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FACULDADE DE MEDICINA

Retinal Vascular Reactivity Study Using Optical Coherence Tomography Angiography

David Samuel Cordeiro Sousa

Orientador: Prof. Doutor Carlos Alberto Matinho Marques Neves

Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, na especialidade de Oftalmologia

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CONTENTS

ABBREVIATIONS

ATP - Adenosine triphosphate

BTS – British Thoracic Society

CDI - Colour Doppler Imaging

D-OCT – Doppler-Optical Coherence Tomography

DAP – Diastolic Arterial Pressure

DCP – Deep Capillary Plexus

DM – Diabetes mellitus

DR – Diabetic Retinopathy

DVC – Deep vascular complex

FAZ – Foveal avascular zone

GCL – Ganglion cell layer

HCT – Hypoxia challenge test

HFL – Henle fiber layer

ICP/MCP – Intermediate/Middle capillary plexus

INL – Inner nuclear layer

IOP – Intraocular pressure

IPL – Inner plexiform layer

LBF – Laser Doppler blood flowmeter

MAP – Mean arterial pressure

MOPP – Mean ocular perfusion pressure

NFL – Nerve fiber layer

OCT – Optical coherence tomography

OCTA - Optical coherence tomography angiography

OLM – Outer/External limiting membrane

ONL – Outer nuclear layer

OPL – Outer plexiform layer

OPP – Ocular perfusion pressure

RPE – Retinal pigment epithelium

SAP – Systolic arterial pressure

SCP – Superficial capillary plexus

T1D – Type 1 diabetes mellitus

VD – Vessel density

VEGF – Vascular endothelial growth factor

ABSTRACT

As the highest oxygen-consuming tissue of the human body, the retina has evolved to have an intricate vascular autoregulatory system. This mechanism ensures adequate supply of oxygen and nutrients to retinal tissues in conditions such as high blood pressure or hypoxia. Most studies on retinal vascular responses used imaging techniques that were limited to the assessment of the larger vessels. Novel and more detailed ways of analysing retinal vascular responses could provide insights on retinal autoregulation in both health and disease. Optical coherence tomography angiography (OCTA) allows a fast, safe, non-invasive and three-dimensional angiographic scan of the retinal microvasculature with unprecedented high-resolution. The main goal of this thesis was to dynamically study retinal vascular reactivity in healthy subjects and in a cohort of patients with type 1 diabetes (T1D) using OCTA, and to explore potential applications in ophthalmology and other medical fields. With this purpose, we developed a series of experimental multidisciplinary works. The first publication constitutes the proof of concept on the capacity of OCTA to detect a retinal vascular response. In the second, OCTA was used to study early structural retinal microvascular changes in patients with T1D before clinically evident retinopathy. The third paper consists of a methodological protocol on the use of OCTA to study retinal vascular responses of both vasodilation and vasoconstriction, using two standardized stress tests – hypoxia and isometric exercise. The last publication applied these methods to the disease setting (i.e. T1D patients with no evidence of retinopathy), and significant changes in retinal autoregulatory responses were found. The studies developed within this thesis highlight the potential of optimizing OCTA technology in order to combine a functional analysis to the currently available single structural angiogram. It provides a basis for future applications of this concept in ophthalmology and many other fields, such as neurological and cardiovascular research.

KEYWORDS: optical coherence tomography angiography; retinal vascular autoregulation; retinal vascular reactivity; isometric exercise; hypoxia.

RESUMO

A retina, o tecido que mais oxigénio consome por unidade de área, possui um complexo sistema de autorregulação vascular. Este mecanismo permite manter o adequado fornecimento de oxigénio e nutrientes à retina em condições de hipóxia ou alterações da pressão arterial, por exemplo. Na maior parte dos estudos existentes, as respostas vasculares da retina foram avaliadas com recurso a técnicas de imagem que se limitam à avaliação dos vasos de maior calibre. A possibilidade de analisar em maior detalhe estes mecanismos poderá ajudar a compreender melhor os processos envolvidos na autorregulação microvascular da retina, tanto em condições fisiológicas como de doença. A angiografia por tomografia de coerência ótica (OCTA) é um exame rápido, seguro, não-invasivo, tridimensional e de alta resolução da microvasculatura retiniana. O objetivo principal desta tese foi estudar a reatividade vascular da retina usando OCTA, em indivíduos saudáveis e com diabetes tipo 1 (DM1), e discutir potenciais aplicações clínicas. Nesse sentido, desenvolvemos uma série de trabalhos experimentais multidisciplinares. A primeira publicação constitui a prova de conceito sobre a capacidade da OCTA detetar uma resposta vascular retiniana. No segundo artigo, utilizou-se OCTA para estudar alterações microvasculares estruturais da retina em pacientes com DM1 sem retinopatia. O terceiro manuscrito consiste num protocolo metodológico em que a OCTA é utilizado para estudar respostas vasculares retinianas a estímulos vasodilatadores e vasoconstritores, utilizando dois testes padronizados - hipóxia e exercício isométrico, respetivamente. Na última publicação, aplicou-se este protocolo a doentes com DM1 sem evidência de retinopatia, e verificou-se que estes apresentam alterações nas respostas autorregulatórias da retina. Os estudos realizados no âmbito desta tese destacam o potencial de otimização da tecnologia OCTA no sentido de combinar uma análise funcional ao angiograma estrutural atualmente disponível. A metodologia desenvolvida nesta tese para o estudo da microcirculação retiniana constitui um ponto de partida para futuras aplicações clínicas da OCTA, não só em Oftalmologia

mas também em outras áreas, como a Medicina Cardiovascular e Neurociências.

PALAVRAS-CHAVE: Angiografia por tomografia de coerência ótica; autorregulação vascular retiniana; reatividade vascular retiniana; exercício isométrico; hipóxia.

THESIS OUTLINE

This Doctoral thesis is divided in three parts.

Part I provides an *Introduction* on the retinal vasculature and imaging methods, including optical coherence tomography angiography. It provides the background information to understand the research questions and purpose of the studies conducted.

Part II comprises four *Original Articles* published in international peer-reviewed journals with an impact factor in the first quartile of the area of specialty and indexed in the *Web of Science* and *Scopus* databases. These manuscripts include the proof of concept of the project, and the detailed methodology and results obtained to answer each of the research questions.

Lastly, **Part III** includes an *Integrated Discussion* on the results of the studies, while addressing future perspectives and potential applications of the concepts developed in this thesis.

PART I

INTRODUCTION

I. Retinal Vasculature Development, Anatomy and Physiology – Overview

Vision is possible through the complex processing of the light stimuli in the retina. By entering the eye, the light is focused by the anterior optics of the eye, crosses the transparent neural retina and eventually reaches the photoreceptors. These cells initiate a cascade of events called phototransduction, by which the photons' energy is converted into an electric stimulus relayed via the inner retinal neurons and the optic nerve to the brain (Figures 1-2).[1]

The energy needed for these processes make the retina the single largest oxygen consumer by weight of any tissue in the human body – for instance, in dark environments, a single photoreceptor may consume up to 10^8 ATP molecules per second. Therefore, the retina is dependent on a dedicated and efficient vascular network to meet its demand.[2–4]

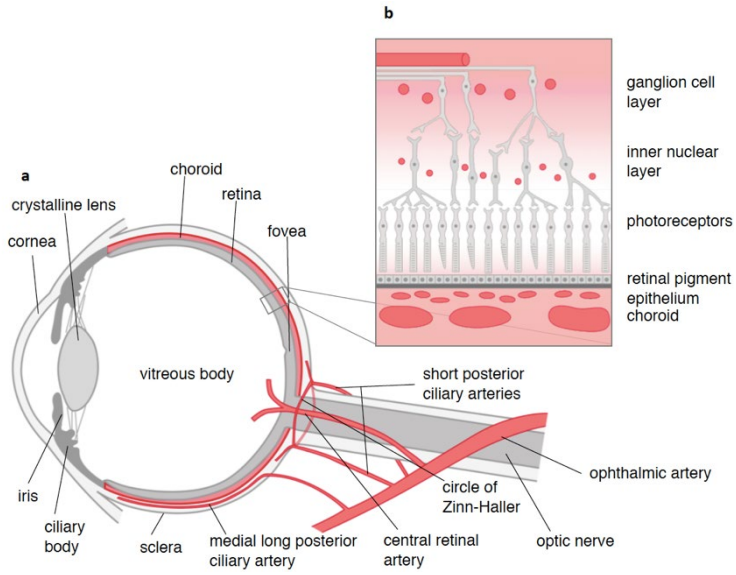

Figure 1. Anatomy of the eye and its vasculature: schematic overview - a. ocular anatomy, including the globe structures, optic nerve and vascular supply; b. cross section of the retinal and superficial choroidal structures. Adapted with permission from the PhD thesis of Karel van Keer [5]

Two different circulations are responsible for maintaining an adequate supply of substrates and oxygen to the retina: i) the retinal and ii) the choroidal system. The first is mostly responsible for supplying the inner and middle retinal layers, while the latter is the main supply of the outer retinal layers and peripheral avascular retina (Figure 2). The retinal circulation has no anastomoses, being considered an end-arterial system. The central retinal artery is a branch of the ophthalmic artery, penetrates the optic nerve and emerges at its head divided in the superior and inferior branches. These progressively subdivide into arterioles that extend centrifugally to supply each quadrant of the retina. The venous system mirrors the arterial distribution, merging into the central retinal vein, which exits the eye also via the optic nerve and drain into the cavernous sinus.[6–8]

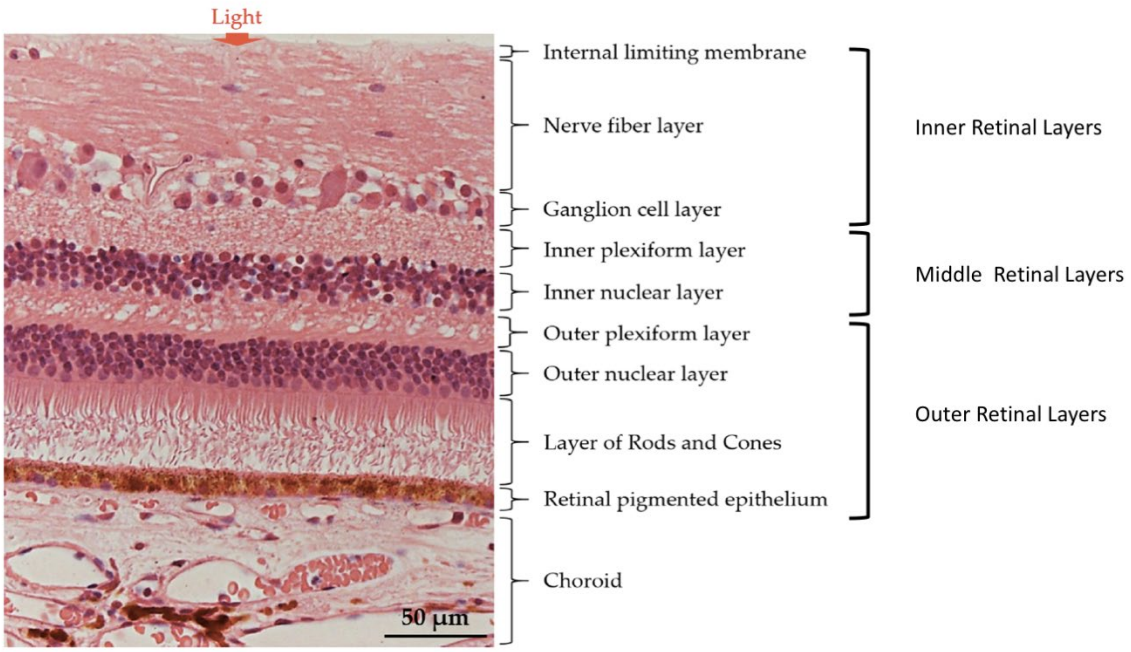

Figure 2. Normal Retinal structure – histological appearance of choroid and retinal layers. The retina is arranged in different layers of cells, from the Retinal Pigment Epithelium to the Nerve Fiber Layer. In these retinal layers five retinal neuronal cells co-exist: i) rod-and cone-photoreceptors, ii) horizontal cells, iii) bipolar cells, iv) amacrine cells, and v) the retinal ganglion cells. The arrow indicates the light transmission into the retina. Adapted with permission from Ding SLS, et al[9] under the terms of the Creative Commons Attribution 4.0 International License. Classification of retinal layers according to Sarraf et al[7].

The embryonic development of the retinal vascular plexuses begins at 15-weeks gestation, from a capillary ring around the optic nerve head.[10,11] Retinal astrocytes are then responsible for the angiogenesis VEGF-rich environment that spread outward from the optic nerve head.[12–14] The growing vessels initially differentiate into the major retinal arteries and veins of the superficial retinal vascular complex. After the 25th week, a precise balance of VEGF secretion from astrocytes, retinal Müller and glial cells ensures a timely and uniform deep retinal vascular complex development. Rods, cones and retinal pigment epithelial cells express an inhibitor of the VEGF receptor, preventing the deep vessels to penetrate the outer retina.[10,11,13]

The superficial and deep vascular complexes continue to grow radially to the periphery, but both circumvent the fovea also due to the expression of anti-angiogenic factors. The development of a fovea with increased photoreceptor concentration and devoid of any vessels creates a structure designed for sharp central visual acuity – the foveal avascular zone.[15–17]

In the parafoveal region, the retinal vascular network is elegantly organized in three levels: i) superficial capillary plexus (SCP), ii) intermediate capillary plexus (ICP), and iii) deep capillary plexus. The SCP runs in the ganglion cell layer, the ICP locates between the inner nuclear layer and the inner plexiform layer, and the DCP runs between the inner nuclear layer and the outer plexiform layer (Figures 3-4). Although well-established as two distinct plexuses, the ICP and DCP are usually collectively named as the deep vascular complex (DVC).[11,18,19]

The retinal arterial system has some specific anatomic features. Compared with similar sized vessels, they tend to have an unusually well-developed smooth muscle layer (five to seven layers near the optic nerve head, three layers at the equator and one to two at the periphery) and lack the internal elastic lamina. The capillary wall is composed of a basement membrane, endothelial cells, and intramural pericytes – which contribute to the regulation of microvascular growth and tone.[20,21] The endothelium of the retinal blood vessels is non-fenestrated, and together with the surrounding pericytes and supporting glial cells, constitute the inner blood-retinal barrier.

This impermeable barrier allows for a tightly controlled retinal microenvironment by limiting the free diffusion of solutes from the bloodstream.[22,23]

Although the structure is well-defined, the dynamic blood flow in these plexuses is complex and yet to be completely understood. It is believed the arterial inflow occurs predominantly at the SCP and ICP, while venous outflow happens in all planes and mainly through the DCP. *Ex-vivo* human studies found that retinal venules are found deeper than arterioles, and that direct arterial supply is only found in the SCP and ICP, with the DCP receiving it from the overlying plexus. Further in-vivo imaging studies suggested the existence of both parallel (i.e. within the same plexus) and in series (i.e. from the superficial to the deep plexuses) circulations responsible for the blood distribution in the retinal vascular network, with substantial predominance of the latter (Figure 4).[24–31]

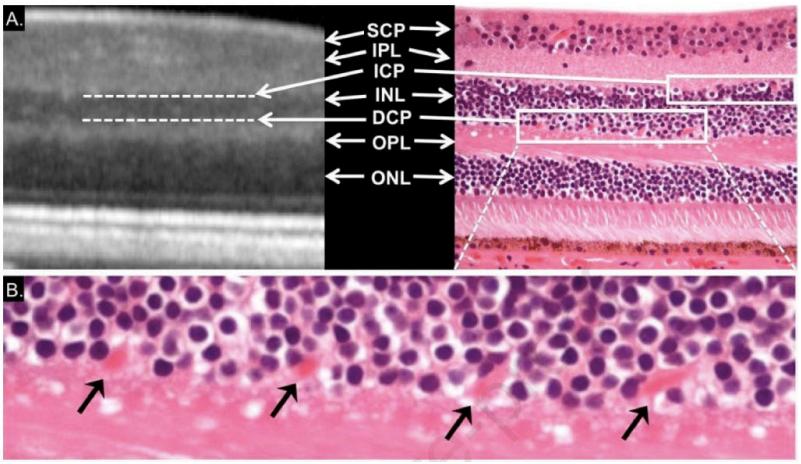

Figure 3. Localization of the retinal capillary plexus networks within the human perifoveal macula. (A): The superficial vascular complex is located predominantly within the ganglion cell layer and referred to as the superficial capillary plexus (SCP) and to a lesser extent within the nerve fiber layer (NFL) where it is most prominent around the optic nerve and referred to as the nerve fiber layer capillary plexus. The deep vascular complex is composed of an intermediate capillary plexus (ICP) within the inner portion of the INL bordering the IPL and a DCP within the outer portion of the INL bordering the OPL. (B): Magnification of the retina stained with hematoxylin and eosin at the lower border of the INL displays the DCP vessels (arrows). DCP: Deep Capillary Plexus; ICP: Intermediate Capillary plexus; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; NFL: Nerve Fiber Layer; OPL: Outer Plexiform Layer; SCP: Superficial Capillary Plexus. Image courtesy of Ehsan Rahimy, MD.

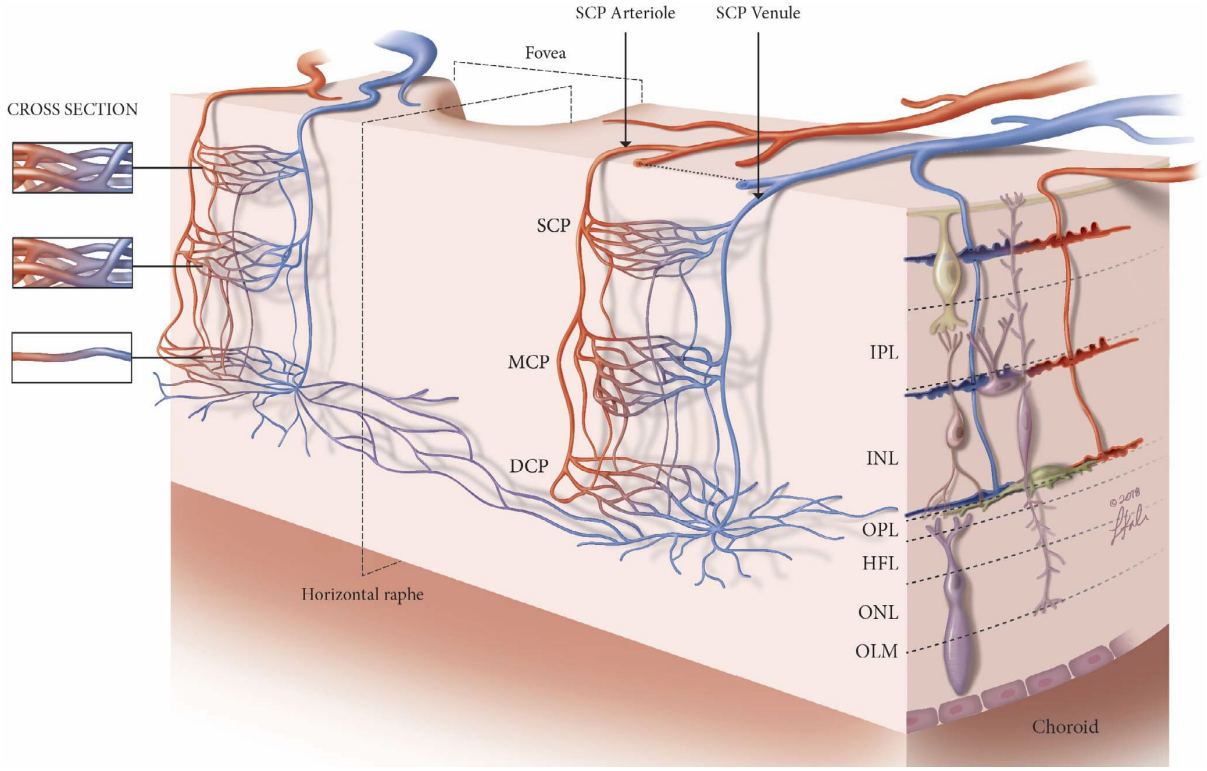

Figure 4. Schematic depiction of the retinal circulation and the *in-series* and *parallel* blood flow distribution. The SCP is located at the level of the ganglion cell layer. The MCP is located between the IPL and the inner aspect of the INL. The DCP is located within the outer aspect of the INL and OPL. The MCP and DCP receive arteriolar supply (red) from SCP arterioles and drain (blue) to SCP venules. Direct anastomotic connections between each of these layers are seen on both the arteriolar and venular sides of the capillary beds. The DCP includes vortices that drain centrally into a venule, and also connect to other venules and arterioles through radially oriented capillaries. The DCP contains channels that traverse the horizontal raphe. All three plexuses form venules that drain into the retinal vein located at the level of the SVP.

GCL, ganglion cell layer; HFL, Henle fiber layer; INL, inner nuclear layer; IPL, inner plexiform layer; OLM, outer limiting membrane; ONL, outer nuclear layer; SCP, superficial capillary plexus; MCP, intermediate (middle) capillary plexus; DCP, deep capillary plexus. Adapted with permission from Nesper and Fawzi 2018 [32] under the terms of the Creative Commons Attribution 4.0 International License.

II. Retinal Vasculature Autoregulation

As one of the most metabolically active tissues in the body, the maintenance of an adequate retinal nutrient and oxygen supply is crucial. This is particularly true for the macula, where the demand is higher than in any other region of the retina. The retinal vascular network has thus evolved with the capacity for blood flow autoregulation. Autoregulation can be defined as the intrinsic capability to maintain an appropriate blood flow over a range of scenarios to ensure adequate supply according to the metabolic demand of a certain tissue. In the retina, this is achieved through an interplay of numerous factors that allow for each capillary plexus to dilate and constrict independently (Figure 4).[3,33–36]

Multiple histological studies and nerve stimulation experiments have revealed a rich supply of autonomic vasoactive nerves to choroidal but not to retinal vessels, which are believed to be relatively independent of neural and hormonal influences. Nevertheless, along the optic nerve head and within the retina, α- and β-adrenergic receptors and receptors for angiotensin II are present.[33,37,38]

The retinal blood flow depends on three major factors: i) perfusion pressure, ii) vascular resistance and iii) blood viscosity. Although limited by multiple factors (e.g. local hematocrit influence on viscosity, shear rate, irregular vascular branching), the Hagen-Poiseuille law[6] relates the blood flow (Q) with the *viscosity* (μ) of an incompressible uniform liquid flowing in a tube with *length* (L), constant *radius* (R) and the pressure difference between the two ends of the tube (ΔP) as such:

$$Q = \frac{\pi R^4 \Delta P}{8 \mu L}$$

The ocular blood flow is thus dependent on the difference between the arterial pressure (inflow)

and the venous pressure (outflow) – i.e. the ocular perfusion pressure (OPP). In sitting and standing positions, the mean ocular arterial pressure is about two thirds of the mean brachial artery blood pressure. As the venous pressure almost equals the intraocular pressure, the mean OPP (MOPP) corresponds to the difference between 2/3 of the mean arterial blood pressure (MAP) and the intraocular pressure (IOP)[6,39]:

$$MOPP = \frac{2}{3} MAP - IOP$$

The vascular resistance (i.e. vessel diameter) is modulated by the interaction of multiple factors affecting the tone of the pericytes and smooth muscle cells. Blood viscosity can be affected by conditions such as high hematocrit, leukemia, and sickle cell anemia. However, at physiologic high shear rates, blood viscosity remains low and constant.[6] Therefore, the blood flow equation may be simplified as:

$$Q = \frac{MOPP}{Vascular\ Resistance}$$

As per the above equation, the MOPP decreases by increasing the IOP. However, the compensatory autoregulation in retinal vascular resistance keeps the blood flow and oxygen supply constant for a range of moderate increments in the IOP.[33,40] On the other hand, the MOPP can be increased by raising the systemic blood pressure and stimuli that increase peripheral vascular resistance, such as isometric exercise and the cold pressor test. In these situations, the blood flow remains relatively unchanged until MOPP is elevated by an average of 40-60% above baseline.[41,42] Retinal vascular autoregulation corresponds to this ability for a change in vascular resistance in order to maintain an adequate ocular blood flow over a range of MOPP – Figure 5.

Given that the retinal vessels are not innervated, these autoregulatory mechanisms are achieved

mainly through local factors. These include both a metabolic and myogenic component. The metabolic factors in play are released by glial, neural or endothelial cells, and can be both tone relaxing (e.g. nitric oxide, lactate and prostacyclin) or tone contracting (e.g. endothelin-1, angiotensin II, thromboxane-A2). The myogenic response corresponds to an endothelial response to sensed mechanical forces.[6,43]

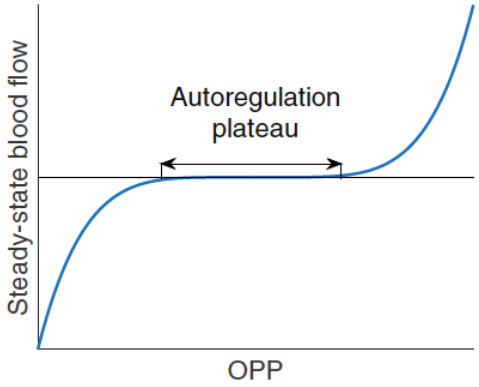

Figure 5. Schematic representation of an ocular blood flow autoregulation in relation to the ocular perfusion pressure (OPP). Autoregulatory responses allow to keep the blood flow constant through a wide range of OPP values. Adapted from Schmidl et al.[44]

The retinal and cerebral blood flow is not only regulated in response to changes in perfusion pressure, but also dependent on local neural activity – a concept termed as neurovascular coupling.[45] This concept is not new,[46] and mostly studied for the brain vasculature, but it has been recently expanded and better understood in the retina too. In this process, neural feedforward mechanisms contribute for an adequate supply of nutrients and oxygen to match the demands of active neurons.[45,47] These neurons, either directly or via other cell types (e.g. glial cells, astrocytes) induce the release of vasoactive substances to increase blood flow locally according to the neural activity – a process also referred as functional hyperemia.[45,47,48] As mentioned above, and although the retinal vessels devoid of intrinsic innervation, they do have receptors for various neurotransmitters that can influence pericytes and smooth muscle cells

tone.[37,38] The well-described pronounced hyperemic response in the retina with flickering light is an example of the effect of neural activity level in retinal blood flow.[49–51] And interestingly, this response appears to be relatively independent of changes in the OPP or vascular resistance.[52,53]

Table 1 summarizes the autoregulatory response of the retinal microvasculature during some commonly evaluated conditions.

Table 1. Retinal Vascular responses according to each stimulus.

Stimulus	**Retinal Vascular Response**
Hypoxia	Vasodilation [54–57]
Hyperoxia	Vasoconstriction [56–60]
Hypercapnia	Vasodilation [61–63]
Hypocapnia	Vasoconstriction [64,65]
Flicker-stimulation	Vasodilation [49,50,66–68]
Isometric Exercise	Vasoconstriction [69–71]
Cold Pressor Test	Vasoconstriction [72]

III. Retinal Vasculature Imaging

The transparency and optical properties of the eye make the ocular fundus the only location in the human body where direct non-invasive monitoring of the vascular network is possible. As part of the central nervous system, retinal *in-vivo* imaging is being particularly important for investigating changes during development, aging, disease progression, and treatment.[73,74] By using visible (380-780 nm) and near-infrared (780-1400 nm) light with ever increasing technical sophistication, retinal imaging allow for a unique combination of safety and histologic-level resolution.[75,76]

For structural analysis, the most relevant innovation in retinal imaging was the development of the optical coherence tomography (OCT).[77] Before that, fundus observation relied on biomicroscopy, ophthalmoscopy, and fluorescein or indocyanine green angiographies. The advent of the OCT made it possible to study the retinal health in-vivo, by providing impressive detail of the various retinal layers – figure 6. It generates cross-sectional images (B-scans) of the retina, such as ultrasonography, but uses reflected light instead of sound waves to obtain an image. The combination of different waves of reflected light from a tissue of interest and a reference path produce the typical interference light pattern of whiter or darker bands. It is also possible to obtain *en-face* images (C-scans), formed on the XY or coronal plane. This allows the study of a specific layer instead of only visualizing the depth in cross-sectional scans. By using light (i.e. with much shorter wavelength than sound waves), the OCT reaches much higher resolutions, typically between 3- to 8-mm of axial resolution. The technology is still evolving, to achieve faster speeds of acquisition and even higher axial resolution.[75,76]

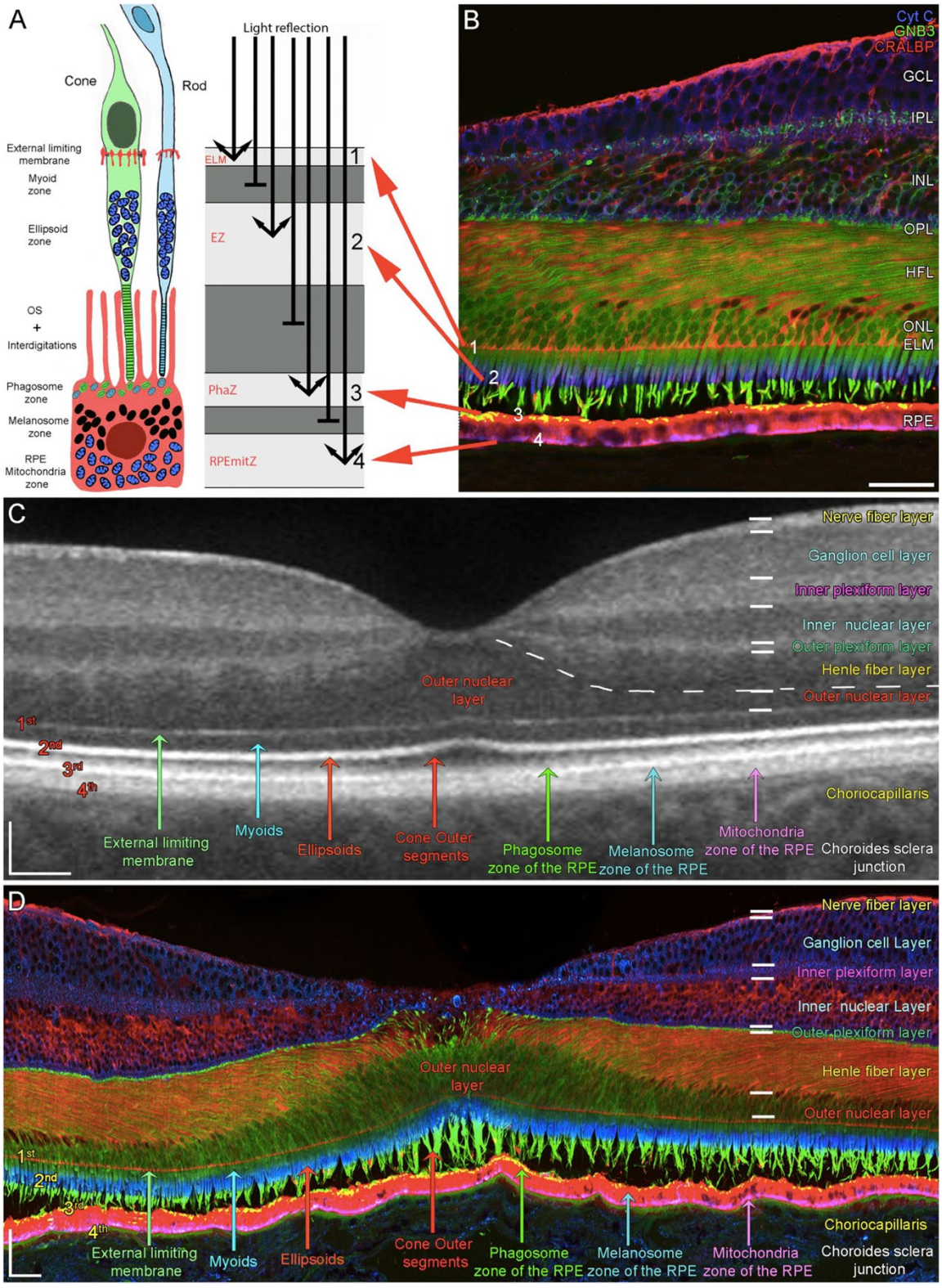

Figure 6. Correlation of optical coherence tomography (OCT) and histology. Graphical depiction showing the hyperreflective / hyporeflective bands at the foveal outer retina and their cellular origin (A). High magnification of a foveal cross-section showing retinal layers (B). Nomenclature of OCT bands (C) and their correspondent layers in a histological section stained with immunohistochemistry (D). Scale bars: (B) 50 µm; (C, D) 50 µm x 50 µm. Adapted with permission from Cuenca N, et al (2020)[76]

As an extension of the OCT, optical coherence tomography angiography (OCTA) is a novel imaging tool with increasing applications for research and clinics in ophthalmology and many other fields. OCTA technology uses infrared wavelengths to provide non-invasive high-contrast high-resolution three-dimensional images of the retinal microvasculature.[34,78,79] This technology generates pictures of unprecedent detail of the retinal capillaries by interferometrically measuring the amplitude and delay of reflected or backscattered light from moving erythrocytes. It does so by performing multiple B-scans at the same location and detecting motion contrast produced by these moving blood cells in the retinal vessels. Since no motion in the retina other than blood flow is expected, stationary objects won't produce much of a change from one image to the next, while moving objects will. By looking at change over time, the generated final image clearly defines retinal microvasculature, with the vessels depicted as white pixels on a black background – figure 7. Unlike fluorescein angiography, OCTA does not require dye injection, which makes it safer and more readily available exam to study the retinal vascular network.[76,79,80] As a three-dimensional imaging technology, one of the main advantages of OCTA is the possibility of vascular depth discrimination. This is limited, though, by the possibility of superficial vessels projecting flow into deeper layers. With recent software advances for the removal of these projection artifacts, the deep retinal vascular layers and not only the superficial plexus can be clearly imaged, overcoming one of its main limitations.[18,29]

The clinical value of OCTA has been demonstrated in several ocular diseases, including diabetic retinopathy, glaucoma, age-related macular degeneration and neurodegenerative disorders.[81–84]

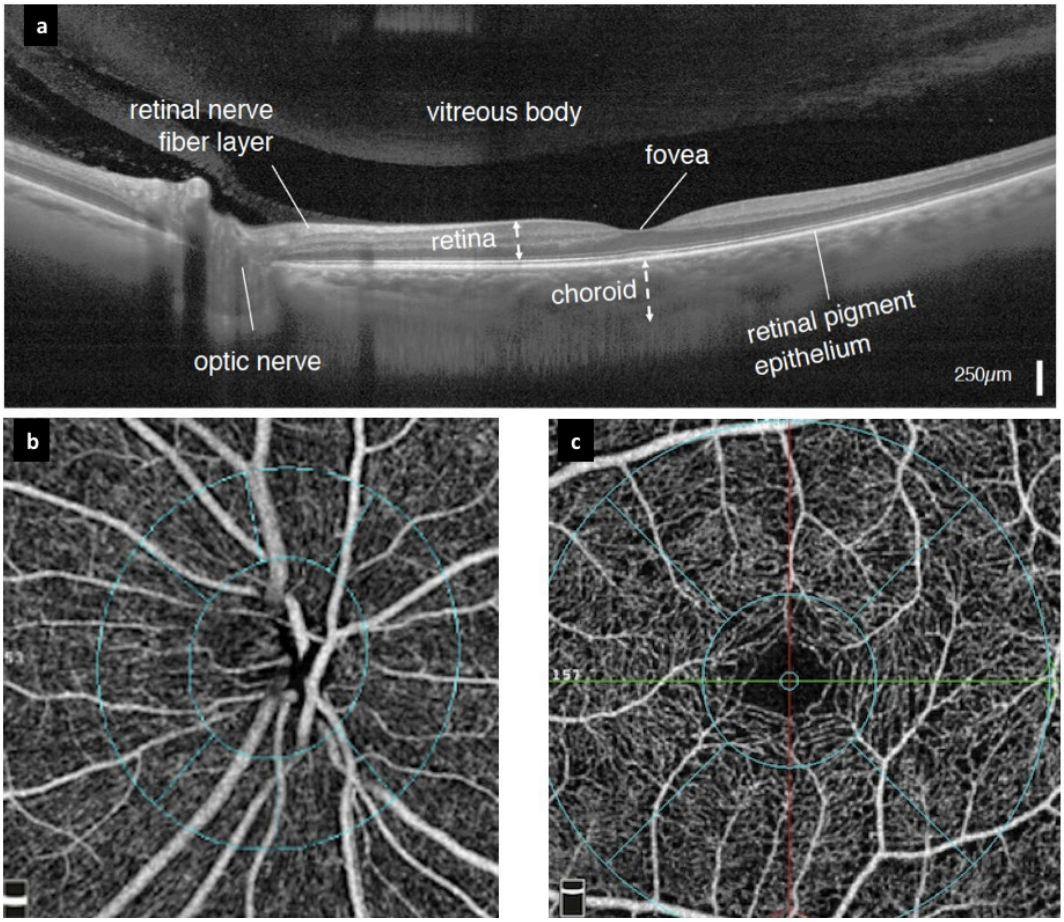

Figure 7. Cross-sectional optical coherence tomography (OCT) of the retina (A) - Adapted with permission from the PhD thesis of Karel van Keer[5]; and OCT-Angiography of the peripapillary vascular network (B) and of the macular vascular network (C) from participant in the studies included in Part II.

Not only the structure, but also the dynamic study of retinal vessels behaviour and blood flow autoregulatory function is important to better understand the (patho)physiological mechanisms behind the retinal supply of nutrients and oxygen in health and disease. A number of non-invasive methods have been used for ocular vessels' assessment, including laser Doppler flowmetry [85,86], colour Doppler imaging [87,88], and laser speckle flowgraphy [89]. However, these techniques are not widely used in clinic, being mostly limited to research purposes.[34,90] Currently, no technology allows a non-invasive measurement of volumetric absolute retinal blood flow, providing at most hemodynamic metrics which are surrogates for the actual blood

flow.[47] Table 2 summarizes the characteristics, advantages and limitations of various methods.

Table 2. Techniques for *in vivo* study of ocular hemodynamics.

Technique	Measurements	Advantages	Limitations
Laser Doppler Flowmetry	Velocity, Volume and Flow in arbitrary units	Multiple hemodynamic parameters	No absolute measurements and limited interindividual comparisons
Colour Doppler Imaging	Velocity	Vessel selective, no need for clear media	Velocity measurements only, operator-dependent
Laser speckle flowgraphy	Velocity and Flow in arbitrary units	Time evolution of velocity at the same site of the same eye	No absolute measurements and limited interindividual comparisons
OCT Angiography	Flow in arbitrary units	Fast, high-quality images	No absolute measurements, motion/projection artifacts

OCT: optical coherence tomography. Adapted from Prada et al[47]

Retinal vascular autoregulation changes and neurovascular coupling dysfunction are reported in the early stages of a number of ocular diseases, such as diabetes[91–94], retinal vein occlusion[95], glaucoma[96–98], and also neurodegenerative disorders[99–102]. For instance, in diabetes, it is estimated that 98% of the sight loss could be prevented by early detection and adequate management.[103] Roughly 400 million people worldwide have diabetes[104] and, of these, type 1 diabetes (T1D) - which tends to be diagnosed in young individuals - accounts for 5-10%.[104,105] Diabetic retinopathy (DR) is a microvascular complication of the systemic disease and a leading cause of acquired visual loss in the middle-aged and economically active population.[106]

As detailed above, retinal imaging provides a unique opportunity to study a central nervous system microvascular network in both physiologic and pathologic scenarios. The available technology may allow for earlier detection of structural or metabolic changes and contribute to prevent sight loss.

The general aim of the works performed within this PhD thesis was to dynamically study the retinal vascular response in healthy subjects and in a cohort of patients with T1D with no clinical evidence of DR, using optical coherence tomography angiography (OCTA).

Two standardized stress tests were used to induce a retinal vascular response potentially detectable using OCTA. These are described in detail in Part II, and briefly mentioned in this section for reader's convenience:

i) *Hypoxia Challenge Test (HCT)***.** The HCT, whose protocol is standardized by the British Thoracic Society[107], consists in a mild hypoxic stimulus performed to people with respiratory disease in order to evaluate their susceptibility to hypoxic environments,

such as a long-haul flight. As described above, the effects of lower levels of partial pressure of arterial oxygen (PaO_2) on retinal vessels are similar to the brain vasculature, with hypoxia triggering *vasodilation*.[54,56–58]

ii) *Handgrip Test*. The handgrip maneuver is an isometric exercise that induces a sympatheticomimetic response with increases in heart rate and arterial pressure.[71,108] It induces an retinal autoregulatory response of *vasoconstriction* that keeps the blood flow unchanged until the ocular perfusion pressure increases above 35-40% compared to baseline.[42,109–111]

Specific aim #1 - Proof of concept

To describe the effects of mild hypoxia in ocular hemodynamics using OCTA.

Research question

Is OCTA able to detect a retinal autoregulatory vascular response to mild hypoxia?

Specific aim #2

To compare, using OCTA, the retinal vessel density between T1D patients without DR and a cohort of age and gender-matched healthy individuals.

Research question

Are there structural changes in the retinal vascular network of T1D patients with no evidence of DR, when compared to healthy subjects?

Specific aim #3

To describe, using OCTA, the retinal vascular response to hypoxia in T1D patients without DR, compared with age and gender-matched healthy individuals.

Research question

Are there functional changes in the retinal vascular response to a hypoxic stress in T1D without DR, when compared to healthy individuals?

Specific aim #4

To describe the retinal vascular response to isometric handgrip test in T1D patients without DR, in comparison with age and gender-matched healthy individuals.

Research question

Are there functional changes in the retinal vascular response to isometric exercise in T1D without DR, when compared to healthy individuals?

PART II

ORIGINAL ARTICLES

A total of four manuscripts are included below, according to the objectives of this PhD Thesis. All the works were published in international peer-reviewed journals with an impact factor in the first quartile of the area of specialty and indexed in the *Web of Science* and *Scopus* databases.

The **first manuscript** corresponds to specific aim #1, constituting the proof of concept on the capacity of OCTA to detect a retinal vascular response to mild hypoxia.

The **second manuscript** corresponds to specific aim #2, analysing with OCTA the structural retinal microvascular changes in patients with type 1 diabetes before clinically evident retinopathy.

The **third manuscript** is a methodological protocol on the use of OCTA to study retinal vascular responses of both vasodilation and vasoconstriction, using the two above-mentioned standardized stress tests. This work validated the methods used and provided a basis for future applications of this concept in ophthalmology and many other fields, such as neurological research.

Lastly, the **fourth manuscript** corresponds to specific aims #3 and #4, in which the tests described in the protocol were applied to both healthy subjects and patients with type 1 diabetes with no clinical evidence of diabetic retinopathy.

As these were individually published works, the figures and tables numbering should be considered within each manuscript. However, references are merged with the ones from the rest of the document for readers' convenience.

I. Proof of concept - Hypoxia challenge test and retinal circulation changes

Hypoxia challenge test and retinal circulation changes - a study using optical coherence tomography angiography

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ABSTRACT

Purpose: Previous studies report that the response of retinal vessels to a decrease in oxygen (hypoxia) is vasodilation, thus increasing blood flow. We aimed to characterize the changes in retinal microvasculature induced by a mild hypoxia stress test in a healthy population, using optical coherence tomography angiography technology (OCTA).

Methods: Interventional repeated-measures study. The standardized hypoxia challenge test (HCT) was performed to all volunteers, according to the British Thoracic Society protocol. OCTA was done at three time-points (baseline, during HCT and 30' post-hypoxia). Macular and peripapillary vessel densities were assessed using built-in software. To minimize confounding bias, analysis were performed separately in right (OD) and left (OS) eyes. Repeated-measures ANOVA and mean comparison analysis were used as statistical tests (STATA v13).

Results: Studied population included 30 healthy subjects (14 women), with a mean age of 28.8 ± 4.2 [range 22-37] years. Baseline vessel density increased in hypoxic conditions and subsequently decreased to near-baseline values in post-hypoxia conditions. This pattern was observed for both eyes in both parafovea (OD: 55.3±2.3 to 56.7±1.9 to 55.8±1.9, $p<0.05$; OS: 56.9±2.1 to 57.9±1.9 to 57.3±1.7, $p<0.05$) and peripillary (OD: 60.5±0.5 to 62.6±0.5 to 60.1±0.4, $p<0.05$; OS: 60.4±0.4 to 62.3±0.5 to 60.7±0.4, $p<0.05$) areas.

Conclusions: To our knowledge, there is no published data specifically addressing mild hypoxia conditions and retinal microvasculature changes, using OCTA. This pilot study may pave way to better understand vascular responses in disease setting.

INTRODUCTION

Published literature on the effects of different PaO_2 levels on retinal vessels report a response similar to the brain vasculature, with increases in oxygen (hyperoxia) causing vasoconstriction and a decrease in blood flow; and reductions in oxygen (hypoxia) triggering dilation of the vessels and an increased blood flow [54,56–58].

A standardized assessment - Hypoxia Challenge Test (HCT) - is performed to people with respiratory disease to evaluate their susceptibility to a hypoxic environment, such as a long-haul flight. According to aviation regulations, flight cabins are pressurized to a value of around 565 mmHg (0,74atm), equivalent to be at an altitude around 8.000 ft., corresponding to breathing 15-16% oxygen at sea level [107,112,113]. While most healthy travelers can easily compensate for this amount of hypoxemia, the same may not apply for patients suffering from circulatory or ischemia-associated conditions with a known impairment in optical vascular regulation. [114–116]. The HCT creates a hypoxic environment by reducing inspired oxygen fraction (FiO_2), and making it equivalent to the flight cabin altitude.

Optical coherence tomography angiography (OCTA) is a novel diagnostic tool with increasing applications in clinical practice, which uses infrared wavelengths to provide non-invasive high-resolution pictures of the retinal microvasculature.[78]

We aimed to characterize the changes induced by a hypoxia stress and retinal microvasculature changes in a healthy population, using OCTA technology. The understanding of the physiologic response of retinal vessels to a hypoxic stimulus may drive new directions in ocular vascular disorders research.

MATERIALS AND METHODS

This study was conducted in Lisbon Academic Medical Center (CAML). The research protocol adhered to the tenets of the Declaration of Helsinki.[117], and was approved in full by the CAML Institutional Review Board (IRB). In accordance with IRB recommendation, recruited patients must have had the intention to fly, so the hypoxic stress test would be applied to individuals who would experience it anyway. Written informed consent was obtained from all enrolled volunteers, after detailed explanation of the objectives, procedures and risks of the study.

Study participants

An open-call was launched by e-mail until the required number of healthy volunteers was reached. Before the study beginning, participants had to fill a questionnaire, including the following information: age, gender, smoking pack years, known diseases and current chronic medication, previous intraocular surgery or trauma, symptoms during previous flights, intention to fly in the future. To be enrolled in the study, all individuals must have to be free from major systemic illnesses or ocular disease, such as high refractive errors (± 4 diopters), glaucoma, macular disease, diabetes, and previous ocular trauma and/or surgery.

Study design and data collection protocol

An interventional, repeated-measures study was conducted in order to characterize and explore the macular and peripapillary vessel density changes induced in flight hypoxia conditions using HCT as a stress test. Three measurements times were considered: #1) baseline, #2) hypoxia and #3) post-hypoxia.

Firstly, a complete ophthalmological examination was performed to all subjects, including best-corrected visual acuity (BCVA), slit-lamp biomicroscopy and fundoscopy, auto-refractometer

(RK-5, Canon Europe®, The Netherlands), intraocular pressure (iCare TA011®, Vantaa, Finland), and eye biometry (Lenstar®, Haag-Streit, Switzerland). Other baseline measurements (timepoint #1) were performed, including arterial pressure (Carescape® V100, GE Healthcare, Portugal), and OCTA examination (AngioVue®, Optovue, CA, USA).

Using OCTA, peripapillary vessel density (ppVD) was assessed in a 0.75mm wide ring around the optic nerve head (figure 1 Foveal vessel density (fVD) was assessed in a circle of 1 mm diameter and parafoveal vessel density (pfVD) calculated by analyzing a 2 mm-wide around the fovea area (figure 2). Both values were calculated using AngioAnalytics®, the built-in software of the OCTA device, as a ratio of the white pixels (i.e. retinal vessels: where erythrocyte movement was detected) by the total number of pixels. Only high-quality images (score higher than 70) were considered.

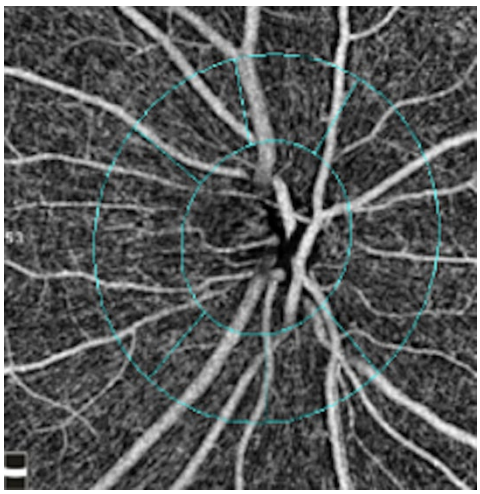


Figure 1 - Optical coherence tomography angiography of the optic disc and peripapillary area. The 0.75mm-wide ring around the optic disc corresponds to peripapillary vessels.

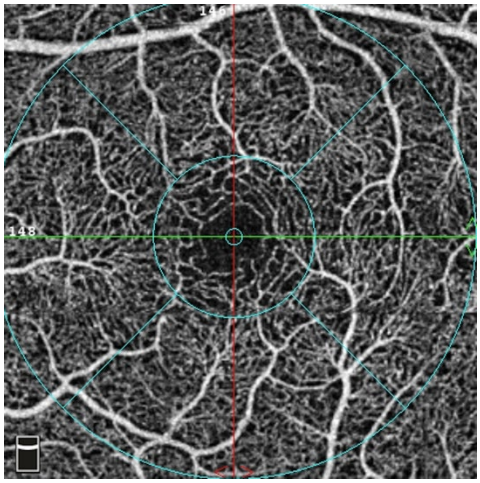
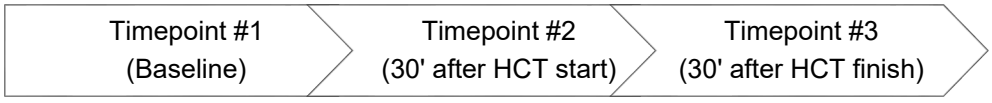


Figure 2 - Optical coherence tomography angiography of the macula. The inner circle and the 2mm-wide ring around the circle correspond to foveal and parafoveal vessels, respectively.

The HCT was performed at sea level in order to create a normobaric hypoxic environment by reducing FiO_2, and making it equivalent to the flight cabin values. The British Thoracic Society (BTS) proposes a practical and inexpensive protocol to perform HCT.[107] Briefly, following this protocol, participants had to breath a FiO_2 of 15% by using a gas mixture with a supply of 99,993% nitrogen (Linde Healthcare®, Portugal) through a 40% flow Venturi mask (Intersurgical®, UK) at 10L/min. Cardiorespiratory monitoring was performed using a polygraph and an oxymeter in a hand finger (Alice PDX, Philips-Respironics®, USA). Parameters monitored included oxygen peripheral saturation, arterial pressure and electrocardiography.

As established by BTS, recommended HCT duration is between 20 to 25 minutes. In accordance, 30 minutes after test start, all measurements were repeated in such hypoxic conditions (timepoint #2, figure 3). Finally, after the recommended 30 minutes recovery time[107], timepoint #3 post-hypoxia measurements were done. The scheme below illustrates the repeated-measures study protocol.



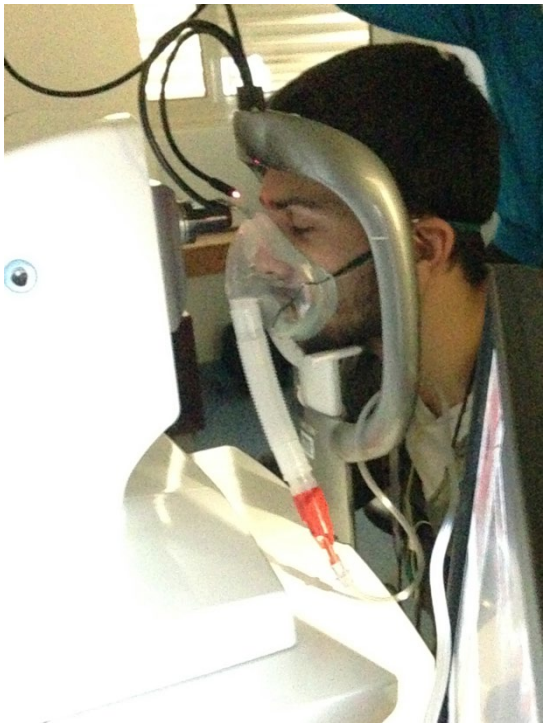


Figure 3 - Volunteer with adapted mask performing optical coherence tomography angiography in hypoxic conditions.

Statistical analysis

Statistical analysis was performed using STATA v13 (Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). ANOVA repeated measures (ANOVA-RM) analysis was used to assess within-subject differences between the three measurements [baseline (#1), hypoxia (#2), post-hypoxia (#3)]. Paired analysis using t-test statistic was also performed to confirm the findings between different time points. Exact p values are reported, rounded to two decimal places, except if p values < 0.01. Since this is the first time the current research protocol is being used, in order to use all the collected data (i.e. from both eyes) and to allow a better evaluation of the consistency of our findings, right and left eyes were analyzed separately. We calculated our required sample size by arbitrarily considering a 10% clinically significant change in retinal vessels diameter and a standard deviation of 5%. Thus, based on these values, with a power of

90%, an alpha value of 0,05 and accounting for subject attrition, 30 volunteers were enrolled.

RESULTS

A total of 60 eyes from 30 Caucasian volunteers (14 women), with a mean age of 28.8 ± 4.2 [range 22-37] years were included in the study. All subjects had a 20/20 BCVA. Mean axial length was 24.0 ± 0.9 mm for both right (OD) and left (OS) eyes. Spherical equivalent was -1,75 ± 1.4 diopters and -1,74 ± 1.5 diopters for right and left eyes, respectively.

Table 1 summarizes the mean values of each repeated variable during HCT and in the three timepoints [baseline (#1), hypoxia (#2) and post-hypoxia (#3)]. During HCT, minimum and mean Spo_2 levels were 88.5 ± 2.4% and 92.8 ± 1.8%, respectively. The systolic, diastolic and mean arterial pressure values decrease significantly in hypoxia conditions, when compared to baseline values ($p<0.05$, paired t-test).

Table 1. Mean values of each repeated variable in the three collected time-points.

Variable	Baseline		Hypoxia		Posthypoxia	
SAP, mmHg	120.4 ± 6.5		114.9 ± 10.0*		119.7 ± 10.3	
DAP, mmHg	77.8 ± 8.4		74.9 ± 8.2*		76.9 ± 9.2	
MAP, mmHg	92.0 ± 7.5		88.2 ± 8.1*		91.2 ± 8.9	
O2 peripheral saturation	97.6 ± 1.0		92.8 ± 1.8*		96.8 ± 1.3	
Foveal VD (OD | OS)	33.5 ± 1.0	35.1 ± 1.0	33.7 ± 1.1	35.6 ± 1.0	33.5 ± 1.2	35.0 ± 1.0
Parafoveal VD (OD | OS)	55.3 ± 2.3	56.9 ± 2.1	56.7 ± 1.9*	57.9 ± 1.9*	55.8 ± 1.9	57.3 ± 1.7
Peripapillary VD (OD | OS)	60.5 ± 0.5	60.4 ± 0.4	62.6 ± 0.5*	62.3 ± 0.5*	60.1 ± 0.4	60.7 ± 0.4

CCT = corneal central thickness, DAP = diastolic arterial pressure, IOP = intra-ocular pressure, MAP = mean arterial pressure, OD = right eye, OS = left eye, SAP = systolic arterial pressure, VD = vessel density.
Mean values ± standard deviation are depicted.
* When compared to baseline, a significant difference in hypoxia conditions was found ($p < 0.05$, paired *t*-test). Further details of statistical analyses between the different time-points are described in results section.

Macular Vessel Density

Parafoveal vessel density (pfVD) was significantly different among the three timepoints (OD: $F(2,29)=3.13$, $p=0.05$; OS: $F(2,29)=3.78$, $p=0.03$). Paired analysis confirmed the increase in mean pfVD in hypoxic conditions (OD: 55.3 ± 2.3 to 56.7 ± 1.9, $p=0.04$; OS: 56.9 ± 2.1 to 57.9 ± 1.9, $p=0.03$), when compared to baseline ($p<0.01$) and a subsequent decrease in post-hypoxia conditions to near-baseline values (OD: 56.7 ± 1.9 to 55.8 ± 1.9, $p=0.01$; OS: 57.9 ± 1.9 to 57.3 ± 1.7, $p=0.02$). Figure 4 illustrates the changes in pfVD.

Foveal vessel density (fVD) did not differ significantly within subjects (OD: $F(2,29)=0.50$, $p=0.61$;

OS: F(2,29)=0.30, p=0.74) throughout the experiment.

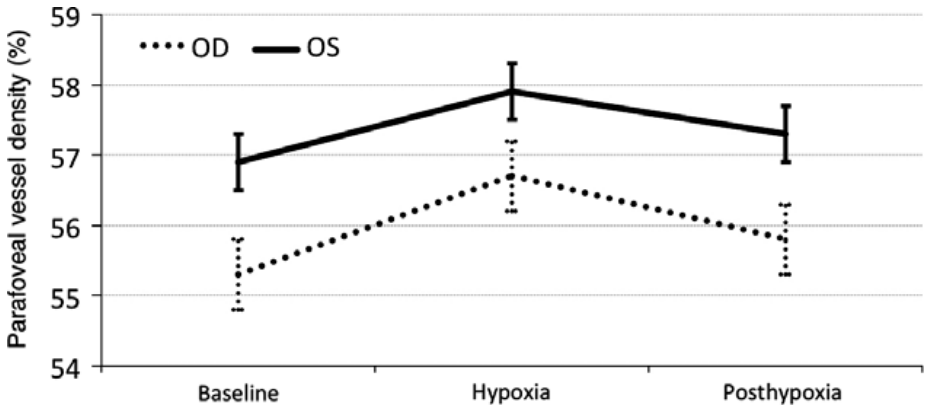


Figure 4 - Graph showing the changes in parafoveal vessel density between the three timepoints. Mean values and standard deviation range are shown. Baseline vessel density increased in hypoxic conditions and subsequently decreased to near-baseline values in post-hypoxia conditions, for both eyes ($p<0.05$).

Peripapillary Vessel Density

A within-subject difference in peripapillary vessel density (ppVD) for both right and left eyes was also found (OD: $F(2,29)=5.27$, $p<0.01$; OS: $F(2,29)=3.21$, $p=0.05$). Paired analysis confirmed the increase in mean ppVD in hypoxic conditions (OD: 60.5 ± 0.5 to 62.6 ± 0.5, $p=0.01$; OS: 60.4 ± 0.4 to 62.3 ± 0.5, $p=0.03$), when compared to baseline ($p<0.01$) and a subsequent decrease in post-hypoxia conditions to values similar to baseline (OD: 62.6 ± 0.5 to 60.1 ± 0.4, $p=0.01$; OS: 62.3 ± 0.5 to 60.7 ± 0.4, $p=0.02$). Figure 5 illustrates the changes in ppVD.

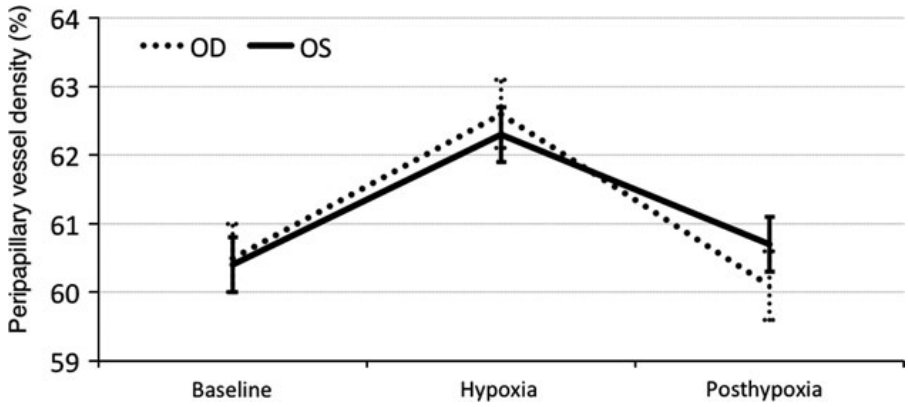


Figure 5 - Graph showing the changes in peripapillary vessel density between the three timepoints. Mean values and standard deviation range are shown. Baseline vessel density increased in hypoxic conditions and subsequently decreased to near-baseline values in post-hypoxia conditions, for both eyes ($p<0.05$).

DISCUSSION

The retina is a tissue with high-energy demands, a single retinal photoreceptor being capable of using up to 10^8 ATP molecules per second.[4] The supply route by the retinal vascular system is thus crucial for visual function.[118]

Vascular reactivity, the response of the vessels to a vasoactive stimulus such as hypoxia, can be used to assess the vascular range of adjustment in which the vessels are able to compensate for fluctuations in PaO_2.[57] Therefore, we were interested in assessing if the OCTA technology would be able to detect differences in vessel density with PaO_2 changes in a healthy population, even with this *mild* hypoxia, using HCT as a standardized test mimicking flight-cabin hypoxia conditions.

Our findings in healthy volunteers suggested that retinal (parafoveal and peripapillary) vessels dilate in response to a normobaric hypoxic stimulus. Moreover, the significant decrease observed for arterial pressure values during the test further confirm the well-recognized hypoxia-induced retinal vessels' vasodilation. These results are in accordance with previous literature and recently published works by Cheng and collaborators who studied the relationship between retinal blood flow and PaO_2 using laser blood flowmeter (LBF) technology.[57] LBF is a non-invasive technique that determines centerline blood velocity (mm/s) and vessel diameter (μm) of the retinal arterioles and venules, then calculating flow in μl/min based on the Poiseuille principle.[119] These authors found that a combination of both a hyperbolic and linear function explains this relationship, with retinal vessels dilation in response to decreased arterial oxygen until an oxygen arterial pressure threshold of 32-37 mmHg, below which no further compensation is observed. Despite being considered a reliable technology, LBF is not widely available in clinic, being considered more a research tool.[120,121]

The results of this study using OCTA are also in accordance with previous findings using other non-invasive devices (Oxymap® retinal oximeter, spectral-domain-OCT/metabolic

hypersprectal camera derived retina blood flow and high-resolution retinal vessel measurement system), which consistently reported a decrease in retinal vessels diameter during hyperoxia, and an increased retinal blood flow under hypoxia conditions.[122–124]

Compared to these equipments, OCTA has the advantage of providing a high-quality morphological image, and allowing function/anatomy correlation.[78,125] However, since OCTA technology is not able to quantify retinal vessels oxygen saturation, it would be interesting to correlate the vascular changes detected with OCTA with the quantitative oxygen saturation obtained with retinal oximetry, for instance.

We specifically studied macular and peripapillary vessel density as a surrogate for the whole retinal vasculature behavior and analyzed the hypoxic response of retinal vasculature as well as the response when baseline conditions were restored. Also, only healthy subjects were included, and thus only physiological responses were presented. However, vascular reactivity is most probably different in patients with ocular disorders, such as normal-tension glaucoma, age-related macular degeneration or diabetic retinopathy. It has been suggested that even small reductions of oxygen in the inspired air limits the capacity of oxidative phosphorylation, with consequent deterioration of visual performance.[126] Therefore, it remains to be understood if individuals with vascular dysregulation disorders may be at risk due to commercial flights related-hypoxia.

Of note, foveal vessel density did not change significantly with oxygen conditions change. The fact that this area in OCTA comprises a 1-mm diameter circle around the foveal center, thus including the foveal avascular zone (FAZ) may explain these results. With the exception of anecdotal reports, the FAZ is completely devoid of blood vessels, being entirely dependent on the choroidal circulation to receive blood supply.[118,127] Therefore, minimal changes with hypoxia would be expected using OCTA.

Contrary to the choroid, which has a prominent autonomic regulation, the retinal vessels'

changes in their diameter and flow are thought to be predominantly mediated by local autoregulatory factors.[6,128,129] Changes in perfusion pressure, oxygen and pH level all contribute to modify smooth muscle tone as a consequence of intraluminal pressure and metabolic activity, respectively.[58,130,131] Facing a hypoxic stress like HCT, these factors interplay and generate the physiologic response depicted above. Nevertheless, as changes in either the upstream or downstream vascular territories may impact the other, the overall effect of these two regulation systems on the observed ocular blood flow are difficult to determine. Published literature inconsistently reports retinal vascular responses to precise PaO_2 levels, which should be better defined in healthy individuals to then be able to evaluate non-physiologic states.[54,57] Given these findings with OCTA, this still-evolving technology may be useful in investigating vascular reactivity, not only ocular diseases such as diabetic retinopathy, age-related macular degeneration and glaucoma, but also in vascular dysregulation associated to central nervous system disorders.

Further studies should be undertaken to quantify changes in vascular behaviors in patients with ocular disease with an underlying compromised vascular component.

Limitations & Conclusions

The results of this study should be interpreted against its limitations.

Firstly, the protocol included a single hypoxic measurement in similar conditions for every participant. The ideal scenario would be to perform constant measurements during the hypoxic stimulus, which was not feasible with the available technology. Moreover, the volunteers were exposed to only 30 minutes of hypoxia. Although in accordance with BTS recommendation of a stable flight cabin hypoxia, this limits the extrapolations of our findings to longer hypoxic stimulus. Thirdly, the built-in software used (AngioAnalytics®, Optovue, USA) calculates vessel

density as a ratio of the white pixels (i.e. retinal vessels: where erythrocyte movement was detected) by the total number of pixels. The vessel density is thus calculated in arbitrary units and its extrapolation to be a valid measurement of blood flow remains to be fully validated for OCTA technology. Future software improvements are desired to more accurately evaluate flow, vessel cross-sectional area and flow velocity. Lastly, the protocol induced a normobaric hypoxic stimulus. If intended to entirely reproduce real flight cabin conditions, a hypobaric stress test should be applied.

In conclusion, this first study evaluating healthy subjects retinal vasculature using a standardized hypoxia stress test and OCTA suggests that hypoxic conditions may be associated with an increase in retinal blood flow. Given the scarce data on this subject, these results may help to understand physiologic responses to a hypoxic stress and design a reliable protocol to investigate this response in disease setting, using the increasingly available and non-invasive OCTA technology.

II. OCTA study of the retinal vascular plexuses in type 1 diabetes without retinopathy

Optical Coherence Tomography Angiography Study of the Retinal Vascular Plexuses in Type 1 Diabetes Without Retinopathy

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Abstract

Aim: Previous data suggest the existence of retinal vascular changes and impaired autoregulation in the very early stages of diabetic retinopathy (DR). We compared the retinal plexuses between patients with type 1 diabetes (T1D) without DR and a demographically similar healthy cohort, using optical coherence tomography angiography (OCTA).

Methods: Patients with T1D and no signs of DR were prospectively recruited from an outpatient clinic. Using OCTA (AngioVue®), the parafoveal superficial (SCP) and deep (DPC) capillary plexus as well as the foveal avascular zone (FAZ) and perimeter were gathered. Mean comparison tests and linear regression analysis were used as statistical tests (STATA v14).

Results: Studied population included 48 subjects (24 T1D). The analysis of SCP revealed an attenuation of the capillary network compared to the control group in both parafoveal (51.8±4.5 vs. 55.8±3.2, p<0.001) and perifoveal (51.9±3.3 vs. 53.9±1.9, p=0.01) regions. A similar finding was observed in the DCP for both parafoveal (56.4±4.3 vs. 60.4±2.2, p<0.001) and perifoveal (54.7±3.9 vs. 60.8±3.4, p=0.001) sectors. Also, a longer time since T1D diagnosis was associated with a larger FAZ area (p=0.055) and perimeter (p=0.03).

Conclusions: Significant differences in the retinal microvasculature were observed between healthy subjects and T1D patients using OCTA, even before clinically detectable disease on fundus biomicroscopy.

Introduction

Diabetes mellitus and specifically diabetic retinopathy (DR) are major public health challenges.[132] Globally, roughly 400 million people have DM, and this number is rise to more than 600 million by 2040.[104,133] Of these, type 1 diabetes (T1D) - which tends to be diagnosed in younger individuals - accounts for approximately 5-10% of the cases.[105] As DR is a leading cause of acquired visual loss in the working-age population, an early and effective management of these patients is of paramount importance.[106] Previous data from studies in animal models[134] and humans[96] suggest the existence of retinal vascular changes and impaired autoregulation in the very early stages of DR.[135]

Previous research with optical coherence tomography (OCT) provided evidence of inner retinal changes in patients with subclinical DR, suggesting that neurodegeneration accompanies early DR development.[136,137] Optical coherence tomography angiography (OCTA) is a novel diagnostic tool that uses infrared wavelengths to provide non-invasive high-contrast high-resolution imaging of the retinal microvasculature.[34,78,79] It is able to detect motion contrast produced by moving blood cells in retinal vessels. Recent advances in projection artefact removal allowed to not only accurately define the superficial plexus, but also the deep retinal vascular layers .[29]

Although some studies report on OCTA findings in type 2 diabetes, currently there is scarce data regarding early changes in younger patients with T1D.[138–141] Therefore, the aim of our study is to compare the superficial and deep retinal vessel plexuses between T1D patients without DR and a cohort of demographically similar healthy subjects using OCTA technology.

Methods

This research protocol followed the tenets of the Declaration of Helsinki[117] and was submitted and approved by the Ethics Committee of Lisbon Academic Medical Center in March 2018. Written informed consent was obtained from all the participants before enrolment, after detailed explanation of the study.

Participants

A total of 27 adult subjects with T1D were prospectively recruited from an adult diabetes clinic. A demographically similar cohort of 24 healthy volunteers served as control group. An anonymous questionnaire was filled out, including age, gender, smoking-pack years, diabetic disease duration and current treatment, other known medical history and current chronic medication. Subjects were asked to abstain from smoking, alcohol and caffeine for at least 6 hours before the study to reduce the possible autonomic effects and measurement bias.[142]

Exclusion ophthalmological criteria for both groups were the presence of any degree of diabetic retinopathy on fundus examination, significant lens opacities, high refractive error (spherical equivalent below -6.50 or above +4.00 diopters), history of glaucoma or ocular hypertension, and neuro-ophthalmic disease. Exclusion systemic criteria were hypertension, nephropathy or other documented microvascular complication, and smokers of more than 5 cigarettes a day. Pregnant women were also not recruited.

Protocol

A complete ophthalmological examination was conducted to all subjects, including best-corrected visual acuity (BCVA), slit-lamp biomicroscopy of the anterior and posterior segment, auto-refractometer and intraocular pressure (RK-5®, Canon Europe®, The Netherlands), colour

fundus photography (CR-2®, Canon, USA) and optical biometry (Lenstar®, Haag-Streit, Switzerland). Glycated haemoglobin (HbA1c) values of T1D arm and time since diagnosis were retrieved from clinical notes.

OCTA examination (AngioVue®, Optovue, CA, USA) was performed by an experienced technician using the standard macular protocol. Parafoveal and perifoveal vessel densities in the superficial and deep plexuses were calculated automatically calculated using AngioAnalytics®, the built-in software of the OCTA device. Foveal avascular zone area and perimeter were also calculated. Only high-quality images (score higher than 8/10) were considered. The device used was the latest version available, which includes the latest projection artefact removal algorithm.[29]

Sample size

Based on previous OCTA studies[143,144], sample size was calculated considering a 10% clinically significant difference in vessel density between groups, and a standard deviation of 5%. Accordingly, considering a power of 90%, an alpha value of 0.05, a minimum of 17 T1D patients and 17 controls are necessary to include.

Statistical analysis

Statistics were performed using STATA v14 (StataCorp LP, College Station, Texas, USA). All quantitative data were expressed as mean ± standard deviation. Comparisons of means between groups was done using Student's t-test. A multivariate linear regression model was used to assess correlation between continuous variables. P-values < 0.05 were considered to be statistically significant.

Results

After excluding three T1D patients due to treated systemic hypertension, 48 subjects (24 T1D without DR and 24 healthy controls) were studied, with similar demographic and baseline ophthalmological characteristics - Table 1. In the T1D group, the time since diagnosis was 13.6 ± 9.7 [range 1-35] years, and mean HbA1c value was 8.0% ± 1.4% [range 6.2%-11.5%]. Mean BCVA was 0.0 LogMar in both groups, and no significant abnormalities were found on ophthalmological examination of the included patients.

The analysis of superficial capillary plexuses revealed a rarefaction of the capillary network compared to the control group in both parafoveal (51.8 ± 4.5 vs. 55.8 ± 3.2, p<0.001) and perifoveal (51.9 ± 3.3 vs. 53.9 ± 1.9, p=0.01) sectors. Similar findings were observed for deep capillary plexuses in both parafoveal (56.4 ± 4.3 vs. 60.4 ± 2.2, p<0.001) and perifoveal (54.7 ± 3.9 vs. 60.8 ± 3.4, p=0.001) sectors – Table 2.

No significant differences were found in mean FAZ area and perimeter when comparing diabetic and control groups. However, in an age-controlled linear regression model, a longer time since T1D diagnosis was associated with a larger FAZ area (p=0.06) and perimeter (p=0.03). Moreover, T1D patients with a longer duration of disease were shown to have decreased vessel densities in the parafoveal (p=0.05) and perifoveal (p=0.04) superficial plexuses, but not in the deep plexuses.

Age, gender, and HbA1c values were not significantly correlated with any of the analysed outcomes.

Table 1 Demographic and baseline data

	Control group	Type 1 diabetes	*p*-value
Age, years	31.8 ± 8.2	35.6 ± 10.4	0.17
Male/female, *n*	10/14	10/14	–
Body mass index, kg/m^2	22.6 ± 3.0	24.4 ± 3.0	0.12
Intraocular pressure, mmHg	13.3 ± 2.1	14.6 ± 3.0	0.14
Axial length, mm	24.1 ± 0.9	23.5 ± 1.0	0.07

The data are expressed as mean ± standard deviation, when applicable
P-values obtained with Student's *t* test

Table 2 OCT-angiography quantitative analysis of retinal vascular plexuses

	Control	Type 1 diabetes without DR	*p*-value
Parafoveal			
Superficial plexus	55.8 ± 3.2	51.8 ± 4.5	< 0.001
Deep plexus	60.4 ± 2.2	56.4 ± 4.3	< 0.001
Perifoveal			
Superficial plexus	53.9 ± 1.9	51.9 ± 3.3	0.01
Deep plexus	60.8 ± 3.4	54.7 ± 3.9	0.001
FAZ area, mm^2	0.23 ± 0.10	0.21 ± 0.07	0.52
FAZ perimeter, mm	1.83 ± 0.44	1.81 ± 0.31	0.83

DR diabetic retinopathy, *FAZ* foveal avascular zone
The data are expressed as mean ± standard deviation
P-values obtained with Student's *t* test

Discussion

In this OCTA study, a quantitative comparative analysis of different retinal vascular plexuses between patients with T1D without DR and a healthy cohort was performed. We were looking for the potential early vascular changes in T1D - even before clinically detectable disease - in order to better understand DR pathophysiology and ultimately improve the management of the disease.

Although OCTA technology allowed us to non-invasively obtain images with unprecedented detail of the retinal microvasculature, [34,78,79] some aspects still need improving. Specifically, one of its main recognised limitations has been the appearance of projection artefacts in deeper layers.[29] It should be mentioned that the device used in our study includes a projection artefact removal software, which allows to accurately define the deep retinal vascular layers and not only the superficial plexus.

Our study reports the existence a significantly decreased macular vascular density in both superficial and deep plexuses in T1D patients without DR, comparatively to a healthy cohort. These early microvascular changes in T1D are in line with two studies published on the subject.[138,145] However, a recent study in a paediatric T1D population did not observe such difference.[139] Such variations are most probably justified by the significant differences in T1D duration among studies (13.6 ± 9.7 years in our study vs. 11.0 ± 4.0[138] vs. 6.4 ± 6.2[139]), being lowest in the latter study, in which no differences were found. Accordingly, we found that longer disease duration was associated with a decreased vessel density in superficial plexuses. Therefore, it is reasonable to think that the same paediatric population will eventually present similar OCTA findings with a similar disease duration in the future.

As one of the most energy-demanding tissues in the body, the retina requires an effective blood flow regulation for its normal functioning [34]. It has the ability for local autoregulation, which is important to keep blood flow relatively constant despite the variations in perfusion pressure [146]. This study suggests OCTA is sensitive enough to detect significant microvascular changes in

both superficial and deep retinal plexuses' before clinically detectable DR. These findings may correspond to an early stage of retinal blood flow autoregulation compromise, which may eventually lead to the sequence of events in the natural history of diabetic eye disease. As a complex disease, DR has been a field of controversy regarding relative contribution of each pathway and cascade into the overall outcome of vascular occlusion and retinal ischemia [147]. The multitude of confounding factors such as advancing age, concomitant vascular conditions, accompanying macrovascular diseases, systemic medications that can interfere with autonomic nervous system response make this area of research a challenging one. Early diagnosed T1D are a subset of diabetic patients where these specific confounding aspects are minimised [148], thus allowing a more direct correlation between the retinal vascular response with the disease. Another strength of our results is the representative sample regarding the population demographics and wide range of time elapsed since T1D diagnosis.

Despite the mentioned differences observed in macular vascular density, FAZ area and perimeter were not different between the two groups. We hypothesise that a rarefaction in vessel density may precede the FAZ enlargement, which may be detectable only later in the course of the disease, as previously reported in type 2 diabetic patients with DR.[149–151] Another finding supporting this argument is the fact that we found a statistically significant association between longer T1D duration and larger FAZ area and perimeter. This suggests that changes in FAZ are also present before clinically detectable DR in patients with T1D. On the other hand, HbA1c values were not linearly associated with any of the outcomes. A clear explanation is lacking, but the fact that we used only one HbA1c value (and not mean values since diagnosis), the small standard deviation (i.e. a relatively homogeneous T1D sample), the fact that HbA1c does not measure glucose variability, and the multifactorial nature of the disease may all have contributed for the lack of significance.

Some limitations should be acknowledged. Firstly, the study is cross-sectional in nature and although the series is larger than the calculated sample size, all patients were Portuguese and

Caucasian (with the exception of two African T1D patients). Therefore, even being very likely to encounter similar findings in different populations and ethnicities, external validity is inherently limited. Secondly, OCTA technology and the built-in analytics software calculate vessel density by image binarization, and its extrapolation as valid blood flow measurement is still to be fully validated, as in every OCTA device. In the future, we suggest that exploring other vascular quantification methods and fractal dimension analysis and compare them with the reported OCTA outcomes would provide new insights in this field.

Conclusion

Our work suggests there are significant differences in the retinal microvasculature between healthy subjects and T1D patients, even before clinically detectable disease on fundus biomicroscopy. OCTA is a promising technology in the early diagnosis and management of diabetic eye disease.

A Protocol to Evaluate Retinal Vascular Response using Optical Coherence Tomography Angiography

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Abstract

Introduction: Optical coherence tomography angiography (OCTA) is a novel diagnostic tool with increasing applications in Ophthalmology clinics that provides non-invasive high-resolution imaging of the retinal microvasculature. Our aim is to report in detail an experimental protocol for analysing both vasodilatory and vasoconstriction retinal vascular responses with the available OCTA technology.

Methods: A commercial OCTA device was used (AngioVue®, Optovue, CA, USA) and all examinations were performed by an experienced technician using the standard protocol for macular examination. Two standardized tests were applied: i) the hypoxia challenge test (HCT) ii) the handgrip test, in order to induce a vasodilatory and vasoconstriction, respectively. OCTA was performed at baseline conditions and during the stress test. Macular parafoveal vessel density of the superficial and deep plexuses was assessed from the *en face* angiograms. Statistical analysis was performed using STATA v14.1 and a $p < 0.05$ was considered for statistical significance.

Results: Twenty-four eyes of 24 healthy subjects (10 male) were studied. Mean age was 31.8 ± 8.2 years [range 18-57 years]. Mean parafoveal vessel density in the superficial plexus increased from 54.7 ± 2.6 in baseline conditions to 56.0 ± 2.0 in hypoxia ($p<0.01$). Mean parafoveal vessel density in the deep plexuses also increased, from 60.4 ± 2.2 at baseline to 61.5 ± 2.1 during hypoxia ($p<0.01$). The OCTA during the handgrip test revealed a decrease in vessel density in both superficial (55.5 ± 2.6 to 53.7 ± 2.9, $p < 0.001$) and deep (60.2 ± 1.8 to 56.7 ± 2.8, $p < 0.001$) parafoveal plexuses.

Discussion: In this work we detail a simple, non-invasive, safe and non-costly protocol to assess a central nervous system vascular response (i.e. the retinal circulation) using OCTA technology. A vasodilatory and vasoconstriction response were observed in two physiologic conditions – mild hypoxia and isometric exercise, respectively. This protocol constitutes a new way of studying retinal vascular changes which may be applied in health and disease of multiple medical fields.

Introduction

In the central nervous system, the possibility of direct visualization of the vascular system is unique to the retina vessels, which derive from the ophthalmic artery, the first branch of the internal carotid artery.[152] As one of the most metabolically active tissues in the body, the retina requires an effective blood flow regulation for its normal functioning [34]. It has the ability for local autoregulation, which is important to keep blood flow relatively constant despite the variations in perfusion pressure [146].

The impairment of the normal retinal vascular response is reported in the early stages of a number of ocular diseases, such as diabetic retinopathy [91–94], age-related macular degeneration and glaucoma [96,97]. Therefore, the study of retinal vessels behavior and blood flow regulatory function is crucial to increase our knowledge about the mechanisms behind several ocular vascular diseases.

A number of non-invasive methods have been used to for retinal vessels' assessment, including laser Doppler velocimetry [153], laser Doppler flowmetry [85,86], laser speckle flowgraphy [89], blue-field entoptoscopy [56,154], and colour Doppler imaging [87,88]. However, these devices and techniques are not widely available in clinic, being mostly limited to research purposes [34,90].

Optical coherence tomography angiography (OCTA) is a novel diagnostic tool with increasing applications in Ophthalmology clinics. OCTA technology uses infrared wavelengths to provide non-invasive high-contrast high-resolution imaging of the retinal microvasculature [34,78,79]. This technology is an extension of the widely used optical coherence tomography (OCT), and generates images of unprecedent detail by interferometrically measuring the amplitude and delay of reflected or backscattered light from moving erythrocytes. It does so by detecting motion contrast produced by moving blood cells in retinal vessels. Retinal blood flow induces a change between sequential B-scans, while no-flow areas produce no variation. Since no motion

in the retina other than blood flow is expected, stationary objects will not produce a significant change in sequential images, while moving objects produce a detectable change. By comparing changes over time, the generated final image clearly defines retinal microvasculature. Recent advances in projection artefact removal allowed to accurately define the deep retinal vascular layers and not only the superficial plexus, overcoming one of its main limitations [29]. Its potential for clinical use is tremendous, not only by allowing clinical evaluation of vascular pathologies with no need for invasive procedures, but also it can allow quantitative assessment of the retinal vascular bed. Furthermore, it unlocks new possibilities in detecting functional changes in what otherwise would be subjects with no visible structural defects.

Our research group has previously reported the potential of OCTA to detect changes in retinal vessels, having recently published a proof of concept in healthy volunteers to characterize the physiologic retinal vascular response under hypoxic conditions. This work confirmed the ability of this technology to non-invasively detect a significant retinal vasodilatory response to a mild hypoxic stress, in healthy volunteers [155].

Given the reported ability of OCTA to assess dynamic retinal vascular changes, this manuscript aims to report in detail a protocol for analysing both vasodilatory and vasoconstriction retinal vascular responses to a standard stimulus with the widely used clinically available OCTA technology.

Material and Methods

i) Ethics and Informed Consent

This research protocol follows the tenets of the Declaration of Helsinki [117] and was submitted and approved by the Ethics Committee of Lisbon Academic Medical Center in March 2018. Written informed consent was obtained from all the participants before enrolment, after detailed explanation of the objectives, procedures and risks of the study. Two standardized tests were applied: i) the hypoxia challenge test (HCT) [156], and ii) the handgrip test [108].

As recommended by the Ethics Committee, in order to minimize ethical concerns regarding the HCT, patients and volunteers recruited must had the intention to fly in the future. All the safety recommendations regarding the handgrip test were also followed and the test stopped if necessary [108]. Only the physicians had access to each subject electronic health records. Medical confidentiality was assured. By agreeing to be part of this study, all the participants had access to a comprehensive ophthalmological exam. At any time, it was granted to all the enrolled subjects the possibility to anonymously withdraw the study.

ii) Participants

Twenty-four healthy volunteers were recruited. An anonymous questionnaire was carried out, including the following questions: age, gender, smoking-pack years, known diseases and current chronic medication, previous intraocular surgery or trauma, symptoms during previous flights and intention to fly in the future. Subjects were also asked to abstain from alcohol and caffeine for at least 6 hours before the study to reduce the possible autonomic effects and measurement bias [142], and were instructed to rest for 10 minutes in a sitting position before the protocol start.

Exclusion ophthalmological criteria were: the presence of significant lens opacities (Lens

Opacities Classification System III equal to or more stage 2), high refractive error (spherical equivalent below -6.50 or above +4.00 diopters), history of glaucoma or ocular hypertension, neuro-ophthalmic disease, and previous intraocular surgery. Exclusion systemic criteria included: hypertension (defined as systolic blood pressure higher than 140 mmHg and diastolic blood pressure higher than 90 mmHg or use of anti-hypertensive drugs), nephropathy or other documented microvascular complication, diabetes mellitus, local or systemic inflammatory diseases, those taking vasoactive drugs, and smokers of more than 20 cigarettes a day. Pregnant women were excluded.

iii) Protocol

Firstly, the study protocol was explained individually to every subject, a written consent given, and the questionnaire filled. Then, a complete ophthalmological examination was conducted to all subjects, including best-corrected visual acuity, slit-lamp biomicroscopy with fundoscopy, auto-refractometer (RK-5®, Canon Europe®, The Netherlands), fundus photography (CR-2®, Canon, USA), intraocular pressure and eye biometry (Lenstar®, Haag-Streit, Switzerland). Other baseline measurements performed included arterial pressure (Carescape® V100, GE Healthcare, Portugal), and pulse oximetry. Room temperature was kept at 22ºC and similar mesopic conditions were adopted throughout the study.

a. *Optical Coherence Tomography Angiography*

A commercial OCTA device was used (AngioVue®, Optovue, CA, USA), which has an A-scan rate of 70.000 A-Scan/second with 5 µm axial resolution, and uses the split-spectrum amplitude-decorrelation angiography (SSADA) algorithm, thus enhancing signal-to-noise ratio of flow detection. The device used also included the latest projection artifact removal algorithm,

allowing for a more precise deep plexus analysis.

All examinations were performed by an experienced technician at the required timepoints using the standard protocol for macular examination (3 x 3 mm^2). Two repeated scans were performed at baseline and during the stress test, respectively. Vessel density of the superficial and deep plexuses was assessed from the *en face* angiograms, by analyzing a predefined annulus with an outer diameter of 3 mm and an inner diameter of 1 mm, corresponding to the parafoveal region. This variable was automatically gathered using AngioAnalytics®, the built-in software of the OCTA device, as a ratio of the white pixels to the total number of pixels (i.e. the proportion of the image occupied by retinal vessels [157]). Only high-quality images (high signal strength, focused, and without movement artifacts) were considered.

b. Vasodilatory response - Hypoxia Challenge Test

The vasodilatory response with retinal blood flow increase in response to a decreased arterial oxygen values has been already reported as a physiologic response, mainly associated with the local release of hypoxia-related metabolites, such as retinal relaxing factor, prostacyclin and lactate [6,57].

The following protocol was designed in order to comparatively characterize with OCTA the retinal vessel density changes induced in hypoxia conditions using HCT as a hypoxic stress test. The HCT is performed at sea level in order to create a normobaric hypoxic environment by reducing FiO_2 and making it equivalent to the flight cabin values. The British Thoracic Society (BTS) proposes a practical and inexpensive protocol to perform HCT [156]. Briefly, participants had to breath a FiO2 of 15% by using a gas mixture with a supply of 99,993% nitrogen (Linde Healthcare®, Portugal) through a 40% flow Venturi mask (Intersurgical®, UK) at 10L/min. Cardiorespiratory monitoring during HCT was performed using a polygraph and an oximeter in a hand finger (Alice PDX, Philips-Respironics®, USA). Other parameters monitored during HCT

were oxygen peripheral saturation, arterial pressure and continuous electrocardiography.

As established by BTS, recommended HCT duration to obtain stable conditions is 20 minutes. Accordingly, OCTA was performed at baseline and then, again, 30 minutes after HCT start, under the described hypoxic conditions. Then, the Venturi mask and cardiorespiratory monitoring devices were withdrawn. All symptoms were recorded, and the test stopped if medically necessary.

c. Vasoconstrictive response - Handgrip Test

The handgrip test, as an isometric exercise, is a sympatheticomimetic test causing steady and safe increases in heart rate and arterial pressure. Therefore, the associated physiologic retinal vascular response consists in a vasoconstriction response [71,108,111].

The following protocol was conducted after previously explained in detail to all participants. Subjects had to be sat in a chair in front of the OCTA device, with the forearm in neutral position, the elbow flexed at 90º, and wrist with the thumb facing upwards. The participants were asked to hold a Jamar hydraulic dynamometer, and maximal grip force (MGF) was calculated with his dominant arm. Motorization of arterial pressure was performed in the contralateral arm. The participants were then instructed to relax and place the chin in the OCTA chinstrap and be prepared for examination. When ready, a voice signal requested the participant to keep a contraction of at least one third of the maximal calculated force for 3 to 5 minutes (monitored by an investigator). After 90 seconds, the OCTA acquisition started, being completed for both eyes within the 3 to 5-minute handgrip test. The arterial pressure in the contralateral arm was measured every minute and registered. According to the handgrip test recommendations [108], if a diastolic blood pressure higher than 120 mmHg and/or any adverse symptom was registered, the test was interrupted. The procedure was repeated after a 15-minute

resting time if any reliability-limiting situation occured.

iv) Statistics

Statistical analysis was performed using STATA v14.1. A repeated-measures ANOVA model was used to assess differences between the baseline and stress measurements. The Shapiro-Wilk and Skewness/Kurtosis test suggested the normal distribution of the variables considered, and the inexistence of significant outlier values was also confirmed. Equality of variances was investigated and the results reported accordingly, applying the Greenhouse-Geisser correction when variables' variances were not equal. A $p < 0.05$ was considered for statistical significance. To guarantee independence of observations, only the right eye of each patient was considered for analysis.

Results

i) Demographics and Baseline data

Twenty-four eyes of 24 healthy subjects (10 male) were studied. Mean age was 31.8 ± 8.2 years [range 18-57 years]. Mean best-corrected visual acuity was 0.0 LogMar, mean intraocular pressure was 13.3 ± 2.1 mmHg (range 10-18 mmHg), with a mean spherical equivalent of -1.3 ± 1.8 D (range -5 to -2 D) and mean axial length of 24.12 ± 0.9 mm (range 22.5-25.9 mm). Mean body mass index was 22.6 ± 3.0 Kg/m² (range 18.6-28.7 Kg/m²).

ii) Vasodilatory response – Hypoxia Challenge Test

The peripheral oxygen saturation decreased from 98±1% to stable minimum values of 87±2% during HCT – Table 1. The mean parafoveal vessel density in the superficial plexus increased from 54.7 ± 2.6 in baseline conditions to 56.0 ± 2.0 in hypoxia ($F_{1,23}$= 15.69, p < 0.001). The mean parafoveal vessel density in the deep plexuses also increased, from 60.4 ± 2.2 at baseline to 61.5 ± 2.1 during hypoxia ($F_{1,23}$= 16.26, p < 0.001) – Table 1. The increase in vessel density was observed in 22 (92%) of the 24 eyes in both plexuses.

TABLE 1 | Systemic variables and retinal vascular response to the hypoxia challenge test.

		Baseline	Hypoxia	*p*-value
SAP (mmHg)		118 ± 11	114 ± 10	0.15
DAP (mmHg)		75 ± 9	75 ± 10	0.29
MAP (mmHg)		89 ± 9	88 ± 9	0.97
Heart rate (bpm)		64 ± 8	76 ± 12	0.03
O_2 Hb saturation (%)		98 ± 1	87 ± 2	<0.0001
Parafoveal vessel density	Superficial plexus	54.7 ± 2.6	56.0 ± 2.0	<0.001
	Deep plexus	60.4 ± 2.2	61.5 ± 2.1	<0.001

Mean values and standard deviations are presented. bpm, beats per minute; DAP, diastolic arterial pressure; Hb, hemoglobin; MAP, mean arterial pressure; SAP, systolic arterial pressure.

iii) Vasoconstrictive response – Handgrip Test

As depicted in table 2, the handgrip test was associated with an expected heart rate, systolic and diastolic blood pressure increase compared to baseline (all p<0.001).

The OCTA during the handgrip test revealed that isometric exercise elicited a decrease in vessel density in both superficial (55.5 ± 2.6 to 53.7 ± 2.9, $F_{1,23}$= 27.37, p < 0.0001) and deep (60.2 ± 1.8 to 56.7 ± 2.8, $F_{1,23}$ = 27.90, p < 0.0001) parafoveal plexuses – Table 2. The decrease in vessel density was observed in 22 (92%) and 23 (96%) of the 24 eyes in the superficial and deep plexuses, respectively.

Regarding the systemic response, the mean percent increase in mean arterial pressure and heart rate was 32 ± 1.7 % and 23 ± 1.7 %, respectively. Table 2 summarizes both OCTA and cardiovascular response findings to the handgrip test.

TABLE 2 | Systemic variables and retinal vascular response to the handgrip test.

		Baseline	Handgrip	*p*-value
SAP (mmHg)		117 ± 12	150 ± 18	<0.0001
DAP (mmHg)		78 ± 10	102 ± 14	<0.0001
MAP (mmHg)		91 ± 10	118 ± 15	<0.0001
Heart rate (bpm)		64 ± 8	78 ± 10	<0.0001
Parafoveal vessel density	Superficial plexus	55.5 ± 2.6	53.7 ± 2.9	<0.0001
	Deep plexus	60.2 ± 1.8	56.7 ± 2.8	< 0.0001

Mean values and standard deviations are presented. Bpm, beats per minute; DAP, diastolic arterial pressure; MAP, mean arterial pressure; SAP, systolic arterial pressure.

Discussion

As an energy-demanding tissue, the retina blood flow autoregulatory mechanisms are crucial to keep blood flow relatively constant despite the variations in perfusion pressure. [34,146]. In this study with OCTA, we report a standardized, reliable and non-invasive way of studying retinal vascular vasodilatory and vasoconstrictive responses to hypoxia and isometric exercise, respectively.

Despite the constant blood flow thought to be provided by the choroidal circulation, retinal vessels are believed to present a large reserve for vasodilation and vasoconstriction in order to balance changes in the arterial pressure of oxygen [57,158]. The encountered vasodilatory response in mild hypoxic conditions are consistent with findings of previous studies using different technologies, such as blue field entoptic phenomenon and scanning laser Doppler flowmetry [56,159]. The stress test used in our study – HCT, consistently induced the expected systemic responses, with an increase in heart rate accompanying the hypoxemia. A few OCTA studies have been published reporting the vasoconstrictive response to hyperoxia [143,160]. However, to the best of our knowledge, our group was the first to report the vasodilatory response to hypoxia, using the standardized HCT as the hypoxic stimulus.

Although it is believed that autonomic innervation of retinal vasculature is not significant [6,161], the vasoconstrictive response to isometric exercise was clearly observed in our study. This response was associated with the expected sympathetic nervous system induced increase in arterial blood pressure and heart rate. As previously described, this regulation should be, at least partially, induced by the local response to the increase in arterial pressure – the Bayliss effect in retinal autoregulation [42,111]. This observed vasoconstrictive response in retinal vessels is similar to the one described in peripheral arteries' behaviour [108]. However, with still so much to unveil about mechanisms behind the retinal autoregulation, we are not able to definitely attribute this retinal vascular response to a specific factor.

For both retinal vascular responses, the differences observed in our sample were striking and quite homogeneous, with more than 90% of the subjects presenting a similar vessel density change in both plexuses. This reinforces the potential of the above-described protocols as a repeatable method for the evaluation of retinal vascular function, in a healthy retina and possibly in a vascular setting as well. Although the clinical significance of the statistically significant findings encountered should be discussed, the magnitude of change we encountered is comparable to other OCTA studies. For example, Simonett and collaborators [145] were able to distinguish a healthy cohort from patients with no or mild diabetic retinopathy with changes in vessel density similar in magnitude to the ones reported in our study.

In a recent study, Hagag et al. have demonstrated a reduction on the flow index and vessel density (-7.8%) of the deep capillary plexus only and in the flow index of the all-plexus slab measured by OCTA with hyperoxia. These authors describe an approach to induce hyperoxia that consists in the fitting of a simple face mask and giving supplemental oxygen for 10 minutes at a flow rate of 15 litters per minute, which delivers 60-90% oxygen in the inspired oxygen, therefore, creating a systemic hyperoxic condition [160]. In contrast, in our work, in order to induce vasoconstriction, we used the handgrip test. We found a significant decrease in the vessel density of both superficial and deep plexus, although with a lower magnitude of change, as detailed in Table 2. The small sample in the work of Hagag et al. and the fact that another vasoconstrictor stimulus (with a different physiologic mechanism) was chosen may have contributed to this difference. These results should be validated by performing both tests (induced hyperoxia and handgrip tests) and comparing the retinal vascular response with OCTA technology.

Regarding the vasodilatory response, an alternative method to induce retinal vessels dilation that could be considered is the hypercapnia test, described elsewhere [162,163]. However, compared to our choice to induce a safe level of hypoxia, this test involves re-inhalation of expired air by inserting a length of corrugated tube between the Y-piece and the endotracheal tube, which

increases the deadspace by a volume similar to the tidal volume (V_T) obtained with a pressure support of 7 cmH_2O [162]. Comparatively, we believe that our work presents a simpler, safer and reproducible methodology to induce a detectable vasodilation in the retina using OCTA.

After establishing a protocol to evaluate retinal vascular response with OCTA, it will be possible to study not only the healthy eye, but also responses in a compromised vascular setting, such as in diabetic retinopathy, age-related macular degeneration and glaucoma. We hypothesize that local factors may play a crucial role when it comes to the retinal vascular regulation and responses in metabolic diseases. In fact, a recent thesis [164] has suggested that retinal autoregulation are mostly due to myogenic and metabolic factors in order to accommodate local blood flow to differences in perfusion pressure and metabolic needs. In this context, we think that this protocol, once established, could be used to assess functional changes in the retinal vascular physiology before clinically detectable diseases, taking the paradigmatic example of DR.

In summary, this work details a simple, non-invasive, safe and non-costly method to assess vascular changes in healthy subjects that can be the stepping-stone for several experiments. It has gone some way towards enhancing our understanding of possible and reproducible methods of induce and detect vasoconstriction and vasodilation in retinal vessels. Because OCTA technology and devices are increasingly used in Ophthalmology, we hope that our research will serve as an encouragement for the devolvement of technology capable of dynamically assessing the vascular response in opposition to static images only. In fact, and contrary to other devices with mainly research purposes, OCTA technology is easy to use and increasingly available in ophthalmology clinical practice worldwide [165] and therefore we think that establishing a protocol with an available tool will have impact in the Ophthalmology community.

Limitations

As a first limitation of our study, we should mention the relatively small sample size, which limits association analysis with demographic and ophthalmic features of the participants. Secondly, the current OCTA technology is still evolving, and one should be careful in the interpretation of the findings and their magnitude. As an example, with the current device, we are not able to estimate absolute blood flow values, but only *perfused vessel densities*. Thirdly, in both protocols, there is an autonomic nervous system activation and, therefore, we are not able to isolate the factor inducing the retinal vascular response from the autonomic-related vascular consequences. Moreover, despite reporting statistically significance in the parafoveal vessel density means in response to both the hypoxic and handgrip tests, we are unsure about the clinical significance of these differences. In fact, there are no current data in literature to indicate what would normal inter-exam variation be when calculating parafoveal vessel density. To be able to comment more accurately about the obtained results, it is essential to know the error associated with the built-in software and then be able to distinguish clinical change from measurement variability. Assessing the reproducibility of these measurements would then be a next step to overcome this caveat [166]. With this study, we expect to promote the development of the technology in order to allow for larger studies to confirm our results with a dynamic (and continuous, ideally) analysis of retinal vascular responses in health and disease, with potentially relevant diagnostic and therapeutic implications.

Conclusion

This study on human volunteers constitutes a proof of concept on how to evaluate a central nervous system response (i.e. the retinal circulation) in two physiologic conditions – hypoxia and isometric exercise. The importance of identifying such response with a rapid, non-invasive, and reliable technology used in clinical practice - OCTA, may be a steppingstone for new lines of research not only in Ophthalmology, but also in physiology and neuroscience.

IV. Retinal Vascular Reactivity in Type 1 Diabetes Patients Without Retinopathy Using OCTA

Retinal Vascular Reactivity in Type 1 Diabetes Patients Without Retinopathy Using Optical Coherence Tomography Angiography

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ABSTRACT

Purpose: We hypothesize that patients with type 1 diabetes (T1D) may have abnormal retinal vascular responses before diabetic retinopathy (DR) is clinically evident. Optical coherence tomography angiography (OCTA) was used to dynamically assess the retinal microvasculature of diabetic patients with no clinically visible retinopathy.

Methods: Controlled non-randomized interventional study. The studied population included 48 eyes of 24 T1D patients and 24 demographically similar healthy volunteers. A commercial OCTA device (AngioVue®) was used and two tests were applied: (i) the hypoxia challenge test (HCT) and (ii) the handgrip test, in order to induce a vasodilatory or vasoconstrictive response, respectively. The HCT is a standardized test that creates a mild hypoxic environment equivalent to a flight cabin. The handgrip test (i.e. isometric exercise) induces a sympathetic autonomic response. Changes in the parafoveal superficial and deep capillary plexuses in both tests were compared in each group. Systemic cardiovascular responses were also comparatively evaluated.

Results: In the control cohort, the median parafoveal superficial and deep plexuses' vessel density increased during hypoxia ($F_{1,23}$=15.69, $p<0.001$ and $F_{1,23}$=16.26, $p<0.001$, respectively). In the T1D group, this physiological response was not observed in neither the superficial, nor the deep retinal plexuses. Isometric exercise elicited a significant decrease in vessel density in both superficial and deep plexuses in the control group ($F_{1,23}$=27.37, $p<0.0001$ and $F_{1,23}$=27.90, $p<0.0001$, respectively). In the T1D group, this response was noted only in the deep plexus ($F_{1,23}$=11.04, $p<0.01$).

Conclusions: Our work suggests there is an early impairment of the physiologic retinal vascular response in patients with T1D without clinical diabetic retinopathy.

Introduction

Diabetes mellitus and diabetic retinopathy (DR) in particular are major public health challenges and a leading cause of blindness in the working age population worldwide.[132,167] Before the first typical signs of DR are detected on retinal examination, it is believed that substantial neural retinal damage and subclinical microvascular changes have already developed.[136,147] In fact, there is cumulative evidence of an altered neurovascular coupling early in the pathophysiology of the disease. Previous studies using the laser Doppler flowmeter, functional magnetic resonance imaging, the dynamic vessel analyser and flicker electroretinography suggest that this abnormal retinal vessels' autoregulation is associated with an increased risk of DR progression.[147,168–172]

Optical coherence tomography angiography (OCTA) is an extension of structural OCT with increasing applications in both clinical and research settings. OCTA technology uses infrared wavelengths to provide non-invasive high-contrast imaging of the retinal microvasculature with unprecedented resolution. It does so by detecting motion contrast produced by moving red blood cells in retinal vessels over sequential B-scans, without any need for a contrast injection. By examining serial images over time, the generated final image clearly defines retinal vascular plexuses.[29,34,78,79,165]

Recently, a few studies using OCTA have been reporting structural quantitative changes (i.e. reduced vessel density and increased foveal avascular zone area) in patients with diabetes before clinically evident DR.[138,173–177] However, little is known about the possibility of using OCTA to evaluate individual functional retinal vascular changes. Our group recently published a safe, reproducible and inexpensive protocol to assess retinal microvasculature reactivity in healthy subjects, detailing how OCTA technology is able to detect retinal vasodilation and vasoconstriction in response to two physiologic conditions — mild hypoxia and isometric exercise, respectively.[178]

We hypothesize that patients with diabetes may have altered physiologic retinal vascular responses early in the natural history of the disease. Therefore, OCTA was used to dynamically study the retinal microvasculature of diabetic patients with no visible signs of retinopathy, thus contributing to the understanding of the earliest processes of DR development.

Material and Methods

i) Ethics and Informed Consent

Our research protocol follows the tenets of the Declaration of Helsinki[117] and was submitted and approved by the Ethics Committee of Lisbon Academic Medical Center. Written, informed consent was obtained from all participants after detailing the aims, procedures and risks of the study. Two standardized tests were applied: i) the Hypoxia Challenge Test (HCT)[156] and ii) the Handgrip Test[108]. As recommended by the Ethics Committee, in order to minimize ethical concerns regarding the HCT, patients and volunteers recruited must had the intention to fly in the future. All the safety recommendations regarding the handgrip test were also followed and the test was discontinued if necessary.[108] Medical confidentiality was assured. At any time, subjects could anonymously withdraw from the study. The study protocol has been registered in the ISRCTN clinical studies online platform with the number #98388473, available online at http://www.isrctn.com/ISRCTN98388473.

ii) Study Design, Participants and Inclusion/Exclusion Criteria

A controlled non-randomized interventional study was conducted, including one group of patients with type 1 diabetes (T1D) without clinical signs of DR, and a demographically similar control group of healthy subjects.

Patients with T1D were recruited from an adult diabetes outpatient clinic, and a demographically (age and gender) similar sample of healthy volunteers was selected as a control group. An anonymous questionnaire was carried out, including the following questions: age, gender, smoking-pack years, known diseases and current chronic medication, previous intraocular surgery or trauma, symptoms during previous flights, and intention to fly in the future. Clinical data available from the electronic health records included demographic

characteristics, time from T1D diagnosis, glycated haemoglobin (HbA1c) level, current medication, comorbidities, and presence of microalbuminuria. Subjects were also asked to abstain from alcohol and caffeine for at least 6 hours before the study to reduce the possible autonomic effects and measurement bias.[142] Patients with T1D were treated with rapid acting insulin analogues (continuous subcutaneous insulin infusion - CSII), or long and rapid acting insulin analogues (multiple daily injections - MDI). All volunteers were asked not to eat or to take insulin (MDI) or insulin boluses (CSII) in the 2 hours preceding the study, in order to minimize its vasodilatory effects in the observed vascular response.[179] It was also confirmed before the start of the experimental protocol that no diabetic patient had hypoglycaemia (<70mg/dL) or level 1 hyperglycaemia (>180mg/dL). Lastly, in order to minimize the effect of diurnal variations in the systemic and ocular measurements, the individuals of each group were evenly distributed among the scheduled morning and afternoon study sessions.

Ophthalmic exclusion criteria for both groups were as follows: the presence of significant lens opacities (Lens Opacities Classification System III equal to or more stage 2), diabetic retinopathy, high refractive error (spherical equivalent below -6.50 or above +4.00 diopters), history of glaucoma or ocular hypertension, neuro-ophthalmic disease, and previous intraocular surgery. Systemic exclusion criteria included the following: hypertension (defined as systolic blood pressure higher than 140 mmHg and/or diastolic blood pressure higher than 90 mmHg), medically treated hypertension, nephropathy or other documented microvascular complications, local or systemic inflammatory diseases, and smokers of more than 20 cigarettes a day. Pregnant women were also excluded.

iii) Study Protocol

Firstly, the study protocol was explained individually to every subject, written consent was obtained, and the health questionnaire completed. Then, all subjects underwent a complete ophthalmological examination including: best-corrected visual acuity, slit-lamp biomicroscopy

with fundoscopy, auto-refraction (RK-5®, Canon Europe, The Netherlands), fundus photography (CR-2®, Canon, United States), intraocular pressure measurement, and ocular biometry (Lenstar®, Haag-Streit, Switzerland). Other baseline measurements performed included arterial blood pressure and pulse oximetry (Carescape V100®, GE Healthcare, Portugal). Room temperature was maintained at 22ºC, and consistent mesopic conditions were maintained throughout the study.

A commercial OCTA device was used (Optovue Avanti XR, version 2017.1.0.151, CA, United States), with an A-scan rate of 70,000 A-Scan/s with 5-mm axial resolution and using a split-spectrum amplitude-decorrelation angiography (SSADA) algorithm, thus giving an enhanced signal-to-noise ratio of flow detection. The device used also included the latest projection artefact removal software, allowing for a more precise analysis of the deep plexus. All examinations were performed by an experienced technician at the determined timepoints using the 6 x 6 mm standard protocol for a macular OCTA examination. Two repeated scans were performed – one at baseline and another during the stress test. Vessel density of the superficial and deep plexuses were assessed from the *en face* angiograms, by analysing a predefined annulus with an outer diameter of 3 mm and an inner diameter of 1 mm, corresponding to the parafoveal region. This vessel density value was automatically generated using built-in AngioAnalytics®. Only high-quality images (signal strength > 8/10, focused, and without movement artefacts) were included in the analysis. No subjects were excluded due to poor imaging quality. However, and of note for future studies, it is worth mentioning that because of the sustained isometric effort required during the handgrip test, a minority of the volunteers found it difficult to keep a completely steady position in the OCTA chinrest. This specific situation affected the imaging mostly with movement artefacts and the examinations needed to be repeated once in 4 of the 48 subjects (two in each group).

a. Vasodilatory Response - Hypoxia Challenge Test (HCT)

The physiologic response to hypoxia has been previously reported. Similarly to the cerebral vasculature, retinal vessels respond to a decrease in PaO_2 with vasodilation and increase in blood flow. This local autoregulatory adaptation contributes to keep a rather stable oxygen pressure in the inner retina until PaO_2 levels are as low as 40 mmHg.[6,57,102,180]

The following protocol has been described in detail in a previous publication.[178] Briefly, the HCT is a standard test[156] performed at sea level in order to create a normobaric hypoxic environment by reducing the FiO^2 and making it equivalent to that of a flight cabin. The parameters monitored during HCT include oxygen peripheral saturation, arterial pressure, and continuous electrocardiography. As established by the British Thoracic Society, the recommended HCT duration to obtain stable conditions is 20 minutes. Accordingly, OCTA was performed at baseline and then, again, 30 min after HCT start (i.e. in plateau hypoxic conditions). All symptoms were recorded, and the test stopped if medically necessary.

b. Vasoconstrictive Response—Handgrip Test

It is known that isometric exercise is used to evaluate sympathetic autonomic response causing steady and safe increases in heart rate and arterial blood pressure, along with physiological peripheral vasoconstriction.[108] In the retina, the blood flow remains relatively unchanged until the mean ocular perfusion pressure increases by 35-60% above baseline. This is achieved through a local autoregulatory increase in vascular resistance - ie. retinal vasoconstriction.[42,71,111]

The following protocol has been described in detail in a previous publication.[178] In brief, subjects sit in front of the OCTA device, with the forearm in neutral position, the elbow flexed at approximately 90º, and the wrist with the thumb facing upward. Using a Jamar hydraulic dynamometer, the participants are asked to keep a steady contraction of at least one-third of the maximal calculated force. The OCTA acquisition starts in the plateau phase – i.e. after 90

seconds, being completed for both eyes within the 3- to 5-minute test period. The arterial pressure in the contralateral arm is measured every 90 seconds. According to the test recommendations, if a diastolic blood pressure reaches values higher than 120 mmHg and/or any adverse symptom is registered, the test is immediately interrupted.

iv) Primary and Secondary Outcomes

A comparative analysis for each group - T1D patients and healthy controls, was undertaken for the following outcomes: i) parafoveal vessel density evaluated using OCTA at baseline and during HCT; ii) parafoveal vessel density evaluated using OCTA at baseline and during the Handgrip test; and iii) systemic cardiovascular response in both scenarios.

v) Statistics

Sample size was calculated considering a 5% clinically significant difference in mean vessel density between groups, and a standard deviation of 5%. Considering a power of 90%, an alpha value of 0.05, a minimum of 17 T1D patients and controls should be included. To account for the attrition rate and to maintain the power of the study, a total of 24 patients were included in each group.

Statistical analysis was performed using STATA v14.1. A repeated-measures ANOVA model was used to assess differences between the baseline and stress tests' measurements. The Skewness-Kurtosis test was used to assess the normal distribution of the variables considered, and the inexistence of significant outlier values was also confirmed. Equality of variances was investigated, and the results were reported accordingly, applying the Greenhouse–Geisser correction when variables' variances were not equal.[181] A p-value < 0.05 was considered for statistical significance. To guarantee independent observations, only the right eye of each patient was considered for analysis.

Results

i) Demographics and Baseline data

Forty-eight eyes of 24 healthy subjects and 24 T1D patients without evidence of diabetic retinopathy were studied. In the T1D group, the mean HbA1c value was 7.9 ± 1.4 % (range 6.2-11.5 %) and the mean time from the diagnosis was 14.8 ± 9.7 years (range 2-36 years). Both groups were similar with respect to the demographic and baseline characteristics, including age, gender, arterial blood pressure, heart rate, body mass index, ocular axial length, intraocular pressure and best-corrected visual acuity – Table 1. Of note, and as previously reported elsewhere[145,182], a baseline rarefaction of the superficial and deep parafoveal plexuses was noted in the group of patients with diabetes, even before clinically evident retinopathy – Table 1.

Table 1. Demographic and Baseline Data

	Control	Type 1 Diabetes	*P* Value
Age, mean (SD), years	31.8 (8.2)	36.9 (10.4)	0.07
Male/Female, n	10/14	10/14	1.00
SAP, mean (SD), mm Hg	117 (12)	117 (9)	0.85
DAP, mean (SD), mm Hg	78 (9)	77 (6)	0.13
Heart rate, median (IQR), beats/min	62 (58–67)	62 (56–71)	0.63
Body mass index, mean (SD), kg/m^2	22.6 (3.0)	25.1 (3.9)	0.06
Axial length, mean (SD), mm	24.1 (0.9)	23.5 (1.0)	0.07
Intraocular pressure, mean (SD), mm Hg	13.3 (2.1)	14.6 (3.0)	0.14
Visual acuity, median (IQR), logMAR	0 (0–0)	0 (0–0)	0.45
Parafoveal vessel density, median (IQR)			
Superficial plexus	55.1 (53.1–56.4)	53.1 (48.9–55.1)	0.016
Deep plexus	60.4 (59.3–61.8)	57.2 (53.3–60.0)	< 0.001
HbA1c, mean (SD), %	—	7.9 (1.4)	—
Time since diagnosis, mean (SD)	—	14.8 ± 9.7	—

P values obtained with Student's *t* test, Wilcoxon rank-sum (Mann-Whitney) test or Pearson's χ^2 test, as appropriate. DAP, diastolic arterial blood pressure; HbA1c, glycated hemoglobin, pressure; SAP, systolic arterial blood pressure; SD, standard deviation; IQR, interquartile range.

ii) Systemic response

a. Hypoxia Challenge Test

The median peripheral oxygen haemoglobin saturation decreased from 97% to 88% in both groups – Table 2. Also, as expected, an increase in the heart rate was noted in hypoxic conditions

in both groups, in order to increase the cardiac output. The arterial blood pressure changes were less notable, with a mild decrease in mean arterial pressure noted in the control group and no significant differences in T1D patients – Table 2.

Table 2. Systemic Response to the Hypoxia Challenge Test

	Control	*P* Value	Type 1 Diabetes	*P* Value
O_2 Hb saturation, median (IQR), %				
Baseline	97 (97–98)	—	97 (97–98)	—
Hypoxia	88 (85–89)	<0.0001	88 (86–89)	<0.001
SAP, mean (SD), mm Hg				
Baseline	117 (12)	—	117 (9)	—
Hypoxia	114 (10)	0.05	118 (11)	0.25
DAP, mean (SD), mm Hg				
Baseline	78 (9)	—	77 (6)	—
Hypoxia	75 (10)	0.045	75 (7)	0.10
MAP, mean (SD), mm Hg				
Baseline	91 (10)	—	90 (6)	—
Hypoxia	88 (9)	0.02	89 (8)	0.40
Heart rate, median (IQR), beats/min				
Baseline	62 (58–67)	—	62 (56–71)	—
Hypoxia	74 (71–80)	<0.001	79 (63–86)	<0.001

P values (versus baseline) obtained with Student's *t* test and Wilcoxon rank-sum (Mann-Whitney) test, as appropriate. DAP, diastolic arterial pressure; Hb, hemoglobin; MAP, mean arterial blood pressure; SAP, systolic arterial pressure; SD, standard deviation; IQR, interquartile range.

b. Handgrip test

As shown in Table 3, the handgrip test was associated with a significant increase of the heart rate, systolic and diastolic arterial blood pressure in both groups.

Table 3. Systemic Response to the Handgrip Test

	Control	*P* Value	Type 1 Diabetes	*P* Value
SAP, mean (SD), mm Hg				
Baseline	118 (11)	—	123 (12)	—
Handgrip	150 (18)	<0.0001	155 (24)	<0.0001
DAP, mean (SD), mm Hg				
Baseline	75 (9)	—	79 (9)	—
Handgrip	102 (14)	<0.0001	98 (13)	<0.0001
MAP, mean (SD), mm Hg				
Baseline	89 (9)	—	94 (9)	—
Handgrip	123 (13)	<0.0001	122 (17)	<0.0001
Heart rate, median (IQR), beats/min				
Baseline	67 (64–73)	—	62 (67–78)	—
Handgrip	77 (71–85)	<0.01	79 (68–87)	<0.01

P values (versus baseline) obtained with Student's *t* test and Wilcoxon rank-sum (Mann-Whitney) test, as appropriate.
DAP, diastolic arterial pressure; MAP, mean arterial blood pressure; SAP, systolic arterial pressure; SD, standard deviation; IQR, interquartile range.

iii) Retinal vascular response

a. Hypoxia Challenge Test (figure 1)

In the healthy cohort, the median parafoveal vessel density in the superficial plexus increased from 55.1 (53.1 – 56.4) in baseline conditions to 56.5 (54.0 – 57.6) in hypoxia ($F_{1,23}$ = 15.69, p < 0.001). The median parafoveal vessel density in the deep plexuses also increased, from 60.4 (59.3 - 61.8) at baseline to 62.0 (59.9 - 62.8) during hypoxia ($F_{1,23}$ = 16.26, p < 0.001) – Table 4.

In the T1D group, there were no statistically significant differences during hypoxia for the superficial ($F_{1,23}$ = 0.06, p = 0.81) or deep ($F_{1,23}$ = 1.08, p = 0.31) parafoveal plexuses – Table 4.

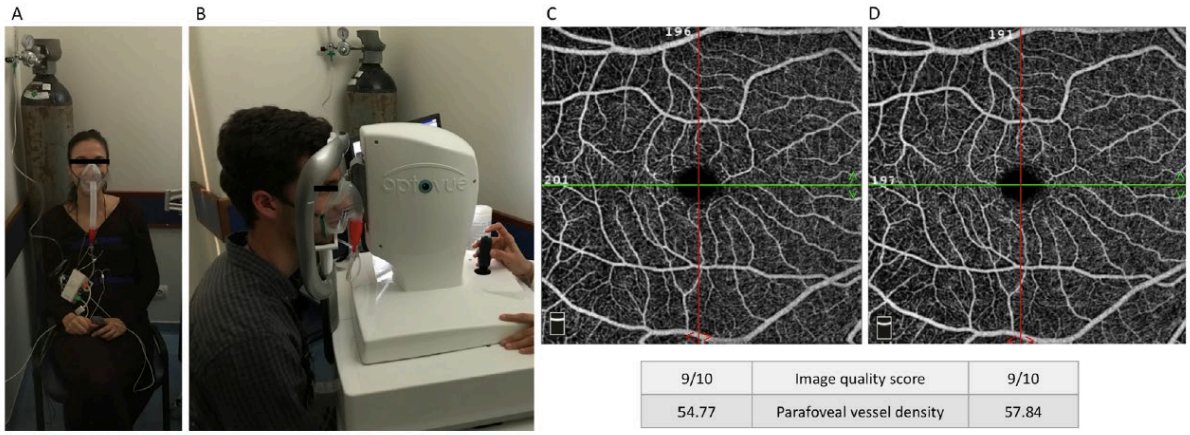

Figure 1. Exemplar of the setup during the hypoxia challenge test and OCT-angiography examination (**A, B**) and macular en-face 6- × 6-mm angiograms obtained in baseline conditions (**C**) and during the hypoxic test (**D**). The angiograms belong to the healthy volunteer depicted in **B**. Vessel density increased in hypoxic conditions as expected. Image quality score and parafoveal vessel density are provided according to the built-in angioanalytics software, as described in the Methods section.

Table 4. Retinal Vascular Response to the Hypoxia Challenge Test

Parafoveal Vessel Density, Median (IQR)	Control	*P* Value	Type 1 Diabetes	*P* Value
Superficial plexus				
Baseline	55.1 (53.1–56.4)	—	53.1 (48.9–55.1)	—
Hypoxia	56.5 (54.0–57.6)	<0.001	52.0 (50.0 - 54.2)	0.81
Deep plexus				
Baseline	60.4 (59.3 - 61.8)	—	57.2 (53.3 - 60.0)	—
Hypoxia	62.0 (59.9 - 62.8)	< 0.001	56.2 (54.4 - 57.9)	0.31

P values (versus baseline) obtained with ANOVA repeated-measures. IQR, interquartile range.

b. Handgrip test (figure 2)

In the control group, isometric exercise elicited a significant decrease in vessel density in both superficial [55.8 (53.5 – 56.9) to 54.1 (52.2 – 54.5), $F_{1,23}$ = 27.37, p < 0.0001] and deep [60.4 (58.7 - 61.2) to 57.1 (53.7 – 58.6], $F_{1,23}$ = 27.90, p < 0.0001) parafoveal plexuses – Table 5.

In the T1D group, the expected vasoconstrictive response with decrease in vessel density during the test was not observed in the superficial plexus ($F_{1,23}$ = 3.86, p = 0.06), being noticed only in the deep ($F_{1,23}$ = 11.04, p < 0.01) parafoveal plexus – Table 5.

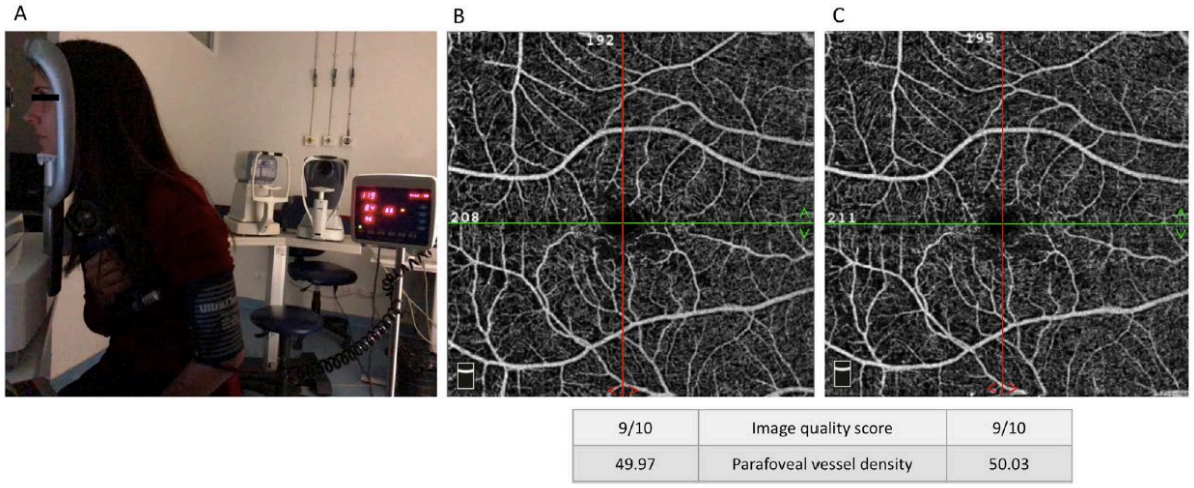

Figure 2. Exemplar of the setup during the handgrip test and OCT-angiography examination (**A**) and macular en-face 6- × 6-mm angiograms obtained in baseline conditions (**B**) and during the handgrip test (**C**). The angiograms belong to a volunteer with type 1 diabetes with no clinical evidence of diabetic retinopathy. The physiological decrease in vessel density during the handgrip test was not observed. Image quality score and parafoveal vessel density are provided according to the built-in angioanalytics software, as described in the Methods section.

Table 5. Retinal Vascular Response to the Handgrip Test

Parafoveal Vessel Density, Median (IQR)	Control	*P* Value	Type 1 Diabetes	*P* Value
Superficial plexus				
Baseline	55.8 (53.5—56.9)	—	53.5 (51.1–55.4)	—
Handgrip	54.1 (52.2—54.5)	<0.0001	52.8 (48.7–54.8)	0.06
Deep plexus				
Baseline	60.4 (58.7–61.2)	—	58.5 (54.1–60.3)	
Handgrip	57.1 (53.7–58.6)	<0.0001	55.9 (52.5–60.3)	<0.01

P values (versus baseline) obtained with ANOVA repeated-measures. IQR, interquartile range.

Discussion

Our study used OCTA to dynamically study the retinal microvasculature functional responses to mild hypoxia and isometric exercise using two standardised tests – the Hypoxia Challenge Test and the Handgrip test, respectively.

A protocol for this functional analysis using OCTA has been previously reported in healthy subjects[178], and this work replicated the same results in terms of retinal responses to isometric exercise and mild hypoxia. Importantly, this study is the first to document using OCTA the impairment of this physiological retinal vascular response in patients with diabetes before any clinical features of DR exists. Our sample of young patients with T1D lacked other vascular co-morbidities, such as hypertension and atherosclerosis that are likely to influence OCTA measurements. Thus, the changes identified are most likely due to diabetes-specific factors influencing retinal vascular behaviour.[148]

The retina is one of the most metabolically active tissues in the body, and for its normal functioning an effective autoregulation is crucial.[34] The lack of the expected vascular response pattern to both stimuli – mild hypoxia and isometric exercise, suggests there is an early impairment of the retinal autoregulatory function in the diabetic group. Our results are supported by previous findings of pre-clinical structural and functional changes in patients with diabetes.[138,145,174–176,183] The altered retinal vascular response that we observed also corroborates previous evidence on the importance of neurovascular coupling dysfunction very early in DR development. These studies used the laser Doppler blood flowmeter, functional magnetic resonance imaging, flicker electroretinography and the dynamic vessel analyser as devices to assess retinal vascular function.[147,168–172] However, these tools are generally used for research-only purposes and therefore not widely accessible in ophthalmology clinics. In our study we used OCTA technology, which is increasingly available in clinical practices worldwide and may be further optimized for this purpose. The ability to evaluate individual functional responses has multiple potential advantages. Firstly, it overcomes the limitations of interpreting single

structural exams that may vary among individuals, including pre-determined OCTA metrics, such as the foveal avascular zone.[184] Also, the retinal functional response may be a more sensitive marker to detect earlier changes, when compared with a structurally normal OCT scan or fluorescein angiography. This may well have potential implications when considering monitoring and managing the metabolic, systemic and ophthalmic manifestations of the disease.[185] We found a significantly altered vascular response in subjects with type 1 diabetes before any clinical evidence of diabetic retinopathy. This abnormal retinal microvascular response may correspond to an earlier marker of endothelial dysfunction and/or changes in the signaling between the retina and the vessels (i.e. neurovascular coupling)[6,186,187], and we provide a novel way of studying it non-invasively using modern OCTA technology.

Another interesting finding of our study is the notion that the cardiovascular systemic response does not appear to be significantly different between the groups. The circumstantial finding of a statistically significant decrease in the arterial blood pressure of the healthy cohort during mild hypoxia (which was not clearly observed in the diabetic group) seems to have limited clinical meaning. However, this may be worth clarifying in future studies. The identified abnormal regional retinal response suggests there is an increased ability of OCTA to sensitively identify vascular changes. As an innovative, non-invasive, and safe technology, able to study a central nervous system (retinal) microvasculature, OCTA is becoming a useful tool for the study of non-ophthalmic conditions. Multiple reports have been published with OCTA applications mainly in neurodegenerative conditions (e.g. Parkinson, Alzheimers's, multiple sclerosis), but also in other diseases.[100,188–194] Therefore, studying a patient's retinal vascular responses can be a useful adjunct to routine structural anatomical evaluation with interesting application also in non-ophthalmic settings. This study highlights the potential to adapt the available OCTA technology to combine this form of functional analysis to the currently available structural angiogram.

With this study, we have demonstrated an attenuated retinal vascular response in patients with

type 1 diabetes with no clinical evidence of ocular disease. However, these findings should be interpreted along with the limitations of our study. Although well-powered for the main outcomes, the young age group, Caucasian population and relatively small sample size may limit the external validity of the study and also the possibility of multivariate analysis with certain demographics and subgroup features. Also, the diurnal changes of the systemic and ocular variable analyzed were minimized as possible but should not be excluded as a potential source of bias. Despite careful recruitment, the wide range of diabetes' duration in our sample may suggest the underestimation of the diabetic retinopathy status, as some patients would be above the usual time for the early manifestations of DR (such as peripheral hemorrhages and/or microaneurysms)[195], that could have been under reported with our methodology. Larger studies, including patients with a range of disease severity through the various ETDRS levels would be useful to add weight to our findings. Secondly, although individuals with type 1 diabetes were selected to minimize the influence of any co-pathologies and systemic medication in the vascular analysis, a small number of subjects were under systemic medication (other than insulin): four patients were on levothyroxine, two were on simvastatin, one patient was taking sertraline, and one patient was on mexazolam and levothyroxine. The patients taking levothyroxine had normal thyroid function tests. Although unlikely to affect the observed patterns of retinal vascular response, we acknowledge the potential vascular effects of these drugs.[196–199] Also, insulin is inherently vasoactive.[179] Despite being asked not to administer insulin boluses in the two hours preceding the OCTA measurements to reduce its influence on our observations, we should not exclude the potential for some degree of measurement bias introduced by its cardiovascular effects. Likewise, current blood glucose concentration itself may have a significant vascular effect.[200,201] As this parameter was not systematically evaluated in both groups, it is also a limitation of our study protocol that we were not able to rigorously control the observed vascular response for the current glycemia. Thirdly, although widely used in clinics and research, the OCTA technology is not yet optimized for these functional analyses,

and it should be remembered that this technology measures *perfused vessel densities,* not absolute blood flows. Lastly, in order to ensure that the protocol for dynamic retinal microvasculature analysis is as reproducible as possible, we used the manufacturer's default software for superficial and deep capillary plexuses analysis. Thus, the inherent bias related to segmentation and differences to other devices and models should be considered.[79] We are certain that this study acts as a valuable contributor to the development of the OCTA technology as a realistic tool in functional retinal analysis.

In conclusion, we used OCTA technology in conjunction with standardized stress tests and observed an early impairment in the physiological retinal vascular response in patients with type 1 diabetes before any clinical evidence of retinopathy. Further work is required to better delineate the process of DR development and to develop tools that optimize its clinical significance.

PART III

INTEGRATED DISCUSSION

As the highest oxygen-consuming tissue of the human body, the retina has evolved with an intricate vascular autoregulatory system. This mechanism ensures adequate supply of oxygen and nutrients to retinal tissues in conditions such as high blood pressure or hypoxia. Contrary to the choroid, however, the retinal vasculature lacks intrinsic innervation and its contractile status is regulated mainly by local factors. [4,6]

Most of the literature that studied retinal vascular responses used imaging techniques that were limited to the assessment of the larger vessels. These also lacked the axial resolution to differentiate the different capillary plexuses of the human retina.[50,51,53,57,66,69,85,202–205] Novel and more detailed ways of analysing retinal vascular responses could thus provide insights on retinal autoregulation in both health and disease.

Recently developed, optical coherence tomography angiography (OCTA) provides a fast, three-dimensional angiographic scan of the retina in a non-invasive way and of unprecedent high-resolution. It does so by detecting motion contrast of moving red blood cells, with sufficient depth-resolution to image retinal superficial and deeper capillary plexuses. By using non-visible infrared light, it also does not disrupt the light/dark conditions of the measurements.[78,79]

The main goal this thesis was to dynamically study the retinal vascular response in healthy subjects and in a cohort of patients with type 1 diabetes (T1D) using OCTA, and to explore potential applications in ophthalmology and other medical fields. With this purpose, we developed a series of experimental multidisciplinary works and the published manuscripts are included in Part II.

Firstly, in order to evaluate if OCTA is able to image an autoregulatory retinal vascular response,

we designed a study that served as the *proof of concept* for the subsequent works.

Research Question #1 - Is OCTA able to detect a retinal autoregulatory vascular response to mild hypoxia?

The retinal autoregulatory system has evolved in a way that increases flow when oxygen supply is reduced, by inducing arteriolar and venular vasodilation. This response aims to maintain adequate oxygenation to retinal tissues during the hypoxic exposure.[128,206]

As detailed in Part II.I, to answer this question we developed a study protocol including a cohort of healthy young subjects who were subjected to the *hypoxia challenge test (HCT).* This test creates a mild hypoxic environment similar to the flight-cabin of commercial airplanes, and was applied in collaboration with the Respiratory Medicine Department. OCTA was performed in baseline, hypoxic and post-hypoxic conditions. During HCT, minimum and mean Spo_2 levels were 88.5 ± 2.4% and 92.8 ± 1.8%, respectively. The results revealed a significant increase in vessel density in the superficial capillary plexuses during hypoxia for both parafoveal and peripapillary locations.

These findings suggested that OCTA was able to detect the retinal autoregulatory response to hypoxia. The documented increase in vessel density is in accordance with the previously documented vasodilatory response.[56,57,207] Most of the previous literature used altitude as the hypoxic stimulus to evaluate the retinal vascular changes.[206,208] By using the HCT, we were able to create a normobaric environment to study the hypoxic response. Therefore, comparisons on the magnitude of response between these studies should be made with caution, not only because of differences in the setting, but also on the time and degree of the stimulus and instrumentation used. For instance, Cheng and collaborators studied the relationship between retinal blood flow and PaO_2 using laser blood flowmeter (LBF) technology.[57] LBF is a non-invasive technique that determines centerline blood velocity (mm/s) and vessel diameter (μm)

of the retinal arterioles and venules, then calculating flow in µl/min based on the Poiseuille principle.[119] Despite being considered a reliable technology, LBF is not widely available in clinic, being considered more a research tool.[120,121] Moreover, OCTA is able to provide depth-resolved and much higher resolution images of the macular microvasculature. Of note, however, at the time of this study the projection artifact removal algorithms were still not widely available, which limited the analysis to the superficial capillary plexus in order to minimize potential measurement bias associated with the study of the deeper plexuses. As discussed below, the possibility of separately analysing different retinal plexuses is of particular interest.

In this proof of concept study, only healthy volunteers were enrolled, and a mild hypoxic stimulus was used. Given the scarce data on this subject, these results contributed to understand the physiologic responses to a hypoxic stress and to design a reliable protocol to investigate this response in disease setting. In fact, one of the main questions raised by this work is if individuals with vascular dysregulation disorders are at risk during commercial flights related hypoxia. As a fast, non-invasive and highly detailed imaging technology, we considered to extend the use of OCTA to quantify retinal vascular behaviours in a very prevalent and relevant systemic vascular disorder – diabetes mellitus.

The majority of the studies using OCTA in diabetes mellitus enrolled patients with type 2 diabetes. As our main goal would be to study the diabetes-specific eventual retinal vascular dysregulation, we decided to include patients with type 1 diabetes (T1D) before the existence of any clinical evidence of diabetic retinopathy (DR). These individuals are in general younger, and present less comorbidities than ones the type 2 diabetes , allowing us to minimize a multitude of confounding factors for retinal vascular analysis, such as age and accompanying cardiovascular diseases.[148]

Research question #2 - Are there structural changes in the retinal vascular network of T1D patients with no evidence of DR, when compared to healthy subjects?

Diabetic retinopathy is the leading cause of visual impairment among the working-age population of developed countries.[209] Retinal structural changes are well-studied in the early stages of DR – such as the disruption of the inner blood retinal barrier and subsequent basement membrane thickening.[210] This pathologic events alter the communication between endothelial cell and pericytes, eventually leading to endothelial cell loss and vasodegeneration, which is clinically seen as vascular dropout.[211–213]

Dilated fundus examination and fluorescein angiography (FA) are the gold standard examinations for DR diagnosis. Although FA is highly sensitive to detect microaneurysms and areas of neovascularization, it is a costly, lengthy and invasive procedure, as it requires dye injection prior to imaging.[214] OCTA is a non-invasive technology that overcomes some of these limitations and presents the additional unique property of analysing capillary plexuses at different levels.[79,214]

To answer this question, we designed a study using OCTA (Part II.II) to compare the retinal vascular network of patients with T1D before any evidence of DR, and a matched cohort of healthy subjects. We enrolled a total of 48 subjects (24 T1D) and observed a consistent rarefaction of both the superficial and deep capillary parafoveal plexuses in the diabetic cohort, when compared to the healthy volunteers. Also, a longer time since T1D diagnosis was associated with a larger foveal avascular zone (FAZ).

Strikingly, for these and subsequent studies, the OCTA included the latest project artifact removal software, thus allowing for depth-resolved imaging of the different retinal plexuses: the superficial capillary vascular complex; and the deep vascular complex (including both the intermediate and deep capillary plexuses).[18]

The decrease in vessel density in both superficial and deep plexus suggest a role for

compromised circulation very early in DR development.[176,215] These results were submitted for publication in early 2019 and added to the limited available literature then[139,145,216–218], by showing that microvascular changes such as FAZ enlargement/remodelling and areas of capillary nonperfusion may exist in T1D before clinicians are able to identify the classical diagnostic features of DR.

Once again, OCTA technology was sensitive enough to detect microvascular structural changes. With this purpose, it has been increasingly used mostly in type 2 diabetes, in which OCTA quantitative and qualitative metrics have proved clinically useful to estimate baseline DR severity, disease progression, and treatment requirements.[144,175,219–221] Large prospective studies would further enable and validate the use of OCTA imaging as a clinically meaningful biomarkers in the diagnosis management of DR. For instance, it is known that there are a subset of T1D patients that do not develop significant micro/macrovascular complications and associated vision loss, even after decades of disease. The better characterization of these "happy few" patients may contribute to understand what drives progression and target future treatments in a more personalised approach.[222] Moreover, with the recent developments in artificial intelligence for DR diagnosis, it is also worth mentioning the potential of *automated* OCTA with this purpose.[223–225]

By conducting a study for this research question, we contributed to further understand the potential role of structural OCTA analysis in "pre-clinical" stages of diabetic eye disease. As a complex disease, DR has been a field of controversy regarding relative contribution of each pathway and cascade into the overall outcome of vascular occlusion and retinal ischaemia.[147] We hypothesised that these OCTA findings may correspond to an early stage of retinal vascular autoregulation compromise, which would then lead to the sequence of events in the natural history of DR.

As a starting point for the OCTA study of retinal vascular reactivity in the disease setting, we

must first have better characterized the response in each capillary plexus in a healthy population. With this purpose, we developed a detailed methodological protocol for analysing both vasodilatory and vasoconstriction retinal vascular responses to a standard stimulus using OCTA technology.

A Protocol to Evaluate Retinal Vascular Response Using OCTA

In the central nervous system, the possibility of direct visualization of the vascular system is unique to the retina vessels. These have the ability for local autoregulation, which is crucial to keep blood flow relatively constant despite the variations in perfusion pressure or reduced oxygen supply.[146]

As detailed in Part II.III, in this study, a young healthy cohort was enrolled and two standardized tests were applied: i) the hypoxia challenge test (HCT), and the ii) the handgrip test, in order to induce a retinal vasodilatory and vasoconstrictive response, respectively. OCTA was performed at baseline conditions and during the stress test.

During the hypoxic stimulus, the mean parafoveal vessel density increased significantly in both the superficial and deep plexuses. The hypoxemia also induced the expected increase in heart rate. The vasodilatory response with retinal blood flow increase in response to a decreased arterial oxygen values has been already reported as a physiologic response, although the mechanisms between the oxygen sensing in the retina and increase in blood flow are not completely understood. It is believed there are oxygen sensitive ion-channels in the retina, which respond directly or induce the local release of hypoxia-related metabolites, such as retinal relaxing factor, prostacyclin, lactate, and adenosine. This metabolic response is thought to be the most likely as the responses tend not to be immediate.[6,57,226–228]

Although our protocol used a single mildly hypoxic stimulus, it has been estimated a lower PaO_2 threshold of 32-37 mmHg, until when the retinal arteriolar and venular dilation is able to

compensate so that oxygen delivery is maintained constant. We also did not analyse separately the arteriolar and venular response.[57] This relationship is still to be understood, as it is not clear if the venules respond to the same metabolites as the arterioles, or if they only dilate passively as a consequence of the increased blood flow given their high capacitance.[57,229]

According to our study protocol, and although the hypoxic stimulus is mild, an increase in the respiratory frequency may contribute for some degree of hypocapnia. Since hypocapnia would induce vasoconstriction,[56,57] this might have contributed to reduce the magnitude of retinal vasodilation and increase in vessel density detected using OCTA.

The stress test used in our study – HCT, consistently induced the expected systemic responses too, with an increase in heart rate accompanying the hypoxemia. A few OCTA studies have been reporting the vasoconstrictive response to hyperoxia.[143,160] However, to the best of our knowledge, our group was the first to report the vasodilatory response to hypoxia, using the standardized HCT as the hypoxic stimulus.

For the handgrip test, a vascular autoregulatory response was also noted, with a corresponding decrease in vessel density in both retinal plexuses. Regarding the systemic response, the mean percent increase in mean arterial pressure and heart rate was 32 % and 23 %, respectively. In order to maintain the retinal blood flow constant, the associated response consists in a retinal vasoconstriction.[71,108,111,230] This protective mechanism of autoregulation ensure the blood flow remains largely unchanged until the mean ocular perfusion pressure increases by an average of 34-60%. As discussed above, although devoid of intrinsic autonomic innervation, the retinal vessels have adrenergic receptors allowing them to respond to neurotransmitters.[6,161] However, the response to increases in blood pressure is triggered not only by local metabolites, but also as a myogenic response to the increase in the intraluminal vascular pressure – Bayliss effect.[41,42,109,111]

Although further studies are needed to better characterize the clinical significance and

applicability of these findings, OCTA appears to have a potential role in the evaluation of retinal vascular function. This protocol was published in Frontiers of Neuroscience and aimed to provide insight on use of OCTA to study a central nervous system (i.e. retinal) vascular response, which could be applied in health and disease of multiple medical fields.

Recently, a few research groups have been publishing studies using OCTA with the purpose of evaluating retinal vascular responses. For instance, Nesper and Fawzi used dark adaptation, transition from dark to light, and flicker stimulation as stimuli to assess retinal microvascular reactivity.[35] In this study, the possibility of high-resolution depth-resolved OCTA imaging, allowed to distinguish responses of the superficial and deeper plexuses, depending on the stimulus. This is of particular relevance for example in the dark environments, in which the energy demand of the photoreceptors is maximal and partially met by diffusion from the deep retinal vasculature and not only from the choroid. They found the expected increase in vessel density in the superficial plexus during ambient light and flicker stimulation. But interestingly, in the transition from dark to light the vessel density of the deeper plexuses was decreased. The authors suggested this is due to the fact that the deep plexus is maximally dilated during dark adaptation, constricting after the transition to light to allow for the blood to be redirected to the more superficial layers. The ability to depth-resolve the maximal dilation of the deep plexus may have important implications in pathologic conditions, in which deep capillary loss may be associated with photoreceptor disruption.[149,231] Moreover, these results further confirm the distinct neurovascular control mechanisms for each plexus and the uniqueness of OCTA in the ability to study them independently.[24,232,233]

As OCTA has the limitation of providing retinal blood flow information in arbitrary units, other groups have been developing ways to measure absolute blood flow. This would be clinically important as changes in blood velocity can occur in nondisease states, which is a limitation of ocular colour Doppler imaging methods. Therefore, many efforts are being made to use Doppler effect in OCT systems, to allow non-invasive quantification of blood flow. However, accurate

quantification remains challenging, with current systems being limited in the maximum velocities measurable, small vessels assessment, and the geometry of the vessel (i.e. the Doppler angle). [234]

In this context, with its advantages and limitations, we believe OCTA technology is promising for the functional assessment of retinal vascular reactivity. Taking the paradigmatic example of a diabetic individual with no clinical evidence of DR, we extended this protocol to the disease setting, in order to answer the remaining research questions.

Research Questions #3 / #4 - Are there functional changes in the retinal vascular response to a hypoxic stress/isometric exercise in T1D patients without DR, when compared to healthy individuals?

For decades, scientists and clinicians have been interested in identifying early retinal microvascular changes in DR, as these are thought to serve as biomarkers for the progression of the disease, assist in management decisions and predict other cardiovascular events.[235–237] In Part II.II, we demonstrated the ability of OCTA to detect "pre-clinical" DR structural changes at the capillary level, with a rarefaction of both superficial and deep retinal plexuses.

The endothelial cells and pericytes, which are affected early in the pathogenesis of DR, are main regulators of retinal vascular contractile status and responsible for an appropriate vascular autoregulation.[6,47] In cases of small shortages of oxygen or nutrients, the inability to adapt the supply to match the demand may lead to tissue hypoxia and permanent damage.[36] Similarly, in response to situations of hyperperfusion, the failure of autoregulatory mechanisms may be responsible for the formation of reactive oxygen species and ultimately cell death.[238]

However, as discussed above, regulation of retinal blood flow occurs not only in in response to changes in oxygen and perfusion pressure, but also in dependence on local neural activity – neurovascular coupling or functional hyperemia.[45] Although this concept was developed by a

landmark paper in 1890, it is not fully understood and most of the literature stems from brain research.[46,239–241] As a neural tissue, the retinal microcirculation status is coupled with neural activity, with a hyperemic response occurring in response to flicker-light stimulation, for example.[45] Recent studies using this stimulus have documented that patients with diabetes have an altered vascular response to flicker and that this correlates with the severity of the DR.[96,242,243]

To answer these questions, we applied the protocol described in Part II.III to dynamically assess retinal vascular responses using OCTA to healthy subjects and patients with T1D and no clinical evidence of DR. As detailed in Part II.IV, both stimuli - hypoxia and isometric exercise - were used, in order to evaluate eventual changes in the vasodilatory or vasoconstrictive retinal autoregulatory responses. The systemic cardiovascular responses were also compared between groups.

Contrary to the healthy subjects, in the T1D group the physiological autoregulatory response to hypoxia (i.e. vasodilation) was not observed in neither the superficial, nor the deep retinal plexuses. During isometric exercise, the expected vasoconstrictive retinal response was observed in both plexuses in the control group but only detected in the deep plexus of the patients with T1D.

These results corroborate previous findings of pre-clinical changes in patients with diabetes, not only on the retinal vascular network, but also of retinal function such as with decreased dark adaptation, reduced light sensitivity, hue discrimination, contrast sensitivity, microperimetry defects, and electroretinogram changes.[138,145,174–176,183,244–250] The reported functional abnormalities in patients with early or no DR suggest that not only the retinal vascular component is affected, but rather the whole system of neural, glial, and vascular elements that form the neurovascular unit. This further highlights the existence of neurovascular coupling dysfunction very early in DR development, and go in accordance with previous works using laser Doppler blood flowmeter, functional magnetic resonance imaging,

and flicker-light stimulation.[147,168–172] These imaging tools are generally used for research-only purposes and therefore not widely accessible in ophthalmology clinics. In our study we used OCTA technology, which is increasingly available and may be further optimized for this purpose.

Strikingly, in the diabetic cohort, the autoregulatory retinal vasoconstriction in response to the handgrip was preserved in the DCP, although in a lesser magnitude. It has been considered the deeper plexuses are more susceptible for damage in early stages of DR, but contradictory data exists is this regard.[7,174] Although presenting more structural changes (such as microaneurysms and capillary loops) than the superficial plexus, the DCP has been found to have higher vessels density and a lower percent area of nonperfusion on OCTA.[232,251,252] It is also known that it is in the outer retinal layers that the oxygen consumption is higher, that the DCP might have to supply the photoreceptors in high-activity conditions (such as dark environments), and that the retinal capillary plexuses autoregulate independently.[3,7,35,36,160,253] Therefore, we hypothesize that the capacity for retinal autoregulation might be preserved in the DCP until later stages, in order to maintain an adequate supply of oxygen and nutrients to the energy-demanding outer retinal layers.

As per the study design, our sample of young patients with T1D lacked other vascular co-morbidities, such as hypertension, dyslipidaemia and atherosclerosis. This suggests these changes in retinal vascular behaviour are most likely due to diabetes-specific factors.[148] The degree of systemic influences on the retinal vascular behaviour was also minimised by: i) asking all subjects to abstain from alcohol and caffeine for at least 6 hours prior to the study, ii) asking all T1D volunteers not to eat or take insulin in the 2 hours before the study and confirming that none had neither hypoglycemia (<70 mg/dL) nor more than level 1 hyperglycemia (>180 mg/dL), and iii) distributing evenly in the morning and afternoon sessions the participants from both groups. In addition, a "relatively healthy" T1D cohort may have also contributed for another interesting finding of our study – the fact that the cardiovascular systemic response (i.e. blood

pressure and heart rate) was similar between groups. During hypoxia, the heart rate increase predominated, in order to increase the cardiac output and blood flow.[57,254] An increase in the blood pressure during hypoxia is also expected, but this was not observed in our studies. The mild hypoxic stimulus and the significant heart rate increase may be potential contributions for this finding, that is worth clarification in future studies. During the sympathomimetic isometric exercise, significant increases in both heart rate and arterial pressure were expected and observed in both groups.[71,108,111] Although the autonomic cardiorespiratory control in diabetes is still not completely understood, imbalances in the baro- and chemoreflex responses have been reported early in the pathogenesis of the disease.[255–258] It is thought that the "autonomic neuropathy" may in many cases be a functional condition of sympathetic activation, driven by many factors such as reduced sensitivity to hypoxia.[135] Even though our study was not designed for this purpose, the changes observed in retinal autoregulatory function were not matched with significant cardiovascular responses to the stress tests. Future studies including continuous and spectral measurements of cardiorespiratory variables may provide a more comprehensive understanding of the relationship between the degree of autonomic dysfunction and the changes in retinal vascular response.

This study was published in IOVS in mid-2020 and was the first to document with OCTA the impairment of both autoregulatory retinal vascular responses (i.e. vasodilation and vasoconstriction) in patients with diabetes before any clinical feature of DR were evident. The identified regional abnormalities in vascular autoregulation suggested there is an important role for retinal imaging to sensitively identify these changes.

OCTA Advantages & Limitations

All imaging methods have advantages and limitations, and OCTA is no exception.

As advantages, OCTA does not require any dye and is able to depth-resolve the retinal microvascular plexuses, with high contrast and impressive resolution. The images obtained are used for software processing, and quantitative data is obtained as potential biomarkers in health and disease. In neovascular diseases, it is able to image the neovascular membranes with detailed morphology and confirm the depth location of the pathology. Lastly, OCTA is a fast and safe exam and can be repeated in short periods of time for the same subject to assess microvascular response to functional stimulation, as performed during our dynamic studies.[79]

However, OCTA has important limitations. Firstly, the quantification of OCTA metrics may be biased by interindividual factors, such as age and systemic vascular risk. The inexistence of normative database for vessel density, foveal avascular zone and other OCTA parameters limits the possibility of classifying a single measurement as normal or abnormal. Therefore, one of the advantages of this innovative concept of using OCTA for dynamic retinal vascular analysis is the ability to study an individual functional responses and not a single structural exam.[184] The *fucntional* reponse is likely to be more accurate in the identification of what is normal or abnormal, than a cross-sectional quantitative metric. Also, the retinal functional response may be a more sensitive marker to detect earlier changes, with potential implications when considering monitoring and managing the metabolic, systemic and manifestations of the various conditions.[79,185]

Secondly, there are limitations inherent to the technology, such as the influence of confounding factors in the metrics. For instance, the axial length affects the magnification of the OCTA images and the vessel density and FAZ measurements. A larger scan area would mean that the FAZ occupies a smaller portion of the whole image, resulting in an erroneously higher vessel density.[259,260] Although there are ways to minimise this bias,[261] in our studies, the axial length

was comparable between groups and without significant outliers. Also, as OCTA uses motion contrast to image the retinal microvasculature, it is dependent on very high A-scan rates and imaging larger fields of the retina may be challenging, since the area and number of A-scans exponentially increase in widefield imaging. Moreover, contrary to fluorescein angiography, OCTA is not able to detect vascular leakage and might not image very slow flow areas as well.[79]

Thirdly, OCTA technology is not yet optimized for functional analyses and blood flow metrics. Current methods and technology measures *perfused vessel densities* and not absolute blood flow. In addition, the algorithms for display and measurements of the OCTA images may vary between different manufacturers, as this depends on the coupled OCT instrument, scan protocols and signal processing methods. And lastly, the OCTA scans may have associated artifacts (e.g. motion, projection) and an understanding of the technology and careful interpretation of the outputs is crucial.[79]

We are confident that these and future studies are valuable contributions to the further development of OCTA technology as a realistic tool in functional retinal analysis.

FUTURE PERSPECTIVES

The studies included in this thesis culminated with the development of an innovative way to use OCTA technology to assess retinal vascular reactivity. In Part II.IV, this concept was applied in pre-clinical stages of patients with DR, and significant differences in retinal autoregulatory responses were found, in comparison to healthy subjects. This constitutes a stepping-stone for several future studies and is tremendously exciting as the applications of OCTA extend well beyond diabetes and ophthalmology. As a non-invasive, and safe technology, able to study a central nervous system microvasculature, OCTA is also becoming a useful tool for the study of many non-ophthalmic conditions. For instance, in neurology, more than 120 published manuscripts used OCTA and detected abnormalities in a panoply of neurodegenerative disorders, finding good correlation between the findings and the severity of the disease.[81,82] The applications of OCTA have also extended to cardiovascular research, and applied in the early detection of systemic microvascular disease.[262] And lastly, also in physiologic conditions, such as studies in sports medicine.[189] Therefore, studying a patient's retinal vascular responses can be a useful adjunct to routine structural anatomical evaluation with interesting applications in many fields. The concept developed in this thesis highlights the potential to optimize the available OCTA technology in order to combine a functional analysis to the currently available structural angiogram. A potential adaptation within the device would be the development of a "built-in" flicker stimulus. Also, other technologies are emerging to assess blood flow using OCT. One of the most promising is Doppler-OCT, which allows to measure total blood flow. However, accurate quantification remains challenging, with current systems being limited in the maximum velocities measurable, small vessels assessment, and the geometry of the vessel (i.e. the Doppler angle). The Doppler-OCT is also not yet commercially available, and its use is currently limited to specialized laboratories.

As a very recent imaging technology, most of OCTA studies are cross-sectional. In DR in

particular, future longitudinal studies will allow for a better characterization of the relationship between structural and functional changes and the severity of the disease, thus providing important management and prognostic information. Moreover, the combination of biochemical and imaging biomarkers might provide further information. In this line, and given the potential contribute of inflammatory mediators to DR development, our group is studying the relationship between OCTA findings and the levels of cytokine such as IL-1β and TNF.[263–265]

Moreover, the incorporation of artificial intelligence in OCTA imaging may be of significant value in the diagnosis and management of multiple conditions.[225,266] Future multicentre studies with a longitudinal design are promising to understand the potential of OCTA metrics as biomarkers of structural and functional retinal changes. Once again, as a central nervous system structure, this information might be useful for the study of neurological and cardiovascular diseases and potentially applied in population-wide programs.[74] We hope that the ever increasing interest in OCTA metrics could also serve as an encouragement for the devolvement of technology and methods to overcome some of its limitations and being optimized for dynamically assessing vascular responses.

While looking forward for the application of OCTA in the evaluation of retinal microvasculature, our group continues involved in the research of new and better ways of studying retinal autoregulatory mechanisms. Recently, we have been invited to lead a research topic for the prestigious *Frontiers in Neuroscience* titled *Retinal Vascular Functional Assessment in Health and Disease*. We are thrilled with the potential submissions under this topic, and glad to work on this with the co-editors Gemmy Cheung, José Cunha-Vaz, Mali Okada, and Giuseppe Querques.

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Learn from yesterday, live for today, hope for tomorrow – Albert Einstein

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If you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise;

If you can dream—and not make dreams your master;
If you can think—and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools;

If you can make one heap of all your winnings
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breathe a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: 'Hold on!'

If you can talk with crowds and keep your virtue,
Or walk with Kings—nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And—which is more—you'll be a Man, my son!

If, Rudyard Kipling