Retinal Blood Flow and Vascular Reactivity in Diabetic Retinopathy

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any final revisions, as accepted by my examiners.

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Abstract

Introduction

Retinal vascular reactivity is impaired in patients with diabetes and is thought to be involved in the onset and progression of diabetic retinopathy (DR). Previous studies that have utilized hyperoxia to assess retinal vascular reactivity have been limited due to confounding factors associated with the administration of oxygen and have used a variety of different instruments to measure retinal blood flow. The influence of blood glucose at the time of blood flow assessment has also not been systemically investigated.

The specific aims of each Chapter are as follows:

Chapter 3: To compare three systems used to administer hyperoxia to human subjects.

Chapter 4: To quantify the magnitude and timeline of change of retinal hemodynamic parameters induced by an isocapnic hyperoxic stimulus.

Chapters 5, 6 and 7: To quantify the magnitude of change of retinal hemodynamic parameters induced by hyperoxia, hyperglycemia and combined hyperoxia / hyperglycemia, respectively, in groups of diabetic patients with no clinically visible, and mild-to-moderate, DR and in age-matched subjects without diabetes.

Methods

Chapter 3: Subjects breathed air followed by oxygen, or oxygen plus carbon dioxide using a non-rebreathing system, or air followed by oxygen using a sequential rebreathing system. The magnitude of change and variability of CO_2 concentrations was compared between systems.

Chapter 4: Baseline retinal blood flow data was acquired while the subjects breathed air using a sequential rebreathing system. An isocapnic hyperoxic stimulus was initiated and maintained for 20 minutes. Air was then re-administered for 10 minutes. Retinal blood flow measurements were acquired every minute over the course of the study. The magnitude of change of each hemodynamic parameter was determined by fitting individual data with a sigmoidal function.

For Chapter 5, 6 and 7 diabetic patients with no clinically visible, and mild-to-moderate, DR were stratified into groups based upon their retinopathy status. Age-matched non-diabetic subjects were recruited as controls. Baseline retinal blood flow data was acquired while subjects breathed air. Retinal blood flow measurements were then acquired after exposure to (a) hyperoxia, (b) hyperglycemia and (c) combined hyperoxic / hyperglycemic stimuli. Change in hemodynamic parameters was compared between groups and correlated with objective measures of retinal edema.

Results

Chapter 3: The difference in group mean end-tidal CO_2 levels between baseline and hyperoxia was significant for oxygen administration using a non-rebreathing system. The sequential rebreathing technique resulted in a significantly lower variability of individual CO_2 levels than either of the other techniques.

Chapter 4: An ~11% decrease of diameter, ~36% decrease of velocity and ~48% decrease of blood flow was observed in response to isocapnic hyperoxia in young, healthy subjects. A response time of 2.30±0.53 minutes and 2.62±0.54 minutes was observed for diameter and

velocity, respectively.

Chapter 5: Retinal blood velocity, flow, and WSR significantly decreased in response to isocapnic hyperoxia in all groups. The magnitude of the reduction of blood flow was significantly reduced with increasing severity of retinopathy. There was a significant relationship between baseline objective edema index values and retinal vascular reactivity.

Chapter 6: A significant change in blood glucose level was observed for all groups. No significant change in any hemodynamic parameter was found in patients with diabetes and in age-matched subjects without diabetes.

Chapter 7: Retinal blood velocity and flow significantly decreased in all groups in response to combined hyperoxic / hyperglycemic provocation. The vascular reactivity response was not significantly different *across* the groups.

Conclusions

Chapter 3: Control of CO_2 is necessary to attain standardized, reproducible hyperoxic stimuli for the assessment of retinal vascular reactivity.

Chapter 4: Arteriolar retinal vascular reactivity to isocapnic hyperoxic provocation occurs within a maximum of 4 minutes. Although there was a trend for diameter to respond before velocity, the response characteristics were not significantly different between diameter and velocity. Different response characteristics of the retinal vasculature to transmural pressure mediated autoregulation as opposed to metabolic mediated vascular reactivity are suggested.

Chapter 5: The vascular reactivity response in terms of the reduction of blood flow relative to baseline was significant in all groups but the magnitude of the change in flow was

significantly reduced with increasing severity of retinopathy. A loss of retinal vascular reactivity is indicated in patients with moderate DR without clinically evident diabetic macular edema (DME), and in patients with DME.

Chapter 6: Unaltered retinal arteriolar blood flow was found 1 hour after glucose ingestion in patients with diabetes and in age-matched subjects without diabetes. These results do not support the theory that retinal blood flow is affected by an acute increase of blood glucose in diabetic patients and in subjects without diabetes.

Chapter 7: The vascular reactivity response to a combined hyperoxic / hyperglycemic provocation produced a pronounced reduction in blood flow. Unlike the response to hyperoxia alone, the vascular reactivity response was not significantly different *across* the groups. This suggests that hyperglycemia may influence the retinal vascular reactivity response to hyperoxia.

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Finally I would like to thank my parents David and Hazel, my family, my fiancée Zora Juričić and her family (my soon-to-be in-laws!) for love, support and encouragement. I could not have done this without you.

Dedication

This work is dedicated to my parents David and Hazel. You are the most hard-working, loving, dedicated, selfless people I have ever met and I am blessed to have such wonderful parents. Thanks for giving me the opportunity to pursue my dreams.

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List of Abbreviations

Advanced glycation end-products (AGE); Average velocity (V_{mean}); Basement membrane (BM); Blood flow (Q); Blood pressure (BP); Blood retinal barrier (BRB); Branch retinal vein occlusion (BRVO); Calcium ion concentration (Ca^{2+}); Canon laser blood flowmeter (CLBF); Carbon dioxide (CO_2); Central inspiratory activity (CIA); Central retinal artery (CRA); Clinically significant DME (CSDME); Closed circuit device (CCD); Concentration of CO_2 in the expired breath ($P_{ET}CO_2$); Diabetic macular edema (DME); Diabetic retinopathy (DR); Diameter (D); Dorsal respiratory group (DRG); Early Treatment Diabetic Retinopathy Study (ETDRS); Endothelial-derived contracting factors (EDCF); Endothelial-derived relaxing factors (EDRF); Endothelin (ET); Fractional inspired oxygen (FiO_2); Frequency (f); Fluorescein angiography (FA); Glucose transporters (GLUT); Glycosylated hemoglobin (A1c); Heidelberg retinal flowmeter (HRF); Heidelberg retinal tomography (HRT); Impaired glucose tolerance (IGT); Internal limiting membrane (ILM); Intra-cellular adhesion molecule (ICAM); Intra-ocular pressure (IOP); Length (L); Lens Opacity Classification System III (LOCS III); Macular edema module (MEM); Maximum frequency shift (Δf_{max}); Maximum to minimum (max:min); Microaneurysms (MAs); Nitric oxide (NO); Optical Coherence Tomography (OCT); Oral glucose tolerance test (OGTT); Oxygen (O_2); Partial pressure of arterial carbon dioxide (PCO_2); Partial pressure of arterial oxygen (PO_2); Positive end-expiratory pressure (PEEP); Pressure gradient (ΔP); Protein kinase C (PKC); Pulsatile ocular blood flow (POBF); Resistance (R); Retinal vessel analyzer (RVA); Retinal pigment epithelium (RPE); Retinal Thickness Analyzer (RTA); Scanning laser tomography (SLT); Short-wavelength automated perimetry (SWAP); Short-wavelength (SW); Smooth muscle

cells (SMCs); Stereo-fundus photography (SFP); Vascular endothelial growth factor (VEGF); Ventral respiratory group (VRG); Viscosity (η); Visual acuity (VA); Wall shear rate (WSR); White-on-white (WW)

1 Introduction

1.1 Retinal blood supply and drainage

Blood reaches the inner retina via the aortic artery, common carotid arteries, internal carotid artery, ophthalmic artery and finally the central retinal artery (CRA). The CRA pierces the optic nerve 10-15mm behind the globe and runs forward in the central portion of the nerve lying alongside the central retinal vein.

Bifurcation of the central retinal artery on the surface of the nerve head provides four major arterioles, one of which supplies each retinal quadrant. Retinal tissue is nourished by two separate vascular systems: the inner retina is supplied by a double-layer of vessels derived from the central retinal artery and the outer retina (posterior to outer plexiform layer) is supplied from the underlying choriocapillaris primarily via the short posterior ciliary arteries and to a lesser extent by the long posterior ciliary arteries (derived from the ophthalmic artery).[1,2] Blood is drained by the retinal venules and into the central retinal vein and finally the ophthalmic vein to leave the orbit.

Retinal blood flow in the inner retina is characterized as a low flow system making the retina vulnerable to interruption in blood supply. Consequently, the extraction of oxygen as blood passes through the capillary beds is very high. The retinal arterio-venous difference in blood oxygenation is 35%.[3,4] This is in contrast to the much faster choroidal blood flow where the

arterio-venous difference in blood oxygen is ~4%.[5]

1.1.1 Structure of the retinal vessels

Retinal arterioles and venules are composed of 3 layers: (1) adventitia, (2) smooth muscle cells, and (3) endothelial cells (Figure 1.1).

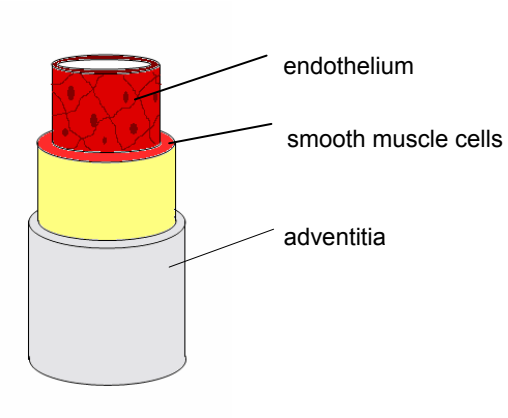

Figure 1.1 Structure of a blood vessel

The central retinal artery and especially the downstream arterioles are thought to be responsible for regulating the flow of blood to the capillaries; the arterioles are termed "resistance" vessels. Retinal capillaries are composed of a single layer of endothelial cells surrounded by a basement membrane (BM) containing pericytes and are arranged in a laminar fashion, the number of layers depending on retinal location (3 or 4 in the central retina decreasing to 2 in the periphery).[6] Capillaries facilitate exchange (and area often termed

"exchange" vessels) of metabolites at the tissue level and, in conjunction with the pericytes, allow "fine tuning" of capillary perfusion.[6] The tight junctions between the endothelial cells (zonula occludens) constitute the inner blood retinal barrier (BRB), preventing leakage of blood constituents into the retinal tissue for optimal retinal functioning.

1.2 Blood flow

Blood flow (Q) within the general circulation is proportional to perfusion pressure and inversely proportional to vascular resistance (referred to as Ohm's law). Perfusion pressure is defined as the difference between arterial and venous pressures (i.e. the pressure gradient; ΔP); it describes the force with which blood is moved through the vascular bed. Friction between the vessel wall and blood produces resistance (R) to flow.

$$Q = \Delta P / R \qquad (1.1)$$

Resistance to flow is dependant on the properties of the fluid and the tube through which it is flowing. Using steady blood flow conditions through a cylindrical rigid tube, resistance is proportional to the viscosity (η) and length (L) and inversely proportional to the fourth power of the radius (r) of the vessel.

$$R = 8\eta L/\pi r^4 \qquad (1.2)$$

Poiseuille combined equations 1 and 2 and formulated a law that now bears his name:

$$Q = \Delta P \pi r^4 / 8 \eta L \qquad (1.3)$$

Thus, large alterations of blood flow can be achieved with small changes in vessel diameter.

The cardiovascular system differs from the rigid tubes studied by Poiseuille in a number of ways. Vessels in the human body are tapering and elastic, heart contractions result in pulsatile not steady flow, laminar flow is not always present in larger vessels and blood is not a perfect Newtonian fluid (i.e. viscosity varies with velocity). Despite these limitations, Poiseuille's equation is a useful approximation for the calculation of blood flow and is believed to exist in the retinal blood vessels that are 80μm in diameter or larger.[7-11]

1.2.1 Ocular perfusion pressure (OPP)

The pressure of blood entering the eye (OPP) is determined by the blood pressure (BP) and the intra-ocular pressure (IOP), in the relationship:

$$OPP = 2/3\ BP_{mean} - IOP \qquad (1.4)$$

where BP_{mean} is the mean arterial blood pressure and IOP is the intra-ocular pressure (both are explained below).[12]

1.2.1.1 Blood pressure

The pressure with which blood is ejected from the heart is transmitted throughout the entire vascular network so that blood is forced from the heart to the capillaries and back to the heart again. The maximum pressure produced during systole (ventricular contraction) is called the systolic pressure and is generally 120mmHg and the minimum during diastole (ventricular relaxation) is generally 80mmHg in young clinically healthy persons. BP is measured using a sphygmomanometer and stethoscope. The area under the pressure wave over time equals the mean BP

$$\text{BP mean} = \int \text{BP}.dt \qquad (1.5)$$

Clinically the mean BP is calculated using the following formula:

$$BP_{mean} = BP_{dias} + (BP_{sys} - BP_{dias} / 3) \qquad (1.6)$$

where BP_{sys} is the systolic pressure and BP_{dias} is the diastolic pressure.

1.2.1.2 Intra-ocular pressure

Aqueous is secreted by the ciliary epithelium of the ciliary processes and flows through the pupil to drain out of the eye via the trabecular meshwork in the anterior chamber angle and subsequently into Schlemm canal (called the conventional route) or across the iris root and

anterior face of the ciliary body and subsequently to the supra-choroidal space (known as the uveoscleral route). The pressure generated as a result of this fluid filling this space is called the IOP and is commonly measured using an applanation tonometer. Normally, the IOP measures between 10 and 21 mmHg.

1.3 Regulation of retinal blood flow

A sympathetic and parasympathetic nerve supply innervates retinal vessels but is ineffective in altering vascular tone anterior to the lamina cribrosa.[13-17] Consequently, changes in vascular tone are initiated from within the retinal tissue itself in response to local demands (Figure 1.3).[18,19] Since the retinal vascular bed can be imaged non-invasively, it presents a unique opportunity to study hemodynamics.

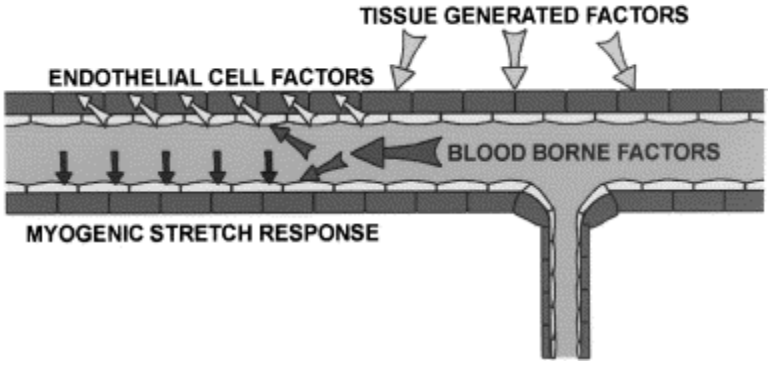

Figure 1.2 Schematic illustration of local control mechanisms in the vascular system.
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1.3.1 Autoregulation

Autoregulation is defined as "the ability of a vascular bed to maintain constant blood flow to the tissues under conditions of varying perfusion pressure". Previously published studies have documented efficient retinal blood flow regulation (within certain limits) both during manipulation of IOP[20,21] and after exercise induced changes in perfusion pressure.[22-28] Retinal blood flow is regulated by myogenic and metabolic stimuli and endothelium-derived vasoactive factors.[19,29] Myogenic blood flow regulation maintains constant blood flow in response to increased blood pressure (perfusion pressure) by vasoconstriction and therefore increased resistance. Calcium ion movement into the smooth muscle cells promotes constriction and reduces blood flow in order to avoid hyper-perfusion, capillary damage and edema.[29] The reverse is also true whereby a reduction in perfusion pressure would have the opposite effect. Metabolic blood flow regulation alters vascular resistance so that the blood supply maintains the concentrations of certain metabolites and waste-products within narrow limits (principally the partial pressure of O_2 and CO_2)[2]

1.4 Endothelial vasoactive substances

The endothelium produces vasoactive substances that are important determinants of retinal blood flow. They can be divided into two groups based on their actions: (a) endothelial-derived relaxing factors (EDRF) and (b) endothelial-derived contracting factors (EDCF). A counter-balancing basal production of EDRFs and EDCFs maintains homeostatic retinal

blood flow. Certain conditions result in increased or decreased production or action of one or both types of endothelial derived factors and an alteration of blood flow.[30]

1.4.1 Endothelium derived relaxing factors

Nitric oxide (NO) is the most important EDRF and is derived from L-arginine by nitric oxide synthase. NO induces vasodilatory effects by increasing cyclic guanidine monophospate that in turn reduces calcium ion concentration (Ca^{2+}) in smooth muscle cells resulting in relaxation and subsequent dilation. NO is also involved in anti-coagulation, vascular remodeling and angiogenesis. In the retinal circulation, NO (together with other endothelium-derived agents) controls basal blood flow by maintaining vasodilation[31,32]; inhibition of NO results in a pronounced vasoconstriction in ocular vascular tissue.[33-36] Increased shear stress (i.e. frictional forces produced on the inner endothelial wall resulting from blood flow) increases NO production[37] causing vasodilation and increased blood flow[38] however, this response may be limited to the larger conduit arteries and may not be primarily responsible for the alteration of blood flow in the resistance arterioles of the retina.

1.4.2 Endothelium derived constricting factors

The endothelin family comprise 3 polypeptides, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), that are important in vascular regulation.[39] ET-1 is the most potent and is only produced by endothelial cells.[39] ET-1 produces vasoconstriction at higher concentrations

(and vasodilation at low concentrations).[40] Vasoconstriction is mediated through the ETa receptor subtype[41] found on vascular smooth muscle cells and pericytes in rats[42] and humans.[43] Vasoconstriction occurs secondary to increased intracellular Ca^{2+}.[44-46] Vasoconstriction and reduction of retinal blood flow has been demonstrated in animals[47,48] and humans[49] after intravenous administration of ET-1. Hyperoxia has been shown to stimulate ET-1 release from retinal vascular endothelial cells *in vitro*,[50] in animal models,[51] and in human tissue.[52] Shear stress down-regulates endothelin synthesis.[53] and NO inhibits the release of ET-1[54] while ET-1 impairs NO bioavailability possibly contributing to impairment of vascular reactivity.[55]

1.5 Quantification of retinal hemodynamics:

1.5.1 Pulsatile ocular blood flow

Pulsatile ocular blood flow (POBF) assessment provides an indirect measurement of choroidal perfusion. Ocular blood volume increases during systole and results in increased IOP while the reverse is true during diastole. The air pressure required to indent the cornea is measured 200 times a second via a pressurized tip in contact with the cornea. Calculation of POBF from the change in IOP is based upon a model eye assuming a standard ocular rigidity.[56-58] An initial decrease followed by an increase in POBF has been documented in patients with diabetes who subsequently develop diabetic retinopathy (DR).[59-61] Increase in blood glucose levels has been demonstrated to result in a higher pulsatile blood flow in patients with type 2 diabetes.[62]

1.5.2 Interferometry

This technique was developed by Schmetterer et al.[63] and assesses change in axial length i.e. position of the fundus relative to the corneal surface with each cardiac cycle. Laser light is directed along the pupillary axis and reflected light from both the cornea and the fundus collected to produce an interference pattern. As a bolus of blood enters the eye during systole the choroidal blood volume increases and the axial length decreases. This produces a change in the interference pattern. The change in the intereference pattern is measured in micrometers. The relationship between pulsation and volumetric flow is unknown. Patients

with proliferative DR exhibited decreased amplitude of fundus pulsation.[64]

1.5.3 Fluorescein angiography

Fluorescein dye was first used by to assess retinal blood flow quantitatively by Hickam and Frayser.[65] Fluorescein is a vegetable based dye and is injected into the antecubital vein of the arm to enter the circulation. As the dye passes through the ocular vasculature, it is illuminated with light of a certain wavelength (~490nm) causing it to fluoresce. As it does so, it emits light of ~530nm. A barrier filter is used to increase the contrast as it passes through the ocular vascular beds. Relative concentrations of fluorescein in the retinal blood vessels are calculated for each successive picture. Over the past decade, scanning laser ophthalmoscopes with confocal optics have improved the quality of images and allow high temporal resolution imaging. Commonly used parameters to describe retinal blood flow include mean circulation time (average time fluorescein stays in a given vascular bed),[65] arterio-venous passage time (time between first appearance of fluorescein in an artery and its corresponding vein)[66] and mean dye velocity (time for fluorescein to travel a certain distance along an arteriole).[66] The main limitations of this technique include the assumption that an artery is completely drained by a corresponding vein, the sum of vessel diameters is directly related to blood volume and that no leakage of fluorescein occurs during measurements.[67] The technique is invasive and metabolic changes associated with diabetes may artifactually alter the successive retinal frames.[68]

1.5.4 Blue field entoptic phenomenon

This technique allows determination of leukocyte velocity in the macular capillaries. The blue field entoptic phenomenon (BFEP) is best perceived by using a narrow band (half-height width of ~20nm) of blue light (430nm). The erythrocytes absorb, while leukocytes reflect, blue light and the subject becomes aware of the movement of the leukocytes to which they compare to a simulated particle field.[69] By asking subjects to match the speed and pulsatility of the movement of their leukocytes with that of the simulated field retinal blood flow[69] and the retinal vascular reactivity response to various stimuli can be quantified.[70] The major limitation of this technique is that it is subjective, has large individual variation,[71] poor reproducibility[68] and requires good visual acuity.[68]

1.5.5 Retinal vessel analyzer (RVA)

The RVA comprises a fundus camera, a charge coupled device (CCD) and a personal computer.[72] An image of the fundus is displayed on a monitor for real time inspection.[27] A region of interest is defined by the examiner and the diameter of the vessel (arteriole, venule, or both) inside this region is determined using an algorithm that detects the vessel edges at a maximum frequency of 50Hz.[73] Measurement of vessel diameter continues during small changes in position of the vessel of interest (i.e. during minor eye movements), however, acquisition of diameter measurements is interrupted during major eye movements or blinks and restarted when a clear retinal image is re-acquired.[74] Change in vessel diameter is expressed as a function of time or relative position along a vessel.[74] This instrument assess

change in diameter (relative units) but is not capable of measuring blood velocity or flow.

1.5.6 Doppler shift theory

In 1842, Christian Doppler related the frequency of sound (or light) reflected from an object to its velocity. This phenomenon later became known as the "Doppler principle". In the context of perfusion measurements, laser light (frequency=f) reflected from a moving particle has a shift in frequency (Δf) that is proportional to the velocity of the moving particle. Light scattered from stationary tissue is not shifted and acts as the reference frequency from which a relative change in retinal blood velocity is measured (Figure 1.4).

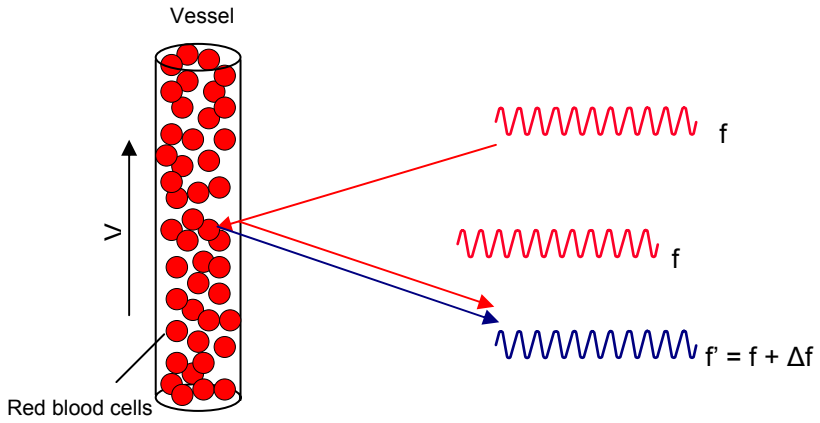

Figure 1.3 Diagramatic representation of the Doppler principle.
Light scattered from stationary tissue is reflected at frequency f. Light reflected from the red blood cells (moving with velocity V) is reflected at frequency f '.

1.5.7 Colour Doppler imaging

This device measures blood velocity in extraocular blood vessels.[75] Sound waves of a certain frequency are emitted and reflection by moving particles produces a frequency shift while stationary tissue reflects the sound wave with the same frequency as the source. By measuring the frequency shift, blood velocity can be determined.[76,77] Published studies in patients with mild-to-moderate DR have demonstrated reduced blood velocities in the central retinal artery relative to subjects without diabetes,[61,78,79] increased resistance[80,81] and decreased vascular response to hyperoxia.[82] This device cannot measure vessel diameter and therefore cannot quantify flow.

1.5.8 Heidelberg Retina Flowmeter (HRF, Heidelberg Engineering, Germany)

The HRF comprises a confocal scanning laser ophthalmoscope and a single photodetector.[83] Briefly, the fundus is illuminated with a 780nm diode laser and measurements of the intensity of back scattered light from a measurement area of 10º by 2.5º are acquired with a resolution of 256 pixels and 64 lines. Each line of 256 pixels is scanned 128 times at a frequency of 4000Hz. The intensity of back-scattered laser light from the retina is measured as a function of time (to produce an intensity-time curve) for each pixel within the image. The incident and reflected light interfere resulting in an oscillation, or "beat", of the measured light intensity. The frequency of the intensity oscillation is proportional to the Doppler frequency shift and derives a measure of volumetric blood flow in arbitrary units.[83] However, experimental tests have revealed a number of

limitations of this technology.[84] Importantly, the Doppler signal depends on the angle between the incident and reflected light.[9] Problems associated with the HRF include susceptibility to eye motion during measurement making some data unusable. Also, there is an artifactual flow reading even when no cells are moving, and an artifactual increase in the total number of cells when in motion.[85] Work from our own laboratory has shown that artificially induced light scatter erroneously elevates HRF values.[86] In summary, the HRF is only capable of providing relative measurements of blood flow at the same sites over time.[85] HRF investigation of blood flow in patients with DR revealed increased blood flow in some patients while others demonstrated no change.[87]

1.5.9 Bi-Directional Laser Doppler Velocimetry

By utilizing *two* photo-detectors separated by a known angle, the absolute quantification of centre-line blood velocity is possible.[11,88,89] Early instruments developed by Riva et al.[90] and Feke et al.[11] comprised a fundus camera or slit lamp, respectively, to which a laser and photo-detectors were attached for the assessment of retinal hemodynamics. These instruments were not capable of maintaining the center-line position of the measurement laser on the vessel during micro-saccades leading to aberrant velocity measurements and was not capable of measuring vessel diameter.[85] Velocity was calculated by determining the difference in the frequency shift in 2 directions (represented by the wave vectors K1 and K2 below) separated by a known angle (Figure 1.5).

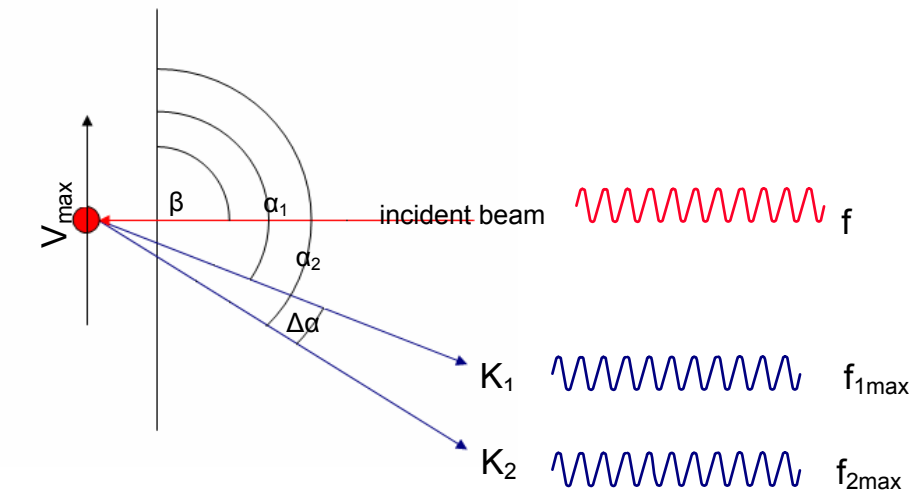

Figure 1.4 Diagrammatic representation of bidirectional laser Doppler velocimetry.
The incident beam is directed perpendicular to the vessel with red blood cells moving with centre-line velocity Vmax. The maximum frequency shift is detected at K1 and K2 that are separated by a known angle.

$$\Delta f = f2\ max - f1\ max = (\alpha 2 - \alpha 1).v/\lambda \qquad (1.7)$$

where α1 and α2 are the angles between the velocity vector Vmax and K1 and K2, respectively. $F1_{max}$ and $f2_{max}$ are the maximum frequency shifts at K1 and K2, respectively.

$$Vmax = \lambda.\Delta f / n.\Delta\alpha.\cos\beta \qquad (1.8)$$

where Δα is the angle between K1 and K2 and β is the angle between the vector Vmax and the incident beam.

Mean velocity is calculated using the following relationship assuming Poiseuille flow[9]

$$V_{mean} = V_{max} / 2 \quad (1.9)$$

Flow is calculated using the following equation[91]

$$Flow = 1/2.\pi.D^2/4.V_{mean}.60 \quad (1.10)$$

where D= vessel diameter and V_{mean}= mean velocity across a cardiac cycle

1.5.10 Canon laser blood flowmeter (CLBF model 100)

The CLBF surpasses all other instruments because it permits the non-invasive bi-directional quantification of blood velocity with simultaneous assessment of retinal vessel diameter and therefore is able to derive blood flow in real units.[11] These features allow intra- and inter-ocular comparisons to be made for each patient and between patients with ocular disease.[85,92]

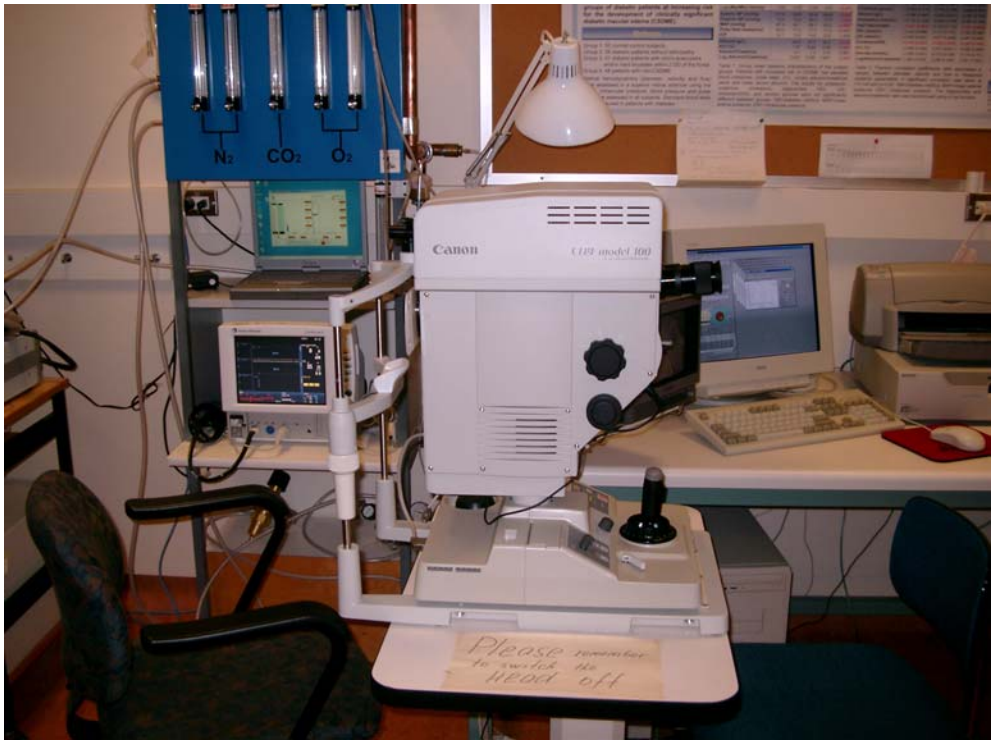

Figure 1.5 Photograph of the Canon Laser Blood Flowmeter

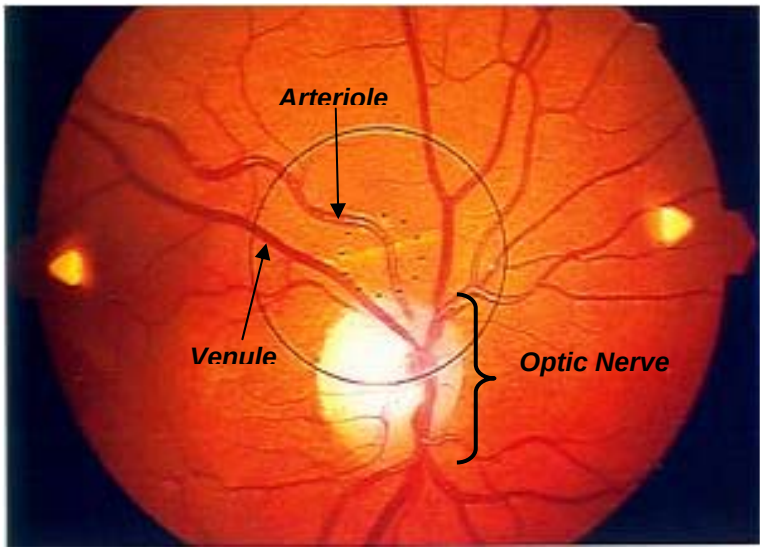

Figure 1.6 Fundus photograph taken using the CLBF

The instrument comprises a fundus camera incorporating a laser and 2 pairs of photo-detectors. A red diode laser (675nm, 80μm x 50μm oval) is used to measure velocity every 0.02 second across a 2 second measurement window resulting in a velocity-time trace. The CLBF also uses a green diode vessel tracking laser system (543nm, 1500μm x 150μm rectangle) that is used to stabilize the laser position at the measurement site and allows rejection of velocity measurements involving significant saccades.[93,94] Diameter readings are acquired every 4 milliseconds during the first and final 60 milliseconds of the 2 second velocity measurement window. Two sequential maximum velocity measurements are determined from the maximum frequency shifts at each photomultiplier along 2 optical paths (path 1 and path 2).

Magnification effects associated with refractive and axial components of ametropia are corrected to provide absolute measurements of diameter (μm), velocity (mm/sec) and flow (μL/min). The technological principles utilized in this device have been described in detail elsewhere.[11,88,91,95] In addition, the CLBF has been extensively evaluated in clinically normal subjects[10,96] and those with various types of retinal diseases.[91,92]

1.6 Respiration

Respiration allows the transfer of oxygen and carbon dioxide between the atmosphere and blood. The trachea is the main air conduit which splits into 2 bronchi as it descends in the

thorax. The right and left bronchi enter the right and left lung respectively and produce many branches to form bronchioles which further sub-divide into alveoli to increase the surface area over which gas exchange can take place. Alveoli allow the venous blood (rich in CO_2 and low in O_2) to come in close proximity to air (rich in O_2, low in CO_2) and each gas diffuses along their concentration gradient across the alveolar epithelium.[97]

The breathing cycle

For the majority of the time, breathing occurs unconsciously. Inspiration is initiated by increased activity of the inspiratory muscles (principally the diaphragm). Contraction of the diaphragm, and with larger breaths the intercostal muscles, increases the thoracic space and moves the ribs up and out allowing alveolar pressure to decrease. Air rushes into the lungs down a pressure gradient until alveolar pressure equals atmospheric pressure. Inspiration ends with a sharp decrease in excitatory activity in the inspiratory muscles. Expiration follows due to the elastic recoil of the ribs and muscles. The alveoli decrease in volume, the diaphragm and intercostals muscles relax forcing air out of the lungs again. During exercise, inspiration and expiration become less passive and other voluntary muscles become involved to increase the respiration rate to meet the metabolic needs of muscle tissue.[97]

1.6.1 Neuronal control of muscles for respiration

Neuronal control of breathing comes from 2 groups of cells in the medulla oblongata, the

dorsal respiratory group (DRG) and the ventral respiratory group (VRG). Inspiration results from reduced inhibition of the central inspiratory activity (CIA) neurons located in the medullary reticular formation. In turn, this excites the DRG / VRG complex driving inspiration. Inspiration ends when a certain threshold is reached within the VRG due to feedback from the CIA and lung receptor neurons. Duration of expiration is determined by the intensity of inhibition of the inspiratory cells of the DRG / VRG complex, after which inspiration starts again.[97]

1.7 Summary

In summary, the retina is a unique vascular bed because the retinal vessels can be visualized directly and non-invasively. Retinal blood flow follows Poiseuille law and is auto-regulated. The retinal arterioles are the resistance vessels of the retina. Arteriolar tonus is regulated principally by NO and ET-1. A number of instruments permit the non-invasive measurement of parameters that reflect retinal blood flow. Of these instruments, the CLBF is unique in that it is able to quantify retinal blood flow in absolute units. The absolute quantification of blood flow in conjunction with a validated technique to produce isocapnic hyperoxia (described later) will provide a novel and potentially powerful technique to investigate retinal vascular reactivity.

1.8 Review of Diabetes

Diabetes is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia resulting from defects of insulin secretion, insulin action or both.[98] The two main classes that have been described are type 1 (10% of individuals; characterized by autoimmune destruction of Islet cells) and type 2 (90% of individuals; characterized by insulin resistance and / or reduced insulin secretion).[99] The World Health Organization has estimated that the number of people diagnosed with diabetes will increase from 143 million in 1997 to 300 million by 2025.[100] Diabetes affects over 16 million people in the United States[101] and approximately 2 million people in Canada (Canadian Diabetes Association). At least US$100 billion is spent annually to treat patients with diabetes and its complications,[102,103] while in Canada the equivalent figure is $13.5 billion (Canadian Diabetes Association).

1.8.1 Complications of diabetes

The Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study identified chronic hyperglycemia as an initiating factor in the complications seen in diabetes that impacts the retina, kidneys and nerves and macro-vascular complications that result in myocardial infarction and cerebro-vascular accident.[104,105] The macro- and micro-vascular complications of diabetes are reduced when strict control of blood glucose is maintained.[104,105] The effects of cellular damage resulting from hyperglycemia has been recently been reviewed (Figure 1.8).[106]

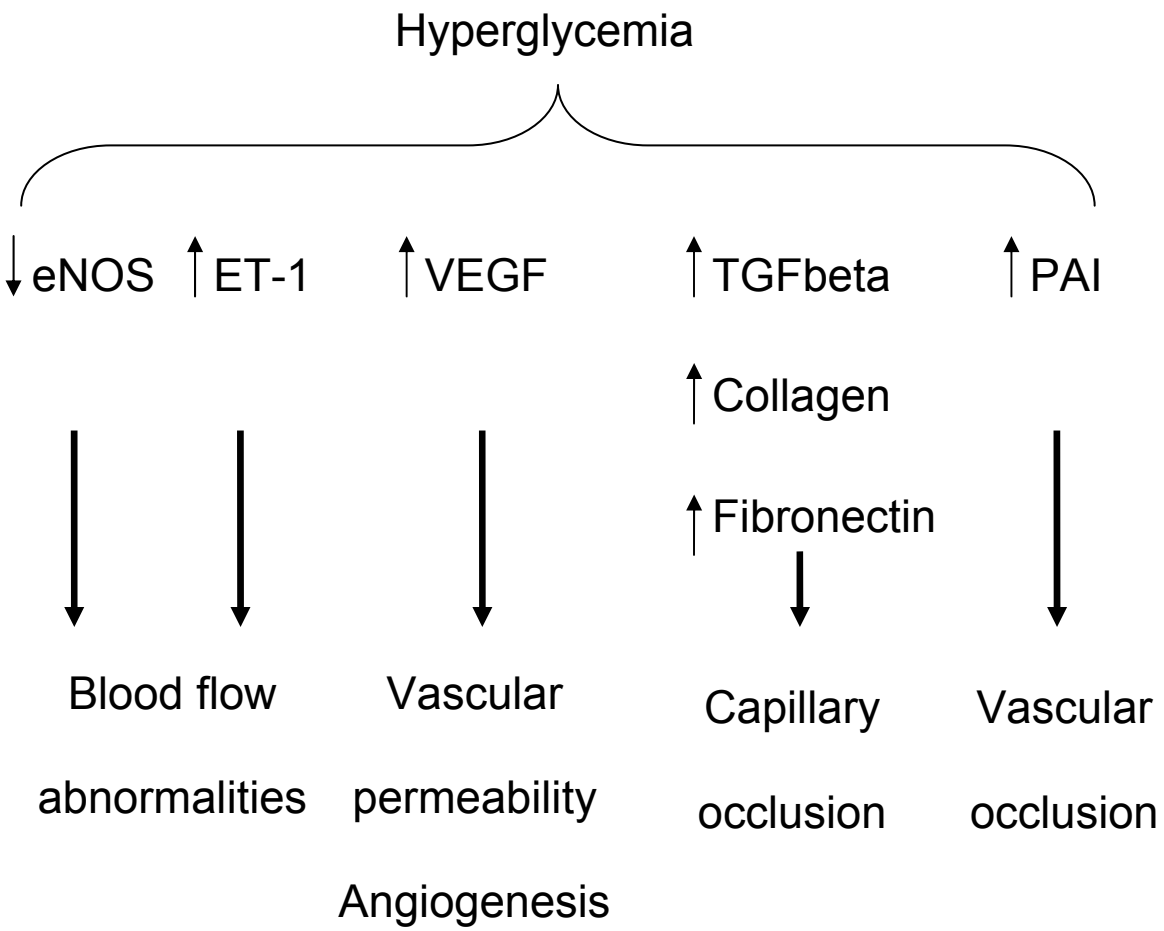

Figure 1.7 Biochemical and morphological consequences of hyperglycemia (eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; VEGF, vascular endothelial growth factor; TGF-beta, transforming growth factor; PAI, plasinogen activator inhibitor).

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1.9 Diabetic retinopathy

DR is one of the most common causes of blindness in North Americans between 20 and 74 years.[107] DR is highly prevalent and is positively associated with duration of diabetes.[108-114] The incidence of development and progression of retinopathy is reduced in those individuals with blood glucose levels that are closest to those of non-diabetic individuals.[104]

1.9.1 Clinical classification and etiology of DR

DR can be categorized as a disorder that preferentially affects the vasculature resulting in capillary closure, ischemia, leakage and eventually neo-vascularization. From a clinical perspective, it can be divided into 3 major categories including non-proliferative, proliferative and maculopathy. Non-proliferative DR is characterized by one or more of the following features: (1) microaneurysms (MAs; localized bulging, or aborted budding, of the capillary endothelial wall) are one of the earliest clinically visible signs of retinopathy,[115,116] (2) hemorrhages may be flame-shaped (if located in the nerve fibre layer) or dot and blot (outer plexiform and inner nuclear layers) and generally occur due to vascular rupture,[97] (3) hard exudates have a glistening appearance, are located in the outer plexiform layer and may be associated with retinal edema,[117] (4) cotton-wool spots appears as fluffy white alterations of the nerve fibre layer and occur as a result of interruption of axoplasmic flow in the ganglion cell layer,[118] (5) venous beading is caused by focal venous dilation and thinning of the vessel wall and is positively associated with the subsequent development of proliferative DR,[119] (6)

intra-retinal microvascular abnormalities describes dilation of capillary segments between arterioles and venules and occur in close proximity to areas of non-perfusion.

Proliferative DR is characterized by the presence of new vessels originating from the retina or optic nerve head and is associated with mid-peripheral retinal ischemia.[120] New vessels grow along the vitreo-retinal interface that may result in pre-retinal fibrosis, pre-retinal hemorrhage, tractional detachments of the retina and severe vision loss.[121]

Diabetic maculopathy is characterized by edematous, exudative or ischemic events, occurring within the retinal arcades. Aspects of all three types of maculopathy frequently occur concomitantly; however, individuals generally exhibit a preponderance of one particular type of maculopathy. Breakdown of the inner or outer blood retinal barrier (BRB) is associated with intra-retinal accumulation of protein, then water (as a result of oncotic pressure) and hard exudates, resulting in increased retinal thickness and disruption of neural components of the retina (producing visual impairment).[122]

Diabetic macular edema (DME) remains the most common cause of visual impairment in diabetes.[123] Over any ten year period, ninety-five thousand diabetic patients will develop sight loss due to DME.[124] Longer duration of diabetes, higher glycemic levels, severity of retinopathy as well as systemic factors such as hypertension, renal disease and / or cardiovascular disease may exacerbate DME.[117,125] DME can be sub-classified in

terms of distribution that is either focal or diffuse. Focal DME results from microaneurysms and / or dilated, leaking capillaries[126] while diffuse DME is thought to result from breakdown of the outer BRB.[117,126-128] Clinically significant DME (CSDME) is defined as retinal thickening within 500µm of the centre of the fovea, hard exudates within 500µm of the fovea associated with retinal thickening, or retinal thickening of 1500µm diameter any part of which lies within 1500µm of the fovea (Figure 1.9). Clinical trials have been undertaken to identify the point at which treatment for DME should be initiated.[129]

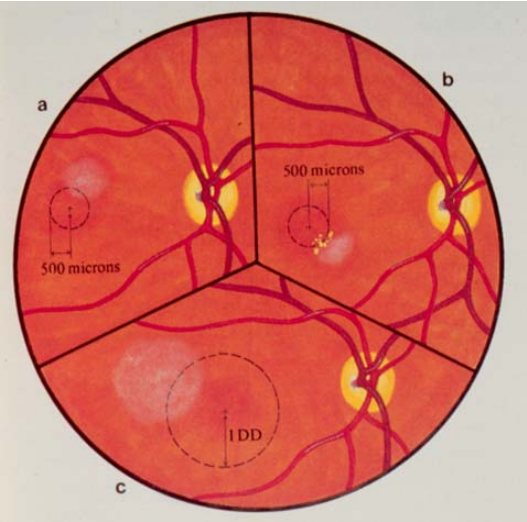

Figure 1.8 Diagrammatic representation of clinically significant diabetic macular edema

1.9.2 Retinal morphological / biochemical changes associated with hyperglycemia

Retinal endothelial cells are particularly susceptible to hyperglycemia. Glucose enters endothelial cells of the inner blood retinal barrier by glucose transporters (GLUT), specifically GLUT-1, which is thought to be exclusively responsible for the movement of

glucose in and through endothelial cells.[130,131] Although glucose transport through GLUT-1 is almost saturated at normal physiological glucose levels,[130] chronic hyperglycemia results in increased intracellular glucose concentrations that may be due to hyperglycemia itself,[132] increase in GLUT-1 density,[131] translocation of GLUT-1[133] or a novel glucose transporter.[132]

After glucose has gained entry to the cell a diverse range of pathophysiological changes take place. Regulation of blood flow is affected by alterations in the concentrations of NO and ET-1. During hyperglycemia, NO levels are reduced due to protein kinase C (PKC) activation, activation of the polyol pathway and increased superoxide production.

Endothelins are important regulators of retinal blood flow during homeostatic conditions[49,134-136] and during hyperoxia.[52] Endothelins are up-regulated in diabetes and mediate structural and functional alterations.[137,138] During hyperglycemia, ET-1 levels are increased as a result of PKC and mitogen-activated protein kinase activation.

In addition, the biochemical pathways involved in hyperglycemia induced damage include: the polyol pathway, increased production of advanced glycation end-product precursors, PKC activation and increased activity of the hexosamine pathway.[106]

Abnormal leukocyte adhesion (i.e. leukostasis) to the retinal vascular endothelial cells occurs

early in diabetic patients and animals.[139-142] Diabetes induces upregulation of adhesion molecules[142-144] and promotes vascular leakage[145-147] by upregulation of intra-cellular adhesion molecule (ICAM), vascular endothelial growth factor (VEGF) or advanced glycation end-products.[148,149] Platelet-fibrin thrombi in retinal capillaries are increased in DR secondary to chronic hyperglycemia and platelet activating factor which contributes to thrombus formation.

Pericytes facilitate the "fine control" of capillary blood flow and are responsible for endothelial cell proliferation.[150] Selective loss of pericytes occurs quite early in the disease process and affects capillary tone and capillary blood flow regulation.[151] Hyperglycemia inhibits pericyte growth and contraction and promotes apoptosis secondary to accumulation of toxic products intracellularly.[152-155]

BM thickening secondary to increased production of collagen, laminin and fibronectin by the endothelial cells and pericytes as well as glycation of proteins under hyperglycemic conditions occurs early in the disease process.[151,156] Decreased compliance of capillaries due to BM thickening may represent an early hemodynamic abnormality.

Smooth muscle cells surround the endothelial cells of arterioles and venules[157] and together with the pericytes are responsible for the maintenance of blood flow and pressure. Depletion of SM cells in diabetes occurs around the same time as pericyte loss,[158] while decreased

vessel contractibility and increased permeability of the blood retinal barrier has been demonstrated in mice deficient in smooth muscle actin (a molecule necessary for cell structural integrity).[159]

The morphological changes described above are involved in the pathogenesis of capillary closure resulting in non-perfused acellular ghost "vessels" of BM. Retinal ischemia secondary to widespread non-perfusion ensues and leads to the expression of inflammatory mediators and angiogenic growth factors and neovascularization.

One of the most important growth factors that is upregulated in DR is VEGF. This growth factor stimulates the degradation of extracellular matrix, the migration, proliferation and tube formation of endothelial cells. VEGF levels are increased in early DR adjacent to areas of hypoxia or neovascularization. Importantly, VEGF increases endothelial cell permeability by alteration of endothelial cell junctions and is therefore important in the development of DME.[160]

Formation or regression of edema is dependent on the net movement of water between the vascular and tissue compartments.[161] Hydrostatic pressure drives fluid into the tissue, oncotic pressure drives fluid out of the tissue; Starling's law states that for no net movement of fluid the hydrostatic pressure must balance the oncotic pressure. As the retinal arterioles control downstream hydrostatic pressure, increased hydrostatic pressure in capillaries and

venules will dilate according to the law of LaPlace (assuming constant oncotic pressure).[162] Nguyen et al.[163] have shown that supplemental oxygen for 3 months reduced retinal thickness substantially in patients with DME presumably secondary to vasoconstriction and decreased hydrostatic pressure (Figure 1.10)

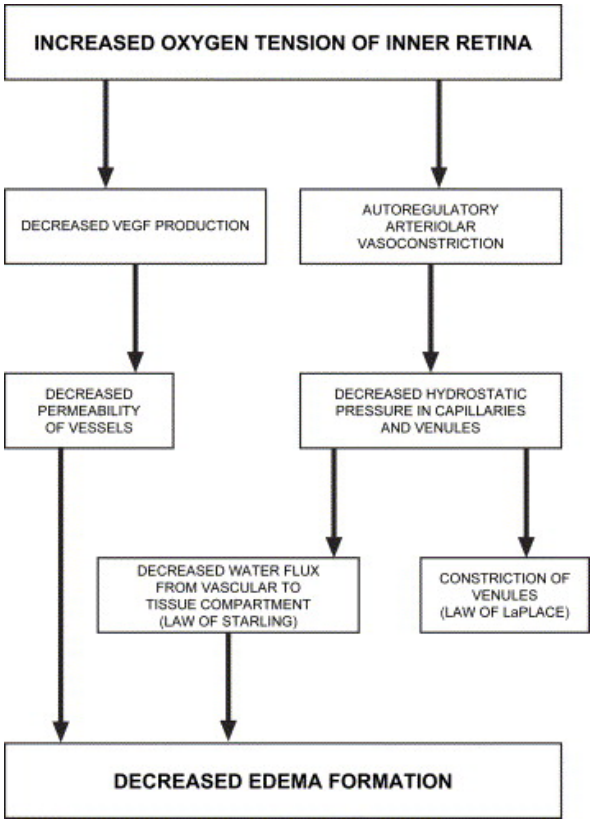

Figure 1.9 Increased retinal oxygenation decreases edema formation by vasoconstriction and reduced VEGF production

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1.10 Clinical detection and monitoring of DME

Stereo-fundus slit lamp biomicroscopy (using a high powered plus lens e.g. 66D, 78D or 90D after pupillary dilation) has traditionally been the *clinical* "gold standard" technique for the detection of DME.[164] This technique relies upon the subjective interpretation of the thickness of the transparent retina.[165,166] Consequently, the clinical estimation of the extent and location of retinal thickening of a given patient is highly variable between examiners and is also highly variable between visits for a single examiner.[167]

1.10.1 Stereo-fundus photography

Stereo-fundus photography (SFP) is regarded as the "gold standard" for the *documentation* of macular edema and was used by the Early Treatment Diabetic Retinopathy Study (ETDRS).[129,166,168] The acquisition of quality images (from which stereoscopic information is available) is affected by pupil dilation, media opacities, and patient co-operation. Stereo images are viewed and the area determined to exhibit thickening is demarcated. Recently published studies have assessed the agreement between SFP and fundus biomicroscopy as well as newer technologies to objectively assess retinal thickness (Retinal Thickness Analyzer (RTA) and Optical Coherence Tomography (OCT)). SFP determination of retinal thickness in terms of location and severity (area of retinal involvement) agreed very favorably with fundus biomicroscopy[169] and OCT[170] but less well with RTA.[171]

1.10.2 Fluorescein angiography

Breakdown of the inner BRB allows transport of electrolytes and plasma into the intra-retinal space, resulting in DME.[172] In patients with diabetic maculopathy, fluorescein angiography (FA) is used to identify areas of capillary non-perfusion (i.e. ischemic maculopathy for which laser treatment is ineffective), cystoid macular edema (i.e. a severe form of edema that typically does not respond well to laser treatment), and identify areas of diffuse or focal leakage.[172-177] One of the limitations of this technique is that FA identifies capillary leakage which may or may not correspond with the location of retinal thickening.[178-180]

1.10.3 Psychophysical detection of DME

A number of psychophysical techniques exist that have been used to detect pre-clinical changes that predict the ultimate development of sight-threatening DR and DME and are described in the following sections.

1.10.4 Color discrimination tests

Selective loss of S-cones has been documented early in diabetes[181-183] that precedes VA loss and is correlated with the presence of sight threatening DR.[184] The foveal S-cone free area is increased in size in diabetes[185] and anomalascope errors are positively correlated with

increasing vascular abnormalities.[186] Reduced short-wavelength cone sensitivity is related to severity of DR.[187]

1.10.5 Short-wavelength automated perimetry

Short-wavelength automated perimetry (SWAP) uses a blue stimulus to preferentially stimulate the SWS visual pathway and a high luminance yellow background to saturate both medium- and long-wavelength sensitive pathways and simultaneously suppress rod activity.[188] A selective loss of short-wavelength (SW) sensitive pathway sensitivity has been demonstrated in patients with no, or minimal retinopathy.[189-194] Indeed, SW field loss is correlated with severity of retinopathy[195] and precedes white-on-white (WW) field loss in DR and maculopathy[196-199] and predicts the development of DME with high sensitivity and specificity suggesting functional loss precedes morphological abnormalities.[196] SWAP is also a useful tool to detect early macular ischemia associated with DR.[198] Although the application of SWAP for the detection of diabetic maculopathy is far more favorable than that of the detection of glaucoma, the inherent variability of the SWS visual pathway is a limiting factor in the clinical application of this technique.[200] Other limitations associated with SWAP include greater long term fluctuation, crystalline lens and macular pigment absorption, longer examination duration and pronounced fatigue effects.[201]

1.11 Imaging technologies for the non-invasive quantification of DME

1.11.1 Optical Coherence Tomography

Optical Coherence Tomography (OCT) provides high resolution cross sectional images of the retina.[202] The longitudinal resolution has been reported to be 10-20μm[203] while the lateral resolution is 25μm.[204] Technically, partially coherent near-infrared light (843nm) is split by an interferometer into a "probe" and "reference" beam.[205] After reflection from the retina, the "probe" beam interferes with the "reference" beam to give retinal thickness and distance information. Processing of the cross sectional scan pseudo-corrects for eye movements before it is displayed in a false color representation. "Warm" colors (red to white) represent structures of high reflectivity, whereas "cool" colors (blue to black) represent structures of low reflectivity. Normal thickness of the fovea is approximately 170μm.[206,207] Published studies have demonstrated retinal thickening in diabetic patients without clinically evident DME[208-211] and in patients with DME,[210-213] the locus of the retinal thickening being primarily in the outer layers of the retina.[203] Retinal structural changes in DME have been described using OCT.[214] Sensitivity and specificity for the detection of DME in the central fovea is 89% and 96%, respectively.[215] Comparison studies with microperimetry,[216] multi-focal electro-retinograms,[217] FA characteristics[218] and stereo fundus photography[170,219] in patients with DME have been undertaken. The limitations of this technique are that image quality is degraded by media opacities and depends on operator technique[220]. Also, longitudinal analysis of change is weakened since it is impossible to register and align successive images and the

image processing algorithms within OCT are unable to truly correct for eye movement.

1.11.2 Retinal Thickness Analyzer

The Retinal Thickness Analyzer (RTA) comprises a laser slit biomicroscope and digital camera attached to an ophthalmic table, a patient headrest, and a personal computer. In brief, a green helium-neon laser light of 543 nm wavelength is scanned across the retina to produce 16 discrete slit images within a 3 mm x 3 mm area of retina. The reflected slit images are recorded digitally. Retinal thickness is derived from the separation between the anterior (that is, at, or close to, the internal limiting membrane, ILM) and posterior (that is, at, or close to, the retinal pigment epithelium, RPE) reflectance interfaces[221] for 16 points along each slit using densitometry). Consequently, the derivation of retinal thickness is dependent upon the clarity of the RTA derived slit image. Patient fixation is aided by means of an internal fixation target that can be moved. Depth resolution and depth precision are reported to be 5–10 μm and 50 μm, respectively.[222] A more detailed explanation of the RTA optical principles has been described elsewhere.[222-225] The repeatability of scan thickness is poorest in the central foveal region[226] and the scan quality is reduced in the presence of lenticular opacities.

The RTA has been used in patients with diabetes to determine the sensitivity and specificity of detecting DME. In comparison studies with OCT, the RTA has been reported to be more specific for the detection of DME.[219] Others have reported that agreement between fundus

biomicroscopy assessment of patients with DME was better with the macular edema module (MEM) of the Heidelberg retinal tomograph (HRT) compared to RTA.[227]

1.11.3 Scanning laser tomography (SLT)

The description of the SLT technique in this thesis is more detailed than that of OCT or RTA because we have used the technique in the study protocol. SLT is a non-invasive technique for topographical assessment of the retina using confocal imaging. A two-dimensional optical section image is produced at the focal plane of the instrument while scattered light is suppressed using a pinhole. The process is repeated for sequential planes of focus along the optical axis (at more posterior positions). This image series is corrected for minor eye movements that occur during image acquisition and the sections are aligned to make a composite image from which retinal topography can be determined. Reflectance as a function of depth for each pixel element is calculated according to the following formula:

$$\text{Relative intensity} = (\text{Measured intensity} / \text{maximum intensity}) * 100 \qquad (1.11)$$

A z-profile is generated when relative intensity is plotted as a function of scan depth. The peak of the z-profile is principally determined by refection occurring at the vitreous / ILM interface.

In normal retina, the z-profile is narrow and symmetrically distributed but is broadened in areas of retina thickening. In DME, the z-profile signal width is correlated with the magnitude of retinal thickening.[228] Z-profile signal width is determined at 50% of the maximum relative intensity. Variation of reflectance intensity between successive images is reduced by employing a normalization procedure.[228] In addition, maximum reflectance intensity is reduced in areas of retinal edema.[96,228]

In the MEM of the HRT I and II, edema index values are determined using:

Edema index = Normalized signal width / Maximum reflectance intensity[229] (1.12)

The units of the macular edema module are therefore arbitrary. Edema index evaluation objectively quantifies DME and has been shown to have high sensitivity and specificity for the detection of DME.[227] The edema index has been shown to correlate with sensitive measures of visual function including SWAP and VA.[230] In addition, Kisilevsky and co-workers[231] investigated the agreement between the HRT MEM and fundus biomicroscopy assessment ("clinical gold standard"), FA and SWAP in patients with and without clinically manifest DME using a sector analysis procedure. MEM demonstrated very good agreement with fundus biomicroscopy assessment and good agreement with FA and SWAP. In addition,

many sectors flagged as being abnormal by MEM but not by fundus biomicroscopy assessment were also flagged as being abnormal by FA and / or SWAP implying that structural or functional abnormalities are occurring in retinal areas that are not yet clinically visibly thickened using fundus biomicroscopy. Using MEM findings with FA and SWAP results allows pre-clinical detection of DME and therefore improved diagnostic test performance.

1.12 Treatment

1.12.1 Laser

Laser photocoagulation is the treatment of choice for focal and diffuse DME as well as for proliferative DR.[129,168,232-235] In DME, it has been shown to result in the long-term stabilization of, or in a small percentage of cases improvement in, visual acuity (VA) relative to untreated eyes,[129,168] is cost effective[236] and offers improved quality-of-life for patients.[237] However, a single session of laser stabilizes VA in approximately only 60% of patients with many requiring repeat laser sessions.[129,168,238,239] Diffuse DME can be difficult to treat with laser photocoagulation since some cases remain unresponsive.[240] The side-effects associated with laser include post laser scotomata,[241,242] altered retinal function,[243] enlarging atrophic laser scars,[244] subretinal neovascularization and fibrosis.[245]. Recent studies have tested the use of "lighter" or "sub-threshold" treatment and micropulse laser,[246] to reduce post laser retinal scarring and loss of visual sensitivity.[247-250] Generally, laser only stabilizes VA rather than

improving it in the majority of cases. Due to its destructive nature, other pharmacological treatment modalities have been developed (described below).

1.12.2 Pharmacological Treatment

Current pharmacological treatments and drugs for future development include aldose reductase inhibitors,[251-254] AGE inhibitors,[255-259] antioxidants,[255,260-262] supplemental O_2,[163,263,264] growth factor modulators including VEGF inhibitors,[265-271] PKC inhibitors,[272-274] corticosteroids,[275-279] or non-steroidal anti-inflammatory drugs.[280,281]

Potential targets for treatment of DME include reduction of blood retinal barrier breakdown with steroids,[282] anti-VEGF factors,[265] and PKC inhibitors.[283] The major complications associated with these treatments include cataracts, ocular hypertension and endophthalmitis.[284-286]

1.12.3 Vitrectomy

Increased macular thickness secondary to refractory DME may be improved with vitrectomy (surgical removal of the vitreous).[287] Vitrectomy is also an effective treatment in patients with neovascularization.[288-290] In both cases, removal of the vitreous allows increased retinal oxygenation by diffusion and therefore VEGF production is downregulated.[162]

1.13 Summary

DME is the most common cause of visual impairment in diabetes. Hyperglycemia induced cellular damage and vascular alteration has been described and results in the formation of DME. A number of techniques have been developed that allow quantification of DME. Among these, SWAP and MEM have been shown to allow pre-clinical detection of DME and offer improved diagnostic performance. Early and precise identification of the location of retinal thickness in conjunction with effective, non-destructive treatment may prevent vision loss in patients with DME in the future. New treatment options are becoming available for the treatment of DR and DME, however it is important that they are initiated early enough to prevent or slow the progression of retinopathy i.e. prior to the development of significant pathological changes. Quantification of the time course and or clinical features associated with impairment of vascular reactivity will permit a fuller understanding of the pathophysiology of DR and DME.

1.14 Retinal hemodynamics in patients with diabetes

Studies investigating change in retinal blood flow in patients with diabetes (relative to that of non-diabetic subjects) show conflicting results. Patients with no DR have been shown to exhibit increased,[87,291-293] decreased,[294-296] or no change[87,297-299] of retinal blood hemodynamics. Patients with non-proliferative DR have been reported to exhibit

increased,[292,293,297,300,301] decreased,[302-304] or no change[292,298,299] of retinal blood hemodynamics. Patients with proliferative DR have been shown to exhibit increased[293] or decreased[305] retinal blood hemodynamics.

Progression of retinopathy has also been investigated. One study detailed increased blood flow in patients progressing from mild-to-moderate non-proliferative DR to severe DR,[306] whilst another study found a bimodal relationship between retinopathy status and blood flow i.e. initially decreasing flow followed by increasing flow as retinopathy progressed.[296,307]

The disparity of the results in the studies described above may be partly attributable to different techniques used to measure retinal hemodynamics, patients with differing morphological and / or glycemic characteristics and the utilization of different retinal locations and vessels.

1.15 Vascular reactivity in diabetes

1.15.1 Physiological vascular reactivity provocation

Vascular reactivity is "the ability of a tissue to regulate its blood flow according to its metabolic needs". Administration of oxygen (O_2) and glucose have previously been used to provoke retinal vascular reactivity. Retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant

level. Most previously published retinal vascular reactivity studies have employed gas delivery systems that utilize a reservoir bag and one-way valves to essentially negate the mixing of inspired and expired gases i.e. a non-rebreathing system. Hyperoxia typically stimulates hyperventilation (faster or deeper respiration) that results in an uncontrolled and variable reduction of the partial pressure of arterial carbon dioxide (PCO_2).[308,309] Any reduction of PCO_2 will produce an exaggerated vasoconstrictive effect on retinal vasculature. A number of authors[310-314] have attempted to correct for the reduction in PCO_2 during hyperoxia by manually adding CO_2 to the inspired gases of the non-rebreathing system. Impairment of retinal vascular reactivity to hyperoxia in DR has been demonstrated previously.[139,315-317]

Published studies investigating the retinal vascular response to hyperglycemia are conflicting. Some authors have reported increased retinal blood flow,[91,294,304,318] while others report no change in retinal blood flow[319,320] in response to elevated blood glucose.

The disparity of the results may be partly attributable to different techniques used to measure retinal hemodynamics, patients with differing morphological and / or glycemic characteristics and the utilization of different retinal locations and vessels as well as the use of non-standardized hyperoxia and glucose stimuli.

1.16 Conclusion

The blood supply to the eye is well documented. The retina is anatomically and functionally unique. Retinal blood flow is believed to be efficiently autoregulated and endothelial derived vasoactive factors are important determinants of retinal blood flow. Blood in the retinal vessels exhibits laminar flow.

Many instruments quantify aspects of retinal hemodynamics. Previously published studies are conflicting regarding the influence of diabetes and on retinal blood flow. The Canon Laser Blood Flowmeter permits the non-invasive quantification of retinal blood flow in real units and presents a novel opportunity to quantify retinal hemodynamic alterations induced by diabetes. In addition, the time course between the development of clinically visible DR and alteration of retinal hemodynamics remains to be elucidated. Previously published studies that have assessed vascular reactivity have not used a standardized hyperoxic stimulus.

Hyperglycemia initiates the biochemical and morphological alterations observed in DR. Strict control of blood glucose levels may delay the development of DR. Retinal perfusion abnormalities may precede the morphological changes in DR. The influence of short-term glycemic control has yet to be determined.

DME is a common cause of visual impairment in patients with DR. Objective techniques for

the quantification of DME have been developed that allow earlier detection and more precise localization of DME. The assessment of retinal hemodynamics in individuals with DME may improve understanding of the pathophysiology of DME.

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2 Rationale

Disturbance of retinal hemodynamics is a surrogate marker of early diabetic retinopathy (DR). Previously published studies are conflicting in terms of the reported change of blood flow attributable to early diabetes. The apparent disparity of these results may be partly attributable to the different techniques used to measure retinal hemodynamics, the inclusion of patients with differing morphological and varying glycemic control characteristics and the use of differing sites to measure blood flow. Many studies have measured either change in diameter or velocity but have been unable to quantify change in flow. Assessment of retinal vascular reactivity (i.e. the vascular response to a given stimulus) has previously been investigated using a variety of instruments and stimuli in patients with diabetes. Previously published studies that have employed an inspired oxygen stimulus in human subjects were unable to maintain homeostatic concentrations of carbon dioxide (CO_2) during hyperoxia. Maintenance of baseline concentrations of CO_2 is termed isocapnia. Alteration of CO_2 alters vascular tone and has a profound impact on retinal blood flow.

The global aim of this work was to assess retinal vascular reactivity in healthy volunteers and groups of patients with diabetes with mild-moderate DR using standardized blood flow assessment and a standardized hyperoxic stimulus. The Canon Laser Blood Flowmeter (CLBF-100) was employed to assess retinal arteriolar hemodynamics. It allows the simultaneous quantification of vessel diameter and centre-line blood velocity to calculate volumetric retinal blood flow in microlitres per minute. In addition, a defined retinal arteriolar

measurement site was employed to acquire blood flow measurements. Furthermore, a unique isocapnic hyperoxic stimulus for the assessment of vascular reactivity was developed. The combination of a standardized blood flow assessment technique and a standardized stimulus to provoke change in retinal hemodynamics, when applied to carefully defined volunteer groups, was anticipated to reveal greater understanding of the physiology of blood flow regulation and its disturbance in DR.

The first aim of this thesis was to detail three different techniques to administer hyperoxia and compare between them in terms of their ability to maintain isocapnia during hyperoxia. The techniques included administration of O_2 only using a non-rebreathing system, the administration of O_2 with added CO_2 using a non-rebreathing system (with CO_2 flow continually adjusted) and the administration of O_2 using a sequential rebreathing system (with O_2 flow set equal to the subjects' minute ventilation). (Chapter 3) The hypothesis was that the sequential rebreathing system would be superior to the other systems and would result in smaller variation of systemic carbon dioxide levels. In addition, a perturbation of $P_{ET}CO_2$ (concentration of CO_2 in the expired breath) was used to determine the influence of change of $P_{ET}CO_2$ on retinal hemodynamics.

Having developed a standardized isocapnic hyperoxic stimulus, the second aim of the thesis was to define the magnitude of change and response characteristics of retinal hemodynamics in a group of young clinically normal subjects. Retinal arteriolar diameter and blood velocity

measurements were acquired every minute over the course of the study using the CLBF-100 while each subject breathed air, followed by isocapnic hyperoxia, and finally air again. The magnitude of change in flow and the recovery to baseline values was characterized for each individual. Change in diameter and velocity relative to the onset of the stimulus and to each other was determined for each individual to allow investigation of the retinal physiological response to oxygen. (Chapter 4) The hypothesis was that administration of hyperoxia via the sequential rebreathing system would produce a pronounced reduction of blood flow that would return to baseline values after the stimulus was removed.

The third aim of the thesis was to detail the magnitude of change in retinal hemodynamics to isocapnic hyperoxia in a group of diabetic patients with mild-to-moderate DR stratified by their retinopathy status and in age matched subjects without diabetes. Isocapnic hyperoxia was administered using the sequential rebreathing technique that was validated in previous experiments. Retinal hemodynamic measurements were acquired during air breathing and during isocapnic hyperoxia using the CLBF-100. The magnitude of change in arteriolar diameter, blood velocity, blood flow, maximum-to-minimum velocity ratio and wall shear rate was calculated and compared between the groups. Analysis of the differences in responses of the groups will provide insight into the pathophysiology of early DR (Chapter 5). The hypothesis was that the magnitude of the vascular reactivity would be impaired in those individuals with more extensive DR.

Previously published studies that have employed a glucose stimulus show conflicting results in terms of its impact upon retinal hemodynamics. The fourth aim of the thesis was to detail the change in retinal hemodynamics to a standardized oral glucose load drink in a group of diabetic patients with mild-to-moderate DR stratified by their retinopathy status and in age matched subjects without diabetes. The stimulus employed was the same as that used to determine oral glucose tolerance in those who may have symptoms of diabetes or fasting blood glucose levels outside the normal range. Retinal hemodynamic measurements were acquired using the CLBF-100 prior to, and 1 hour after glucose drink ingestion. The magnitude of change in arteriolar diameter, blood velocity, blood flow, maximum-to-minimum velocity ratio and WSR was calculated and compared between the groups. Alteration of blood hemodynamics during acute hyperglycemia would necessitate the determination of blood glucose levels prior to assessment of retinal hemodynamics (Chapter 6). The hypothesis was that hyperglycemia would result in an increase of retinal blood.

Previously published studies that have employed a combined hyperglycemic / hyperoxic provocation also show conflicting results. The fifth aim of the thesis was to detail the change in retinal hemodynamics to a combined hyperglycemic / hyperoxic provocation in a group of diabetic patients with mild-to-moderate DR stratified by their retinopathy status and age matched subjects without diabetes. The stimuli employed were identical to that developed in previous chapters. Retinal hemodynamic measurements were acquired using the CLBF-100 prior to, and 1 hour after, glucose ingestion. The magnitude of change in arteriolar diameter,

blood velocity, blood flow, maximum-to-minimum velocity ratio and WSR was calculated and compared between the groups. Alteration of the vascular reactivity response to isocapnic hyperoxia during acute hyperglycemia would necessitate the determination of blood glucose levels prior to assessment of retinal vascular reactivity using isocapnic hyperoxia (Chapter 7). The hypothesis was that glucose would impair the vascular reactivity response to hyperoxia.

3 Comparison of different hyperoxic paradigms to induce vasoconstriction - Implications for the investigation of retinal vascular reactivity

Gilmore ED, Hudson C, Venkataraman ST, *et al*. Comparison of different hyperoxic paradigms to induce vasoconstriction: implications for the investigation of retinal vascular reactivity. *Investigative ophthalmology & visual science* 2004;**45**(9):3207-3212.

	Concept / Design	Recruitment	Acquisition of data	Analysis	Write-up / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
Venkataraman			Y		
Preiss					Y
Fisher					Y

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

3.1 Abstract

Purpose: To compare 3 different techniques used to induce hyperoxia. The impact of each technique on end-tidal CO_2 ($P_{ET}CO_2$) was assessed since change in $P_{ET}CO_2$ will confound the interpretation of the vascular response to manipulation of the partial pressure of O_2 ($P_{ET}O_2$).

Methods: The sample comprised 9 subjects (mean age 25 years; range 21-49 yrs). Each subject attended for 3 sessions. At each session, subjects initially breathed air followed by O_2 only, or O_2 plus CO_2 using a non-rebreathing circuit (with CO_2 flow continually adjusted to negate "drift" of $P_{ET}CO_2$), or air followed by O_2 using a sequential re-breathing circuit (with O_2 flow set equal to the subjects' alveolar ventilation). O_2 and CO_2 concentrations in the inspired and expired breath were compared across the different techniques.

Results: The difference in group mean $P_{ET}CO_2$ values between baseline and elevated O_2 breathing was significantly different (*t*-test, p=0.0038) for O_2 only administration using a non-rebreathing system but not for the other two techniques. The sequential rebreathing technique resulted in a significantly lower difference (i.e. pre- and during hyperoxia) of individual $P_{ET}CO_2$ values (*t*-test, p=0.0317) than either of the other two techniques.

Conclusion: The sequential rebreathing technique resulted in a reduced variability of $P_{ET}CO_2$ values compared to the two techniques using a non-rebreathing system. In turn, the sequential rebreathing technique should result in reduced variability of retinal blood flow values and thereby reduce the required sample size of blood flow studies.

Keywords: Hyperoxia, isocapnia, oxygen, carbon dioxide, vascular reactivity

3.2 Introduction

Administration of oxygen (O_2) has previously been used as a stimulus to provoke retinal vascular reactivity. Vasoconstriction of retinal vessels[1,2] and the resulting reduction of retinal blood flow[3-16] has been demonstrated using a variety of measurement techniques, including laser Doppler and blue-field entoptic phenomena. Retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant level[16]; however, retinal blood flow also varies directly with the partial pressure of arterial carbon dioxide (PCO_2).[12] The change of end-tidal CO_2 concentration ($P_{ET}CO_2$; the maximum concentration of CO_2 during each expiration) reflects the change in arterial PCO_2.[17] Indeed, CO_2 is thought to represent a more potent vasoactive agent than O_2.[7] Change of retinal perfusion, measured using laser Doppler blood flow techniques, induced by perturbation of O_2 or CO_2 can be used to provide a measure of the magnitude of retinal vascular reactivity.

All previously published retinal vascular reactivity studies have employed gas delivery systems that utilize a reservoir bag and one-way valves to essentially negate the mixing of inspired and expired gases i.e. a non-rebreathing system. Hyperoxia typically stimulates hyperventilation (faster or deeper respiration), however, this results in an uncontrolled and variable reduction of PCO_2.[18,19] Any reduction of PCO_2 will produce an exaggerated vasoconstrictive effect on retinal vasculature. The vasoconstrictive effect previously attributed to O_2 when administered by non-rebreathing circuits likely represents the combined effect of elevated PO_2 and reduced PCO_2. Harris et al.,[16,20,21] Roff et al.[14] and Chung et al.[15] have

recognized this potential artifact and have attempted to correct for the reduction in PCO_2 during hyperoxia by adding CO_2 to the inspired gases of the non-rebreathing system.[14-16,20,21] The maintenance of homeostatic PCO_2 is termed "isocapnia". Another method to prevent reduction of PCO_2 involves the use of a sequential rebreathing circuit that provides a feedback loop to compensate any hyperventilation induced reduction in PCO_2.[22] This system has the advantage that it passively adjusts the inspired CO_2 to the minute ventilation in order to stabilize PCO_2.

The magnitude of change and the variability of $P_{ET}CO_2$ need to be quantified and compared across the various techniques used to induce hyperoxia. Three different techniques were compared i.e. administration of O_2 only using a non-rebreathing system, O_2 with added CO_2 using a non-rebreathing system (with CO_2 flow continually adjusted to negate "drift" of $P_{ET}CO_2$) and O_2 using a sequential rebreathing system (with O_2 flow set equal to the subjects' minute ventilation). In addition, the relationship between change in $P_{ET}CO_2$ and retinal blood flow was assessed to determine the clinical research relevance of this parameter.

3.3 Materials and Methods

3.3.1 Sample

The study received approval by the University of Waterloo Office of Research Ethics. 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 comprised 4 males and 6 females of average age 25 years (range 21-49 years). In order to determine the relationship between $P_{ET}CO_2$ and retinal blood flow, a second sample of 6 males and 2 females of average age 26.5 years (range 24-36 years) was subsequently recruited. Subjects with any cardiovascular or respiratory disorders were excluded from the study.

3.3.2 Procedures

Each subject attended for 3 sessions of approximately 30 minutes each. The group mean number of days between each of the 3 sessions was 11 days. At each session, subjects initially breathed air followed by O_2 only or O_2 plus CO_2 using a non-rebreathing system, or compressed air followed by O_2 using a sequential rebreathing system. Each gas condition (air or O_2) was administered for 15 minutes. The non-rebreathing system comprised a silicone mouthpiece and two low resistance one-way valves (Figure 3.1) connected to the gas supply via a reservoir bag. The sequential rebreathing system comprised fresh gas and rebreathed gas reservoirs that were interconnected by two one-way valves and a single peep valve (Figure 3.2). It was assembled by adding a gas reservoir to the expiratory port of a commercial 3-valve oxygen delivery system (Hi-Ox^{80}, ViasysHealthcare, Loma Linda, CA). For the purpose of this study, a silicone mouthpiece was attached to the sequential rebreathing system that, in turn, was connected to the gas supply. For both systems, flow from the gas tanks was controlled using standard rotometers as flowmeters. O_2 and CO_2 were mixed in a baffled

container prior to being administered to the subject via the non-rebreathing circuit.

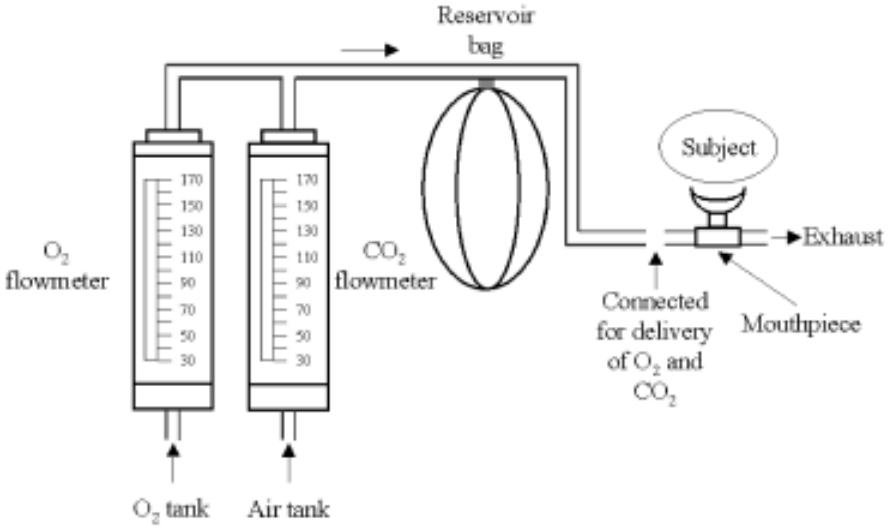


Figure 3.1 Schematic diagram showing the components of the non-rebreathing system

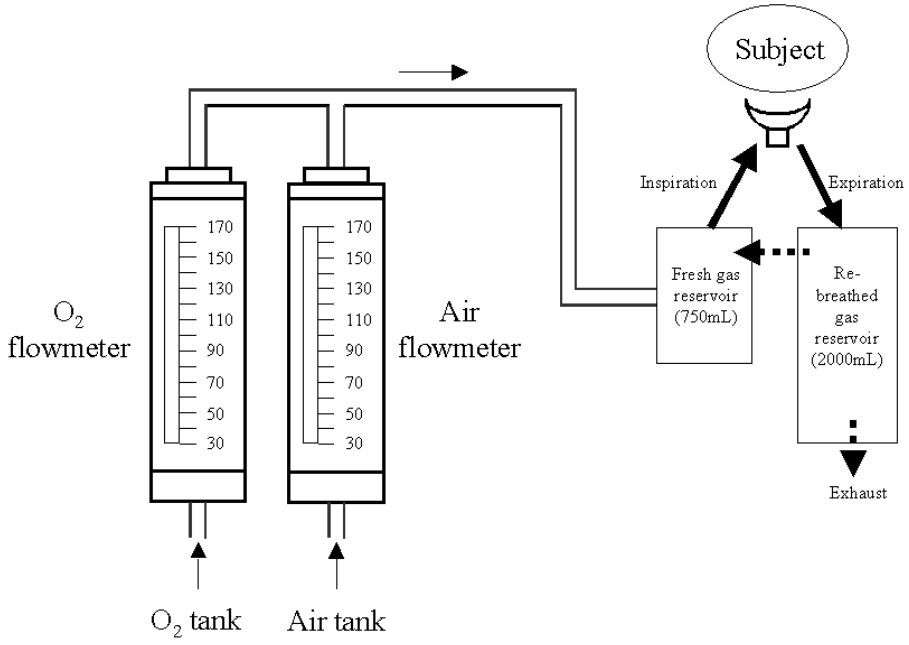


Figure 3.2 Schematic diagram showing the components of the sequential rebreathing system

Each subject was seated for 5 minutes prior to commencing the study. For every situation, an initial air breathing period was employed to allow stabilization of baseline breathing parameters e.g. respiration rate. For the O_2 plus CO_2 using a non-rebreathing system (Figure 3.1), CO_2 flow was continually adjusted to negate "drift" of $P_{ET}CO_2$. For the O_2 using a sequential rebreathing system (Figure 3.2), O_2 flow was set equal to the subjects' minute ventilation (determined while breathing air).

In order to determine the impact, if any, of perturbation of $P_{ET}CO_2$ on retinal blood flow, the sequential rebreathing system was utilized to manipulate $P_{ET}CO_2$ while quantifying retinal blood flow with the CLBF. A steady state perturbation of $P_{ET}CO_2$ was produced since the time between $P_{ET}CO_2$ fluctuation and its impact upon retinal hemodynamics is unknown and since the CLBF does not provide a continuous measurement of retinal blood flow. A methodology that initially raised $P_{ET}CO_2$ values and then returned to homeostatic levels was employed. Following stabilization of cardiovascular and respiratory parameters, air flow delivered to the subject via the sequential rebreathing system was reduced to elevate $P_{ET}CO_2$ by approximately 5mmHg (i.e. volunteers were compelled to rebreath). At this point in time, a minimum of 10 CLBF readings was acquired. Air flow was subsequently returned to baseline levels and a further 6 CLBF readings were acquired.

3.3.3 Data acquisition and analysis

Tidal gas concentrations were continuously sampled from the mouthpiece using a rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA). In addition, hemoglobin oxygen saturation via pulse oximetry, respiratory and pulse rate were also continually recorded. All data outputs were downloaded to an electronic data acquisition system (S5 Collect, Datex-Ohmeda, USA). Data was analyzed using box plots that depicted the median, upper 25^{th} and lower 75^{th} percentiles, SD and outliers of inspired- and end-tidal gas concentrations. Data points lying outside the upper 25^{th} or lower 75^{th} percentiles were excluded from the analysis since all of these values were found to be erroneous i.e. these

points resulted from inappropriate interpretation of tidal waveforms by the gas monitor.

Group mean inspired and expired O_2 (F_IO_2 and $P_{ET}O_2$, respectively), inspired and expired CO_2 (F_ICO_2 and $P_{ET}CO_2$, respectively) and respiration rates (RR) as a function of delivery system are shown in Table 3.1. The group mean F_IO_2 was greater than 90% for all techniques.

	Inspired O_2 (SD)		Expired O_2 (SD)		Inspired CO_2 (SD)		End-tidal CO_2 (SD)		Heart rate (beats/min) (SD)	
	Air (%)	O_2 (%)	Air (%)	O_2 (%)	Air (%)	O_2 (%)	Air (%)	O_2 (%)	Air	O_2
Pure O_2	20.33 (0.05)	91.51 (2.27)	14.73 (0.56)	81.92 (7.06)	0.07 (0.07)	0.07 (0.08)	5.42 (0.19)	5.17 (0.19)	72.96 (4.00)	66.96 (4.55)
O_2+CO_2	20.28 (0.14)	92.06 (1.01)	14.82 (0.68)	82.19 (3.16)	0.08 (0.12)	0.97 (0.28)	5.34 (0.16)	5.29 (0.17)	77.03 (5.77)	71.39 (7.03)
O_2 (SRB)	19.91 (0.46)	93.50 (2.48)	15.30 (0.63)	87.21 (5.82)	0.42 (0.33)	0.74 (0.39)	5.13 (0.16)	5.07 (0.13)	76.91 (4.51)	70.54 (5.58)

Table 3.1: Group mean and SD of inspired O_2, expired O_2, Inspired CO_2, end-tidal CO_2, and heart rate as a function of technique.
(SD - standard deviation; SRB - sequential rebreathing).

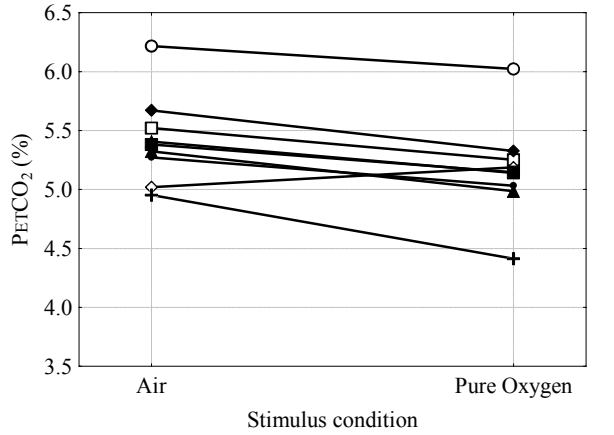
3.4 Results

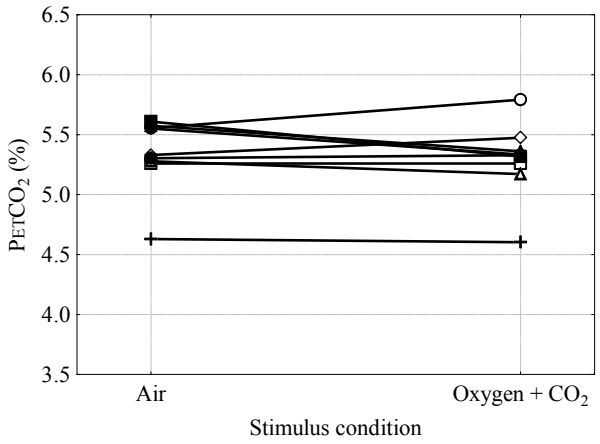
The *group mean* difference of $P_{ET}CO_2$ (and SD) between baseline and elevated O_2 breathing for each of the three techniques is shown in Table 3.2. Figure 3.3 shows $P_{ET}CO_2$ for each *individual* at baseline (i.e. air) and during hyperoxia for each of the three techniques.

P_{ET} CO_2 (%)	Pure O_2	O_2 + CO_2	SRB
Group Mean Difference (O_2-Air)	-0.21	-0.06	-0.06
Group Mean SD	0.24	0.17	0.08

Table 3.2: Group mean difference in $P_{ET}CO_2$ between baseline and oxygen breathing using 3 different techniques (SRB – sequential rebreathing; SD – standard deviation).

For O_2 breathing only using a non-rebreathing system, *group mean* $P_{ET}CO_2$ reduced from 5.32% (SD 0.18) at baseline to 5.06% (SD 0.18) during hyperoxia (Figure 3.3a). For O_2 with added CO_2 using a non-rebreathing system, *group mean* $P_{ET}CO_2$ was 5.34% (SD 0.16) at baseline and 5.29% (SD 0.17) during hyperoxia (Figure 3.3b). For a sequential rebreathing system, *group mean* $P_{ET}CO_2$ was 5.13% (SD 0.15) at baseline and 5.07% (SD 0.13) during hyperoxia (Figure 3.3c).





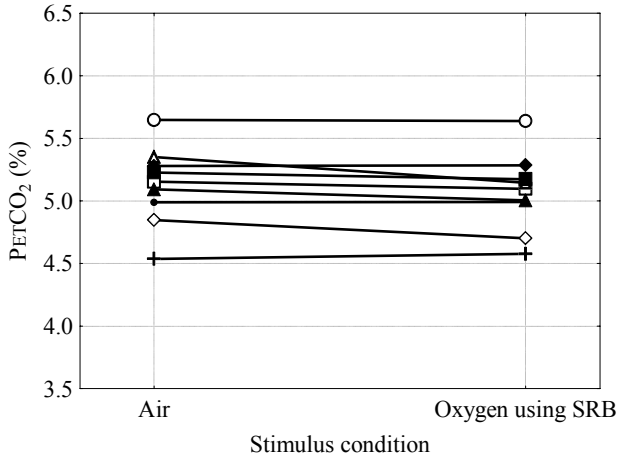
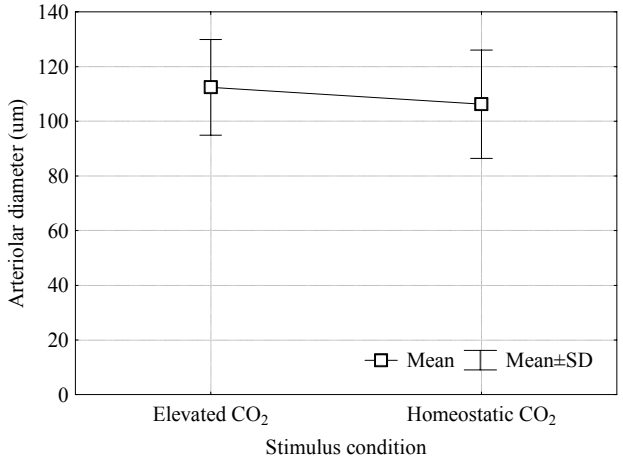


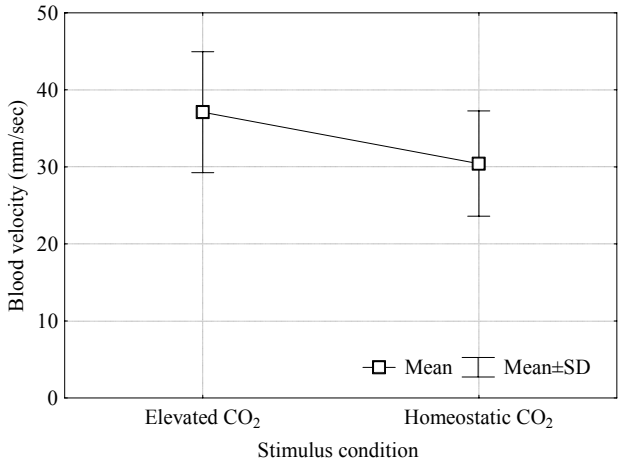
Figure 3.3 Change in end-tidal CO_2 for each individual using (A) pure O_2 delivered by a non-rebreathing system, (B) O_2 with added CO_2 delivered by a non-rebreathing system and (C) O_2 delivered via a sequential rebreathing system.

The difference in *group mean* $P_{ET}CO_2$ values between baseline and hyperoxia was significantly different for O_2 only and a non-rebreathing system (*t*-test, p=0.0038) but not for the other two techniques.

The sequential rebreathing technique resulted in a significantly lower difference (i.e. a smaller difference between baseline and during hyperoxia) of *individual* $P_{ET}CO_2$ values (as reflected in the SD values, Table 3.2) than either of the other two techniques (*t*-test, p=0.0008 and p=0.0317 for O_2 only and O_2 with added CO_2, respectively).

The group mean difference in blood flow between the elevated (group mean 5.69%, SD 0.44) and homeostatic (group mean 5.03%, SD 0.59) $P_{ET}CO_2$ conditions was 0.66%, SD 0.21 i.e. a 5.00mmHg (SD 1.58mmHg) change. This perturbation of $P_{ET}CO_2$ resulted in a significant reduction (i.e. in response to a lowering of $P_{ET}CO_2$) in retinal arteriolar diameter, blood velocity and blood flow of 6.18 μm ($p<0.0050$), 6.68 mm/sec (p=0.0005), and 3.04 uL/min ($p<0.0005$), respectively (Figure 3.4).





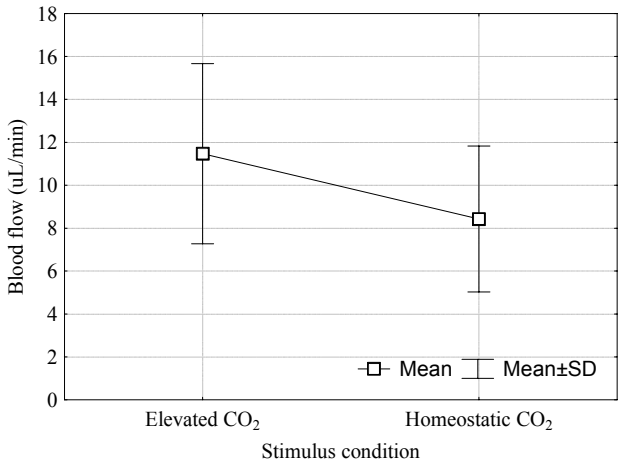


Figure 3.4 Change in (A) arteriolar diameter, (B) blood velocity and (C) flow induced by a change in end-tidal CO_2

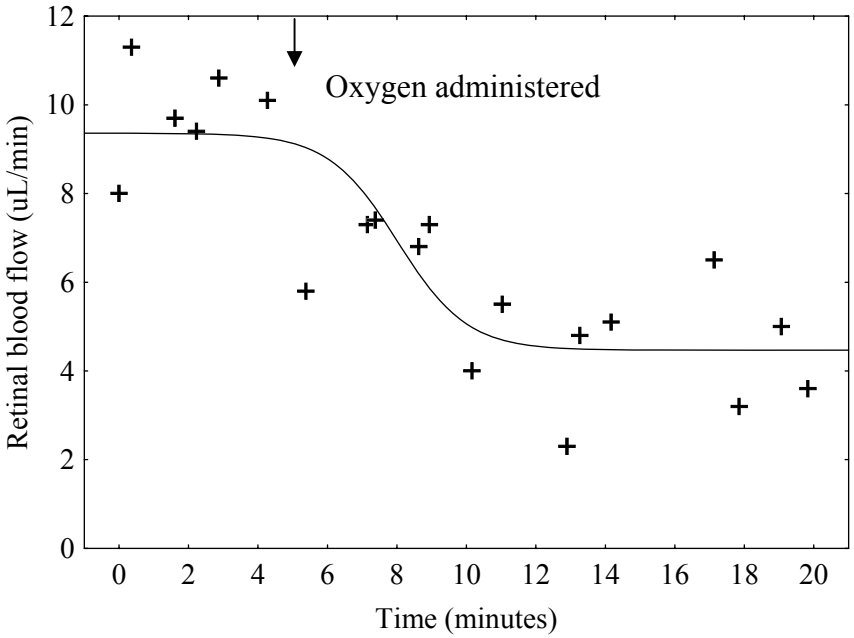


Figure 3.5 Change in retinal blood flow (as measured by the Canon Laser Blood Flowmeter, CLBF-100) induced by O_2 delivered using the sequential rebreathing circuit.

Oxygen was administered at 5 minutes. The data has been fit with a sigmoid type function; equation y=(((v1-v2)/(1+euler^(x-v3)/(v4)))+v2) where v1 and v2 are the upper and lower asymptotes, v3 and v4 localize the midpoint of the descending part of the function on the x-axis (R=0.85). $PetCO_2$ Air = 5.00%; $PetCO_2$ O_2 = 4.88%. FiO_2 Air = 20.31% ; FiO_2 O_2 = 93.88%.

3.5 Discussion

Elevating PO_2 by simply raising the FIO_2 without taking any measures to control PCO_2 resulted in a significant reduction from baseline in mean $PETCO_2$. This group mean reduction could be ameliorated using the co-administration of O_2 and CO_2 or the sequential rebreathing technique. Of the latter two methods, the sequential rebreathing technique had a significantly smaller variability of individual $PETCO_2$ measurements.

Non-rebreathing techniques involve the administration of gas using a reservoir bag via a one-way "demand" valve i.e. a valve that opens at the onset of each inspiration. Expired gas leaves the system via a second one-way valve. Riva et al.[4] were the first to describe retinal vascular effects using 100% O_2 and laser Doppler velocimetry. In terms of vision science based studies, Harris et al.[16,20,21] were the first to consider the potential confounding factor of change in $PETCO_2$ during hyperoxia by co-administering O_2 and CO_2 using a non-rebreathing system. Roff et al.[14] and Chung et al.[15] also employed this technique in their ocular blood flow studies. Interestingly, these studies have not reported the magnitude of individual variability of $PETCO_2$. This study demonstrates that the maintenance of homeostatic $PETCO_2$ levels using non-rebreathing techniques apply to groups as a whole, but are less reliable for individual subjects.

Furthermore, the link between change in $PETCO_2$ and retinal hemodynamics (namely retinal

arteriolar diameter, blood velocity and blood flow) has been demonstrated. The group mean change of $P_{ET}CO_2$ of 0.66% produced a group mean 27% change in retinal blood flow i.e. a 2mmHg "drift" of $P_{ET}CO_2$, that invariably occurs using non-rebreathing techniques, results in a 10 to 12% artifactual change in blood flow. This emphasizes the importance of using breathing circuits that facilitate control of $P_{ET}CO_2$ measurements during administration of elevated O_2.

An alternative method of preventing reduction of $P_{ET}CO_2$ with increases in ventilation (as induced by exposing subjects to O_2) is to increase the dead space of the circuit so that rebreathing occurs. Adding circuit dead space may not limit the reduction of $P_{ET}CO_2$ with hyperventilation as spontaneously breathing subjects (as opposed to those being mechanically ventilated) will overcome the effects of rebreathing by increasing respiratory volume. The sequential rebreathing method developed by Sommer et al.[22] and by Banzett et al.[23] utilized in this study, passively matches the inhaled CO_2 to increases in minute ventilation thereby preventing the expected reduction in PCO_2 (Figure 3.2). The sequential rebreathing technique is effective irrespective of the pattern of breathing. Compared to a non-rebreathing system and adding CO_2 to inspired gas, this system has the advantage of avoiding the risk of raising $P_{ET}CO_2$ and consequently eliciting subject discomfort. The flow of fresh gas (air or O_2, not containing CO_2) is set to just match the patient's minute ventilation during resting conditions while breathing air. This flow is identified by observing that the fresh gas reservoir just collapses at the end of each breath. The gas exhaled by the subject is "stored" in a second

reservoir bag and is available for re-breathing on the next inspiration. As the flow of the fresh gas is fixed, any increases in ventilation will proportionally increase the volume of previously exhaled gas that is rebreathed. Only the fresh gas (O_2 in this case) contributes to the elimination of CO_2. As the flow of fresh gas is equal between air and O_2, the rate of elimination of CO_2 is constant across the two conditions. The constant $P_{ET}CO_2$ also maintains the subjects breathing comfort. Figure 3.5 shows a typical example of the change in retinal blood flow (i.e. the magnitude of retinal vascular reactivity) as measured by the Canon Laser Blood Flowmeter, CLBF-100, induced by O_2 delivery via the sequential rebreathing circuit. The CLBF-100 has been described in detail elsewhere.[24]

Impaired vascular reactivity has been implicated in the pathogenesis of diabetic retinopathy and glaucoma.[6,16,20,21,25-28] Hyperoxia has been frequently used to provoke vascular reactivity. A recent study has shown that endothelin-1 plays a major role in hyperoxia-induced vasoconstriction in humans.[29] Other factors that may be responsible for regulating vascular tone in the retina include the endothelins,[30-36] nitric oxide,[37] prostacyclins[38] and angiotensin.[39] Regulation of blood flow is necessary to maintain structure and function of tissue. This is achieved using systemic controls (nervous influences) and / or local factors (metabolic or myogenic). The vascular response to metabolic factors, sometimes loosely described as autoregulation, stabilizes local blood flow by making the necessary adjustments to ensure a steady supply of metabolites to tissues by altering blood flow as appropriate.

In this study, the group mean reduction of $P_{ET}CO_2$ was ameliorated by the co-administration of O_2 and a small amount of CO_2 using the non-rebreathing system. CO_2 is a potent vasoactive agent. Retinal blood flow varies directly with the arterial PCO_2, as reflected in the $P_{ET}CO_2$. However, with this method there were significant intra-subject variations of $P_{ET}CO_2$. In addition, individuals responded in different ways and thus it would be more difficult to standardize hyperoxia using the co-administration of O_2 and CO_2. In contrast, the sequential rebreathing technique was shown to allow the administration of elevated O_2 levels without a reduction in $P_{ET}CO_2$ and, importantly, reduced variability of $P_{ET}CO_2$ measurements when compared to the non-rebreathing techniques. Steady state manipulation of $P_{ET}CO_2$ unequivocally demonstrated that relatively modest change in $P_{ET}CO_2$ resulted in significant change of retinal hemodynamics.

3.6 Conclusion

Published retinal vascular reactivity studies have employed a non-standardized hyperoxic stimulus without control of $P_{ET}CO_2$ making the results difficult to interpret. Compounded vasoconstrictive effects occur when no control for the reduction of systemic PCO_2 levels are made. In addition, blood flow measurements taken under these conditions may exhibit exaggerated variability due to continuous alterations in PCO_2. Rigorous control of $P_{ET}CO_2$ using a modified commercially available sequential rebreathing circuit will allow the establishment of a standardized, reproducible hyperoxic stimulus for the investigation of vascular reactivity of the human retina.

3.7 References

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4 Retinal arteriolar diameter, blood velocity and blood flow response to an isocapnic hyperoxic provocation.

Gilmore ED, Hudson C, Preiss D, and Fisher J. American Journal of Physiology (Heart and Circulation Physiology). 2005;288:H2912-2917.

	Design	Recruitment	Acquisition of data	Analysis	Writing / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
Preiss					Y
Fisher					Y

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

4.1 Abstract

Purpose: To simultaneously quantify the magnitude and response characteristics of retinal arteriolar diameter and blood velocity induced by an isocapnic hyperoxic provocation in a group of clinically normal subjects.

Methods: The sample comprised 10 subjects (mean age 25 years; range 21-40 yrs). Subjects initially breathed air for 5 to 10 minutes followed by oxygen (O_2) for 20 minutes, and then air for a final 10 minute period using a sequential re-breathing circuit (Hi-Ox^{SR}, Viasys). Retinal arteriolar diameter and blood velocity measurements were simultaneously acquired with the Canon Laser Blood Flowmeter (CLBF-100). The magnitude of the response, the response time and response lag of diameter and velocity were calculated.

Results: In response to hyperoxic provocation, retinal diameter was reduced from control values (mean ± SD) of 111.6 ± 13.1μm to 99.8 ± 10.6μm ($p<0.001$) and recovered after withdrawal of hyperoxia. Retinal blood velocity and flow concomitantly reduced from control values of 32.2 ± 6.4mm/sec and 9.4 ± 2.5μL/min to 20.7 ± 3.4mm/sec and 5.1 ± 1.3μL/min, respectively ($p<0.001$ for both velocity and flow), and recovered after withdrawal of hyperoxia. The response time and response lag were not significantly different for each parameter between effect and recovery. The response time and response lag were not significantly different between diameter and velocity.

Conclusions: Arteriolar retinal vascular reactivity to hyperoxic provocation is rapid with a maximal vasoconstrictive effect occurring within a maximum of 4 minutes. Although there

was a trend for diameter to respond before velocity to the isocapnic hyperoxic provocation, the response characteristics were not significantly different between diameter and velocity.

Keywords: Vascular reactivity, Laser Doppler velocimetry, retinal blood flow.

4.2 Introduction

The blood supply to the inner retina is derived from the central retinal artery while the choriocapillaris supplies the outer retina and photoreceptors. The retinal tissue is one of the most metabolically active in the body and, as a result, an uninterrupted nutrient supply is essential.[1] The inner retinal blood vessels (i.e. past the lamina cribrosa) are thought to be unique due to the absence of an autonomic nerve supply to regulate vascular tone.[2] Blood supply to the inner retina is regulated via local feedback signals that alter retinal perfusion in response to changes in systemic blood pressure or the concentration of certain metabolites.[3,4] In particular, retinal blood flow is strongly dependent on the partial pressure of oxygen (PO_2).[5-9]

The retinal vasculature can be non-invasively visualized and, consequently, its hemodynamic parameters quantified. Impairment of vascular reactivity has been demonstrated in the pathogenesis of various ocular diseases including diabetic retinopathy.[6,10-12] Administration of O_2 has previously been employed as a stimulus to provoke and assess the magnitude of the retinal vascular response. Vasoconstriction of retinal vessels[13,14] and the resulting reduction of retinal hemodynamic parameters has been demonstrated using a variety of measurement techniques.[5,6,7,8,9,15-24] However, none of these studies have utilized a technique that is capable of absolute quantification of retinal blood flow.

The aim of this study was to quantify the magnitude and response characteristics of retinal arteriolar diameter, blood velocity and blood flow induced by a hyperoxic provocation in a group of clinically normal subjects. There are two unique aspects to this study. First, we used a technique that allows the simultaneous quantification of vessel diameter and centre-line blood velocity to calculate retinal blood flow in μL/min. Second, we used a unique system validated in our laboratory[25,26] to administer isocapnic hyperoxia. This overcomes the drawbacks of previous studies due to inadequate control of PCO_2 when implementing hyperoxia. The precise sequence of hemodynamic events underlying retinal vascular reactivity will be elucidated by simultaneously investigating the change in diameter and velocity relative to the onset of the stimulus and to each other.

4.3 Materials and Methods

4.3.1 Sample

The sample comprised 10 clinically normal subjects (5 male: 5 female; mean age 25 years, SD 6 years). Only subjects who were 40 years or younger with no media opacities were included i.e. NO<1, NC<1, P <1, C<1 (Lens Opacity Classification System III).[27] All subjects had a LogMAR visual acuity of 0.00, or better. Subjects were excluded if they exhibited any eye disease, any cardiovascular or respiratory disorders, a refractive error greater than ±6.00 DS or ±2.00 DC, glaucoma or diabetes in a first degree relative, or medications with known effects on blood flow (e.g. anticonvulsants, muscle relaxants, or anti-inflammatory

medications). None of the subjects were smokers. All participants were asked to refrain from caffeine-containing drinks or snacks for at least 12 hours prior to their study visit. The study was approved by the University of Waterloo Office of Research Ethics and the University Health Network Research Ethics Board, Toronto. 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.

4.3.2 Gas Delivery System

The sequential rebreathing system comprised a fresh gas reservoir and an expiratory gas reservoir, each connected to the patient by one-way valves. The inspiratory and expiratory limbs were interconnected by a single PEEP valve, allowing exhaled gas to be rebreathed when the gas in the inspiratory limb was depleted. This system was assembled by adding a gas reservoir to the expiratory port of a commercial 3-valve oxygen delivery system (Hi-Ox^{SR}, ViasysHealthcare, Yorba Linda, CA). Flow from the gas tanks was controlled using standard rotometers as flowmeters. This method has been described in detail in previous publications.[25,26]

4.3.3 Canon Laser Blood Flowmeter

The principal underlying the CLBF-100 is based on the Doppler effect. Laser light (frequency=f) reflected from a moving particle is shifted in frequency (Δf) that is proportional

to the velocity of the moving particle. A vessel that exhibits Poiseuille flow will have a range of velocities and thus a range of frequency shifts up to a maximum frequency shift (Δf_{max}) that corresponds to the maximum velocity of the blood moving at the centre of the vessel (since resistance is developed at the vessel wall). Light scattered from stationary tissue is unshifted and acts as the reference frequency from which a relative change in retinal blood velocity is measured.[28] By utilizing two photomultipliers separated by a known angle, the maximum frequency shift is subtracted to allow the absolute quantification of centre-line blood velocity irrespective of the angle between the moving particle and reflected beam.[29,30] A red diode laser (675nm, 80μm x 50μm oval) is used to measure velocity every 0.02 seconds across a 2 second measurement window resulting in a velocity-time trace. The CLBF-100 also uses a green diode vessel tracking laser system (543nm, 1500μm x 150μm rectangle) that is used to stabilize, and measure the diameter of the vessel of interest.[31,32] The vessel tracking system stabilizes measurement site position and allows rejection of velocity measurements involving significant saccades. Diameter readings are acquired every 4 milliseconds during the first and final 60 milliseconds of the 2 second velocity measurement window. Two sequential measurements utilizing different optical paths (path 1 and path 2) are taken to ensure consistency and averaged to give one reading. In combination with the average velocity (V_{mean}) over a pulse cycle and diameter (D), flow through the vessel can be calculated from $1/2.\pi.D^2/4.V_{mean}.60$ (for technical summary see Kida and co-workers[33]). Magnification effects associated with refractive and axial components of ametropia are corrected to provide absolute measurements of diameter (μm), velocity (mm/sec) and flow (μL/min). The technological principles utilized in this device have been described in detail

elsewhere.[29,30,33,34] In addition this device has been extensively evaluated in clinically normal subjects[35,36] and those with various types of retinal pathologies.[33,37]

4.3.4 Procedures

Each subject was seated for at least 5 minutes prior to commencing measurements to allow stabilization of heart rate and blood pressure. An initial air breathing period was employed to allow stabilization of baseline parameters e.g. respiration rate, PO_2 and PCO_2. Retinal arteriolar diameter and centreline blood velocity measurements were simultaneously acquired from either the supero- or infero-temporal arteriole in one eye of each subject using the CLBF-100. A minimum of 5 baseline measurements were acquired while the subject breathed air (5 to 10 minutes). The isocapnic hyperoxic stimulus was then initiated and maintained for 20 minutes. Subsequently, air was re-administered for a further 10 minutes maintaining isocapnia to baseline levels. Retinal blood flow measurements were acquired every minute over the course of the study (Figure 4.1).

4.3.5 Gas analysis and systemic responses

A rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA) was used to quantify the relative concentrations of O_2 and CO_2 in both the inspired and expired gases on a breath-by-breath basis. The relative concentrations O_2 and CO_2 are sampled continuously by the gas analyzer whilst the inspired and end-tidal concentrations of O_2 and CO_2 were

downloaded to a personal computer every 5 seconds (S5 Collect software, Datex-Ohmeda, USA). In addition, finger-oxygen saturation, respiration rate and pulse rate were also recorded continuously. The fractional concentration of oxygen in the expired breath (FeO_2) was chosen as the parameter that most closely reflects the change in arterial PO_2. Gas data was analyzed using box plots that depicted the median, upper 25^{th} and lower 75^{th} percentiles, and outliers of end-tidal gas concentrations. Data points lying outside the upper 25^{th} or lower 75^{th} percentiles were excluded from the analysis since all of these values were found to be erroneous i.e. these points resulted from inappropriate interpretation of tidal waveforms by the gas monitor. Blood pressure was measured non-invasively once every 3 minutes over the course of the experiment (Cardiocap 5, Datex-Ohmeda, USA).

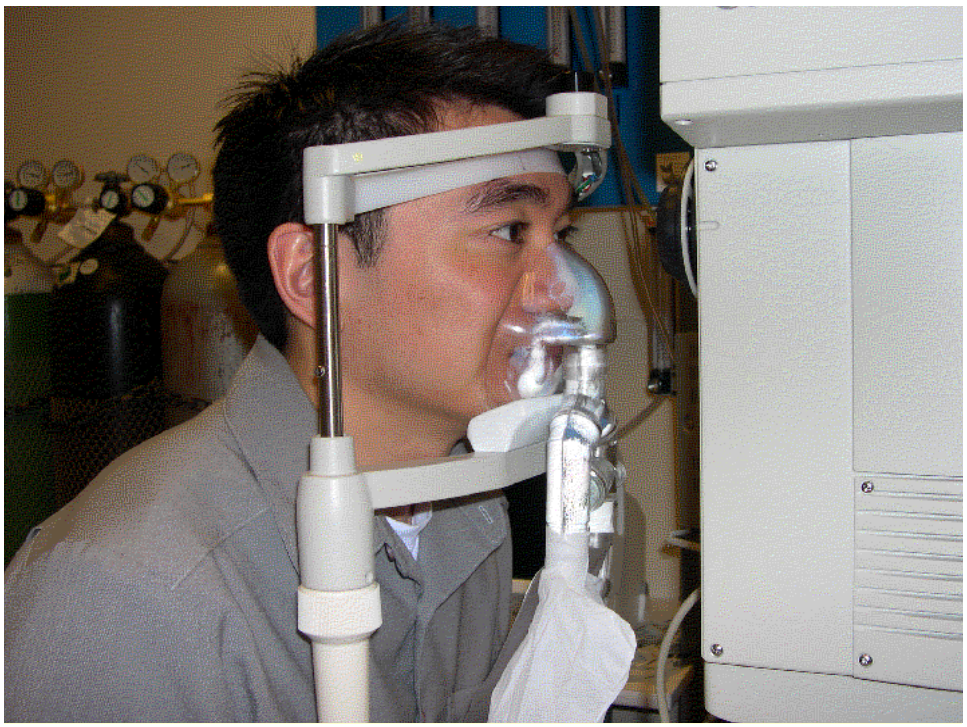


Figure 4.1 Photograph of sequential rebreathing system fitted to participant.

4.3.6 Function fitting

Arteriolar diameter and velocity data was fit using a double sigmoidal function of the form:

$$y = [(t<20)*(((\alpha-\beta)/(1+\eta^{(t-\gamma)}))+\beta)] + [(t>20)*(((\beta-\delta)/(1+\theta^{(t-\varepsilon)}))+\delta)] \qquad (4.1)$$

where y is the magnitude of the hemodynamic parameter (i.e. diameter, velocity or flow) at a certain time, t, from the initial measurement (t=0). An arbitrary time point (t=20, i.e. approximately midway through the procedure) was used to divide the data into 2 sections. The exponents η and θ were constrained so that the inflexion points of the function could not occur before the O_2 had been turned on (effect phase) or before the O_2 had been turned off (recovery phase), respectively. For $t<20$, α and β are the upper and lower asymptotes, respectively. γ is the value of t that corresponds to a value halfway between α and β i.e. the midpoint of the effect phase of the function. For $t>20$, β is set as the lower asymptote and δ is the upper asymptote (independent of α). ε is the value of t that corresponds to a value halfway between β and δ i.e. the midpoint of the recovery phase of the function. α, β, γ, δ and ε were varied using the "non-linear regression" module in Statistica (Statsoft, Inc.) to produce a least-squares fit. As a result, the same mathematical model was utilized for all subjects and all hemodynamic parameters but the coefficients of the model varied between subjects and hemodynamic parameters of a given subject. An example of the function fitting is shown (Figure 4.2).

Goodness of fit (r-value) was determined. Fitted functions with r-values less than 0.6 were

excluded from the analysis. The velocity data from 2 subjects and diameter data from 1 subject were not included in the analysis due to either a low r-value (less than 0.60) or inappropriate fit of the data. The magnitude of the retinal vascular response of diameter and velocity was calculated (i.e. α - β for effect phase and δ - β for recovery phase). The time interval between the 5^{th} and 95^{th} percentile of the change in diameter and velocity was quantified and referred to as the retinal vascular "response time". The time interval between onset (or cessation) of the hyperoxic stimulus and the midpoint of the effect (i.e. γ), or recovery (i.e. ε), phase of the function was determined and referred to as the "response lag".

4.4 Results

There was an abrupt reduction in vessel diameter, blood velocity and blood flow on initiation of hyperoxia. All parameters returned to control values when hyperoxia was discontinued (Figures 4.3a and b). Group mean retinal arteriolar diameter was 111.6μm (SD=13.1μm; range 85-129μm) prior to isocapnic hyperoxic provocation, decreased to 99.8μm (SD=10.6μm) during provocation (two-tailed paired *t*-test $p<0.001$) and recovered to 109.9μm (SD=11.7μm) on removal of the stimulus. Group mean retinal blood velocity was 32.2mm/sec (SD=6.4mm/sec; range 22-42mm/sec) prior to provocation, decreased to 20.7mm/sec (SD=3.4mm/sec) during provocation (two-tailed paired *t*-test $p<0.001$) and recovered to 33.3mm/sec (SD=5.0mm/sec). Group mean retinal blood flow was 9.4μL/min (SD=2.5μL/min; range 5.4-13.4μL/min) prior to provocation, decreased to 5.1μL/min (SD=1.3μL/min) during provocation (two-tailed paired *t*-test $p=0.001$) and recovered to 9.2μL/min (SD=1.7μL/min).

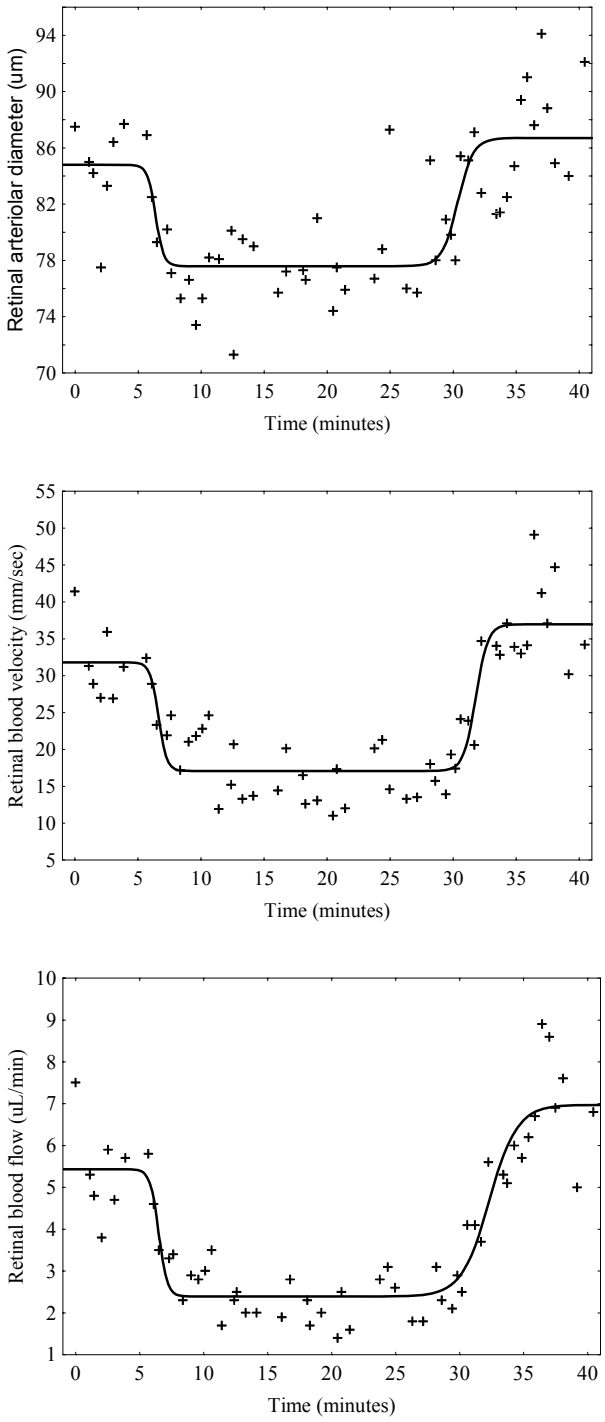
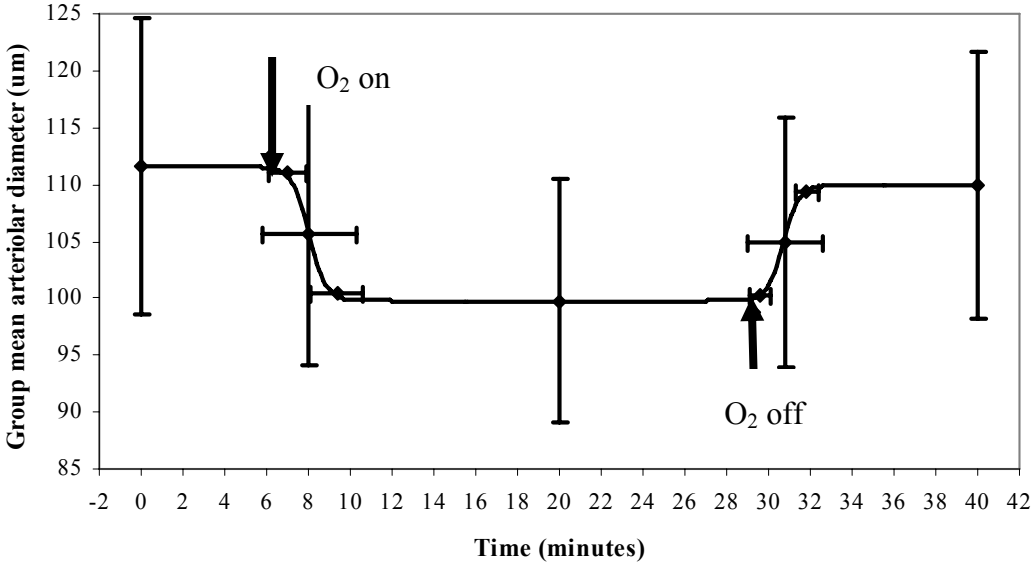


Figure 4.2 Change in retinal arteriolar diameter (upper left), blood velocity (upper right) and blood flow (lower left) for a single participant induced by isocapnic hyperoxia using the sequential rebreathing circuit (fit with sigmoidal function).

The r-values were 0.77, 0.89 and 0.92 for diameter, velocity and flow respectively. The concentration of expired CO2 was 4.53%, 4.47% and 4.30% during air, oxygen and air breathing periods respectively.



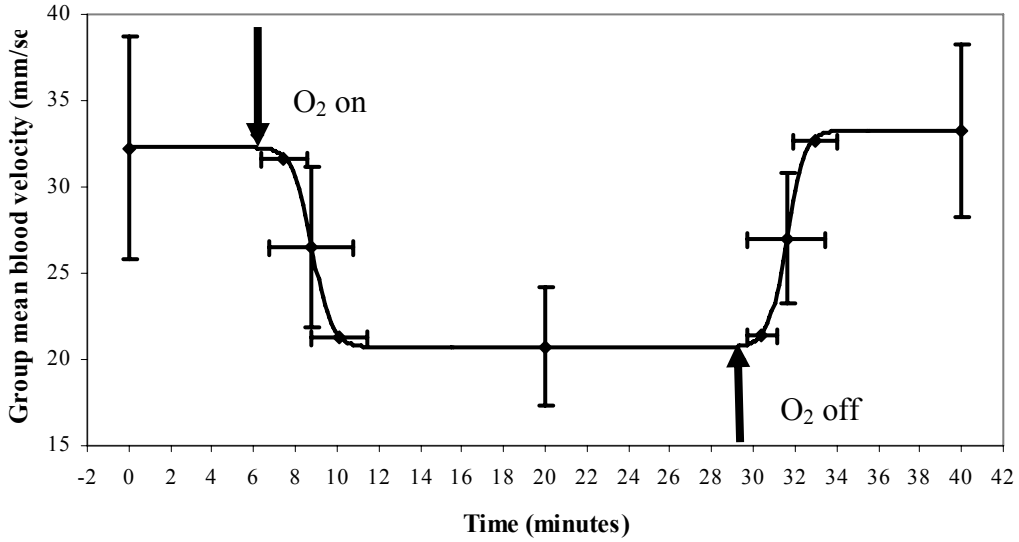


Figure 4.3a Upper; Group mean magnitude of retinal arteriolar diameter before, during and after the isocapnic hyperoxic provocation. Lower; Group mean magnitude of retinal blood velocity before, during and after the isocapnic hyperoxic provocation.

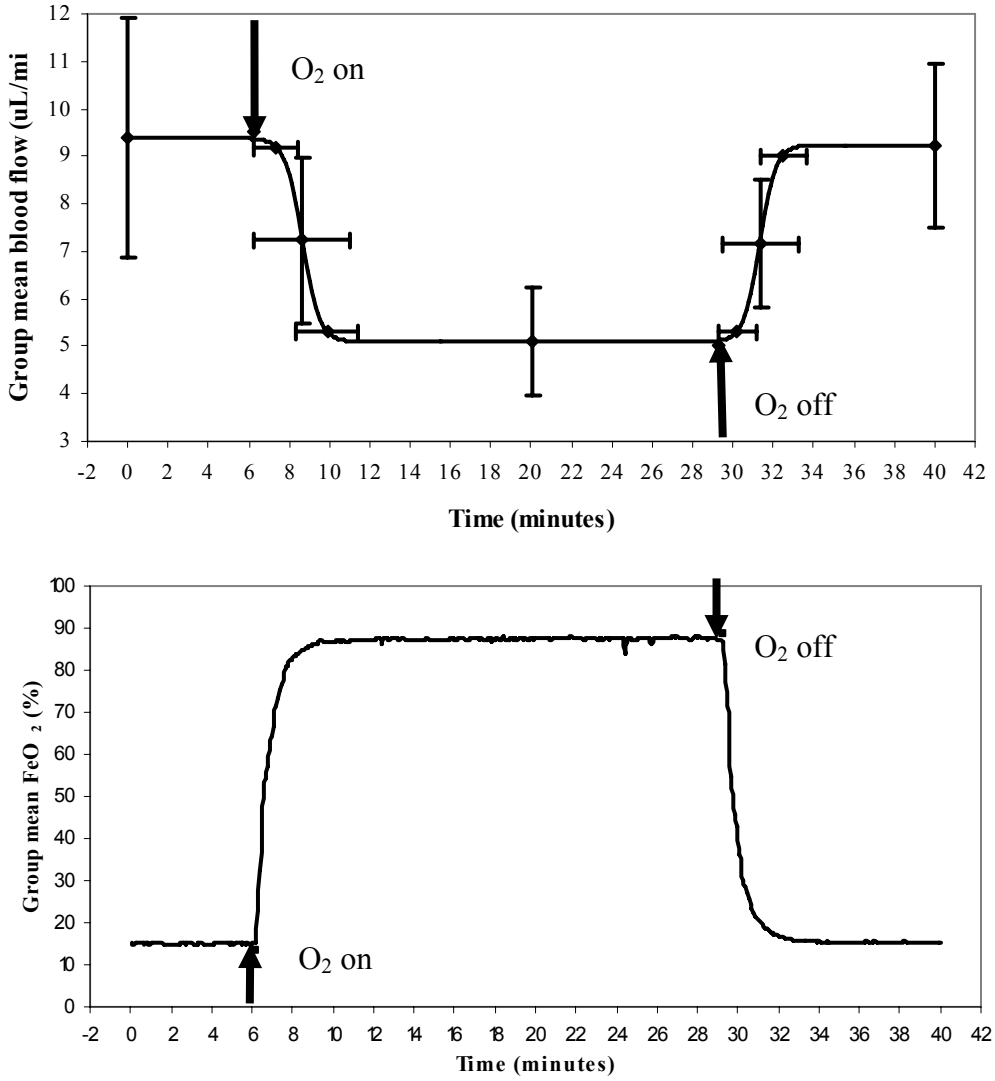


Figure 4.3b Upper; Group mean magnitude of retinal blood flow before, during and after the isocapnic hyperoxic provocation. Lower; Group mean magnitude of FeO_2 before, during and after the isocapnic hyperoxic provocation.

All participants "oxygen on" and "oxygen off" points are coincident. Diameter, velocity and flow data have been fit using a sigmoidal function. The time points detailed include (from left to right) group mean baseline magnitude, 5% point, midpoint and 95% point (effect function), group mean magnitude during hyperoxia, 5% point, midpoint and 95% point (recovery function), final group mean magnitude. Error bars represent ± 1 standard deviation.

The group mean response time of diameter was 2.37 minutes (SD=0.46 minutes) and 2.22 minutes (SD=0.60 minutes) for the effect (i.e. after initiation of hyperoxia) and recovery (i.e. after cessation of hyperoxia) phases, respectively (Figure 4.3). The group mean response time of velocity was 2.68 minutes (SD=0.55 minutes) and 2.55 minutes (SD=0.53 minutes) for the effect and recovery phases, respectively (Figure 4.3). The group mean response time of flow was 2.54 minutes (SD=0.56 minutes) and 2.28 minutes (SD=0.43 minutes) for the effect and recovery phases, respectively (Figure 4.3). The response time during the effect phase was not significantly different to the response time during the recovery phase for both diameter and velocity (two-tailed paired *t*-test). Also there was no significant difference between the response times of diameter versus velocity.

The group mean response lag of diameter was 2.00 minutes (SD=1.07 minutes) and 1.38 minutes (SD=0.45 minutes) for the effect (i.e. after initiation of hyperoxia) and recovery (i.e. after cessation of hyperoxia) phases, respectively. The group mean response lag of velocity was 2.60 minutes (SD=1.19 minutes) and 2.29 minutes (SD=0.87 minutes) for the effect and recovery phases, respectively. The group mean response lag of flow was 2.44 minutes (SD=1.32 minutes) and 2.03 minutes (SD=1.02 minutes) for the effect and recovery phases, respectively. The response lag during the effect phase was not significantly different to the response lag during the recovery phase for both diameter and velocity (two-tailed paired *t*-test). The diameter response lag was not significantly different than the velocity response lag for the effect phase and for the recovery phase.

A correction factor was calculated since the change in arterial PO_2 was not square wave i.e. the time from the onset (or cessation) of oxygen until 50% of the observed change in FeO_2 had taken place. The group mean correction factor was 0.50 minutes for effect and 0.49 minutes for recovery and was not significantly different between effect and recovery. The magnitude of the correction factor relates to a group mean response lag of 1.7 minutes for diameter and 2.6 minutes for velocity (mean of effect and recovery phases).

The group mean r-value for diameter and velocity of the fitted functions were 0.843 and 0.700, respectively.

The inspired and end-tidal gas parameters and relevant systemic measures for air, isocapnic hyperoxia and air are detailed in Table 4.1. Only heart rate, $FiCO_2$, FiO_2 and FeO_2 changed significantly as a result of the hyperoxic provocation. The group mean mean arterial blood pressure (MAP; [(2/3 * diastolic BP) + (1/3 * systolic BP)] was 81.7 mmHg (SD=10.1mmHg) prior to hyperoxic provocation, 80.9 mmHg (SD=6.0mmHg) during provocation and 81.8mmHg (SD=7.9mmHg) after provocation. There was no significant difference in MAP across the stimulus conditions (two-tailed paired *t*-test).

		Inspired O_2 (%)	End-tidal O_2 (%)	Inspired CO_2 (%)	End-tidal CO_2 (%)	Respiration rate (breaths/min)	Pulse rate (beats/min)	Saturation O_2 in blood (%)
Air	Mean	19.84	15.10	0.62	5.10	17.22	67.30	97.80
	SD	0.34	0.58	0.31	0.50	1.59	7.81	1.46
O_2	Mean	91.69	86.23	0.90	4.98	17.61	63.99	98.38
	SD	7.90	3.29	0.57	0.45	2.45	6.48	1.05
Air	Mean	19.88	16.25	0.67	5.00	17.40	68.89	97.72
	SD	0.43	0.53	0.38	0.46	2.79	8.16	2.11

Table 4.1: Group mean and SD of inspired and end-tidal O_2, inspired and end-tidal CO_2, respiration rate, pulse rate and saturation of O_2 in blood.
(SD- standard deviation)

4.5 Discussion

Retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant level[6,38] and also varies directly with the partial pressure of arterial carbon dioxide (PCO_2).[19] Numerous studies have investigated retinal vascular reactivity using a hyperoxic stimulus. Some have investigated change of retinal vessel diameter,[13,14,17] whilst others have measured change in aspects of hemodynamics using a variety of techniques.[5,6,9,15,18,23,24] Alternatively, others have measured retinal vessel diameter and separately employed a bi-directional laser Doppler system to measure centre-line retinal blood velocity in order to calculate flow.[7,8,16,19-22] The technique utilized in this study measures centre-line retinal blood velocity using a bi-directional photodetector and *simultaneously* acquires retinal vessel diameter measurements for the absolute quantification of retinal blood flow. All of these studies have employed 100% O_2 or co-administered O_2 (>90%) and CO_2 (~5%). None have used a truly isocapnic hyperoxic stimulus. Administration of enriched O_2 concentrations typically results in the reduction of the partial pressure of arterial carbon dioxide (PCO_2).[39] The change of end-tidal CO_2 concentration ($P_{ET}CO_2$; the maximum concentration of CO_2 during each expiration) reflects the change in arterial PCO_2.[40] The method of gas delivery used in this study minimizes alterations in systemic PCO_2 concentration.[26] We have previously shown that this delivery system results in stabilization of end-tidal CO_2 during hyperoxic provocation, thereby isolating the retinal vascular reactivity response to oxygen alone.[25] Additionally, a recent paper has demonstrated separate vasoconstrictive effects of hyperoxia (i.e. O_2 mediated) and arterial hypocapnia (i.e. CO_2 mediated) in the cerebral vasculature.[41]

In general, previous studies utilizing 100% O_2 to assess retinal vascular reactivity have tended to find a greater magnitude of vasoconstriction, that we attribute to a compounded effect of elevated arterial O_2 and reduced CO_2.[8,16,20,21] Most of these studies have measured vascular reactivity in venules possibly because the derived velocity profile is non-pulsatile. Arterioles were used in this study since they are thought to be primarily responsible for the vascular reactivity response and obey Poiseuille flow principles to a greater extent (given the more circular cross section).

Three previously published studies have investigated the time course of the change in diameter or velocity using a hyperoxic stimulus (referred to in this paper as "response time" and "response lag").[14,16,19] To the best of our knowledge, this is the first time that the response characteristics of arteriolar diameter and blood velocity have been *simultaneously* quantified due to hyperoxic provocation. In addition, the characteristics of the effect and recovery phases (i.e after onset and cessation of the hyperoxic stimulus respectively) have not been investigated concomitantly. The response characteristics of the retinal arterioles reported in this study are comparable to those of previous studies. There was a trend for diameter to respond before velocity to the hyperoxic stimulus but neither the response time nor the response lag was significantly different between diameter and velocity. Although direct comparison is difficult due to difference in study design, Nagaoka et al.[42] found that retinal arteriolar velocity responded approximately 1.3 minutes prior to diameter in response to cold pressor provocation. When considered alongside the results reported in this manuscript,

different response characteristics of the retinal vasculature to transmural pressure mediated autoregulation as opposed to metabolic mediated vascular reactivity are suggested. Overall, there is remarkable similarity in retinal arteriolar response times to two totally different perturbations (isocapnic hyperoxia arteriolar constriction occurred at 2.4 minutes while cold pressor induced constriction occurred at 2.9 minutes) suggesting a common mechanism of hemodynamic alteration.

The change in arterial PO_2 was not square wave and as a result a correction factor was calculated to compensate for this effect. A finite time is required for O_2 to reach the retinal vasculature due to physiological delay of gas exchange in the lungs and lung-to-eye circulation time. The corrected response lag was therefore the measured response lag of diameter and velocity reported above less the influence of the "correction factor". Nevertheless the impact of the correction factor does not influence the differential relationship between diameter and velocity.

Homeostatic oxygen supply is primarily maintained during hyperoxia by a reduction of vessel diameter,[38] although the exact governing mechanism has yet to be fully elucidated. Microelectrode animal studies indicate that inner retinal PO_2 is well regulated during hyperoxia.[43] Various biochemical factors that may be responsible for retinal hyperoxia-induced vasoconstriction include endothelin-1[44-48] and prostanoids.[49] In addition other mechanisms have been investigated in the cerebral vasculature involving superoxide generation and nitric oxide[50-53] or red blood cell physiological changes.[54-57]

4.6 Conclusion

This study is novel in that it utilized an isocapnic hyperoxic stimulus to provoke retinal vascular reactivity. Previous studies have been unable to avoid a concomitant reduction in PCO_2 during hyperoxia. In addition, the measurement technique utilized to assess retinal hemodynamics provided the unique ability to simultaneously quantify retinal blood velocity and vessel diameter for the absolute quantification of retinal blood flow. Although there was a trend for diameter to respond before velocity to the hyperoxic stimulus neither the response time nor the response lag was significantly different between diameter and velocity.

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5 Retinal arteriolar diameter, blood velocity and blood flow response to an isocapnic hyperoxic provocation in early sight-threatening diabetic retinopathy

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	Design	Recruitment	Acquisition of data	Analysis	Writing / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
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Harvey		Y			
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Lam		Y			
Devenyi		Y			

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5.1 Introduction

Purpose: The aim was to quantify the magnitude of retinal arteriolar vascular reactivity in diabetic patients stratified by severity of retinopathy and age-matched controls.

Methods: The sample comprised 21 non-diabetic controls (Group 1), 19 patients with no clinically visible DR (Group 2), 19 patients with mild-to-moderate non-proliferative DR and without clinically evident diabetic macular edema (DME) (Group 3) and 17 patients with DME (Group 4). Subjects initially breathed air, followed by O_2, whilst maintaining isocapnia. Retinal arteriolar diameter and blood velocity measurements were simultaneously acquired.

Results: Change in blood velocity and wall shear rate (WSR) was significantly less in Groups 3 and 4 ($p<0.0001$ and $p=0.0002$, respectively) than that of Groups 1 and 2. Change in blood flow was significantly less in Group 4 ($p<0.004$) than that of Groups 1 and 2. The change in max:min ratio was significantly less in Groups 2 and 4 than Group 1 ($p=0.001$). There was a significant relationship between baseline objective edema index values and vascular reactivity.

Conclusion: The magnitude of vascular reactivity in response to isocapnic hyperoxia was reduced in those individuals with clinically evident DR relative to subjects without diabetes. The differences in vascular reactivity occurred in the absence of any difference in baseline hemodynamic values. Vascular reactivity is impaired in early sight-threatening DR and this impairment is related to the objectively defined magnitude of retinal edema.

Keywords: Retinal blood flow, Laser Doppler velocimetry, hyperoxia, diabetic retinopathy, diabetic macular edema.

5.2 Introduction

The blood supply to the inner retina is derived from the central retinal artery. The inner retinal blood vessels are thought to be unique due to the absence of an autonomic nerve supply to initiate changes in vascular tone.[1] Blood supply to the inner retina is regulated via local feedback signals that alter retinal perfusion.[2,3] Vasoconstriction of retinal vessels in response to hyperoxia,[4-6] and the resulting reduction of parameters that reflect flow, has been demonstrated using a variety of measurement techniques.[4,6-10]

Vascular reactivity represents the response of the vasculature to a given stimulus, such as hyperoxia.[4] Impairment of retinal vascular reactivity to hyperoxia in diabetic retinopathy, as reflected by a reduced hemodynamic response, has been demonstrated previously.[7,11-13] However, previous studies are limited because many have not utilized simultaneous diameter and velocity measurements, all did not control for systemic variation in arterial CO_2 during hyperoxic provocation and none have focused on changes associated with the development of early sight-threatening diabetic retinopathy.

The aim of this study was to quantify the magnitude of change of retinal arteriolar diameter, blood velocity and blood flow induced by an isocapnic hyperoxic provocation in diabetic patients clinically stratified by retinopathy status and in age-matched subjects without diabetes. In addition, volunteers underwent non-invasive, objective assessment of diabetic macular edema (DME) using the Macular Edema Module (MEM) of the Heidelberg Retina

Tomograph II.[14] There are four unique aspects to this study. First, we used a technique that allowed the simultaneous quantification of vessel diameter and centre-line blood velocity to calculate volumetric retinal blood flow in micro-liters per minute. Second, we used a unique standardized system[15] to administer isocapnic hyperoxia. In addition, a larger and clinically defined sample was used in this study. Finally, we correlated the retinal hemodynamic response to isocapnic hyperoxia with the objective assessment of retinal edema.

5.3 Materials and Methods

5.3.1 Sample

Using previously published data from our laboratory,[4] the vascular reactivity response of healthy young subjects in terms of change of retinal blood flow in response to isocapnic hyperoxia was found to be 4.3 μL/min and the SD of the difference between baseline and recovery was 0.85 μL/min. Assuming a 50% reduction in vascular reactivity response when comparing healthy subjects to our most advanced diabetic retinopathy group,[13] the difference between groups in order to reach statistical significance would need to be 0.72 μL/min (i.e. [50% of 4.3 μL/min] / 3). Therefore, the standardized effect size (difference between means / SD) was calculated to be 0.85 and the resulting sample size using an alpha of 0.05 and power of 0.9 was 16 per group. The sample comprised 21 non-diabetic, age-matched controls (Group 1; mean age 49 yrs, SD 10 yrs), 19 patients with no clinically visible DR (Group 2; mean age 52 yrs, SD 11 yrs), 17 patients with mild-to-moderate non-proliferative DR as defined by the ETDRS[16] (Group 3; mean age 51 yrs, SD 12 yrs) and 17 patients with diabetic macular edema (DME) (Group 4; mean age 55 yrs, SD 8 yrs)(Table 5.1). The diabetic groups were stratified for increasing risk for the development of DME (Groups 2 to 4). The number of patients classified as having type 1 diabetes as a function of group was 2, 2, and 1 for groups 2 to 4, respectively.

Group	Group mean age (years) (SD)	Group mean duration diabetes (years) (SD)	Number treated with insulin	Male to female ratio	Group mean A1c value (SD)	Group mean random glucose (mmol/L)
1	49 (10)	--	--	7 M:14 F	--	5.7 (0.9)
2	52 (11)	10 (9)	7	8 M: 11 F	0.072 (0.014)	10.5 (4.7)
3	51 (12)	14 (10)	12	9 M: 8 F	0.083 (0.019)	7.8 (3.8)
4	55 (8)	13 (8)	7	13 M: 4 F	0.084 (0.014)	10.0 (3.8)

Table 5.1: Group mean age, duration of diabetes, number treated with insulin, male to female ratio, A1c and random glucose as a function of group (A1c: glycosylated hemoglobin. M; male, F; female)

Volunteers were allocated into groups according to their retinal status using dilated stereo fundus biomicroscopy (by agreement of 2 clinicians). All volunteers were aged between 30 and 70 years and had a LogMAR VA of 0.3 or better. Volunteers were excluded if they exhibited any eye disease (apart from DR for Groups 2, 3 and 4) or had undergone ocular surgery, any cardiovascular (except well controlled systemic hypertension) and respiratory (except treated asthma) disorders, a refractive error greater than ±6.00 DS or 2.00 DC and glaucoma in a first degree relative. None of the volunteers were regular smokers or had undergone retinal laser treatment. All volunteers were asked to refrain from caffeine-containing drinks or snacks for at least 8 hours prior to their study visit. Lens clarity was graded using the Lens Opacity Classification System III (LOCS III).[17] The study was approved by the University Health Network Research Ethics Board, Toronto and the University of Waterloo Office Research Ethics. Informed consent was obtained from each volunteer after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki.

5.3.2 Isocapnic hyperoxia delivery system

The isocapnic hyperoxia delivery system comprised a sequential re-breathing circuit made up of a fresh gas reservoir, an expiratory gas reservoir and a face-mask (Hi-Ox^{SR}, ViasysHealthcare, Yorba Linda, CA). The inspiratory and expiratory limbs were interconnected by a single positive end-expiratory pressure (PEEP) valve, allowing exhaled gas to be re-breathed when the gas in the inspiratory limb was depleted. Flow from gas tanks containing air (baseline) or oxygen (hyperoxia) respectively was controlled using standard

rotometers as flowmeters. This method has been described in detail in a previous publication.[15]

5.3.3 Quantification of retinal vessel diameter, blood velocity and flow

The principal underlying the quantification of retinal hemodynamics is based on the Doppler effect. Laser light (frequency=f) reflected from a moving particle is shifted in frequency (Δf) that is proportional to the velocity of the moving red blood cells. A vessel that exhibits Poiseuille flow will have a range of velocities and thus a range of frequency shifts up to a maximum frequency shift (Δf_{max}) that corresponds to the maximum velocity of the blood moving at the centre of the vessel. By utilizing two photomultipliers separated by a known angle, the maximum frequency shift detected by each photomultiplier is subtracted to allow the absolute quantification of centre-line blood velocity irrespective of the angle between the moving particle and reflected beam.[18,19]

The Canon Laser Blood Flowmeter (CLBF; Canon, Tokyo, Japan) utilizes a red diode laser (675nm, 80µm x 50µm oval) to measure velocity every 0.02 seconds across a 2 second measurement window resulting in a velocity-time trace. The CLBF also uses a green diode laser system (543nm, 1500µm x 150µm rectangle) that is used to measure vessel diameter and maintain centration of the laser at the measurement site.[20,21] The vessel tracking system allows post-acquisition rejection of velocity measurements impacted by significant saccades. Diameter readings are acquired every 4 milliseconds during the first and final 60 milliseconds

of the 2 second velocity measurement window. Two sequential measurements utilizing different optical paths (path 1 and path 2) are taken to ensure consistency and averaged to give one reading. In combination with the average velocity (V_{mean}) over a pulse cycle and diameter (D), flow through the vessel can be calculated using $1/2.\pi.D^2/4.V_{mean}.60$. Magnification effects associated with refractive and axial components of ametropia are corrected to provide absolute measurements of diameter (μm), velocity (mm/sec) and flow (μL/min). The technological principles utilized in this device have been described in detail elsewhere.[18,19,22,23] In addition, this device has been extensively evaluated in volunteers with,[23-25] and without,[4,26-28] retinal diseases.

5.3.4 Quantitative assessment of retinal edema

A confocal scanning laser tomograph that sequentially acquires two-dimensional section images along the optical axis was employed. The distribution of reflected light intensity along the optical axis for a given pixel is described by the *z*-profile or confocal intensity profile. Studies have demonstrated a broadening of the *z*-profile signal width and a decrease in peak reflectance intensity in areas of retinal edema.[29] Normalization of the reflectance values reduces the variation in intensity between successive scans. The MEM technique of the Heidelberg Retina Tomograph II (HRT; Heidelberg Engineering, Heidelberg, Germany) determines the z-profile signal width (at half peak height) and peak reflectance intensity.[29] MEM has been demonstrated to have high sensitivity and good specificity for the detection of DME.[14]

5.3.5 Procedures

One eye of each subject was randomly assigned to the study if both eyes met study criteria. Volunteers attended for 2 visits. Visit 1 was used to establish eligibility and baseline characteristics, determine group assignment, undertake objective assessment of DME and to familiarize the volunteer with the technique used to quantify retinal hemodynamics. Three sets of MEM images centered on the fovea were acquired at visit 1 for each volunteer. Visit 2 was used to quantify retinal vascular reactivity to isocapnic hyperoxia (detailed in the next paragraph). Refraction, LogMAR visual acuity, resting blood pressure and random blood glucose level were assessed prior to dilation of the study eye with 1% Tropicamide (Alcon Canada Inc). At least five retinal hemodnamic measurements were attempted using the CLBF at baseline and then also during isocapnic hyperoxia. Goldmann intraocular pressure assessment was undertaken after retinal blood flow measurements had been acquired. Axial length was measured by I^3 System ABD A-scan ultrasound (I^3 Innovative Imaging Inc, Sacramento, CA) to correct blood flow measurements for magnification effects due to ametropia. The median time interval from initial visit to hyperoxia visit was 8 days.

Volunteers initially breathed air for 10 minutes followed by oxygen (O_2) for 10 minutes, using a sequential re-breathing circuit (Hi-Ox^{SR}) to maintain isocapnia. An initial air breathing period was employed to allow stabilization of baseline parameters e.g. respiration rate, partial pressure of arterial oxygen (PO_2) and the partial pressure of arterial carbon dioxide (PCO_2), as indicated by measurements of end-tidal oxygen ($P_{ET}O_2$) and carbon dioxide ($P_{ET}CO_2$). Retinal

hemodynamic measurements were simultaneously acquired from an arteriole within 1 disc diameter from the optic nerve head using a straight vessel segment in one eye of each volunteer. Measurements were acquired during the second 5 minute period of each paradigm (i.e. a 5 minute stabilization period was employed during air and oxygen breathing prior to acquisition of retinal hemodynamic measurements).

5.3.6 Gas analysis and systemic vascular responses

A rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA) was used to quantify the relative concentrations of O_2 and CO_2 in both the inspired and expired gases on a breath-by-breath basis. The relative concentrations of O_2 and CO_2 were sampled continuously by the gas analyzer and the inspired O_2, inspired CO_2, $P_{ET}O_2$ and $P_{ET}CO_2$ were downloaded to a personal computer every 5 seconds (S5 Collect software, Datex-Ohmeda, USA). In addition, finger-oxygen saturation, respiration rate and pulse rate were also recorded continuously. $P_{ET}CO_2$ was analyzed by calculating the upper 10^{th} and lower 90^{th} percentiles. Data points lying outside the upper 10^{th} or lower 90^{th} percentiles were excluded from the analysis since all of these values were found to be erroneous i.e. these points resulted from inappropriate interpretation of tidal waveforms by the gas monitor. Blood pressure was also measured non-invasively once every minute over the course of the hyperoxic paradigm (Cardiocap 5, Datex-Ohmeda, USA).

5.3.7 Analysis

A post acquisition analysis of the CLBF velocity waveforms was performed using a standardized protocol to remove aberrant waveforms affected by eye movement, tear film breakup, or improper tracking of the measurement laser. The maximum number of acceptable pulse cycles was used in the data analysis for each measurement (with a minimum of 1 complete velocity waveform required). In addition, maximum to minimum (max:min) velocity ratio was calculated during air breathing and compared to that during oxygen breathing for each individual. This ratio reflects vascular compliance, where an elevation of max:min ratio indicates increased vascular rigidity (the site of this change can be up-stream of, down-stream of, or at the CLBF measurement site). In the physiological situation, compliance is expected to reduce and rigidity increase during hyperoxia due to increased tonus of the vessel wall. In addition, wall shear rate (WSR= mean velocity * 8 / diameter)[30] was calculated because change is shear stress is believed to alter blood flow and this mechanism is thought to be disturbed in diabetes and atherosclerosis.[30,31]

The normality of each hemodynamic parameter as a function of group and condition was confirmed prior to the use of parametric statistics. A normal distribution was confirmed for all parameters apart from max:min velocity ratio which was log transformed for statistical analysis. The change in each of the hemodynamic parameters in response to provocation within each group was determined using paired two-tailed t-tests. Repeated measures ANOVA was used to determine any differences between the baseline hemodynamic parameters between groups and any difference in the response of the hemodynamic

parameters between the groups. The dependant variables were diameter, velocity, blood flow, max:min velocity ratio and WSR. The within subject factor was isocapnic hyperoxia and the between subject factor was group. The magnitude of change of each of the homodynamic parameters was correlated with systemic mean arterial blood pressure, duration of diabetes, A1c values and the edema index values within 500μm and 1500μm radii of the fovea. Two-tailed *t*-tests were utilized to determine differences between testing conditions where appropriate.

5.4 Results

There were no significant differences between the groups for all primary outcome measures at baseline.

Group mean baseline and effect magnitudes of retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR for each group are shown in Table 5.2. The magnitudes of change of each of these parameters in response to isocapnic hyperoxia are shown in Figure 5.1. Retinal arteriolar diameter significantly decreased in response to isocapnic hyperoxia in groups 1, 3 and 4 ($p<0.005$); group 2 exhibited a non-significant trend towards vasoconstriction ($p=0.090$). Retinal blood velocity, flow, and WSR significantly decreased in response to isocapnic hyperoxia in all groups ($p\leq0.0002$, $p<0.0001$ and $p\leq0.005$, respectively). Max:min velocity ratio significantly increased in groups 1, 2 and 3 ($p\leq0.007$).

	Group 1	Group 2	Group 3	Group 4
Diameter air (µm)	110.7 (12.8)	114.8 (8.0)	113.5 (10.4)	115.9 (12.6)
Diameter O_2 (µm)	106.6 (13.2)	111.9 (8.2)	109.3 (11.9)	109.5 (13.2)
Velocity air (mm/sec)	34.8 (8.2)	36.4 (7.6)	31.9 (7.0)	32.4 (7.8)
Velocity O_2 (mm/sec)	21.0 (5.8)	23.9 (5.7)	24.7 (6.3)	26.4 (5.0)
Flow air (µL/min)	10.2 (3.5)	11.4 (2.8)	9.9 (3.3)	10.4 (3.2)
Flow O_2 (µL/min)	5.8 (2.3)	7.1 (2.0)	7.3 (3.1)	7.6 (2.5)
Max:min air	3.1 (0.8)	3.9 (1.9)	4.1 (2.5)	4.1 (2.2)
Max:min O_2	5.1 (2.7)	4.6 (1.9)	5.3 (3.7)	3.8 (1.4)
WSR air (s^{-1})	1280 (350)	1262 (286)	1142 (247)	1159 (331)
WSR O_2 (s^{-1})	807 (242)	838 (142)	911 (202)	970 (210)

Table 5.2: Group mean diameter, velocity, flow, max:min velocity ratio and WSR during air and oxygen breathing as a function of group

(max:min; maximum to minimum ratio of velocity, WSR; wall shear rate).

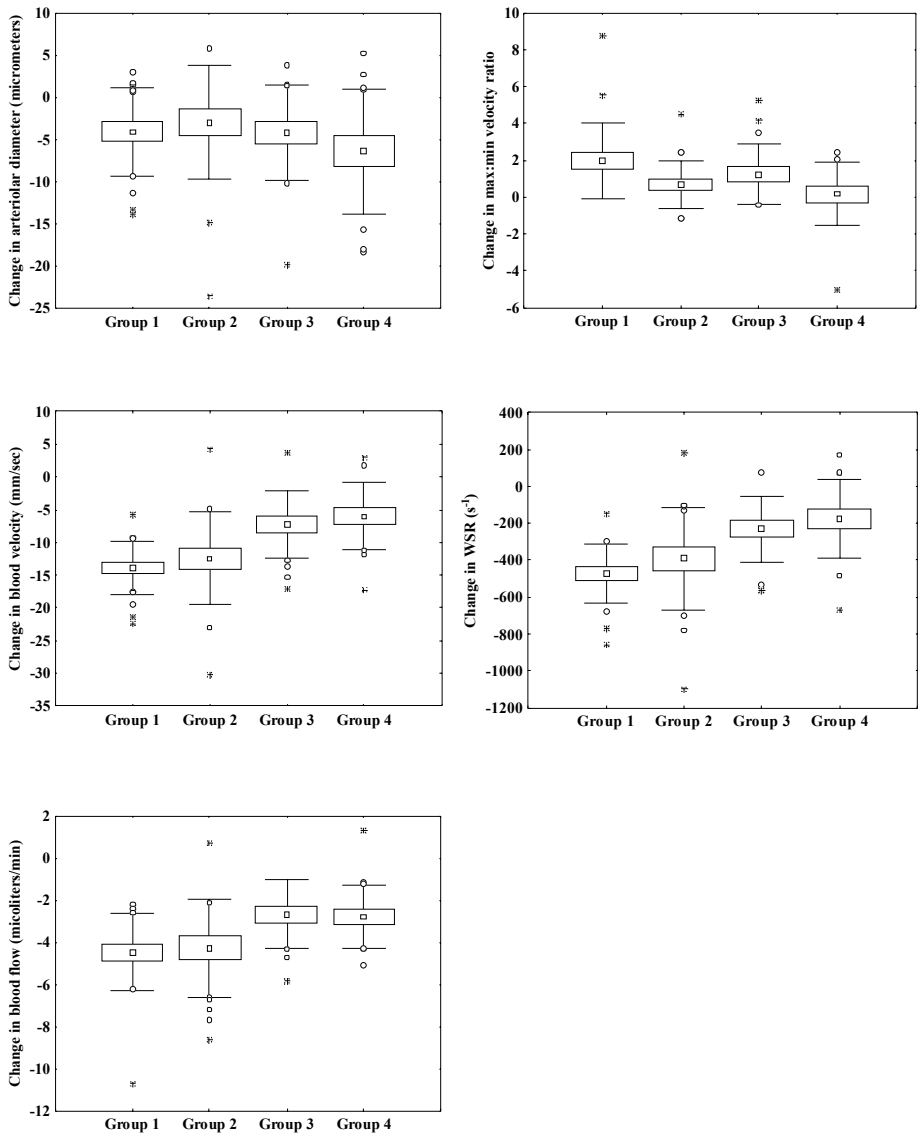

Figure 5.1 Change in retinal arteriolar diameter (upper left), blood velocity (middle left), blood flow (lower left), max:min velocity ratio (upper right), and wall shear rate (middle right) with isocapnic hyperoxia provocation as a function of group. For each graph the centre of the box represents the group mean response, the limits of the box represent ±1 SE and the whiskers represent ±1 SD standard deviation. Open circles represent outlier values and stars represent extreme values. Extreme values are outside the 3 box length range from the upper and lower value of the box. Group 1; non-diabetic, age-matched controls. Group 2; patients with no clinically visible DR. Group 3; patients with mild-to-moderate non-proliferative DR in the absence of clinically evident DME. Group 4; patients with diabetic macular edema.

Group mean reduction in diameter, velocity and flow shown as percentage change in response to isocapnic hyperoxia as function of group are shown in Table 5.3. The magnitude of the reduction of blood velocity in response to isocapnic hyperoxia was significantly reduced with increasing severity of retinopathy ($p<0.0001$). The responses of groups 3 and 4 were significantly less than that of groups 1 and 2 and the response of group 3 was significantly less than that of group 2. The magnitude of the reduction of blood flow was significantly reduced with increasing severity of retinopathy ($p<0.004$). The responses of groups 3 and 4 were significantly less than that of group 1 and the response of group 3 was significantly less than that of group 2.

Group mean reduction (%)	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Diameter	3.7% (4.8%)	2.5% (5.4%)	3.7% (4.8%)	5.5% (6.4%)
Velocity	40% (8%)	33% (15%)	22% (13%)	17% (13%)
Flow	44% (9%)	36% (15%)	28% (14%)	26% (16%)

Table 5.3: Group mean reduction in diameter, velocity and flow in percentage change due to isocapnic hyperoxia as function of group.

Considering the patients with diabetes as a whole, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin (one tailed t-test; $p>0.05$).

The magnitude of the increase of max:min velocity ratio in response to isocapnic hyperoxia was significantly reduced with increasing severity of retinopathy ($p<0.001$). The response of group 1 was significantly greater than that of groups 2 and 4. The magnitude of the decrease of WSR in response to isocapnic hyperoxia was significantly reduced with increasing severity of retinopathy ($p=0.0002$). The response of group 1 was significantly greater than that of groups 3 and 4 and the response of group 2 was significantly greater than that of group 4.

There was no correlation between the magnitude of change of each of the homodynamic parameters and age, systemic mean arterial blood pressure, duration of diabetes and A1c values.

Group mean baseline and effect values for relevant respiratory and systemic parameters as a function of group are shown in Table 5.4. Fractional inspired oxygen (FiO_2) changed significantly in each group with isocapnic hyperoxic provocation ($p<0.0001$; paired two-tailed t-test). Expired carbon dioxide ($P_{ET}CO_2$) did not change in any group. The group mean mean arterial blood pressure (MAP; [(2/3 * diastolic BP) + (1/3 * systolic BP)] was not significantly different between baseline and isocapnic hyperoxia for any of the groups. Pulse rate did not change significantly in any group with isocapnic hyperoxic provocation.

	Group 1	Group 2	Group 3	Group 4
$P_{ET}CO_2$ air (%)	4.9 (0.4)	4.9 (0.5)	4.8 (0.5)	4.9 (0.4)
$P_{ET}CO_2$ O_2 (%)	4.8 (0.4)	4.8 (0.4)	4.7 (0.5)	4.9 (0.4)
FiO_2 air (%)	20.0 (0.5)	20.1 (0.2)	20.1 (0.3)	20.1 (0.4)
FiO_2 O_2 (%)	92.9 (2.9)	92.8 (2.8)	91.4 (5.1)	92.1 (4.2)
MAP air (mmHg)	90.7 (10.3)	91.5 (6.8)	92.0 (9.8)	102.9 (8.2)
MAP O_2 (mmHg)	92.3 (10.4)	91.9 (7.2)	92.8 (9.9)	105.2 (9.1)
PR air (bpm)	65.3 (9.2)	73.2 (11.4)	74.3 (12.9)	78.8 (11.0)
PR O_2 (bpm)	60.8 (8.4)	60.0 (11.1)	71.9 (13.0)	75.8 (11.8)

Table 5.4: Group mean baseline and effect values for fractional expired carbon dioxide ($P_{ET}CO_2$), inspired oxygen (FiO_2), mean arterial blood pressure (MAP) and mean pulse rate (PR) during air and isocapnic hyperoxia as a function of group.

Group mean edema index values within 500μm and 1500μm radii of the fovea as a function of group are shown in Table 5.5. Edema index values were significantly greater for group 4 than group 1 for both the 500μm and 1500μm radii circles ($p \leq 0.0005$; paired two-tailed t-test). Edema index values were significantly greater for group 3 than group 1 for the 1500μm circle only ($p=0.0005$; two-tailed t-test). Group 4 was significantly greater than group 2 for both the 500μm and 1500μm radius circles ($p \leq 0.0005$; two-tailed t-test). There was a significant correlation between baseline edema index values within the 500μm radius circle and the magnitude of change in velocity in response to isocapnic hyperoxia ($r=0.3$; $p=0.03$). Baseline edema index values within the 1500μm radius circle also correlated with the magnitude of change in velocity ($r=0.3$; $p=0.03$) and flow ($r=0.3$; $p=0.04$), in response to isocapnic hyperoxia.

Edema Index values	Group 1	Group 2	Group 3	Group 4
500 µm radius	1.12 (0.28)	1.18 (0.38)	1.34 (0.24)	1.55*† (0.36)
1500 µm radius	1.19 (0.21)	1.19 (0.24)	1.39* (0.20)	1.48*† (0.23)

Table 5.5: Group mean edema index values within 500µm and 1500µm radius of the fovea as a function of group (* indicates significantly different from Group 1; † indicates significantly different from Group 2).

Group mean renal function indicators as a function of group are shown in Table 5.6. Group mean blood albumin was significantly lower in group 3 than that of group 2 (two-way, paired t-test $p<0.01$).

Group	Blood sodium (mmol/L)	Blood potassium (mmol/L)	Blood creatinine (µmol/L)	Blood albumin (g/L)	Urine creatinine (µmol/L)	Urine albumin (mg/L)
1	--	--	--		--	--
2	139 (2.0)	4.1 (0.3)	73 (23)	43.1 (2.0)	8885 (5008)	28.0 (32.8)
3	138 (2.0)	4.3 (0.6)	77 (36)	40.8* (2.8)	9132 (5918)	99.8 (112.8)
4	138 (2.0)	4.2 (0.4)	86 (23)	41.4 (2.7)	8388 (6902)	89.2 (104.9)

Table 5.6: Group mean blood sodium, potassium, creatinine and albumin, urine creatinine and urine albumin as a function of group

(* indicates significantly different from Group 2).

5.5 Discussion

The present study investigated change in retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR induced by an isocapnic hyperoxic provocation in groups of diabetic patients stratified by severity of retinopathy and compared to age-matched subjects without diabetes. At baseline, there were no differences in the hemodynamic values between the groups. The vascular reactivity response in terms of the reduction of blood flow relative to baseline was significant in all groups but the magnitude of the change in flow was significantly reduced with increasing severity of retinopathy. DME patients and patients with moderate DR without clinically evident DME demonstrated significantly reduced vascular reactivity compared to age-matched subjects without diabetes and patients with no clinically visible DR. Max:min velocity ratio increased significantly as a result of isocapnic hyperoxia in each group except the DME group. The magnitude of change of max:min and WSR was significantly less in the DME group compared to that of age-matched subjects without diabetes. The correlation between the objective assessment of retinal edema and retinal vascular reactivity has not been reported previously. Taken as a whole, these results indicate a loss of retinal vascular reactivity in patients with moderate DR without clinically evident DME, and in patients with DME. Furthermore, the results indicate an inability to increase vessel tonus and reduce shear stress (as indicated by the absence of change in max:min velocity ratio and WSR, respectively), in response to isocapnic hyperoxic provocation in patients with DME.

Retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant level[6,32] and also varies directly with the partial pressure of arterial carbon dioxide (PCO_2).[33] Oxygen supply to the retina during hyperoxia is controlled by either a direct reduction of vessel diameter,[32] or by change in WSR via an up-stream flow-induced mechanism that initiates a secondary retinal diameter response;[30] however, the exact mechanism by which retinal vessels respond to changes in PO_2 have yet to be fully elucidated. Hyperoxia stimulates ET-1 release from retinal vascular endothelial cells *in vitro* and is the primary factor modulating retinal vascular reactivity induced by hyperoxia,[34] in animals[35] and humans.[36]

Previously published studies have investigated retinal vascular reactivity using diabetic patients and a non-isocapnic hyperoxic stimulus.[7,12,13] A reduced magnitude of vascular reactivity to hyperoxia relative to subjects without diabetes has been demonstrated in patients with a spectrum of diabetic retinopathy severity up to that of proliferative retinopathy. These studies have been limited because many have not utilized simultaneous diameter and velocity measurements, all did not control for systemic variation in arterial CO_2 during hyperoxia and none have focused on changes associated with the development of early sight-threatening diabetic retinopathy culminating in DME. Most of these studies have measured vascular reactivity in venules. We studied the retinal arteriolar response since the arterioles are known to be primarily responsible for the regulation of vascular reactivity and more closely obey Poiseuille flow principles (given their more circular cross section).

Work form our laboratory[37] has assessed homeostatic arteriolar diameter, velocity and flow in groups of patients that were very similar to the present study. There were no significant differences found in diameter, velocity or flow across the groups, however, there was a significant elevation of max:min velocity ratio with increasing risk of development of DME reflecting decreased vascular compliance with increasing severity of DR. Considering the results of this study, impairment of vascular reactivity would seem to occur concomitantly with the loss of vessel compliance reported by Guan et al.[37] Importantly, this study found that the vascular reactivity response between the groups was significantly different but the baseline hemodynamic parameters were not different. These findings probably resulted from the relative differences in sample size between this study and that of Guan et al.[37] and an increased sensitivity of the vascular reactivity parameters to detect hemodynamic disturbance. The present study investigated change in max:min velocity ratio and WSR across groups before and during isocapnic hyperoxia. Functional hemodynamic indices such as max:min velocity ratio, resistivity index, pulsatility index and WSR have been investigated previously using the retinal vasculature.[25,37,38] Increased peripheral arterial stiffness (measured in the arm and ankle) has been positively correlated with severity of DR.[39] Resistivity and pulsatility indices were not used in this study because of the extraneous influence of downstream impedance.[40,41]

WSR is a measure of shear stress i.e. shear stress = WSR * viscosity. To the best of our knowledge, this study is the first to detail WSR in groups of diabetic patients and age-matched subjects without diabetes, and the change of WSR in response to isocapnic

hyperoxia. A short-term increase in WSR using a hypoxic stimulus in cats has previously been demonstrated.[30] Our work agrees with Nagoaka et al.[30] by demonstrating a decrease in WSR during a hyperoxic stimulus in all groups except those with DME. Our results show that patients with DME are unable to regulate WSR in response to isocapnic hyperoxic provocation. It has previously been shown that increased shear stress results in increased hydraulic conductivity across the vessel wall[42] and the propensity for edema formation.

MEM allows the objective assessment of retinal edema and has previously been shown to have a high sensitivity and good specificity for detection of DME.[14,43] No previous publications investigating change in vascular reactivity in DR have used objective techniques for the assessment of retinal edema. In addition, the method used to deliver isocapnic hyperoxia in this study has previously been shown to reduce variability of end-tidal CO_2 measurements.[15] The correlation between the magnitude of the vascular reactivity response and the edema index suggests a continuum of edema formation and impairment of vascular reactivity with increasing severity of DR.

The reason for impairment of vascular reactivity in patients with diabetes is uncertain but may include structural alterations to the smooth muscle cells (SMCs) or pericytes and functional alterations of the endothelial cells.[44,45] The endothelium determines, in part, the regulation of retinal blood flow. This is achieved principally via ET-1 and nitric oxide. ET-1 is upregulated in diabetes,[46] however the sensitivity and / or response to ET-1 is impaired in diabetes.[47,48] ET-1 upregulation results in increased media-to-lumen ratio, matrix accumulation and

vascular remodeling.[46] These influences may be important in the altered vascular responsiveness to endothelin-1 noted by some authors.[49,50] Nitric oxide (NO) also plays an important role in regulation of retinal blood flow.[51] NO bioactivity is reduced in diabetes due to decreased production, or inactivation,[52] resulting in increased arterial stiffness due to alteration of the collagen / elastin ratio of the vessel wall.[53] Taken as a whole, these vascular remodeling changes will result in reduced compliance and vascular reactivity. Alternatively, the method of control of blood glucose may impact the vascular reactivity response since insulin is known to have vasoactive properties.[54] However, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin.

In diabetes, SMCs undergo abnormal growth, proliferation and migration[55] thereby preventing normal function. In addition, pericytes are capable of responding to changes in oxygen concentration[56] and can regulate endothelin-1 and iNOS release from endothelial cells.[57] As pericytes are progressively reduced in number during DR, impairment of vascular reactivity may be due, in part, to loss of pericytes.

We hypothesize that increasing arterial stiffness and a reduced vascular reactivity response are important in the development of retinal edema. Retinal arterioles are the resistance vessels of the retina and control downstream hydrostatic pressure.[58] An absence of an effective vascular reactivity control mechanism will result in increased hydrostatic pressure at the level of the capillary bed. According to Starling's law, increased hydrostatic pressure will result in

the net movement of fluid out of the vascular compartment into the extra-cellular space and the formation of edema.[58]

5.6 Conclusion

This study utilized an isocapnic hyperoxic stimulus to assess retinal vascular reactivity in patients stratified by retinopathy status and subjects without diabetes. Isocapnic hyperoxia resulted in a significant reduction of retinal arteriolar blood velocity and flow for all participants. The magnitude of change in blood velocity and flow in response to isocapnic hyperoxia was reduced in those individuals with clinically evident diabetic retinopathy relative to subjects without diabetes. A reduced change in max:min velocity ratio and WSR in response to isocapnic hyperoxia in patients with DME was also demonstrated. The differences in vascular reactivity occurred in the absence of any difference in baseline hemodynamic values. Vascular reactivity is impaired in early sight-threatening DR and this impairment is related to the magnitude of edema. Altered production or sensitivity to various biochemical factors and / or structural / functional changes of the endothelial cells, smooth muscle cells or pericytes may be involved in the relatively early development of DR. A prospective study investigating change in the retinal vascular reactivity response and functional hemodynamic indices with the development of DR and DME is required.

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6 Retinal arteriolar hemodynamic response to an acute hyperglycemic provocation in early and sight-threatening diabetic retinopathy

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	Concept / Design	Recruitment	Acquisition of data	Analysis	Write-up / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
Nrusimhadevara		Y	Y		
Ridout	Y		Y		
Harvey		Y	Y		
Mandelcorn		Y	Y		
Lam		Y	Y		
Devenyi		Y	Y		

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

6.1 Abstract

Purpose: To quantify the magnitude of change of retinal arteriolar diameter, blood velocity, blood flow, maximum to minimum (max:min) velocity ratio and wall shear rate (WSR) induced by a hyperglycemic provocation in a group of diabetic patients stratified by retinopathy and compare to age-matched subjects without diabetes.

Methods: The sample comprised 20 non-diabetic controls (Group 1; mean age 49 yrs, SD 10 yrs), 19 patients with no clinically visible DR (Group 2; mean age 52 yrs, SD 11 yrs), 18 patients with mild-to-moderate non-proliferative DR (Group 3; mean age 51 yrs, SD 12 yrs) and 18 patients with diabetic macular edema (Group 4; mean age 55 yrs, SD 9 yrs). Retinal hemodynamic measurements using the Canon Laser Blood Flowmeter (CLBF-100) were acquired before and 1 hour after drinking a standardized oral glucose load drink. The magnitude of the retinal vascular response as well as max:min velocity ratio and WSR was calculated and compared across groups.

Results: A significant change in blood glucose level was observed for all groups ($p<0.05$). The change in blood glucose elevation was significantly less in Group 1 compared to the other Groups. No significant change in arteriolar diameter, blood velocity, blood flow, max:min velocity ratio and WSR was found in patients with diabetes and in age-matched subjects without diabetes.

Conclusion: The results of this study indicate that retinal blood flow is unaffected by acute elevation of blood glucose using an oral glucose load drink in patients with diabetes and age-matched subjects without diabetes.

Keywords: Retinal blood flow, Laser Doppler velocimetry, hyperglycemia, diabetic retinopathy, diabetic macular edema.

6.2 Introduction

Disturbance of retinal hemodynamics is an accepted surrogate marker of early diabetic retinopathy (DR).[1-6] In the context of this paper, hemodynamics is a term that covers all indices of blood flow assessment. Published studies investigating change in homeostatic retinal blood flow in patients with diabetes (relative to that of non-diabetic subjects) show conflicting results.[2,3,7-16] More recently, our group has reported a loss of retinal arteriolar vascular compliance[17] and a reduced magnitude of vascular reactivity (Gilmore et al., In Submission 2006) as representing some of the earliest and most prominent hemodynamic disturbances in early DR.

Published studies investigating the retinal vascular response to hyperglycemia are also conflicting. Some authors have reported increased retinal blood flow[2,3,18,19] while others report no change in retinal blood flow[20,21] in response to elevated blood glucose. The apparent disparity of these results may be partly attributable to the different techniques used to measure retinal hemodynamics, the inclusion of patients with differing morphological and varying glycemic control characteristics, the use of different methodologies to provoke hyperglycemia and the use of differing sites to measure blood flow.

The aim of this study was to quantify the magnitude of change, if any, of retinal arteriolar diameter, blood velocity, blood flow, max:min velocity ratio and wall shear rate (WSR) induced by an acute hyperglycemic provocation in groups of diabetic patients with early and

sight-threatening DR and in age-matched subjects without diabetes. The diabetic patients were clinically stratified by retinopathy status. In addition, volunteers underwent non-invasive, objective assessment of diabetic macular edema (DME) using the Macular Edema Module (MEM) of the Heidelberg Retina Tomograph II.[22] There are unique aspects to this study. (1) We used a technique that allowed the simultaneous quantification of vessel diameter and centre-line blood velocity to calculate volumetric retinal blood flow in microlitres per minute and utilized a defined retinal arteriolar measurement site. (2) We used a standardized oral glucose load drink to elevate blood glucose. (3) Relative to previously published studies, a large sample of clinically defined patients with early and sight-threatening DR was recruited. (4) We quantified change in functional hemodynamic indices in response to hyperglycemia as a function of severity of DR. (5) Change in retinal blood flow parameters in response to hyperglycemic provocation were correlated with non-invasive, objective measures of DME.

6.3 Materials and Methods

6.3.1 Sample

Kida et al.[19] demonstrated a 30% elevation of retinal arteriolar blood flow in healthy subjects in response to elevated blood glucose and found a 66% reduction in that response when comparing healthy subjects to patients with impaired glucose tolerance and branch retinal vein occlusion. Using previously published data from our laboratory,[23] group mean homeostatic blood flow values in healthy young subjects was found to be 9.4 μL/min (SD=2.5 μL/min) and the SD of the difference between baseline and recovery in response to hyperoxic provocation was 0.85 μL/min. Considering the combined results of these studies, healthy subjects would be expected to manifest a 30% increase of the homeostatic blood flow value i.e. 9.4 μL/min * 30% = 3.13 μL/min. The difference between groups in order to reach statistical significance would need to be 0.70 μL/min (i.e. [33% of 9.4 μL/min] / 3). Therefore, the standardized effect size (difference between means / SD) was calculated to be 0.82 and the resulting sample size using an alpha of 0.05 and power of 0.9 was 18 per group. The sample comprised 22 non-diabetic controls (Group 1; mean age 49 yrs, SD 10 yrs), 18 patients with no clinically visible DR (Group 2; mean age 52 yrs, SD 11 yrs), 18 patients with mild-to-moderate non-proliferative DR and an absence of clinically evident DME (Group 3; mean age 51 yrs, SD 12 yrs) and 17 patients with diabetic macular edema as defined by the ETDRS[24] (Group 4; mean age 55 yrs, SD 9 yrs)(Table 6.1).

Group	Group Mean Age (SD)	Male to female ratio	Group Mean duration diabetes (years)	Group mean A1c value (SD)	Number treated with insulin
1	49 (10)	7 M:15 F	--	--	--
2	52 (11)	8 M: 10 F	10 (9)	0.072 (0.014)	7
3	51 (12)	9 M: 9 F	14 (10)	0.083 (0.019)	12
4	55 (9)	12 M: 5 F	13 (8)	0.084 (0.014)	7

Table 6.1: Group mean age, duration of diabetes, number treated with insulin, male to female ratio, A1c and random glucose as a function of group.
(A1c: glycosylated hemoglobin. M; male, F; female)

Diabetic patients were stratified into groups according to their retinal status using dilated stereo fundus biomicroscopy. Volunteers were aged between 30 and 70 years and had a LogMAR VA of 0.3 or better. Volunteers were excluded if they exhibited any eye disease (apart from DR for groups 2, 3 and 4) or had undergone ocular surgery, any cardiovascular (except well controlled systemic hypertension) and respiratory (except treated asthma) disorders, a refractive error greater than ±6.00DS or ±2.00DC and glaucoma in a first degree relative. None of the volunteers were regular smokers or had undergone retinal laser treatment. All volunteers were asked to refrain from caffeine-containing drinks or snacks for at least 8 hours prior to their study visit. Lens clarity was graded using the Lens Opacity

Classification System III (LOCS III).[25] The study was approved by the University Health Network Research Ethics Board, Toronto and the University of Waterloo Office of Research Ethics. Informed consent was obtained from each volunteer after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki.

6.3.2 Oral glucose tolerance test (glucose load drink)

An oral glucose load drink (75 grams glucose suspended in 300ml water) was given to all participants after fasting for a minimum of 8 hours. Diabetic patients were asked to omit their usual doses of insulin or oral hypoglycemic agents during this fasting period.

6.3.3 Quantification of retinal vessel diameter, blood velocity and flow

The principal underlying the quantification of retinal hemodynamics is based on the Doppler effect. Laser light (frequency=f) reflected from a moving particle is shifted in frequency (Δf) that is proportional to the velocity of the moving red blood cells. A vessel that exhibits Poiseuille flow will have a range of velocities and thus a range of frequency shifts up to a maximum frequency shift (Δf_{max}) that corresponds to the maximum velocity of the blood moving at the centre of the vessel. By utilizing two photomultipliers separated by a known angle, the maximum frequency shift at each photomultiplier is subtracted to allow the absolute quantification of centre-line blood velocity irrespective of the angle between the moving particle and reflected beam.[26,27]

The Canon Laser Blood Flowmeter (CLBF; Canon, Tokyo, Japan) utilizes a red diode laser (675nm, 80μm x 50μm oval) to measure velocity every 0.02 seconds across a 2 second measurement window resulting in a velocity-time trace. The CLBF also uses a green diode laser system (543nm, 1500μm x 150μm rectangle) that is used to measure vessel diameter and maintain centration of the laser at the measurement site.[28,29] The vessel tracking system allows post-acquisition rejection of velocity measurements impacted by significant saccades. Diameter readings are acquired every 4 milliseconds during the first and final 60 milliseconds of the 2 second velocity measurement window. Two sequential measurements utilizing different optical paths (path 1 and path 2) are taken to ensure consistency and averaged to give one reading. In combination with the average velocity (V_{mean}) over at least one pulse cycle and diameter (D), flow through the vessel can be calculated using $1/2.\pi.D^2/4.V_{mean}.60$. Magnification effects associated with refractive and axial components of ametropia are corrected to provide absolute measurements of diameter (μm), velocity (mm/sec) and flow (μL/min). The technological principles utilized in this device have been described in detail elsewhere.[19,26,27,30] In addition, this device has been extensively evaluated in volunteers with,[19,31,32] and without,[23,33-35] retinal diseases and following therapeutic intervention.[36]

6.3.4 Quantitative assessment of retinal edema

A confocal scanning laser tomograph that sequentially acquires two-dimensional section images along the optical axis was employed. The distribution of reflected light intensity along the optical axis for a given pixel is described by the *z*-profile or confocal intensity profile. The

MEM technique of the Heidelberg Retina Tomograph II (HRT; Heidelberg Engineering, Heidelberg, Germany) determines the z-profile signal width (at half peak height) and peak reflectance intensity. Studies have demonstrated a broadening of the z-profile signal width and a decrease in peak reflectance intensity in areas of retinal edema.[37] Normalization of the reflectance values reduces the variation in intensity between successive scans. Edema index values are generated that represent the z-profile signal width divided by normalized reflectance intensity for each pixel within the image. MEM has been demonstrated to have high sensitivity and good specificity for the detection of DME.[22] The average edema index of all pixels within the 500μm and 1500μm radius circles was calculated.

6.3.5 Procedures

One eye of each subject was randomly assigned to the study if both eyes met the study criteria. Volunteers attended for 2 visits (median interval between first and second visit was 21 days). Visit 1 was used to establish eligibility and baseline characteristics, determine group assignment, and undertake objective assessment of DME and to familiarize the volunteer with the technique used to quantify retinal hemodynamics. Three sets of MEM images centered on the fovea were acquired at visit 1 for each volunteer. Visit 2 was used to quantify retinal vascular response to hyperglycemia. Refraction, logMAR visual acuity, resting blood pressure and random blood glucose level were assessed prior to dilation of the study eye with 1% tropicamide (Alcon Canada). Retinal hemodynamic measurements were acquired from a retinal arteriole approximately 1 disc diameter from the optic nerve head at a straight vessel segment and distant from bifurcations in one eye of each volunteer. At least five retinal

hemodynamic measurements were attempted using the CLBF at baseline and 1 hour after consuming the glucose load drink i.e. the expected peak of glucose profile. Random blood glucose levels were assessed prior to, and directly following, the second set of CLBF measurements. Axial length was measured by I^3 System ABD A-scan ultrasound (I^3 Innovative Imaging Inc, Sacramento, CA) to correct blood flow measurements for magnification effects due to ametropia.

6.3.6 Analysis

A post acquisition analysis of the CLBF velocity waveforms was performed using a standardized protocol to remove aberrant waveforms affected by eye movement, tear film breakup, or improper tracking of the measurement laser. The maximum number of acceptable pulse cycles was used in the data analysis for each measurement (with a minimum of 1 complete velocity waveform required). In addition, maximum to minimum (max:min) velocity ratio was calculated before and during hyperglycemia for each individual. This ratio reflects vascular compliance, where an elevation of max:min velocity ratio indicates increased vascular rigidity (the site of this change can be up-stream of, down-stream of, or at the CLBF measurement site). In addition, WSR (mean velocity * 8 / diameter)[38] was calculated because change is shear stress is believed to alter blood flow and this mechanism is thought to be disturbed in diabetes and atherosclerosis.[38,39]

The normality of each hemodynamic parameter as a function of group and condition was

confirmed prior to the use of parametric statistics. A normal distribution was confirmed for all parameters apart from max:min velocity ratio and WSR which were log transformed for statistical analysis. The change in each of the hemodynamic parameters in response to provocation within each group was determined using paired two-tailed t-tests. Repeated measures ANOVA was used to determine any differences between the baseline hemodynamic parameters between groups and any difference in the response of the hemodynamic parameters between the groups. The dependent variables were diameter, velocity, blood flow, max:min velocity ratio and WSR. The within subject factor was hyperglycemia and the between subject factor was group. The magnitude of change of each of the hemodynamic parameters was correlated with systemic mean arterial blood pressure, duration of diabetes, A1c values and the edema index values within 500μm and 1500μm radii of the fovea. Two-tailed *t*-tests were utilized to determine differences between testing conditions where appropriate.

6.4 Results

There were no significant differences between the groups for all retinal hemodynamic outcome measures at baseline.

Table 6.2 details blood glucose before and 1 hour after ingestion of glucose. At baseline, group 1 blood glucose was significantly lower than groups 2, 3, and 4 ($p \leq 0.016$). Blood glucose increased significantly in all groups ($p < 0.0001$) and this increase was significantly different between the groups ($p < 0.0001$). The change in blood glucose was significantly less

in group 1 compared to that of the other 3 groups.

Group mean blood glucose	Group 1	Group 2	Group 3	Group 4
Baseline	5.8 mmol/L (1.1)	7.7 mmol/L (2.9)	9.0 mmol/L (3.8)	10.1 mmol/L (2.5)
Effect	8.7 mmol/L (2.8)	16.1 mmol/L (4.8)	16.3 mmol/L (4.4)	17.3 mmol/L (3.3)
Change	2.9 mmol/L (2.8)	8.4 mmol/L (3.9)	7.4 mmol/L (2.0)	7.2 mmol/L (2.8)

Table 6.2: Group mean (SD) baseline, effect and change in glucose as function of group.

Group mean baseline and effect magnitudes of retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR for each group are shown in Table 6.3. Retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR did not change from baseline as a result of the glucose load drink in any of the groups ($p>0.05$) (Figure 6.1).

	Group 1	Group 2	Group 3	Group 4
Diameter pre-glucose (µm)	110.0 (12.4)	114.3 (7.4)	115.0 (9.4)	113.9 (13.5)
Diameter post-glucose (µm)	108.2 (12.5)	113.3 (8.7)	114.6 (9.1)	109.1 (10.0)
Velocity pre-glucose (mm/sec)	34.0 (6.2)	35.9 (7.6)	33.9 (10.1)	33.6 (7.8)
Velocity post-glucose (mm/sec)	35.1 (7.0)	35.8 (6.4)	34.9 (9.4)	34.5 (8.2)
Flow pre-glucose (µL/min)	9.8 (2.7)	11.2 (3.0)	10.9 (4.6)	10.0 (3.3)
Flow post-glucose (µL/min)	9.8 (2.8)	10.9 (2.7)	11.0 (4.1)	10.2 (3.4)
Max:min pre-glucose	3.3 (0.7)	3.8 (1.9)	3.8 (1.3)	3.9 (2.1)
Max:min post-glucose	3.5 (0.7)	4.1 (1.7)	4.2 (1.7)	4.2 (2.2)
WSR pre-glucose (s^{-1})	1263 (272)	1264 (263)	1156 (308)	1198 (300)
WSR post-glucose (s^{-1})	1327 (317)	1253 (239)	1186 (276)	1228 (343)

Table 6.3: Group mean mean functional hemodynamic indices before and after ingestion of glucose as a function of group

(max:min; maximum to minimum ratio of velocity, WSR; wall shear rate).

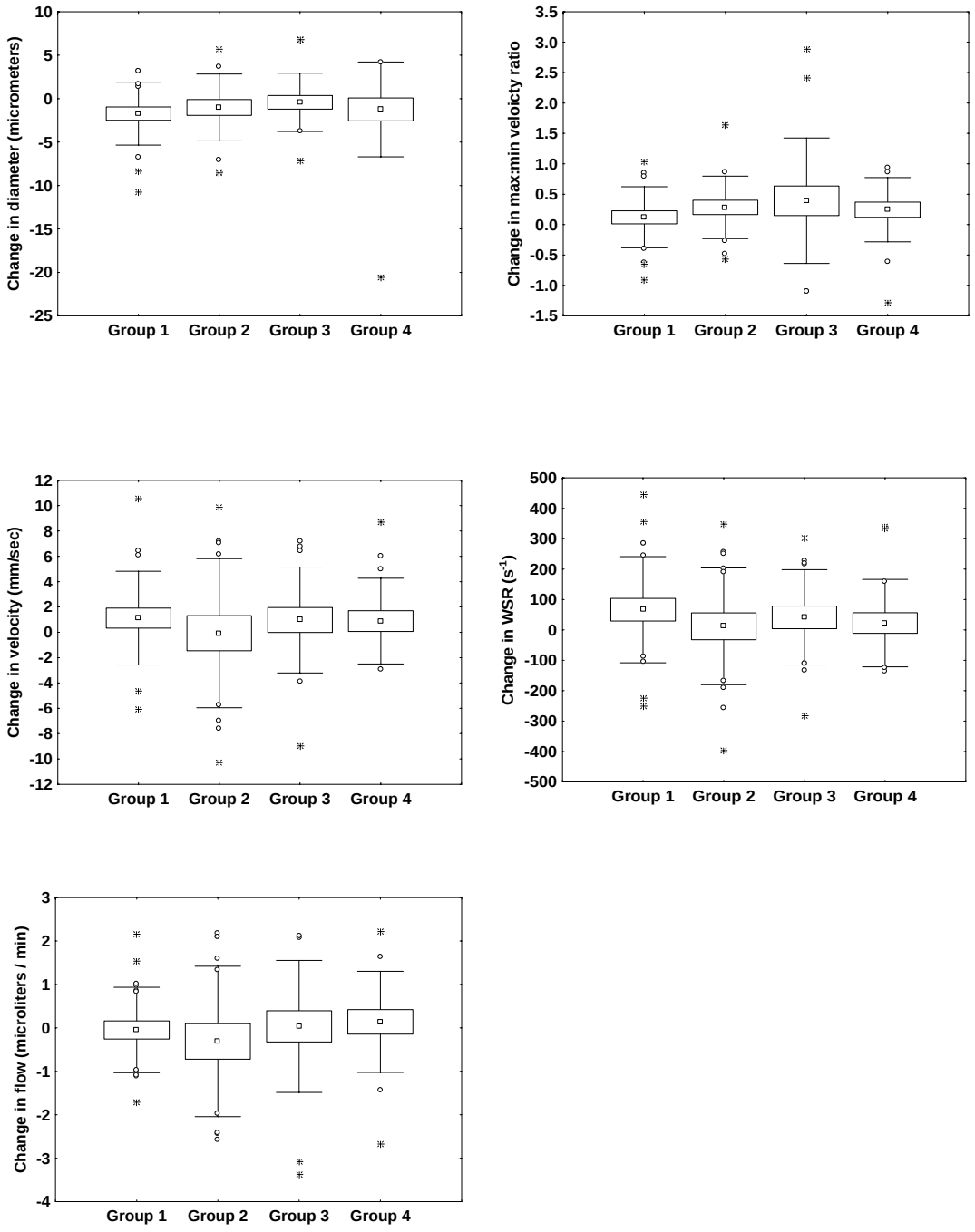

Figure 6.1 Change in retinal arteriolar diameter, blood velocity, blood flow, max:min velocity ratio, wall shear rate with isocapnic hyperoxia as a function of group. For each group the centre of the box represents the group mean response, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. Open circles represent outlier values and stars represent extreme values. Extreme values are outside the 3 box length range from the upper and lower value of the box. Group 1; non- diabetic, age-matched controls. Group 2; patients with

no clinically visible DR. Group 3; patients with mild-to-moderate non-proliferative DR in the absence of clinically evident DME. Group 4; patients with DME.

Considering the patients with diabetes as a whole, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin (one tailed t-test; $p>0.05$).

Change of flow was significantly correlated with change in blood glucose for the non-diabetic group only ($r=0.52$; $p=0.027$). Change in flow was not correlated with change in blood glucose for any of the diabetic patient groups.

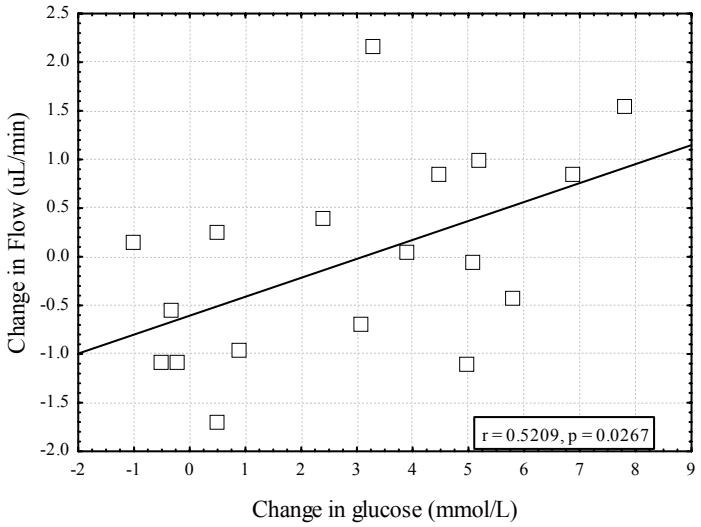

Figure 6.2 Graph showing relationship between blood flow and blood glucose in the non-diabetic group.

Group mean edema index values within 500μm and 1500μm radii of the fovea as a function of group are shown in Table 6.4. Edema index values were significantly larger for group 4 compared to groups 1 and 2 for both the 500μm and 1500μm radius circles ($p \leq 0.0005$). Edema index values of group 3 were also significantly larger than those of group 1 for the 1500μm circle only ($p=0.0005$). There was no correlation between baseline edema index values within the 500μm or 1500μm radius circle and the magnitude of change in diameter, velocity or flow in response to the oral glucose load drink.

Edema Index values	**Group 1**	**Group 2**	**Group 3**	**Group 4**
500 μm radius	1.12 (0.28)	1.18 (0.38)	1.34 (0.24)	1.55*† (0.36)
1500 μm radius	1.19 (0.21)	1.19 (0.24)	1.39* (0.20)	1.48*† (0.23)

Table 6.4: Group mean edema index values within 500μm and 1500μm radius of the fovea as a function of group.

(* indicates significantly different from Group 1; † indicates significantly different from Group 2).

6.5 Discussion

There were no significant differences between the groups for all retinal hemodynamic outcome measures at baseline and none of the parameters changed as a result of glucose ingestion in any of the groups. Interestingly, change of flow was significantly correlated with change in blood glucose for the non-diabetic group only. There was no correlation between baseline edema index values and the magnitude of change in diameter, velocity or flow in response to the oral glucose load drink.

Chronic hyperglycemia is an established risk factor for the development of DR.[40,41] Chronic hyperglycemia increases the influx of glucose into the retina[42-44] that causes altered retinal biochemistry and subsequently precipitates the morphological changes of DR. Both fasting blood glucose levels and the response to an oral glucose tolerance test are typically used to detect diabetes. At baseline, 3 participants in the clinically normal group had blood glucose levels greater than 7.0mmol/L.[45] Two of these individuals had an oral glucose tolerance test response that was inside normal limits. The third individual showed a marginally elevated oral glucose tolerance test response at 1 hour post load (11.9mmol/L, reference value 11.1mmol/L)[45]; however, the normal time point for assessment of OGTT is 2 hours post load when blood glucose values are expected to be lower for non-diabetic individuals.

Published studies investigating differences in homeostatic retinal blood flow in patients with diabetes (relative to that of non-diabetic subjects) show conflicting results. Patients with

background diabetic retinopathy have been reported to exhibit either increased,[7-10] or decreased,[2,3,11-14] blood hemodynamics. Others reported no significant change in blood hemodynamics.[15] Recent work from our own laboratory has shown a significant elevation of max:min velocity ratio with increasing risk of development of DME suggesting decreased vascular compliance with increasing severity of DR; interestingly, there were no significant differences found in diameter, velocity or flow across the groups.[17]

Studies investigating the retinal vascular response to acute hyperglycemia are also conflicting.[2,18-21] Bursell et al.[2] measured blood flow using cine fluorescein angiography and a glucose clamp methodology in a group of diabetic subjects without retinopathy. They found a significant increase in flow between the various glucose levels employed in their study. Kida et al.[19] evaluated arteriolar retinal blood flow using the CLBF after ingestion of a glucose load drink in clinically normal subjects and those who had been diagnosed with branch retinal vein occlusion (BRVO). Patients were separated into either normal or impaired glucose tolerance groups based on their 2 hour post-load glucose level.[46] At the 1 hour time point, retinal blood flow had significantly increased in the clinically normal group relative to baseline and the response was significantly greater than that of the patient group. At the 2 and 3 hour time points, the retinal blood flow had significantly increased from baseline for both the clinically normal group and the BRVO patients with normal glucose tolerance but, interestingly, not those patients with impaired glucose tolerance. Garhofer et al.[18] found increased retinal blood flow in a group of non-diabetic subjects in response to an acute elevation of blood glucose. Blood flow was calculated using velocimetry measurements

(Oculix, Berwyn, PA, USA) and venular diameter determination using the Retinal Vessel Analsyer (Imedos, Germany). According to these authors, an increase in velocity (rather than diameter) precipitated the observed change in blood flow.

Grunwald et al.[47] measured changes in retinal blood velocity and combined venular diameter measurements to calculate flow in a group of diabetic patients with no or mild DR. These poorly controlled patients were initially hyperglycemic (mean glucose measurement 21.7mmol/L). The patients' blood glucose levels were returned to 'normal' values (mean 5.8mmol/L) following exogenous insulin injection and blood flow values were seen to decrease by approximately 15%. However, the authors point out that the administration of glucose (to elevate blood glucose) may not be directly comparable to that of administration of insulin (to reduce blood glucose) which is known to have vasoactive effects.[48]

Sullivan et al.[21] found no change in blood flow in response to acute hyperglycemia. However, the diabetic subjects in their study had a relatively long duration of type 1 diabetes and therefore were unable to react to the glucose stimulus (due to a loss of vascular compliance). This is in keeping with other studies that have found a significant change of flow in patients without diabetes[18] and in patients with normal glucose tolerance.[19] Recent work for our laboratory has shown a increase of max:min velocity ratio (suggesting a loss of retinal vascular compliance) and a loss of vascular reactivity with increasing severity of non-proliferative diabetic retinopathy (Gilmore et al., *In Submission* 2006). In this study, we found a significant correlation between change in blood glucose and change in blood flow for the

non-diabetic subject group only.

The results of the present study show that retinal arteriolar blood flow is unaltered during acute increase of blood glucose in patients with diabetes and in age-matched subjects without diabetes. To the best of our knowledge, this is the first published study to employ a bi-directional laser Doppler technique to quantify blood velocity and simultaneously quantify arteriolar diameter in a group of patients with diabetes stratified by retinopathy status and a group of age-matched subjects without diabetes. The results of this study are in agreement with Fallon et al[20] and Sullivan et al.[21] The present study also investigated change in max:min velocity ratio and WSR in response to the hyperglycemic provocation. These indices have been shown to be sensitive to alterations in retinal hemodynamics in patients with diabetes[17,49] (Gilmore et al., *In Submission* 2006), and other patient populations.[32] Neither the max:min velocity ratio nor WSR changed as a result of the hyperglycemic provocation in any of the groups. In addition, the method of control of blood glucose may impact the vascular reactivity response since insulin is known to have vasoactive properties.[50] However, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin.

In support of our results, is the fact that glucose has a low basal retinal influx[51] and glucose transport operates near saturation level at normal physiological glucose concentrations.[52] Glut-1 is one of the most important glucose transporters and is responsible for the movement of glucose across the inner and outer blood retinal barriers.[53] Glut-1 expression is unchanged

during acute increase of blood glucose preventing increased glucose uptake across bovine retinal endothelial cells.[51]

Conversely, other published studies have documented increased retinal blood hemodynamics during short-term hyperglycemic episodes in cats and mini-pigs.[54,55] By contrast, Ben-Nun et al.[56] and Puchowitz et al.[57] reported no change in retinal blood flow during hyperglycemia in cats and rats, respectively. Although these studies have employed different species and methodologies to quantify retinal blood flow, there is clearly no consensus as to whether blood flow changes during an acute increase of blood glucose.

The limitations of this study are that a single set of retinal blood flow measurements were acquired 1 hour after ingestion of a glucose-load drink. There may be retinal hemodynamic changes induced by hyperglycemia at shorter,[18] or longer,[19] time periods than that measured in this study. We chose the 1 hour post-ingestion time point to acquire blood flow measurements at peak blood glucose values.[58] The response to elevated glucose levels was only measured in major arterioles of the retina; however, this site represents the resistance vessels of the retinal vasculature. In addition, the insulin status of the diabetic patients was unknown. The power of the study, calculated using the largest difference between the groups was 13%. Although we cannot say for certain that we have not committed a type II error, the lack of response in any of the groups leads us to believe that acute changes in blood glucose does not change retinal blood flow.

6.6 Conclusion

The main finding of our study is unaltered retinal arteriolar blood flow 1 hour post acute blood glucose increase in patients with diabetes and in age-matched subjects without diabetes. In addition, retinal arteriolar diameter, blood velocity, max:min velocity ratio and WSR were unchanged as a result of the acute hyperglycemic provocation. The results of this study do not support the view that retinal blood flow is affected by an acute increase of blood glucose in diabetic patients and in subjects without diabetes.

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7 Retinal arteriolar diameter, blood velocity and blood flow response to a combined isocapnic hyperoxia and glucose provocation in early and sight-threatening diabetic retinopathy

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	Concept / Design	Recruitment	Acquisition of data	Analysis	Write-up / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
Nrusimhadevara		Y	Y		
Ridout	Y		Y		
Harvey		Y	Y		
Mandelcorn		Y	Y		
Lam		Y	Y		
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7.1 Abstract

Purpose: To quantify the magnitude of change of retinal hemodynamics induced by a combined isocapnic hyperoxic / hyperglycemic provocation in groups of diabetic patients and in age-matched volunteers without diabetes and to compare the response to that of an isocapnic hyperoxic provocation alone.

Methods: The sample comprised 17 non-diabetic controls (Group 1), 15 patients with no clinically visible diabetic retinopathy (DR) (Group 2), 16 patients with mild-to-moderate non-proliferative DR (Group 3) and 15 patients with diabetic macular edema (Group 4). Retinal hemodynamic measurements were acquired at baseline (homeostatic blood glucose levels and breathing air) and 1 hour after drinking a standardized oral glucose load drink using isocapnic hyperoxia. The magnitude of change of retinal arteriolar diameter, velocity, blood flow, max:min velocity ratio and WSR was calculated and compared across groups.

Results: Retinal blood velocity and flow significantly decreased in all groups ($p \leq 0.001$ and $p \leq 0.0002$, respectively). Max:min velocity ratio significantly increased ($p \leq 0.005$), and WSR significantly decreased ($p \leq 0.0002$), in groups 1, 2 and 3 but not group 4. The vascular reactivity response was not significantly different *across* the groups. The age-matched non-diabetic group demonstrated a reduced response of change in flow ($p=0.009$) and WSR ($p=0.010$) to the combined hyperoxic / hyperglycemic provocation compared to that of hyperoxia alone.

Conclusions: The vascular reactivity response to a combined hyperoxic / hyperglycemic provocation produced a pronounced reduction in blood flow. Unlike the response to hyperoxia

alone, the vascular reactivity response was not significantly different *across* the groups. This suggests that hyperglycemia may influence the retinal vascular reactivity response to hyperoxia.

Keywords: Retinal blood flow, Laser Doppler velocimetry, hyperglycemia, diabetic retinopathy, diabetic macular edema.

7.2 Introduction

Vascular reactivity represents the hemodynamic response of the vasculature to a given stimulus, such as hyperoxia[1] (Gilmore et al., *In Submission* 2006) or hypercapnia.[2] Vascular reactivity in response to hyperoxia with simultaneous hyperglycemia has previously been reported to be impaired in humans.[3,4] By contrast, other authors have reported no effect of hyperglycemia on hyperoxia induced retinal vascular reactivity in humans.[5] However, previous studies are limited because many have not utilized simultaneous diameter and velocity measurements to calculate retinal blood flow in absolute units and all did not control for systemic variation in arterial CO_2 during hyperoxic provocation. In addition, a unique aspect of this study is the focus on changes associated with the development of early sight-threatening diabetic retinopathy (DR) culminating in diabetic macular edema (DME). Impairment of vascular reactivity during acute hyperglycemia may yield important information about the pathophysiology associated with the development of early DR.

Previous work from our laboratory has shown that hyperoxia induced change of blood flow in early DR is impaired and this impairment precedes change in homeostatic blood flow parameters; vascular reactivity is a more sensitive marker of vascular dysfunction in early DR than homeostatic blood flow assessment (Gilmore et al., *In Submission* 2006). Following on from this work, we used the same techniques to investigate the impact of acute hyperglycemia on vascular reactivity in early DR; we were unable to reveal any impact of hyperglycemia on vascular reactivity. This study advances the work by investigating the effect of a combined

hyperoxic / hyperglycemic stimulus on vascular reactivity. We hypothesize that hyperglycemia reduces the retinal vascular reactivity response to a hyperoxic stimulus.

The aim of this study was to quantify the magnitude of change of retinal hemodynamics induced by a combined isocapnic hyperoxia / hyperglycemic provocation in groups of diabetic patients clinically stratified by retinopathy status and in age-matched subjects without diabetes. The response to the combined hyperoxia / hyperglycemia provocation will be compared to that of an isocapnic hyperoxic provocation alone using volunteers common to both paradigms (Gilmore et al., *In Submission* 2006). Volunteers also underwent non-invasive, objective assessment of diabetic macular edema (DME) using the Macular Edema Module (MEM) of the Heidelberg Retina Tomograph II.[6] We correlated the retinal vascular reactivity response to a combined hyperoxic / hyperglycemic provocation with the objective assessment of retinal edema.

7.3 Materials and Methods

7.3.1 Sample

Using previously published data from our group,[1] the vascular reactivity response of healthy young subjects in terms of change of retinal blood flow in response to isocapnic hyperoxia was found to be 4.3 μL/min and the SD of the difference between baseline and recovery was 0.85 μL/min. Assuming an approximate 50% reduction in vascular reactivity response when comparing healthy subjects to our most advanced diabetic retinopathy group[7] (Gilmore et al., *In Submission* 2006), the difference between groups in order to reach statistical significance would need to be 0.72 μL/min (i.e. [50% of 4.3 μL/min] / 3). Therefore, the standardized effect size (difference between means / SD) was calculated to be 0.85 and the resulting sample size using an alpha of 0.05 and power of 0.9 was 16 per group. The sample comprised 17 non-diabetic controls (Group 1; mean age 49 yrs, SD 10 yrs), 15 patients with no clinically visible DR (Group 2; mean age 54 yrs, SD 11 yrs), 16 patients with mild-to-moderate non-proliferative DR [8] (Group 3; mean age 51 yrs, SD 12 yrs) and 15 patients with DME (Group 4; mean age 55 yrs, SD 8 yrs) (Table 7.1).

Group	Group mean age (years) (SD)	Group mean duration diabetes (years) (SD)	Number treated with insulin	Male to female ratio	Group mean A1c value (SD)
1	49 (10)	--	--	6 M:11 F	--
2	54 (9)	6 (5)	5	5 M: 10 F	0.070 (0.010)
3	51 (12)	15 (10)	12	8 M: 7 F	0.084 (0.019)
4	55 (8)	13 (9)	6	11 M: 4 F	0.087 (0.014)

Table 7.1: Group mean age, duration of diabetes, number treated with insulin, male to female ratio, and A1c value.
(A1c: glycosylated hemoglobin. M; male, F; female)

Volunteers were stratified into groups according to their retinal status using dilated stereo fundus biomicroscopy. All volunteers were aged between 30 and 70 years and had a logMAR VA of 0.3 or better. Volunteers were excluded if they exhibited any eye disease (apart from DR for groups 2, 3 and 4) or had undergone ocular surgery, any cardiovascular (except well controlled systemic hypertension) and respiratory (except treated asthma) disorders, a refractive error greater than ±6.00 Dioptres sphere or ±2.00 Dioptres cylinder and glaucoma

in a first degree relative. None of the volunteers were regular smokers or had undergone retinal laser treatment. All volunteers were asked to refrain from caffeine-containing drinks or snacks for at least 8 hours prior to their study visit. Lens clarity was graded using the Lens Opacity Classification System III[9] (LOCS III). The study was approved by the University of Waterloo Office of Research Ethics and the University Health Network Research Ethics Board, Toronto. Informed consent was obtained from each volunteer after explanation of the nature and possible consequences of the study according to the tenets of the Declaration of Helsinki.

7.3.2 Oral glucose tolerance test (glucose load drink)

An oral glucose load drink (75 grams glucose suspended in 300ml water) was given to all participants. Diabetic patients were asked to fast for a minimum of 8 hours and to omit their usual doses of insulin or oral hypoglycemic agents during this fasting period.

7.3.3 Isocapnic hyperoxia delivery system

The isocapnic hyperoxia delivery system comprised a sequential re-breathing circuit made up of a fresh gas reservoir, an expiratory gas reservoir and a face-mask (Hi-Ox^{SR}, ViasysHealthcare, Yorba Linda, CA). The inspiratory and expiratory limbs were interconnected by a single positive end-expiratory pressure (PEEP) valve, allowing exhaled gas to be re-breathed when the gas in the inspiratory limb was depleted. Flow from gas tanks containing oxygen and air respectively was controlled using standard rotometers as

flowmeters. This method has been described in detail in a previous publication.[10]

7.3.4 Quantification of retinal vessel diameter, blood velocity and flow

The principal underlying the quantification of retinal hemodynamics is based on the Doppler effect. Laser light (frequency=f) reflected from a moving particle is shifted in frequency (Δf) that is proportional to the velocity of the moving red blood cells. A vessel that exhibits Poiseuille flow will have a range of velocities and thus a range of frequency shifts up to a maximum frequency shift (Δf_{max}) that corresponds to the maximum velocity of the blood moving at the centre of the vessel. By utilizing two photomultipliers separated by a known angle, the maximum frequency shift at each photomultiplier is subtracted to allow the absolute quantification of centre-line blood velocity, irrespective of the angle between the moving particle and reflected beam.[11,12]

The Canon Laser Blood Flowmeter (CLBF; Canon, Tokyo, Japan) utilizes a red diode laser (675nm, 80µm x 50µm oval) to measure velocity every 0.02 seconds across a 2 second measurement window resulting in a velocity-time trace. The CLBF also uses a green diode laser system (543nm, 1500µm x 150µm rectangle) that is used to measure vessel diameter and maintain centration of the laser at the measurement site [13,14]. The vessel tracking system allows post-acquisition rejection of velocity measurements impacted by significant saccades. Diameter readings are acquired every 4 milliseconds during the first and final 60 milliseconds of the 2 second velocity measurement window. Two sequential measurements utilizing

different optical paths (path 1 and path 2) are taken to ensure consistency and averaged to give one reading. In combination with the average velocity (V_{mean}) over a pulse cycle and diameter (D), flow through the vessel can be calculated using $1/2.\pi.D^2/4.V_{mean}.60$. Magnification effects associated with refractive and axial components of ametropia are corrected to provide absolute measurements of diameter (μm), velocity (mm/sec) and flow (μL/min). The technological principles utilized in this device have been described in detail elsewhere.[11,12,15,16] In addition, this device has been extensively evaluated in volunteers with,[16-18] and without,[1,19-21] retinal diseases and following therapeutic intervention.[22]

7.3.5 Quantitative assessment of retinal edema

A confocal scanning laser tomograph that sequentially acquires two-dimensional section images along the optical axis was employed. The distribution of reflected light intensity along the optical axis for a given pixel is described by the *z*-profile or confocal intensity profile. The MEM technique of the Heidelberg Retina Tomograph II (HRT; Heidelberg Engineering, Heidelberg, Germany) determines the z-profile signal width (at half peak height) and peak reflectance intensity. Studies have demonstrated a broadening of the *z*-profile signal width and a decrease in peak reflectance intensity in areas of retinal edema.[23] Normalization of the reflectance values reduces the variation in intensity between successive scans. MEM has been demonstrated to have high sensitivity and good specificity for the detection of DME.[6]

7.3.6 Procedures

One eye of each subject was randomly assigned to the study if both eyes met study criteria. Volunteers attended for 2 visits. Visit 1 was used to establish eligibility and baseline characteristics, determine group assignment, and undertake objective assessment of DME and to familiarize the volunteer with the technique used to quantify retinal hemodynamics. Three sets of MEM images centered on the fovea were acquired at visit 1 for each volunteer. Visit 2 was used to quantify the retinal vascular response to a combined hyperoxic / hyperglycemic provocation. Refraction, logMAR visual acuity, resting blood pressure and random blood glucose level were assessed prior to dilation of the study eye with 1% tropicamide (Alcon Canada). Retinal hemodynamic measurements were simultaneously acquired from an arteriole approximately 1-2 disc diameters from the optic nerve head using a straight vessel segment in one eye of each volunteer. At least five retinal hemodynamic measurements were acquired using the CLBF at baseline and 1 hour after glucose ingestion (while breathing oxygen under isocapnic conditions). Intraocular pressure was measured by Goldmann applanation tonometry after retinal blood flow measurements had been acquired. Axial length was measured by I^3 System ABD A-scan ultrasound (I^3 Innovative Imaging Inc, Sacramento, CA) to correct blood flow measurements for magnification effects due to ametropia.

The response to the combined hyperoxia / hyperglycemia provocation was compared to the response of an isocapnic hyperoxic provocation alone determined at a separate visit (Gilmore et al., *In Submission* 2006). The median time between the 2 provocations was 7 days.

7.3.7 Gas analysis and systemic vascular responses

A rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, USA) was used to quantify the relative concentrations of O_2 and CO_2 in both the inspired and expired gases on a breath-by-breath basis. The relative concentrations of O_2 and CO_2 were sampled continuously by the gas analyzer and the inspired O_2, inspired CO_2, $P_{ET}O_2$ and $P_{ET}CO_2$ were downloaded to a personal computer every 5 seconds (S5 Collect software, Datex-Ohmeda, USA). In addition, finger-oxygen saturation, respiration rate and pulse rate were also recorded continuously. $P_{ET}CO_2$ was analyzed by calculating the upper 10^{th} and lower 90^{th} percentiles. Data points lying outside the upper 10^{th} or lower 90^{th} percentiles were excluded from the analysis since all of these values were found to be erroneous i.e. these points resulted from inappropriate interpretation of tidal waveforms by the gas monitor. Blood pressure was also measured non-invasively once every minute over the course of the hyperoxic paradigm (Cardiocap 5, Datex-Ohmeda, USA).

7.3.8 Analysis

A post acquisition analysis of the CLBF velocity waveforms was performed using a standardized protocol to remove aberrant waveforms affected by eye movement, tear film breakup, or improper tracking of the measurement laser. The maximum number of acceptable pulse cycles was used in the data analysis for each measurement (with a minimum of 1 complete velocity waveform required). In addition, maximum to minimum (max:min)

velocity ratio was calculated during air breathing and compared to that during oxygen breathing for each individual. This ratio reflects vascular compliance, where an elevation of max:min ratio indicates increased vascular rigidity (the site of this change can be up-stream of, down-stream of, or at the CLBF measurement site). In the physiological situation, compliance is expected to reduce and rigidity increase during hyperoxia due to increased tonus of the vessel wall. In addition, wall shear rate (WSR= mean velocity * 8 / diameter)[24] was calculated because change is shear stress is believed to alter blood flow and this mechanism is thought to be disturbed in diabetes and atherosclerosis.[24,25]

The normality of each hemodynamic parameter as a function of group and condition was confirmed prior to the use of parametric statistics. A normal distribution was confirmed for all parameters apart from max:min velocity ratio which was log transformed for statistical analysis. The change in each of the hemodynamic parameters in response to provocation within each group was determined using paired two-tailed t-tests. Repeated measures ANOVA was used to determine any differences between the baseline hemodynamic parameters between groups, any difference in the response of the hemodynamic parameters between the groups and any difference in the response between combined hyperoxia / hyperglycemia and hyperoxia alone within each group. The dependant variables were diameter, velocity, blood flow, max:min velocity ratio and WSR. The within subject factor was glucose and isocapnic hyperoxia and the between subject factor was group. The magnitude of change of each of the homodynamic parameters was correlated with systemic mean arterial blood pressure, duration of diabetes, A1c values and the edema index values

within 500μm and 1500μm radii of the fovea. Two-tailed t-tests were utilized to determine differences between testing conditions, where appropriate.

7.4 Results

There were no significant differences between the groups for all the retinal hemodynamic outcome measures at baseline.

Group mean baseline and effect magnitudes of retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR for each group are shown in Table 7.2. The magnitudes of change of each of these parameters in response to combined hyperoxic / hyperglycemic provocation are shown in Figure 7.1.

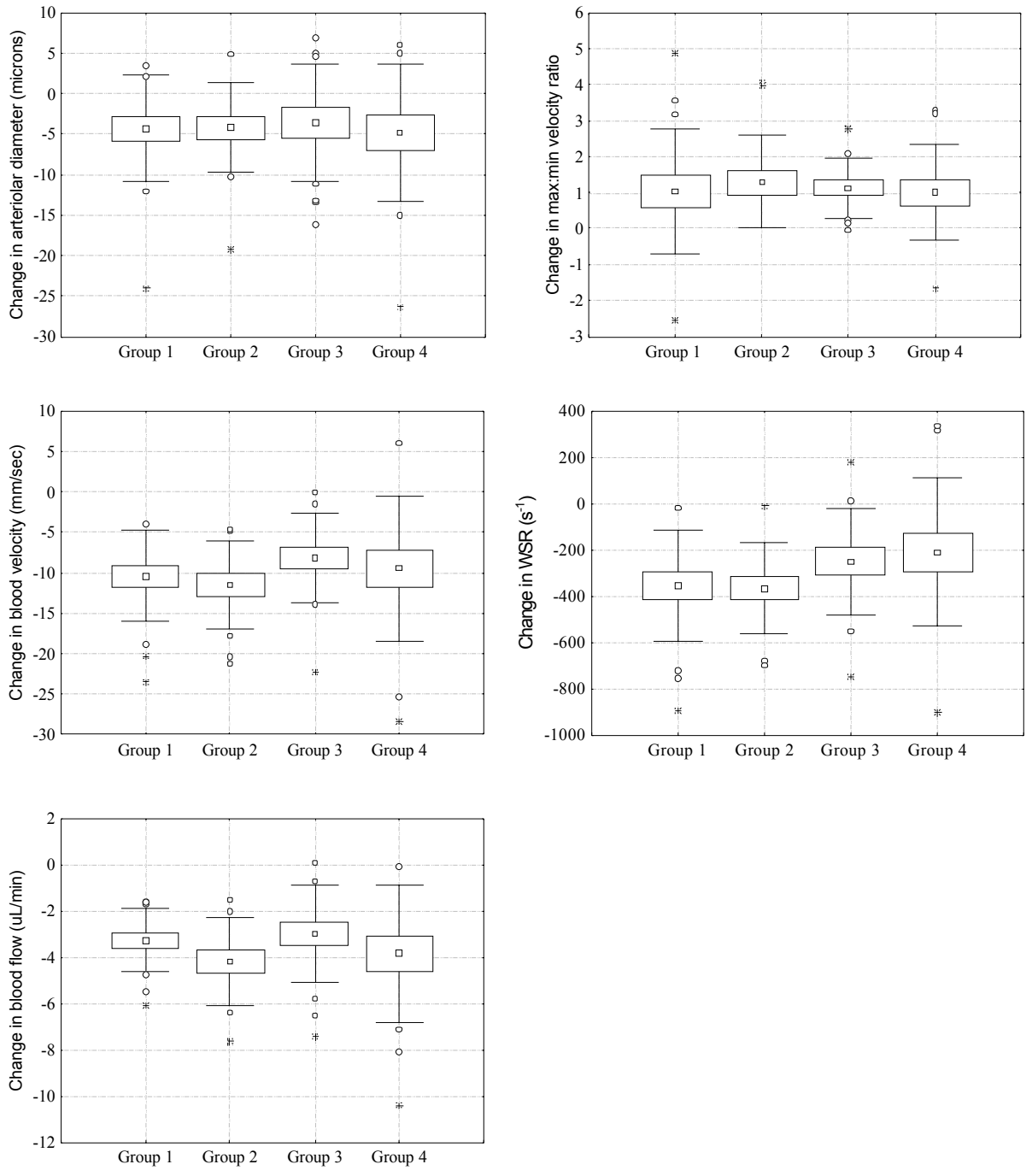

Figure 7.1Change in retinal arteriolar diameter (upper left), blood velocity (middle left), blood flow (lower left), max:min velocity ratio (upper right), and wall shear rate (middle right) following combined hyperglycemic / hyperoxic provocation as a function of group.

For each graph the centre of the box represents the group mean response, the limits of the box represent ±1 SE

and the whiskers represent ±1 SD standard deviation. Open circles represent outlier values and stars represent extreme values. Extreme values are outside the 3 box length range from the upper and lower value of the box. Group 1; non-diabetic, age-matched controls. Group 2; patients with no clinically visible DR. Group 3; patients with mild-to-moderate non-proliferative DR in the absence of clinically evident DME. Group 4; patients with DME.

Retinal arteriolar diameter did not change significantly in any group (Bonferroni corrected $p<0.01$) in response to a combined hyperoxic / hyperglycemic provocation. Retinal blood velocity and flow significantly decreased in all groups ($p\leq0.001$ and $p\leq0.0002$, respectively). Max:min velocity ratio significantly increased ($p\leq0.005$), and WSR significantly decreased ($p\leq0.0002$), in groups 1,2 and 3 but not group 4.

The magnitude of change of the retinal hemodynamic outcome measures in response to combined hyperoxic / hyperglycemic provocation was not significantly different between the groups.

	Group 1	Group 2	Group 3	Group 4
Diameter air / pre-glucose (µm)	108.4 (10.0)	115.7 (8.3)	113.2 (9.8)	113.9 (13.5)
Diameter O_2 / post-glucose (µm)	104.0 (11.3)	111.5 (8.4)	109.6 (12.4)	109.1 (14.2)
Velocity air / pre-glucose (mm/sec)	33.4 (7.0)	37.0 (8.1)	34.5 (7.3)	35.0 (11.0)
Velocity O_2 / post-glucose (mm/sec)	23.0* (5.7)	25.5* (6.1)	26.3* (6.2)	25.5* (7.1)
Flow air / pre-glucose (µL/min)	9.3 (2.4)	11.8 (3.1)	10.7 (3.8)	11.0 (4.4)
Flow O_2 / post-glucose (µL/min)	6.0* (2.3)	7.6* (2.3)	7.7* (3.2)	7.1* (2.1)
Max:min air / pre-glucose	3.5 (1.6)	4.1 (2.2)	3.6 (1.3)	3.5 (0.9)
Max:min O_2 / post-glucose	4.5* (1.8)	5.3* (3.1)	4.7* (2.0)	4.5 (1.8)
WSR air / pre-glucose (s^{-1})	1242 (290)	1285 (275)	1216 (206)	1221 (362)
WSR O_2 / post-glucose (s^{-1})	886* (220)	918* (202)	966* (204)	1010 (298)

Table 7.2: Group mean diameter, velocity, flow, max:min ratio and WSR during air / pre-glucose and during oxygen / post-glucose as a function of group.
(max:min; maximum to minimum ratio of velocity, WSR; wall shear rate). *; indicates significantly different from baseline (air).

Using data from volunteers common to both paradigms, the magnitude of change of flow

(p=0.009) and WSR (p=0.010) was significantly less for the age-matched non-diabetic group to the combined hyperoxic / hyperglycemic provocation than that of hyperoxia alone (Bonferroni corrected p=0.01). For the non-diabetic group, flow reduced by 43.8% in response to isocapnic hyperoxia but only reduced by 36.0% in response to the combined hyperoxic / hyperglycemic provocation, while WSR reduced by 36.8% in response to hyperoxia and by 27.3% in response to the combined hyperoxic / hyperglycemic provocation. There were no significant differences in any of the outcome parameters between the combined hyperoxic / hyperglycemic provocation and hyperoxia alone for any of the diabetic groups.

Group mean reduction in diameter, velocity and flow shown as percentage change in response to the combined hyperoxic / hyperglycemic provocation as function of group are shown in Table 7.3.

Group mean change (%)	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Diameter	-4.0% (6.0%)	-3.6% (4.4%)	-3.2% (6.7%)	-4.1% (4.7%)
Velocity	-30.5% (12.0%)	-30.6% (11.4%)	-23.1% (13.8%)	-24.2% (10.6%)
Flow	-36.0% (11.8%)	-35.5% (11.1%)	-27.8% (14.6%)	-31.5% (12.6%)

Table 7.3: Group mean change in diameter, velocity and flow in percentage change due to combined hyperoxic / hyperglycemic provocation as function of group.
(negative value indicates reduction from baseline)

Considering the patients with diabetes as a whole, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin (one tailed t-test; $p>0.05$).

There was a significant correlation between the magnitude of change in blood glucose and age ($r=0.339$; $p=0.007$). There were no other correlations evident between change in the hemodynamic parameters and age, systemic mean arterial blood pressure, duration of diabetes and A1c values.

Group mean baseline and effect values for relevant respiratory and systemic parameters as a function of group are shown in Table 7.4. Fractional inspired oxygen (FiO_2) changed significantly in each group with combined hyperoxic / hyperglycemic provocation ($p<0.0001$; paired two-tailed t-test). Expired carbon dioxide ($P_{ET}CO_2$) did not change in any group. The group mean mean arterial blood pressure (MAP; [(2/3 * diastolic BP) + (1/3 * systolic BP)] was not significantly different between baseline and combined hyperoxic / hyperglycemic provocation for any of the groups. Pulse rate did not change significantly in any group with combined hyperoxic / hyperglycemic provocation.

	Group 1	Group 2	Group 3	Group 4
$P_{ET}CO_2$ air / pre-glucose (%)	4.8 (0.4)	5.0 (0.4)	5.1 (0.3)	5.1 (0.4)
$P_{ET}CO_2$ O_2 / post-glucose (%)	4.8 (0.4)	5.0 (0.5)	5.1 (0.4)	5.0 (0.4)
FiO_2 air / pre-glucose (%)	20.5 (1.7)	20.1 (0.2)	20.0 (0.5)	20.2 (0.6)
FiO_2 O_2 / post-glucose (%)	92.5* (4.5)	92.8* (2.5)	92.8* (2.8)	93.1* (2.5)
MAP air / pre-glucose (mmHg)	89.4 (7.5)	94.9 (8.3)	93.1 (8.8)	99.2 (9.8)
MAP O_2 / post-glucose (mmHg)	90.7 (7.2)	94.4 (8.1)	94.7 (8.4)	102.7 (8.8)
PR air / pre-glucose (bpm)	66.3 (7.0)	72.9 (8.3)	74.3 (12.9)	78.8 (11.0)
PR O_2 / post-glucose (bpm)	63.0 (6.7)	70.1 (9.2)	71.9 (13.0)	75.8 (11.8)

Table 7.4: Group mean fractional expired carbon dioxide ($P_{ET}CO_2$) and inspired oxygen (FiO_2) mean arterial blood pressure (MAP) and mean pulse rate during air / pre-glucose and during oxygen / post-glucose as a function of group.

*; indicates significantly different from baseline.

Table 7.5 details blood glucose before and 1 hour after ingestion of glucose. At baseline, group 1 blood glucose was significantly lower than groups 3 and 4 ($p=0.001$). Blood glucose increased significantly in all groups ($p\leq0.0005$). The increase in blood glucose was significantly less in group 1 compared to that of the other 3 groups ($p<0.0001$).

Group mean blood glucose	Group 1	Group 2	Group 3	Group 4
Baseline (mmol/L)	5.5 (0.7)	7.7 (2.3)	8.1 (3.7)	9.4 (2.6)
Effect (mmol/L)	8.9* (3.0)	14.8* (4.7)	16.0* (4.1)	18.2* (3.8)
Change (mmol/L)	3.4 (3.0)	7.1 (4.5)	7.9 (2.4)	8.7 (2.6)

Table 7.5: Group mean (SD) baseline, effect and change in glucose as function of group.
*; indicates significantly different from baseline.

Group mean edema index values within 500μm and 1500μm radii of the fovea as a function of group are shown in Table 7.6. Edema index values were significantly greater for group 4 than group 1 and 2 for both the 500μm and 1500μm radii circles ($p \leq 0.0005$; paired two-tailed t-test). Edema index values were significantly greater for group 3 than group 1 for the 1500μm circle only ($p=0.0005$; two-tailed t-test).

Edema Index values	Group 1	Group 2	Group 3	Group 4
500 µm radius (arbitrary units)	1.09 (0.29)	1.14 (0.40)	1.32 (0.24)	1.56*† (0.39)
1500 µm radius (arbitrary units)	1.17 (0.21)	1.16 (0.23)	1.39* (0.20)	1.48*† (0.24)

Table 7.6: Group mean edema index values within 500µm and 1500µm radius of the fovea as a function of group.
(* indicates significantly different from Group 1; † indicates significantly different from Group 2)

There was no correlation between baseline edema index values within the 500µm or 1500µm radius circle and the magnitude of change in diameter, velocity or flow in response to combined hyperoxic / hyperglycemic provocation.

Group mean renal function indicators as a function of group are shown in Table 7.7. Group mean blood albumin was significantly lower in group 3 than that of group 2 (two-way, paired t-test $p<0.01$).

Group	Blood sodium (mmol/L)	Blood potassium (mmol/L)	Blood creatinine (µmol/L)	Blood albumin (g/L)	Urine creatinine (µmol/L)	Urine albumin (mg/L)
1	--	--	--		--	--
2	139 (2.0)	4.1 (0.3)	72 (23)	43.1 (2.1)	8245 (4286)	26.1 (36.1)
3	138 (2.4)	4.3 (0.6)	77 (36)	40.8* (2.8)	9132 (5918)	49.6 (43.2)
4	138 (2.6)	4.3 (0.4)	90 (24)	41.0 (2.6)	6708 (4408)	70.1 (66.2)

Table 7.7: Group mean blood sodium, potassium, creatinine and albumin, urine creatinine, and urine albumin as a function of group.

*; indicates significantly different from Group 2.

7.5 Discussion

The present study investigated change in retinal arteriolar diameter, blood velocity, flow, max:min velocity ratio and WSR induced by a combined isocapnic hyperoxic / hyperglycemic provocation in a group of diabetic patients stratified by severity of retinopathy and compared to age-matched subjects without diabetes. The vascular reactivity response in terms of the reduction of blood flow relative to baseline was significant in all groups but the magnitude of the change in flow was not significantly different *across* the groups. The magnitude of change of flow and WSR was significantly less for the age-matched non-diabetic group to the combined hyperoxic / hyperglycemic provocation than that of hyperoxia alone i.e. *within* group comparison. There were no significant differences in any of the hemodynamic outcome parameters between the combined hyperoxic / hyperglycemic provocation and hyperoxia alone *within* any of the diabetic groups.

An intriguing finding of this study was that the age-matched non-diabetic group demonstrated a reduced response of change in flow and WSR to the combined hyperoxic / hyperglycemic provocation compared to that of hyperoxia alone. Careful examination of the characteristics of the age-matched non-diabetic group showed that 3 of them had possible impaired glucose tolerance (IGT). When these subjects were excluded from the analysis, the difference in flow and WSR response between the combined hyperoxic / hyperglycemic provocation and that of hyperoxia alone was not significant (note this finding was based on a sample size of 14). This suggests that hyperglycemia may influence the retinal vascular reactivity response to

hyperoxia in patients with IGT.

Unlike the response to hyperoxia alone (Gilmore et al., *In Submission* 2006) which showed a step profile of reduced reactivity with increasing severity of retinopathy, the vascular reactivity response to a combined hyperoxic / hyperglycemic provocation was not significantly different *across* the groups. This suggests that hyperglycemia may influence the retinal vascular reactivity response to hyperoxia.

Previous work from our laboratory has demonstrated that blood flow significantly decreases in patients with diabetes and subjects without diabetes in response to isocapnic hyperoxia alone; however, the magnitude of the change in flow is significantly less in response to hyperoxia in patients with more severe mild-to-moderate non-proliferative DR (Gilmore et al., *In Submission* 2006), indicating a loss of vascular reactivity with increasing severity of retinopathy. Also, we have shown that retinal blood flow is not affected by short-term increases of blood glucose in patients with diabetes and subjects without diabetes (Gilmore et al, *In Submission* 2006). In agreement with our previous work, we showed that WSR decreased significantly in response to hyperoxic / hyperglycemic provocation in each group except the DME group. WSR is a measure of shear stress i.e. shear stress = WSR * viscosity. To our knowledge, this study details for the first time WSR in groups of diabetic patients and age-matched non-diabetic subjects, and the change of WSR in response to a combined hyperoxic / hyperglycemic provocation. This finding suggests that patients with DME have impaired ability to regulate shear stress in response to provocation. Max:min velocity ratio

significantly increased in all group of the present study except that of the DME group. This finding is the same as that of the response to isocapnic hyperoxia alone (Gilmore et al., *In Submission* 2006) and indicates increased pulsatility characteristics of retinal arteriolar blood flow in patients with DME.

Previously published studies have investigated retinal vascular reactivity using a non-isocapnic oxygen stimulus in patients with diabetes during homeostatic conditions and hyperglycemia.[3-5] These studies have reported either no difference of the magnitude of vascular reactivity response to hyperoxia during normoglycemic and hyperglycemic conditions,[5] or an impaired vascular reactivity response during hyperglycemia.[3,4] However, these studies are limited because of small sample sizes, many have not utilized simultaneous diameter and velocity measurements and all did not control for systemic variation in arterial CO_2 during hyperoxic provocation. In contrast to our study, none have focused on changes associated with the development of early sight-threatening DR, culminating in DME. Most of the previous studies have measured vascular reactivity in venules; however, we studied the retinal arteriolar response since the arterioles are primarily responsible for the regulation of vascular reactivity. The method of control of blood glucose may impact the vascular reactivity response since insulin is known to have vasoactive properties.[26] However, the vascular reactivity response in terms of change in retinal blood flow in patients treated with insulin was not different from those treated without insulin.

Patel et al.[3] assessed the vascular reactivity response to a non-isocapnic hyperoxic stimulus in

patients with diabetes under conditions of normoglycemia and hyperglycemia. They concluded that patients with diabetes have impaired vascular reactivity that is exacerbated by hyperglycemia (>15mmol/L). A possible explanation for the differences in results between that of Patel et al.[3] and our study is that a 60% FiO_2 stimulus (as opposed to >90% FiO_2) may reveal smaller alterations in retinal vascular reactivity between groups. Grunwald et al.[4] induced a reduction in blood glucose level in poorly controlled patients using exogenous insulin. Due to the vasoactive properties of insulin,[27] the study of Grunwald et al.[4] is not directly comparable to our study. In agreement with our results, Davies et al.[5] reported that vascular reactivity was not affected by acute changes of blood glucose. In support of our observation is the fact that glucose has a low basal retinal influx[28] and glucose transport operates near saturation level at normal physiological glucose concentrations.[29] Glut-1 is one of the most important glucose transporters and is responsible for movement of glucose across the inner and outer blood retinal barriers.[30] Glut-1 expression is unchanged during short-term elevations of glucose preventing increased glucose uptake across bovine retinal endothelial cells.[28]

Vascular reactivity has also been assessed in other vascular beds in animals[31] and humans[32,33]. Hamaty et al.[31] reported that short-term hyperglycemia *per se* did not result in abnormal vascular responses in rat tail artery. Houben et al.[32] reported that endothelium-dependent or independent vasoreactivity was not affected by moderate-to-severe hyperglycemia in humans assessed by measuring skin and forearm blood flow. In addition, Capaldo et al.[33] were unable to detect a difference in change of velocity to a vasodilatory agent in the coronary circulation

in normal subjects under baseline and hyperglycemic conditions. Previously published studies in animals have investigated preretinal oxygen tension using hyperoxia during hyperglycemia. Ernest et al.[34] reported increased oxygen tension, whilst others have reported no change in pre-retinal tension.[35,36] In addition, retinal oxygen consumption has been reported in increase[37] or remain unchanged[38] during hyperglycemia.

7.6 Conclusion

In summary, the results of this study indicate that the vascular reactivity response to a combined hyperoxic / hyperglycemic provocation produces a pronounced reduction in blood flow. Unlike the response to hyperoxia alone (Gilmore et al., *In Submission* 2006), the vascular reactivity response to a combined hyperoxic / hyperglycemic provocation was not significantly different *across* the groups. The age-matched non-diabetic group that included 3 suspect IGT individuals, however, demonstrated a reduced response of change in flow and WSR to the combined hyperoxic / hyperglycemic provocation compared to that of hyperoxia alone (Gilmore et al., *In Submission* 2006); these effects were not present when the 3 suspect IGT individuals were excluded from the analysis. Comparing the vascular reactivity response between the combined hyperoxic / hyperglycemic provocation to that of hyperoxia alone, no significant differences were found for any of the outcome parameters *within* any of the diabetic groups. The results suggest that individuals with IGT may exhibit reduced vascular reactivity to hyperoxia during acute hyperglycemia. Future work will investigate the vascular reactivity response in IGT using graded rather than >90% inspired hyperoxic stimuli.

7.7 References

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8 General Discussion

Disturbance of retinal hemodynamics is a surrogate marker of early diabetic retinopathy (DR). Impairment of vascular reactivity to various stimuli has been established. However, studies investigating retinal vascular reactivity have been limited by various factors including non-standardized methods to induce vascular reactivity, different techniques to measure retinal hemodynamics, the inclusion of patients with differing morphological and varying glycemic control characteristics and the use of differing sites to measure retinal blood flow. Many have investigated change in vessel diameter or blood velocity but have not used techniques that simultaneously measure diameter and velocity to quantify blood flow in absolute units.

The comparison of 3 different techniques to administer hyperoxia to human subjects demonstrated that the sequential rebreathing system stabilized PCO_2 and, importantly, reduced the variability of an individual's PCO_2 measurement during hyperoxia. Also, we demonstrated that modest changes of PCO_2 resulted in change of retinal blood flow. Overall, we conclude that the sequential rebreathing system is superior to other techniques for the provocation, and thereby the assessment, of retinal vascular reactivity (Chapter 3).

Chapter 4 details the vascular reactivity response to hyperoxia using the sequential rebreathing system in young, healthy subjects. The Canon Laser Blood Flowmeter (CLBF-100) was employed to assess retinal arteriolar hemodynamics. The study used an isocapnic hyperoxic stimulus to determine retinal vascular reactivity. There was a pronounced reduction in diameter, velocity and flow with a maximal effect evident within 4 minutes from stimulus

onset. All parameters returned to baseline values when the stimulus was removed. The change in retinal blood flow was slightly less than previously published studies using 100% O_2, a response we attribute to elevated O_2 levels and compounded reduction of CO_2. The response characteristic of the change in diameter was not significantly different from that of velocity. Comparing to cold pressor provocation, the results of this study suggest that different response characteristics of the retinal vasculature occur to transmural pressure mediated autoregulation as opposed to metabolic mediated vascular reactivity.

Chapter 5 details the change in retinal hemodynamics to isocapnic hyperoxia in a group of diabetic patients with mild-to-moderate non-proliferative retinopathy DR and age matched subjects without diabetes. DME patients and patients with moderate DR without clinically evident DME demonstrated significantly reduced vascular reactivity compared to age-matched subjects without diabetes and patients with no clinically visible DR. These results indicate a loss of retinal vascular reactivity in patients with moderate DR without clinically evident DME, and in patients with DME. Furthermore, the results indicate an inability to increase vessel tonus and reduce shear stress in response to isocapnic hyperoxic provocation in patients with DME. The reasons for this impairment may be structural alterations of the smooth muscle cells in the vessel wall, the supporting pericytes, functional alterations of the endothelial cells or increased vascular stiffness.

Chapter 6 details the change in retinal hemodynamics to a standardized oral glucose load drink in a group of diabetic patients with mild-to-moderate non-proliferative retinopathy DR

and age matched subjects without diabetes. None of the hemodynamic parameters changed as a result of glucose ingestion in any of the groups. There was no correlation between baseline edema index values and the magnitude of change in diameter, velocity or flow in response to the oral glucose load drink. Interestingly, change of blood flow was significantly correlated with change in blood glucose for the non-diabetic group only. Careful examination of the control group revealed that 3 participants had suspect impaired glucose tolerance (IGT). When these three participants were excluded from the analysis, there was no significant correlation between change in blood glucose and change in flow. This finding suggests that patients with IGT may respond differently to acute hyperglycemia than either age-matched controls or patients with diabetes.

Chapter 7 details the change in retinal hemodynamics to a combined hyperglycemic / hyperoxic provocation in a group of diabetic patients with mild-to-moderate non-proliferative retinopathy DR and age matched subjects without diabetes. The vascular reactivity response in terms of the reduction of blood flow relative to baseline was significant in all groups but, unlike the response to hyperoxia alone, the magnitude of the change in flow was not significantly different *across* the groups. The magnitude of change of flow and WSR was significantly less for the age-matched non-diabetic group to the combined hyperoxic / hyperglycemic provocation than that of hyperoxia alone i.e. *within* group comparison. Careful examination of the characteristics of the age-matched non-diabetic group showed that 3 participants had suspected IGT. When these subjects were excluded from the analysis, the difference in flow and WSR response between the combined hyperoxic / hyperglycemic

provocation and that of hyperoxia alone was not significant. This suggests that hyperglycemia may influence the retinal vascular reactivity response to hyperoxia, especially in patients with IGT.

8.1 Future work

Future work should investigate the vascular reactivity response of patients with IGT. These patients may exhibit responses that are different from patients with established diabetes or subjects without diabetes. We hypothesize that individuals with IGT manifest distinct changes of retinal hemodynamics in response to change of blood glycemic levels since, unlike patients with established diabetes, they retain vascular reactivity function. In addition, a graded stimulus (i.e. incremental increases in PO_2) and / square wave change in PO_2 may be better able to determine subtle changes in vascular reactivity and the timeline of the vascular reactivity response between groups of patients, respectively. In addition, the vascular reactivity response could be used in conjunction with retinal oximetry measurements to assess the degree of physiological impairment in groups of patients with diabetes and in response to new therapies. A fuller understanding of biochemical influences to the vascular reactivity response can be obtained by measuring certain blood markers in patients with e.g. intracellular adhesion molecule. Investigation of vascular reactivity in patients with diabetes using stimuli other than hyperoxia e.g. carbon dioxide may also provide more useful information about the pathophysiology of diabetic retinopathy.

9 Appendix A: Frequency-of-seeing characteristics of the short-wavelength sensitive visual pathway in clinically normal subjects and diabetic patients with focal sensitivity loss.

Gilmore ED, Hudson C, Nrusimhadevara RK, *et al*. Frequency of seeing characteristics of the short wavelength sensitive visual pathway in clinically normal subjects and diabetic patients with focal sensitivity loss. *Brit J Ophthalmol* 2005;**89**(11):1462-1467.

	Concept / Design	Recruitment	Acquisition of data	Analysis	Write-up / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y
Nrusimhadevara		Y	Y		
Harvey		Y	Y		

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

9.1 Abstract

Aims: To define the frequency-of-seeing (FOS) characteristics of the short-wavelength (SW) sensitive visual pathway in normal subjects and in diabetic patients with focal SW sensitivity loss.

Methods: For clinically normal subjects, FOS was assessed at two retinal locations (4.24° and 9.90° eccentricity) for both white-on-white (WW) and SW stimulus parameters. Inter-examination variability was quantified for the clinically normal subjects only. For patients with diabetes, FOS was assessed inside an area of focal SW sensitivity loss, and at the same eccentricity in the quadrant diametrically opposite, using SW stimulus parameters only.

Results: For clinically normal subjects, the group mean SW FOS slope was significantly flatter ($p<0.0001$) than that of WW at both locations. The co-efficient of repeatability for SW FOS slope was $\pm 41.55 dB^{-1}$ (relative to a group mean sensitivity of $23.98 dB^{-1}$) and $\pm 19.98 dB^{-1}$ (group mean sensitivity $16.15 dB^{-1}$) for 4.24° and 9.90°, respectively. For the patients with diabetes, the group mean SW FOS slope was significantly flatter ($p=0.02$), and group mean SW threshold significantly higher ($p=0.007$) in the area of focal SW sensitivity loss than that of the non-focal sensitivity loss location.

Conclusions: The results of this study suggest that the clinical utility of SW automated perimetry will be limited by a greater magnitude of measurement variability, as indicated by a flatter FOS slope compared to conventional perimetry.

Keywords: Short-wavelength perimetry (SW), frequency-of-seeing (FOS), short-term fluctuation (SF), psychometric function

9.2 Introduction

Short-wavelength automated perimetry (SWAP) detects glaucomatous visual field damage earlier than white-on-white (WW).[1-4] Other studies have investigated progression of visual field defects using SWAP[5-8] and also demonstrated SW field loss in diabetic retinopathy and maculopathy[9-14] prior to the occurrence of WW loss. SWAP may represent a useful clinical tool to aid the management of various ocular diseases.

SWAP exhibits a greater between-subject variability and a greater short-term fluctuation (SF; i.e. variation of sensitivity within an examination), than WW perimetry.[15-19] These two factors will reduce the potential of SWAP to detect abnormality and progression i.e. visual field loss will have to be greater to reach significance in areas of high variability.[17] For WW perimetry, the lower the sensitivity at a given stimulus location the higher the SF.[20] SF only provides an estimate of within-examination variability. The magnitude of within-examination variability is determined by the frequency-of-seeing (FOS) function[21] that describes the probability of detecting a stimulus as a function of intensity. The flatter the slope of the FOS function, the greater the within-examination variability. Previous studies have shown shallower slopes in areas of visual field loss in glaucoma patients for WW and motion stimuli.[22-25]

The influence of sensitivity level and stimulus location on SWAP SF has to be established. This factor limits the accuracy of threshold determination and also governs the optimum bracketing procedure employed to estimate threshold. The aims of the study were to define the influence of; 1) Stimulus location on the FOS characteristics of the WW and SW sensitive

visual pathways in clinically normal subjects; and 2) Localized sensitivity loss on the FOS characteristics of the SW sensitive visual pathway in a diabetic patient group with focal DME.

9.3 Materials and Methods

9.3.1 Sample

Sixteen clinically normal subjects (10 males) and 10 patients with diabetes (6 males) participated in the study. The study was approved by the Research Ethics Board of the University Health Network, Toronto. Written informed consent was obtained from all volunteers.

9.3.2 Clinically normal group

The average age of the sample was 26 years (SD 8 years, 20-47 years). Ten right eyes and 6 left eyes were chosen at random. Inclusion criteria consisted of a logMAR visual acuity of 0.00, or better, a normal fundus appearance (stereo-biomicroscopy using dilated pupils) and normal visual fields (assessed by a minimum of one perimetry examination using Humphrey Field Analyzer (HFA) II program 24-2). Exclusion criteria comprised (i) a distance refractive error of > ±6.00DS or ±1.50DC, (ii) family history of glaucoma, (iii) a Goldmann IOP greater than or equal to 22mmHg (iv) CNS disorders or psychiatric illness, (v) systemic medication with known CNS effects and (vi) significant lenticular opacities (graded by the Lens Opacity Classification System III i.e. NO>1, NC>1, P>1, C>1).[26]

9.3.3 Patients with diabetes

The average age of the sample was 55 years (SD 9 years, 38-66 years). Five right eyes and 5 left eyes were used. Inclusion criteria consisted of a logMAR visual acuity of 0.50, or better, clinically evident focal diabetic macular edema and repeatable sensitivity loss (i.e. ≥5 contiguous stimulus locations of significantly reduced sensitivity as assessed by SWAP and horizontal hemifield asymmetry analysis[9]) on each of 2 separate occasions. Exclusion criteria were the same as those imposed for the clinically normal group, apart from LOCS III criteria.

9.3.4 Visits

All volunteers attended for two visits. Only the results from the second visit were analysed, thereby minimizing learning effects for both perimetric paradigms.[27] Visit 1 was used to undertake refraction, VA and fundus examination and to perimetrically train volunteers (using WW and SW program 10-2). For the clinically normal group, visit 2 comprised two sessions comprising 4 FOS runs each (one before and one after lunch). The order of stimulus condition was randomised between subjects and retained for the post-lunch session. At visit 2, nine clinically normal subjects underwent WW stimulus parameters first. For the patients with diabetes, visit 2 comprised program 10-2 SWAP followed by 2 FOS test runs using SW stimulus parameters only. Volunteers were given rests every 5 minutes to minimize fatigue.[28] The same FOS program was used for both clinically normal subjects and patients with diabetes.

9.3.5 Procedures

A HFA II model 740 (Carl Zeiss Meditec, Dublin, U.S.A.) and custom FOS software was utilized. The patient's correction was adjusted for a viewing distance of 30cm. For the WW stimulus parameters, a 10cd/m^2 background luminance and a Goldmann III (0.431° subtense) white stimulus were utilized. For the SW stimulus parameters, a high intensity yellow background (100cd/m^2) in conjunction with a Goldmann V (1.724° subtense) blue stimulus were utilized.[29] Maximum stimulus intensity (i.e. 0dB) was 10,000 apostilbs and 65 apostilbs for WW and SW stimulus parameters, respectively. Stimulus duration was 200ms. Fixation was assessed using the corneal reflex monitor and the Heijl-Krakau technique.

9.4 Frequency-of-seeing (FOS)

When performance is expressed as probability, psychometric functions are ogival, or S-shaped, in form. Volunteers were given five minutes to adapt to the background luminance prior to starting each FOS determination. For the clinically normal group, sensitivity was assessed at the fovea and five other retinal locations (at 5.66° eccentricity along the 135°, 225° and 315° meridians, and at 4.24° and 9.90° along the 45° meridian) using an initial 4dB crossing of threshold and then a 2dB reversal. FOS functions were assessed at the 4.24° and 9.90° locations (one per location) along the 45° meridian at each session (Figure 10.1). For the patients with diabetes sensitivity was assessed for a location inside an area of focal SW sensitivity loss and at the same eccentricity in the quadrant diametrically opposite and at 2

other locations (one in each of the 2 remaining quadrants). A hemifield asymmetry analysis using SWAP program 10-2 identified localized areas of focal SW sensitivity loss.[9] The hemifield asymmetry analysis compared individual asymmetry across the midline to asymmetry values of a database of normal values.

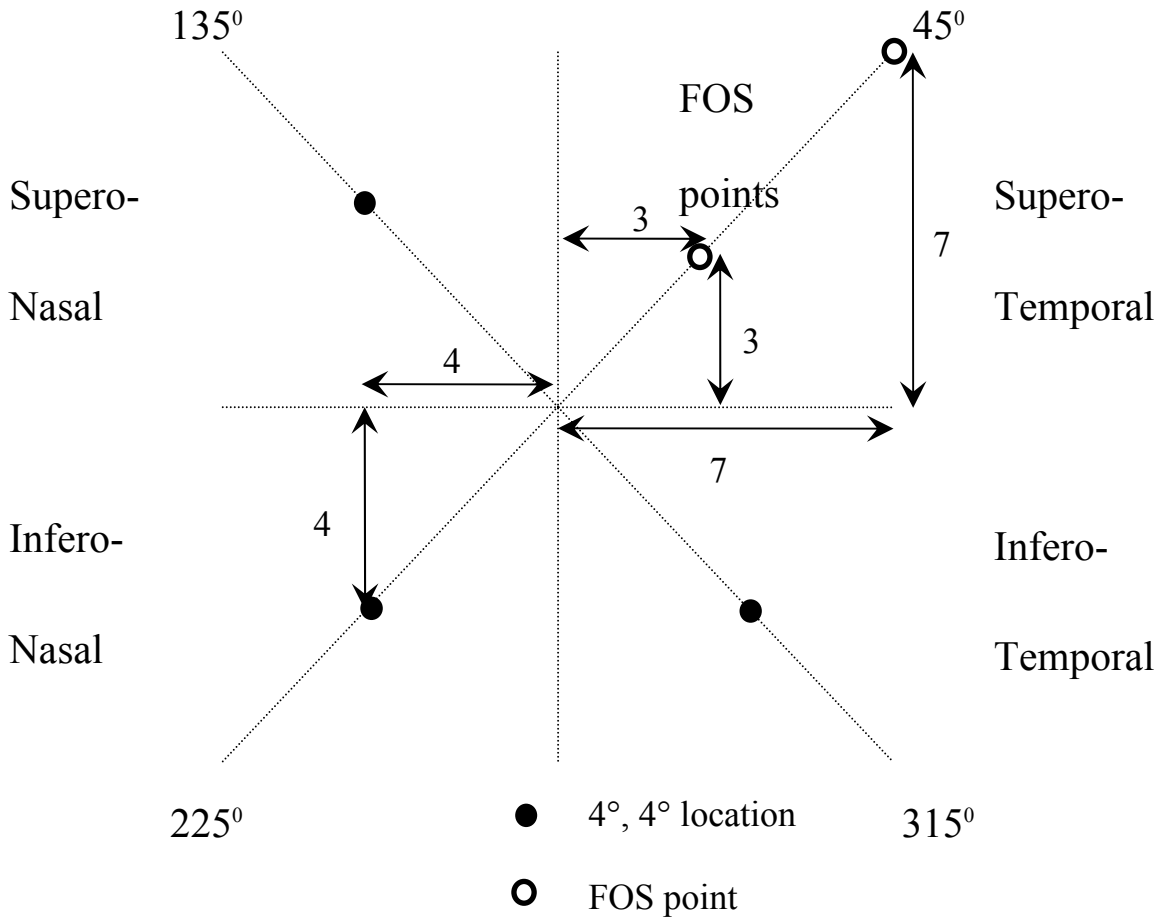

Figure 9.1 Schematic diagram showing position of FOS stimulus locations for clinically normal subjects (right eye).

Frequency-of-seeing stimulus locations were fixed for all subjects at 4.24º (i.e. polar co-ordinates 3º, 3º) and 9.90º (i.e. polar co-ordinates 7º, 7º) along the 45º meridian. Supra-threshold stimuli were presented at 5.66º (i.e. polar co-ordinates 4º, 4º) along the 135º, 225º and 315º meridians.

For FOS testing, stimuli were presented randomly at pre-set sensitivity levels (selected by the operator) above and below the estimated threshold. The FOS functions were determined using 8 sensitivity levels (two separate FOS test runs comprising 4 sensitivity levels each). Ten presentations were made at each sensitivity level. Sensitivity levels were selected for the first FOS run at ±1dB & ±3dB relative to the estimated threshold. The sensitivity levels for the second FOS run were then empirically chosen based upon the results of the first run. Volunteers who exhibited more than 25% false positive / negative responses or fixation losses were excluded from the analysis. Supra-threshold stimuli were randomly presented in order to introduce spatial uncertainty with the aim of maintaining global attention of the subject. No subjects were excluded due to excessive false positive / negative or fixation losses.

9.4.1 FOS function fitting

The FOS data was fit using the following function:

$$P(I)= 1- [0.5^{(- (S / \alpha)^{\beta})}]$$

where, P(I) is the probability of stimulus detection, S is sensitivity (dB), α is a point on the x-axis (i.e. sensitivity) that corresponds to a certain performance level.[30] For this study, α was taken at 0.5 or 50% FOS. β is the slope of the central portion of the function. Statistica (Statsoft, Inc.) was used to produce a least-squares fit of the function. Sensitivity was compared within a given stimulus parameter (since comparison of sensitivity between WW and SW parameters is invalid due to differing dynamic ranges). Sensitivity was taken at 50% FOS.

9.5 Statistical analysis

For the clinically normal group, slope and r-value (i.e. goodness of fit) of the FOS functions were compared between the different stimulus parameters (WW / SW). Sensitivity (i.e. 50% FOS) was also included in the analysis to determine significant interactions with FOS slope and to monitor systematic change in sensitivity across the two sessions (i.e. fatigue). A repeated measures Analysis of Variance (ANOVA) was undertaken on the data with slope and sensitivity as dependent variables and stimulus condition, eccentricity and session as the within-subject factors (SPSS Inc, Chicago, IL). For the diabetic patient group, slope and sensitivity were compared between locations for SW stimulus parameters using Student's t-test (two-tailed).

Inter-session variability was quantified using the co-efficient of repeatability (COR)[31] for the clinically normal group using visit 2 session 1 and session 2 data i.e. 1.96*SD of the differences across sessions.

9.6 Results

9.6.1 Clinically normal group

FOS functions of each clinically normal individual are shown for WW and SW stimulus parameters in Figures 9.2 and 9.3, respectively. Test times to complete each session were not significantly different between sessions or parameters.

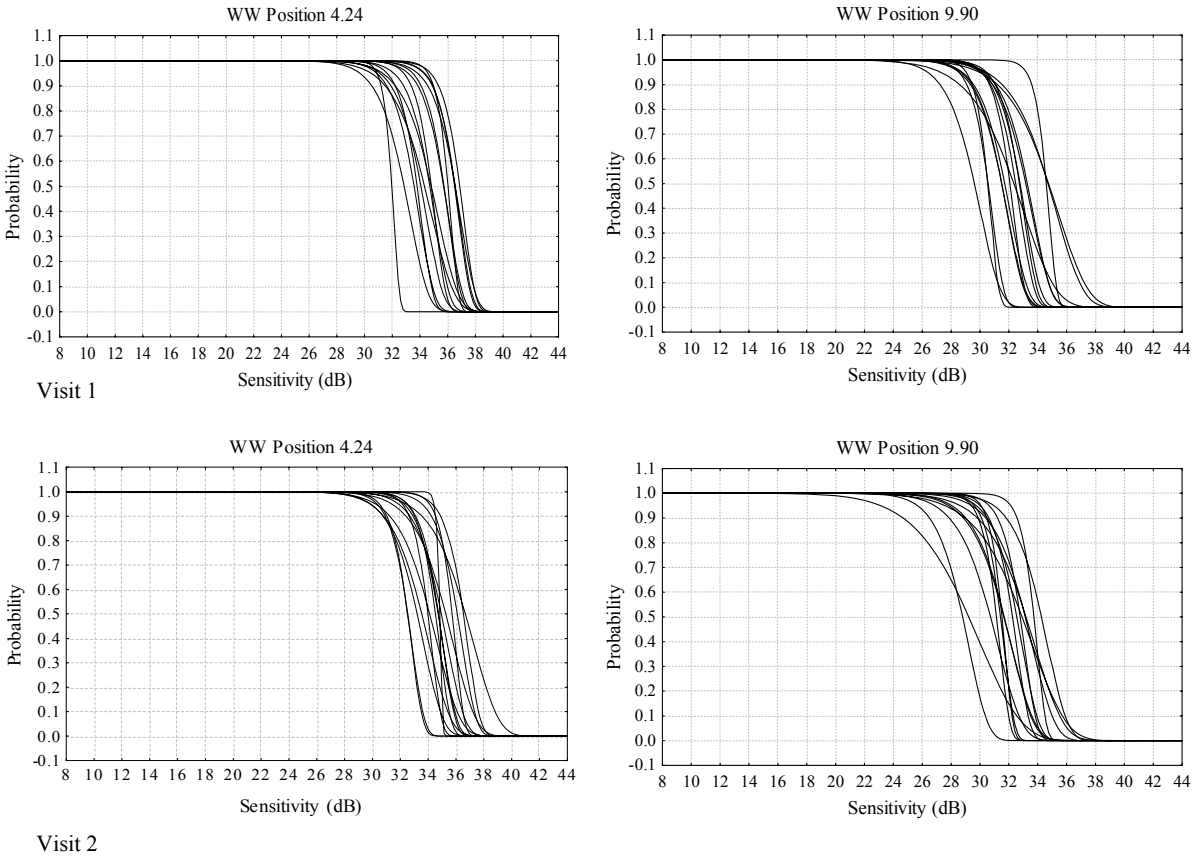

Figure 9.2 Individual FOS functions using WW stimulus parameters for clinically normal subjects (Upper; Visit 1. Lower; Visit 2. Left; 4.24° eccentricity. Right; 9.90° eccentricity).

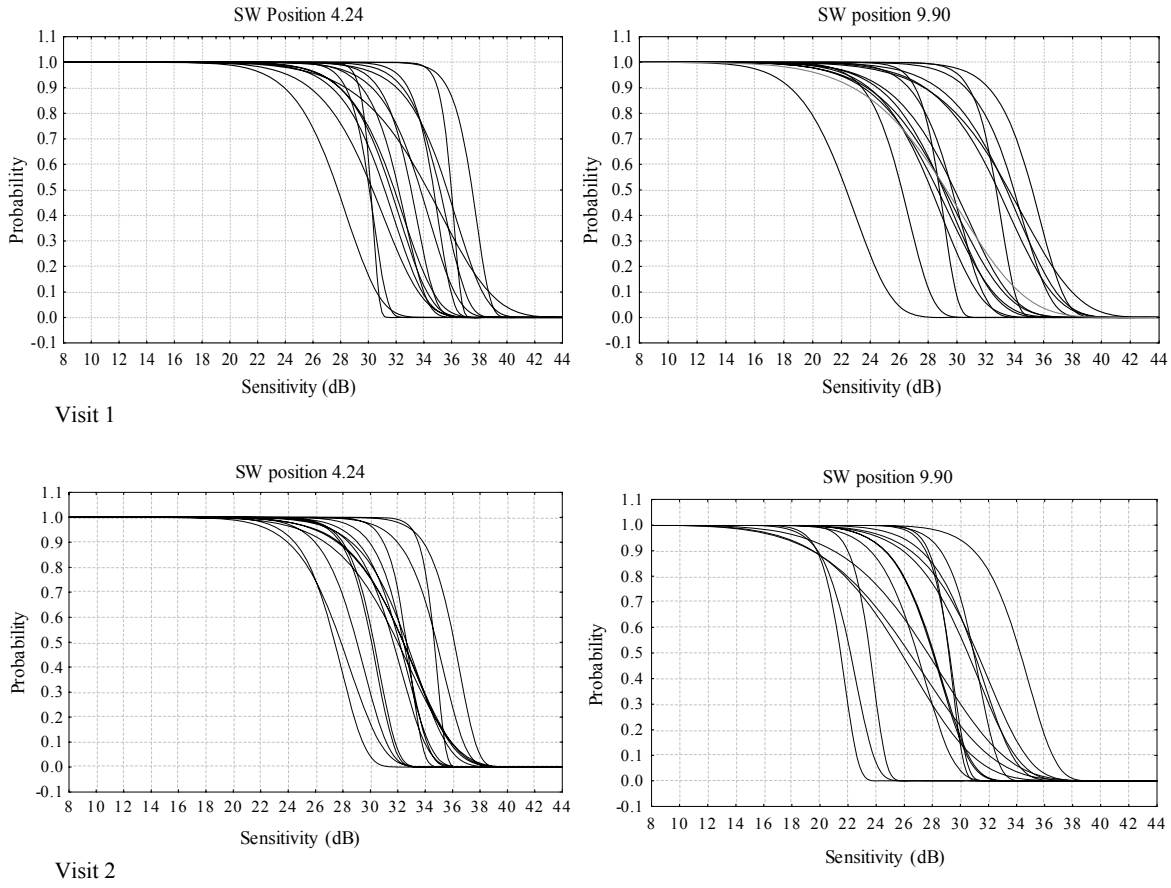

Figure 9.3 Individual FOS functions using SW stimulus parameters for clinically normal subjects

(Upper; Visit 1. Lower; Visit 2. Left; 4.24° eccentricity. Right; 9.90° eccentricity).

9.6.2 Group mean slope

Group mean FOS slope data is shown in Table 9.1. The SW slopes were found to be significantly flatter than those attained using WW parameters ($p<0.0001$) and the slopes for both stimulus conditions were significantly flatter at 9.90° than at 4.24° ($p=0.0198$) (Figures 9.2 and 9.3). The interaction of slope, stimulus parameter and eccentricity was not significant.

	WW 4.24º		WW 9.90º		SW 4.24º		SW 9.90º	
	Session1	Session2	Session1	Session2	Session1	Session2	Session1	Session2
Sensitivity (dB)								
Mean	34.78	34.59	32.17	31.93	32.83	31.78	29.65	28.54
SD	1.76	1.28	1.88	1.50	2.63	2.40	3.41	2.89
Slope (dB^{-1})								
Mean	39.15	34.19	31.68	29.74	26.40	21.56	14.94	17.35
SD	14.20	10.52	12.70	13.85	17.80	11.36	8.78	9.00

Table 9.1 Mean sensitivity and FOS slope for WW and SW stimulus parameters at 4.24º and 9.90º eccentricity for clinically normal subjects.

9.6.3 Group mean sensitivity

Group mean FOS sensitivity data is shown in Table 9.1. Sensitivity was significantly higher at 4.24° than at 9.90° ($p<0.0001$). Sensitivity was not significantly different across sessions.

9.6.4 Group mean r-values

Group mean FOS r-value data is shown in Table 9.2. r-values were consistently lower using SW stimulus parameters and at the more eccentric stimulus location.

	WW 4.24º		WW 9.90º		SW 4.24º		SW 9.90º	
	Session 1	Session 2	Session 1	Session 2	Session 1	Session 2	Session 1	Session 2
r-value Mean	0.99	0.98	0.99	0.97	0.96	0.97	0.94	0.94
SD	0.02	0.02	0.03	0.03	0.05	0.04	0.06	0.04

Table 9.2 Mean r-value for WW and SW stimulus parameters at 4.24º and 9.90º eccentricity for clinically normal subjects.

9.6.5 Repeatability of FOS determination

Inter-session variability was quantified using the co-efficient of repeatability (COR) (Table 9.3) The COR was calculated for the clinically normal group using the session 1 and session 2 data gathered at visit 2.

	W 4.24º	W 9.90º	S 4.24º	W 9.90º
Sensitivity (dB)				
COR	+/-2.85	+/-3.38	+/-3.86	+/-4.19
Mean	34.68	32.05	32.31	29.09
Slope (dB^{-1})				
COR	+/-30.76	+/-29.91	+/-41.55	+/-19.98
Mean	36.67	30.71	23.98	16.15

Table 9.3 COR of sensitivity and FOS slope for WW and SW stimulus parameters at 4.24º and 9.90º eccentricity for clinically normal subjects.

9.6.6 Patients with diabetes

Figure 9.4 shows typical FOS functions for a location within, and distant from, an area of focal sensitivity loss for a patient with diabetes.

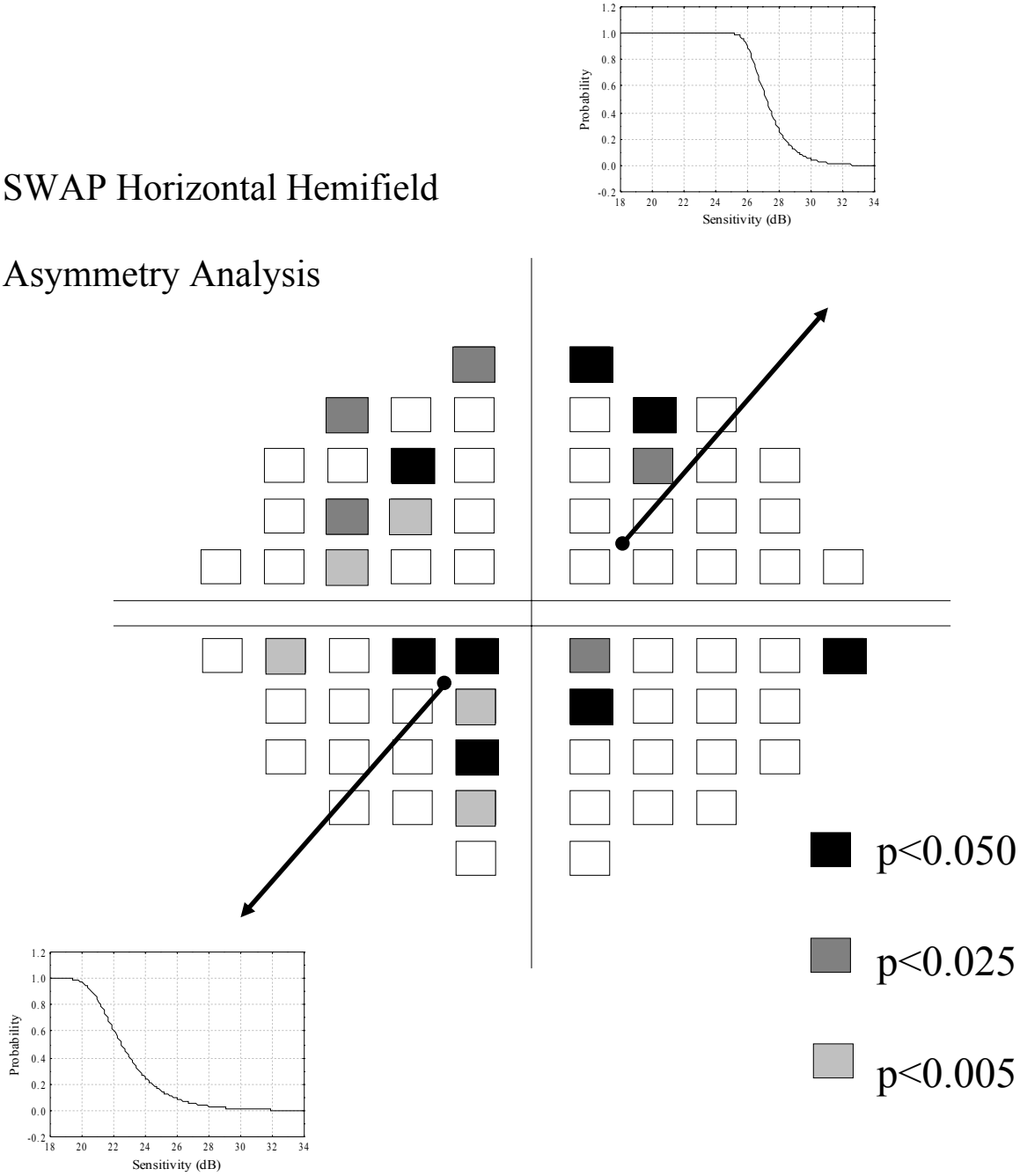

Figure 9.4 Individual horizontal hemifield analysis (right eye) and FOS functions using SW stimulus parameters for a patient with diabetes

(Upper; location distant from area of focal SW sensitivity loss. Lower; location within area of focal SW sensitivity loss. SWAP; Short-wavelength automated perimetry).

9.6.7 Group mean slope

The group mean SW slope values for the diabetic patients are shown in Table 9.4. The SW slopes derived from the focal sensitivity loss location were found to be significantly flatter than those from the non-focal sensitivity loss location (p=0.007).

	SW stimulus parameters	
	Non-DME location	DME location
Sensitivity (dB) Mean	23.29	18.07
SD	4.80	5.05
Slope (dB^{-1}) Mean	16.59	7.87
SD	8.61	4.12

Table 9.4 Mean sensitivity and FOS slope for SW stimulus parameters at the DME and non-DME location for patients with diabetes.

9.6.8 Group mean sensitivity

The group mean SW sensitivity values for the diabetic patients are shown in Table 9.4. SW sensitivity attained at the focal sensitivity loss location were significantly lower than those attained at the non-focal sensitivity loss location (p=0.02).

9.6.9 Group mean r-values

Using SW stimulus parameters, the group mean r-value at the non-focal sensitivity loss location was 0.964 (SD 0.04). At the focal sensitivity loss location, the group mean r-value was 0.925 (SD 0.06). r-values were consistently lower at the focal sensitivity loss location.

Figure 9.5 illustrates the relationship between SW sensitivity (@ 50% FOS) and SW FOS slope for the clinically normal subjects and patients with diabetes. It clearly shows a curvilinear relationship between sensitivity and slope, with FOS slope becoming flatter in locations of lower sensitivity.

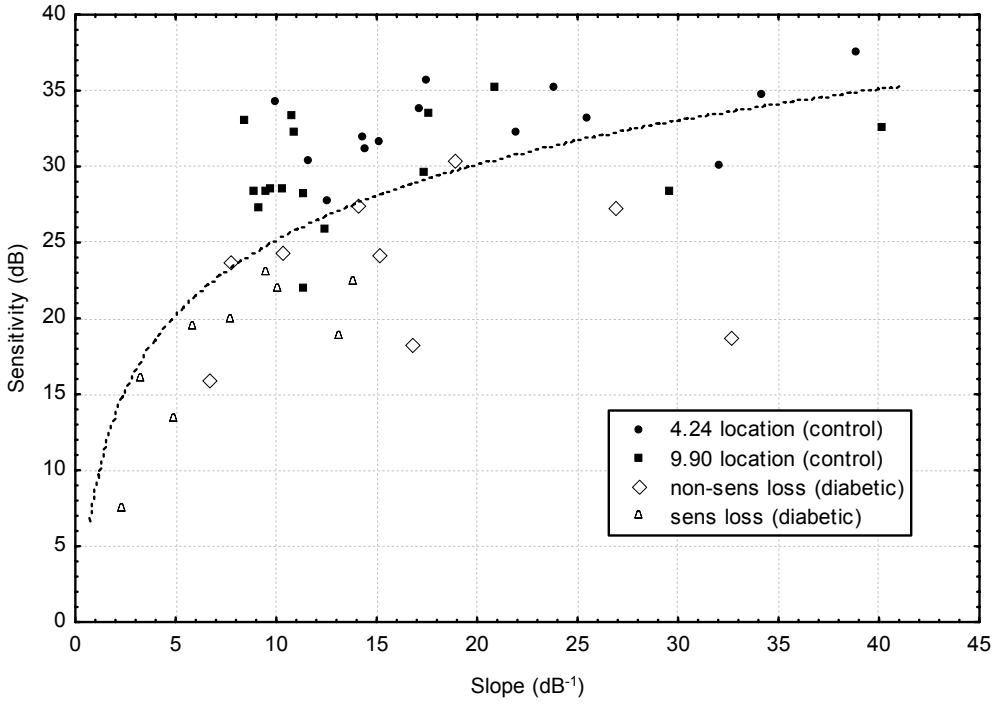

Figure 9.5 Relationship between SW sensitivity (@ 50% FOS) and SW FOS slope for all volunteers.

Clinically normal group: 4.24° location (filled circles); 9.90° location (filled squares). Patients with diabetes: location distant from area of focal sensitivity loss (open diamonds); location within area of focal sensitivity loss (open triangles). A logarithmic best fit line has been fit to all the data (r=0.6).

9.7 Discussion

For the clinically normal group, the group mean slope of the FOS function using SW stimulus parameters was significantly flatter than that of WW ($p<0.0001$). For the patients with diabetes, SW FOS slope was flatter in locations of focal sensitivity loss compared to the non-focal sensitivity loss locations ($p=0.007$) (Figure 9.5).

It has been established that SWAP exhibits greater SF than that of WW perimetry[15,18,32]; however, these studies have been based upon double determinations of staircase estimations of threshold. This study found FOS slope in clinically normal subjects to be approximately 38% and 53% flatter for SW than WW perimetry at 4.24° and 9.90°, respectively. Previous studies have tended to underestimate the magnitude of threshold variability for SWAP.[15,18,32] This underestimation may be attributed to the staircase estimation of threshold that in turn is used to estimate SF.

For the clinically normal group, the group mean COR for sensitivity was found to be greater for the more eccentric locations and for SW perimetry, indicating greater variability. The magnitude of COR for slope was found to be higher than group mean slope for SW perimetry i.e. the magnitude of variation of the measurement was found to be higher than magnitude of the measurement *per se*. The absolute sensitivity value was lower for SW automated perimetry parameters. For WW, the magnitude of the COR for slope was found to be lower than the group mean slope.

For WW perimetry, decrease in sensitivity is accompanied by a flattening of the slope of the FOS function[20] and glaucomatous patients show enhanced variability when compared to clinically normal subjects.[33] For the clinically normal group, the FOS slopes for both stimulus conditions were significantly flatter at 9.90°, while sensitivity was significantly higher at 4.24°. For the patients with diabetes, the results demonstrated that the slope of the SW FOS function was significantly flatter in locations within focal sensitivity loss. Consideration of all the SW data in terms of a plot of sensitivity versus FOS slope clearly showed a curvilinear relationship, with slope becoming flatter in locations of lower sensitivity.

In summary, the results of this study confirm that the clinical utility of SWAP will be limited by an increased magnitude of threshold variability compared to that of WW automated perimetry. Despite evidence that SWAP improves the detection of early visual field loss, its utility as a routine clinical tool needs to be treated with caution as a result of exaggerated threshold variability.

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10 Appendix B: Eccentricity and measurement variability and repeatability with the Retinal Thickness Analyzer

Gilmore ED, Hudson C. Eccentricity and measurement variability and repeatability with the retinal thickness analyser. *Brit J Ophthalmol* 2004;**88**(1):62-65.

	Concept / Design	Recruitment	Acquisition of data	Analysis	Write-up / publication
Gilmore	Y	Y	Y	Y	Y
Hudson	Y			Y	Y

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

This work was presented as a poster at the 2002 annual meetings of the Association for Research in Vision and Ophthalmology and of the American Diabetes Association.

10.1 Abstract

Aims: To define the variability and repeatability of retinal thickness measurements using the Retinal Thickness Analyzer (RTA) and to elucidate any interaction between eccentricity (i.e. position relative to the fovea) and variability and repeatability.

Methods: The sample comprised 20 normal subjects of mean age 33 years. Each subject attended for 2 visits. Repeated RTA scans were acquired centred on the fovea and for any one of the four possible non-foveal scan areas. The mean retinal thickness (SD) was calculated for a series of concentric circular bands centred on fixation. A repeated measures analysis of variance (ANOVA) was used to determine any significant interaction between the variability of RTA thickness values and eccentricity.

Results: The group mean co-efficient of variation and co-efficient of repeatability were highest at the fovea. The repeated measures ANOVA revealed that the within-test variability of RTA measurements varied significantly with eccentricity ($p<0.0001$). Similarly, the between-test repeatability varied significantly with eccentricity ($p=0.045$).

Conclusion: The significantly elevated within-test variability, and between-test repeatability, in the foveal area needs to be considered when using the RTA to evaluate patients with macular disease.

Keywords: Eccentricity, variability, repeatability, RTA, retinal thickness, retinal topography.

10.2 Introduction

Ocular diseases that result in alterations of retinal thickness include diabetic macular edema (DME), glaucoma, age-related maculopathy, vascular occlusion and macular hole. Established clinical techniques are non-quantitative and relatively insensitive to small changes in retinal thickness because they rely on the subjective assessment of the clinician[1]. Reliable, quantitative and sensitive methods to determine retinal thickness will lead to more accurate diagnosis and effective management. For example, the Retinal Thickness Analyzer (RTA; Talia Technology Ltd, Neve-IIan, Israel) has been extensively evaluated in clinically normal subjects[2-8], patients with various retinal diseases[9-27] and following various interventions[28-31]. Retinal thickness measurements using the RTA have been shown to correlate with other techniques[7, 27, 32] and histological assessment[4,7]. Importantly, the within-session global (as opposed to local) variability of the RTA has been estimated to be approximately 11-23μm, while the between-session repeatability was approximately 11-31μm[2-5,8,10,16]. Despite previous anecdotal comments about a possible effect[2], no studies have systematically examined the relationship between RTA measurement variability and repeatability and eccentricity. To the best of our knowledge, this study is the first study to elucidate an interaction between eccentricity and RTA measurement variability and repeatability. For the purposes of this study, eccentricity was defined as the distance of the retinal thickness measurement (co-ordinates x, y) relative to the position of the fovea (co-ordinates 0, 0).

10.3 Materials and Methods

10.3.1 Sample

The sample comprised 20 normal subjects of mean age 33 years (SD 8 years, range 22-51 years). Informed consent was obtained from each subject. The study followed the tenets of the Declaration of Helsinki and was approved by the University of Waterloo Office of Research Ethics and the Toronto University Health Network Research Ethics Board. One eye of each subject was assigned to the study: ten right, and ten left, eyes were selected. Inclusion criteria comprised a logMAR visual acuity of 0.0, or better, and a normal fundus appearance. Visual acuity was assessed using a 96% contrast Regan logMAR chart. Stereo fundus biomicroscopy, through a dilated pupil, was carried out to ensure the exclusion of significant ocular pathology. Exclusion criteria included a distance refractive error of greater than ±6.00 dioptres sphere and / or ±1.50 dioptres cylinder, a history of ocular disease, or surgery, and a family history of glaucoma, or diabetes, in a first degree relative. Subjects with significant lenticular opacities, as assessed by the Lens Opacity Classification System III[33] were excluded: significant lenticular opacity was defined as nuclear colour >2; nuclear opalescence >2; cortical cataract >1; and posterior sub-capsular cataract >1. Recent publications have demonstrated that RTA measurement can be adversely affected by lenticular opacity[8, 27].

10.3.2 Retinal Thickness Analyzer

The RTA comprises a laser slit biomicroscope and digital camera attached to an ophthalmic table, a patient headrest and a personal computer (software version 4.075). In brief, a green helium-neon laser light of 543nm wavelength is scanned across the retina to produce sixteen discrete slit images within a 3mm x 3mm area of retina. The reflected slit images are recorded digitally. Retinal thickness is derived from the separation between the anterior (i.e. at, or close to, the internal limiting membrane, ILM) and posterior (i.e. at, or close to, the retinal pigment epithelium, RPE) reflectance interfaces[34] for 16 points along each slit using densitometry. Consequently, the derivation of retinal thickness is dependent upon the clarity of RTA slit image. Patient fixation is aided by means of an internal fixation target that can be moved. Depth resolution and depth precision are reported to be 5-10μm and 50μm, respectively[3]. A more detailed explanation of the RTA optical principles have been described elsewhere[3-5,23].

10.3.3 Procedures

Each subject attended for 2 visits within a maximum 4 week period (mean interval 7 days, range 1-30 days). At both visits, the study eye of each subject was dilated using 1% Mydriacyl. Retinal thickness was initially assessed using the 5 default fixation locations of the RTA (i.e. centred on the fovea, supero-temporal, supero-nasal, infero-temporal and infero-nasal). Subsequently, the fovea-centred scan area, and any one of the four possible non-foveal scan areas (since RTA thickness values are not significantly different between the 4 meridians[5]) were each repeated 6 times using an alternating paradigm. The position of the

non-foveal scan was constant for a given subject but was systematically varied between subjects. Only the fovea-centred and the selected non-foveal scans, from the initial 5 default scans, were included in the analysis. A single, experienced RTA operator was used throughout (EG).

10.3.4 Analysis

Circular band analysis. This method of analysis was chosen since it was relatively robust to misalignment of successive RTA images. The scanned area of each image was divided into concentric circular bands using the radial analysis feature. Radii ranged from 200 to 3000μm and successive circles, centred on the fixation target, were separated by 200μm (Figure 10.1). The mean retinal thickness value within each concentric circular band was calculated for each individual. The variability of the mean thickness values was compared during, and between visits, for each concentric circular band, using the co-efficient of variation (COV = SD / mean) and co-efficient of repeatability (COR = 1.96 x SD of the differences between visits 1 and 2), respectively. The number of retinal thickness values used to calculate the mean varied between 4 (0 to 200μm band) and 61 (1400 to 1600μm band) according to the position and area of the circular band relative to the fovea.

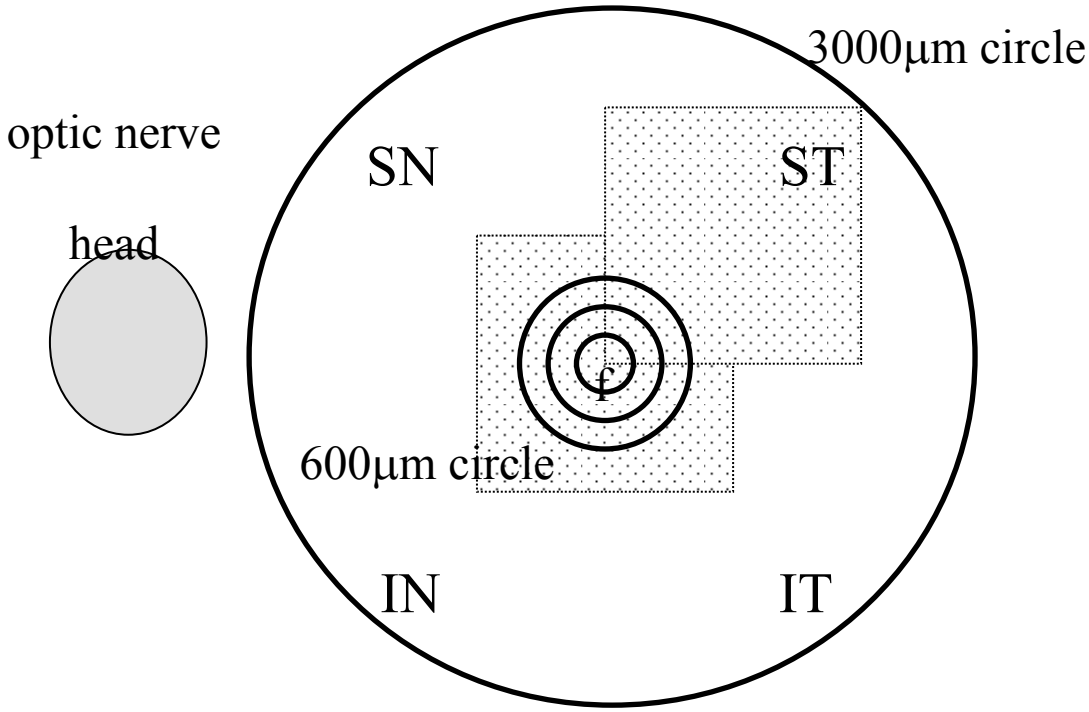

Figure 10.1 Schematic diagram showing the default RTA scan locations.

(f = fovea, SN = superonasal, ST = superotemporal, IN = inferonasal, IT = inferotemporal) and the radii used for the circular band analysis (ranging from 200–3000 μm radii, centred on the fixation target, in 200 μm steps).

Spoke analysis. A spoke analysis was also undertaken to negate the influence, if any, of the number of retinal thickness values used to calculate the mean in each circular band. This approach addressed the unequal number of data points used to generate the means for the circular band analysis. Using the spoke analysis, the mean of three retinal thickness values in each band was calculated extending from the fovea along one of the principal meridians (i.e. 45°, 135°, 225°, or 315°). The COV was then calculated for each band of the spoke as a function of visit.

Point-wise analysis. A point-wise analysis was undertaken to determine if the magnitude of retinal thickness *per se* was related to the variability of thickness measurements. Images were registered across visits (i.e. aligned) and the mean and SD of thickness values of all points was calculated. The mean and SD of the point-wise thickness data in the seven scans within each session were plotted to elucidate any relationship between measurement variability and retinal thickness.

10.3.5 Statistics

A repeated measures analysis of variance (ANOVA) was used to determine any significant interaction between the variability of RTA thickness values and eccentricity. Variability of retinal thickness was the dependent variable; visit (i.e. 1 or 2) and eccentricity were the within-subject factors. Intra-class correlation coefficients were also calculated to determine the reliability of the RTA thickness measurements across visits.

10.4 Results

The group mean profile of retinal thickness is shown in Figure 10.2, upper. The group mean retinal thickness was thinnest at the fovea (136.2μm, SE 6.9), thickest at 1000-1800μm from the fovea (186.7μm, SE 4.8) and then declined with further increase in eccentricity (163.6μm, SE 4.7). Individual retinal thickness values ranged from 86.7μm to 219.8μm (median value 133.5μm) for the central radius (0-200μm from the fovea).

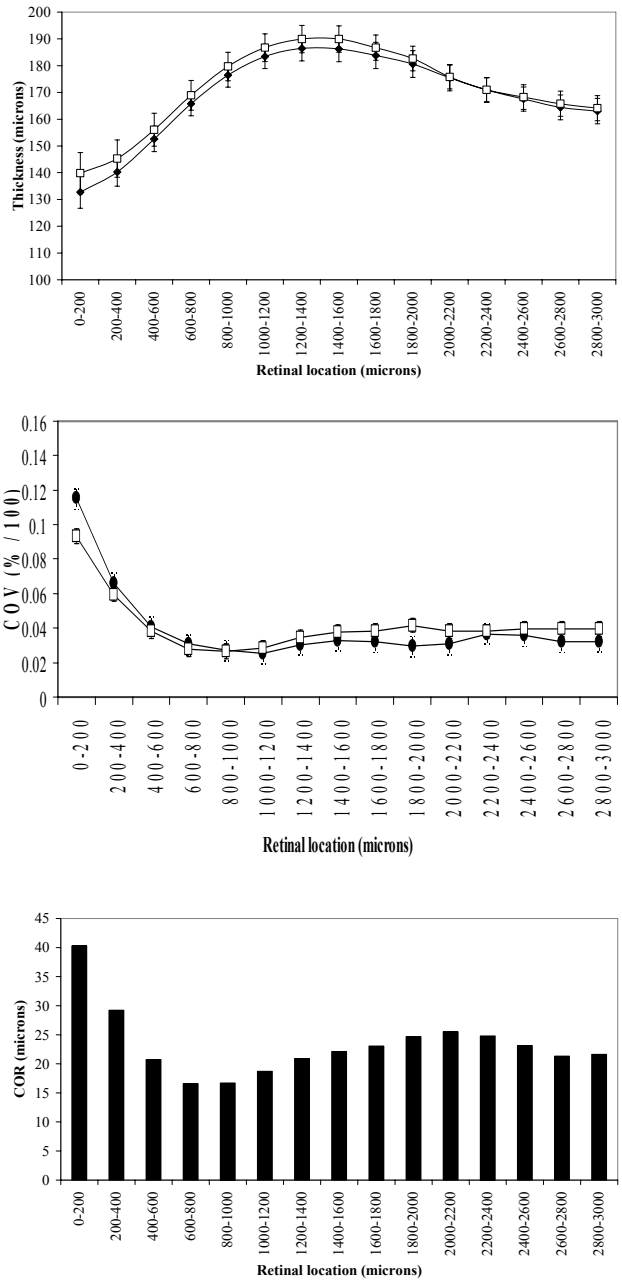

Figure 10.2 Upper; Group mean profile of retinal thickness (0-3000μm) derived using the circular band analysis for visits 1 (filled circle) and 2 (open square). Middle; Group Mean profile of COV derived using the circular band analysis for visits 1 (filled circle) and 2 (open square). Lower; Bar chart showing group mean COR derived from the circular band analysis.

Error bars represent ± 1 standard error of the mean (COV ; co-efficient of variation. COR ; co-efficient of repeatability).

The group mean COR of retinal thickness is shown in Figure 10.2, lower. It was highest at the fovea (40.3μm compared to a mean effect of 136.2μm), lowest at 600-1000μm (16.6μm compared to a mean effect of 172.7μm) eccentricity, then increased up to 2000-2200μm (25.5μm compared to a mean effect of 175.6μm) eccentricity and subsequently decreased with further increase in eccentricity (21.5μm @ 2600-3000μm compared to a mean effect of 164.3μm).

The repeated measures ANOVA revealed that the variability in retinal thickness measurements across visits was not significant ($p=0.455$). The variability in retinal thickness measurements (i.e. within-visit) as a function of eccentricity was significant ($p<0.0001$). Similarly, the variability in retinal thickness measurements across visits (i.e. repeatability) as a function of eccentricity was significant ($p=0.045$). Intra-class correlation coefficients calculated for each eccentricity ranged between 0.79 (1800-2000 μm) and 0.91 (2600-2800 and 2800-3000μm) and were significantly correlated across visits ($p<0.0001$).

The spoke analysis demonstrated a similar relationship between COV and eccentricity, and similar magnitudes of COV, to that of the circular band analysis. The point-wise analysis showed that there was no relationship between retinal thickness and the variability of the retinal thickness measurements.

10.5 Discussion

This study describes the local variability and repeatability of retinal thickness measurements using the RTA in a group of normal subjects with minimal, if any, media opacities. It is the first study to elucidate the interaction between eccentricity and RTA measurement variability and repeatability. Within-test variability was found to vary significantly with change in eccentricity from the fovea ($p<0.0001$). Similarly, the between-test repeatability was found to vary significantly with eccentricity ($p=0.045$). The within-test variability, and between-test repeatability, were both most pronounced at the fovea.

The retinal thickness values obtained from our sample are generally in good agreement with previous histological studies[35,36]. Hogan and co-workers[35] reported mean foveal thickness values of 130μm and while Straatsma and co-workers[36] reported values for foveal thickness of 100μm. Conversely, Fine and Yanoff[37] reported mean foveal thickness values of 200μm based upon histological techniques. Generally, with the exception of Konno and co-workers[7], published RTA studies have tended to find higher retinal thickness values when compared to our retinal thickness values. The difference in the magnitude of retinal thickness between the results reported in this, and previous RTA studies, may be attributed to the strict exclusion criteria employed in this study in relation to lens opacity. The light scattering effect induced by media opacities will result in blurred retinal slit images and an artifactual increase of retinal thickness values. Interestingly, a more recent study has found values of mean retinal thickness that are in very close agreement with our findings[8].

The variability and repeatability of RTA measurements have been reported in various published studies[2-5,8,10,16]. These authors found global mean COV values ranging from 10.6μm to 23μm. The retinal thickness values of any individual need to exceed the normal range of values to an extent greater than the magnitude of the COV before a significant difference in retinal thickness can be claimed. However, these studies have assessed variability over relatively large scan areas and did not address the issue of the interaction of RTA measurement variability with eccentricity. The magnitudes of COV found in this study are similar to those previously reported when relatively large scan areas are compared (by interpolation of areas under the COV profiles in Figure 10.2, middle).

Change in retinal thickness over time of any individual needs to exceed the magnitude of the COR before significance can be claimed. Previous studies[2-4,10, 16] evaluated repeatability by calculating the standard deviation of RTA measurements divided by the mean (across two visits). These studies found repeatability values ranging from 10.8μm to 19μm but did not consider any possible interaction of RTA repeatability and eccentricity and therefore calculated average indices that reflected repeatability for relatively large scan areas. The magnitudes of COR found in this study are high compared to those previously reported. This can be explained in part by the use of the COR index (which represents 1.96 x SD of the differences in RTA measurements across visits) and, more importantly, by the local variation in COR revealed in this study. In addition, the intra-class correlation coefficients demonstrated that the data was highly correlated within- and between-visits.

The circular band analysis resulted in a different number of data points within each circular band area i.e. 28 (i.e. 4 points x 7 scans) to 427 (i.e. 61 points x 7 scans). The spoke analysis (which resulted in 3 data points within each circular band) demonstrated a similar relationship between COV and eccentricity, and similar magnitudes of COV, with that of the circular band analysis. We conclude that the variation in COV as a function of eccentricity is not attributable to the differences in sampling rate across the circular bands.

The point-wise analysis demonstrated no relationship between retinal thickness and the variability of the retinal thickness measurements (as assessed by the SD of the 7 scans). The COV and COR are most prominent in areas of greatest change in retinal topography, particularly in the region of the foveal pit. This may in part be explained by the possible impact of involuntary physiological eye movements, and reduced nerve fibre layer (NFL) thickness at the fovea resulting in a localized reduction of reflectance intensity. Also, the ability of the RTA software algorithm to identify the anterior and posterior reflecting slit interfaces may be limited in areas of minimal retinal thickness. The possible impact of involuntary physiological eye movements as a further contributor to this local variation in RTA measurement variability and repeatability cannot be excluded. Interestingly, the test-retest measurement variability of confocal scanning laser tomography has also been demonstrated to be significantly greater in areas of greatest change of retinal topography[38,39]. Such an effect can be anticipated to impact upon other reflectance based scanning laser imaging systems.

We report a significant interaction between eccentricity and RTA measurement variability and repeatability. The group mean COV (11% at fovea and 3.5 to 5.0% elsewhere) and COR (40.3μm at the fovea and 16.6 to 25.5μm elsewhere) were highest at the fovea. Measurement variability and repeatability was not attributable to the magnitude of retinal thickness *per se* but instead, was most evident in areas of greatest change of retinal topography. The exaggerated within-test variability and between-test repeatability in the foveal area needs to be considered when using the RTA to evaluate patients with macular pathology. Confidence limits to determine abnormality relative to a normal database, and change in retinal thickness relative to baseline for a given individual, need to take into account the increased variability and repeatability in areas of greatest rate of change in retinal topography in order to maximize the sensitivity of the RTA.

10.6 References

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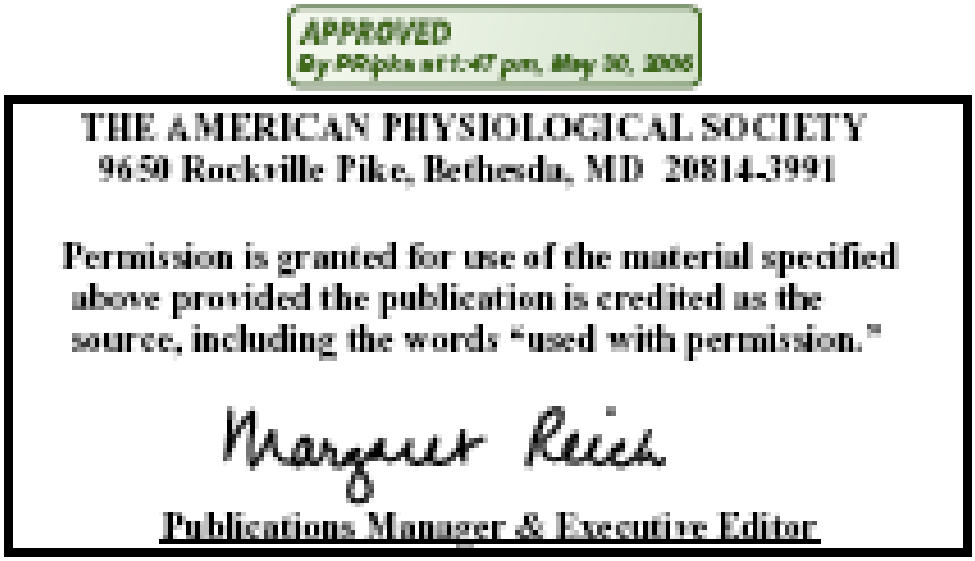

11.3 British Journal of Ophthalmology

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