis[1]		Multivariate Analysis[2]	
	β (95% CI)	P	β (95% CI)	P
Control	–0.31 (–0.97, 0.35)	0.352	0.04 (–0.44, 0.52)	0.877
No DR	1.46 (0.52, 2.41)	0.002	0.99 (–0.02, 2.00)	0.054
NPDR	0.58 (0.12, 1.03)	0.013	0.57 (0.11, 1.03)	0.016

β. Regression Coefficient. BCVA. Best-Corrected Visual Acuity. DR. Diabetic Retinopathy. IOP. Intraocular Pressure. NPDR. Non-Proliferative Diabetic Retinopathy. SE. Spherical Equivalent.

[1] In the mixed-effects multilevel univariate linear model, only annular RPC density was included as predictor of RNFL thickness.

[2] In the mixed-effects multilevel multivariate linear model, age and spherical equivalent were also included as predictors.

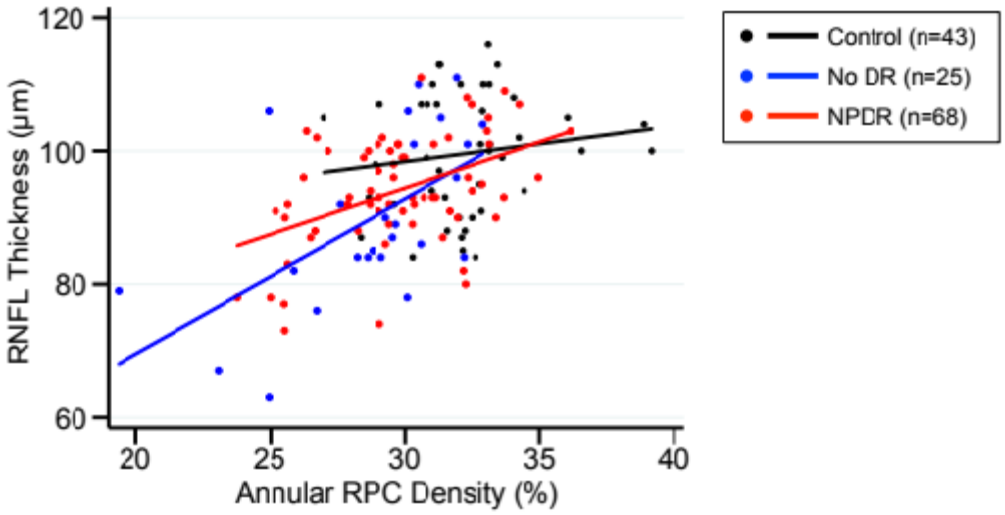

Figure 5. Association between RNFL thickness and Annular RPC Density, by Study Group. Scatter plots and fitted lines demonstrating the association between RNFL thickness and annular RPC density according to study group. As depicted, when reading the plots from right to left, decreasing values of RPC density do not significantly affect RFNL thickness in the control group (black dots and line). On the other hand, for both No DR and NPDR eyes, as RPC decreases, RNFL thickness is seen to decrease significantly.

Association of Diabetes-Specific Factors with RNFL Thickness and RPC Density

Given the aforementioned associations between diabetes and peripapillary neurovascular changes, we next performed analyses where only eyes of diabetic patients were considered, in order to assess whether diabetes-specific factors (e.g., time since diagnosis and HbA1c level) were independently associated with either RNFL thickness and/or annular RPC density.

Regarding RNFL thickness, none of the diabetes-specific factors were found to be significantly associated (Table 6). Advancing age and the spherical equivalent were shown to be associated with RNFL thickness, as previously demonstrated when controls were included in the analysis (Table 3).

Table 6. Univariate and Multivariate Mixed-Effects Multilevel Assessment of Factors Associated with RNFL Thickness in Diabetic Patients (n=93).

	Univariate Analysis[1]		Multivariate Analysis[2]	
	β (95% CI)	P	β (95% CI)	P
Age	–0.33 (–0.59, –0.07)	0.011	–0.36 (–0.62, –0.11)	0.005
Female Gender	–0.29 (–5.01, 5.59)	0.915		
Hypertension	3.80 (–4.81, 12.41)	0.387		
Study Group				
NPDR	1.70 (–2.15, 5.56)	0.387		
BCVA	–9.02 (–24.56, 6.53)	0.256		
IOP	–0.28 (–0.91, 0.34)	0.375		
SE	1.85 (0.50, 3.19)	0.007	2.02 (0.74, 3.30)	0.002
HbA1c	0.63 (–1.18, 2.44)	0.495		
Time with DM	–0.20 (–0.49, 0.09)	0.177		
Insulin	1.15 (–4.06, 6.36)	0.665		

β. Regression Coefficient. BCVA. Best-Corrected Visual Acuity. DM. Diabetes Mellitus. DR. Diabetic Retinopathy. HbA1c. glycosylated hemoglobin. IOP. Intraocular Pressure. NPDR. Non-Proliferative Diabetic Retinopathy. SE. Spherical Equivalent.

[1] In the mixed-effects multilevel univariate linear model, the demographic/clinical variables were analyzed, including one covariate at a time.
[2] In the mixed-effects multilevel multivariate linear model, only the significant (P<0.05) variables from the univariate model were included.

Regarding RPC density, we found that the number of years since the diagnosis of DM was significantly and independently associated with decreased annular RPC density in the RNFL slab (β = –0.09, P = 0.011), even after adjusting for patient age (β = –0.07, P = 0.037)

Table 7. Univariate and Multivariate Mixed-Effects Multilevel Assessment of Factors Associated with Annular RPC Density in Diabetic Patients (n=93).

	Univariate Analysis[1]		Multivariate Analysis[2]	
	β (95% CI)	P	β (95% CI)	P
Age	–0.08 (–0.14, –0.01)	0.018	–0.06 (–0.13, –0.003)	0.062
Female Gender	–0.04 (–1.41, –1.32)	0.952		
Hypertension	0.43 (–1.82, 2.67)	0.709		
Study Group				
NPDR	0.75 (–0.65, 2.15)	0.294		
BCVA	–1.99 (–7.90, 3.91)	0.508		
IOP	–0.14 (–0.38, –0.10)	0.248		
SE	0.36 (–0.06, 0.79)	0.095		
HbA1c	–0.06 (–0.55, 0.42)	0.794		
Time with DM	–0.09 (–0.16, –0.02)	0.011	–0.07 (–0.14, –0.005)	0.037
Insulin	0.62 (–0.72, 1.96)	0.365		

β. Regression Coefficient. BCVA. Best-Corrected Visual Acuity. DM. Diabetes Mellitus. DR. Diabetic Retinopathy. HbA1c. glycosylated hemoglobin. IOP. Intraocular Pressure. NPDR. Non-Proliferative Diabetic Retinopathy. SE. Spherical Equivalent.
[1] In the mixed-effects multilevel univariate linear model, the demographic/clinical variables were analyzed, including one covariate at a time.
[2] In the mixed-effects multilevel multivariate linear model, only the significant (P<0.05) variables from the univariate model were included.

Discussion

This study evaluated the peripapillary capillary network in the early stages of DR in an attempt to determine if there was evidence of compromise of RPCs and, in turn, if such compromise could underlie the previously identified subclinical structural neuroretinal damage of the RNFL. Firstly, we found that both peripapillary RNFL thickness and the density of RPCs within the RNFL layer were significantly decreased in diabetic eyes, even in those with no signs of DR. We further demonstrated that RNFL thickness and RPC density displayed an abnormal association profile, with RNFL thickness of diabetic eyes being less resistant to decreases in capillary density. Finally, we have ascertained that time since the diagnosis of diabetes is the only diabetes-specific predictor to independently correlate with RNFL thickness.

To our knowledge, this is the first study to evaluate the integrity of the peripapillary capillary network in DR. Previous studies have addressed changes in the area of the foveal avascular zone (FAZ) and in parafoveal capillary density [20-23], but changes in circumpapillary microvasculature have not been reported. Partially, this is because the study of peripapillary capillary networks has been hampered by methodological constraints, due to confounding by the larger blood vessels in the OCT-A image.

To overcome this issue, we have developed an innovative customized macro on an open-source platform (Fiji, SciJava Consortium) that is able to reliably exclude the larger blood vessels, and consider only the capillaries for analysis. The output values attained by our custom-developed algorithm are within the range of values reported by studies that also isolated capillary networks [13, 19]. Furthermore, even though the output values from the custom-developed analysis largely agrees with the output from the in-built software (AngioVue version 2017.1.0.116, Optovue Inc, Fremont, CA, USA) (Figure 4), the custom-developed macro allows to adjust for inter-patient variations in the area occupied by the larger blood vessels and, ultimately, allows to isolate the capillaries. Therefore, any detected changes between groups can be specifically ascribed to differences in capillary density, and potential confounding from larger blood vessels is eliminated.

On univariate analysis, we found that both diabetic eyes (with or without signs of DR) displayed decreases both in RNFL thickness and in capillary density (Tables 3 and 4). The decrease in RNFL thickness herein reported is consistent with the results from a large meta-analysis of 668 diabetic eyes without DR, where a pooled average reduction of 2.88 μm was found, when compared with healthy control eyes [10]. On multivariate analysis, while adjusting for other factors associated with the outcome measures of interest, we found that although

RPC density was decreased in diabetic eyes, RNFL thickness was significantly decreased only in the NPDR group. This result suggests that, in DR progression, decreases in RPC density may precede RNFL thinning, hinting that the former may be in the causal pathway of the latter. Furthermore, our data suggest that the circumpapillary RNFL of diabetic eyes is less robust to decreases in RPC density, since it seems to experience concomitant greater decreases, as compared to control eyes (Figure 5).

Overall, our data are highly suggestive that the neurovascular peripapillary coupling is compromised in the early clinical stages of DR, and even before the onset of clinically detectable vascular changes (preclinical DR). As stated in the Introduction section, the peripapillary region is of particular interest in vascular retinal diseases, since it may unveil changes already in the early stages of disease progression. Accordingly, despite the decreases in peripapillary capillaries here demonstrated, a previous report showed no qualitative or quantitative differences in parafoveal capillaries of diabetic patients without DR, when comparison to nondiabetic controls [22]. Therefore, our work also lays ground for the hypothesis that RPC density may be an early predictor of DR in diabetic patients and, perhaps, it may even have prognostic value. DR progression is protracted [24] and we are currently unable to identify the patients more likely to progress, which greatly hampers the feasibility of early intervention studies. Therefore, such an early predictor of DR damage would be of the utmost importance.

Previous work has reported consistent reductions in RPC density in eyes of both normal-tension and open-angle glaucoma patients [19,25,26]. Other studies have detailed peripapillary capillary involvement in high myopia [27], congenital disc anomalies [28], an even in neurodegenerative diseases, such as multiple sclerosis [29]. The effect size of RPC density reduction in glaucoma studies appears slightly larger than that herein reported (approximately 5 to 7% decrease [19], cf. Table 2). However, we had anticipated that any difference we could detect would be smaller than those reported in glaucoma studies. First, these studies mostly included patients with advanced glaucoma (while we included patients with early DR). Secondly, due to its pathophysiology, glaucoma is expected to target the optic nerve head more that DR. Because still little data is available on RPC density, the value of a clinically meaningful difference is yet unknown. However, in patients with glaucoma, the data herein presented should be interpreted with caution. Having been diagnosed with glaucoma was an exclusion criteria for this study. Therefore, it is currently unknown how glaucoma, in diabetic patients, interacts with DR to produce neuro-vascular peripapillary damage.

In our studiy, we analyzed data of two eyes of the same patients by performing multilevel mixed models [30]. By definition, these models are appropriate to accommodate nested data (as described above, in the Materials and Methods section) and also enabled us to control for confounding. Therefore, despite some demographic imbalances between groups (e.g., age; Table 1), we were still able to ascertain if having diabetes (with or without DR) was independently associated with the main outcome measures (RNFL thickness and RPC density). Interestingly, we found that age per se was an independent predictor of decreased RNFL thickness and RPC density, which is in agreement with previous data [31,32]. Similarly, despite our exclusion criteria of spherical equivalent superior to six diopters, we found that the spherical equivalent was significantly associated with the outcome measures, probably as a surrogate marker of the axial length of the eye. It would have been even more accurate to correct for axial length, but we did not have access to these measurements in this study, which can be considered as a limitation. Furthermore, and as expected, the proportion of insulin use, HbA1c level and time since the diagnosis of diabetes were all higher in the NPDR group, relative to diabetic patients without DR (Table 1). However, with the exception of time since diagnosis, these diabetes-specific factors were not associated with the outcome measures (Tables 6 and 7). One hypothesis was that our sample size is underpowered to detect these specific differences. Alternatively, if peripapillary neuro-vascular changes occur already in the early course of DR, as disease progresses, diabetes-specific factors may not be as tightly associated with these early changes.

In this regard, the data herein presented does not detail how subsequent stages of DR (diabetic macular edema and proliferative DR) affect peripapillary RPC density. These conditions were deliberately pre-specified as exclusion criteria, because macular edema distorts OCT-A segmentation (which is paramount for precise slab definition, as detailed in the Materials and Methods section) and the retinal thickening in macular edema falsely increases peripapillary RNFL thickness [33]. Furthermore, panretinal photocoagulation (PRP) and focal laser treatment are independently associated with decreases in RNFL thickness [34,35]. Therefore, if we had recruited these patients, confounding impossible to control for would have been introduced.

Although our initial premise was to determine whether circumpapillary capillary compromise could underlie the RNFL thinning consistently described in diabetic patients with no clinical evidence of DR [10], previous studies have identified an association between RNFL thinning and the severity of diabetic polyneuropathy [11,12]. Although our data firmly supports that early capillary involvement may underlie the reported peripapillary RNFL structural

changes, we cannot exclude that diabetic polyneuropathy will not also contribute towards these changes, since we have not assessed polyneuropathy severity, as part of our pre-specified clinical assessment.

This study also has many strengths. Seven standard 45º-field photographs of the eye fundus were taken for every diabetic patient, and a certified grader, blinded to all study variables, performed the DR grading. The sample is well sized, clinical evaluation was detailed, sophisticated and innovative image acquisition and analysis algorithms were employed and careful statistical planning allowed to control for potential confounders.

Ultimately, our study provides novel mechanistic insight into DR, demonstrating that the peripapillary capillary network is decreased even before the onset of overt vascular changes, which may subsequently lead to neuroretinal thinning. Furthermore, our data hints that RPC density may become of clinical value when predicting which diabetic patients are more likely to progress to DR and, perhaps, which are more likely to progress to severe forms of DR. For this purpose, further prospective longitudinal studies are warranted.

Agradecimentos

Quero expressar a minha gratidão ao meu Orientador, Dr João Pedro Marques e Co-Orientador, Professor Doutor Rufino Martins da Silva, por toda a disponibilidade, apoio, atenção e colaboração que demonstraram ao longo da realização deste trabalho.

Expresso também o meu agradecimento ao Professor Doutor Joaquim Neto Murta, Diretor do Serviço de Oftalmologia dos Hospitais da Universidade de Coimbra, por me ter permitido realizar a tese nesta área e me ter disponibilizado todos os recursos necessários do Serviço de Oftalmologia.

Ao Dr Tiago Rodrigues agradeço toda colaboração prestada, tendo sido essencial e decisiva para a realização de todo este trabalho.

Ao Professor Doutor Michael-John Dolan pelo desenvolvimento da macro customizada que permitiu introduzir inovação, precisão e reprodutibilidade a este estudo.

Por último, deixo o meu agradecimento aos Técnicos Mário Soares e Pedro Melo, responsáveis pela execução dos Exames necessários à investigação clínica, bem como à Técnica Sílvia Simão, certificada pelo Coimbra Ophthalmology Reading Centre (CORC) e responsável pelo grading ETDRS das retinografias.

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