

**RETINAL BLOOD OXYGEN SATURATION AND ANGIOGENIC
AND INFLAMMATORY BIOMARKERS IN TYPE 2 DIABETES**

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Introduction: Diabetic retinopathy (DR) is a major source of visual loss in the world, including North America. A number of hyperglycemia-related pathways have been associated with DR onset and progression. Disturbances in the retinal vasculature appear to play a vital role in DR, resulting in biochemical and functional vascular changes. Therefore, this study investigated retinal blood oxygen saturation and angiogenic and inflammatory biomarkers in DR.

Methods: Chapter 3 and 5: FD-OCT Doppler blood flow was non-invasively measured using a prototype system based on the RTVue (Optovue Inc., USA). A minimum of six separate FD-OCT Doppler measurements was acquired. Chapter 4, 5, 7: Non-invasive hyperspectral retinal (HR) imaging was acquired in participants with mild-to-moderate NPDR and age-matched controls. For each subject, six repeated HRC images were acquired at wavelengths of 586 and 605nm.

Results: Chapter 3: The individual COV medians for retinal blood flow were 7.5% and 9.2% for young and elderly subjects, respectively. The group mean CORs for retinal blood flow for young participants were $6.4\mu\text{l}/\text{min}$ and for elderly subjects were $10.5\mu\text{l}/\text{min}$. Chapter 4: Retinal blood oxygen saturation in the arterioles of healthy controls was $92.97\pm 1.6\%$, and in the venules was $55.90\pm 4.8\%$. Retinal blood oxygen saturation for diabetic subjects with NPDR was significantly higher at $94.65\pm 2.2\%$ ($p=0.015$) in the arterioles and $64.13\pm 4.3\%$ ($p<0.001$) in the venules. Chapter 5: Total retinal blood flow was significantly lower in NPDR when compared to controls (42.66 vs 32.97 ; $p=0.004$). There was no relationship between total retinal blood flow and venular oxygen saturation ($r=0.2$). Chapter 6: Angiopoietin 2, IL-8, HGF was significantly higher

in NPDR patients than in control patients ($p=0.005$, $p=0.034$, $p=0.018$, respectively) and EGF was significantly lower in NPDR patients when compared to controls ($p=0.025$).

Chapter 7: The study demonstrated a correlation between retinal blood oxygen saturation and Ang 2, HGF and EGF but did not find any correlation for IL-8, TGF- β even though these biomarkers were significantly higher in the diabetic group.

Conclusions: Chapter 3: Doppler OCT gave consistent and repeatable blood flow measurements within retinal venules in normal subjects. Chapter 4: A higher blood oxygen saturation could be the result of less oxygen consumption due to cell death. Chapter 5: There is no correlation between retinal blood flow and retinal blood oxygen saturation. Chapter 6: Further investigation of Ang 2, HGF, IL-8, EGF, TGF- β could be used to better understand the pathophysiology of DR. Chapter 7: The result of this study revealed a relationship between the biomarkers that might result in cell death and higher retinal blood oxygen saturation.

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Dedication

This thesis is dedicated to my loveing parents, Ebtehaj and Faramarz.

For their endless love, encouragement and support throughout my life.

Table of Content

Abstract	iii
Acknowledgments	v
List of Figures	xi
List of Tables	xii
List of Abbreviations	xiii
1 Introduction	1
1.1 Retinal Oxygen Supply.....	1
1.2 Structure of the Retinal Vessels.....	2
1.3 Blood-Ocular Barrier	2
1.4 Retinal Blood Flow.....	3
1.5 Laminar Blood Flow.....	4
1.6 Poiseuille’s Law	4
1.7 Autoregulation	5
1.8 Quantification of Retinal Blood Flow	5
1.9 Retinal Blood Oxygen Saturation.....	6
1.10 Quantification of Blood Oxygen Saturation (Oximetry).....	7
1.10.1 Oxygen Transport	7
1.10.2 Pulse Oximeter Principle	8
1.10.3 Cerebral Oximetry	8
1.10.4 Retinal Oximetry	8
1.10.4.1 Invasive Measurements of Oxygen Saturation.....	9
1.10.4.2 Non-invasive Measurements of Oxygen Saturation.....	10
Haemoglobin Contribution in Oxygen Saturation Measurement	10
Haemoglobin Light Absorbance	11
Light Absorbance Assessment	12
1.10.4.2.1 Dual-Wavelength Retinal Oximetry	13
1.10.4.2.2 Three-Wavelength Retinal Oximetry	14
1.10.4.2.3 Multi-Wavelength Retinal Oximetry	14
1.10.4.2.4 Hyperspectral Approach	15
1.10.4.2.4.1 Hyperspectral Retinal Camera (HRC), Optina Inc.....	16
1.11 Methods Used to Measure Oxygen Saturation in Diabetic Retinopathy (DR).....	17
1.12 Advantage and Disadvantage of Non-invasive Retinal Oximetry Techniques	18

1.13	Extraneous Factors Affecting Retinal Oximetry Results	19
1.14	Clinical Applications of Retinal Oximetry	21
1.15	Review of Diabetes.....	22
1.15.1	Complications of Diabetes.....	22
1.15.2	Retinal Blood Flow in Diabetic Retinopathy.....	22
1.15.2	Vascular Reactivity (VR) in DR.....	23
1.15.3	Retinal Responses to Flicker in DR.....	23
1.15.3	Retinal Oxygen Saturation in DR	24
1.15.4	Clinical Classification and Etiology of DR	26
1.16	Retinal Biochemical/Morphological Changes Associated with Hyperglycemia 26	
1.16.1	Inflammatory Biochemical Biomarkers	26
1.16.1.1	Vascular Endothelial Growth Factor (VEGF)	26
1.16.1.2	Transforming Growth Factor Beta (TGF-β)	27
1.16.1.3	Angiopoietins-2 (Ang 2)	28
1.16.1.4	Leptin	29
1.16.1.5	Tumor Necrosis Factor (TNF-α)	30
1.16.1.6	Intercellular Adhesion Molecule-1 (ICAM-1)	30
1.16.1.7	Hepatocyte Growth Factor / Scatter Factor (HGF / SF)	30
1.16.1.8	Heparin-Binding Epidermal Growth Factor (HB-EGF)	31
1.16.1.9	Interleukin-1 β (IL-1 β)	31
1.16.1.10	Endothelial Growth Factor (EGF)	32
1.17	Summary.....	32
2	Rationale	34
2.1	General Objective.....	36
2.2	Specific Aims.....	36
2.3	Hypotheses	37
3	Variability and Repeatability of Quantitative, Fourier-Domain OCT Doppler Blood Flow in Young and Elderly Healthy Subjects	38
3.1	Introduction	39
3.2	Methods	41
3.2.1	Sample	41
3.2.2	Doppler Fourier-Domain Optical Coherence Tomography (Doppler FD- OCT)	43
3.2.3	Procedures	46

3.2.4	Image Grading	46
3.2.4.1	Semi-automated Doppler OCT of retinal circulation (DOCTORC).....	46
3.3	Results	49
3.4	Discussion	55
4	Retinal Blood Oxygen Saturation Disturbances in Patients with Type 2	
	Diabetes	60
4.1	Introduction	61
4.2	Methods	64
4.2.1	Sample	64
4.2.2	Assessment of Retinal Blood Oxygen Saturation.....	64
4.2.3	Statistical Analysis	67
4.3	Results	68
4.4	Discussion	71
5	The Association of Retinal Blood Flow and Retinal Blood Oxygen Saturation in	
	Mild-to-Moderate Diabetic Retinopathy	74
5.1	Introduction	75
5.2	Methods	78
5.2.1	Sample	78
5.2.2	Assessment of Retinal Blood Flow	78
5.2.3	Assessment of Retinal Blood Oxygen Saturation.....	79
5.2.4	Procedures	80
5.2.5	Analysis	80
5.3	Results	81
5.4	Discussion	84
6	Multiplex Analysis of Inflammatory and Angiogenic Biomarkers in Aqueous	
	Humor in Mild-to-Moderate Non-Proliferative Diabetic Retinopathy	86
6.1	Introduction	87
6.2	Methods	93
6.2.1	Sample	93
6.2.2	Assessment of Cytokines in Aqueous Humor (AH).....	93
6.2.3	Measurement of Cytokines Using Multiplex Analysis.....	94
6.2.4	Statistical Analysis	94
6.3	Results	95

6.4 Discussion.....	97
7 Retinal Blood Oxygen Saturation and Aqueous Humor Biomarkers in Early Diabetic Retinopathy	101
7.1 Introduction	102
7.2 Methods	107
7.2.1 Sample	107
7.2.2 Assessment of Retinal Blood Oxygen Saturation.....	108
7.2.3 Procedures	109
7.2.4 Measurement of Cytokines Using Multiplex Analysis.....	109
7.2.5 Statistical Analysis	110
7.3 Results	111
7.4 Discussion	119
8 General Discussion	123
8.1 Future work	126
Appendix A	128
Methodology	128
Procedure for sample collection	128
Analysis of angiogenic and inflammatory biomarkers	128
Letters of Copyright Permission.....	130
References	133

List of Figures

Figure 1.1 This graph represents absorption spectra of Hb and HbO ₂ . ³⁸ Reprinted by permission from Macmillan Publishers Ltd: [Eye] ³⁸ , copyright 2011.	11
Figure 3.1 FD-OCT Doppler image acquisition page. A double circular scan pattern with concentric radii is used for the measurement, which is typically centered at the optic nerve head.	45
Figure 3.2 Doppler FD-OCT image for beam passing through the superior nasal quadrant of the pupil. The peak of the sinusoidal should be positioned either within superior nasal or inferior nasal quadrants..	45
Figure 3.3 Cross-sectional of retinal blood vessels displaying Doppler signal. The grader adjusts the vessel circumference with a dotted circle; and the diameter with a horizontal solid line.	48
Figure 3.4 Box plot of individual COVs for blood flow in young and elderly participants. The error bars show the non-outlier range (± 1.5 times the height of the box).	49
Figure 3.5 Bland and Altman plot showing the difference in total blood flow as a function of mean blood flow between sessions for young subjects. MoD: Mean of Differences; 95% Conf. Limit: 1.96 x SD of the MoD (also known as Limits of Agreement).	51
Figure 3.6 Bland and Altman plot showing the difference in total blood flow as a function of mean blood flow between sessions for elderly subjects. MoD: Mean of Differences; 95% Conf. Limit: 1.96 x SD of the MoD (also known as Limits of Agreement)	52
Figure 3.7 Scatter plot illustrating test-retest characteristics of the Doppler FD-OCT for measuring total retinal blood flow ($\mu\text{l}/\text{min}$).....	53
Figure 3.8 Fundus image of an elderly subject with tortuous vessels and high COR value.....	59
Figure 4.1 Oxygen saturation in first-degree arteriole. The box plots illustrate retinal arteriolar blood oxygen saturation in control and NPDR participants. The error bars show the non-outlier range.	69
Figure 4.2 Oxygen saturation in first-degree venules. The box plots illustrate retinal venular blood oxygen saturation in control and NPDR participants. The error bars show the non-outlier range.....	70
Figure 5.1 Box plots representing venous retinal blood flow in control and NPDR patients (42.66 vs 32.97 $\mu\text{l}/\text{min}$; $p=0.004$). Box plots illustrating group mean retinal blood flow. The * represents extremes and the small circles represent outliers. The error bars show the non-outlier range.	81
Figure 5.2 Oxygen saturation as a function of retinal venous blood flow in controls ($r=0.243$, $p=0.34$)	82
Figure 5.3 Oxygen saturation as a function of retinal venous blood flow in NPDR group ($r=0.228$, $p=0.45$)	83
Figure 7.1 Retinal blood oxygen saturations as a function of biomarkers. Please note the difference in horizontal axis scaling for HGF. The number of subjects varies across assays.	117

List of Tables

Table 3.1 Individual subject / group characteristics for the young and elderly groups. (IOP, intraocular pressure (mmHg); Sys, systolic blood pressure (mmHg); Dias, diastolic blood pressure (mmHg); HR, heart rate per minute; F, Female; M, Male.)	43
Table 3.2 Co-efficient of variation (%) for venous area, velocity and blood flow.....	54
Table 3.3 Co-efficient of repeatability (COR) for individuals as a group (Young).....	54
Table 3.4 Co-efficient of repeatability (COR) for individuals as a group (Elderly).....	54
Table 3.5 Comparison of Co-efficient of Variation (COV) using different Doppler based technologies.	58
Table 4.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy) *(difference between control and NPDR group p<0.001).....	64
Table 5.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy). *(difference between control and NPDR group p<0.001).....	78
Table 5.2 Group mean values for blood oxygen saturation and total retinal blood flow (RBF) for non-proliferative diabetic retinopathy (NPDR) and aged-matched controls.	81
Table 6.1 AH levels of Cytokines in subjects. Cytokine levels in pg/mL \pm SD (range in cytokine values). OOR represents Out of range (in this case was lower in the range). *The values reported were below the range but detectable.	96
Table 7.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy) *(difference between control and NPDR group p<0.001).....	107
Table 7.2 Group mean values for blood oxygen saturation and aqueous humor biomarkers. OOR represents out of range. Bold values are significant.	111
Table 7.3 Pearson correlation values r and statistical significance (p) between baseline levels of biomarkers. The p-values are reported in ()......	111
Table 7.4 This table represents the repeat measurement values reported for one control and one NPDR. The sample volumes were low and this made it impossible to run subjects other than the two in the Table more than once. * One of the values was out of range (OOR) and the other one was reported in the standard range. NA; not applicable.....	118

List of Abbreviations

AGE	Advance Glycation End-products
ALK	Activin Receptor-like Kinase
Ang 2	Angiopoietin-2
AR	Aldose Reductase
A-V	Arteriole-Venule
BL	Basal Lamina
b-FGF	basic Fibroblast Growth Factor
BRB	Blood Retinal Barrier
BTF	Bragg Tunable Filter
CCD	Charge-Coupled Device
COR	Co-efficient of Repeatability
COV	Co-efficient of Variation
CRA	Central Retinal Artery
DR	Diabetic Retinopathy
ECM	ExtraCellular Matrix
ERK	Extracellular signal-Regulated Kinase
FD-OCT	Fourier-Domain optical coherence tomography
Hb	Deoxyhaemoglobin
HbO ₂	Oxyhaemoglobin
HB-EGF	Heparin-Binding Epidermal Growth Factor
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HIF	Hypoxia-Inducible Factor
HRC	Hyperspectral Retinal Camera
ICAM-1	Intercellular Adhesion Molecule-1
IGF-I	Insulin-like Growth Factor I
IL	Interleukin
MAPK	Mitogen-Activated Protein Kinases
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NPDR	Non-Proliferative Diabetic Retinopathy
NDR	No Diabetic Retinopathy
OCT	Optical Coherence Tomography
OD	Optical Density
ODR	Optical Density Ratio
p38 MAPK	p38 Mitogen-Activated Protein Kinases
PEDF	Pigment Epithelium-Derived Factor
PDR	Proliferative Diabetic Retinopathy
PKC	Protein Kinase C
RBF	Retinal Blood Flow
RPE	Retinal Pigment Epithelial
ROS	Reactive Oxygen Species
SO ₂	Oxygen Saturation
SLO	Scanning Laser Ophthalmoscope
TGF- β	Transforming Growth Factor Beta
TLS	Tunable Laser Source

TNF- α
VEGF
VR

Tumor Necrosis Factor Alpha
Vascular Endothelial Growth Factor
Vascular Reactivity

1 Introduction

1.1 Retinal Oxygen Supply

The retina is a tissue with extraordinary metabolic demand, especially for key molecules such as oxygen^{1,2}. The retina is constantly active and converts light into neural signals and this activity increases in the dark as well (with increase in oxygen consumption). The retinal cells demand a continuous oxygen supply. Nonetheless, a vascular network situated in front of the photoreceptors would affect with transmission of light to the photoreceptors. The outer 1/3 to 1/2 of the retina collects most of its oxygen demand via the much greater choroidal microcirculation. The choroid nourishes the outer retinal layers, including the photoreceptors^{3,4}. The oxygen diffusion from choroid to the layer of photoreceptors results in an extraordinary concentration of oxygen². The uveal system, specifically the choriocapillaries, feeds the outer retinal layers closest to Bruch's membrane. The short posterior ciliary arteries directly supply the choroid posteriorly, whereas the long posterior ciliary arteries travel in the suprachoroidal space anteriorly then supply the choroid anteriorly via recurrent branches. The retinal inner layers are nourished by the retinal blood vessels. The inner retina maintains its blood via the aortic artery, common carotid arteries, internal carotid artery, ophthalmic artery and finally the central retinal artery (CRA). The CRA enters the optic nerve 10-15 mm behind the globe and runs forward in the central section of the nerve along with the central retinal vein. The CRA supplies the inner two thirds of the retina, the furthestmost anterior share of the superficial nerve fiber layer of the optic nerve head and to some extent the retrolaminar optic nerve^{3,4}.

1.2 Structure of the Retinal Vessels

Retinal vessels are made of 3 layers: (1) adventitia, (2) smooth muscle cells, and (3) endothelial cells.

The CRA and specifically the downstream arterioles are considered to be responsible for blood flow regulation to the capillaries; the arterioles govern downstream retinal blood flow and as a result are termed as “resistance” vessels. Retinal capillaries are composed of a basement membrane containing pericytes covered by a single layer of endothelium. Capillaries are responsible for facilitating exchange (often termed “exchange” vessels) of metabolites at the tissue level ⁵. The tight junctions between the endothelial cells create the inner blood retinal barrier (BRB), inhibiting leakage of blood components into the retinal tissue for ideal retinal functioning.

1.3 Blood-Ocular Barrier

The blood-ocular barrier system is designed in 2 forms: the blood-aqueous barrier and the BRB. The blood-aqueous barrier is made of an epithelial barrier found in the non-pigmented layer of the ciliary epithelium and in the posterior iridial epithelium, and by the iridial vessel’s endothelium. The BRB is a physiologic barrier that controls flux of water, ion, and protein into and out of the retina. It contains inner and outer modules, the inner BRB is designed with tight junctions between retinal capillary endothelial cells and the outer BRB is made of tight junctions between retinal pigment epithelial cells.

The BRB is critical for sustaining normal visual function. Variations of the BRB present a vital role in the development of retinal diseases such as diabetic retinopathy and age-related macular degeneration. Diabetic retinopathy is commenced by a variation of the inner BRB and Macular edema is a direct result of changes of the BRB ⁶.

1.4 Retinal Blood Flow

A constant circulation of blood in the cardiovascular network is termed as blood flow. This is necessary for the transportation of nutrients, hormones, oxygen, carbon dioxide, metabolic waste in the body to sustain metabolism at cellular level, pH, body temperature, and the protect from microbial and mechanical disturbances. The principles that manage the flow within a cylindrical tube can be applied to the movement of blood cells within a blood vessel. Particularly, the flow of fluid within a tube is determined by two factors: 1) the perfusion pressure difference between the two ends of the vessel 2) vascular resistance, since the impediment to blood flow in a vessel is resistance. But there is not any straight method to measure the resistance. Resistance to blood flow within a vascular system differs upon the size factors of individual vessels (i.e. length and diameter), the way they are prearranged in the vascular system (i.e. series and parallel arrangements), physical individualities of the blood (i.e. viscosity, laminar flow versus turbulent flow) and extravascular mechanical forces acting upon the vasculature (in terms of the eye, the intraocular pressure)⁷.

Ohm's law explains the same physical issues that impact the flow of any fluid, and are based on a fundamental law of physics⁸.

$$Q = \frac{\Delta P}{R}$$

Flow (Q) is related to the difference in pressure (ΔP) between the two ends of the tube and inversely correlated to the resistance (R).

The rate of the blood flow across a vessel for a particular pressure difference is termed the conductance. Conductance equals the reciprocal of resistance:

$$\text{Conductance} = \frac{1}{\text{Resistance}}$$

Once the blood flow is unstable, minor changes in the diameter of a vessel creates substantial changes in the vessel's ability to conduct blood. The conductance of the vessel increases in proportion to the fourth power of the diameter, in agreement with the following formula: ⁷

$$\text{Conductance} \propto \text{Diameter}^4$$

$$R \propto \frac{\eta L}{r^4}$$

Where R is vessel resistance, L is length, η is blood viscosity, and r^4 is the fourth power of radius.

1.5 Laminar Blood Flow

The behaviour of blood flow throughout most of the circulatory system is termed laminar flow. It is distinguished by concentric layers of blood moving at different velocities in parallel down the length of a blood vessel. Basically, laminar flow is defined as when a fluid flows in parallel levels, with no distraction between the levels. The peak velocity (V_{max}) is located in the center of the vessel and the lowest velocity ($V=0$) is seen along the vessel wall ⁷.

1.6 Poiseuille's Law

Poiseuille's law is a physical law that states that the velocity of a liquid flowing through a capillary is directly proportional to the pressure of the liquid and the fourth power of the radius of the capillary and is inversely proportional to the viscosity of the liquid and the length of the capillary.

The formula below, known as Poiseuille's law, can be derived by integrating the velocities of all the concentric rings of flowing blood and multiplying them by the areas of the rings:

$$Q = \frac{\pi \Delta P r^4}{8 \eta l}$$

In which Q is the rate of blood flow, ΔP is the pressure difference between the two ends of the vessel, r is the radius of the vessel, l is length of the vessel, and η is viscosity of the blood ⁷. Therefore, huge alterations of blood flow can be achieved with small changes of vessel diameter.

1.7 Autoregulation

Autoregulation is the intrinsic regulation of retinal blood flow, such that a change in the arterial blood pressure produces a compensatory change in diameter so that the blood flow is kept constant. Autoregulation is defined as “the ability of a vascular bed to maintain blood flow to the tissues under conditions of varying perfusion pressure” ⁹.

Metabolic autoregulation is the ability to change perfusion in response to altered tissue needs ¹⁰. For example, when metabolic activity increases blood flow will proportionally increase. Autoregulation and metabolic autoregulation represent different mechanisms involved in the overall regulation of retinal blood flow.

1.8 Quantification of Retinal Blood Flow

The methods for measuring retinal blood flow have improved but still require further development. Some of these methodologies are described briefly here.

Previous techniques for assessing retinal perfusion have several restrictions, such as being invasive (e.g. Fluorescein angiography), or are subjective (e.g. Blue field entoptic phenomenon), or are incapable of calculating blood flow (e.g. Retinal Vessel Analyzer)

since a surrogate factor of flow is actually measured. Furthermore, the Canon Laser Blood Flowmeter is an instrument that utilizes bi-directional laser Doppler velocimetry and simultaneous vessel densitometry to determine volumetric retinal blood flow and is currently restricted to relatively large vessels ($>60\mu\text{m}$) and a single measurement site.

Fourier-Domain optical coherence tomography (FD-OCT) is a non-contact imaging method that mixes micrometer-scale resolution with millimeter image penetration depth¹¹⁻¹³. It is similar to the ultra sound technique with the exception of using light energy in the form of laser instead of sound. FD-OCT is based on low coherence interferometry, a classic optical measurement technique. FD-OCT makes high-resolution imaging possible. It is clinically used in the diagnosis and management of retinal diseases.

To overcome some the previous limitations of retinal blood flow measurements, a new prototype termed Doppler FD-OCT blood flow (e.g. Optovue RTVue) has been developed. Doppler FD-OCT measures the intensity of light back-reflected from various features within the imaged object resulting from spatial variations of the tissue refractive index and then detects the Doppler signal in each point within the object^{14,15}.

Another OCT based technology, termed bi-directional OCT, has been designed to measure retinal blood flow independent from the angle of incidence¹⁶. This is based on the illumination of a retinal vessel by two lasers from two different angles (α_1 and α_2), separated by a known angle $\Delta\alpha$ ¹⁷.

1.9 Retinal Blood Oxygen Saturation

A critical aspect of the circulation is the overall oxygen delivery to the tissues. Measurement of oxygen in the blood (SO_2) is called oximetry; in other words, oximetry is a technique in which the blood oxygen saturation level is quantified. A constant supply of

oxygen to the retina is required and this demand is regulated primarily by the choroidal blood vessels^{1,2,18} and to a lesser extent by the inner retinal vessels. Serious pathological conditions arise when this supply is disturbed. For example, the major hallmarks of proliferative diabetic retinopathy (e.g. retinal neovascularization and macular edema) are thought to partly result from hypoxia and defective vascular autoregulation^{19,20}. Therefore, it is critical to study the regulation of oxygen supply to the retina in order to learn more about the pathophysiology of diseases such as diabetes and age-related macular degeneration.

The combination of retinal oxygen saturation and retinal blood flow can be used to extract more information about retinal metabolism since:

$$\text{Retinal O}_2 \text{ Delivery} = [1.39 \times \text{Hb} \times \text{SaO}_2 + (0.003 \times \text{PaO}_2)] \times \text{blood flow}$$

Theoretically, each gram of Hemoglobin binds 1.39 ml of oxygen. The oxygen saturation curve represents a PaO₂ (arterial oxygen tension) as a function of SaO₂ (Arterial SO₂). The hemoglobin concentration is calculated by production, destruction and loss. 0.003 stands for the oxygen solubility coefficient in human plasma²¹.

There are some methods that simultaneously measure retinal blood SO₂ and retinal blood flow measurements, which are restricted to animals.

1.10 Quantification of Blood Oxygen Saturation (Oximetry)

1.10.1 Oxygen Transport

Oxygen is one of the most required substrates in virtually all tissues. Oxygen diffuses into the pulmonary blood in the lung's alveoli and then will be transported to the tissues. Haemoglobin is responsible for carrying almost all of the oxygen in the blood. It is

composed of four subunits; each accompanied with a haeme group plus a globin chain. The heme group is composed of a porphyrin ring that contains an iron (Fe) atom in its center. Typically, the Fe is in the +2 redox state (ferrous) and can reversibly bind oxygen ²².

1.10.2 Pulse Oximeter Principle

Pulse oximetry is a non-invasive technique to measure the oxygen saturation in arterial blood. Pulse oximetry is based on the different absorption characteristics of oxyhaemoglobin (HbO₂) and deoxyhaemoglobin (Hb) for red and infrared light ²³. A pulse oximeter is based on spectral analysis and brings together two technologies, spectrophotometry (a technique which measures haemoglobin oxygen saturation) and optical plethysmography (a technique which measures changes in pulsatile of arterial blood volume where the sensor is located) ²³.

1.10.3 Cerebral Oximetry

Similar technology to pulse oximetry applies for cerebral oximetry in that it uses different light absorption between oxygenated and deoxygenated hemoglobin to quantify oxygen saturation (650–900nm) ²⁴. Cerebral oximeters release consecutively pulsed near-infrared light that is capable of penetrating the skull, and detect photons reflected back through the skull to define oxygen saturation. It represents a balance between oxygen delivery and consumption of the brain.

1.10.4 Retinal Oximetry

The ocular fundus provides a direct view of retinal vessels. Imaging these vessels can deliver information on retinal morphology, retinal blood flow and even blood oxygen saturation. Retinal blood oxygen saturation might be associated with metabolic changes in ocular diseases. The notion of retinal oximetry has been acknowledged since the 18th

century. Attempts to non-invasively measure the retinal blood oxygen saturation date back to the 1950's. Different retinal oximetry techniques are described in the literature, the primary ones are: photographic, digital and spectroscopy methods. Each method has its advantages and disadvantages. Generally, the techniques used to study retinal oxygen saturation can be divided into invasive and non-invasive methods. Invasive measurements are defined as techniques that involve penetration to the body (probe insertion, dye injection).

1.10.4.1 Invasive Measurements of Oxygen Saturation

Generally two types of probes exist: oxygen sensitive polarographic electrodes and probes containing oxygen sensitive dye.

The electrode method is established on the basis of an electrochemical reaction. This reaction involves oxygen as a substrate. This is achieved by using a polarising voltage across the two electrodes. The reaction rate correlates with the concentration of dissolved oxygen in the vicinity of the electrodes and a current ²⁵⁻²⁹.

The other generation of the probes holds an oxygen sensitive dye, palladium-mesotetra-(4-carboxyphenyl)-porphyrin. The dye is excited by specific wavelength light and then re-radiates light with a different wavelength, which can be detected. The reradiated light is influenced by the concentration of oxygen ³⁰⁻³².

Another invasive technique exists which is based on injecting an oxygen sensitive dye into the blood stream instead of adhering the dye to the optic probe and inserting it to the eye. The injected dye will reach the retinal and choroidal vasculature as well as the rest of the vasculature in the body. Subsequently the retina and the choroid will be illuminated with a specific wavelength light and the reemitted light will have the information on the oxygen

concentration in the vicinity of the dye. Unfortunately, this technique is not safe to be used for human retina since the dye is not risk-free (e.g. toxicity). Several studies have used oxygen sensitive techniques³³⁻³⁶ but their use is confined to patients who are undergoing surgery (e.g. vitrectomy). Generally, the invasive techniques are either limited to animal studies or patients with a restricted population. However, these studies contributed a wealth of information on retinal and choroidal oxygenation, mainly operating on animal models, in health and ocular diseases.

1.10.4.2 Non-invasive Measurements of Oxygen Saturation

Haemoglobin Contribution in Oxygen Saturation Measurement

Non-invasive retinal oximetry demands an imaging system, which captures retinal images at multiple wavelengths of light. Retinal non-invasive measurements of oxygen saturation are based on measurements of haemoglobin color / absorbance. This makes it compulsory to study the key characteristics of haemoglobin and oxygen transportation in blood. Oxygen is transported in the blood either bound to haemoglobin or dissolved in the plasma (9 mmol of oxygen is carried by binding to haemoglobin in each liter of arterial blood and about 0.1mmol/L is dissolved in plasma considering haemoglobin concentration is 150g/L, assuming standard pH)³⁷. The percentage of dissolved oxygen is insignificant in both arterial and venous blood. Therefore measuring the magnitude of oxygen bound to haemoglobin could be a useful assessment of the oxygen content in blood in the retina. Oxygen saturation is usually defined as the proportion of haemoglobin that is bound to oxygen.

$$\text{Oxygen Saturation} = \frac{[HbO_2]}{[Hb] + [HbO_2]} \times 100\%$$

[HbO₂] is the concentration of oxygenated haemoglobin and [Hb] is the concentration of deoxyhaemoglobin

Haemoglobin Light Absorbance

As mentioned above, non-invasive oxygen saturation measurements are based upon the absorbance of haemoglobin. Oxygenated and deoxygenated haemoglobin represent different absorbance properties.

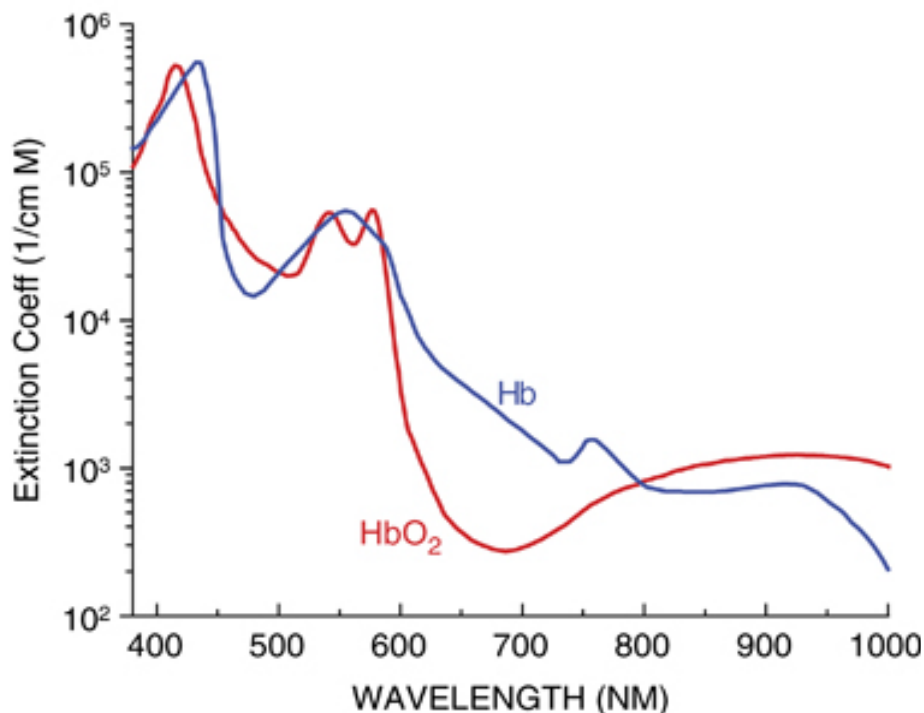


Figure 1.1 This graph represents absorption spectra of Hb and HbO₂.³⁸ Reprinted by permission from Macmillan Publishers Ltd: [Eye]³⁸, copyright 2011.

Light absorption of oxygenated and deoxygenated haemoglobin is different at most wavelengths of light. Although as can be seen in the above figure (Figure 1.1), the absorption of light in oxy- and deoxyhemoglobin is the same at certain wavelengths, and these are termed isosbestic wavelengths.

Light Absorbance Assessment

Light absorbance of a solution can be described with optical density; and optical density is defined as:

$$OD = \log \frac{I_0}{I}$$

OD is optical density

I_0 is the original light intensity before reduction of light due to absorbance

I is the light intensity after absorbance in the sample / solution has weakened the light intensity.

Looking at the optical density equation, it can be concluded that a greater optical density results in greater absorbance. A blood vessel optical density measured at an isosbestic (oxygen insensitive) wavelength will be related to vessel diameter and other factors but not oxygen saturation. Also, optical density at a non-isosbestic wavelength (oxygen sensitive) will rely on similar factors as the optical density at an isosbestic wavelength but also on oxygen saturation. Therefore the ratio optical densities at a non-isosbestic and isosbestic wavelength will be sensitive to oxygen saturation while the influence of other factors such as vessel diameter will neutralise. Several studies^{39,40} showed that for a solution of haemoglobin in water there is a roughly linear relationship between such ratio and oxygen saturation;

$$\text{Oxygen Saturation} = a + b \times \frac{OD_{\text{non-isosbestic}}}{OD_{\text{isosbestic}}}$$

Where a and b are constant and ODR is the ratio of optical densities.

$$ODR = \frac{OD_{\text{non-isosbestic}}}{OD_{\text{isosbestic}}}$$

It is more difficult to measure blood in retinal vessels than a solution of haemoglobin in a test tube. The above equation is imprecise as the light is scattered from blood cells and vessel walls⁴¹⁻⁴⁵. Moreover, the light intensities, I and I_0 have to be assessed from reflected light rather than transmitted light and the differences in pigmentation in the retina (within and between subjects) can affect the measurement^{39,41}.

The light intensity is estimated by the brightness assessment on the vessel. This brightness is influenced by light absorbance by blood in the vessel. I_0 , the reference light intensity, is estimated by the brightness of the image next to the vessel. Using brightness near the vessel for reference (I_0), the light intensities I and I_0 , are influenced by comparable factors apart from light absorbance by the vessel.

1.10.4.2.1 Dual-Wavelength Retinal Oximetry

Hickam and Frayser were the first to noninvasively measure the retinal oxygenation in human using photographic methods by using particular filters to acquire fundus images with two wavelengths of light^{40,46}. Laing and co-workers followed their work and established two-wavelength photographic densitometry to obtain oxygen saturation in retinal vessels in rabbits⁴⁷. This technique is restricted due to its laborious nature. It is fundamentally restricted by the nonlinearity and inconstant reproducibility of photographic film.

Beach and co-workers introduced another version of two wavelengths to measure oxygen saturation³⁹. The key difference was the use of digital camera technology and optical solutions that was not used by Hickam. Beach and co-workers also tried to compensate for the eye movement by capturing both images (one with a wavelength sensitive to oxygen saturation and one insensitive) at the same time and with a single flash. They also tried to

eliminate the effect of vessel diameter and fundus pigmentation. Hardarson and co-workers used a technology based on the technology of Beach and co-workers. They improved the computer programs, which made it more repeatable ⁴⁸. Hammer and co-workers also developed an instrument, which uses two wavelengths of light simultaneously but using a different optical approach ^{49,50}.

1.10.4.2.2 Three-Wavelength Retinal Oximetry

An instrument that utilised the scanning of three wavelengths and the detection of reflected light electronically was built to compensate for the effect of light scatter. The technique uses scanning fundus reflectometry to calculate the optical density of a retinal vessel at three wavelengths (558, 569, and 686 nm). A vessel tracking system was used to reduce the effects of eye movements during scanning ⁴². One of the drawbacks of this method is that it only measures a small retinal area at a time. Later, another scanning technique was introduced with four wavelengths ⁵¹.

1.10.4.2.3 Multi-Wavelength Retinal Oximetry

It is difficult to obtain absolute oxygen saturation because of the approximation made in two-wavelength oximetry. The result of two-wavelength oximeters might rely on factors such as vessel diameter and fundus pigmentation. Some groups attempted to measure absolute retinal oxygen saturation with more wavelengths and different modelling to estimate the saturation from light intensities. Some of these techniques are challenged by long exposure time and therefore limited to immobilized eyes in animal studies.

The simultaneous capture of light at multiple wavelengths (less than 2nm apart over a range of several hundred nanometres) was performed by coupling an imaging spectrograph with a fundus camera ^{52,53}. This method might produce absolute values but it is restricted to

only a slit of 1.5 mm x 40 μ m at a time. This permits simultaneous assessment of a retinal arteriole and venule if they are close together. The retina of patients with central retinal vein occlusion was illuminated by bright light for six seconds and separated multiple wavelengths with interferometry⁵⁴. The method is thought to produce oxygen saturation in every pixel of a 35° fundus image. Later, a hyperspectral system with a spectral resolution of 2.5 nm and a range of 410 nm to 950 nm was used^{55,56}. Measurements were acquired at the optic nerve head of monkeys and each scan took eight seconds.

Acquiring information on multiple wavelengths in a short time is challenging because of not exceeding safe light levels. However, reducing the area of measurement can solve this. Schweitzer and co-workers⁵³ achieved this by measuring only a slit at the fundus.

A lens-let array and different filters behind each lens-let has been used by Ramella-Roman and co-workers⁵⁷. This makes it possible to simultaneously capture an image at several different wavelengths. An instrument that captures information on eight wavelengths simultaneously by using spectral demultiplexing was established by Harvey and co-workers (2005)⁵⁸.

Soon after, the multi- / hyper-spectral retinal oximetry approach was performed using a technique based upon a hologram of up to 50 distinct wavelengths in snapshot images⁵⁹. This technique has been mostly limited to animal models.

1.10.4.2.4 Hyperspectral Approach

Spectral retinal imaging is a fairly new technique that combines the capabilities of spectroscopy and imaging. Spectroscopy is a well-established tool used to investigate and analyze materials by means of obtaining and finding the spectral signatures of their elements using various combinations or sequences of wavelengths. Spectral imaging

expands the efficacy of spectroscopy benefiting both spectroscopy and imaging, thus offering both spectral and spatial communication. A stack of monochromatic images is projected onto a two-dimensional detector array, such as a charge-coupled device (CCD); the data are filed and multiple images are gathered over multiple wavelengths forming a “spectral data cube”^{60,61}.

At present, conventional imaging techniques provide a digital camera (e.g. CCD) to record data. A multispectral or hyperspectral imaging system is capable of generating a reflectance or transmission spectrum of each pixel within the image allowing the identification of substances by recognizing its corresponding spectral signature. The spectral imaging system is principally reliant on a combination of its spectral and spatial resolution.

A more detailed pixel spectrum is delivered with hyperspectral imaging compared to a multispectral pixel spectrum. Consequently, this detailed spectrum allows more information to be acquired for accurate classification and quantification. The accuracy of the spectral analysis is affected by the higher spatial resolution. Generally, hyperspectral imaging of the retina enables direct and quantitative mapping of retinal biochemistry.

1.10.4.2.4.1 Hyperspectral Retinal Camera (HRC), Optina Inc.

A prototype custom-built fundus camera (H-8.5 HR Camera, Optina, QC, Canada) is the foundation of the hyperspectral imaging system that incorporates a tunable laser source (TLS) as the light source. The TLS makes it possible to transfer wavelengths within a spectral range of 400-1000nm (visible to IR) and with a half peak bandwidth of 2nm. The TLS is built on Photon etc’s Bragg grating filtering technology which permits rapid wavelength presentation from the steady and powerful super-continuum light source (Leukos-SM-30-OEM, Leukos Innovative Optical Systems, Limoges, France). The TLS

can be electronically tuned with an operating ambient temperature of 10-30°C. An automatic spectral system is incorporated into the system to achieve exact and accurate (<1nm) wavelength choice. The sensitive 1.3MPixel (1392 x 1040 pixel) 14 bit CCD camera (Pixelfly USB, PCO AG, Kelheim, Germany) used in this system is for high definition imaging. The imaging system is operated by using PhySpec (Photon Etc, Montreal, QC, Canada), a software programme that manipulates the Bragg tunable filter (BTF) and CCD camera to allow aspects such as operator defined wavelength range and wavelength interval. A low power white light source (delivering about 100mW over 420nm to 2400nm) and the BTF are used to exclude the use of conventional flash lamp, and therefore all images are acquired at low light levels, thereby minimizing any potential change of the metabolites and photopigment status in the eye. A diagonal field of view of approximately 37.4° is sustained by the opto-mechanics of the system (Personal Communication, Dr Jean-Philippe Sylvestre, 2013) ⁶².

1.11 Methods Used to Measure Oxygen Saturation in Diabetic Retinopathy (DR)

Tiedeman and co-workers ⁶³ used a dual wavelength retinal imaging system in diabetic subjects without clinical evidence of diabetic retinopathy during normoglycaemia and hyperglycaemia. They reported evidence of significantly higher retinal oxygen consumption during hyperglycemia. Hammer and co-workers showed higher venular oxygen saturation with the severity of diabetic retinopathy in subjects with mild non-proliferative DR and in subjects with proliferative DR ⁴¹. An insignificant increase in retinal oxygen saturation was also reported comparing healthy controls to non-proliferative diabetic retinopathy subjects, though significance was only reported for severe- non-proliferative diabetic retinopathy and proliferative DR ⁵⁶. This suggests that imaging

techniques such as hyperspectral retinal imaging may provide improved methods of predicting the probability of onset, differentiating the severity and of observing progression of diabetic retinopathy in vivo. A recent study by Man and co-workers⁶⁴ suggested that eyes with longer axial length show lower retinal function and hence lower O₂ consumption; therefore eyes with longer axial length are fairly less hypoxic when diabetes exists, which may partly explain the reduced risk of DR in these eyes. Hardarson also reported increased retinal blood oxygen saturation in patients with diabetic retinopathy⁶⁵.

1.12 Advantage and Disadvantage of Non-invasive Retinal Oximetry Techniques

The first methods introduced by Hickam used photographic densitometry of large retinal vessels in vivo to monitor arterial and venous retinal blood oxygen saturation⁴⁰. This method depended on external calibration by means of independent arterial oxygen saturation measurement. This technique is laborious and restricted by the nonlinearity and variable reproducibility of photographic film.³⁹

Delori established a retinal oximetry technique based on retinal photoelectronic measurements of optical density at three wavelengths. A mechanical scanner in a modified fundus camera equipped with a photomultiplier detector to sequentially monitor the light reflected inside and outside vessels at three wavelengths was used. This method could compensate for the effect of light scatter and minimize the eye movements during scanning. However, this technique was limited by measuring a small area at a time. The multi-wavelength oximeter introduced by Oxymap (Reykkavik, Iceland) has the advantage of measuring a larger area and little expertise to acquire pseudo-color fundus image of retinal vessel oxygenation. The disadvantage of this method is that they used a flashlight that can in turn cause metabolic / photochemical changes in the retina. Flash intensity

significantly affects retinal vessel oxygen saturation measurements using dual-wavelength retinal oximetry ⁶⁶. In terms of retinal oxygen saturation imaging, the technology provided by Optina Inc (previously Photon etc) permits the acquisition of hyperspectral retinal images for numerous wavelengths in a fraction of a second (*i.e.* minimizing eye movement effects) at background light levels (BTF) far lower than any of the competing technologies, thereby avoiding disturbance of retinal metabolic status.

The other drawback of the Oxymap oximeter is that it can only give relative oximetry values and this explains why sometimes the reported values are over 100%. However, it has been reported that the results are repeatable and sensitive to changes in oxygen saturation. The best interpretation of the results would be the oxygen saturation is probably slightly higher in diabetic patients than normal ⁶⁷.

1.13 Extraneous Factors Affecting Retinal Oximetry Results

To date, no technique has been definitively able to quantify retinal blood oxygen saturation independent of vessel diameter, haemoglobin concentration, and fundus pigmentation. One of the puzzling characteristics of whole-blood spectroscopic oximetry techniques is the scattering of light. The refractive-index discontinuity between red blood cells and the plasma in which they are suspended causes light scattering ^{43,68}.

Two optical spectroscopic techniques have been introduced to determine the retinal oxygen saturation. One method illuminates an area of the retina and the retinal images are formed on film or a CCD detector array. The other method scans multiple monochromatic beams across a retinal vessel. The light that reflects back out of the eye is collected at distinct points along the scan. Both methods are similar in that the reflected light from the center of

the vessel is compared with the reflected light from the fundus on each side of the vessel to calculate the transmittance of the blood within the vessel.

Generally, a beam of light passes through the pupil of the eye and is focused onto a retinal vessel. Then the fraction of light that is reflected back out of the pupil of the eye is collected by retinal oximeter. There are several light paths that influence this collected power. Some quantity of the incident light may be directly backscattered from the cornea, lens or vitreous. Also, there is a specular reflection from the apex of the vessel. This reflection may derive from the inner limiting membrane ⁶⁹ or from the vessel wall. The beam flux decreases according to the Lambert–Beer Law due to the differential light absorption by hemoglobin and oxy-hemoglobin within the red blood cells contained in the vessel. Light is also backscattered by red blood cells. A portion of this scattered light is distributed at angles that cannot be collected by the instrument and this unquantified scattered light component results in an apparent rise in absorption. The beam that emerges from the other side of the vessel is amplified due to scattering and attenuation of absorption. The beam passes through the retinal rods and cones and ultimately reaches the scattering and also absorbing layers of the retinal pigment epithelium and choroidal plexus ⁷⁰. Strong melanin and hemoglobin absorption restricts lateral diffusion and causes a tight localized point spread function on the retinal pigment epithelium for visible wavelengths shorter than 575 nm. For wavelengths above 575 nm, the light enters the choroid deeply and reflects off the sclera and scatters back through the choroid ⁷¹. Concerning retinal oxygen saturation imaging, the conventional flash used to adequately illuminate the fundus will change the metabolites and photopigments of the retina.

There are also other technical problems associated with the oximetry techniques such as poor image quality as a result of small vessel branches and haemorrhages. The oximeters can only quantify large first or second-degree vessels reliably ($> 50 \mu\text{m}$ diameter) and vessel diameter may disturb the outcomes. Haemorrhages close to the measured vessel segment can also affect the results of the measurement by affecting the measured brightness, used as a reference in the saturation calculation.

1.14 Clinical Applications of Retinal Oximetry

Accumulating evidence suggests that vascular dysfunction plays a role in several prominent ophthalmic diseases such as diabetic retinopathy, glaucoma, etc. These studies provide data on ocular blood flow using a relatively small number of complicated ocular blood flow imaging techniques. Although these techniques are not used clinically, they can potentially offer the earlier detection of ocular diseases and possibly be utilized to sensitively monitor new therapeutic strategies.

Clinically, retinal oxygen saturation can be used in the early detection of changes in autoregulation⁷ and blood flow in the retina that have been linked to the onset of diabetic retinopathy (DR). Reduced oxygenation and the subsequent abnormal angiogenesis results in loss of retinal tissue and resulting vision impairment⁷². Another useful application of retinal oxygen saturation is the assessment of central venous oxygen saturation as an indicator of oxygen delivery to vital organs such as the brain⁷³. There are invasive procedures for monitoring oxygen delivery, for instance fiber optic sensors inserted into the heart and pulmonary artery⁷⁴, they can be used in a hospital environment but they are not suitable for ambulatory medical care. Therefore, the eye could be the ideal candidate as a

window to the body and the retina for monitoring blood oxygen levels since retinal vessels are easily accessible and readily imaged.

1.15 Review of Diabetes

Diabetes is a metabolic disorder of multiple etiologies considered as a persistent hyperglycemia resulting from defects of insulin secretion, insulin action or both ⁷³. There are two main types of diabetes: are type 1 (10% of individuals; categorized by autoimmune destruction of Islet cells) and type 2 (90% of individuals; categorized by insulin resistance and / or reduced insulin secretion) ⁷⁶.

1.15.1 Complications of Diabetes

Diabetes is often accompanied by several complications that share the same aetiology and a major of these complications is vascular related. The Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study acknowledged chronic hyperglycemia as an initiating cause in the complications presented in diabetes that influence the retina, kidneys, nerves that result in myocardial infarction and cerebrovascular accident. Both macro- and micro- vascular complications of diabetes are decreased when rigorous control of blood glucose is maintained ^{77,78}.

1.15.2 Retinal Blood Flow in Diabetic Retinopathy

Even though hyperglycemia is thought to be the initial insult in the pathogenesis of DR ⁷⁹, the effects on hemodynamic parameters should not be underestimated in the development of DR. Retinal blood flow impairment is one of the earliest functional abnormalities detected in diabetes ^{80,81}.

The notion of altered retinal blood flow in diabetes is not new⁸⁰. Many studies have been dedicated to assess retinal hemodynamics. The commencement of clinical signs of evident

DR has been proposed to take place after retinal vasodilation. Elevated retinal blood flow has been classically proposed to ultimately cause the progress of DR, probably because of amplified frictional forces (i.e., shear stress) on the endothelial cells lining the walls of retinal vessels ⁸¹. However, the exact nature of the blood flow disruption is debatable, perhaps due to the variety of methods used to measure retinal hemodynamics, the diverse stages of retinopathy considered, and the diversity of the diabetic groups ⁸². From a clinical perspective, the assessment of ocular hemodynamics requires an earlier detection of DR and improved DR severity differentiation and perhaps a flag for new treatment opportunities. Retinal hypoxia is thought to promote the production of vascular endothelial growth factor (VEGF) and the development of macular edema and neovascularization. Measurement of retinal oxygenation in patients with diabetes may help to assess the severity of DR and the response to novel “prophylactic” treatments. Ultimately, knowing the blood flow to, and the oxygenation of, the retinal tissue will permit the assessment a metabolic parameter of the retinal tissue, namely oxygen utilization (also termed “retinal oxygen delivery”).

1.15.2 Vascular Reactivity (VR) in DR

VR characterizes the hemodynamic response of the vasculature to a stimulus, such as hyperoxia or hypercapnia ⁸³. VR has been reported to be impaired in response to hyperoxia with simultaneous hyperglycemia ^{84,85}. However, another study has shown that hyperglycemia has no effect on hyperoxia induced retinal vascular reactivity ⁸⁶⁻⁸⁸.

1.15.3 Retinal Responses to Flicker in DR

While the particular mechanism behind flicker-induced vasodilatation is still controversial, it has been used to test the ability of retinal vessels to adjust to different metabolic

circumstances in health and disease. Garhöfer and co-workers showed that flicker responses of retinal arterioles and venules are significantly decreased in patients with diabetes with no or mild NPDR ⁸⁹. More recently a study by Lasta and co-workers confirmed the previous study in type 1 diabetes with no or mild NPDR ⁹⁰.

1.15.3 Retinal Oxygen Saturation in DR

It has been suggested that a change in blood circulation leads to functional damage and broad retinal tissue impairment and disturbance during diseases such as DR ⁴². In this respect, it will be critical to study the oxygen distribution or consumption of the retina and its alteration in response to DR since RBF or SO_2 alone represent only part of the required information to calculate retinal oxygen utilization. Measurement of these alterations could be used to improve the capability of early detection of diseases such as diabetes / diabetic retinopathy. Most of the non-invasive optical techniques that are used to measure the retinal oxygen saturation rely on measuring differences between hemoglobin and oxygenated hemoglobin (Hb and HbO_2) light absorption (see Section procedures for complete methodology explanation). Simultaneous retinal blood flow measurement and retinal hemoglobin oxygenation are required to assess absolute values of oxygen delivered to the retina ^{45,49}. The non-invasive quantification of oxygenation of the retina will be feasible by retinal oximetry, which in turn may offer a sensitive and predictive biomarker. Quantification of blood flow and oxygenation of the retina will permit the assessment of the tissue oxygen utilization.

Assessing oxygen concentration in the retina has been achieved using O_2 -sensitive microelectrodes introduced into the eye ⁹¹⁻⁹⁴. While this technique is precise and can accurately define oxygen saturation, the invasive nature of the technique limits its' usage to

animal models and disqualifies it from clinical purpose. Alternative methods using the introduction of a phosphorescent dye has been utilized to derive oxygen concentration in the optic nerve head and retinal vessels⁹³⁻⁹⁵. Nonetheless, the use of such a dye in humans is not accepted due to toxicity concerns. Imaging techniques established on oxygenated hemoglobin and reduced hemoglobin (i.e. deoxyhemoglobin) and their spectral changes have been used in humans to non-invasively assess oxygen saturation in retinal vessels^{39,42,46,52,53,63}. Hickam and co-workers first proposed a non-invasive measurement of the retinal oxygen saturation using a two-wavelength (510, 640 nm) method. Following that, another two-wavelength (470, 515 nm) technique was developed to measure oxygen saturation. These photographic based techniques, however, were laborious. Later, Pittman and Duling⁴⁵ introduced a three-wavelength spectrophotometer method for oximetry. Delori introduced the first scanning retinal oximeter illuminating with multiple wavelengths⁴². Another group⁵³ used a grating spectrograph to acquire spectra and applied a multi-wavelength curve fitting technique for saturation estimation. Assessment of oxygenation in the retina may facilitate the early detection of diabetic retinopathy. Oxygen saturation in retinal vessels has been found to be greater in diabetic retinopathy in contrast to control subjects⁶⁷. A study by Man and co-workers using the oximetry module of the Vesselmap system (Imedos UG, Jena, Germany) showed an association between eyes with DR and increased venular SO_2 and decreased A-V difference in the DR group when compared with eyes without DR, suggesting an altered metabolic state in DR⁹⁶. Another study by Jørgensen and co-workers showed increased SO_2 in arterioles and venules and a normal A-V difference in PDR patients. They also showed that immediately after retinal photocoagulation treatment, the SO_2 increases in venules but not in arterioles resulting in

the A-V difference decrease when compared to pre-treatment; however, they showed that three months after retinal photocoagulation treatment, the SO_2 is elevated in arterioles and venules but the A-V difference stays the same as the pre-treatment level ⁹⁷.

1.15.4 Clinical Classification and Etiology of DR

DR is a potential blinding disease. It has a vaso-obliteration phase, which eventually can result in proliferative phase. DR is categorized as non-proliferative (including: microaneurysms, exudate, capillary nonperfusion, permeability or leakage) which leads to hypoxia and the final phase as proliferative retinopathy (preretinal neovascularization). Its classification is traditionally established on ophthalmoscopically visible signs of increasing severity graded from no retinopathy through different steps of non-proliferative or pre-proliferative disease to advanced proliferative disease.

1.16 Retinal Biochemical/Morphological Changes Associated with Hyperglycemia

1.16.1 Inflammatory Biochemical Biomarkers

1.16.1.1 Vascular Endothelial Growth Factor (VEGF)

Normal retinal vascular development appears not to be determined by hypoxia-induced VEGF expression ⁹⁸. However, VEGF signaling is critical for the development of normal vasculature ⁹⁹. VEGF is a well-known persuasive angiogenic factor that stimulates retinal neovascularization, leading to tractional retinal detachment and vitreous hemorrhage, which are important because they represent the most severe causes of visual loss in patients with PDR ^{100,101}. There is a wealth of evidence that during retinal and choroidal neovascularization and the formation of diabetic macular edema, hypoxia intensely stimulates VEGF expression by stabilization of HIF-1 (hypoxia-inducible factor-1) transcription ¹⁰²⁻¹⁰⁴.

Previous studies also specify the important role of VEGF in the breakdown of the blood-retinal barrier in DR. Vascular hyper-permeability secondary to tight junction impairment between vascular endothelial cells and neovascularization are bold symbols of vascular endothelial cell dysfunction induced by diabetes. VEGF plays an important role to mediate these early and late vascular alterations¹⁰⁵⁻¹⁰⁷. It has been reported that VEGF is significantly increased in diabetes and a correlation between the levels of VEGF and DR severity has been shown¹⁰¹. Angiogenesis is caused by disruption between the production of angiogenic activator such as VEGF and angiogenic inhibitors such as angiostatin and pigment epithelium-derived factor (PEDF)^{108,109}. The amplified production of angiogenic activators and reduced production of angiogenic inhibitors caused by local hypoxia, disturbs the balance between the desirable and undesirable regulators of angiogenesis. Subsequently, over proliferation of capillary endothelial cells occurs which leads to neovascularisation¹⁰⁹.

1.16.1.2 Transforming Growth Factor Beta (TGF- β)

Anomalous interactions between the endothelial cell and pericytes might be implicated in diabetic microangiopathy¹¹⁰. There are some pathways of interaction between the endothelial cells and pericytes. One of these communication paths is TGF signaling, which is important for pericyte differentiation^{111,112}. Pericytes make the vasculature stable, modulate (rather than majorly control) blood flow at the local tissue level and control endothelial proliferation. One of the hallmarks of early changes in diabetic retinopathy is pericyte loss¹¹³. Another structural hallmark is thickening of the capillary basement membrane. These changes along with others seen in diabetes such as increased blood viscosity might result in occlusive angiopathy and a consequent tissue hypoxia. Growth

factors such as TGF- β and VEGF might be involved in basement membrane thickening ¹¹⁴. It has been suggested that increase of serum proteins due to microvascular disturbances and hypoxia might be a cause for vitreous alterations of Insulin-like growth factor I (IGF-I) and of active TGF- β . These changes appear to take place late in the sequence of events resulting in PDR and are not restricted to diabetes; they were also detected in other retinal hypoxic diseases ¹¹⁵. TGF- β 1 has been shown to participate in the pathogenesis DR ^{116,117}. A role for TGF- β 1 has been shown in the microvascular complications of type 1 diabetes ^{118,119}. In addition, hyperglycemia induces protein kinase C (PKC) activation, which results in increased microvascular protein buildup by up-regulating TGF- β 1, fibronectin and type IV collagen ^{120,121}. Serum protein influx due to hypoxia is likely the reason for vitreous alterations of active TGF- β ¹¹⁵.

It has been shown that TGF- β appears to decrease the tightness of the inner blood retinal barrier (iBRB) ¹²². A study showed that an intravitreal level of Angiopoietin-2 is significantly associated with VEGF and TGF- β 1 concentration in DR undergoing vitrectomy. This suggests that these factors could be involved in promoting retinal angiogenesis synergistically ¹¹⁶.

1.16.1.3 Angiopoietins-2 (Ang 2)

Ang 2, one of the important growth factors of hypoxia-induced microvascular changes, is significantly involved in the initiation of retinal neovascularization. Angiopoietins binds to the receptor tyrosine kinase, Tie2. The angiopoietins are growth factors that regulate the development of physiological angiogenesis and pathological neovascularization ^{123,124}. Vitreal increase in Ang 2 and VEGF levels as well as a correlation between Ang 2 and VEGF has been suggested in PDR ¹²⁵. Another study proposed that Ang 2 is expressed by

hypoxia and VEGF in bovine aortic endothelial cells ¹²⁶. Another study showed that Ang 2 is playing a role in pericyte recruitment and preretinal vessel formation under physiological and pathological disorders¹²⁷. There is also evidence that Ang 2 is involved in pericyte apoptosis by $\alpha_3\beta_1$ integrin signaling in DR ¹²⁸.

Ang 2 has been shown to be elevated in the vitreous of patients with clinically significant macular edema (CSME) ¹²⁹ suggesting a possible role of Ang 2 in the alteration of the blood-retinal barrier. Another study suggested that Ang 2 might be involved in elevated vasopermeability in DR¹³⁰.

1.16.1.4 Leptin

Leptin, an adipocyte-derived hormone, is an energy metabolism regulator and is associated with diabetes mellitus through its metabolic actions. Leptin stimulates endothelial proliferation and angiogenesis ¹³¹. Results describing the association between serum leptin and diabetic microangiopathies are variable. Sari and co-workers ¹³² didn't find any difference in the leptin levels in serum between patients with and without diabetic nephropathy, retinopathy and neuropathy. Furthermore, Asakawa and co-workers ¹³³ reported no relationship in the leptin level and microangiopathies in Japanese patients of type 2 diabetes. However, Freuhwald-Schultes and co-workers ¹³⁴ reported high serum leptin amount in type 2 diabetes patients with early stages of renal disease, (i.e., microalbuminuric and macroalbuminuric nephropathy), while serum leptin level did not significantly show any association with diabetic neuropathy. Jung and co-workers ¹³⁵ didn't find any relationship between leptin concentrations and diabetic retinopathy.

1.16.1.5 Tumor Necrosis Factor (TNF- α)

Elevated levels of TNF α have been reported in the vitreous of diabetic patients ¹³⁶ and a robust association between plasma levels of TNF- α and severity of DR has been shown ¹³⁷. Furthermore, increased levels of TNF- α in an animal model of diabetes prompted NADPH oxidase and production of reactive oxygen species (ROS) causing endothelial dysfunction ¹³⁸. In vivo studies have also shown that TNF- α plays a role in angiogenesis ¹³⁹. There is also evidence that blocking TNF- α reduces leukocyte adhesions as well as ICAM-1 expression ¹⁴⁰. TNF- α -mediated endothelin-1 release and cell proliferation is enhanced by hypoxia in human optic nerve head astrocytes in glaucoma ¹⁴¹.

1.16.1.6 Intercellular Adhesion Molecule-1 (ICAM-1)

Inflammatory leukocyte recruitment constitutes one of the key pathological stages in DR. Diabetes has been shown to up-regulate several pro-inflammatory mediators in the retina, such as ICAM-1, and this localized inflammatory process is considered to be part of the development of DR ¹⁴²⁻¹⁴⁵. Retinal vascular ICAM-1 expression is elevated by VEGF. This may stimulate retinal leukostasis in diabetic eyes ¹⁴⁶. Increased expression of ICAM-1 has been demonstrated to show an association with the severity of retinopathy ¹⁴⁷. There is also a direct relationship between ICAM-1 and VEGF-induced vascular permeability ¹⁴⁸, as well as evidence that pigment epithelium-derived factor (PEDF) decreases the hypoxia-induced ICAM-1 expression ¹⁴⁹.

1.16.1.7 Hepatocyte Growth Factor / Scatter Factor (HGF / SF)

Complicated events of interactions between retinal cells with growth factors and cytokines, extracellular matrix proteins and metallo-proteinases trigger the development of proliferative retinal diseases ¹⁵⁰⁻¹⁵². HGF is one of these growth factors that have been

studied. Its receptor is the c-met, a transmembrane tyrosine kinase ¹⁵³. An elevated HGF level in patients with type 1 diabetes mellitus with PDR and the relationship between progression of DR and the concentration of HGF was suggested as a role of HGF in the pathogenesis of PDR ¹⁵⁴.

It has been reported that HGF is an endothelium specific growth factor with more potent mitogenic activity than that of basic fibroblast growth factor (b-FGF), VEGF, interleukin 6 (IL-6), and interleukin 1 (IL-1) ^{155,156}. Furthermore, the HGF concentration in the vitreous of PDR has been shown to be higher when compared to non-diabetic patients ¹⁵⁷. In addition, it has been presented that HGF might affect the progression of DR ¹⁵⁸.

1.16.1.8 Heparin-Binding Epidermal Growth Factor (HB-EGF)

It has been reported that the retinal pigment epithelial (RPE) cells migration can be synergistically stimulated by HGF coupled with HB-EGF or EGF, which are mediated by an increased activation of protein kinase C and extracellular signal-regulated kinase (ERK) phosphorylation ¹⁵⁹.

1.16.1.9 Interleukin-1 β (IL-1 β)

IL-1 β appears to trigger the neuro-inflammatory cascade ¹⁶⁰ and is a multifunctional pro-inflammatory cytokine ¹⁶¹. One of the earliest events after ischemic conditions is IL-1 β release, which leads to inflammatory response and eventually tissue damage ¹⁶². There is a growing body of evidence that IL-1 β is a retinal damage mediator in DR. IL-1 β appears to be up-regulated in the retina in experimental diabetes ¹⁶²⁻¹⁶⁶. More recently, it has been reported that IL-1 β might up-regulate IL-8 in Müller cells through the p38 mitogen-activated protein kinases (p38 MAPK) and ERK1/2 pathways ¹⁶⁷ (ERK1/2 is a significant

member of mitogen-activated protein kinases that control a broad range of cellular activities and physiological processes).

1.16.1.10 Endothelial Growth Factor (EGF)

It has been reported that the insulin-induced vascular leakage can be prevented by EGF inhibition ¹⁶⁸. It has been reported that EGF has a potent mitogen role ¹⁶⁹. It has been reported that AGE could trigger EGF receptor (EGFR) ^{170,171}. The EGRF can bind to multiple growth factors such as EGF, HB-EGF ¹⁷². ROS might block EGFR dephosphorylation ¹⁷³.

1.17 Summary

Retinal blood oxygen saturation could represent a valuable and sensitive biomarker of DR potentially prior to any vision loss, although direct evidence to support this statement is currently lacking. Current techniques for the assessment of oxygen saturation disturbance are limited to the utilisation of only a few specific wavelengths. The oximeter used in this study will provide the ability to *non-invasively* and measures the oxygen saturation of the major retinal vessels using multiple wavelengths. The instrument used in this study has the capacity to measure full field reflectance for 100s of wavelengths in a matter of seconds using a half power bandwidth of only 2nm. This instrument will permit the acquisition of high-speed hyperspectral retinal reflection data at background light levels far lower than any similar technologies, thereby avoiding disturbance of retinal metabolic status due to high ambient light levels. Therefore, it will minimize the influence of eye movements on image quality. Retinal oximetry will allow the non-invasive quantification of oxygenation of the retina, which in turn may offer a sensitive and predictive non-invasive biomarker. Moreover, quantification of blood flow and oxygenation of the retina will permit the

assessment of the tissue oxygen utilization. A number of novel inflammatory biomarkers predicting onset or progression of retinopathy in patients with type 2 diabetes have been identified. Some of these biomarkers such as VEGF, Hb-EGF, HGF, Ang 2, leptin, ICAM-1, IL-8 and TGF- β , will be investigated in this study.

To date, VEGF is the most attractive candidate responsible for an early biomarker of DR. Elevated blood glucose and retinal hypoxia are both thought to stimulate production of VEGF and the development of macular edema and neovascularization within the eye, respectively. It has been also shown that ICAM expression is upregulated in the absence of oxygen¹⁷⁴. A relationship between oxidative stress and hypoxia has also been shown¹⁷⁵. An association between TGF- β and Ang 2 and retinal angiogenesis is thought to exist¹¹⁶ while there are evidence showing that TNF- α is stimulated by hypoxia¹⁴¹.

The study of the relationship between inflammatory biomarkers and retinal oxygenation in individuals with type 2 diabetes represents fertile scientific ground to be investigated and would greatly expand our understanding of the development of DR by defining the interaction of inflammatory mediators and retinal oxygen saturation.

In summary, quantifying retinal blood oxygen saturation could help us better understand the pathophysiology behind retinal diseases such as DR and possibly better techniques to earlier diagnose of such diseases.

The combination of retinal oxygen saturation and retinal blood flow can be used to extract more information about retinal metabolism since:

$$\text{Retinal O}_2 \text{ Delivery} = [1.39 \times \text{Hb} \times \text{SaO}_2 + (0.003 \times \text{PaO}_2)] \times \text{blood flow}$$

2 Rationale

The assessment of retinal blood flow is important because its perturbation has been suggested to play a role in many ocular diseases such as diabetic retinopathy¹⁻³ and glaucoma⁴⁻¹¹. Several noninvasive methods have been established to measure ocular blood flow in humans. Previous techniques for evaluating retinal perfusion have many limitations, such as being invasive (e.g. Fluorescein angiography), or are subjective (e.g. Blue field entoptic phenomenon), or are incapable of calculating blood flow (e.g. Retinal Vessel Analyzer) since a surrogate parameter of flow is truly measured. To overcome these limitations, a new technique termed Doppler spectral Fourier-domain optical coherence tomography (Doppler FD-OCT) blood flow (e.g. Optovue RTVue) has been developed. No one has properly looked at reproducibility and variability of FD-OCT, there is a need to study the reproducibility and variability of FD-OCT.

DR results in disturbed oxygen delivery to retinal cells, which in turn will cause hypoxia in retinal tissue. A relationship between the pathogenesis in DR and retinal hypoxia has been found. Hypoxia is thought to provoke neovascularization and retinal edema¹².

Retinal SO₂ could represent a valuable and sensitive biomarker of DR potentially prior to any vision loss, although direct evidence to support this statement is currently lacking. Current techniques for the assessment of oxygen saturation disturbance are limited to the utilisation of only a few specific wavelengths. Ultimately, the retinal oximeter used in this study will provide the ability to non-invasively and quantitatively measure the oxygen saturation of the blood in the major retinal vessels using multiple wavelengths. However, the oximetry data presented in this study is derived from two selective wavelengths in order to easily compare and validate my oximetry results with those of other studies, as explained

in Beach and co-workers¹³. The instrument used in this study has the capacity to measure full field reflectance for 100s of wavelengths in a matter of seconds using a half power bandwidth of only 2nm and no flash. This instrument permits the acquisition of high-speed hyperspectral retinal reflection data at background light levels far lower than any similar technologies, thereby avoiding disturbance of retinal metabolic status due to high ambient light levels. Therefore, it will minimize the influence of eye movements on image quality and will avoid any influence of flash illumination on optical density values. Retinal oximetry allows the non-invasive quantification of oxygenation of the retina. Quantification of blood flow and oxygenation of the retina will permit the assessment of the tissue oxygen utilization, also termed oxygen metabolism.

A number of novel inflammatory biomarkers predicting onset or progression of retinopathy in patients with type 2 diabetes have been identified. Some of these biomarkers such as VEGF, ICAM-1, IL-8, TGF- β , Leptin, Angiopoietin 2, EGF, Hb-EGF, and HGF will be investigated in this study.

To date, VEGF is the most attractive candidate biomarker reflecting the severity and development of DR. Initially elevated blood glucose and then retinal hypoxia are both thought to stimulate production of VEGF and the development of blood retinal barrier leakage in the form of macular edema and neovascularization seen clinically as proliferative DR. A relationship between oxidative stress and hypoxia has also been shown¹⁴. An association between TGF- β and Ang 2 and retinal angiogenesis is thought to exist¹⁵ while TNF- α has been reported to be stimulated by hypoxia¹⁶.

The study of the relationship between inflammatory biomarkers and retinal oxygenation in individuals with type 2 diabetes represents fertile scientific ground to be investigated and

would greatly expand our understanding of the development of DR by defining the interaction of inflammatory mediators and retinal oxygen saturation.

2.1 General Objective

The general objective of my work was to aid the development of new objective metabolic measures and biomarkers of DR and then to apply these outcomes to better understand the pathophysiology of DR.

This study determined the within-session variability and between-session repeatability of FD-OCT, an instrument that allows the noninvasive measurement of retinal blood flow.

The study determined any difference in oxygen saturation and inflammatory vascular biomarkers in the aqueous humor in healthy age-matched controls and type 2 diabetic patients with mild-to-moderate non-proliferative DR undergoing routine uncomplicated cataract extraction. The study investigated the diagnostic value of these early biomarkers to distinguish between healthy age-matched controls and type 2 diabetic patients with relatively early DR.

2.2 Specific Aims

1- The purpose of the first study was to determine the within-session variability and between-session repeatability of spectral Fourier-domain optical coherence tomography (Doppler FD-OCT) Doppler retinal blood flow measurements in young and elderly subjects.

2- The aim of the second study was to assess oxygen saturation disturbances in the retina that occur in patients with type 2 diabetes and to compare the magnitude of retinal SO_2 in arterioles and venules in healthy age-matched controls and in type 2 patients with relatively early (no or mild-to-moderate NPDR).

3- The aim of the third study to investigate the relationship between retinal blood flow and retinal blood oxygen saturation in NPDR.

4- The aim of the fourth study was to determine disturbance of vascular biomarkers of angiogenesis / inflammation (VEGF, Angiopoietin 2, HB-EGF, HGF, Leptin, EGF, IL-8, TGF- β and ICAM-1) in aqueous humor in patients with relatively early DR and in healthy age-matched controls.

5- The aim of the fifth study was to assess the relationship between the retinal oxygen saturation and retinal blood flow in order to determine net oxygen values also termed oxygen utilization or metabolism (i.e. Retinal O₂ Delivery = [1.39 x Hb x SaO₂ + (0.003 x PO₂)] x blood flow).

2.3 Hypotheses

1- The FD-OCT will be repeatable in terms of measuring total retinal blood flow.

2- Disturbance of retinal blood oxygen saturation will occur in relatively early mild-to-moderate nonproliferative DR in the absence of clinical sight-threatening signs.

3- There is a direct correlation between retinal blood flow and retinal oxygen saturation.

4- Disturbance of AH biomarkers will occur in relatively early mild-to-moderate nonproliferative DR in the absence of clinical sight-threatening signs.

5- The relationship between retinal oxygen saturation and vascular biomarkers of inflammation will be different between the two groups (NPDR and control).

3 Variability and Repeatability of Quantitative, Fourier-Domain OCT

Doppler Blood Flow in Young and Elderly Healthy Subjects

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D. Huang				x	x
J. Flanagan	x				x
C. Hudson	x			x	x

Table detailing role of each author in this publication (x denotes significant contribution)

3.1 Introduction

The human eye is perfused mostly from the ophthalmic artery, the first branch of the internal carotid artery. There are two distinct vascular systems that support the mature neural retina: the inner retinal vessels and the choroidal vessels. The two beds vary in both their embryonic differentiation pattern and functionally in the adult human ¹. The retinal vasculature is spread mainly within the inner retina and nourishes most inner retinal structures. The pigment epithelium and photoreceptors, however, are primarily nourished by the choroidal vasculature. The contraction of the arterioles determines the blood flow into the inner retinal capillary bed ². Moreover, the downstream retinal capillaries are believed to be able to further fine tune blood flow via the actions of the contractile pericytes in response to local tissue demands, such as the level of oxygenation ³. All together, these mechanisms sustain constant blood flow over an extensive range of perfusion pressures (i.e., autoregulation).

The assessment of retinal blood flow is important because its perturbation has been suggested to play a role in many ocular diseases such as diabetic retinopathy ⁴⁻⁶ and glaucoma ⁷⁻¹⁴. Several techniques have been developed to quantify retinal blood flow in humans. Previous techniques for evaluating retinal perfusion have numerous limitations, such as being invasive (e.g. Fluorescein angiography), or are subjective (e.g. Blue field entoptic phenomenon), or are incapable of calculating blood flow (e.g. Retinal Vessel Analyzer) since a surrogate parameter of flow is truly measured. In addition, a technique that truly measures volumetric retinal blood flow, that is bi-directional laser Doppler velocimetry and simultaneous vessel densitometry, is currently limited to relatively large vessels and a single measurement site. To overcome these limitations, a new technique

termed Doppler spectral Fourier-domain optical coherence tomography (Doppler FD-OCT) blood flow (e.g. Optovue RTVue) has been developed. FD-OCT is a non-contact imaging method that mixes micrometer-scale resolution with millimeter image penetration depth¹⁵⁻¹⁷. It is similar to the ultra sound technique with the exception of using light energy in the form of laser instead of sound. FD-OCT measures the intensity of light, back-reflected from various features within the imaged object resulting from spatial variations of the tissue refractive index. FD-OCT is based on low coherence interferometry, a classic optical measurement technique. FD-OCT makes high-resolution imaging possible. It is generally used in the diagnosis and management of retinal diseases^{18,19}.

FD-OCT has the ability to detect motion within a sample from the back reflected light, which delivers information on movement^{20,21}. In respect of terminology, Huang and co-workers and many other groups have generally used the term “Doppler shift” to describe the same phenomenon that other groups describe as a phase shift, or Doppler phase, between two subsequent A-scans which represents a difference in position of a given point within the waveform between the two scans. From this point forward, we will use the term “Doppler phase shift” to describe this phenomenon.

The variability and repeatability of this new retinal blood flow measurement technique needs to be established in order to characterize a significant change and to use it as a clinical technique to detect abnormal deviations in blood flow. The aim of this study is to investigate the within-session variability and between-session repeatability of the Doppler FD-OCT morphologic blood flow technology in young and elderly healthy subjects.

3.2 Methods

3.2.1 Sample

This study received approval by the University of Waterloo Office of Research Ethics and the Research Ethics Board of the University Health Network, University of Toronto, Canada. Informed consent was obtained from each subject after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki. The sample consisted of thirty six healthy volunteers in two groups of young and elderly subjects. One eye of each of 20 healthy young (mean age 24.7; SD 2.7 years) and 16 healthy elderly (mean age 64.6; SD 5.1 years) subjects was randomly selected for the study. All subjects had a corrected visual acuity of 20/40 or better. Subjects were excluded for any ocular or systemic disease, refractive error greater than ± 6.00 DS or ± 2.50 DC, glaucoma or diabetes in a first degree relative, history of central nervous system disorders, or medications with known effects on blood flow (e.g., anti-convulsants or anti-inflammatory medications). Subject, and appropriate summary group, characteristics (age, gender, and right or left eye, intraocular pressure, and blood pressure) are detailed in Table 3.1.

Young Group								
Name	Age	M/F	IOP		Visit 1		Visit 2	
			Visit 1	Visit 2	Sys	Dias	Sys	Dias
1	27	F	11.00	10.33	116	68	121	71
2	24	F	11.33	12.00	115	75	111	74
3	29	F	12.33	11.33	125	80	121	82
4	27	F	12.00	12.33	110	69	113	68
5	28	F	11.00	11.66	97	63	110	64
6	19	F	15.00	14.00	109	69	100	68
7	24	F	13.00	12.66	113	67	124	71
8	26	F	12.66	12.00	106	67	115	73
9	28	F	11.00	12.00	113	78	102	81
10	26	F	12.00	11.66	119	81	122	69
11	22	M	15.00	14.00	128	78	119	69
12	23	M	14.33	13.66	125	65	111	78
13	25	M	9.00	10.33	128	71	120	81
14	26	M	18.33	16.66	128	82	135	87
15	24	M	13.66	12.33	131	76	129	75
16	25	M	17.00	15.33	128	67	121	71
17	20	M	12.00	12.66	129	69	125	73
18	23	M	13.33	13.00	125	78	119	75
19	26	M	17.66	16.33	127	81	125	78
20	22	M	16.00	16.00	129	60	128	61
Mean	24.7		13.38	13.01	120.05	72.2	118.55	73.45
SD	2.7		2.48	1.86	9.59	6.69	8.77	6.4
Elderly Group								
Name	Age	M/F	IOP		Visit 1		Visit 2	
			Visit 1	Visit 2	Sys	Dias	Sys	Dias
21	64	F	15.00	15.33	145	56	139	59
22	63	F	13.33	14.00	105	69	111	70
23	62	F	13.00	15.00	119	84	121	83
24	62	F	14.00	13.00	136	83	129	86
25	67	F	15.00	16.00	142	79	134	81
26	61	F	14.00	13.66	138	83	145	84
27	68	F	16.00	15.66	133	86	135	85
28	70	F	11.00	12.00	150	79	145	87
29	59	F	15.00	14.66	124	78	132	85
30	78	F	12.66	12.00	124	78	129	75
31	70	F	14.00	13.33	128	79	135	79
32	60	M	15.00	14.33	131	72	122	76
33	60	M	13.33	13.66	120	76	128	75
34	67	M	14.00	15.33	122	74	124	78
35	62	M	14.00	13.66	125	79	131	81
36	60	M	14.00	14.33	128	78	112	73
Mean	64.6		13.64	13.51	125.58	74.36	123.49	75.72
SD	5.1		2.00	1.68	12.07	7.21	10.42	7.17

Table 3.1 Individual subject / group characteristics for the young and elderly groups. (IOP, intraocular pressure (mmHg); Sys, systolic blood pressure (mmHg); Dias, diastolic blood pressure (mmHg); HR, heart rate per minute; F, Female; M, Male.)

3.2.2 Doppler Fourier-Domain Optical Coherence Tomography (Doppler FD-OCT)

Doppler FD-OCT is a novel imaging method that provides in vivo non-invasive assessment of retinal structure and also retinal blood flow using a physical phenomenon called Doppler phase shift²². The principle of the Doppler phase shift has been integrated into the commercially available Doppler FD-OCT (Optovue Inc, Fremont, CA). Doppler FD-OCT generates high resolution cross sectional images of the retina. This instrument utilizes a laser light source of 841nm with bandwidth of 49nm with an incident power of 500 μ W on the cornea. These parameters result in an axial resolution of 5.4 μ m in tissue²³. System transverse resolution was 20 μ m, as determined by the maximum aperture of the eye²⁴.

Unlike morphological FD-OCT systems that produce just structural images, the prototype Doppler FD-OCT analyzes the Doppler phase shift between two consecutive A-scans. Light reflected from moving particles undergoes Doppler phase shift.

Flow velocity is determined by:

$$v = \frac{\Delta\Phi \cdot \lambda_0}{4\pi \cdot T \cdot n \cdot \sin(\theta)}$$

Where v is the flow velocity in an OCT voxel, and $\Delta\Phi = \Phi_1 - \Phi_2$ is the Doppler phase shift. Φ_1 and Φ_2 are the phase of voxels in the same position in consecutive OCT axial scans, λ_0 is the source center wavelength, n is the refractive index of the medium, T is the time

interval between consecutive scans and θ is the Doppler angle defined by the OCT beam axis relative to the line perpendicular to blood vessel flow axis.

The maximum detectable Doppler phase shift of 8.9 KHz is determined by the acquisition speed of the CCD camera. Given this set up, the maximum measurable velocity in the retinal vessels was 2.8 mm/s²⁴.

Phase detection caused by retinal blood flow was incorporated into the prototype system by creating two circular scans centered on the optic nerve head. The retinal blood flow protocol consists of a double circular Doppler scan comprising two concentric rings of diameters 3.4 and 3.75 mm centered on the optic nerve head²⁵ (Figure 3.1). The double circular FD-OCT beam passes through the pupil nasally with two sets of scans (inferior and superior). Two sets of scans are taken to achieve optimum flow measurements from at least one of the two sets of scans. The circular scan is displayed as the sinusoidal variation in retinal height / morphology (Figure 3.2).

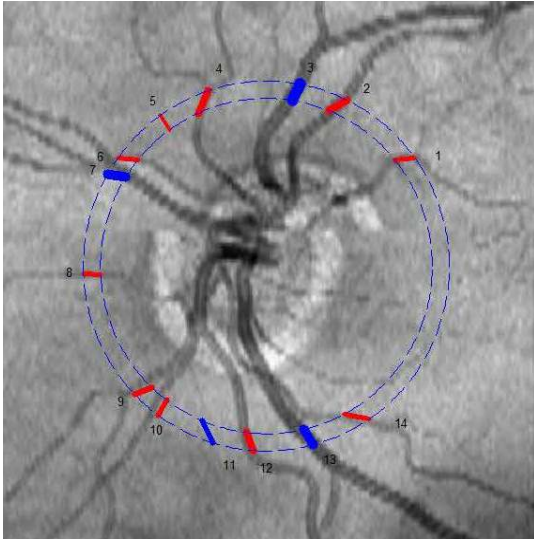


Figure 3.1 FD-OCT Doppler image acquisition page. A double circular scan pattern with concentric radii is used for the measurement, which is typically centered at the optic nerve head.

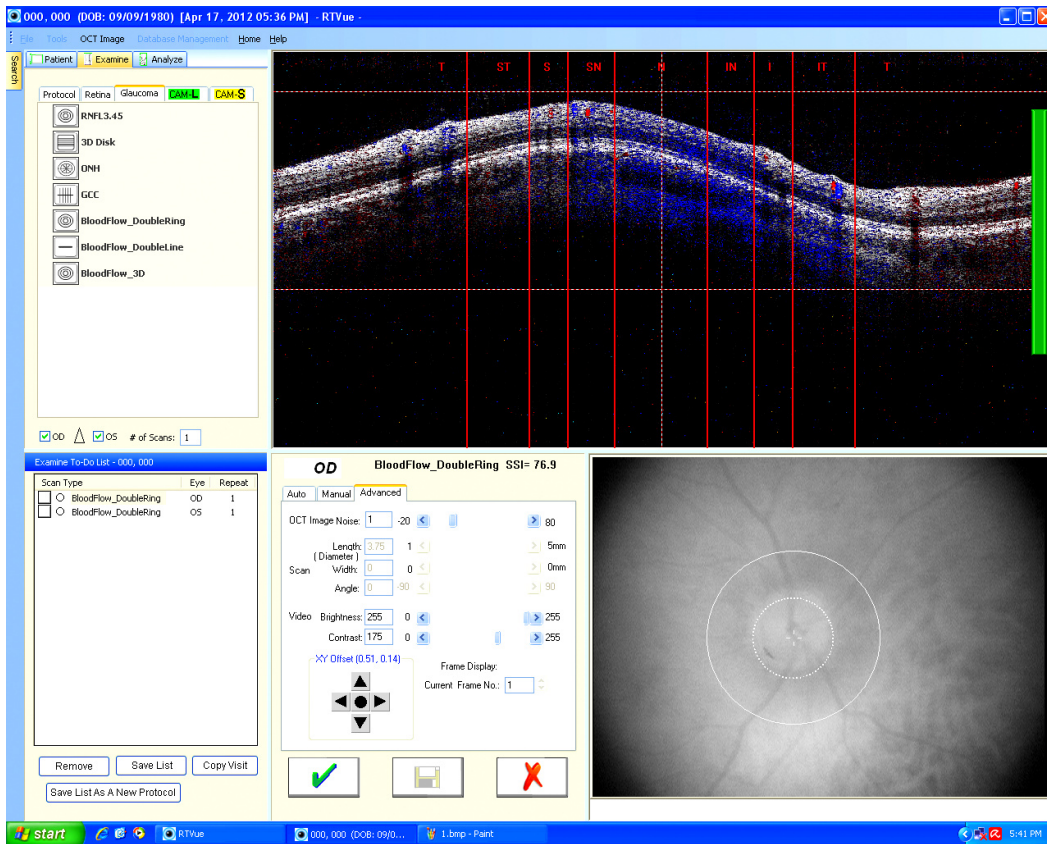


Figure 3.2 Doppler FD-OCT image for beam passing through the superior nasal quadrant of the pupil. The peak of the sinusoidal should be positioned either within superior nasal or inferior nasal quadrants.

The incident angle is estimated by vessel center depth difference within the two consecutive circular OCT scans from the Doppler FD-OCT images. The measured Doppler phase shift, the incident angle calculation and vessel area are used to compute absolute red blood cell velocity and retinal blood flow.

3.2.3 Procedures

Refraction, logMAR visual acuity, Goldmann applanation tonometry and resting blood pressure were assessed prior to dilation of the study eye. The pupil of the study eye was dilated using Tropicamide 1% (Alcon, Mississauga, Canada) at the beginning of each visit to achieve an adequate view of the fundus for the retinal blood flow image acquisition. Visual fields were also tested in subjects using the Humphrey Field Analyzer II (Carl Zeiss Meditec, Inc., Dublin, CA) with the 24–2 automated static threshold test. The clinical fundus view and the automated perimetry results were all confirmed to be normal. Subjects rested for 10 minutes before the start of each study visit to stabilize baseline cardiovascular and respiratory parameters. Each subject attended for two visits. The two visits were undertaken within two weeks and the second visit was at the same time of day and under the same conditions as the initial visit. A minimum of six separate FD-OCT Doppler measurements (i.e. each separate measurement comprising an upper nasal pupil scan and a lower nasal pupil scan) was acquired at each session.

3.2.4 Image Grading

3.2.4.1 Semi-automated Doppler OCT of retinal circulation (DOCTORC)

Acquired Doppler FD-OCT scans and a SLO en-face image were exported as raw data using RTVue Doppler transfer output software. The exported raw data was converted into

DOCTORC grading software (version 2.1.1.4) compatible data for image grading and retinal blood flow calculation²⁵. After loading the Doppler scan into DOCTORC, the initial automated assessment of the loaded scans underwent software processing that identified the vessel type based on the Doppler signal characteristics (i.e. arteriole or venule). Grading also requires a color fundus image of the optic disc to confirm that the automated vessel identification undertaken by DOCTORC is correct by comparing the DOCTORC assignment (i.e. venule or arteriole) with that of a color fundus photograph. The Doppler scans with 3D OCT image and SLO image were then registered to allow the grader to correlate the vessels that are identified on the en-face image with those seen on the cross-sectional Doppler OCT B-scan. DOCTORC then computed the blood flow from the Doppler phase data after manual assessment of scan validity. The blood flow data was then automatically exported into the appropriate subject folder.

The FD-OCT scans were manually graded based on its Doppler signal, size and location between inner and outer circle, clarity of the vessel boundary and finally the type of the vessel. The grader adjusted the dotted circle in order to make it the same size as the Doppler signal (Figure 3.3). There was also a confidence score ranging from 0 to 5 with a confidence score guidance sheet (Doheney Eye Institute, CA). The grader of each vessel on every scan assigned this score. After completion of all these procedures for all the vessels in the Doppler FD-OCT image, the software verified whether the flow calculation for each graded vessel was valid. Subsequently, the total retinal venous blood flow was automatically computed by summing all calculated flow values from all valid retinal venules and the estimated flow from venules²⁵ using an excel file.

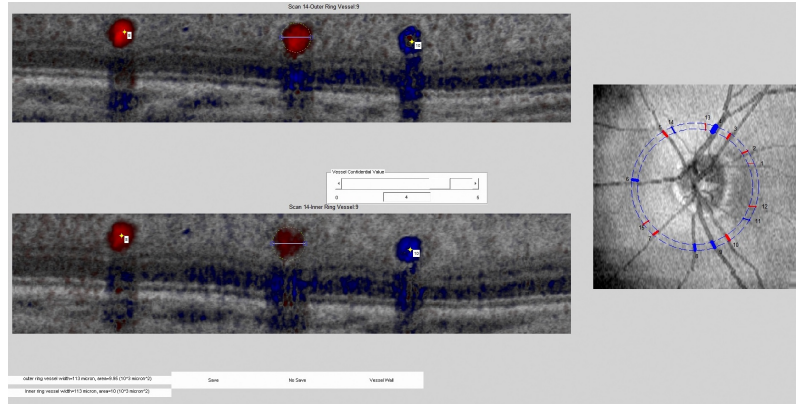


Figure 3.3 Cross-sectional of retinal blood vessels displaying Doppler signal. The grader adjusts the vessel circumference with a dotted circle; and the diameter with a horizontal solid line.

The Co-efficient of Variation (COV) is defined as the ratio of the standard deviation σ to the mean μ , such that $COV = \frac{\sigma}{\mu}$. The Bland-Altman plot²⁶ is a graphical approach to

compare two measurements methodologies of the same object. The differences between the two methods are mapped as the function of the averages of the two. This plot may be used to assess the repeatability of a method. The Co-efficient of Repeatability (COR) can be

calculated as 1.96 times the standard deviation of the differences between the two measurements (d_2 and d_1), such that $COR = 1.96 * \sqrt{\frac{\Sigma(d_2 - d_1)^2}{n-1}}$. Repeatability or test-retest

reliability is defined as the variation in measurements taken by a single operator and instrument under the consistent conditions.

3.3 Results

Typically, it takes between 2 to 5 minutes to analyze each vessel. Depending upon a given patient's vascular tree characteristics, the total time required to undertake the analysis of a data set acquired at a single visit varied between 20 to 60 minutes. Box plots of the COVs for blood flow in both young and elderly participant groups are displayed in Figure 3.4. The individual COVs for blood flow in the young ranged from 0.4 to 20.4% (median 7.5%) and for the elderly subjects ranged from 0.6 to 34.6% (median 9.2%). The individual COR values for flow ranged from 0.2 to 13.8 $\mu\text{l}/\text{min}$ (median 4.5 $\mu\text{l}/\text{min}$) for young participants and from 0.4 to 38.8 $\mu\text{l}/\text{min}$ (median 9.2 $\mu\text{l}/\text{min}$) for elderly participants.

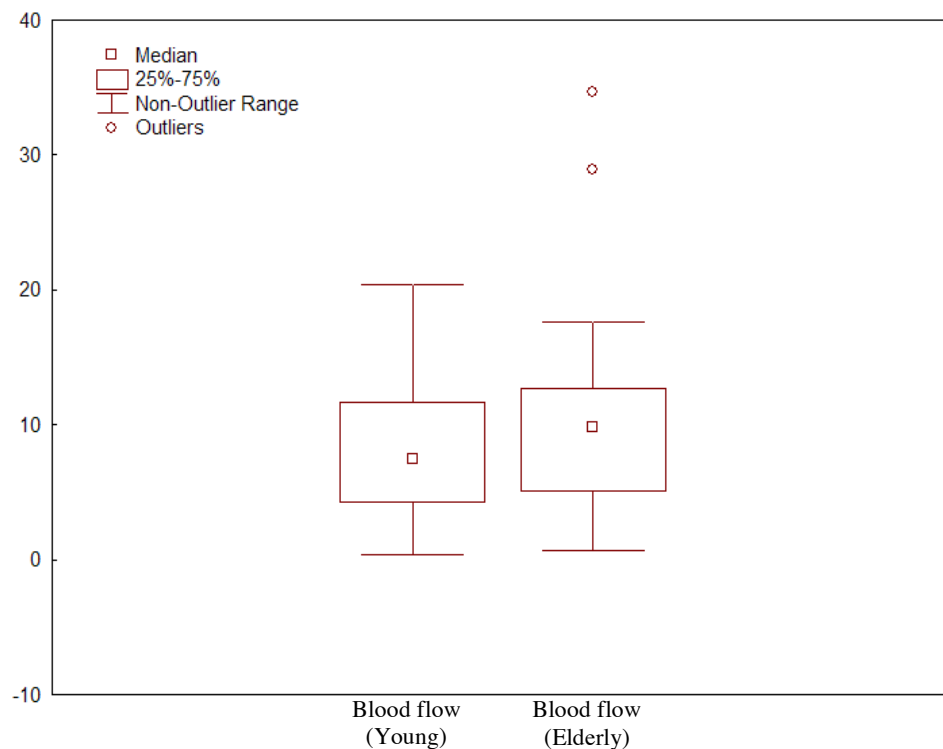


Figure 3.4 Box plot of individual COVs for blood flow in young and elderly participants. The error bars show the non-outlier range (± 1.5 times the height of the box).

The group mean CORs for retinal blood flow for young participants were 6.4 $\mu\text{l}/\text{min}$ (median 5.9 $\mu\text{l}/\text{min}$, relative to a mean effect 39.8 $\mu\text{l}/\text{min}$) and for elderly subjects were

10.5 μ l/min (median 9.2 μ l/min, relative to a mean effect 46.4 μ l/min). Difference versus mean plots of retinal blood flow (Figures 3.5 & 3.6) revealed two clear outliers in the elderly group data (Bland and Altman, 1986). Removal of these outliers reduced the elderly group mean COR for blood flow to 9.88 μ l/min (median 8.3 μ l/min relative to a mean effect 42.9 μ l/min).

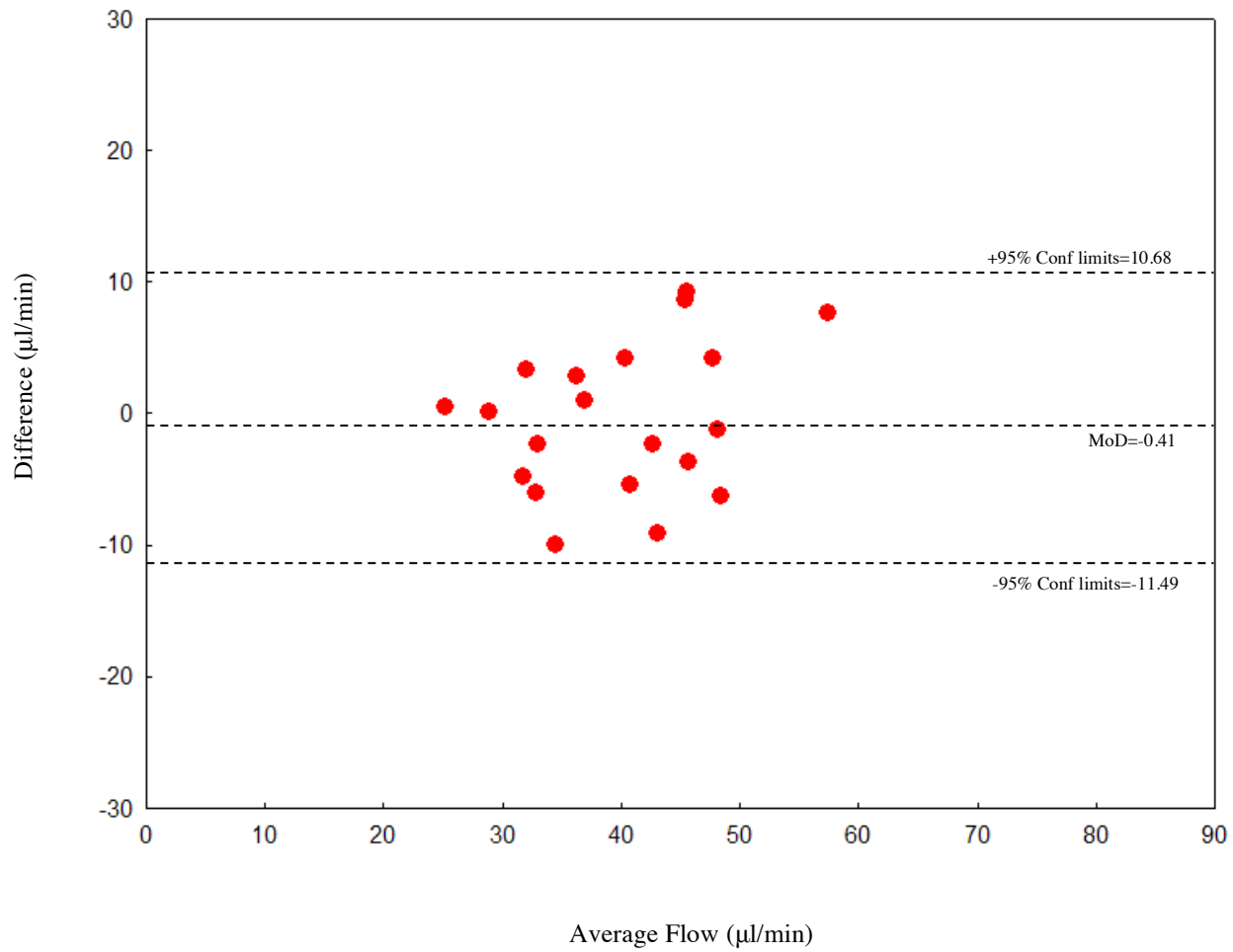


Figure 3.5 Bland and Altman plot showing the difference in total blood flow as a function of mean blood flow between sessions for young subjects. MoD: Mean of Differences; 95% Conf. Limit: $1.96 \times \text{SD}$ of the MoD (also known as Limits of Agreement).

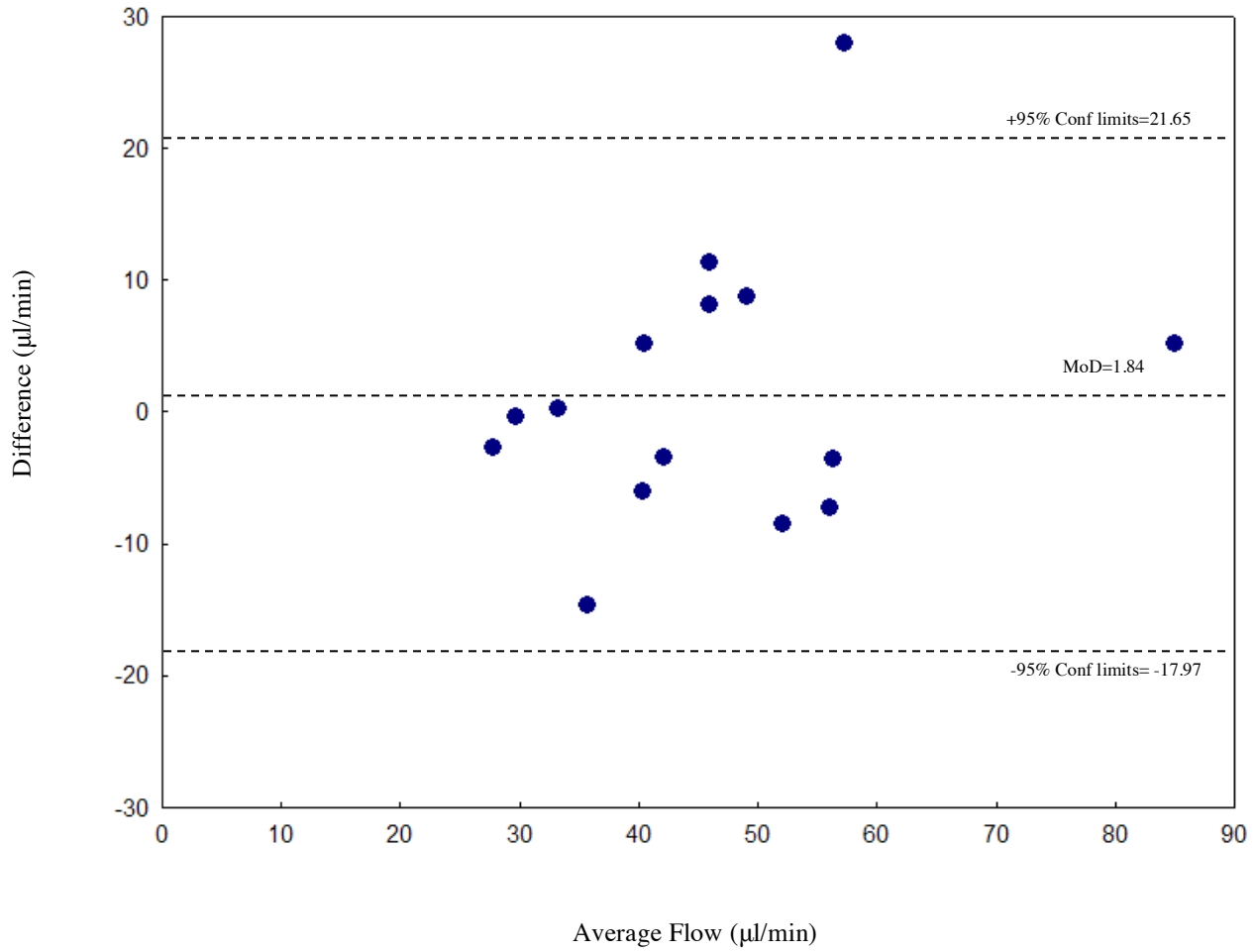


Figure 3.6 Bland and Altman plot showing the difference in total blood flow as a function of mean blood flow between sessions for elderly subjects. MoD: Mean of Differences; 95% Conf. Limit: 1.96 x SD of the MoD (also known as Limits of Agreement)

A scatter plot was used to illustrate test-retest characteristics of the Doppler FD-OCT measurement of total retinal blood flow ($\mu\text{l}/\text{min}$) (Figure 3.7).

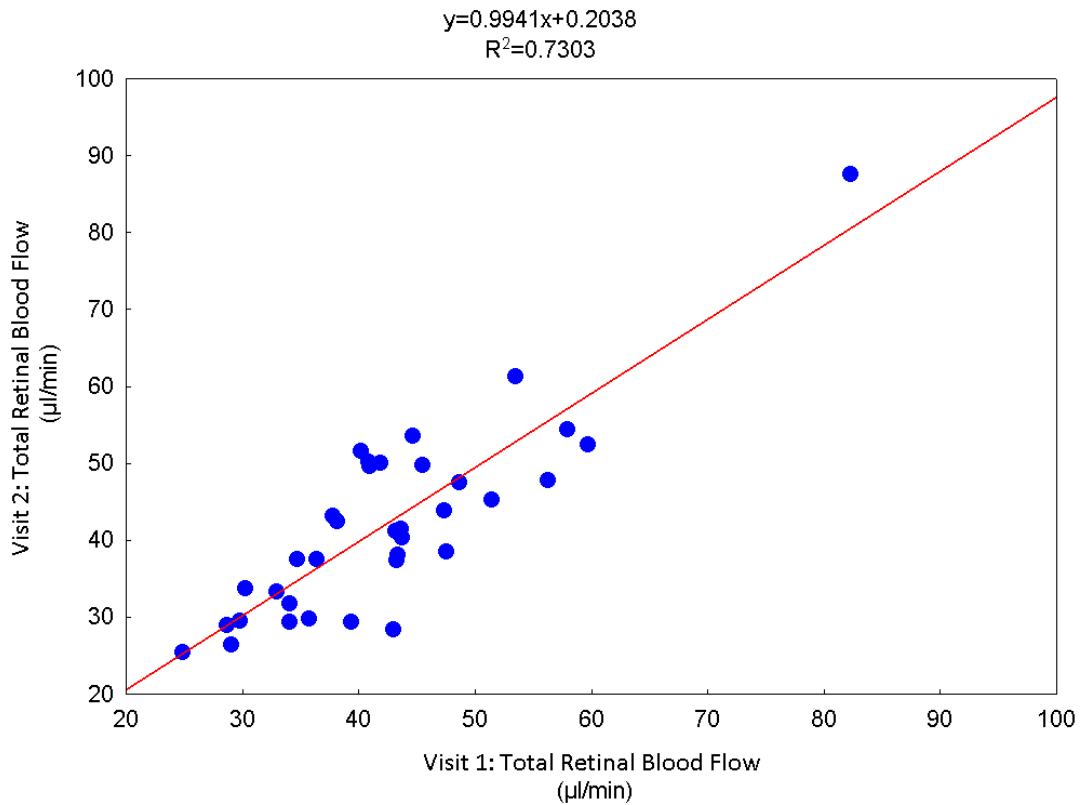


Figure 3.7 Scatter plot illustrating test-retest characteristics of the Doppler FD-OCT for measuring total retinal blood flow ($\mu\text{l}/\text{min}$).

The COV values for venous area, velocity and flow can be found in Table 3.2. The COR values for both young and elderly subjects has been reported in Table 3.3 and 3.4, respectively.

Co-efficient of Variation (%)						
	Venous Area		Velocity		Flow	
	Median	Range	Median	Range	Median	Range
Young	4.7	0.8-16.8	10.4	0.1-28.9	7.5	0.4-20.4
Elderly	4.8	0.4-20.4	10.8	1.1-43.8	9.2	0.6-34.6

Table 3.2 Co-efficient of variation (%) for venous area, velocity and blood flow.

Co-efficient of Repeatability in Young Subjects				
	Individual COR		Overall COR Mean	Effect Size
	Median	Range		
Venous area	0.003	0.0003-0.017	0.005	0.033
Velocity	4.25	1.11-17.53	6.12	21.90
Flow	5.91	0.23-13.76	6.43	39.76

Table 3.3 Co-efficient of repeatability (COR) for individuals as a group (Young).

Co-efficient of Repeatability in Elderly Subjects				
	Individual COR		Overall COR Mean	Effect Size
	Median	Range		
Venous area	0.005	0.001-0.02	0.006	0.035
Velocity	7.19	2.22-18.12	8.20	21.77
Flow	9.16	0.37-38.83	10.53	46.93

Table 3.4 Co-efficient of repeatability (COR) for individuals as a group (Elderly).

3.4 Discussion

Several studies have reported reduced ocular blood flow and poor blood flow regulation in glaucomatous eyes⁷⁻¹⁴. There is also evidence that primary open angle glaucoma is linked to factors related to ocular blood flow regulation and a breakdown of autoregulation⁷. Our group has demonstrated a reduction in the magnitude of retinal arteriolar vascular reactivity in both untreated primary open angle glaucoma and progressive primary open angle glaucoma²⁷. There are also studies that show diabetic retinopathy is preceded by sub-clinical disturbances in the retinal vasculature. Hence, many studies have considered the quantification of inner retinal blood flow as a promising investigation approach for the early detection and improved monitoring of various diseases. However, the results of inner retinal blood flow disturbance in DR have been contradictory²⁸⁻³⁷, although virtually all indicate some aspect of disturbance.

This study shows that the Doppler FD-OCT can reliably and consistently measure retinal blood flow in healthy participants, despite the subjectivity of the vessel area determination. The Doppler FD-OCT offers a quantifiable and repeatable method of assessing retinal blood flow. Blue field entoptic and fluorescein angiographic techniques deliver mostly subjective or semi-quantitative measurements of retinal blood flow. Laser Doppler flowmetry offers flow values only in arbitrary units and readings between subjects are dependent on interpretation. Pulsatile ocular blood flow assessments are dependent on controversial assumptions of ocular physiology and measure total ocular blood flow, a majority of which arises from the choroidal circulation.

Wang and co-workers used a single-beam FD-OCT to measure the total retinal blood flow of all vessels around the optic nerve head. Their results showed values of $45.6 \pm 3.8 \mu\text{l}/\text{min}$

for TRBF with the co-efficient of variation of 10.5%²³. Our results showed a range of variability in individual blood flow with a COV ranging from 0.4% in young subjects (median 7.5%) to 34.6% in elderly subjects (median 9.2%). Our data is in agreement with Wang and co-workers data²³. There are several sources that might contribute to the individual variability including the impact of eye motion, tear film quality or normal biological variations. The individual variability may also reflect the quality of measurement and possibly the analysis method. Subjects with higher COV values could be excluded from study based upon their outlier status.

The differences in COV between the two groups (median 7.5% young group versus 9.2% elderly group) are negligible and the differences in COR between groups are relatively small (median 5.91 μ l/min, relative to a mean effect of 39.76 μ l/min for the young group versus median 9.16 μ l/min, relative to a mean effect of 46.39 μ l/min for the elderly group). This represents a difference in RBF calculations between sessions/days of approximately 4 μ l/min in real terms or in terms of the known range of TRBF values a difference of approximately 8%.

The co-efficient of variation relates the group mean SD of a given parameter as a function of the group mean effect (on a percentage scale). Therefore, comparison of the COV for young and elderly participants between venous area and venous velocity can be used to determine which of the two parameters contributes the greatest variability to the RBF value. We found that the COV for venous area to be approximately half that of the COV for venous velocity (i.e. 4.7 to 4.8% for area versus 10.4 to 10.8% for velocity). We conclude that the velocity measurement is the greater contributor to blood flow variability, a finding that is in agreement with that of the established CLBF methodology^{38,39}. A

mathematical perspective of this issue would be to argue that a summary velocity value is derived by dividing the measured area by the measured flow value. Consequently, the fundamental error components are Doppler angle error caused by motion error, vessel boundary segmentation error, Doppler phase error due to phase wrapping, residual bulk motion error (after compensation) and system phase noise (Huang D. Personal Communication).

One of the advantages of OCT is the exceptional level of resolution used to discern the retinal vasculature. Another more established method to assess RBF is Laser Doppler velocimetry (LDV), which has been applied to investigate retinal blood velocities in the major arterioles and venules of the retina⁴⁰. The most established instrument to utilize the LDV methodology is the Canon Laser Blood Flowmeter (CLBF), which simultaneously measures vessel diameter along with centerline blood velocity in order to derive blood flow values in absolute units⁴¹.

Using the CLBF, the individual COVs for flow were reported to be from 4.8 to 37.3% (median 19.3%). The group mean CORs for flow were found to be 2.6 μ l/min (relative to a mean effect of 8.8 μ l/min). These data are from CLBF measurements of blood flow within retinal arterioles in normal subjects³⁸. Our group studied the retinal arteriolar and venular blood flow in healthy subjects and concluded that the level of variability between the two visits was equivalent pre- and during hyperoxic provocation of vascular reactivity. This study showed the COV for venular flow was 9.9% during the baseline period³⁹. These findings can be found in the Table 3.5.

Co-efficient of Variation (%)						
	CLBF Diameter or FD-OCT Doppler Area		Velocity		Flow	
	Median	Range	Median	Range	Median	Range
CLBF ³⁹	2.0	0.5-6.5	19.9	4.8-39.7	19.3	4.8-37.3
CLBF/Arteriole ⁴⁰	1.8	0.6-3.3	10.7	0.8-22.1	11.8	1.0-23.2
CLBF/Venule ⁴⁰	1.4	0.1-7.1	9.8	1.2-20.8	9.96	1.6-20.8
FD-OCT Doppler /Young	4.7	0.8-16.8	10.4	0.1-28.9	7.5	0.4-20.4
FD-OCT Doppler /Elderly	4.8	0.4-20.4	10.8	1.1-43.8	9.2	0.6-34.6

Table 3.5 Comparison of Co-efficient of Variation (COV) using different Doppler based technologies.

Doppler FD-OCT repeatability for young and elderly subjects, in terms of the COR, was found to be 6.43 μ l/min and 10.53 μ l/min, respectively; the acceptable level of between-session variability needs to be interpreted based upon the magnitude of the effect size. The difference in repeatability between sessions was also greater in subjects with crowded vascular beds, which resulted in difficulty distinguishing the arteriolar from the venular Doppler phase shift signals (Figure 3.8). Other factors such as tortuous vessels or curved and tapering vessels can also play a role in the variability through the generation of irregular, or non-Poiseuille flow conditions. The within-session variability of blood flow measurements could be a result of eye motion, tear film or the anatomy of the vessels or intrinsic variation of RBF. The tear break up can alter the laser intensity projected onto the retina and may result in displacement of the laser from the center of the vessel due to optical blurring effects. We attempted to keep all parameters such as diet in both sessions the same. Other factors such as consuming red meat or caffeine could play a role in the difference as well^{42,43}. These factors can contribute to the individual blood flow variation measured by Doppler FD-OCT. Another limitation of this study is that the maximum

detectable volume of the Doppler FD-OCT system is slower than the maximum flow velocity especially in retinal arterioles assuming equal flow in arteriole and venule; as a result, all reported values were based upon venular measurements only.

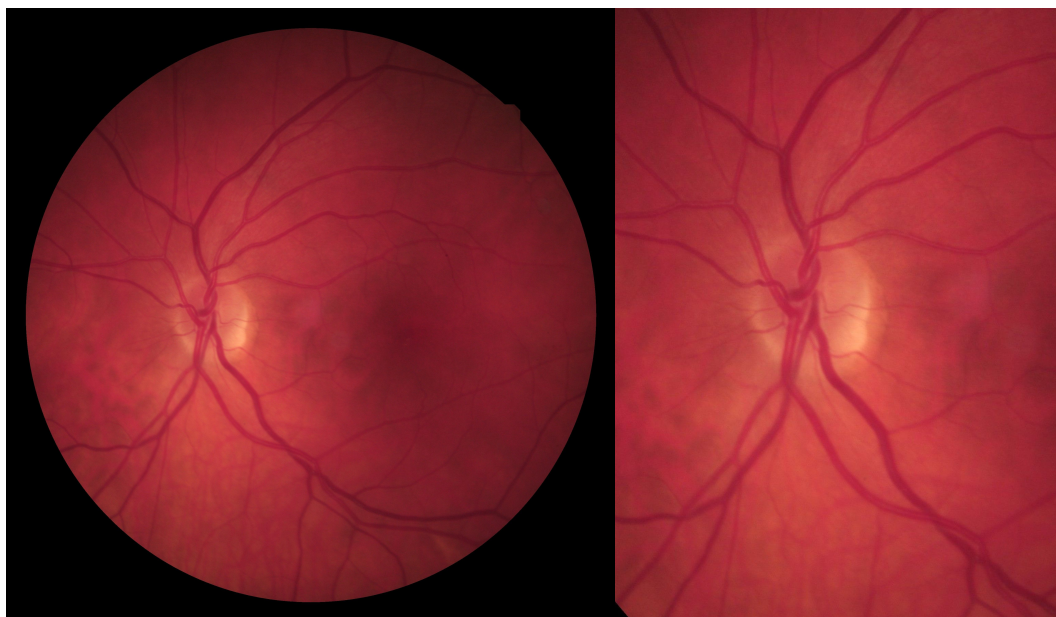


Figure 3.8 Fundus image of an elderly subject with tortuous vessels and high COR value.

Factors that will cause Doppler FD-OCT measurements to be invalid fall into three main causes: Objects that are close to perpendicular to the incident OCT beam will result in a relatively weak Doppler signal because the Cosine of 90° is zero. Also, errors in the measurement of vessel lumen area will greatly influence the estimation of flow and this effect is compounded by the fact that the area estimation requires the subjective determination of the Doppler signal boundary and then the subjective assignment of confidence in the area estimation. In addition, the impact of eye movements will introduce further error into the estimation of vessel lumen area.

To summarize, the degree of variability and repeatability can differ significantly between individuals but overall Doppler FD-OCT gave consistent and repeatable blood flow measurements within retinal venules in normal subjects.

4 Retinal Blood Oxygen Saturation Disturbances in Patients with Type 2 Diabetes

Faryan Tayyari, Lee-Anne Khuu, Shaun Singer, Michael Brent, John G Flanagan,
Christopher Hudson

	Design	Recruitment	Acquisition Of Data	Analysis	Writing / Publication
F. Tayyari	x	x	x	x	x
L. Khuu	x	x		x	
S. Singer		x	x		
M. Brent		x	x		
J. Flanagan	x				
C. Hudson	x			x	x

Table detailing role of each author in this publication (x denotes significant contribution)

4.1 Introduction

The retina is a tissue with extraordinary metabolic demand, especially for key molecules such as oxygen, glucose and amino acids ^{1,2}. This makes the retina more susceptible to hypoxia. Retinal blood flow disturbance in the early stages of diabetes along with hypoxia is thought to play a role in pathological retinopathy. Retinal oxygen consumption has been thought to decrease in diabetes, which might be due to reduction in metabolism of neurons, or to cell death. In other words, diabetic retinopathy (DR) is a microvascular and neurodegenerative disorder characterised by damage to the retinal capillaries and retinal glial, Müller and associated neuronal cells ³⁻⁶. DR results in disturbed delivery of oxygen to retinal cells, which in turn will cause hypoxia in retinal tissue. A relationship between the pathogenesis of DR and retinal hypoxia has been found. Hypoxia is thought to provoke neovascularization and retinal edema ⁷. Therefore, it is critical to study the regulation of oxygen supply to the retina in order to learn more about the pathophysiology of DR and diabetes.

Studies have shown a reduction in retinal oxygen consumption in diabetes-induced rabbits ⁸ and cats ⁹, although there are conflicting results about arterio-venous oxygen differences and consumption in the retina of diabetic rats ¹⁰⁻¹². However, the arterio-venous difference has been shown to decrease in all the stages of retinopathy¹³.

One study indicated a reduction in retinal venular blood oxygen saturation during hyperglycemia in diabetic patients without retinopathy and also found a correlation between the amount of venous oxygen saturation reduction and the duration of diabetes ¹⁴. Holekamp and co-workers (2006) found lower partial pressure of oxygen in the vitreous of a patient with proliferative diabetic retinopathy than in the vitreous of non-diabetic patients

immediately prior to vitrectomy ¹⁵. Likewise, another group found a lower partial pressure of oxygen in the mid-vitreous in diabetic patients when compared to non-diabetic patients ¹⁶ and also reported higher partial pressures of oxygen at the posterior pole in patients with proliferative diabetic retinopathy compared to non-diabetic patients undergoing vitrectomy ¹⁷. Others have found retinal oxygen saturation in arterioles and venules to be higher in diabetic patients ¹⁸. A trend of increasing arterial and venous oxygen saturation correlating to the severity of retinopathy in diabetic patients has been reported, though the arterial increase is not significant ¹³. The same group showed that flicker light stimulation leads to a rise in both central retinal arterial and venous diameter while only resulting was a rise in venous, and not in arterial, oxygen saturation. They presented a reduced flicker response for NPDR patients compared to controls ¹⁹. Another study indicated a trend for higher retinal oxygen saturation in NPDR group but this was insignificant when compared to controls, while significance was only observed when comparing controls to severe-NPDR, PDR ²⁰.

Tiedeman and co-workers ¹⁴ used a dual wavelength retinal imaging system in diabetic subjects without clinical evidence of diabetic retinopathy during normoglycaemia and hyperglycaemia. They reported evidence of significantly higher retinal oxygen consumption during hyperglycemia. Hammer and co-workers showed higher venular oxygen saturation with the severity of DR in subjects with mild non-proliferative DR and in subjects with proliferative DR but the difference in mild-to-moderate NPDR didn't reach significance ²¹. An insignificant increase in retinal oxygen saturation was also reported comparing healthy controls to non-proliferative diabetic retinopathy subjects, though significance was only reported for severe-non-proliferative NPDR and proliferative DR ²¹.

This suggests that imaging techniques such as hyperspectral retinal imaging may provide improved methods of predicting the probability of onset, differentiating the severity and of observing progression of diabetic retinopathy in vivo. A recent study by Man and co-workers²² suggested that eyes with longer axial length show lower retinal function and hence lower O₂ consumption; therefore eyes with longer axial length are fairly less hypoxic when diabetes exists, which may partly explain the reduced risk of DR in these eyes. Hardarson and co-workers also reported increased retinal blood oxygen saturation in patients with DR²³.

In terms of retinal oxygen saturation imaging, the technology provided by Optina Inc (formally known as Photon etc) permits the acquisition of hyperspectral retinal images at background light levels (Bragg tunable filter; BTF) far lower than any of the competing technologies, thereby avoiding flashing induced disturbance of retinal metabolic status.

Oxygen saturation in diabetic patients could offer valuable information for better understanding of changes in metabolism in DR.

4.2 Methods

4.2.1 Sample

This study received approval by the University of Waterloo Office of Research Ethics and the Research Ethics Board of the University Health Network, University of Toronto, Canada. Informed consent was obtained from each subject after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki. The sample consisted of 31 volunteers in two groups of subjects with mild-moderate NPDR (n=14; mean age 66.3; SD 9.1 years) and aged matched healthy controls (n=17; mean age 69.70; SD 6.26 years) (Table 4.1). One eye of each subject was selected for the study. All subjects had a corrected visual acuity of 20/40 or better. Subjects were excluded for a family history of any other ocular disease, apart from diabetic retinopathy. None of the patients with NPDR had received treatment of any kind for their retinopathic changes or had any evidence of diabetic macular edema or any other sight-threatening characteristic. Subjects abstained from caffeine for at least 12 hours before the study.

Group	Group Mean Age (SD)	Male to Female Ratio	Group Mean A1c (SD)
Control	69.1 (5.7)	8 M : 9 F	5.5 (0.4)*
NPDR	66.3 (9.1)	5 M : 9 F	7.5 (1.5)

Table 4.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy) *(difference between control and NPDR group $p < 0.001$)

4.2.2 Assessment of Retinal Blood Oxygen Saturation

A prototype custom-built fundus camera (H-8.5 HR Camera, Optina, QC, Canada) is the foundation of the hyperspectral imaging system that incorporates a tunable laser source (TLS) as the light source. The TLS makes it possible to change wavelengths within a spectral range of 400-1000nm (visible to IR) and with a half peak bandwidth of 2nm. The

TLS is built on Photon etc's Bragg grating filtering technology which permits rapid wavelength presentation from the steady and powerful super-continuum light source (Leukos-SM-30-OEM, Leukos Innovative Optical Systems, Limoges, France). The TLS can be electronically tuned with an operating ambient temperature of 10-30°C. An automatic spectral system is incorporated into the system to achieve exact and accurate (<1nm) wavelength choice. The sensitive 1.3MPixel (1392 x 1040 pixel) 14-bit CCD camera (Pixelfly USB, PCO AG, Kelheim, Germany) used in this system is for high definition imaging. The imaging system is operated by using PhySpec (Photon Etc, Montreal, QC, Canada), a programme that manipulates the Bragg tunable filter (BTF) and CCD camera to allow aspects such as operator defined wavelength range and wavelength interval. A low power white light source (delivering about 100mW over 420nm to 2400nm) and the Bragg tuneable filter are used to exclude the use of conventional flash lamp, and therefore all images are acquired at low light levels, thereby minimizing any potential change of the metabolites and photopigment status in the eye. A diagonal field of view of approximately 37.4° is sustained by the opto-mechanics of the system²⁴.

After pupil dilation with one drop of Tropicamide 1% (Alcon, Mississauga, Canada) each subject had six repeated sets of retinal images captured at 586 and 605nm using the HRC. Optical densities were extracted for first-degree arterioles and venules manually and the repeatability of retinal reflectance was compared sequentially. PHySpec (Photon etc.), a custom-designed software, is used to acquire hyperspectral images and to extract reflected light intensity of the retinal vessels. The final spectral cubes were normalized and registered independently after spectral image acquisition.

The normalized spectral cubes underwent a registration procedure to correct for wavelength-dependent optical deformations (scaling) and fine eye movement (translation). The scaling correction is continual in time and is determined during the instrument set-up and qualification (Photon etc.). PHySpec comprises a registration procedure permitting translation correction between images in the cube. Details of the PHySpec™ software have been published elsewhere ²⁴.

ImageJ™ software was used to provide reflectance profiles across a retinal vessel at a predetermined site; then ODR was calculated using the Minimum-Maximum methodology. In the suggested Minimum-Maximum Method, optical density (OD) of a retinal vessel is derived from the log ratio of minimum and maximum intensity values along a line drawn perpendicular to the long axis of the retinal vessel.

$$OD = -\log_{10} \frac{\textit{minimum intensity}}{\textit{maximum intensity}}$$

Retinal blood oxygen saturation was measured within one disc diameter from optic nerve head in first degree arterioles and venules. The analysis was performed by equating the reflected light intensity within a vessel to the light intensity of the adjacent retina at isosbestic (oxygen insensitive) and non-isosbestic wavelengths.

The light intensity from the retinal area adjacent to the vessel is used to determine the maximum reflected intensity (i.e. a measurement site assumed to be unaffected by hemoglobin absorption) and the center of the vessel (i.e. assumed to be maximally impacted by hemoglobin absorption) is used to determine the minimum reflected intensity. These measurements are used to calculate the OD ratio (ODR) value ($=OD_{605}/OD_{586}$), which

represents the OD value of oxygen sensitive wavelength as a function of the OD value oxygen insensitive wavelength²⁵.

The ODR and SO₂ values were calculated for both retinal arterioles and venules. The arteriovenous differences were in the blood oxygen saturation between the arterial blood and the venous blood.

The measurements were taken at 6 different wavelengths (hemoglobin wavelength insensitive at: 548nm, 569nm, 586nm and hemoglobin wavelength sensitive at 600nm, 605nm, 610nm). The most consistent results in terms of retinal blood SO₂ repeatability were achieved by the wavelength combination OD₆₀₅/OD₅₈₆, 586nm as insensitive to differences in blood oxygenation and gives better results compared to other hemoglobin insensitive wavelength because of the proximity to the hemoglobin sensitive wavelengths. The 605nm wavelength also provided improved repeatability of results.

4.2.3 Statistical Analysis

Two-tailed t-test was performed to determine the significance of any change. The difference in retinal oxygen saturation was considered significant when the p-value was less than or equal to 0.05.

4.3 Results

In diabetic patients, the mean retinal venular blood oxygen saturation (SO₂) was higher than in the healthy controls (94.65±2.2% vs 92.97±1.6%, p=0.0151, Figure 4.1).

Similarly, the mean retinal arteriolar blood SO₂ was higher in diabetic patients than in controls (64.13±4.3% vs 55.90±4.8%, p<0.001, Figure 4.2), while the mean arteriovenous SO₂ difference was lower for the diabetic patient group (30.57±4.76 vs. 37.05±4.95, p=0.001).

Both arteriolar and venular SO₂ values were significantly different between the groups.

The difference between the groups in SO₂ of the venules was higher than the difference in the arteriolar SO₂ between the two groups. This resulted in a significant difference in arteriovenous difference between two groups.

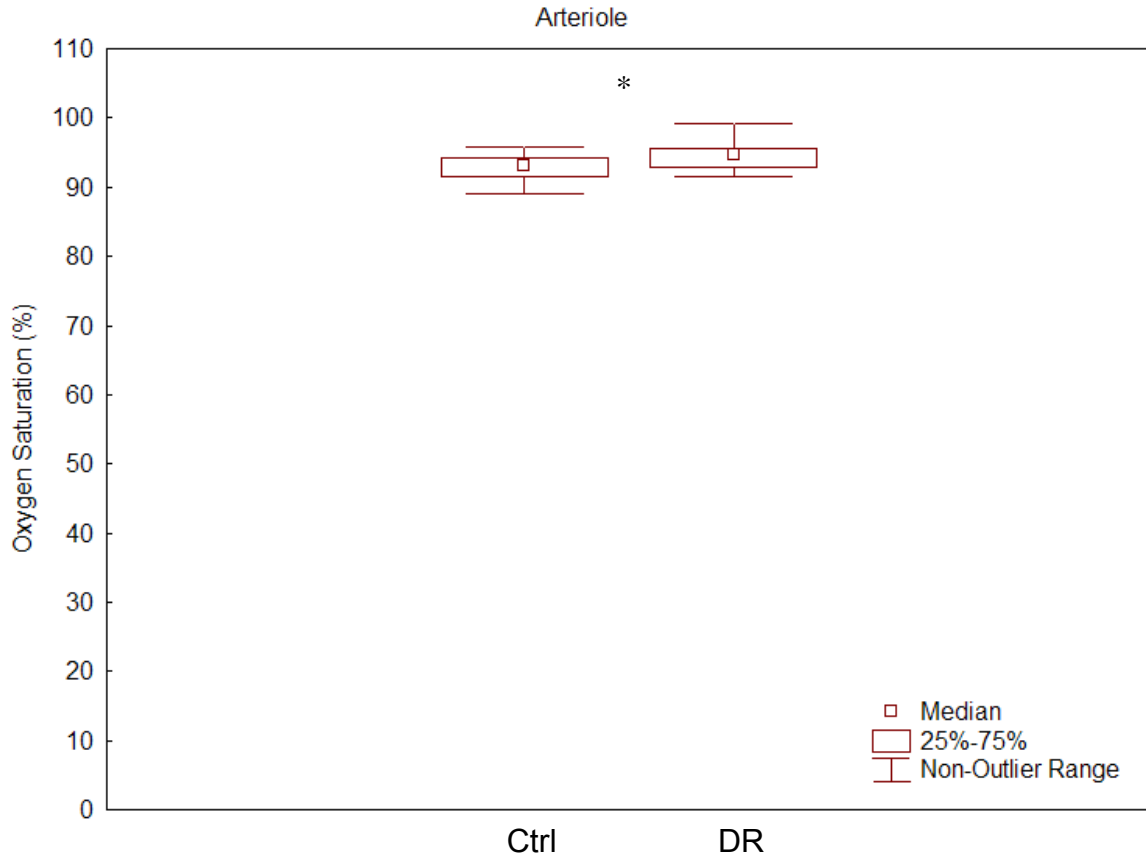


Figure 4.1 Oxygen saturation in first-degree arteriole. The box plots illustrate retinal arteriolar blood oxygen saturation in control and NPDR participants. The error bars show the non-outlier range.

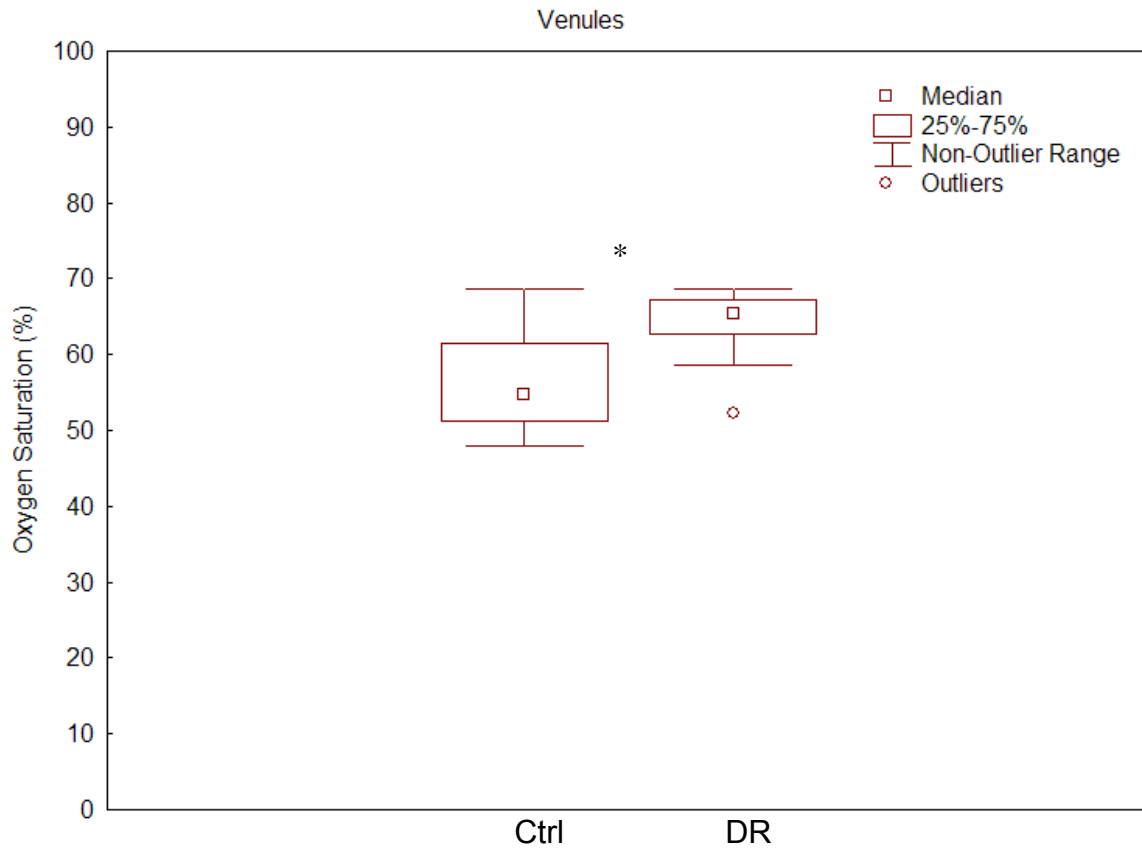


Figure 4.2 Oxygen saturation in first-degree venules. The box plots illustrate retinal venular blood oxygen saturation in control and NPDR participants. The error bars show the non-outlier range.

4.4 Discussion

The results of this study demonstrate a higher retinal SO_2 in the major retinal arterioles and venules in patients with untreated, non sight-threatening, mild-to-moderate NPDR than when compared to healthy controls. This difference was much more evident in venules rather than arterioles. Hardarson and co-workers data is in strong agreement with the results of our study, since they also showed higher retinal SO_2 values in diabetic patients¹⁸. Using different technology and a smaller sample, Hammer and co-workers found a trend for an increase of retinal SO_2 for both arterioles and venules but they could not show a significant difference between controls and patients with mild-moderate NPDR²¹. Khoobehi and co-workers showed that retinal SO_2 increased in diabetic patients but these changes were only significant in severe NPDR and PDR groups when compared to controls²⁰. Using correlation analysis Khoobehi and co-workers showed that the severity of DR had an impact on the increase of SO_2 in diabetic patients²⁰. There was a drawback in the study of Khoobehi and co-workers because their groups were not age-matched. In my study, patients with relatively mild-to-moderate NPDR which did not yet require treatment were shown to have higher retinal arteriolar and venular SO_2 values when compared to controls.

This study showed that the SO_2 values in the major retinal vessels were greater in patients with mild-to-moderate NPDR than in controls. The higher retinal venule SO_2 may be explained by lower oxygen release to, or lower consumption by, the retinal tissues secondary to retinal cell death. However, SO_2 values alone are difficult to interpret since:

$$\text{Retinal } O_2 \text{ Delivery} = [1.39 \times \text{Hb} \times \text{SaO}_2 + (0.003 \times \text{PaO}_2)] \times \text{blood flow}$$

Theoretically, each gram of Hemoglobin binds 1.39 ml of oxygen. The oxygen saturation curve represents a PaO₂ (arterial oxygen tension) as a function of SaO₂ (Arterial SO₂). The haemoglobin concentration is calculated by production, destruction and loss. 0.003 stands for the oxygen solubility coefficient in human plasma

The implications of the factors in the equation above are that if retinal O₂ metabolism increases in NPDR then the results may be explained either by an increase in SO₂ or an increase in blood flow or an increase in both SO₂ and retinal blood flow. This emphasizes the need to measure retinal blood flow and retinal SO₂, ideally simultaneously.

As mentioned in other papers ²¹, the higher retinal SO₂ in NPDR might be a result of capillary occlusion and permanent closure of blood vessels in conjunction with other vessel changes in NPDR ³. This will result in decreased capillary density in some areas and, consequently, hypoxia. However, whether or not retinal SO₂ derives the development of DR, or simply changes subsequently, is undetermined. Prospective studies need to be undertaken which assess the change in oxygen metabolism and retinopathy development over time to determine which parameter is disturbed first.

An elevated SO₂ in retinal arterioles has been suggested to reflect the magnitude of glycated hemoglobin (A1c). However, in this study, no relationship was found between arteriolar SO₂ and A1c. Similarly, Gilmore and co-workers ^{26,27} reported no correlation between change in hemodynamic flow parameters and blood glucose, A1c and duration of diabetes. This finding was in agreement with other studies which did not find correlation between blood flow and blood glucose ²⁸. However, Pemp and co-workers ²⁹ reported decreased retinal blood flow with decreasing plasma glucose using the glucose clamp technique in subjects with type 1 diabetes with no or mild DR. Other studies have also

shown higher blood flow with increased blood glucose^{30,31}. Protein glycation and production of advanced glycation end products (AGEs) may contribute to reduction of the rate of oxygen diffusion out of the arterioles and hence a higher magnitude of retinal blood SO₂³².

The mean arterio-venous difference was 36.89±7.02 for healthy subjects and 30.78±4.76 in NPDR subjects (p=0.009). The lower arterio-venous difference in the NPDR group could be the result of less oxygen demand that especially impacts the venules in NPDR. Hardarson and co-workers reported the arteriovenous difference as insignificant. This could be explained by the fact that their population was not age-matched²³.

Retinal vessel blood oxygen saturation was higher in NPDR when compared to control participants. This could be as a result of reduced oxygen utilization, probably as a result of neuronal cell atrophy, in the diabetic group with mild-to-moderate NPDR.

5 The Association of Retinal Blood Flow and Retinal Blood Oxygen Saturation in Mild-to-Moderate Diabetic Retinopathy

Faryan Tayyari, Lee-Anne Khuu, Shaun Singer, Michael H Brent, John G Flanagan,
Christopher Hudson

	Design	Recruitment	Acquisition Of Data	Analysis	Writing / Publication
F. Tayyari	x	x	x	x	x
L. Khuu	x	x	x	x	
S. Singer		x			
M. Brent		x			
J. Flanagan	x				
C. Hudson	x			x	x

Table detailing role of each author in this publication (x denotes significant contribution)

5.1 Introduction

Diabetic retinopathy (DR) is a primary source of visual loss in the world, including North America¹. DR appears to be allied closely to disturbances in the vasculature. Hence, many studies have considered the inner retinal blood flow. However, the results of inner retinal blood flow disturbance in DR have been generally contradictory²⁻¹¹, although many studies indicate some aspect of disturbance. The study of metabolic disturbance as a functional diagnosis is a potentially encouraging biomarker for the detection of early reversible pathological changes before they become clinically evident.

Elevated retinal blood flow has been classically proposed to ultimately cause the progress of DR, probably because of amplified frictional forces (i.e., shear stress) on the endothelial cells lining the walls of retinal vessels¹². However, the exact nature of the blood flow disruption is debatable, perhaps due to the variety of methods used to measure retinal hemodynamics, the diverse stages of retinopathy considered, and the diversity of the diabetic groups¹³. From a clinical perspective, the assessment of ocular hemodynamics proposes an earlier detection of DR and improved DR severity differentiation and perhaps a flag for new treatment opportunities. Retinal hypoxia is thought to promote the production of VEGF and the development of macular edema and neovascularization. Measurement of retinal oxygenation in patients with diabetes may help to assess the severity of DR and the response to novel “prophylactic” treatments. Ultimately, knowing the blood flow to, and the oxygenation of, the retinal tissue will permit the assessment a metabolic parameter of the retinal tissue, namely oxygen utilization (also termed “retinal oxygen delivery”).

It has been suggested that change in blood circulation leads to functional damage and broad retinal tissue impairment and disturbance during diseases such as DR¹⁴. In this respect, it

will be critical to study the oxygen distribution or consumption of the retina and its alteration in response to DR since RBF or SO_2 alone represent only part of the required information to calculate retinal oxygen utilization. Measurement of these alterations could be used to improve the early detection and management of diseases such as diabetes / diabetic retinopathy. Most of the non-invasive optical techniques that are used to measure the retinal oxygen saturation (SO_2) rely on measuring differences between hemoglobin and oxygenated hemoglobin (Hb and HbO_2) light absorption (see Section procedures for complete methodology explanation). Simultaneous retinal blood flow measurement and retinal hemoglobin oxygenation are required to assess absolute values of oxygen delivered to the retina ^{15,16}. The non-invasive quantification of oxygenation of the retina will be feasible by retinal oximetry, which in turn may offer a sensitive and predictive biomarker. Quantification of blood flow and oxygenation of the retina will permit the assessment of the tissue oxygen utilization.

Assessing oxygen concentration in the retina has been achieved using O_2 -sensitive microelectrodes introduced into the eye ¹⁷⁻²⁰. While this technique is precise and can accurately define oxygen saturation, the invasive nature of the technique limits its' usage to animal models and disqualifies it from clinical purpose. Alternative methods using the introduction of a phosphorescent dye has been utilized to derive oxygen concentration in the optic nerve head and retinal vessels^{19,21}. Nonetheless, the use of such a dye in humans is not accepted due to toxicity concerns. Imaging techniques established on oxygenated hemoglobin and reduced hemoglobin (i.e. deoxyhemoglobin) and their spectral changes have been used in humans to non-invasively assess oxygen saturation in retinal vessels ^{18,22-26}. Hickam and co-workers first proposed a non-invasive measurement of the oxygen

saturation using a two-wavelength (510, 640nm) photographic-based method. Following that, another two-wavelength (470, 515 nm) technique was developed to measure oxygen saturation. These techniques, however, were laborious. Later, Pittman and Duling¹⁶ introduced a three-wavelength spectrophotometer method for oximetry. Delori¹⁴ introduced the first scanning retinal oximeter illuminating with multiple wavelengths. Another group²⁵ used a grating spectrograph to acquire spectra and applied a multi-wavelength curve fitting technique for saturation estimation. Oxygen saturation in retinal vessels has been found to be greater in diabetic retinopathy in contrast to control subjects²⁷. Assessment of oxygenation in the retina may facilitate the early detection of diabetic retinopathy. The aim of this study was to investigate the relationship between retinal blood flow and retinal blood oxygen saturation in NPDR.

5.2 Methods

5.2.1 Sample

This study received approval by the University of Waterloo Office of Research Ethics and the Research Ethics Board of the University Health Network, University of Toronto, Canada. Informed consent was obtained from each subject after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki. The sample consisted of 28 volunteers in two groups of subjects with mild-to-moderate NPDR (n=13; mean age 67.3; SD 10.2years; Table 5.1) and aged matched healthy controls (n=15; mean age 68.1±years; SD 6.0 years; Table 5.1). One eye of each subject was selected for the study. All subjects had a corrected visual acuity of 20/40 or better. Subjects were excluded for family history of any other ocular disease, apart from DR. None of the patients with NPDR had received treatment of any kind for their retinopathic changes or had any evidence of diabetic macular edema or any other sight-threatening characteristic. Subjects abstained from consuming caffeine 12 hours before the study.

Group	Group Mean Age (SD)	Male to Female Ratio	Group Mean A1c (SD)
Control	68.1 (6.0)	5 M : 10 F	5.4 (0.3)
NPDR	67.3 (10.2)	5 M : 8 F	7.5 (1.6)*

Table 5.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy). *(difference between control and NPDR group p<0.001)

5.2.2 Assessment of Retinal Blood Flow

Doppler Fourier-domain optical coherence tomography (Doppler FD-OCT) is used to measure retinal blood flow. This novel imaging method offers retinal blood flow assessment using a physical phenomenon called Doppler phase shift²⁸. The principle of the

Doppler phase shift has been incorporated into the commercially available Doppler FD-OCT (Optovue Inc, Fremont, CA). Doppler FD-OCT produces high resolution cross sectional images of the retina. This instrument uses a laser light source of 841nm with bandwidth of 49nm with an incident power of 500 μ W on the cornea. These factors deliver an axial resolution of 5.4 μ m in tissue²⁹. System transverse resolution was 20 μ m³⁰.

Contradictory to morphological FD-OCT systems that generates just structural images, the prototype Doppler FD-OCT evaluates the Doppler phase shift between two consecutive A-scans. Light reflected from moving particles undergoes Doppler phase shift.

Flow velocity is determined by:

$$v = \frac{\Delta\Phi \cdot \lambda_0}{4\pi \cdot T \cdot n \cdot \sin(\theta)}$$

Where v is the flow velocity in an OCT voxel, and $\Delta\Phi = \Phi_1 - \Phi_2$ is the Doppler phase shift. Φ_1 and Φ_2 are the phase of voxels in the same position in consecutive OCT axial scans, λ_0 is the source center wavelength, n is the refractive index of the medium, T is the time interval between consecutive scans and θ is the Doppler angle defined by the OCT beam axis relative to the line perpendicular to blood vessel flow axis³¹.

5.2.3 Assessment of Retinal Blood Oxygen Saturation

The principal of the oximeter has been described elsewhere³² but briefly a prototype custom-built fundus camera (H-8.5 HR Camera, Optina, QC, Canada) is the foundation of the hyperspectral imaging system that incorporates a tunable laser source (TLS) as the light source. The TLS, built on Photon etc's Bragg grating filtering technology, makes it

possible to transfer wavelengths with a half peak bandwidth of 2nm (ranged from 400nm to 1000nm). The TLS permits rapid wavelength presentation from the steady and powerful super-continuum light source (Leukos-SM-30-OEM, Leukos Innovative Optical Systems, Limoges, France). The imaging system is administered by using PhySpec (Photon Etc, Montreal, QC, Canada), a software programme that operates the Bragg tunable filter (BTF) and CCD camera to permit factors such as operator defined wavelength range and wavelength interval. A low power white light source (delivering about 100mW over 420nm to 2400nm) and the Bragg Tuneable Filter are utilized to eliminate the use of conventional flash lamp, and consequently all images are obtained at low light levels, thus decreasing any potential change of the ocular metabolites and photopigment status ³².

5.2.4 Procedures

Refraction, logMAR visual acuity, Goldmann applanation tonometry and resting blood pressure were assessed prior to dilation of the study eye. The pupil of the study eye was dilated using Tropicamide 1% (Alcon, Mississauga, Canada) at the beginning of each visit to achieve an adequate view of the fundus for the retinal blood flow image acquisition and retinal blood oxygen saturation measurements. A minimum of six separate FD-OCT Doppler measurements (i.e. each separate measurement comprising an upper nasal pupil scan and a lower nasal pupil scan) and six blood oxygen saturation measurements were acquired.

5.2.5 Analysis

A Pearson correlation analyses and linear regression were performed to examine the relationship between retinal blood flow and retinal blood oxygen saturation.

5.3 Results

The results of this study showed that the retinal blood flow was significantly lower in diabetic patients (before removing extreme or outlier points, 42.66 vs 32.97 $\mu\text{l}/\text{min}$; $p=0.004$ and after removing the extreme and outlier points 42.08 vs 33.86 $\mu\text{l}/\text{min}$; $p=0.005$, Figure 5.1)

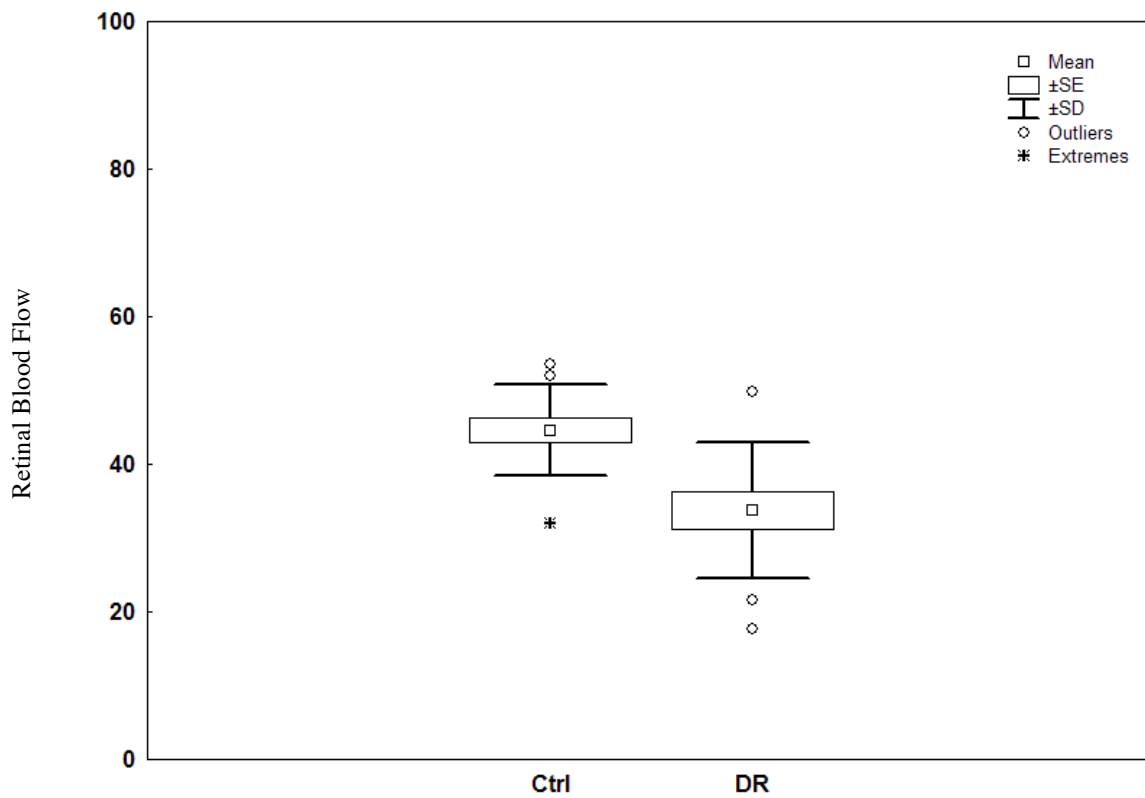


Figure 5.1 Box plots representing venous retinal blood flow in control and NPDR patients (42.66 vs 32.97 $\mu\text{l}/\text{min}$; $p=0.004$). Box plots illustrating group mean retinal blood flow. The * represents extremes and the small circles represent outliers. The error bars show the non-outlier range.

	Control	NPDR	T-test (p-value)
SO₂ (Venule)	56.29±4.7	62.55±5.7	p=0.003
Total RBF	42.66±7.55	32.97±9.2	p=0.004

Table 5.2 Group mean values for blood oxygen saturation and total retinal blood flow (RBF) for non-proliferative diabetic retinopathy (NPDR) and aged-matched controls.

Figure 5.2 shows retinal venous blood flow as a function of retinal blood oxygen saturation in controls and Figure 5.3 illustrates retinal venous blood flow as a function of retinal blood oxygen saturation in NPDR group. The result did not show any relationship between retinal blood flow and oxygen saturation in either of the groups.

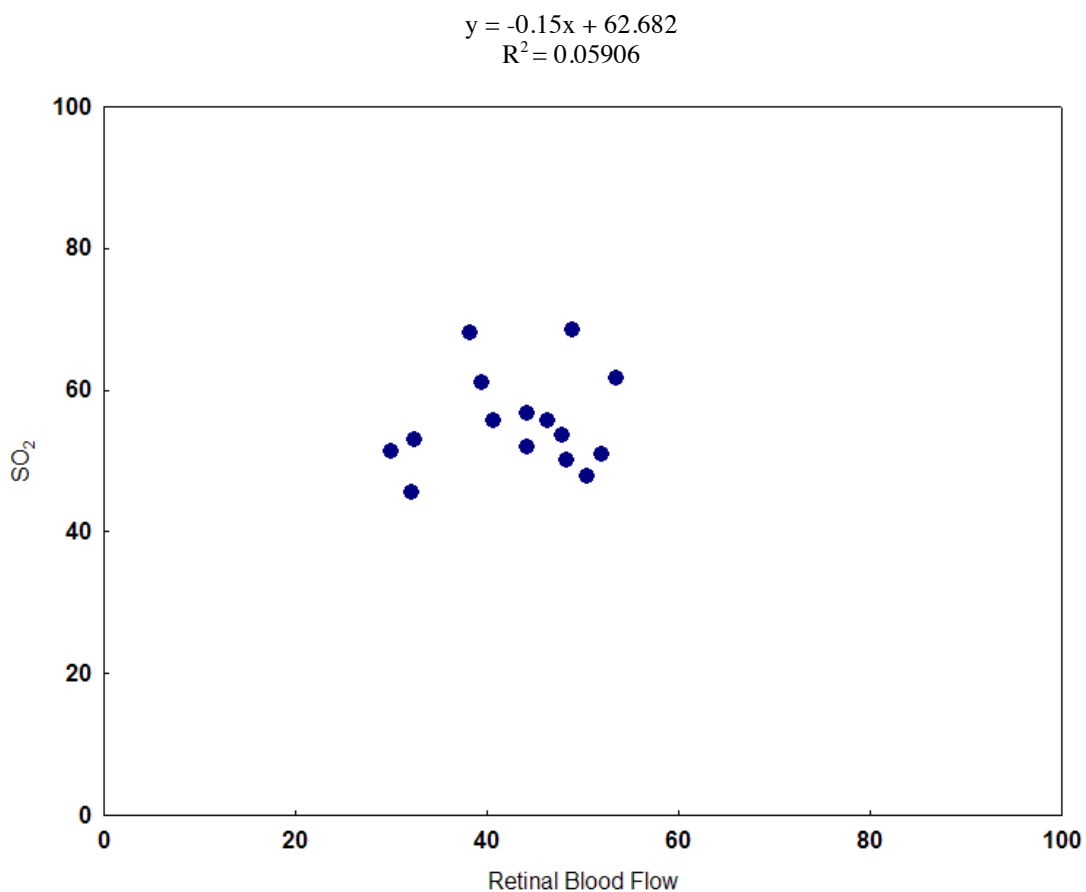


Figure 5.2 Oxygen saturation as a function of retinal venous blood flow in controls ($r=0.243$, $p=0.34$)

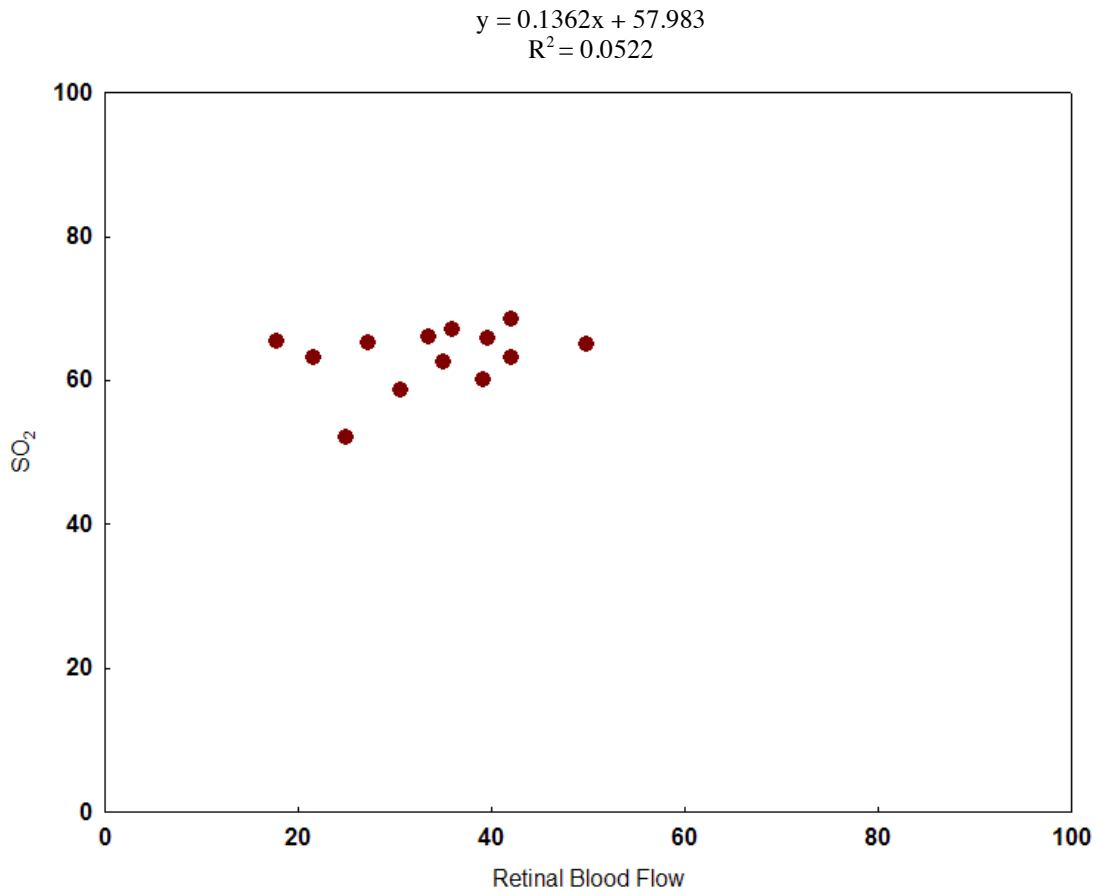


Figure 5.3 Oxygen saturation as a function of retinal venous blood flow in NPDR group ($r=0.228$, $p=0.45$)

5.4 Discussion

It has been shown that the DR is associated with early retinal vascular dysregulation. Therefore, many studies have investigated the inner retinal blood flow. Nevertheless, the results of inner retinal blood flow disturbance in DR have been contradictory²⁻¹¹, although virtually most indicate some aspect of disturbance. It has been reported that NPDR patients or patients without retinopathy have fluctuated retinal blood flow at different times of the day due to fluctuation in their plasma glucose which was suggested as a possible cause of microvascular changes in DR³³. Our results showed lower retinal blood flow in NPDR when compared to controls ($p=0.004$). There was no correlation between total retinal blood flow and retinal venular blood oxygen saturation. This might be as a result of small sample size.

The direction of the change in total retinal blood flow in DR is controversial^{5,34-36}. The present study showed a reduction in retinal blood flow and increased in retinal blood oxygen saturation when comparing NPDR subjects with controls. Our oximetry data is in agreement with Hardarson's results³⁷. Hardarson interpreted this result to occur due to impaired diffusion of oxygen to the adjacent tissue through the thickened arteriolar walls of diabetic subjects. Higher blood flow would be inclined to reduce the release of oxygen from haemoglobin and therefore would result in reduced diffusion of oxygen across the arteriolar walls³⁷.

The blood flow in NPDR group is lower when compared to controls. This might be explained by the concept of mobile oxygen sensor introduced by Ellsworth and co-workers³⁸. This idea is based on the fact that hemoglobin in red blood cells (RBC) becomes deoxygenated and consequently a changes form which transmits to the RBC membrane and

results in the release of ATP. The released ATP binds to the endothelial cell purinergic receptor and leads to arteriolar smooth muscle cell relaxation, higher blood flow and more oxygen delivery ³⁹. High SO_2 represents a greater magnitude of oxyhemoglobin to deoxyhemoglobin in the vessels, which would result in less binding between ATP and the receptors on the endothelial cells. Therefore there might be decreased blood flow and lower oxygen delivery to the tissue.

In summary, the data from this study suggest that there was no association between retinal blood flow and retinal blood oxygen saturation. Given that there is no correlation between SO_2 and RBF, the results of this pilot work suggest that there is a need to measure both SO_2 and RBF in order to calculate retinal oxygen utilization.

The combination of retinal oxygen saturation and retinal blood flow can be used to extract more information about retinal metabolism since:

$$\text{Retinal O}_2 \text{ Delivery} = [1.39 \times \text{Hb} \times \text{SaO}_2 + (0.003 \times \text{PaO}_2)] \times \text{blood flow}$$

Theoretically, each gram of Hemoglobin binds 1.39 ml of oxygen. The oxygen saturation curve represents a PaO_2 (arterial oxygen tension) as a function of SaO_2 (Arterial SO_2). The haemoglobin concentration is calculated by production, destruction and loss. 0.003 stands for the oxygen solubility coefficient in human plasma.

The retinal oxygen saturation without simultaneous retinal blood flow measurement represents the circulation delivery for the cell ⁴⁰. There are some methods that simultaneously measure retinal blood SO_2 and retinal blood flow measurements, which are restricted to animals.

6 Multiplex Analysis of Inflammatory and Angiogenic Biomarkers in Aqueous Humor in Mild-to-Moderate Non-Proliferative Diabetic Retinopathy

Faryan Tayyari, Lee-Anne Khuu, Jeremy Sivak, Michael Brent, Shaun Singer, John G Flanagan, Christopher Hudson

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Table detailing role of each author in this publication (x denotes significant contribution)

6.1 Introduction

Diabetes triggers a number of retinal metabolic and physiologic abnormalities that can contribute to diabetic retinopathy (DR). Many of these retinal abnormalities in diabetes are consistent with inflammation. DR is illustrated by damage to the retinal microvascular causing vascular leakage and ischemia-induced retinal neovascularization as a result of the classic pathways: the polyol pathway, protein kinase C (PKC) activation, increased advanced glycation end-product (AGE), and the superoxide pathway ¹. Each of these pathways can promote expression of angiogenic biomarkers such as vascular endothelial growth factor (VEGF), Leptin, Angiopoietin 2 (Ang 2), Epidermal growth factor (EGF), Transforming growth factor beta (TGF- β), Heparin-binding EGF-like growth factor (HB-EGF), Hepatocyte growth factor/scatter factor (HGF/SF), and Interleukin-8 (IL-8) ^{2,3}.

VEGF is a well-known persuasive angiogenic factor that stimulates retinal neovascularization, leading to tractional retinal detachment and vitreous hemorrhage, which are important because they represent the most severe causes of visual loss in patients with PDR ^{4,5}. There is a wealth of evidence that during retinal and choroidal neovascularization and the formation of diabetic macular edema, hypoxia intensely stimulates VEGF expression by stabilization of HIF-1 (hypoxia-inducible factor-1) transcription ⁶⁻⁸. Previous studies also specify the important role of VEGF in the breakdown of the blood-retinal barrier in DR. Vascular hyper-permeability secondary to tight junction impairment between vascular endothelial cells and neovascularization are obvious of vascular endothelial cell dysfunction induced by diabetes. VEGF plays an important role to mediate these early and late vascular alterations ⁹⁻¹¹. VEGF has also been found markedly increased in DR and was significantly correlated with DR severity ⁵. Angiogenesis is

caused by disruption between the production of angiogenic activator such as VEGF and angiogenic inhibitors such as angiostatin and pigment epithelium-derived factor (PEDF)^{12,13}. The amplified production of angiogenic activators and reduced production of angiogenic inhibitors caused by local hypoxia, disturbs the balance between the desirable and undesirable regulators of angiogenesis. Subsequently, over proliferation of capillary endothelial cells occurs which leads to neovascularisation¹³.

Ang 2 is one of the important growth factors of hypoxia-induced microvascular changes and is significantly involved in the initiation of retinal neovascularization. Angiopoietin molecules bind to the receptor tyrosine kinase, Tie2. The Angiopoietin molecules are growth factors that regulate the development of physiological angiogenesis and pathological neovascularization^{14,15}. Another study revealed that vitreal Ang 2 increased in PDR and they have suggested a relationship of Ang 2 and VEGF with angiogenic activity in PDR¹⁶. Another study proposed that Ang 2 is expressed by hypoxia and VEGF in bovine aortic endothelial cells¹⁷. Others showed that Ang 2 is playing a role in pericyte recruitment and pre-retinal vessel formation under physiological and pathological disorders¹⁸. There is also evidence that Ang 2 is involved in pericyte apoptosis by $\alpha_3\beta_1$ integrin signaling in DR¹⁹. Ang 2 has been shown to be elevated in the vitreous of patients with clinically significant macular edema (CSME)²⁰ suggesting a possible role in the alteration of the blood-retinal barrier. Another study suggested that increased Ang 2 changes result in dysfunction of VE-cadherin causing in greater vascular permeability. Consequently, Ang 2 might be involved in elevated vaso-permeability in DR²¹.

Leptin, an adipocyte-derived hormone, is an energy metabolism regulator and is associated with diabetes mellitus through its metabolic actions. Leptin stimulates endothelial

proliferation and angiogenesis²². Results describing the association between serum leptin and diabetic micro-angiopathies are variable. Sari and co-workers²³ did not find any difference in the leptin levels in serum between patients with and without diabetic nephropathy, retinopathy and neuropathy. Furthermore, Asakawa and co-workers²⁴ reported no relationship in the leptin level and micro-angiopathies in Japanese patients of type 2 diabetes. However, Freuhwald-Schultes and co-workers²⁵ reported high serum leptin amounts in type 2 diabetes patients with early stages of renal disease, (i.e., macroalbuminuric nephropathy and microalbuminuric), while serum leptin levels did not significantly show any association with diabetic neuropathy. Jung and co-workers²⁶ did not find any relationship between leptin concentrations and diabetic retinopathy.

Complicated events of interactions between retinal cells with growth factors and cytokines, extracellular matrix proteins and metallo-proteinases trigger the development of proliferative retinal diseases²⁷⁻²⁹. HGF is one of these growth factors that has been studied. Its receptor is the c-met, a transmembrane tyrosine kinase³⁰. An elevated HGF level in patients with type 1 diabetes mellitus with PDR and the relationship between progression of DR and the concentration of HGF was suggested as a role of HGF in the pathogenesis of PDR³¹.

HGF is an endothelium specific growth factor with more potent mitogenic activity than that of basic fibroblast growth factor (b-FGF), VEGF, interleukin 6 (IL-6), and interleukin 1 (IL-1)^{32,33}. The HGF concentration in the vitreous of patients with PDR has been shown to be higher when compared to non-diabetic patients³⁴. In addition, it has been suggested that HGF might affect the progression of DR³⁵.

Inflammatory leukocyte recruitment constitutes one of the key pathological stages in DR. Diabetes has been shown to up-regulate several pro-inflammatory mediators in the retina, such as ICAM-1, and this localized inflammatory process is considered to be part of the development of DR³⁶⁻³⁹. Retinal vascular ICAM-1 expression is elevated by VEGF. This may stimulate retinal leukostasis in diabetic eyes⁴⁰. Increased expression of ICAM-1 has been demonstrated to show an association with the severity of retinopathy⁴¹. There is also a direct relationship between ICAM-1 and VEGF-induced vascular permeability⁴², as well as evidence that pigment epithelium-derived factor (PEDF) decreases the hypoxia-induced ICAM-1 expression⁴³. Nowak and co-workers also reported high concentration of ICAM-1 in DR⁴⁴.

Anomalous interactions between the endothelial cell and pericytes might be implicated in diabetic microangiopathy⁴⁵. There are some pathways of interaction between the endothelial cells and pericytes. One of these communication paths is TGF signaling, which is important for pericyte differentiation^{46,47}. Pericytes make the vasculature stable; modulate (rather than majorly control) blood flow at the local tissue level and control endothelial proliferation. One of the hallmarks of early changes in diabetic retinopathy is pericyte loss⁴⁸. Another structural hallmark is thickening of the capillary basement membrane. These changes along with others seen in diabetes such as increased blood viscosity might result in occlusive angiopathy and a consequent tissue hypoxia. Growth factors such as TGF- β and VEGF might be involved in basement membrane thickening⁴⁹. It has been proposed that increase of serum proteins due to microvascular disturbances and hypoxia might be a cause for vitreous alterations of Insulin-like growth factor I (IGF-I) and of active TGF- β . These changes appear to take place late in the sequence of events

resulting in PDR and are not restricted to diabetes; they were also detected in other retinal hypoxic diseases⁵⁰. TGF- β 1 has been shown to participate in the pathogenesis of DR^{51,52}. A role for TGF- β 1 has been shown in the microvascular complications of type 1 diabetes^{53,54}. In addition, hyperglycemia induces protein kinase C (PKC) activation, which results in increased microvascular protein buildup by up-regulating TGF- β 1, fibronectin and type IV collagen^{55,56}. Serum protein influx due to hypoxia is likely the reason for vitreous alterations of active TGF- β ⁵⁰.

It has been indicated that TGF- β appears to decrease the tightness of the inner blood retinal barrier (iBRB)⁵⁷. Intravitreal levels of Angiopoietin-2 have been shown to be significantly associated with VEGF and TGF- β 1 concentration in DR patients undergoing vitrectomy. This suggests that these factors could be involved in promoting retinal angiogenesis synergistically⁵¹.

Numerous studies have demonstrated that markers of angiogenesis and inflammation associate with the incidence of diabetes. In this study, VEGF, Leptin, Ang 2, HGF/SF, EGF, IL-8, ICAM-1, HB-EGF and TGF- β have been investigated. One of the initial events in diabetic retinal inflammation is the adhesion of leukocytes to the microvascular endothelium. Increased leukocyte adhesion leads to loss of endothelial cells and breakdown of the blood-retinal barrier^{37,58}. The leukocyte adhesion in the diabetic retina is assisted by the increased upregulation of adhesion molecules such as ICAM-1³⁶. VEGF has been the most extensively investigated biomarker in relation to the alteration of the blood retinal barrier. VEGF also triggers elevated leukostasis in the retinal microvessels⁵. A cellular experiment study showed that Ang 2 stimulates monocytes adhesion by sensitizing endothelial cells for TNF- α and modulates the TNF- α -induced up-regulation of ICAM-1⁵⁹.

HGF is an endothelium specific growth factor with more potent mitogenic activity than that of basic fibroblast growth factor (b-FGF), VEGF, interleukin 6 (IL-6), and interleukin 1 (IL-1) ^{32,33}.

The aim of this study was to determine disturbance of inflammatory and angiogenic vascular biomarkers in the aqueous humor in patients with relatively early DR and in healthy age-matched controls. This will help to better understand the pathophysiology of DR.

6.2 Methods

6.2.1 Sample

This study received approval by the University of Waterloo Office of Research Ethics and the Research Ethics Board of the University Health Network, University of Toronto, Canada. Informed consent was obtained from each subject after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki. The sample consisted of 33 volunteers in two groups of subjects with mild-to-moderate NPDR (n=15; mean age 69.11; SD 6.58 years) and aged matched healthy controls (n=18; mean age 67.86; SD 6.17 years) who were listed for routine cataract extraction with pseudo-intraocular lens implantation. The eye of each subject that underwent cataract surgery was selected for the study. All subjects had a corrected visual acuity of 20/40, or better, post-surgery. Subjects were excluded for a family history of any other ocular disease, apart from diabetic retinopathy. None of the patients with NPDR had received treatment of any kind for their retinopathic changes or had any evidence of diabetic macular edema or any other sight-threatening DR characteristic.

6.2.2 Assessment of Cytokines in Aqueous Humor (AH)

Aqueous humor is essential for the metabolic homeostasis, physiologic and nutritional maintenance in the anterior chamber. Components of the aqueous humor can likely reflect the state of intraocular tissues and represent any biochemical alteration due to ocular diseases. Aqueous humor samples can provide information with minimal risk as an outpatient process for the evaluation of abnormal cytokines or inflammatory biomarkers in various ocular diseases with or without a systemic association³⁶.

Undiluted samples of AH were obtained (by Drs. Singer and Brent) from patients proceeding to cataract surgery. Briefly, a paracentesis was made in the peripheral cornea next to the limbus at the onset of surgery. A Sautter hydrodissection cannula (27G, 0.4x22mm; Geuder, Heidelberg, Germany) was introduced into paracentesis tract to deliver suction by pulling up the plunger. The tip of the cannula was placed in the mid anterior chamber and 20-150 μ l of AH was slowly aspirated. The AH was collected into the attached syringe and transferred consequently into an eppendorf tube of 1.5ml where it was assessed.

6.2.3 Measurement of Cytokines Using Multiplex Analysis

Multiplexed bead-based immunoassay was utilized for the simultaneous measurement of 10 cytokines (VEGF-A, VEGF-C, VEGF-D, Leptin, Ang 2, HGF/SF, IL-8, EGF, TGF- β 1 and TGF- β 1) within each AH sample (Bio-Rad Laboratories, Inc., Hercules, CA). The multiplex technology is based on internally color-coded microspheres coupled to analyte-specific antibodies, permitting the simultaneous measurement of multiple analytes for each sample. Wherever the measured values dropped below the threshold of detection, we set the recorded concentration at the limit of detection. Cytokines for which the majority of concentrations were at or below the limit of detection were considered non-measurable.

6.2.4 Statistical Analysis

AH cytokine levels are reported as mean (pg/mL) \pm standard deviation. Statistical significance was considered when $p \leq 0.05$. Differences in the distribution of cytokines between groups were analyzed by the Student's t-test.

6.3 Results

Using a multiplexed magnetic bead immunoassay (Luminex), the levels of 15 cytokines within AH samples of NPDR patients and their aged-matched controls were measured. The complete dataset of cytokine expression is summarized in Table 6.1.

Of the 12 cytokines and chemokines analyzed in the vitreous of controls and NPDR patients, the levels of 2 cytokine (VEGF-D and ICAM-1) were considered unmeasurable, as the majority of AH sample concentrations were at or below the limit of detection. Of the 12 cytokines with measurable levels, there were 6 cytokines with a mean AH concentration significantly higher in NPDR patients when compared to controls (Table 6.1). Ang 2, IL-8, HGF and HGF were significantly higher in NPDR patients than in control patients ($p=0.005$, $p=0.034$, $p=0.017$, $p=0.018$, respectively) but EGF was significantly lower ($p=0.025$) in NPDR patients than in control patients. No significant difference was found in VEGF-A, VEGF-C, Leptin, and HB-EGF levels between the two groups ($p=0.342$, $p=0.757$, $p=0.151$, $p=0.957$, respectively). The magnitudes of the difference in range between the two groups for each cytokine are reported in Table 6.1.

The aqueous humor concentrations of ICAM-1 were reported to be either out of range or below the range for most of the subjects in both groups suggesting that AH was much diluted to pick up the values.

The aqueous humor concentration of ICAM-1 for the subjects that were not out of range was: mean NPDR group 474.92 ± 470.02 ranged from 4.90 to 944.94 with only two NPDR patients showing ICAM-1 concentrations. However, the result indicated a significant increase in the aqueous humor levels of both TGF- β 1 and TGF- β 2.

Cytokine	Control	NPDR	p-value
Angiopoietins-2	12.46±5.37	21.84±11.58	0.005
VEGF-A	82.02±47.38	127.33±192.98	0.342
VEGF-C	4.87±2.31	5.14±2.57	0.757
VEGF-D	OOOR	OOOR	–
HGF/SF	121.68±53.37	250±201.57	0.018
IL-8	1.14±0.51	2.46±2.40	0.034
EGF	0.24±0.096*	0.17±0.074*	0.025
Leptin	34.82±27.33	73.00±96.00	0.150
TGF-β1	5.94±2.43	18.46±13.74	0.004
TGF-β2	2336.21±1201.99	4689.38±1426.36	<0.001
ICAM-1	OOOR	470.92±470.02	–
Hb-EGF	0.80±0.24	0.81±0.49	0.956

Table 6.1 AH levels of Cytokines in subjects. Cytokine levels in pg/mL ± SD (range in cytokine values). OOR represents Out of range (in this case was lower in the range). *The values reported were below the range but detectable.

6.4 Discussion

VEGF is a well-known persuasive angiogenic factor that stimulates retinal neovascularization, leading to tractional retinal detachment and vitreous hemorrhage, which are important because they represent the most severe causes of visual loss in patients with PDR^{4,5}. Previous studies have indicated that VEGF concentrations are higher in the aqueous humor and the vitreous of diabetic patients and that the severity of DR is intensely associated to the VEGF concentrations. To add up, VEGF plays an important role in both NPDR and PDR^{5,60-64}. However, our study found no significant difference in VEGF concentrations between NPDR patients and age-matched controls. This finding is consistent with other studies. Selim and co-workers did not find any difference between controls and diabetic patients without DR; however, they found a difference between controls and diabetic patients with DR⁶⁵. Shinoda and co-workers also did not find a significant difference in the AH levels of VEGF⁶³. Basically, this might be explained by the wide variation in severity of mild-to-moderate NPDR in our study sample. It has been reported that TGF- β controls release of VEGF by glioma cells in an activin-receptor-like-5-dependent (ALK-5-dependent) approach involving SMAD2, SMAD3, and SMAD1/5/8 signaling⁶⁶. It has also been reported that VEGF gene expression at a transcriptional level can be synergized by both TGF- β and hypoxia signaling pathways⁶⁷. It has been shown that TGF- β 1 down-regulated VEGF-D expression in human lung fibroblast⁶⁸. Although the reason for this discrepancy is uncertain, one possibility is that the methods for measurement and the tissues of interest are different. It has been shown that hyperglycemia does not have any impact on hypoxia-inducible factor (HIF-1 α)⁶⁹. It is thought that HIF-1 α activation plays a role in the up-regulation of angiogenic factors causing neovascularization

Hypoxia, VEGF⁷¹ and retinal ischaemia¹⁷ induce Ang 2. VEGF has been shown to initiate neovascularization and to increase the effect of the angiopoietins⁷². The present study is the first report of the concentration of angiopoietins in the AH samples of mild-to-moderate NPDR. However, there are studies in vitreous²⁰ and serum samples⁷³ that have shown significant increase in the level of Ang 2 of NPDR patients. Ang 2 promotes pericyte loss under high glucose conditions in DR¹⁹. It is hypothesized that Ang 2 stimulates neovascularisation and induces vascular permeability²⁰. Hypoxia-inducution Ang 2 might explain the higher concentration of Ang 2 within the NPDR group.

Leptin is a circulating hormone, which is secreted mostly from adipose tissues. It has been reported that leptin is an angiogenic factor and its level in vitreous have been shown to be higher in patients with proliferative diabetic retinopathy³. Another study implied the possibility of a role for leptin in angiogenesis through VEGF induction as well as PEDF suppression in pericytes. Therefore, leptin could be a factor in the development and progression of DR, especially in obese insulin-resistant patients⁷⁴. Nonetheless, this study did not find a significant difference in leptin levels between mild-to-moderate NPDR and controls. This might be explained by the VEGF levels, which were not significantly higher in NPDR patients either.

In the present study, aqueous HGF levels have been shown to increase in NPDR group when compared to controls. HGF has been crucially indicated in the development of proliferative retinal diseases^{75,76}. Katsura and co-workers found increased HGF levels in the vitreous fluid of patients with proliferative diabetic retinopathy³⁴. HGF levels were significantly increased in the NPDR group when compared to control group⁷⁷. HGF as well

as VEGF have been introduced as the key factors playing a role in retinal vascular permeability^{78,79}. It has been reported that HGF can prevent hypoxia-induced apoptosis by inhibiting caspase-8 through: 1) p38 mitogen-activated protein kinase (MAPK) phosphorylation and 2) blocking Bax translocation⁸⁰. HGF is a multi-potential cytokine that can promote bioactivities, including junctional breakdown, cell survival, migration, cell scattering, and invasive behavior. It has been involved in various events in tissue development/maintenance, homeostasis, and wound healing^{81,82}. Increased HGF might be explained as either body's feedback to fight the insult produced by hyperglycemia⁸³.

It has been reported that IL-1 β might up-regulate IL-8 in Müller cells through the p38 mitogen-activated protein kinases (p38 MAPK) and ERK1/2 pathways⁸⁴ (ERK1/2 is a significant member of mitogen-activated protein kinases that control a broad range of cellular activities and physiological processes). Aqueous concentrations of IL-8 have been shown to be higher in the NPDR patients⁸⁵, which is consistent with our result.

EGF levels were significantly lower in NPDR patients. The lower EGF concentrations were lower than the range which might be explained by diluted environment of the AH samples. Any change in the signalling pathway of EGF might cause apoptosis and hence cell death. It has been also shown that EGF signaling inhibition protects the retina against insulin-induced vascular leakage in diabetes⁸⁶. The EGF decrease might also be explained as body's immune system protective response in diabetes.

A possible explanation for HB-EGF not being upregulated might be that HB-EGF might be translocated to nuclear envelope⁸⁷.

TGF- β can induce angiogenesis when bind to ALK1, whereas ALK5 develops reverse outcomes on endothelial cell behavior. However, it has been reported that endoglin

(transmembrane accessory receptor for TGF- β) promotes ALK1 signaling⁸⁸. Endoglin was not upregulated in this study (p=0.261), which might suggest an anti-angiogenic role of TGF- β during mild-to-moderate NPDR. TGF- β can also bind to EGFR. An immunohistochemical study on human colon adenocarcinoma showed an inverse correlation between TGF- β -EGFR/ErbB signaling⁸⁹, which might indicate anti-angiogenic behaviour from TGF- β when adhered to EGFR.

Altogether, our findings of increased concentrations of Ang 2, HGF, TGF- β and IL-8 or decreased levels of EGF in NPDR patients suggest an important role of these molecules in the pathophysiology of DR before the clinical advent of neovascularization and DME.

Biomarker of Interest	Reason for Biomarker	Evidence / Motivation	Reference
VEGF	Permeability / Neovascularization/ Hypoxia/ Leukostasis/ HIF-1 has crucial role in VEGF production / basement membrane thickening	Human / Animal	4-13 49,90
ICAM-1	Leukostasis / Increased during hypoxia	Human / Animal	36
Leptin	Angiogenesis stimulator	Human / Animal	74
Ang 2	Neovascularization / Pericyte loss / Vascular permeability / monocytes adhesion	Human / Animal	19,20,59,71-73
HGF	DR progression / vascular permeability / anti- and pro-apoptosis	Human / Animal	75,78-82
HB-EGF	Possible transactivation of HGF/c-met / Microcirculatory blood flow regulator	Human / Animal	91,100
EGF	Insulin-induced vascular leakage in DR / Potent mitogen role	Human / Animal	92,99
IL-8	Neutrophil transmigration / neovascular growth / retinal glial cells reactively secrete IL-8 in response to hypoxia / mediate angiogenesis <i>via</i> both VEGF dependent and independent mechanisms	Human / Animal	93-97
TGF-β	Inhibition of proliferation of müller glial cells / basement membrane thickening / decrease the tightness of iBRB	Human / Animal	49,57,98

7 Retinal Blood Oxygen Saturation and Aqueous Humor Biomarkers in Early Diabetic Retinopathy

Faryan Tayyari, Lee-Anne Khuu, Jeremy Sivak, Shaun Singer, Michael Brent, John Flanagan, Christopher Hudson

	Design	Recruitment	Acquisition Of Data	Analysis	Writing / Publication
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L. Khuu	x	x	x	x	
J. Sivak	x			x	
S. Singer		x	x		
M. Brent		x	x		
J. Flanagan	x				
C. Hudson	x			x	x

Table detailing role of each author in this publication (x denotes significant contribution)

7.1 Introduction

Diabetic retinopathy (DR) remains the most common microvascular complication of diabetes and despite significant and advancing research efforts the precise pathology of DR is still not fully understood. One of the suggested pathologies of DR is a direct consequence of hypoxia initiated by hyperglycemia¹⁻³.

The retinal high oxygen demand is thought to contribute to the particular vulnerability of the retina to vascular disease. DR appears to be allied closely to disturbances in the vasculature. Hence, a variety of studies have considered the inner retinal blood flow as a potential biomarker of DR. However, the results of inner retinal blood flow disturbance in DR have been contradictory⁴⁻¹³, although virtually all indicate some aspect of disturbance.

The study of metabolic disturbance as a functional diagnosis is an encouraging conduit for improved understanding the pathophysiology behind the disease. One of the practical factors of the microcirculation that reflects metabolic activity is the blood oxygen saturation. There is a need to study the oxygen supply to the retinal vasculature in diabetes as an improved way to understand the metabolic disturbances associated with this disease.

The early phase of the disease, clinically labeled non-proliferative diabetic retinopathy (NPDR), is characterized in part by retinal swelling consequent to the leakage and increase of extracellular fluid and proteins in the macula. Exudation develops from structural transformations in the retinal vascular endothelium that causes the breakdown of the blood-retina barrier (BRB) and an escalation in vascular permeability¹⁴⁻¹⁶.

It is thought that change in blood circulation may lead to functional damage and broad retinal tissue impairment and disturbance during diseases such as DR¹⁷.

Aqueous humor (AH) is essential for the metabolic homeostasis, physiologic and

nutritional maintenance in the anterior chamber. Components of the AH can likely reflect the state of intraocular tissues and can represent any biochemical alteration due to ocular disease. AH samples can provide information with minimal risk as an outpatient process for the evaluation of abnormal cytokines or inflammatory biomarkers in various ocular diseases with or without a systemic association.¹⁸

Vascular endothelial growth factor (VEGF) is a key regulator of vascular growth in angiogenesis and vasculogenesis¹⁹ and hypoxia is the crucial regulator of VEGF-stimulated neovascularization²⁰. This established persuasive angiogenic factor also plays a role in blood-retina-barrier (BRB) permeability in DR. Vascular hyper-permeability secondary to tight junction impairment between vascular endothelial cells and also neovascularization are bold symbols of vascular endothelial cell dysfunction induced by diabetes. VEGF plays an important role to mediate these early and later vascular alterations²¹⁻²³. Previously, VEGF has been reported to be significantly increased in diabetes and a correlation between the levels of VEGF and DR severity has been shown²⁴. Angiogenesis is caused by disruption between the production of angiogenic activator such as VEGF and angiogenic inhibitors such as angiostatin and pigment epithelium-derived factor (PEDF)^{25,26}. The amplified production of angiogenic activators and reduced production of angiogenic inhibitors caused by local hypoxia, disturbs the balance between the desirable and undesirable regulators of angiogenesis. Subsequently, over proliferation of capillary endothelial cells occurs which leads to neovascularisation²⁶.

Transforming growth factor-beta (TGF- β) is another angiogenic factor that participates in the pathogenesis DR²⁷⁻²⁸. The role of TGF- β 1 has been shown in microvascular complications with type 1 diabetes^{29,30}. In addition, hyperglycemia induces PKC activation,

which results in increased microvascular protein by up-regulating TGF- β 1, fibronectin and type IV collagen^{31,32}. Serum proteins influx due to hypoxia and this probably explains the vitreous alterations of active TGF- β ³². Pfeiffer and co-workers reported that TGF- β 2 was reduced 30% and 70% in PDR and non-diabetic retinal ischemia patients, respectively³³. TGF- β , especially TGF- β 2, is anti-angiogenic and is an anti-inflammatory cytokine in the eye³⁴.

The angiopoietins are growth factors that regulate the development of physiological angiogenesis and pathological neovascularization^{35,36}. Another study suggested a relationship between Ang 2 and VEGF with angiogenic activity in PDR³⁷. Others implied that Ang 2 is expressed by hypoxia and VEGF in bovine aortic endothelial cells³⁸.

Leptin, an adipocyte-derived hormone, is an energy metabolism regulator and is associated to diabetes mellitus through its metabolic actions. Leptin stimulates endothelial proliferation and angiogenesis³⁹. Results describing the association between serum leptin and diabetic micro-angiopathies are variable. Sari and co-workers⁴⁰ did not find any difference in the leptin levels in serum between patients with and without diabetic nephropathy, retinopathy and neuropathy. Furthermore, Asakawa and co-workers⁴¹ reported no relationship in leptin levels and micro-angiopathies in patients with type 2 diabetes of Japanese descent. However, Freuhwald-Schultes and co-workers⁴² reported high serum leptin amounts in type 2 diabetic patients and early stages of renal disease, (i.e., micro-albuminuric and macro-albuminuric nephropathy), while serum leptin levels did not significantly show any association with diabetic neuropathy. Jung and co-workers⁴³ did not find any relationship between leptin concentrations and diabetic retinopathy.

A complicated series of interactions between retinal cells with growth factors and cytokines, extracellular matrix proteins and metallo-proteinases trigger the development of proliferative retinal diseases⁴⁴⁻⁴⁶. HGF is one of these growth factors that has been studied. Its receptor is the c-met, a transmembrane tyrosine kinase⁴⁷. An elevated HGF level in patients with type 1 diabetes mellitus with PDR and the relationship between progression of DR and the concentration of HGF was suggested as a role of HGF in the pathogenesis of PDR⁴⁸.

It has been reported that HGF is an endothelium specific growth factor with more potent mitogenic activity than that of basic fibroblast growth factor (b-FGF), VEGF, interleukin 6 (IL-6), and interleukin 1 (IL-1)^{49,50}. Furthermore, the HGF concentration in the vitreous of patients with PDR has been shown to be higher when compared to non-diabetic subjects⁵¹. In addition, it has been suggested that HGF might affect the progression of DR⁵².

It has been stated that the insulin-induced vascular leakage can be prevented by EGF inhibition⁵³. It has been reported that AGE could trigger EGF receptor (EGFR)^{54,55}. The EGFR can bind to multiple growth factors such as EGF, HB-EGF⁵⁶. ROS might block EGFR dephosphorylation⁵⁷.

The aim of this study was to evaluate retinal blood oxygen saturation levels and aqueous humor concentrations of inflammatory cytokines in diabetic patients with non-proliferative diabetic retinopathy (NPDR) and to compare them with those of control subjects. The non-invasive quantification of retinal oxygenation is feasible using spectral imaging techniques and will be compared to ocular angiogenesis biomarkers, which in turn may offer an alternative sensitive and predictive biomarker of DR severity.

Numerous studies have demonstrated that markers of angiogenesis and inflammation associate with the incidence of diabetes. It is critical to note that inflammation occurs before ophthalmoscopic signs of DR but assessment of inflammatory markers is invasive and not a routine procedure, therefore, it is important to find a technique, which is non-invasive to be able to detect DR before its morphological signs.

7.2 Methods

7.2.1 Sample

This study received approval by the University of Waterloo Office of Research Ethics and the Research Ethics Board of the University Health Network, University of Toronto, Canada. Informed consent was obtained from each subject after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki. The sample consisted of 32 volunteers in two groups of subjects with mild-moderate NPDR (n=14; mean age 69.11; SD 6.58 years) and aged matched healthy controls (n=17; mean age 69.70; SD 6.26 years). The eye of each subject that underwent cataract surgery was selected for the study. All subjects had a corrected visual acuity of 20/40 or better. Subjects were excluded for family history of any other ocular disease, apart from diabetic retinopathy. None of the patients with NPDR had received treatment of any kind for their retinopathic changes or had any evidence of diabetic macular edema or any other sight-threatening characteristic.

Group	Group Mean Age (SD)	Male to Female Ratio	Group Mean A1c (SD)	Duration of DR (SD)
Control	69.70 (6.26)	8 M : 9 F	5.5 (0.4)	-
NPDR	69.11 (6.58)	6 M : 8 F	7.5 (1.5)*	13.2 (8.3)*

Table 7.1 Group mean age, male to female ratio and A1c (A1c: glycosylated hemoglobin. M; male, F; Female. NPDR; Non-proliferative diabetic retinopathy) *(difference between control and NPDR group p<0.001)

7.2.2 Assessment of Retinal Blood Oxygen Saturation

Details of the SO₂ measurement and cube registration have been described elsewhere (Chapter 4). A prototype custom-built fundus camera (H-8.5 HR Camera, Optina, QC, Canada) is the foundation of the hyperspectral imaging system that incorporates a tunable laser source (TLS) as the light source. The TLS makes it possible to transfer wavelengths within a spectral range of 400-1000nm (visible to IR) and with a half peak bandwidth of 2nm. The TLS is built on Photon etc's Bragg grating filtering technology which permits rapid wavelength presentation from the steady and powerful super-continuum light source (Leukos-SM-30-OEM, Leukos Innovative Optical Systems, Limoges, France). The TLS can be electronically tuned with an operating ambient temperature of 10-30°C. An automatic spectral system is incorporated into the system to achieve exact and accurate (<1nm) wavelength choice. The sensitive 1.3MPixel (1392 x 1040 pixel) 14-bit CCD camera (Pixelfly USB, PCO AG, Kelheim, Germany) used in this system is for high definition imaging. The imaging system is operated by using PhySpec (Photon Etc, Montreal, QC, Canada), a software programme that manipulates the Bragg tunable filter (BTF) and CCD camera to allow aspects such as operator defined wavelength range and wavelength interval. A low power white light source (delivering about 100mW over 420nm to 2400nm) and the Bragg Tuneable Filter are used to exclude the use of conventional flash lamp, and therefore all images are acquired at low light levels, thereby minimizing any potential change of the metabolites and photopigment status in the eye. A diagonal field of view of approximately 37.4° is sustained by the opto-mechanics of the system⁵⁸.

7.2.3 Procedures

This was a cross-sectional study and all participants attended for two visits. The eye selected to undergo surgery was assigned to be the study eye. The first visit was at the time of cataract surgery to collect the aqueous humor. The second visit was to assess the magnitude of retinal oxygen saturation and retinal blood flow. The follow-up imaging visit was 4-6 weeks after the surgery to ensure that participants are not using NSAIDs and / or topical steroids.

Visit 1: Undiluted samples of AH were obtained from patients proceeding to cataract surgery. Concisely, a paracentesis was made in the peripheral cornea next to the limbus at the onset of surgery. A Sautter hydrodissection cannula (27G, 0.4x22mm; Geuder, Heidelberg, Germany) was introduced into the paracentesis tract to deliver suction by pulling up the plunger. The tip of the cannula was placed in the mid anterior chamber and 50-150 μ l of AH was slowly aspirated. The AH was collected into the attached syringe and transferred consequently into an eppendorf tube of 1.5ml.

Visit 2: refraction, logMAR visual acuity, Goldmann applanation tonometry and resting blood pressure were assessed prior to dilation of the study eye. The pupil of the study eye was dilated using Tropicamide 1% (Alcon, Mississauga, Canada) at the beginning of second visit to achieve an adequate view of the fundus for the image acquisition. A minimum of six oximetry measurements was acquired.

7.2.4 Measurement of Cytokines Using Multiplex Analysis

Multiplexed bead-based immunoassay was utilized for the simultaneous measurement of 5 cytokines (Angiopoietin 2, HGF/SF, IL-8, TGF- β 1 and TGF- β 2) within each AH sample (Bio-Rad Laboratories, Inc., Hercules, CA). The multiplex technology is based on

internally color-coded microspheres coupled to analyte-specific antibodies, permitting for the simultaneous measurement multiple analytes for each sample. Wherever the measured values dropped below the threshold of detection, we set the recorded concentration at the limit of detection. Cytokines for which the majority of concentrations were at or below the limit of detection were considered non-measurable.

7.2.5 Statistical Analysis

AH cytokine levels are reported as mean (pg/mL) \pm standard deviation. Statistical significance was considered when $p \leq 0.05$. Significant correlations between biomarkers and retinal oxygen saturation were analyzed using bivariate Pearson correlation and linear regression. All significant associations are inserted into a multiple regression model to assess whether the dependent variable (SO_2) could be predicted from biomarkers (independent variables). A Pearson correlation coefficient value of $r > 0.3$ and $p \leq 0.05$ was considered statistically significant.

7.3 Results

Group mean values for the SO₂ and biomarkers are found in Table 7.2.

Oxygen Saturation	Control	NPDR	T-test (p-value)
SO ₂ (Arteriole)	92.97±1.6	94.65±2.2	0.015
SO ₂ (Venule)	55.90±4.8	63.30±5.6	<0.001
AV Difference	37.05±4.95	30.57±4.76	0.001
Biomarkers	Control	NPDR	T-test (p-value)
VEGF-A	82.02±47.38	127.33±192.98	0.342
HGF	121.68±53.37	250±210.57	0.018
IL-8	1.14±0.51	2.46±2.40	0.034
Ang 2	12.46±5.37	21.85±11.16	0.005
Leptin	34.82±27.34	73.00±96.00	0.151
Hb-EGF	0.80±0.24	0.81±0.49	0.956
EGF	0.24±0.09	0.17±0.07	0.025
ICAM-1	OOOR	470.92±470.02	–
TGF-β1	5.94±2.43	18.46±13.74	0.004
TGF-β2	2336.21±1201.99	4689.38±1426.36	<0.001

Table 7.2 Group mean values for blood oxygen saturation and aqueous humor biomarkers. OOR represents out of range. Bold values are significant.

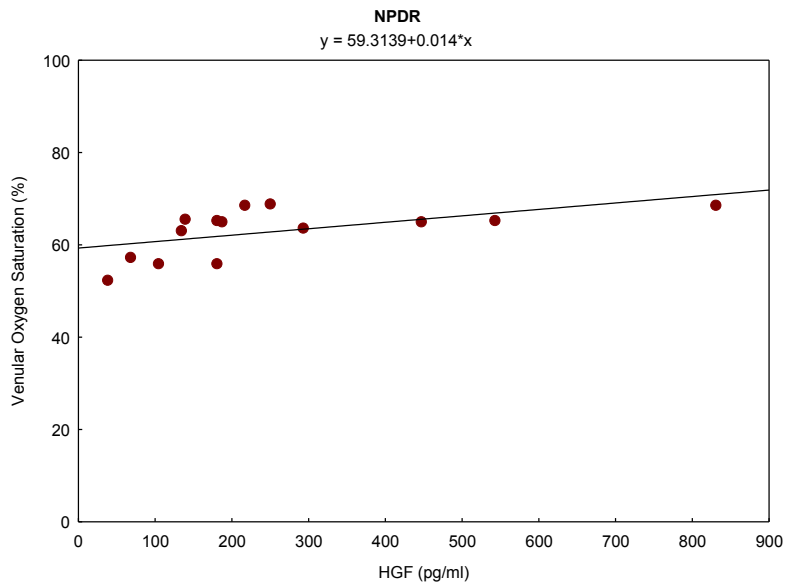
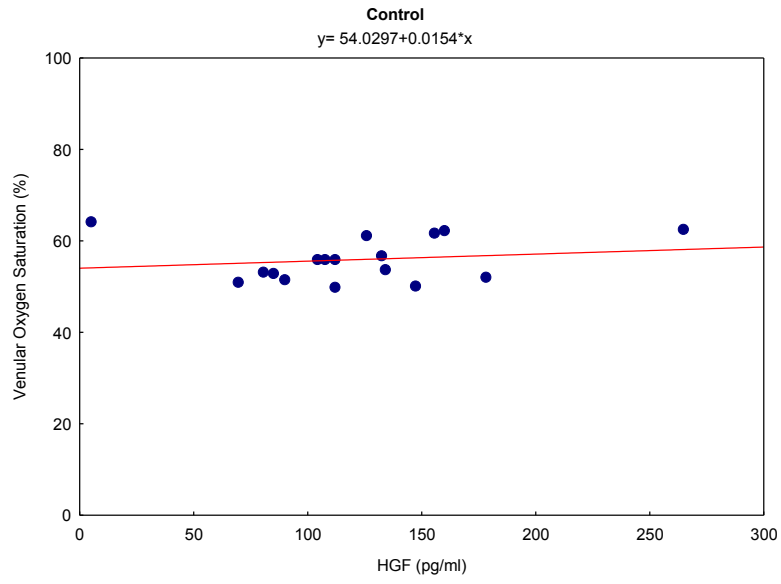
Retinal blood oxygen saturations as a function of biomarkers are illustrated in Figure 7.1.

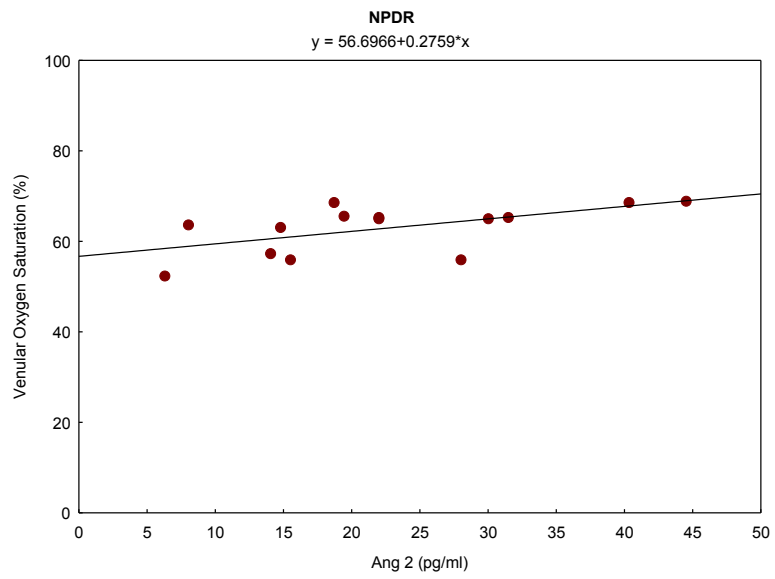
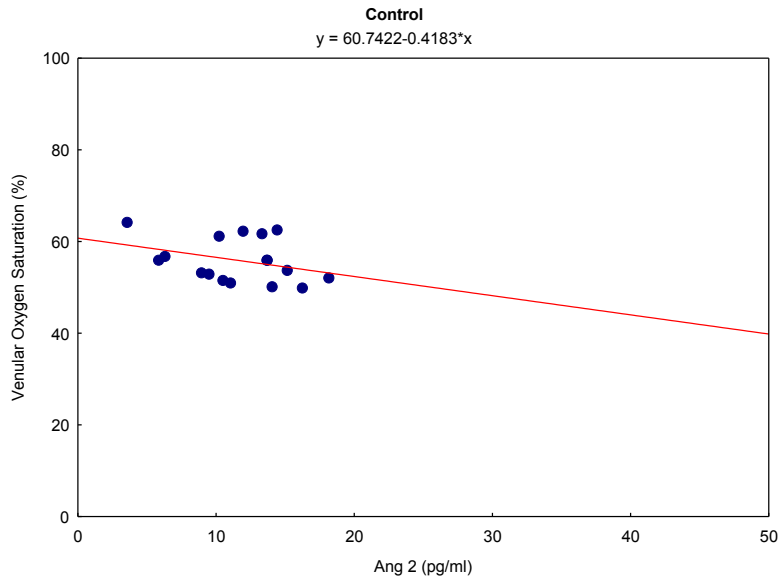
Please note that due to a limitation of AH amount some of the biomarkers are of limited predictive value (for example TGF- β1, TGF- β2 are only performed on 8 NPDR subjects).

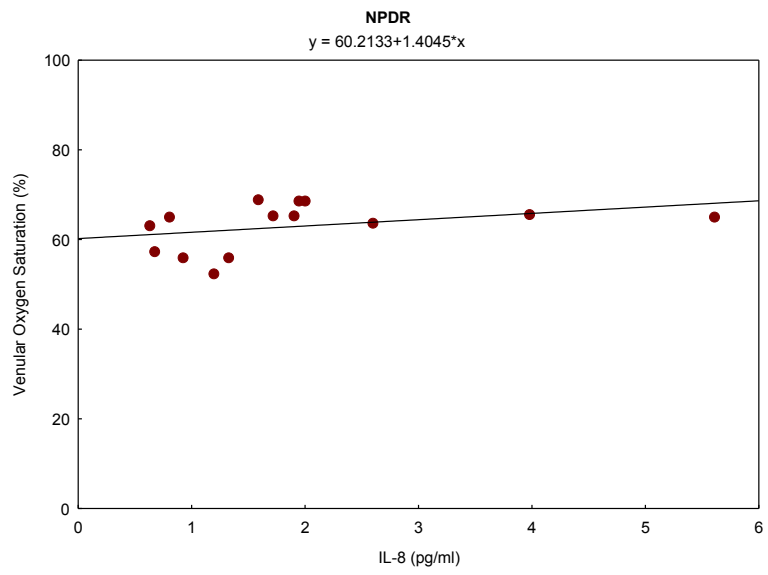
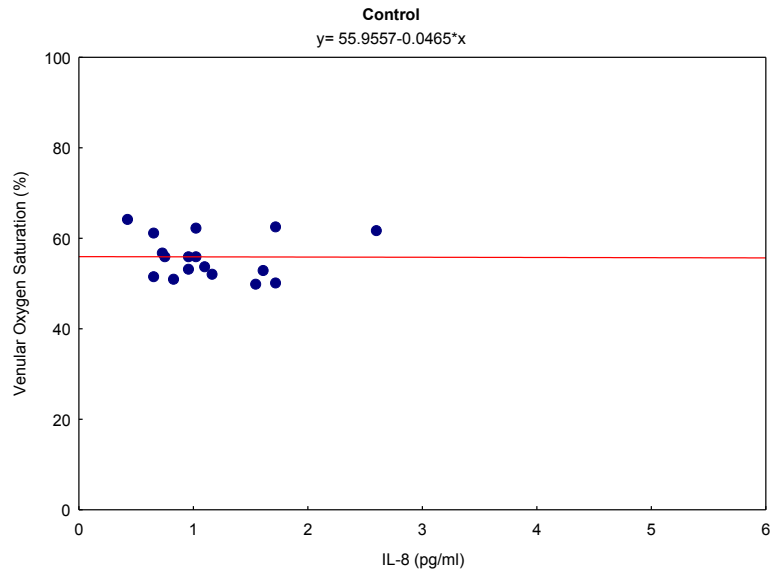
A significant correlation was taken at $r \geq \pm 0.30$ and $p < 0.05$. The correlation was investigated for biomarkers that are significantly different between the groups of controls and NPDR. The r scores are summarized in Table 2.

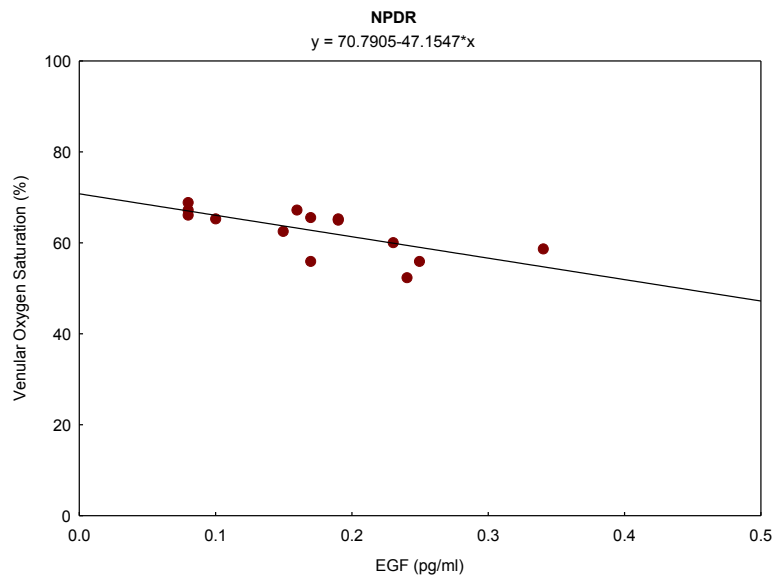
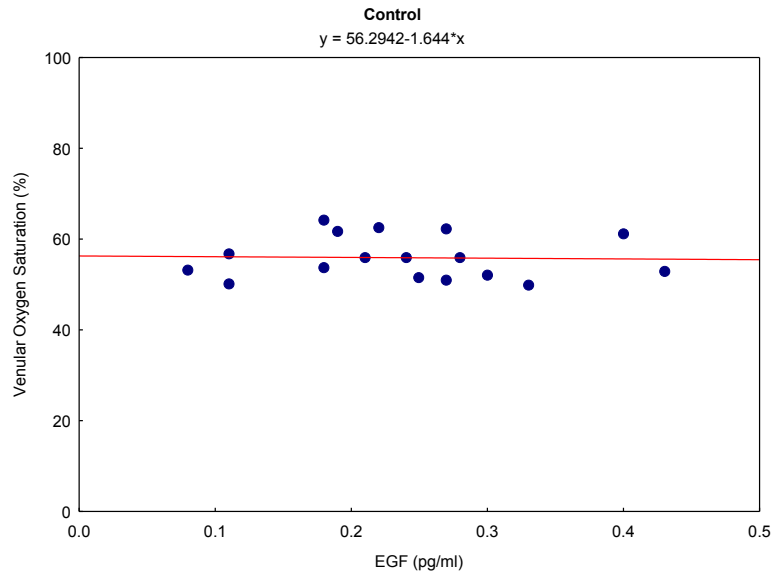
Controls	HGF	IL-8	Angiopietin 2	EGF	TGF-β1	TGF-β2
SO ₂ (Venule)	0.1774 (0.496)	0.4337 (0.082)	0.1507 (0.564)	-0.1665 (0.523)	-0.1952 (0.643)	0.0723 (0.806)
SO ₂ (Arteriole)	-0.0654 (0.804)	0.0284 (0.914)	-0.2166 (0.404)	0.1526 (0.559)	0.01150 (0.768)	0.1116 (0.704)
NPDR	HGF	IL-8	Angiopietin 2	EGF	TGF-β1	TGF-β2
SO ₂ (Venule)	0.5196 (0.050)	0.2337 (0.4213)	0.5477 (0.042)	-0.5520 (0.040)	0.5512 (0.156)	0.2029 (0.692)
SO ₂ (Arteriole)	0.2897 (0.315)	0.2589 (0.371)	0.4803 (0.082)	0.102 (0.717)	0.348 (0.398)	0.3419 (0.231)

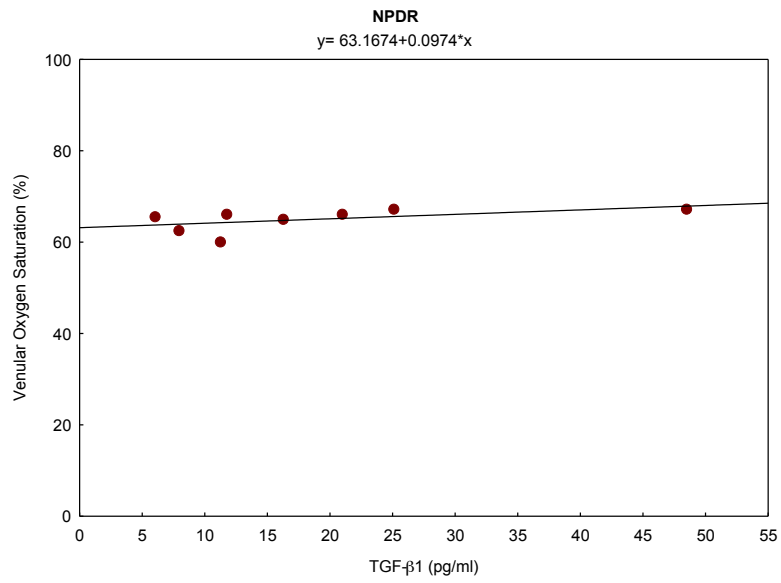
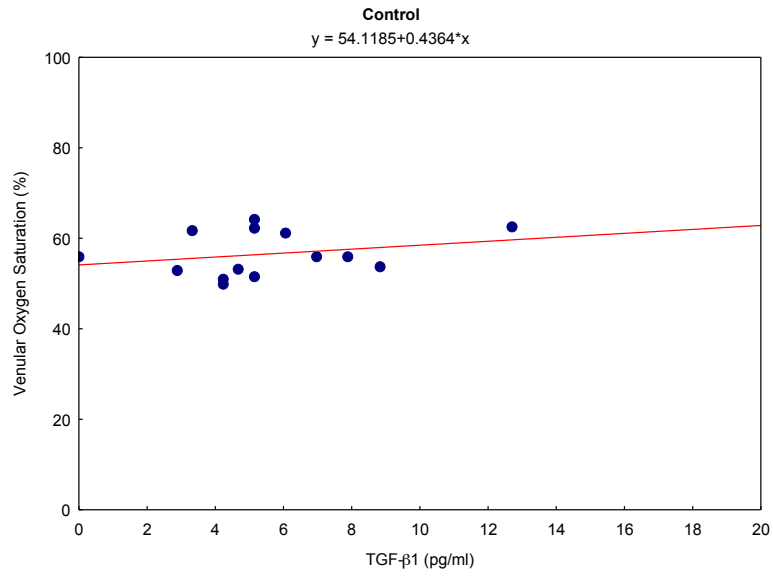
Table 7.3 Pearson correlation values r and statistical significance (p) between baseline levels of biomarkers. The p-values are reported in ().











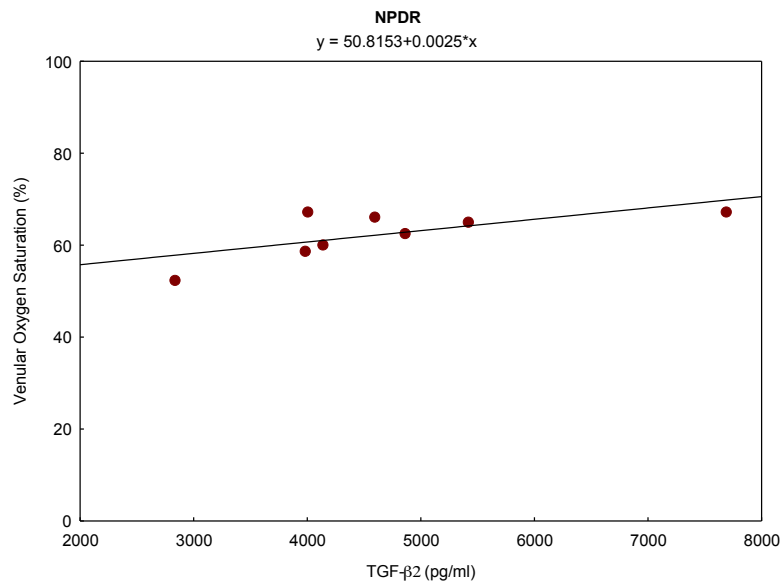
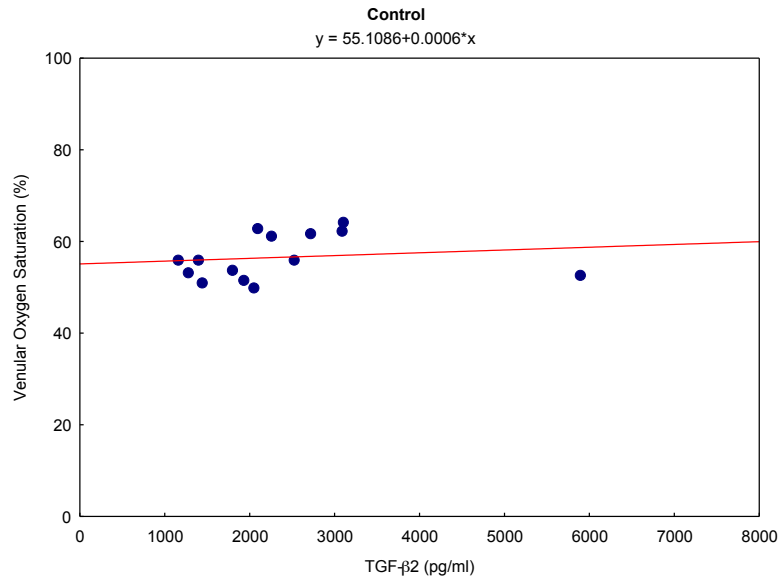


Figure 7.1 Retinal blood oxygen saturations as a function of biomarkers (NPDR; Non-proliferative diabetic retinopathy). Please note the difference in horizontal axis scaling for HGF. The number of subjects varies across assays.

Repeat measurement range values	Control	NPDR
Ang 2	5.58 to 7.72	22.00 to 24.18
EGF	0.13 to 0.24	0.08 to 0.15
Hb-EGF	0.13 to 0.76	0.29 to 0.49
IL-8	0.48 to 1.02	0.69 to 0.81
HGF	58.05 to 104.51	181.17 to 186.70
Leptin	*OOR to 67.57	67.57 to 110.79
TGF-β 1	NA	16.25 to 16.76
TGF-β 2	NA	5415.97 to 5559.45

Table 7.4 This table represents the repeat measurement values reported for one control and one NPDR (NPDR; Non-proliferative diabetic retinopathy). The sample volumes were low and this made it impossible to run subjects other than the two in the Table more than once. * One of the values was out of range (OOR) and the other one was reported in the standard range. NA; not applicable.

7.4 Discussion

Simultaneous retinal blood flow measurement and retinal hemoglobin oxygenation are required to assess absolute values (i.e. net oxygen delivery) of oxygen delivered to the retina^{59,60}.

The increasing number of people with diabetes in the world indicates that DR remains an on-going major sight-threatening consideration. The pathogenesis of DR is a common cause of visual loss worldwide and a number of hyperglycemia-related pathways have been associated with the onset and progressions of DR. Disturbances in the vasculature appear to play a vital role in DR. Therefore, many studies have investigated the inner blood flow. Due to the contradictory nature of previous work on retinal blood flow⁴⁻¹³; this study investigated retinal blood oxygen saturation as a function of biomarkers in AH in DR. This might aid a better understanding of the pathophysiology of DR.

To our knowledge, this is the first study that investigates the association between retinal blood oxygen saturation and aqueous humor angiogenic / inflammatory biomarkers in human NPDR. The result of the present study showed a correlation between SO_2 and HGF, Ang 2 and EGF in NPDR (Table 7.2).

Even though the Pearson r for TGF- β_2 as a function of SO_2 was 0.5512, the associated p -value (0.156) did not reach significance. This might be explained with the low number of subjects in that group; the AH amount was not sufficient for some subjects to allow valid assay.

This study showed a relationship between SO_2 and HGF. It has been found that local release of HGF has a regulatory effect on the glucose metabolism via Met activation⁶¹. Met is the surface receptor for HGF and is associated to the insulin receptor (INSR) tyrosine

kinase ⁶¹. It has been indicated that HGF can prevent hypoxia-induced apoptosis by inhibiting caspase-8 by: 1) p38 mitogen-activated protein kinase (MAPK) phosphorylation and 2) blocking Bax translocation ⁶². HGF is expressed following hypoxic conditions and the hypoxic tissues consume less oxygen and hence more oxygen remains in the blood. In other words, HGF might be expressed to reduce the apoptosis as an immune response (high volumes of HGF). This is thought to occur under hypoxic conditions. Cells are insulted by hypoxia and are then thought not to function fully and therefore do not use oxygen to its' usual capacity. This also could mean that since apoptosis is reduced, there is a chance that some of these cells that are malfunctioning, consume / "steal" oxygen at an exaggerated rate and this could result in the death of normal / less affected cells. The proapoptotic aspect of HGF appears to be associated to HIF-1 deficiency with loss of signaling functions ⁶³. These factors might play a role in the relationship between SO₂ and HGF concentrations in AH.

The other biomarker that has a relationship with the amount of retinal blood oxygen is Angiopoietin 2. Ang 2 promotes pericyte loss under high glucose conditions in DR ⁶⁴. The pericyte loss will result in less oxygen demand and hence increased oxygen in the vessels. There is also evidence that up-regulation of Ang 2 in the absence of VEGF results in endothelial cell death and vascular regression. This leads to hypoxic retinal conditions and in turn less oxygen demand by the tissue, which will result in higher SO₂ in the vessels.

Another biomarker of interest that showed an association with SO₂ levels was EGF. It has been reported that AGE could trigger EGF receptor (EGFR) ^{54,55}. The EGRF can bind to multiple growth factors such as EGF, HB-EGF ⁵⁶. ROS might block EGFR dephosphorylation ⁵⁷. This might lead to elevated EGF release by the body as a feedback to

blockade EGFR. It has also been reported that EGF exerts anti-apoptosis benefits to podocytic injury affected by high glucose⁶⁵. This study revealed that EGF levels in NPDR are lower when compared to controls that result in apoptosis. This might explain the inverse relationship between EGF and SO₂ and more retinal blood oxygen saturation.

Limitations: One of main challenges of the AH results is that only a small amount of AH can be attained from human eyes. Normally 50-150 μ l of AH can be acquired from one eye, which is hardly adequate to assess a limited number of cytokines using traditional ELISA. However, with the flow cytometric bead-based technology, several cytokine analytes can now be measured simultaneously in a single sample. Even so, more sample volume was required for performing all the different assays described in this study.

Another limitation of this study was the delay between the acquiring AH and imaging visit. However, this is unlikely because the patient sample has slowly progressing, early DR. There might be other factors affecting the results, such as diet, when compared to the first visit (surgery day), despite advice from the student investigator to minimize such effects.

In summary, HGF, EGF and Ang 2 showed a correlation with retinal blood oxygen saturation. Hypoxic conditions lead to HGF expression and less oxygen is consumed due to cell death in hypoxic tissues. The proapoptotic characteristic of HGF might be associated to HIF-1 deficiency⁶³. EGF reduction might cause apoptosis or distressed cell growth resulting in cell death and hence less oxygen consumption. Ang 2 stimulates pericyte loss under high glucose conditions in DR⁶⁴. There is also evidence that up-regulation of Ang 2 in the absence of VEGF results in endothelial cell death and vascular regression. The pericyte loss and endothelial cell death might result in less oxygen demand and hence increased oxygen in the vessels. Pericytes are known as blood flow regulators, and their

loss will result in vasodilation and increased blood flow which might be involved in increased SO_2 due to less time to release O_2 to the tissues. The repeat measurement values for some samples were very poor based upon results from only two subjects (one control and one NPDR); however, most repeat values were acceptable to excellent (Table 7.3).

8 General Discussion

Several non-invasive techniques have been developed to quantify ocular blood flow in humans. Previous techniques for measuring retinal blood flow have numerous limitations, such as being invasive (e.g. Fluorescein angiography), or are subjective (e.g. blue field entoptic phenomenon), or are incapable of calculating blood flow (e.g. Retinal Vessel Analyser) since a surrogate parameter of flow is measured. In addition, the only technique that truly measures volumetric retinal blood flow is limited to relatively large vessels and a single measurement site. To overcome these limitations, a new technique has been developed, generally known as Optical Coherence Tomography (OCT) blood flow technology. At this moment in time, however, no agreement has been reached regarding the exact clinical significance of change in retinal capillary blood flow. Indeed, the variability and repeatability of retinal capillary blood flow measurement needs to be established in order to define significant change and to use this technique in a clinical setting. OCT is a noncontact, non-invasive diagnostic technique that can be used to allow measurement of retinal blood flow. The development of Fourier-domain OCT (FD-OCT) considerably improved retinal imaging. Overall, we conclude that the FD-OCT gave repeatable measurements of retinal blood flow in normal subjects. The data was more repeatable for the younger age group (Chapter 3).

Chapter 4 details the magnitude of retinal blood oxygen saturation (SO_2) in DR and aged-matched control subjects. A non-invasive prototype hyperspectral retinal camera (HRC, Optina, QC, Canada) was employed to assess the retinal blood oxygen saturation in NPDR and aged matched controls. For each subject, six repeated retinal images were acquired at wavelengths of 586 and 605nm using the HRC. There was a pronounced increase in SO_2

values in both arterioles and venules in NPDR group. The A-V difference was lower in NPDR group. The pronounced increase in retinal blood SO_2 in NPDR group might be explained possibly secondary to neuronal cell atrophy, in the diabetic group with mild-to-moderate NPDR.

Chapter 5 investigated the relationship between total retinal blood flow and retinal blood oxygen saturation. This study did not find any relationship between retinal blood flow and retinal blood SO_2 .

The study of the relationship between retinal blood flow and retinal oxygenation in individuals with type 2 diabetes represents fertile scientific ground to be investigated and would greatly expand our understanding of the development of DR by enabling to calculate oxygen metabolism. Quantifying retinal blood oxygen saturation could help us better understand the pathophysiology behind retinal diseases such as DR; however, either oxygen saturation or retinal blood flow cannot be used to predict one another. This study emphasizes the need for quantifying both retinal blood flow and retinal oxygen saturation in order to calculate oxygen metabolism.

The combination of retinal oxygen saturation and retinal blood flow can be used to extract more information about retinal metabolism since:

$$\text{Retinal } O_2 \text{ Delivery} = [1.39 \times \text{Hb} \times \text{SaO}_2 + (0.003 \times \text{PaO}_2)] \times \text{blood flow}$$

Chapter 6 looks into the multiplex analysis of angiogenic and inflammatory biomarkers in AH of NPDR. Our findings suggest that up-regulation of Ang 2, IL-8, TGF β , EGF and HGF in NPDR may play important roles in the pathophysiology of DR. These molecules could be considered to be studied further as therapeutic targets for the treatment and prevention of microvascular complications of DR. These findings help to better

understand pathophysiology behind DR and validate oxygen saturation measurements.

Chapter 7 studied the relationship between SO_2 and angiogenic/inflammatory biomarkers in NPDR and compared it to control subjects. The findings of this study expand the existing knowledge to another level by adding the biomarker association. This study found a likely association between the biomarkers and SO_2 . This might be helpful for better understanding the pathophysiology behind DR. This study aids better understanding the pathophysiology of DR and validation of oxygen saturation measurements. The study of the relationship between inflammatory biomarkers and retinal oxygenation in individuals with type 2 diabetes emphasizes the need to expand our understanding of the development of DR by defining the interaction of inflammatory mediators and retinal oxygen saturation. In summary, quantifying retinal blood oxygen saturation could help us better understand the pathophysiology behind retinal diseases such as DR and possibly better non-invasive techniques to earlier diagnose of such diseases.

Limitations:

1. One of main challenges of the AH results is that only a small amount of AH can be attained from human eyes. Normally 50-150 μ l of AH can be acquired from one eye, which is hardly adequate to assess a limited number of cytokines using traditional ELISA. However, with the flow cytometric bead-based technology, several cytokine analytes can now be measured simultaneously in a single sample. Even so, more sample volume was required for performing all the different assays described in this study.
2. Another limitation of this study was the delay between the acquiring AH and imaging visit. However, this is unlikely because the disease is early DR. There might be other factors affecting the results, such as diet, when compared to the first visit (surgery day),

despite advice from the student investigator to minimize such effects.

3. The recruitment was challenging. Participants were already nervous due to surgery and did not want to make it more complicated.

4. Another restriction was our sample size. It was challenging to recruit more subjects. Participants with DR usually had more complications and were excluded from the study.

5. Due to small volume of AH, the assays were limited as well. Each assay needed at least 40 μ l of AH.

6. Another limitation was to simultaneously measure retinal blood flow and retinal oxygen saturation as opposed to sequential methods.

8.1 Future work

Future work should examine the simultaneous disturbance in SO_2 and vascular inflammatory activity over a comprehensive longitudinal study. It is important to understand whether extended exposure to hyperglycemia could have a different effect on retinal blood oxygen saturation.

Longitudinal studies that track changes in retinal blood oxygen saturation are important to better understand its relationship with the progression of DR. It will be also of significance to investigate the retinal blood oxygen saturation in different stages of DR and look at its association with the angiogenic biomarkers.

The future work can expand the investigation to find a better way to understand the mechanism behind the receptors and look for the possible explanation for the behavior of biomarkers such as explore receptor for EGF and see if HB-EGF is binding to the EGFR instead of EGF during NPDR.

FD-OCT was found to be repeatable and reliable when compared with other blood flow measurement instruments in other studies. However there is still a great need for a better instrument. The COV in young healthy participants was 7.5%, which is not negligible when it is required in clinical follow-up of patients. The difference between sessions of BF measurement will only be noticed if it is large during diseases.

Appendix A

Methodology

Procedure for sample collection

Undiluted samples of AH were obtained from patients proceeding to cataract surgery. Briefly, a paracentesis was made in the peripheral cornea next to the limbus at the onset of surgery. A Sautter hydrodissection cannula (27G, 0.4x22mm; Geuder G-34245) was introduced into paracentesis tract to deliver suction by pulling up the plunger. The tip of the cannula was placed in the mid anterior chamber and 20-150 μ l of AH was slowly aspirated. The AH was collected into the attached syringe and transferred consequently into an eppendorf tube of 1.5ml where it was assessed.

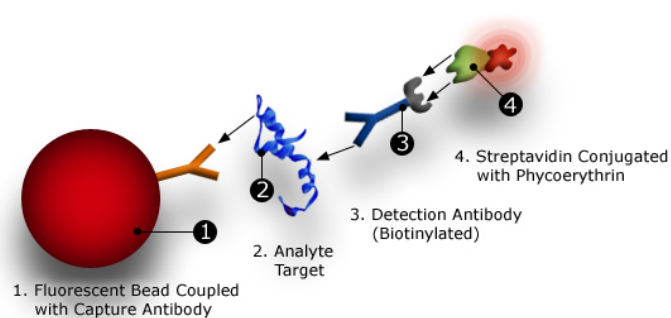
Analysis of angiogenic and inflammatory biomarkers

Multiplex Immunoassays (Luminex, Bio-Rad Laboratories, Inc., Hercules, CA, Eve Technologies)

Multiplex analysis was accomplished by the Bio-Plex 200 suspension array system. This system is built on a flow-based dual laser detector with real time digital signal processing and is capable of distinctive up to 100 diverse internally colour coded beads in a single well of a 96-well plate. This method is similar to sandwich-ELISA. Each polystyrene bead is conjugated with a distinctive reagent, particular to a specific bioassay. Each reactant is explicit for a different target analyte and this can comprise; antigens, antibodies, oligonucleotides, enzyme substrates, or receptors. A particular antibody bound to a bead aims a specific biomarker, which was again bind to a detection antibody with a fluorescent

reporter dye label. The simultaneous detection of many other analytes in the same sample is possible by different colored beads.

To accomplish a multiplex assay, sample and reporter molecules are permitted to react with the conjugated bead mixture in microplate wells. The flow-based Bio-Plex 200 system recognizes each definite reaction based on bead color, and measures it. The dual detection flow cytometer is operated to settle the different assays by bead colors in one channel, and define of the analyte concentration by assessing the reporter dye fluorescence in another channel. The degree of the reaction is assessed using fluorescently-labeled reporter molecules also specific for each target analyte. Bio-Plex Manager software programmes data analysis and producing of thorough summary information.



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