The Relationship between Retinal Vascular Reactivity and Arteriolar Diameter

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 required final revisions, as accepted by my examiners.

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ABSTRACT

Purpose: The primary aim of the study (i.e. Chapter 3) was to compare the magnitude of retinal vascular reactivity in arterioles of varying diameter in healthy, young subjects. The secondary aims were to determine: a) if there are any order effects in terms of provoking vasoconstriction or vasodilation first; and b) the repeatability of the vascular reactivity measurements. An additional aim (i.e. Chapter 4) was to determine the effect of healthy aging on the relationship between retinal vascular reactivity and vessel diameter. **Method:** The sample comprised 10 healthy, young subjects (mean age 26.5 years, SD 4.04) and 7 healthy, older subjects (mean age 55.43 years, SD 5.41). Each subject from the young age group attended for three sessions. The first session was used to determine eligibility and select hemodynamic measurement sites. At sessions 2 and 3, O_2 and CO_2 were sequentially administered to the subjects using a face mask and sequential re-breathing circuit (to maintain standardized hyperoxia and hypercapnia). The order of vasoconstriction and vasodilation was varied across sessions 2 and 3. The design of the protocol was simplified for the subjects from the older age group. Each subject from the older group attended for one visit. O_2 and CO_2 were administered to the subjects using a face mask and sequential re-breathing circuit. The order of gas provocation was varied among the subjects (i.e. hyperoxia or hypercapnia first). For both groups, measurements of vessel diameter, centerline blood velocity and derived blood flow were acquired at each condition (i.e. baseline, during stabilized vasoconstriction, vasodilation, and recovery) at two *discrete* measurement sites along the supero-temporal arteriole. **Results:** The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for the younger group for flow ($p \leq 0.0003$) and a significant influence of

inspired gas provocation on flow for both protocols ($p<0.0001$). In addition, the interaction of measurement site and inspired gas provocation was significant ($p<0.0001$). The magnitude of retinal vascular reactivity showed a significantly greater blood flow response for the wide measurement site ($p<0.0001$). O_2 provocation resulted in vasoconstriction that was still present up to 10 minutes after cessation of the stimulus (order effect of O_2; $p\leq0.046$). No such order effect was apparent for CO_2 provocation (order effect of CO_2; $p=0.352$). The group mean blood flow Coefficient of Repeatability (COR) for the narrow measurement site was 0.74 µl/min (relative to group mean flow of 4.85 µl/min ± SD 1.31) and for the wide measurement site was 1.49 µl/min (relative to group mean flow of 11.29 µl/min ± SD 3.55). There was no difference between the young and the older age groups in retinal vascular reactivity for both the narrow (two-tailed Student t-test, $p=0.8692$) and wide (two-tailed Student t-test, $p=0.2795$) measurement sites. **Conclusion:** This study demonstrated that the magnitude of retinal vascular reactivity was greater for arteriolar measurement sites with *wider* baseline vessel diameters. In addition, it demonstrated that hyperoxic provocation resulted in a persistent vasoconstriction up to 10 minutes after cessation of the stimulus. The study demonstrated that the repeatability of retinal blood flow measurements in absolute terms is lower for smaller diameter vessels. Finally, this study also suggests that age does not affect the relationship between retinal vascular reactivity and vessel diameter.

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1 Introduction

The retina is able to regulate blood flow in response to different metabolic demands. There are several animal and human studies demonstrating a change in retinal vessel diameters[1-3] as well as in retinal [4] and optic nerve head blood flow [5-7] as a result of provocation with diffuse luminance flicker, O_2, CO_2, etc. Like in the brain, blood flow in retinal vessels is strongly coupled to neural activity, which has been recently established using laser Doppler flowmetry [5] and retinal vessel diameter measurements [1;2;8].

Hemodynamics is the study of the properties and mechanisms that control the flow of blood. Blood flow within an organ or vascular network is driven by the difference between the arterial and venous pressures across the organ which is termed perfusion pressure (i.e. pressure gradient). The vascular smooth muscle cells have an intrinsic ability to either constrict or dilate in response to increase or decrease of the perfusion pressure, respectively. The smooth muscles respond by contracting when the lumen of a blood vessel is suddenly expanded, as occurs when intravascular pressure is suddenly increased. Conversely, a reduction in intravascular pressure results in vasodilation. Therefore the arterioles respond to intravascular pressure as a stimulus [9].

Vascular reactivity is the magnitude of change of hemodyamic factors to a provocative stimulus (e.g. an increase in partial pressure of oxygen or carbon dioxide in the blood). There are several studies demonstrating constriction to oxygen and dilation to carbon dioxide [10;11]. Hyperoxia, increased partial pressure of oxygen (PaO_2) in systemic arterial blood, is known to be a potent vasoconstrictor stimulus. Conversely, hypercapnia, increased

partial pressure of carbon dioxide ($PaCO_2$) in systemic arterial blood, is known to be a potent vasodilatory stimulus.

1.1 Anatomy and Physiology of Retinal Vessels

The microcirculation of the retina is composed of arterioles, capillaries and venules. Arteries transport the blood to the tissues under a high pressure; therefore, the arteries have strong vessel walls. Arterioles are small resistance vessels (20-150 μm) composed of an endothelium surrounded by one or more layers of smooth muscle cells. The function of the arterioles is to regulate how much blood is delivered to the downstream capillary bed. The diameter of the arterioles depends on the contractile state primarily of the smooth muscle, which is regulated by multiple factors, such as endothelium derived constricting factors (EDCF), endothelium derived relaxing factors (EDRF), and circulating vasoactive hormones. Capillaries (6-10 μm diameter) are composed of highly attenuated (very thin) endothelial cells surrounded by basement membrane and manifest an absence of smooth muscle. Capillaries represent the site of exchange for fluids, nutrients, electrolytes, hormones, and other substances between the blood and the interstitial fluid. Hence, the walls of the capillaries have several very small pores permeable to water and other small molecules, as well as being very thin. Capillaries are classified as continuous, fenestrated, or discontinuous. Continuous capillaries are present in the retina. Continuous capillaries are the most impermeable type and result in the formation of the blood retinal barrier, similar to that of the brain-blood barrier. The venules collect the blood from the capillaries. These venules become progressively thicker in diameter and become veins. The blood pressure in the veins is very low, therefore, their vessel walls are very thin so the surrounding skeletal muscle can

either constrict to aid transport of the blood back to the heart or dilate so that the veins act as a reservoir for the blood; this mechanism permits modulation of the volume of blood in the circulation. The cross-sectional area of the veins is therefore four times larger than arteries, to enable the storage of blood [12].

Physically, the first barrier between blood and tissue in the retinal circulation are endothelial cells which prevent plasma from leaking out of the vessel. They are also important in the autoregulation of the blood flow, which will be described later. The endothelium consists of a monolayer of cells covering the inner wall of the vasculature. The vascular endothelium is strategically located between the circulating blood and the vascular smooth muscle cells [13]. The second vascular layer is the smooth muscle which can constrict and dilate. The smooth muscle cells are enclosed by an internal and an external elastic lamina. The internal elastic lamina is located between the smooth muscle cells and the endothelium and transmits signals between the two layers. The outer layer protects the vessel and attaches the vessel loosely to the surrounding tissues [14].

In summary, retinal arterioles are composed of endothelial cells and smooth muscle. By contrast, retinal capillaries are made up of tight, non-fenestrated endothelial cells surrounded by a basement membrane containing pericytes and mural cells. The endothelial cells are joined together by zonula occludens, thereby preventing leakage from arterioles, capillaries and venules and preserving optimal retinal function [14;15].

1.1.1 The Vascular Systems of the Human Retina

The human eye obtains its blood mainly from the ophthalmic artery (OA), which is the first branch of the internal carotid artery and the only branch of the internal carotid outside the cranium [16]. The adult neural retina is supported by two distinct vascular systems, the inner retinal vessels and the choroidal vessels. The two beds vary in both their embryonic differentiation pattern and functionally in the adult organism. The retinal vasculature has barrier properties similar to those observed in the brain, whereas the choroidal vessels demonstrate a greatly fenestrated phenotype [17].

A. The retinal blood vessels.

The retinal blood vessels nourish the inner layers of the retina. The inner retina maintains its blood via the aortic artery, common carotid arteries, internal artery, ophthalmic artery and finally central retinal artery (CRA). The CRA enters the optic nerve 10-15 mm behind the globe and runs forward in the central section of the nerve along the central retinal vein. CRA supplies the inner two third of the retina, the most anterior portion of the superficial nerve fiber layer of the optic nerve head and to some extent the retrolaminar optic nerve.

B. The choroidal vessels.

The outer retinal layers, including the photoreceptors, are nourished by the choroid. The uveal system, specifically the choriocapillaries supply the deeper outer layers, including photoreceptors and bipolar cells. The short posterior ciliary arteries directly supply the choroid and the long posterior ciliary arteries travel in the suprachoroidal space anteriorly then supply the choroid anteriorly via recurrent branches.

In summary, retinal tissue is nourished by two vascular systems of blood vessels that differ anatomically and physiologically: the inner retina is nourished by the distribution of the central retinal artery. The outer retina is nourished by the underlying choriocapillaries via the short and long posterior ciliary arteries. Blood is drained by the retinal venules into the central retinal vein and finally the ophthalmic vein.

1.1.2 Venous Outflow

Retinal venous drainage takes place via the central retinal vein, which leaves the eye through the optic nerve and parallels the CRA. The central retinal vein pours into the superior ophthalmic vein or, rarely, directly into the cavernous sinus.

1.2 Blood Flow

The human cardiac cycle comprises of a systolic (i.e. contraction) and diastolic (i.e. relaxation) components. The time in which the ventricle contracts and the blood is driven out into the aorta is called systole. Systole generates a dicrotic wave which is secondary to valve closure that generates a brief pressure rise. The intervening dip is called the dicrotic notch. The ventricle receives blood while relaxed during diastole. Arterial pressure is pulsatile because the heart pumps blood intermittently [18;18].

The rate of blood flow defines the amount of blood that passes a point in the vascular system at a given point in time. Normally the blood flow is expressed in microlitres per minute (µl/min). To determine the flow of the blood through a vessel it is important to know two

factors. First, the difference of the blood pressure (ΔP) at the two ends of the vessel and second the resistance (R) that originates from the inner frictional forces, also termed shear stress [19]. Based on Ohm's Law (sometimes referred as Darcy's Law):

$$Q = \frac{\Delta P}{R} \quad \textbf{(1.1)}$$

The blood flow (Q) is proportional to the perfusion pressure difference between two points ($P_1 - P_2$) and inversely proportional to the resistance (R) [19]. This equation demonstrates that there are only two ways to increase the flow, either increase the perfusion pressure (difference between arterial and venous pressure) or decrease the vascular resistance. Changes in vascular resistance result from the contraction and relaxation of the narrow, terminal branches of the arterial system (i.e. resistance arterioles) [14].

Resistance to flow is dependent on the properties of the fluid and the lumen through which the fluid is flowing. Using steady blood flow conditions through a cylindrical 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.

$$R = 8\eta L / \pi r^4 \quad \textbf{(1.2)}$$

The velocity of the blood is greater in the center of the vessel because the friction created by the vessel wall is higher. According to Poiseuille's law the rate of the blood flow (Q) in the

vessel taking into account the cross sectional velocity profile can be calculated with this formula[19]:

$$Q = \frac{\pi \cdot \Delta P \cdot r^4}{8\eta l} \quad \textbf{(1.3)}$$

where ΔP is the difference of the blood pressure between to ends of the vessel, r is the radius of the vessel, and η is the viscosity of the blood [19]. Due to the r^4 effect, small changes in vascular tone greatly influence blood flow, and if widespread, also impact total peripheral resistance and mean arterial blood pressure [14].

1.2.1 Resistance to Blood Flow

The impediment to blood flow in a vessel is resistance, but there is not any direct means to measure the resistance. Resistance to blood flow within a vascular network depends upon the size factors of individual vessels (i.e. length and diameter), the way they are organized in the vascular network (i.e. series and parallel arrangements), physical characteristics of the blood (i.e. viscosity, laminar flow versus turbulent flow) and extravascular mechanical forces acting upon the vasculature (in terms of the eye, the intraocular pressure) [19].

1.2.2 "Conductance" of Blood Flow in a Vessel and its Relation to Resistance

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

$$\text{Conductance} = \frac{1}{\text{Resistance}} \quad \textbf{(1.4)}$$

When the blood flow is turbulent, minor changes in the diameter of a vessel causes substantial changes in the vessel's ability to conduct blood. The conductance of the vessel increases in proportion to the fourth power of the diameter, in accordance with the following formula: [19]

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

$$R \propto \frac{\eta L}{r^4} \quad \textbf{(1.5)}$$

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

1.2.3 Laminar Blood Flow

The normal condition for blood flow throughout most of the circulatory system is defined as laminar flow. It is differentiated by concentric layers of blood moving at different velocities in parallel down the length of a blood vessel. Basically, laminar flow is when a fluid flows in parallel layers, with no disruption between the layers. The highest velocity (V_{max}) is located in the center of the vessel and the lowest velocity (V=0) is seen along the vessel wall[19].

1.2.4 Poiseuille's Law

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

Cross sections of a large and a small vessel are shown in the Figure 1.1. The concentric rings inside the vessel point out that there are different velocities in each ring and the central ring displays the highest velocity because of *laminar* flow. That is, the blood in the ring contacting the wall in the vessel hardly flows because of its adherence to vascular

endothelium. The next ring of blood toward the center of the vessel is in contact with the first ring and, therefore flows more rapidly. The third, fourth and fifth ring similarly flow at successively greater velocities. Thus the blood that is next to the wall of vessel flows extremely slowly, where as that in the middle of the vessel flows extremely fast. In a small vessel, all the blood is near the wall so that a rapidly flowing central stream of blood simply does not exist.

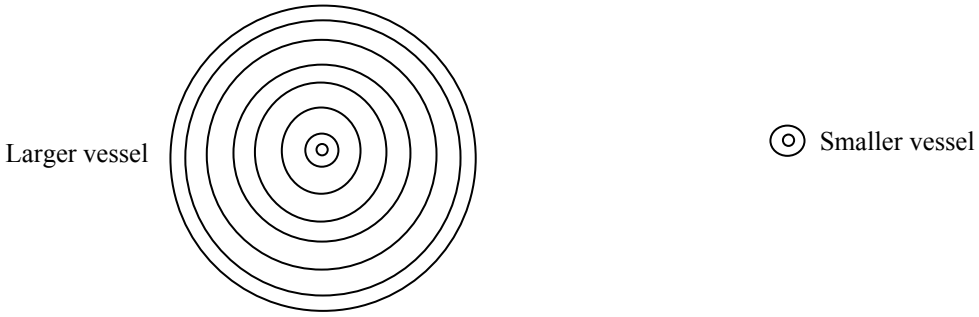


Figure 1. 1 Cross section of large and small vessel

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

$$Q = \frac{\pi \Delta \mathrm{Pr}^4}{8\eta l} \qquad \textbf{(1.6)}$$

In which Q stands for the rate of blood flow, ΔP is the pressure difference between the ends of the vessel, r is the radius of the vessel, l is length of the vessel, and η is viscosity of the blood. Note that the rate of blood flow is directly proportional to the fourth power of the

radius of the vessel and therefore the radius plays by far the greatest role of all factors in determining the rate of blood flow through a vessel [19].

1.2.5 Blood Flow in the Normal Human Retina

The determination of parameters that reflect local blood flow in the eye can be acquired by measurement of perfusion pressure, resistance, vessel diameter and vascular blood velocity. Blood flow in the human retina is relatively constant but can be influenced by factors such as moderate variations in perfusion pressure (i.e. mean arterial pressure - intraocular pressure). The perfusion pressure can be varied by either changes in blood pressure or intraocular pressure. Unlike in the choroid, where CNS controlled sympathetic nerves control blood flow, inner retinal blood flow is autoregulated by changes in the vascular resistance depending on the contractile state of the retinal arterioles. The relationship between flow rate and vessel diameter applies to arterioles and venules, although the blood flow rate in temporal retinal vessels is much greater than in nasal vessels. For a fixed vessel diameter, the blood flow rate as well as the blood speed in a retinal arteriole is greater than in the retinal venule [19]. The retinal volumetric blood flow rate can be determined [20;21,25] using the formula

$$Q = V_{mean} \times S \quad \textbf{(1.7)}$$

Where S is the cross-sectional area of the measurement site which is equal to $\pi D^2 / 4$ and

$$V_{mean} = \frac{V_{\max}}{2} \quad \textbf{(1.8)}$$

1.3 Ocular Perfusion Pressure (OPP)

The perfusion pressure is the pressure within the vasculature. The ocular perfusion pressure can be defined as the local arterial blood pressure minus the intraocular pressure [22]. A reduction in OPP can be achieved by either a reduction in arterial blood pressure or an increase in IOP [12]. The OPP can be calculated with the equation:

$$OPP = \tfrac{2}{3} BP_{mean} - IOP \quad \textbf{(1.9)}$$

where BP_{mean} is the mean arterial blood pressure and IOP is the intraocular pressure [23].

1.4 Vascular Tone Regulation

The degree that a blood vessel constricts relative to the maximally dilated state is defined as vascular tone. Vascular tone is a protection mechanism to guard against transient increases in perfusion pressure. Vascular tone is maintained by a balance between the endothelial vasodilators (e.g., nitric oxide) and vasoconstrictors (e.g., endothelin-1) so that, for example, reduced formation of vasodilators would result in vasoconstriction. A number of regulatory systems and factors, such as circulating hormones, as well as metabolic, myogenic and neurogenic factors, participate in the overall regulation of vascular tone [24,25].

1.5 Autoregulation and Metabolic Autoregulation

Autoregulation is the major regulatory mechanism of retinal blood flow, such that a change in the arterial blood pressure induces a compensatory change in diameter so that the blood flow is kept constant. In other words, change in perfusion pressure results in the manifestation of local blood flow regulation, termed as autoregulation. Autoregulation is defined as "the ability of a vascular bed to maintain blood flow to the tissues under conditions of varying perfusion pressure" [26].

Metabolic autoregulation is defined as the ability to increase perfusion in response to altered tissue needs [27]. For example, when metabolic activity increases blood flow will proportionally increase. Autoregulation and metabolic autoregulation represent distinct mechanisms involved in the regulation of retinal blood flow.

1.6 Endothelial Regulation of the Retinal Vasculature

Retinal circulation displays an effective autoregulation. Local mediators released by endothelial cells and surrounding retinal tissue play an important role in the regulation of retinal circulation. The endothelium can be activated by different stimuli, such as oxygen and carbon dioxide transported by the circulating blood, to release potent relaxing (nitric oxide, etc.) or contracting factors (endothelin, etc.). These endothelium-derived vasoactive factors act upon the smooth muscle cells that surround the endothelial cells (and to a lesser extent the pericytes that surround the capillaries) to regulate blood flow locally. In many vascular diseases, such as hypercholesterolemia, athero-/arterio-sclerosis, hypertension, diabetes, vasospastic syndrome, ischemia and reperfusion, the function of the endothelium can be disrupted. The secretion of vasoactive substances by the vascular endothelium can be manipulated by therapeutic agents. The vascular endothelium plays a critical role in the modulation of blood flow in both physiological and patho-physiological situations. The manipulation of this regulatory system by therapeutic agents might help with new approaches to treat vascular disorders [13]. Such therapeutic agents might include β-adrenergic blockers, angiotensin-converting-enzyme (ACE) inhibitors, various platelet inhibitors, such as aspirin, AT_1-receptor antagonists, Ca^{2+}-channel antagonists, platelet-inhibitors, anti-serotonergic drugs.

1.6.1 Vasodilators

1.6.1.1 Nitric Oxide

The endothelium controls vasodilation by releasing endothelium-derived relaxing factors (EDRF), principally nitric oxide (NO). NO, with a very short half-life, is a strong endothelium-derived vasodilator [28-33]. NO is synthesized by three distinct isoforms of nitric oxide synthase (NOS). Endogenous NO is derived from the metabolism of L-arginine by the catalyzing action of nitric oxide synthase. The activity of eNOS is controlled by concentration of intracellular calcium [34].

Recent studies strongly support the role of NO in the modulation of retinal blood flow in humans. NO synthase inhibition significantly reduces retinal arteriole and venule diameter, which indicates that basal NO secretion contributes to retinal vascular tone [1]. In addition, NO plays an important role in flicker-induced vasodilatation of the human retinal vasculature [1]. Nitric oxide also plays a role in hypercapnia-induced vasodilatation in the choroid and is a modulator of pressure autoregulation in this vascular bed [35;36]. However, the underlying mechanism of hypercapnia-induced vasodilatation has yet to be clearly defined. The release of NO in both vascular smooth muscle cells and pericytes triggers the release of cyclic guanosine monophosphate leading to dephosphorylation of myosin light chain resulting in vasodilatation. Basal release of NO balances the vasoconstrictive effect of endothelin-1 (ET-1).

1.6.1.2 Other Vasodilators

There are other vasodilators that are produced from endothelium such as prostacyclins (PGI_2, ecoprostenol). Prostacyclin induces vasodilation and inhibits the aggregation of platelets. It also induces inhibition of vascular cell migration and proliferation.

1.6.2 Vasoconstrictors

1.6.2.1 Endothelin

Endothelins are the most potent vasoconstrictors. Endothelin is a large peptide (21-amino-acid) [37;38]. The circulating endothelin levels are low indicating that it primarily acts as a local vascular regulatory factor; the peptide is cleared from the circulation by the lungs, the kidneys, and the liver [39-41]. Endothelins consist of endothelin-1, -2 and -3, which act through the endothelin receptors A and B. Endothelin receptor A is located on the vascular smooth muscle and B on the vascular endothelial cell wall. One of the ways that endothelin receptor A mediates vasoconstriction is by the amplified influx of calcium ions. In the general circulation, the endothelial cells discharge vasoactive substances both spontaneously and after chemical stimulation e.g., circulating hormones, or physical stimulation e.g. shear stress. The retinal vasculature demonstrates pronounced vasoconstriction in response to hyperoxia, which appears to be related to the constant oxygen demand of the retina [42]. Endothelin-1 is the main EDCF involved in hyperoxia-induced vasoconstriction in the human retina [43].

1.6.2.2 Norepinephrine and Epinephrine

Norepinephrine is an especially strong vasoconstrictor hormone; however, epinephrine is less potent and, in some vessels, even causes mild vasodilation [19]. Norepinephrine is released from the adrenal glands as a hormone into the blood, but it is also a neurotransmitter in the nervous system where it is released from noradrenergic neurons during synaptic transmission. As a *stress hormone*, it affects parts of the human brain where attention and impulsivity are controlled. Along with epinephrine, this compound affects the fight-or-flight response, activating the sympathetic nervous system to directly increase heart rate, release energy from fat, and increase muscle readiness. Epinephrine is also a hormone and a neurotransmitter which is secreted from the adrenal medula. Epinephrine is a catecholamine, a sympathomimetic monoamine derived from the amino acids phenylalanine and tyrosine [25].

1.6.2.3 Angiotensin

Small arterioles are effectively constricted by the effect of angiotensin. If constriction happens in an isolated tissue area, the blood flow to that area can be rigorously deprived. The primary precursor is renin, made by the kidneys, and elevated when blood volume is low; the next substance needed for this reaction is a liver protein, angiotensinogen. When both rennin and angiotensinogen are present in the blood, local factors can then form this pressor substance. Production of angiotensin is controlled by renin [19;25].

1.6.2.4 Vasopressin

Vasopressin is a more powerful vasoconstrictor than angiotensin. Vasopressin is a antidiuretic hormone. It is secreted from nerve endings projecting into the posterior pituitary gland and also by nerve endings in the hypothalamus [19;25].

1.6.2.5 Cyclooxygenase Products

The endothelial cyclooxygenase pathway produces many contracting factors, including thromboxane A_2, prostaglandin H_2 or superoxide anions. These factors are chiefly produced in the cerebral circulation and in the veins under physiological conditions [44-47].

1.7 Regulation in Retinal Vasculature

The retinal microcirculation is specialized to distribute oxygen and nutrients to areas of metabolic need. Retinal arterioles do not have an autonomic nerve supply but they are thought to be intrinsically able to constrict or dilate, that is autoregulate, in order to keep local perfusion constant or adapted to the local metabolic needs. Thus, within certain limits, vascular perfusion remains independent of the local perfusion pressure. It is believed that the retina is effectively autoregulated and that blood flow regulation is mainly brought about via metabolic and myogenic influences on smooth muscle cells in the arteriolar wall. Pericytes also participate in retinal blood flow regulation at the capillary level. Pericyte regulatory responsibility in capillaries has been demonstrated in vivo. Pericytes may adjust lumen size by contracting and relaxing and thereby control capillary perfusion [48].

1.7.1 Metabolic, Myogenic and Neurogenic "Autoregulation" of Blood Flow

The myogenic theory of autoregulation suggests that increase in blood pressure (perfusion pressure) and hence transmural (i.e. across the vessel wall) pressure results in contraction of the smooth muscle cell. Conversely, a reduction in intravascular pressure results in smooth muscle relaxation and vasodilation. Endothelial derived vasoactive agents are thought to be responsible for initiating the myogenic contraction [49-51] in response to increase in perfusion pressure. The myogenic response is inhibited by calcium-channel inhibitors [49].

The metabolic theory states that vascular resistance is adjusted so that blood flow maintains the concentration of certain metabolites within narrow limits. In other words, local arteriolar

smooth muscle tone is regulated by the local concentration of metabolic products, such as the partial pressure of oxygen and carbon dioxide (pO_2 and pCO_2) in the arterial blood supply. This response occurs independently of perfusion pressure and is sometimes referred to as metabolic autoregulation or vascular reactivity.

It has been accepted that myogenic and metabolic autoregulation are involved in the regulation of ocular blood flow [9].

In 1999 Orgul and co-workers discussed the probable mechanisms underlying autoregulation [25]:

1. Metabolic hypothesis: According to this theory, local arteriolar smooth muscle tone is regulated by the local concentration of metabolic products, such as the partial pressure of oxygen and carbon dioxide (pO_2 and pCO_2) in the arterial blood supply. This response occurs independently of perfusion pressure and it sometimes referred to as metabolic autoregulation or vascular reactivity.

2. Myogenic hypothesis: In general, as local blood pressure and, hence the transmural tension, are increased, arterioles respond actively with constriction. Endothelial derived vasoactive agents are thought to be responsible for initiating the myogenic contraction [49-51] in response to change in perfusion pressure. The myogenic response is inhibited by calcium-channel inhibitors [49].

3. Neurogenic autoregulation: Studies have found that there is no vasoactive autonomic nerve supply to the retina. Sympathetic nerves innervate the central retinal artery up to the lamina cribosa, but not further, and therefore do not have effect on the retinal blood flow.

1.7.2 Shear Stress

Rapid flow of blood through a vascular segment causes a viscous drag at the luminal surface of endothelial cells, which can be expressed as **shear stress** [19]. This stress contorts the endothelial cells in the direction of flow (i.e. re-modeling) and also results in greatly increased release of nitric oxide. In other words, shear stress is the pulling force applied by the blood against the inner vascular endothelial wall. The magnitude of shear stress depends on the shear rate (rate of blood flow) and the blood viscosity. The magnitude of shear stress modulates diameter adaptive responses which are aimed to keep the shear stress value constant. An increase in shear stress will result in vasoconstriction thereby reducing blood flow and reducing shear stress. Changes in endothelial wall morphology occur if shear stress is chronically increased [52] such as in diabetes and arterio-/athero-sclerosis.

1.8 Retinal Vascular Response to O_2 and CO_2

It has been demonstrated in several studies that retinal blood flow depends on arterial oxygen tension [53;53-55]. Administrating oxygen has been used as a stimulus to induce retinal vascular reactivity. Vascular reactivity is the change of hemodynamic parameters in response to a given provocation. It has been shown in both animal [56-58] and human [54;55;59;60] experiments that hyperoxia induces a pronounced decrease in retinal blood flow. To maintain retinal tissue oxygen concentration relatively constant, retinal blood flow varies inversely with the partial pressure of arteriolar oxygen (pO_2). Principally, vascular reactivity is regulated via NO and endothelin-1. The mechanism of hyperoxia is ET-dependent [43]. Hyperoxia has been shown to stimulate ET-1 release from retinal vascular endothelial cells in animal [58] and in human [43]. Oxygen promotes local vasoconstriction of the arterioles, venules, and capillaries in the retina whereas carbon dioxide results in vasodilatation [10]. Conversely, hypercapnia induces an increase in retinal blood flow and this mechanism is thought to be primarily nitric oxide dependent. In the retinal circulation, NO regulates basal blood flow by maintaining vasodilation [35].

1.9 Relationship between Vessel Diameter and Vascular Reactivity

Kiss and co-workers (2002) showed an inverse relationship between the magnitude of vascular reactivity and vessel diameter. That is, smaller diameter vessels constricted to a greater degree than larger vessels in response to hyperoxia [42]. Nagel and co-workers (2003) didn't find any correlation between retinal vascular reactivity and vessel diameter in response to retinal flicker [61]. Similarly, Polak and co-workers (2002) noticed no difference in the flicker response of the peripapillary retinal arterioles [3]. More recently, Knudtson and co-workers (2004) noticed a greater magnitude of vessel wall pulsatility as a result of the pulse cycle in smaller compared to larger retinal arterioles [62]. Clearly, the relationship between vessel diameter and the magnitude of vascular reactivity remains ambiguous.

1.10 Influence of Age on Retinal Blood Flow and Vascular Reactivity

Various organs in the human body are influenced by aging. Although it has been suggested that retinal blood flow and autoregulation decreases in healthy people above 40 years of age [63], the age-related change of these two distinct parameters in elderly healthy subjects is still equivocal. Boehm and co-workers (2005) found that there is a correlation between age and optic nerve head blood flow; the perfusion of the optic nerve head decreased with increasing age [64]. Furthermore, Groh and co-workers (1996) noticed a significant decrease of blood flow of the retina with increasing age and also reported a decrease in blood velocity in the central retinal artery [65]. Conversely, Harris and co-workers (2000) suggested that aging does not affect the central retinal arterial flow velocity; however, they suggested that ophthalmic arterial end-diastolic velocity is age-dependent [66]. They also reported that the flow velocities in the posterior ciliary arteries in men were independent of age; however, the end-diastolic velocity of the posterior ciliary arteries was found to decrease with age in women. Grunwald (1998) reported no significant correlation between choroidal blood velocity and the subjects age [67]. There is also limited evidence to suggest that age may have an impact on the autoregulatory response of the retinal arterioles [68]. Blum and co-workers (2000) found that age had a significant negative effect on the myogenic response of retinal arterioles.

1.11 Techniques for Measuring Ocular Blood Flow in Humans

The eye allows the noninvasive study of vascular hemodynamics in the human body. There are various factors that may influence the control and regulation of ocular blood flow, such as metabolic demands, blood nutrients, and metabolic by-products, perfusion pressure, and blood gasses. The numerous interactions among these factors are complex, and therefore they must be studied to understand the physiology of hemodynamics and possible hemodynamic alterations in disease.

There are a number of techniques for measuring ocular blood flow. Only one commercially available technique can provide volumetric blood flow measurements in absolute units, and it is restricted to the measurement of flow in large retinal vessel. At this time, volumetric flow measurements in absolute units from retinal capillaries and from the choroid are not possible. However, a number of hemodynamic measurement techniques that measure parameters that reflect blood flow in the retina are available. Each of these techniques is discussed in detail below.

1.11.1 Angiographic Techniques

Novotny and Alvis originally introduced fluorescein angiography [39]. Clinically, this method has become an important tool in the investigation of blood-retinal barrier leakage but can also be used to extract qualitative blood flow information from the angiograms. Considerable efforts have been made to quantify aspects of retinal blood flow using

angiographic techniques, whereby the measurement of the time required for the dye to pass through the retinal circulation is determined.

Relative concentrations of fluorescein in retinal vessels are calculated for each consecutive angiographic picture. Dye dilution curves can then be derived for arterioles and venuls of the retina. The mean circulation time of the retina, defined as the difference between venous and arterial times, has a negative relationship with retinal blood velocity. Retinal blood flow can be derived if retinal vessel diameter is simultaneously measured. This technique has been modified employing video-angiography [40;41;69] and scanning laser ophthalmoscopy [70-71;72]. As an alternative to mean retinal circulation time, retinal hemodynamics can be assessed by arterio-venous passage time. Arterio-venous passage time is the time between the first appearance of the dye in a retinal artery and in the corresponding vein [41].

1.11.2 Blue Field Entoptic Technique

The method is based on the subjective assessment of the blue field entoptic phenomenon [73;74]. By illuminating the retina with blue light, the density and velocity of leucocytes flowing in the macular capillaries can be determined. The blue field phenomenon can be seen by looking into a homogenous blue field and fixating upon a central target. The blue light needs to be a narrow optical spectrum with a wavelength of around 430nm. Only the red blood cells (RBCs) absorb short-wavelength light where as the white blood cells (WBCs) will reflect the light. The subject will perceive WBCs moving under the blue light in a 10° to 15° radius central area relative to the fovea [42;73,75]. By comparing the subject's

perceived WBCs velocity to reference velocities, the V_{mean} and the pulsatility of motion can be subjectively determined. The parameters provide information about the retinal blood flow and the flow pulsatility. The major disadvantages of this technique are that the reproducibility is often poor which is due to the technique being subjective and the results are influenced by patient fatigue. Furthermore, people with low visual acuity are unable to see their own leucocytes in the blue light [73].

1.11.3 Color Doppler Imaging (CDI)

CDI obtains valuable diagnostic information by projecting sound into the body and observing how it is reflected and scattered. The CDI probe sends out sound waves and utilizes the time taken for echoes to return to map structure and movement. Ultrasound imaging devices can apply this information to quantify the exact location of the sources of reflection within the body [16]. Furthermore, CDI measures the frequency of reflected sound waves to determine the velocity of blood flow within the ophthalmic artery, long posterior ciliary arteries, short posterior ciliary arteries and central retinal artery. By adding color to the B-scan output, the velocity of the blood can be more readily visualized. Particles moving towards the probe are pictured red-to-white and particles moving away from the probe are pictured blue-to-white. Using the Doppler equation, the shift of frequency can be calculated [76]. Color Doppler imaging enables the measurement of blood flow velocity in the CRA, CRV and PCA. The measurements can be implemented without a clear view and is therefore applicable for patients with poor ocular media. However, the technique requires an experienced operator to acquire meaningful results. Also, the pressure of the probe on the

eye can increase the IOP and provide inaccurate velocities. With this device it is not possible to measure the vessel diameter and therefore, the blood flow can not be calculated [13;77].

1.11.4 Laser Speckle Technique

The laser speckle technique can be used for investigating aspects of retinal blood flow [73;78;79]. The laser speckle phenomenon occurs when coherent light (i.e. laser light) is scattered by a diffusing surface. The waves interfere and induce a pattern due to constructive and destructive interference. The laser speckle device consists of a fundus camera and a diode laser with a wavelength of 808 nm. An extra halogen lamp illuminates the area of interest on the fundus [79]. The magnitude of blood flow through the vessel is indicated by the changes of spackle pattern [16]. The speckle patterns are used to derive hemodynamic parameters. Photographs taken in laser light show different contrast between areas where flow occurs and areas without flow. While areas without flow have high contrast, the areas with flow are blurry and therefore have a lower contrast [80]. Statistics are used to quantify the changes in hemodynamics, like the standard deviation of the intensity distribution in the speckle pattern. For perfect coherent light and an ideal diffuser, the standard deviation is equal to the mean intensity. The ratio of mean intensities (I_{mean}) to the difference of the I_{mean} and the intensity for each measurement is the normalized blur rate (NB), which quantifies the blood velocity [16;78]. The patterns can have 50 different colors, where red distinguishes a fast blood velocity [16;79]. It has been shown that this technique is reproducible [16;79]. The disadvantages of this technique are that is not an absolute measurement and therefore it is not possible to measure the blood flow [81].

1.11.5 Pulsatile Ocular Blood Flow Techniques

Blood enters the ocular blood vessels during systole and continues to flow more slowly during diastole and this causes a change in eye pressure proportional to the ocular volume. Pulsatile ocular blood flow (POBF) can be determined by pneumotonometry. The technique is based on the fact that blood volume increases during the systole and decreases with the diastolic pulse. Using POBF, a formula can be applied to calculate the ocular blood flow from the pulse amplitude [16;82]. As a result, the technique provides immediate data but doesn't measure blood flow directly.

Alternatively, POBF can be assessed by measuring the distance changes between the cornea and the retina during the cardiac cycle using an interferometric method [82]. The measurement point on the retina is illuminated with a coherent laser beam. The light that is reflected from the cornea acts as a reference wave. The fundus pulsation amplitude (FPA) is the maximum amplitude between the fundus and cornea during the cardiac cycle. Once again, the limitation of this technique is that it measures the pulsatile amplitude of blood flow rather than blood flow directly [82].

1.11.6 Laser Doppler Technique

The Doppler effect is a change in the observed frequency of a wave, such as sound or light, occurring when the source and or observer are in motion. This technique is applied in various disciplines, such as acoustics, microwave physics, optics, and astronomy. Measuring the blood velocity in retinal vessels of human subjects is definitely one of the most elegant and challenging application of the Doppler effect.

1.11.6.1 Laser Doppler Velocimetry (LDV)

Laser Doppler velocimetry measures the shift of frequency of laser light when reflected from moving particles. Laser light gets scattered by a stable target (e.g. vessel wall) and moving particles (e.g. red blood cells (RBCs)) in the vessel (Figure 1.2) [83]. The light scattered from the moving particles is shifted in frequency, while the scatter from stable tissues is unaffected.

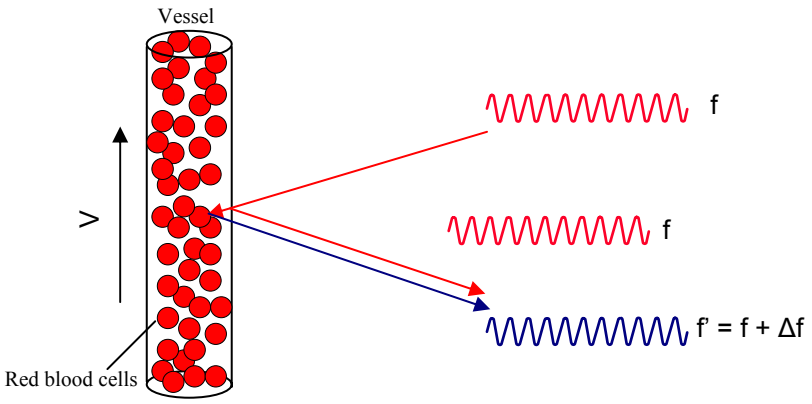


Figure 1. 2 Doppler technique (f = non-shifted light, f' = shifted light, Δf = frequency)

The diffuse light in the vessel spreads the spectrum of scattered light and this is considered the Doppler Shift Power Spectrum (DSPS) [53]. The backscattered DSPS contains the shifted (f') and the non shifted (f) light. The difference between the frequencies (Δf) is:

$$\Delta f = f - f' = \frac{2v}{\lambda} \qquad \textbf{(1.10)}$$

Where υ is the velocity and λ is the wavelength in the medium. It is not considered in this equation that the incident beam and the reflected light can come from any direction. Consequently, the angle between the moving particle and photodetector needs to be considered:

$$\Delta f = (\cos\alpha + \cos\beta)\frac{\upsilon}{\lambda} \qquad \textbf{(1.11)}$$

where α is the angle between the vector V_{max} and the reflection and β is the angle between the incident beam and surface of the target (Figure 1.3)[84;85].

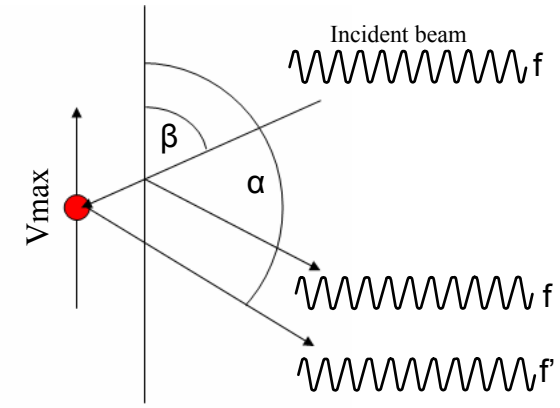


Figure 1. 3 Principle of the laser Doppler velocimetry (V_{max} =maximal velocity, f=non-shifted light, f'= shifted light, α = the angle between incident beam and f, β=the angle between the surface and the incident beam

The maximal velocity can be determined by the maximum Doppler frequency shift, f_{max}, and the intraocular scattering geometry.

$$V_{\max} = \frac{\lambda f \max}{n(\cos\alpha - \cos\beta)} \quad \textbf{(1.12)}$$

where λ is the wavelength of the incident laser in a vacuum and n the refractive index of the flowing medium [86].

1.11.6.2 Bidirectional Laser Doppler velocimetry (BLDV)

The absolute quantification of centerline blood velocity is possible by using *two* photo-detectors [87;88]. BLDV enables the measurement of the maximum blood cell velocity in larger retinal vessels [89]. To determine the V_{max}, it is important to consider that the incident beam should be perpendicular to the vessel so that the angle β (between incident beam and the vessel surface) can be set to zero (Cos β=1) [90]. This angle is critical to obtain accurate values. To prevent incorrect measurements due to angular dependence, the back scattered light is measured from two different directions using two photodetectors [84]. Those two directions are characterized by wave vectors K_1 and K_2 (Figure 1.4). The shift of frequency can be determined by the difference between the wave vectors. The two directions get scattered by two different amounts.

$$\Delta f = f_{2\max} - f_{1\max} = (\alpha_2 - \alpha_1)\frac{\upsilon}{\lambda} \quad \textbf{(1.13)}$$

Where f_1 is the frequency shift from the first wave vector and f_2 is the shift from the second wave vector. The angles α_1 and α_2 are the associated angles to the frequencies $f_{1\max}$ and $f_{2\max}$, respectively [84]. Two separate photodetectors with a known angle of separation record the reading. This allows the absolute quantification of center-line blood velocity independent of the angle between the moving particle and reflected beam [91]. The velocity can be determined by using the equation:

$$V_{\max} = \frac{\lambda \Delta f}{n \Delta \alpha \cos \beta} \quad \textbf{(1.14)}$$

Where $\Delta\alpha$ is the angle between K_1 and K_2 and β is the angle between the vector V_{max} and incident beam.

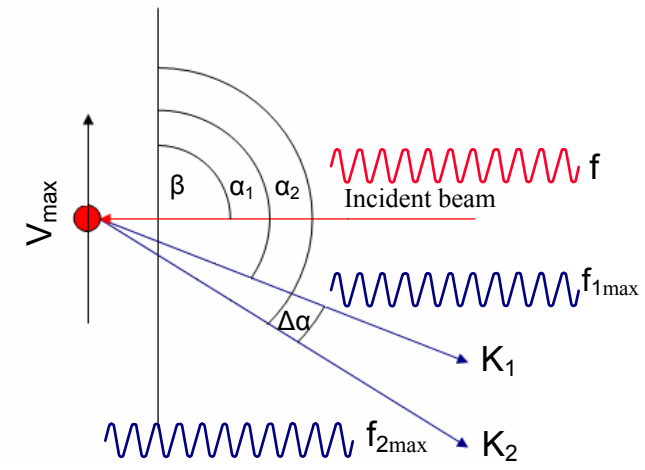


Figure 1. 4 Bidirectional laser Doppler velocimetry (Δα = the angle between K_1 and K_2, ß = the angle between the incident beam and the surface, K_1 and K_2 = wave vectors, f_{1max} and f_{2max} = frequencies)

A vessel exhibits Poiseuille flow and shows different velocities in the vessel [92]. The volumetric blood flow rate, Q, in retinal vessels can be derived from:

$$Q = \frac{\Pi D^2}{4} V_{mean} \quad \textbf{(1.15)}$$

Where D is the vessel diameter and V_{mean} is the ratio of V_{max} to the constant of proportionality of 1.6 between the velocity in the center and the mean velocity. Poiseuille found that the velocity in the center of the vessel is higher than the velocity next to the vessel wall.

1.11.6.3 Laser Doppler Flowmeter (LDF)

The laser Doppler flowmeter (LDF) consists of a modified fundus camera and a computer with special software to analyze the measurements [16]. A single vessel is illuminated by a laser beam. The bidirectional laser Doppler technique is used to determine the maximum center velocity. Using this technique, it is possible to determine localized volumetric flow measurements from capillary beds within the fundus [16]. The difference between the maximum Doppler shift for each of the two directions determines V_{max} in real units [93]. The LDF is similar to LDV, but LDF has a device to perform the additional signal analysis which is required to calculate blood flow as opposed to simply measuring velocity. It displays blood velocity, volume, and flow through the tissue sampled by the laser beam.

1.11.6.4 Scanning Laser Doppler Flowmeter

The Scanning Laser Doppler Flowmeter (SLDF) is a non-invasive device that quickly scans the fundus. Each scan contains 256 individual points and the Doppler shift is calculated for each point separately [81]. The SLDF provides a two-dimensional map of the capillary bed and it can be used to quantify the capillary blood flow. For each pixel in the image, the intensity of back scattered laser light of the retina is measured. The Heidelberg Retina Flowmeter utilizes this technique to derive parameters of blood flow, volume, and velocity [36]. There is a frequency shift shown by the moving red blood cells while light reflected from the surrounded tissue is unchanged. SLDF measures the intensity of back-scattered laser light from the retina as a function of time (to produce an intensity–time curve) for each pixel within the image. The two coherent components of light interfere resulting in an oscillation, or "beat", of the measured light intensity. The Doppler frequency shift demonstrates the frequency of the intensity oscillation. Fast Fourier transforms of the intensity–time curve produces a power spectrum of the Doppler shift to derive parameters of blood flow, volume and velocity [94] at each pixel within the image. Studies have shown that the SLDF is sensitive to small changes in blood flow. The SLDF measures volumetric flow only in arbitrary units, and the measurement cannot therefore be directly compared with the findings of other devices [81].

1.11.7 Heidelberg Retina Flowmeter (HRF)

The HRF is a confocal version of the LDF. Its laser scans the fundus *quickly* and each scan line is divided into 256 individual points. The laser scanning system of the HRF allows the measurement of a 10° horizontal × 2.5° vertical (i.e. approximately 2.7 mm × 0.7 mm) field with a resolution of 256 pixels × 64 lines, respectively. Each line of 256 pixels is scanned 128 times at a repetition rate of 4000 Hz. The resulting image acquisition time is 2.048 s [34;34,81]. It considers the Doppler shift from each point independently. HRF quantifies scatter light from each point; however, it just analyzes the scatter light from the point of illumination. The intensity of back-scattered laser light from the retina is measured as a function of time for each pixel within the image (to produce an intensity-time curve). The oscillation, or "beat" results from the interference of incident and reflected light. The frequency of intensity oscillation is proportional to the Doppler frequency shift.

There are some disadvantages associated with HRF: Firstly, susceptibility to eye motion during measurement [95], which makes some data unusable. Secondly, there is an artifactual flow reading even when no cells are moving, and an artifactual increase in total number of cell parameters when cells are in motion [95]. Also, the characteristics of the retinal microcirculation may result in an infringement of the Bonner and Nossal assumption for quantification of blood flow and hence results in unreliable measurements [95]. Work from our lab has found that artificially induced light scatter incorrectly raises HRF values [36]. In brief, the HRF is able to provide relative blood flow measurements and therefore should only be used for "comparative measurements over time at the same sites" [95].

1.11.8 Canon Laser Blood Flowmeter (CLBF-100)

Non-invasive, quantitative assessment of retinal arteriolar blood flow was achieved using the Canon Laser Blood Flowmeter, model 100 (CLBF). The principal underlying the CLBF is based on the Doppler effect. The CLBF utilizes fundus camera optics that incorporate two lasers and two photo-detectors. A red diode measurement laser (675 nm, 80 μm × 50 μm oval) is used to measure velocity every 0.02 sec across a 2 second measurement window resulting in a velocity-time trace. Velocity readings are determined using the bi-directional photo-detector technique i.e. the difference in Doppler shift between the two detectors is calculated to determine the absolute velocity of the moving red blood cells. Two sequential bi-directional velocity readings (i.e., path 1 and path 2) are taken by the CLBF to ensure consistency and are averaged to give a single velocity reading. The CLBF also uses a green diode vessel tracking laser system (543 nm, 1500 μm × 150 μm rectangle) to stabilize the measurement site, and measure the vessel diameter [96;97]. It acquires multiple diameter readings during the first and final 60 ms of the 2-s velocity measurement window every $4\,ms$. A vessel tracking system permits a graph of eye position to be superimposed on the velocity time curve to aid in artifact rejection. Flow through the vessel is calculated using the equation: $F = V_{\max} S/2$, where S is the cross-sectional area of the vessel at the measurement site. 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). Poiseuille flow and a circular vessel cross section are assumed in the calculation of blood flow.

Compared to other methods of assessing retinal blood flow, the CLBF offers a quantifiable and repeatable method of assessing retinal hemodynamics. The major advantage of the CLBF 100 is that it can be used to measure blood velocity and blood flow in actual units of mm/s and µl/min, respectively. This permits the comparison within an eye and between the eyes of a patient, e.g. in clinical examples, as well as between patients. Laser speckle instruments and scanning laser Doppler flowmetery instruments can only provide information in relative units. They are limited in use to comparative measurements over time at the same sites because their measurement values depend on local tissue light scattering properties [95;98]. In additional, the waveforms of the time variation of the blood velocity during the cardiac cycle can be analyzed by using techniques that have long been used with ultrasound Doppler techniques to assess circulatory characteristics in the carotid system, in cerebral vessels, and in the larger extraocular vessels [98].

The disadvantage of the CLBF is that measurements on vessels less than 60 µm in diameter are difficult to achieve. In addition measurements are limited to a specific arteriolar or venular site and are relevant for a specific acquisition time only. A practical limitation of the CLBF 100 is that it requires relatively clear ocular media [98].

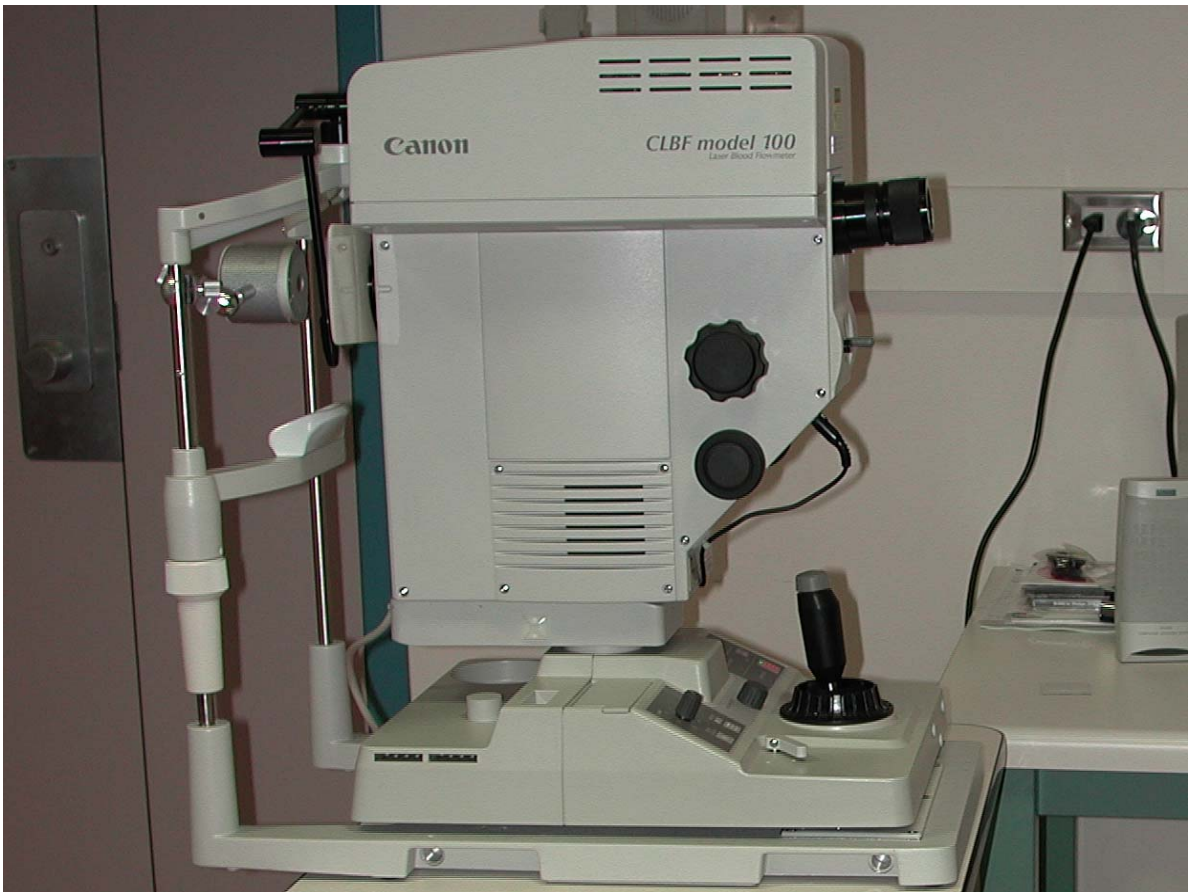


Figure 1. 5 Canon Laser Doppler Flowmeter (CLBF-100)

1.12 Summary

The retina is effectively autoregulated. Small changes in vascular tone greatly influence blood flow, and if widespread, also impact total peripheral resistance and mean arterial blood pressure. The regulation of retinal blood flow is chiefly brought about via metabolic and myogenic autoregulation. Circulating hormones also participate in the blood flow regulation. NO controls blood flow in the retinal circulation by maintaining vasodilation. In addition, endothelins are one of the most important factors in the regulation of tone in the retinal vasculature by promoting vasoconstriction [43]. The relationship between the magnitude of retinal vascular reactivity and vessel diameter is unclear. Some studies have shown that smaller diameter retinal vessels respond to a greater degree than larger vessels [42;62] while others did not find any relationship [3;61].

All techniques for assessing ocular blood flow have limitations. The choice of the optimal technique for the assessment of ocular hemodynamics depends upon the aim of the study as well as the protocol design. In particular, the reproducibility and the sensitivity of each method have to be taken into consideration. There are numerous publications about the reproducibility and sensitivity values for a number of the techniques [53;53;82;99-102]. The Canon Laser Blood Flowmeter permits non-invasive quantification of retinal blood flow in absolute units and represents the ideal technique to determine the relationship between the magnitude of retinal vascular reactivity and vessel diameter by measuring the change between all aspects of retinal hemodynamics in response to a standardized inspired gas challenge.

2 Rationale, Hypotheses and Aims

2.1 Rationale

The relationship between the magnitude of retinal vascular reactivity and vessel diameter is uncertain. Some have found that smaller diameter retinal vessels respond to a greater degree than larger vessels [42;62], while others have found no relationship [3;61]. This information might be useful in the standardization of retinal arteriolar blood flow assessment and provide information that can be used to amplify signal-to-noise ratio (i.e. defined as the ratio of a given transmitted signal to the background noise, or variability, of the signal). Also, elucidation of the relationship between the magnitude of retinal vascular reactivity and vessel diameter might be helpful in understanding the physiological processes of healthy aging and the patho-physiological processes of diseases influencing ocular perfusion, such as diabetes, arterio- / arthero-sclerosis, retinal vessel occlusion and glaucoma.

Furthermore, previous studies [3;42;61;62] have not systematically assessed the relationship between retinal vascular reactivity and vessel diameter. An additional problem with these studies is that they used techniques that assessed hemodynamic parameters that reflected vascular reactivity, rather than techniques that quantified blood flow per se. Using the CLBF, we had the opportunity to take this area of work further by measuring the change in the relationship between all aspects of hemodynamics, that is vessel diameter, centerline blood velocity and volumetric blood flow, in response to an inspired gas challenge. Unlike previous studies, vascular reactivity was quantified by calculating the change in volumetric blood flow that occurred between baseline and the inspired gas challenge.

In Chapter 3, the influence of hypercapnia and hyperoxia on vessel diameter was determined to establish the overall magnitude of vascular reactivity and the resulting change in retinal arteriolar diameter, velocity and blood flow was quantified, in young healthy subjects. In addition, we set out to determine the repeatability of the retinal vascular reactivity measurements and to investigate if there were any order effects in terms of provoking vasoconstriction or vasodilation first (since both of these factors could influence and / or confound signal-to-noise ratio).

The hypercapnic and hyperoxic gas challenges were administered using a sequential rebreathing circuit [103,104]. This allowed the generation of an isocapnic hyperoxic challenge [103] and a hypercapnic challenge that resulted in physiologically insignificant disturbance of arterial oxygen concentration [36] . Previous work from our lab has clearly demonstrated that our isocapnic hyperoxic and hypercapnic gas challenges result in minimal, if any, change in blood pressure and heart rate [36;103;105;106]. Interestingly, other research groups have had limited success in the attainment of isocapnic hyperoxia [103], while others have completely ignored any possible change of arterial oxygen concentration during hypercapnia [106].

The change, if any, in the retinal vascular reactivity response in healthy aging is also unclear. It has been found that retinal autoregulation in response to weight lifting exercise decreases in healthy individuals above 40 years of age [63]. However, this study only assessed change in vessel diameter in response to provocation rather than change in volumetric blood flow. In Chapter 4, the relationship between retinal vascular reactivity and vessel diameter was investigated in elderly healthy subjects and compared to that of the young healthy

subjects. The protocol used for the elderly subjects was considerably shorter than that used for the young subjects but was shortened taking into account information gathered about order effects pertaining to the gas challenges in Chapter 3.

2.2 Hypotheses

For Chapter 3, the hypothesis was that the magnitude of vascular reactivity (as indicated by the overall change in retinal blood flow between the isocapnic hyperoxic and hypercapnic challenges) is inversely proportional to vessel diameter.

Although the relationship between retinal vascular reactivity and vessel diameter is equivocal, there is strong evidence to support our hypothesis from the field of cerebral blood flow research [107;108]. There is also evidence which demonstrates that the magnitude of microvascular autoregulatory adjustments is inversely related to the size of the coronary blood vessel [109].

For Chapter 4, the hypothesis was that the magnitude of retinal vascular reactivity in older healthy subjects is reduced relative to that of healthy young subjects resulting in an alteration of the relationship between baseline arteriolar diameter and vascular reactivity.

To the best of our knowledge, the impact of healthy aging upon the relationship between retinal vascular reactivity and vessel diameter has not been addressed. There is limited evidence to suggest that age may have an impact on the myogenic response on the retinal arterioles [68].

2.3 Aims

In Chapter 3, the primary aim was to determine the magnitude of retinal vascular reactivity in arterioles of varying diameter in healthy, young subjects. The secondary aims of Chapter 3 were: a) To determine if there were any order effects in terms of provoking vasoconstriction or vasodilation first; and b) To determine the repeatability of the retinal vascular reactivity measurements.

In Chapter 4, the aim of this *pilot study* was to determine the effect of healthy aging on the relationship between retinal vascular reactivity and vessel diameter.

3 The Relationship between Retinal Vascular Reactivity and Arteriolar Diameter

3.1 Introduction

The comprehensive study of hemodynamics in the human retina requires the noninvasive, quantitative measurement of blood flow. Using state of the art techniques, it is now possible to simultaneously measure retinal vessel diameter and centerline blood velocity and thereby derive volumetric retinal blood flow in absolute units. Vascular reactivity is defined as the magnitude of change of hemodynamic parameters shown to provocative stimuli, such as that initiated by the manipulation of the partial pressure of oxygen or carbon dioxide in inspired gases. Hyperoxia (i.e. elevated partial pressure of O_2 in systemic arterial blood, PaO_2) induces a pronounced decrease in retinal blood flow in both retinal and cerebral vessels as a result of vascular constriction[10;53-55;60;110-119]. Conversely, hypercapnia (i.e. elevated partial pressure of CO_2 in systemic arterial blood, $PaCO_2$) induces an increase in retinal arteriolar blood flow as a result of vessel dilation [11;110;113;118-121] . In summary, oxygen promotes local vasoconstriction of the arterioles, venules, and capillaries in the retina whereas carbon dioxide results in vasodilatation [10].

Although it is generally accepted in the literature that smaller diameter vessels demonstrate a greater magnitude of vascular reactivity [107], the relationship between *retinal* vascular reactivity and vessel diameter is uncertain. The retinal arterioles represent the resistance vessels of the inner retina and therefore determine the overall blood flow to the capillary bed. Some studies have found that smaller diameter retinal venules and arterioles respond to a greater degree than larger vessels [62;42] while others have found no relationship between vessel diameter and the magnitude of vascular reactivity [3;61]. None of these studies,

however, have systematically assessed the relationship between retinal vascular reactivity and vessel diameter and have invariably used techniques that measured hemodynamic parameters that reflected vascular reactivity but failed to quantify blood flow change *per se*. Understanding of the relationship between retinal vascular reactivity and vessel diameter might be useful in the standardization of retinal blood flow assessment and provide information that can be used to amplify signal-to-noise ratio. Also, it will aid understanding of the physiological processes of healthy aging and the patho-physiological processes of diseases that influence retinal perfusion, such as diabetes, arterio- / arthero-sclerosis, vascular occlusive disease and glaucoma.

The primary aim of the study was to compare the magnitude of retinal vascular reactivity in arterioles of varying diameter in healthy, young subjects. The secondary aims were: a) To determine if there were any order effects in terms of provoking vasoconstriction or vasodilation first; and b) To determine the repeatability of the retinal vascular reactivity measurements. *Unlike previous studies*, vascular reactivity was quantified by calculating the change in volumetric blood flow that occurred between standardized hyperoxic and hypercapnic inspired gas challenges.

3.2 Materials and Methods

3.2.1 Sample

The sample comprised five healthy females and five healthy males of average age 26.2 years (range 21 to 33; SD 3.49). The sample size calculation was determined after estimating the standardized effect size, i.e. SD/Mean effect, for retinal arteriolar blood flow. The change in arteriolar blood flow relative to baseline during hyperoxia is approximately -4 µl/min whereas blood flow typically increases by +3 µl/min during hypercapnia (data obtained from Hudson lab). A mean standard deviation for retinal blood flow would be approximately 3 µl/min. Therefore, the standardized effect size = SD / Mean effect = 3/7 = 0.43. Using a two-tailed α of 0.05 and β of 0.10 (i.e. 90%), the minimum required sample size is n = 3.

One eye of each subject was randomly chosen for this study. All subjects had a logMAR visual acuity of 0.0, or better. Exclusion criteria included any refractive error > ±6.00 Diopters sphere and / or ±1.50 Diopters cylinder, habitual smoking, treatable respiratory disorders (e.g. asthma), cardiovascular disease, systemic hypertension, family history of glaucoma or diabetes, or a history of any ocular disease. All the participants were asked to abstain from caffeine and red meat for 24 hours prior to their study visits. This 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.

3.2.2 Visits

Each subject attended for three visits. The first visit was used to determine eligibility, to define the retinal measurement sites for quantitative blood flow assessment, to acquire baseline data and to familiarize the subjects with the blood flow measurement technique. The second and third visits were used to assess the magnitude of retinal vascular reactivity in response to hyperoxic and hypercapnic stimuli. The inspired gas stimuli were administered consecutively at each visit, but the order of hyperoxia and hypercapnia were systematically reversed across the two visits (in order to identify any residual effects of one gas stimulus versus the other). The order of stimuli was also systematically varied between volunteers.

3.2.3 Quantitative Assessment of Retinal Arteriolar Blood Flow

Non-invasive, quantitative assessment of retinal arteriolar blood flow was achieved using the Canon Laser Blood Flowmeter, model 100 (CLBF). The principal underlying the CLBF is based on the Doppler effect. The CLBF utilizes fundus camera optics that incorporate two lasers and two photo-detectors. A red diode measurement laser (675 nm, 80 μm × 50 μm oval) is used to measure velocity every 0.02 sec across a 2 second measurement window resulting in a velocity-time trace. Velocity readings are determined using the bi-directional photo-detector technique i.e. the difference in Doppler shift between the two detectors is calculated to determine the absolute velocity of the moving red blood cells. Two sequential bi-directional velocity readings (i.e., path 1 and path 2) are taken by the CLBF to ensure

consistency and are averaged to give a single velocity reading. A second green diode vessel tracking laser system (543 nm, 1500 μm × 150 μm rectangle) is used to stabilize, and measure the vessel diameter [96;97]. Multiple diameter readings are acquired during the first and final 60 *ms* of the 2-s velocity measurement window every 4 *ms*. A vessel tracking system provides a graph of eye position to be superimposed on the velocity-time curve to aid in artifact rejection. Flow through the vessel is calculated using the equation: $F = V_{\max} S/2$, where S is the cross-sectional area of the vessel at the measurement site [92;105;123]. 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). Poiseuille flow and a circular vessel cross section are assumed in the calculation of blood flow.

3.2.4 Gas Provocation

A face mask and sequential re-breathing circuit was used to manipulate inspired and expired gases [103]. The sequential re-breathing system comprised fresh gas and re-breathed gas reservoirs that were interconnected by two one-way valves and a single peep valve (Figure 3.1). The sequential re-breathing system is assembled by adding a gas reservoir to the expiratory port of a commercial 3-valve oxygen delivery system (Hi-Ox80, Viasys Healthcare, Yorba Linda, CA) (Figure 3.1). Flow from the gas tanks was controlled using standard rotometers as flowmeters. Gases were mixed in a baffled container prior to being administered to the subject. Tidal gas concentrations, including inspired and expired O_2 and

CO_2 levels, were continuously sampled from the face mask using a rapid response critical care gas analyzer (Cardiocap 5; Datex-Ohmeda, Louisville, CO). Any periods of transitional (i.e. non-stable) gas parameters were excluded from the gas provocation analysis and also from the analysis of CLBF outcome measures (since hemodynamic parameters would be anticipated to be similarly unstable during these transition periods) [103]. A proven advantage of the sequential re-breathing system is that it permits the manipulation of inspired O_2 while maintaining a constant expired CO_2 i.e. isocapnic hyperoxia can be readily achieved [103]. Similarly, any change of expired O_2 during manipulation of inspired CO_2 is physiologically insignificant, although isoxic hypercapnia cannot be attained using a manually controlled sequential re-breathing system [106].

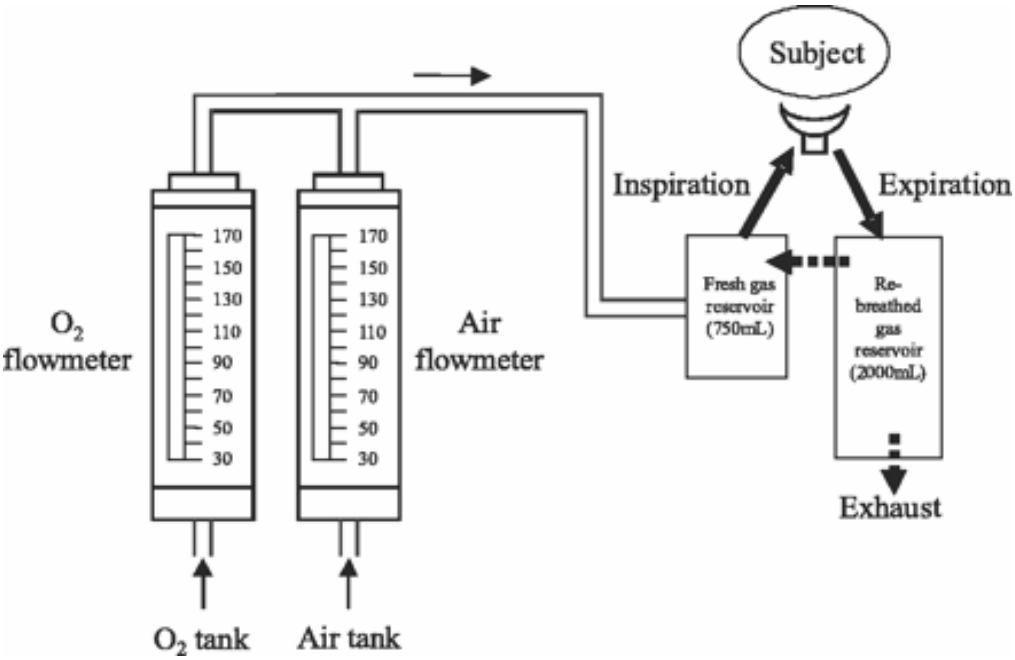


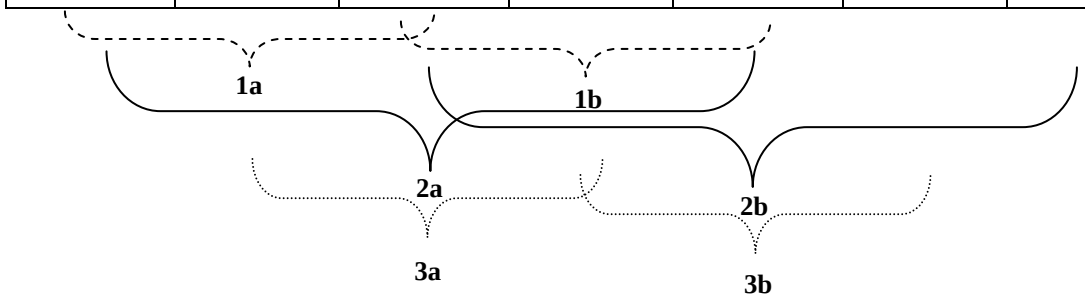
Figure 3. 1 Schematic of sequential re-breathing circuit [103] *Baseline:* An initial air breathing period (@ a rate of 6 to 8 litres per minute, LPM) was employed to allow stabilization of baseline breathing parameters. *Hypercapnia:* The rate of flow of bottled air was reduced (@ a rate of 5 to 6 LPM) so that the subject partially breathed gas from the re-breathed bag. *Isocapnic Hyperoxia:* Subjects breathed O_2 at a flow rate set equal to the subjects' minute ventilation (determined while breathing air).

The gas provocation protocol was designed to reveal any order effects that might occur as a result of hyperoxic and hypercapnic gas challenge, to determine the repeatability of the vascular reactivity measurements and to determine the relationship between arteriolar diameter and the overall magnitude of retinal vascular reactivity. Volunteers underwent assessment over two separate visits in which hyperoxia (Figure 3.2) or hypercapnia (Figure 3.3) were initiated after a baseline period of 10 minutes. The initiation of hyperoxia or hypercapnia after baseline was reversed for each individual across the two visits in order to identify any order effects of one gas challenge versus the other, and was systematically varied between individuals i.e. 5 subjects underwent hyperoxic challenge after baseline at visit 2 and 5 underwent hypercapnic challenge. A minimum of three minutes was allowed following initiation of each gas stimulus and recovery period prior to retinal blood flow assessment.

After the initial gas challenge, a recovery period of 10 minutes was included prior to initiating a challenge which resulted in the opposite physiological vascular response to that of the initial challenge. During each recovery period, subjects breathed air to return their end-tidal O_2 and CO_2 to baseline values. Recovery periods were included between all hyperoxic and hypercapnic challenges and at the end of the protocol. Comparison of the various recovery periods across the protocol permitted the assessment of repeatability of the vascular reactivity measurements, irrespective of any identified order effects (Figures 3.2 and 3.3). Comparison of the arteriolar responses to hyperoxia and hypercapnia, or vice versa, permitted the assessment of the overall magnitude of retinal vascular reactivity (Figures 3.2 and 3.3). The gas provocation protocol was designed to permit the assessment

of the overall magnitude of vascular reactivity irrespective of any order effects associated with one or the other of the gas challenges. For example, if the hyperoxic gas challenge resulted in a residual effect, while hypercapnia did not, it would be appropriate to assess vascular reactivity as the difference in response to CO_2 and subsequently O_2, respectively, and vice versa, if a residual effect was evident for hypercapnia.

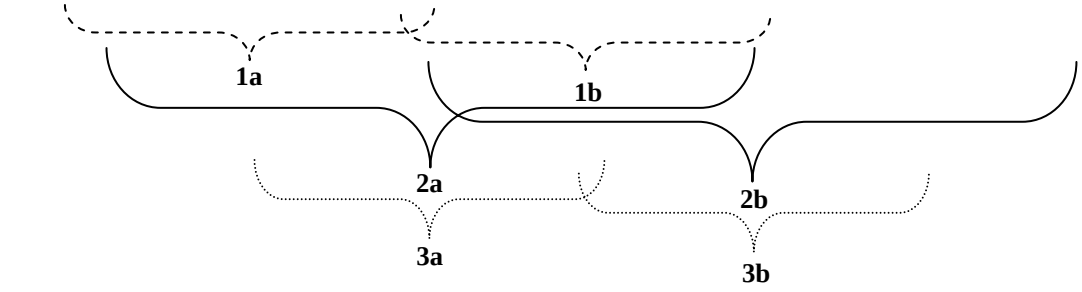
Baseline	O_2	Recovery 1	CO_2	Recovery 2	O_2	Recovery 3
10min	10min	10min	10min	10min	10min	10min



Order Effect; 1a or 1b
Repeatability; 2a or 2b
Vascular reactivity, 3a or 3b

Figure 3. 2 Hyperoxia repeat gas provocation protocol utilized at visit 2 or 3 (O_2; isocapnic hyperoxia. CO_2; hypercapnia). One narrow and one relatively wide diameter measurement site along the superior temporal arteriole were selected for each individual. A minimum of 3 minutes was allowed following initiation of each condition prior to CLBF acquisition. O_2 and CO_2 order were systematically varied between volunteers and across visits.

Baseline	CO_2	Recovery 1	O_2	Recovery 2	CO_2	Recovery 3
10min	10min	10min	10min	10min	10min	10min



Order Effect; 1a or 1b
Repeatability; 2a or 2b
Vascular Reactivity; 3a or 3b

Figure 3. 3 Hypercapnia repeat gas provocation protocol utilized at visit 3 or 2. (O_2; isocapnic hyperoxia. CO_2; hypercapnia). One narrow and one relatively wide diameter measurement site along the superior temporal arteriole were selected for each individual. A minimum of 3 minutes was allowed following initiation of each condition prior to CLBF acquisition. O_2 and CO_2 order were systematically varied between volunteers and across visits.

3.2.5 Procedures

The pupil of the study eye was dilated using Tropicamide 1 % (Alcon, Mississauga, Canada) at the beginning of each visit to achieve an adequate view of the fundus for the retinal blood flow image acquisition. Subjects rested for 10 minutes prior to start of each study visit to stabilize baseline cardiovascular and respiratory parameters. At visit 1, two separate measurement locations, representing different diameter values along the superior temporal arteriole were sought for each subject. Relatively straight segments, distant from vessel branches, of the superior temporal arteriole were selected. Arterioles were used in this study because they are primarily responsible for determining vascular resistance and because they obey Poiseuille flow to a greater extent than venules. Five retinal blood flow measurements were acquired at each location for each condition and the order of measurement location was randomized between subjects with respect to distance from the optic nerve head.

At visits 2 and 3, subjects breathed air at a rate equivalent to their minute ventilation, thereby ensuring a stable $F_{ET}CO_2$ (fractional percent end-tidal CO_2 i.e. the maximum concentration of CO_2 during each expiration), for the first 10 minutes to establish a baseline. At least five retinal blood flow measurements at the two distinct predetermined arteriolar measurement sites were acquired for each gas stimulus and recovery period following an initial 3 minute period of stabilization. For the hypercapnic gas challenge, the rate of flow of bottled air was reduced (typically to a rate of 5 to 6 Liters per minute, LPM) so that the subject breathed a proportion of gas from the re-breathed bag for 10 minutes. The stimulus was designed to result in approximately a 10% increase of $F_{ET}CO_2$ relative to baseline. For

the hyperoxic gas challenge, subjects breathed O_2 at a flow rate equal to their minute ventilation (determined while breathing air at baseline) for 10 minutes. The stimulus was designed to result in approximately a fractional (percent) inspired O_2 (FiO_2) of 95%. If hyperoxic provocation was undertaken following hypercapnia, retinal blood flow measurements were only acquired when end-tidal CO_2 values had returned to baseline levels. At the completion of the protocol, subjects were returned to bottled air for 10 minutes and retinal blood flow measurements were acquired when end-tidal CO_2 values had returned to baseline levels.

3.2.6 CLBF Velocity Waveform Analysis

CLBF analysis software was used to analyze each acquired velocity waveform. A standardized protocol was used to remove aberrant portions of each waveform i.e. due to eye movement or improper vessel tracking. For each acquisition, the maximum number of acceptable cycles was included in the analysis, while a minimum of one complete systolic-diastolic cycle was essential for including a given waveform.

3.2.7 Statistical Analysis

Mean hemodynamic parameters were calculated for each condition (i.e. baseline, O_2, CO_2, recovery, etc.) of each individual as a function of gas provocation protocol (Figure 3.2 & 3.3). Data points which represented "outliers", that is any data point that fell outside the 25th to 75th percentile, were retained in the analysis since the sample size was relatively small and, as a result, likely to generate outliers.

A repeated measures analysis of variance (ANOVA) was undertaken on the group arteriolar diameter, blood velocity and flow to determine the significance of any change over the course of each protocol. Arteriolar measurement site (i.e. narrow or wide) was the within-subject factor. In those situations that revealed a significant ANOVA result, post hoc testing was undertaken using Tukey's HSD (Honestly Significant Difference) test. Tukey's HSD test is "protected" from the probability of making type I experimental errors for any pairwise comparison. This analysis was designed, in part, to reveal any order effects in relation to the provocation of vasoconstriction or vasodilation (i.e. addressing one *secondary aim* of the study). The results of this analysis, if positive, would dictate which conditions were compared to determine the repeatability of, and the influence of vessel diameter on, retinal vascular reactivity.

The repeatability of the vascular reactivity measurements were determined for volumetric blood flow using the Coefficient of Repeatability (COR=1.96*SD of the differences) for both the narrow and wide measurement sites. Any evidence of a systematic order effect

associated with the provocation of vasoconstriction or vasodilation would dictate which conditions were compared to validly determine the repeatability.

In order to test the influence of vessel diameter on the magnitude of retinal vascular reactivity (i.e. *primary aim* of the study), the difference in vascular response in terms of blood flow between hypercapnic and hyperoxic provocation was calculated. A repeated measures ANOVA was used to determine the significance of any difference in flow response between the narrow and wide measurement sites (in this analysis, gas provocation protocol was considered to be a within-subject factor). Any evidence of a systematic order effect associated with the provocation of vasoconstriction or vasodilation would dictate which conditions were compared to validly determine the influence of vessel diameter on the magnitude of vascular reactivity.

3.3 Results

3.3.1 Diameter

The baseline diameter for the narrow arteriolar measurement site was 92.40 μm (SD 13.64) and for the wide measurement site was 116.68 μm (SD 12.68) (re-ANOVA $p<0.0001$).

3.3.1.1 Hyperoxia Repeat Gas Provocation Protocol (see Figure 3.2)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for diameter ($p<0.0001$) and a significant influence of inspired gas provocation on diameter ($p<0.0001$). In addition, the interaction of measurement site and inspired gas provocation was significant ($p=0.0083$).

The *first* hyperoxic provocation resulted in a significant decrease in diameter relative to baseline (Tukey HSD, $p<0.0001$) and relative to recovery 1 ($p<0.0001$) for both the wide and narrow measurement sites (see Figure 3.5 & 3.6). Hypercapnia resulted in significantly larger diameter values for the narrow measurement site relative to recovery 1 ($p<0.0001$) and relative to recovery 2 ($p=0.0037$). Similarly, the wide measurement site exhibited significantly larger diameter values in response to hypercapnia relative to recovery 1 ($p=0.0050$) but was not different relative to recovery 2 ($p=0.0901$). The *repeat* hyperoxic provocation resulted in a significant decrease in diameter relative to recovery 2 ($p<0.0001$) and relative to recovery 3 ($p<0.0001$) for both the narrow and wide measurement sites.

Diameter values *following* the first hyperoxic (i.e. at recovery 1) provocation were significantly less for the narrow (p=0.0070) and wide (p=0.0191) measurement sites compared to baseline (see Figure 3.4 and Table 3.1). Diameter values *following* hypercapnia (i.e. at recovery 2) were not significantly different from baseline (p=0.7115, p=0.2306 for narrow and wide measurement sites, respectively) (see Table 3.2). Diameter values *following* the second hyperoxic provocation (i.e. at recovery 3) were not different for the narrow measurement site (p=0.0972) compared to baseline but were significantly less for the wide measurement site (p=0.0415).

	Hyperoxia Repeat		Hyperoxia Repeat		Hypercapnia Repeat	
DIAMETER	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	92.67	88.15	92.67	90.95	92.12	92.10
(SD)	(13.47)	(13.33)	(13.47)	(11.76)	(14.54)	(14.52)
p-value	p=0.0070		p=0.0972		p=0.6833	
Wide						
Mean	117.31	113.42	117.31	113.94	116.04	114.05
(SD)	(11.98)	(11.93)	(11.98)	(13.90)	(13.96)	(13.14)
p-value	p=0.0191		p=0.0415		p=0.6833	

Table 3. 1 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hyperoxic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

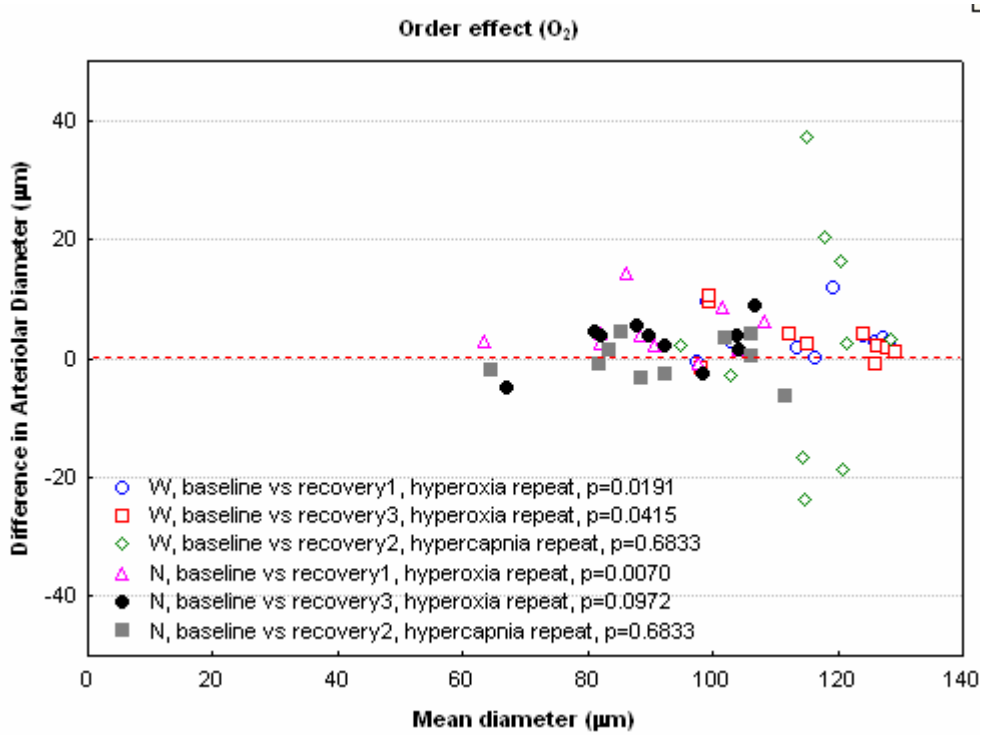


Figure 3. 4 Difference in arteriolar diameter (i.e. between baseline and recovery following O_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean diameter for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following O_2 administration are shown for each situation / plot legend.

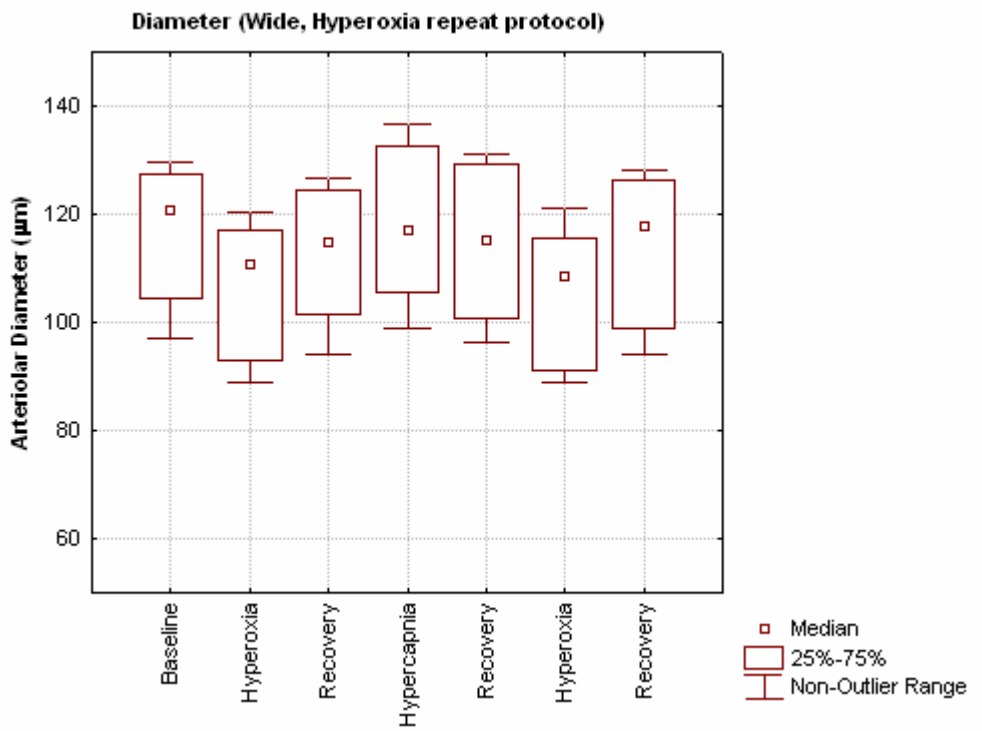


Figure 3. 5 Box plots illustrating group change in retinal arteriolar diameter during the hyperoxia repeat protocol for the wide measurement site.

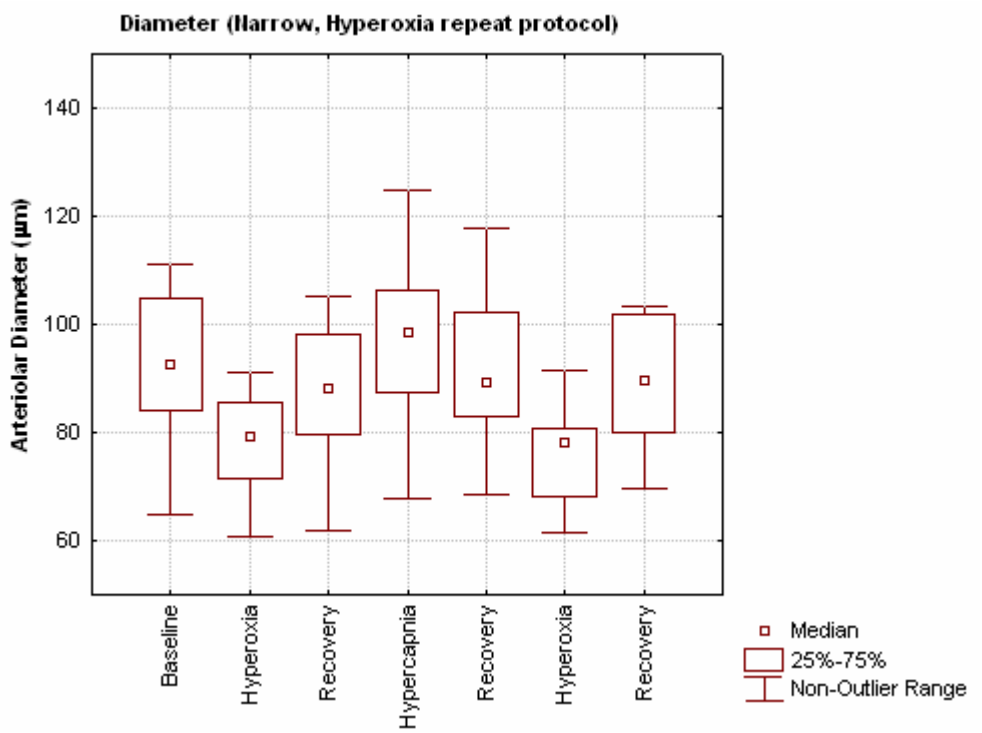


Figure 3. 6 Box plots illustrating group change in retinal arteriolar diameter during the hyperoxia repeat protocol for the narrow measurement site.

3.3.1.2 Hypercapnia Repeat Gas Provocation Protocol (see Figure 3.3)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for diameter ($p<0.0001$) and a significant influence of inspired gas provocation on diameter ($p<0.0001$). However, the interaction of measurement site and inspired gas provocation was not significant ($p=0.7517$).

The *first* hypercapnic provocation resulted in a significant increase in diameter relative to baseline (Tukey HSD, $p=0.0444$) and relative to recovery 1 ($p=0.0324$; see Figure 3.8 & 3.9). Hyperoxia resulted in a significant reduction in diameter ($p<0.0001$) relative to recovery 1 and relative to recovery 2 ($p<0.0001$). The *repeat* hypercapnic provocation was not different relative to recovery 2 ($p=0.0836$) and relative to recovery 3 ($p=0.1063$).

Diameter values *following* hyperoxia (i.e. at recovery 2) were not significantly different ($p=0.6833$) compared to baseline and similarly *following* hypercapnia (i.e. at recovery 1 and 3) were not significantly different compared to baseline ($p=0.8905$, $p=0.7731$ for recovery 1 and 3, respectively) (see Figure 3.7 and Tables 3.1 and 3.2).

	Hypercapnia Repeat		Hypercapnia Repeat		Hyperoxia Repeat	
DIAMETER	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	92.12	91.36	92.12	91.08	92.67	92.31
(SD)	(14.54)	(11.98)	(14.54)	(15.06)	(13.47)	(14.10)
p-value	p=0.8905		p=0.7731		p=0.7115	
Wide						
Mean	116.04	116.13	116.04	115.67	117.31	115.15
(SD)	(13.96)	(13.27)	(13.96)	(12.84)	(11.98)	(13.65)
p-value	p=0.8905		p=0.7731		p=0.2306	

Table 3. 2 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hypercapnic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

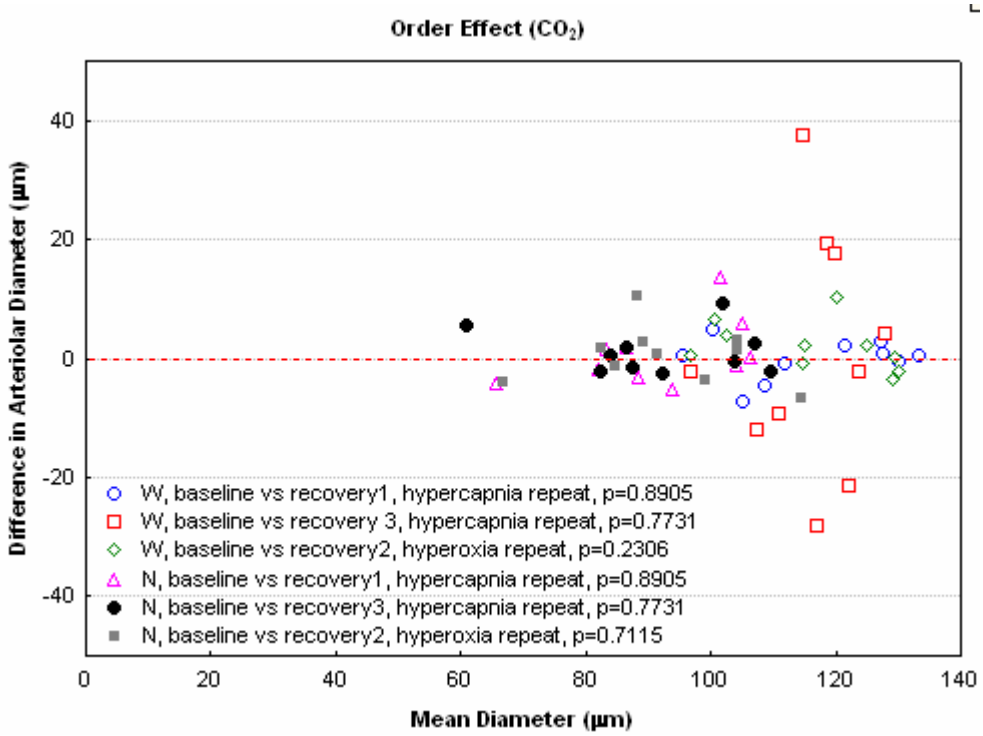


Figure 3. 7 Difference in arteriolar diameter (i.e. between baseline and recovery following CO_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean diameter for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following CO_2 administration are shown for each situation / plot legend.

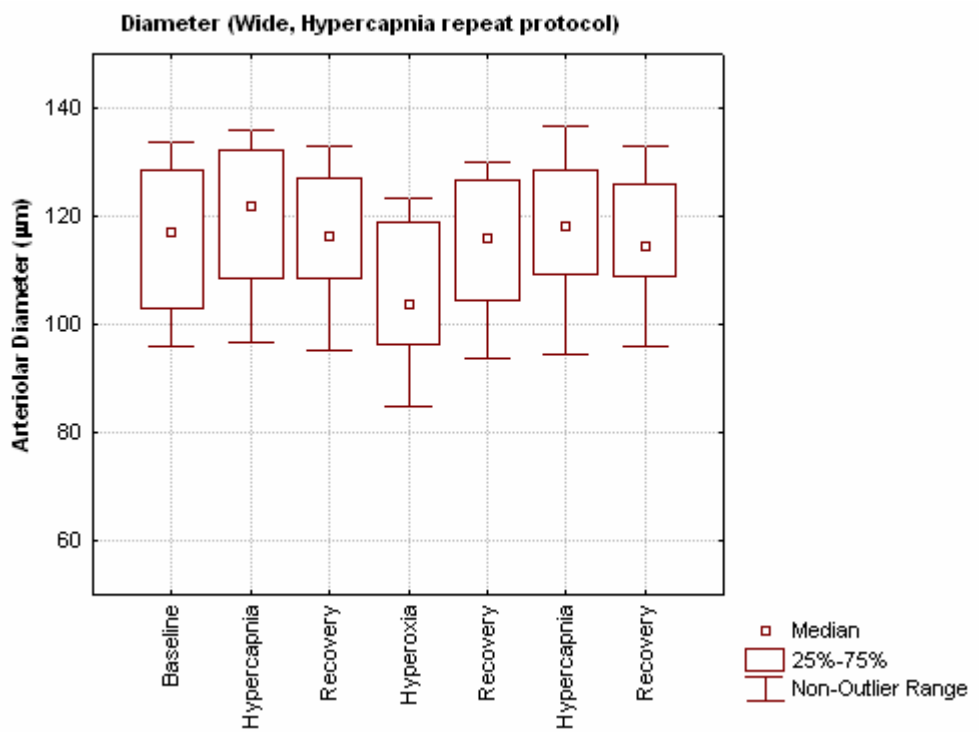


Figure 3. 8 Box plots illustrating group change in retinal arteriolar diameter during the hypercapnia repeat protocol for the wide measurement site.

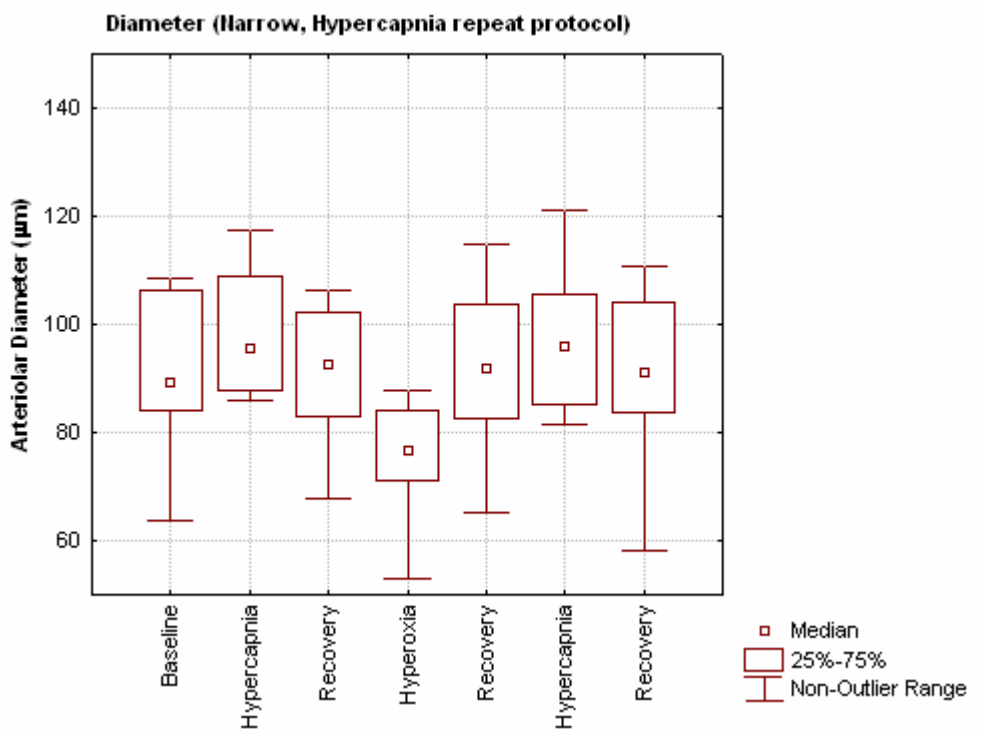


Figure 3. 9 Box plots illustrating group change in retinal arteriolar diameter during the hypercapnia repeat protocol for the narrow measurement site.

3.3.2 Velocity

3.3.2.1 Hyperoxia Repeat Gas Provocation Protocol (see Figure 3.2)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for velocity ($p=0.0018$) and a significant influence of inspired gas provocation on velocity ($p<0.0001$). However, the interaction of measurement site and inspired gas provocation was not significant ($p=0.0810$).

The *first* hyperoxic provocation resulted in a significant decrease in velocity relative to baseline (Tukey HSD, $p<0.0001$) and relative to recovery 1 ($p<0.0001$) for both measurement sites (see Figure 3.11 & 3.12). Hypercapnia resulted in a significant increase in velocity relative to recovery 1 and recovery 2 ($p\leq 0.0020$). The *repeat* hyperoxic provocation resulted in a significant decrease in diameter relative to recovery 2 ($p<0.0001$) and relative to recovery 3 ($p<0.0001$).

Velocity values *following* the first hyperoxic provocation (i.e. at recovery 1) were not different compared to baseline ($p=0.4554$) (see Figure 3.10 and Table 3.3). Similarly, velocity values *following* hypercapnia (i.e. at recovery 2) were not different from baseline ($p=0.9095$) (see Table 3.4). Velocity values *following* the repeat hyperoxic provocation (i.e. at recovery 3) were not different compared to baseline ($p=0.2512$).

	Hyperoxia Repeat		Hyperoxia Repeat		Hypercapnia Repeat	
VELOCITY	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	20.86	20.66	20.86	19.43	20.57	20.75
(SD)	(4.74)	(3.26)	(4.74)	(3.09)	(5.48)	(5.06)
p-value	p=0.4554		p=0.2512		p=0.8961	
Wide						
Mean	28.75	27.12	28.75	27.34	32.93	32.90
(SD)	(3.73)	(8.06)	(3.73)	(6.03)	(7.44)	(5.91)
p-value	p=0.4554		p=0.2512		p=0.9811	

Table 3. 3 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hyperoxic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

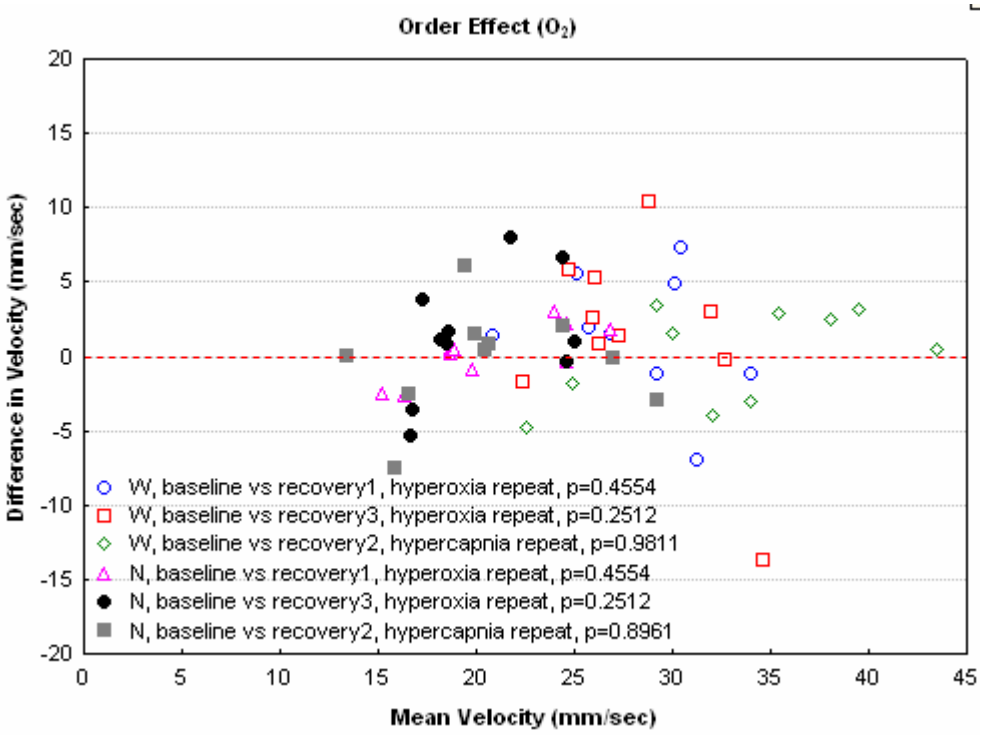


Figure 3. 10 Difference in arteriolar velocity (i.e. between baseline and recovery following O_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean velocity for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following O_2 administration are shown for each situation / plot legend.

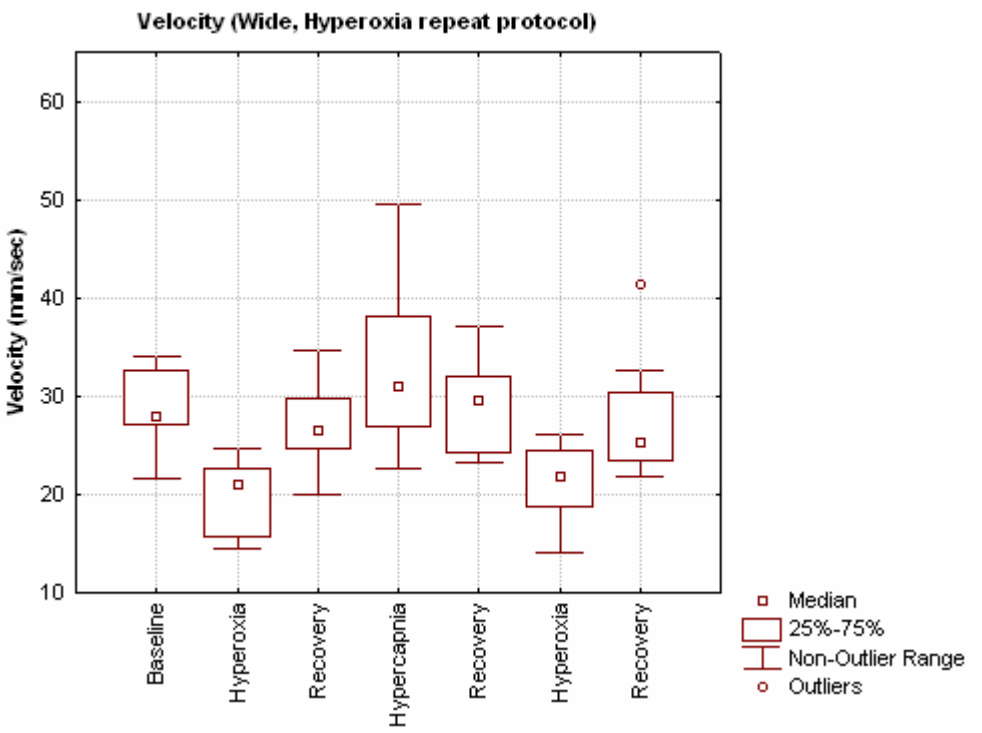


Figure 3. 11 Box plots illustrating group change in retinal arteriolar velocity during the hyperoxia repeat protocol for the wide measurement site.

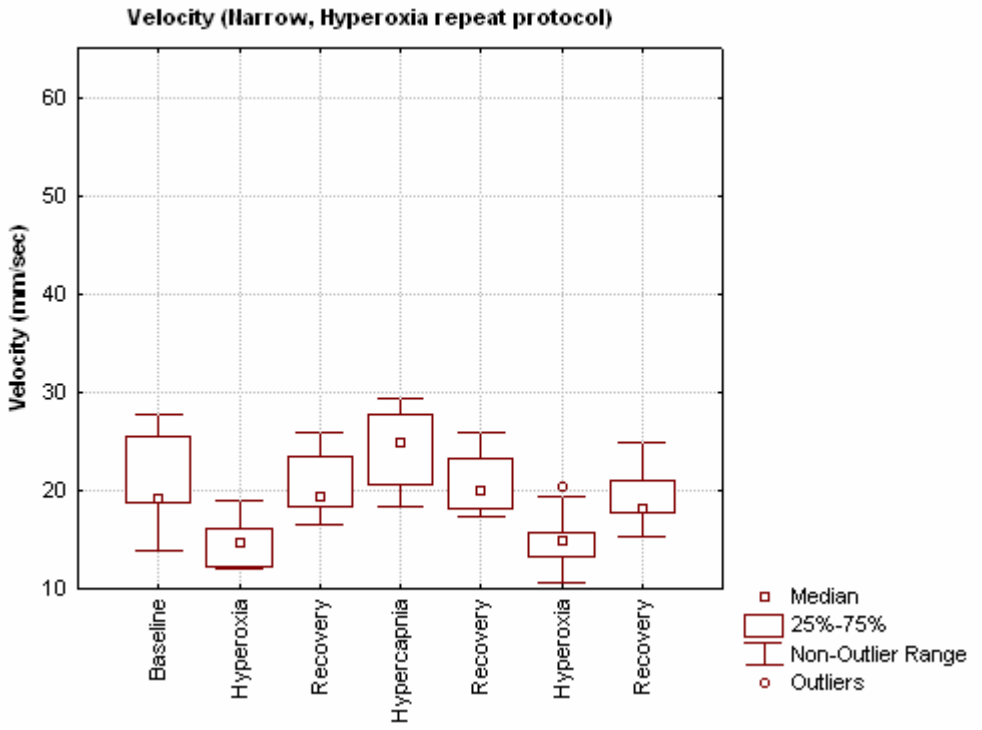


Figure 3. 12 Box plots illustrating group change in retinal arteriolar velocity during the hyperoxia repeat protocol for the narrow measurement site.

3.3.2.2 Hypercapnia Repeat Gas Provocation Protocol (see Figure 3.3)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for velocity ($p=0.0006$) and a significant influence of inspired gas provocation on velocity ($p<0.0001$). In addition, the interaction of measurement site and inspired gas provocation was significant ($p=0.0231$).

The *first* hypercapnic provocation resulted in a significant increase in velocity relative to baseline (Tukey HSD, $p=0.0096$ and $p=0.0003$) and relative to recovery 1 ($p=0.0066$ and $p<0.0001$) for the narrow and wide measurement sites, respectively (see Figure 3.14 & 3.15). Hyperoxia resulted in a significant reduction in velocity relative to recovery 1 and relative to recovery 2 for the narrow ($p=0.0020$ and $p<0.0001$) and wide ($p=0.0009$ and $p<0.0001$) measurement sites, respectively. The *repeat* hypercapnic provocation was not different relative to recovery 2 ($p=0.1557$ and $p=0.2124$) and relative to recovery 3 ($p=0.3096$ and $p=0.0891$) for the narrow and wide measurement sites, respectively.

Velocity values *following* first hypercapnia (i.e. at recovery 1) were not different ($p=0.8904$ and $p=0.4501$ for narrow and wide measurement sites, respectively) compared to baseline (see Figure3.13 and Table 3.4). Velocity values *following* hyperoxia (i.e. at recovery 2) were not different ($p=0.8961$ and $p=0.9811$ for narrow and wide measurement sites, respectively) compared to baseline (see Table 3.3). Velocity values *following* the repeat hypercapnic provocation (i.e. at recovery 3) were not different ($p=0.5878$ and $p=0.6238$ for the narrow and wide measurement sites, respectively) compared to baseline.

	Hypercapnia Repeat		Hypercapnia Repeat		Hyperoxia Repeat	
VELOCITY	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	20.57	20.38	20.57	19.32	20.86	20.81
(SD)	(5.48)	(4.36)	(5.48)	(4.53)	(4.74)	(3.09)
p-value	p=0.8904		p=0.5878		p=0.9095	
Wide						
Mean	32.93	31.88	32.93	32.25	28.75	29.08
(SD)	(7.44)	(8.06)	(7.44)	(7.86)	(3.74)	(4.52)
p-value	p=0.4501		p=0.6238		p=0.9095	

Table 3. 4 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hypercapnic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

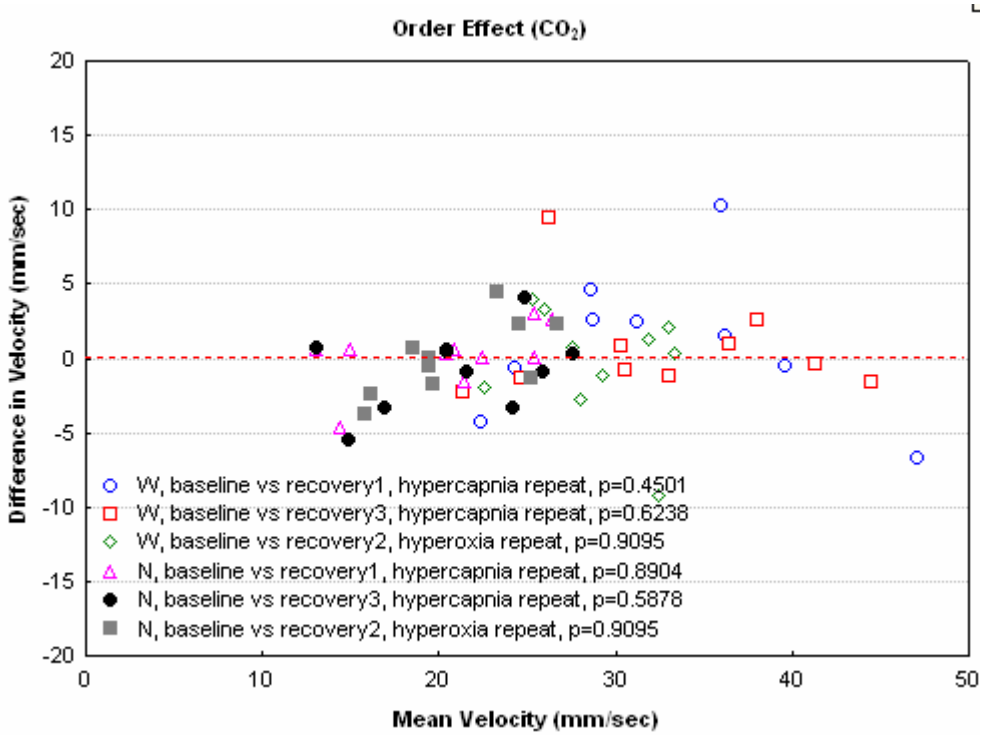


Figure 3. 13 Difference in arteriolar velocity (i.e. between baseline and recovery following CO_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean velocity for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following CO_2 administration are shown for each situation / plot legend.

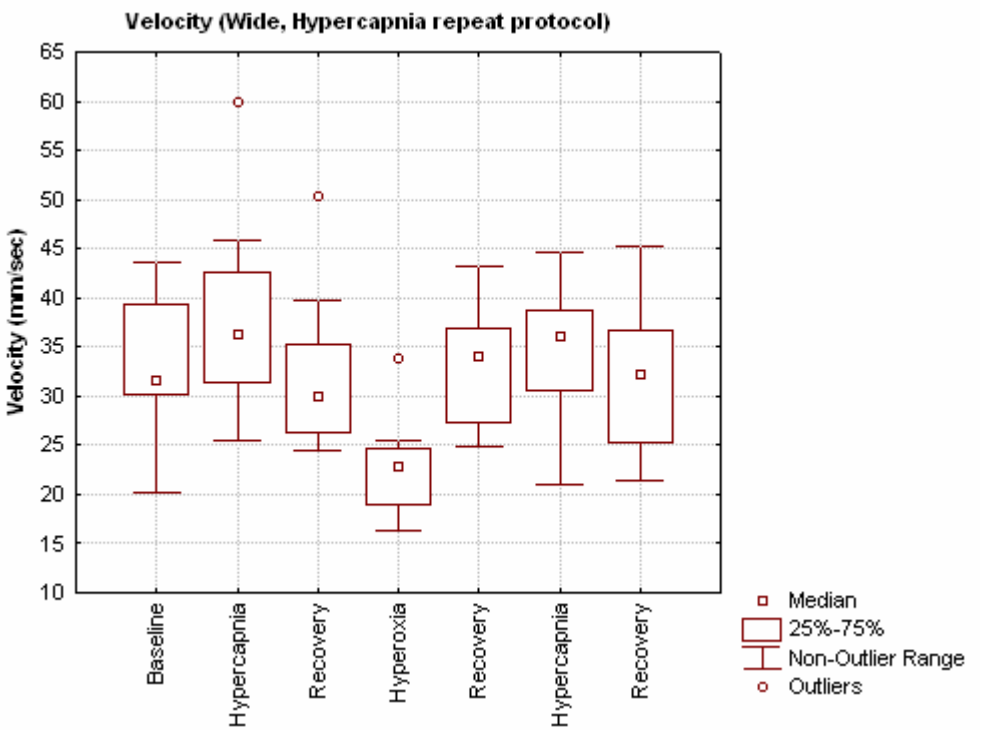


Figure 3. 14 Box plots illustrating group change in retinal arteriolar velocity during the hypercapnia repeat protocol for the wide measurement site.

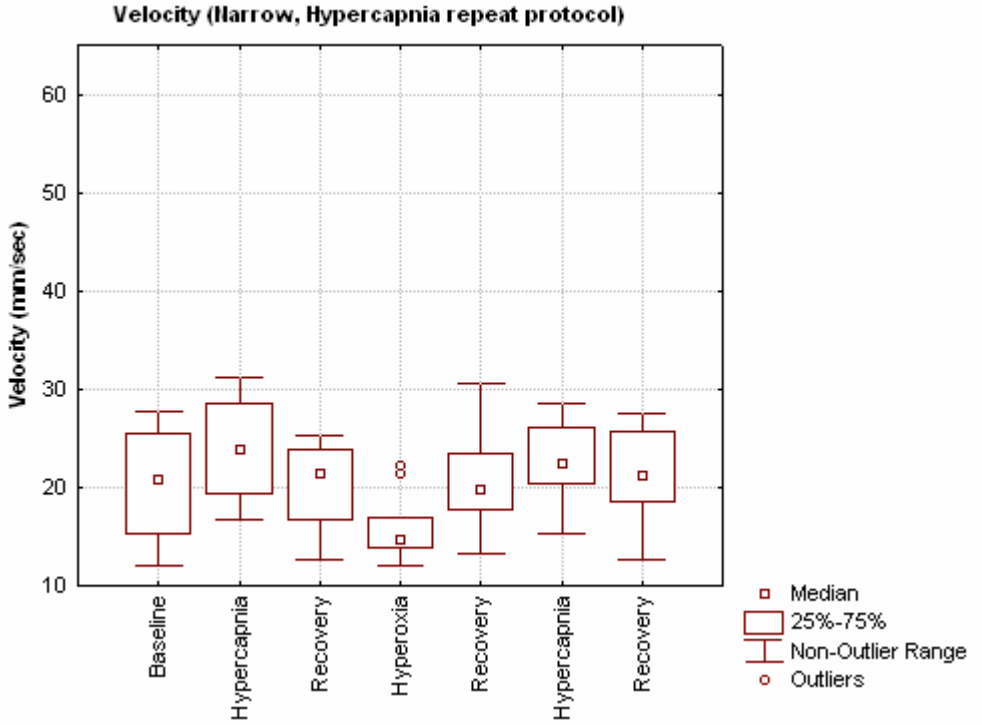


Figure 3. 15 Box plots illustrating group change in retinal arteriolar velocity during the hypercapnia repeat protocol for the narrow measurement site.

3.3.3 Flow

3.3.3.1 Hyperoxia Repeat Gas Provocation Protocol (see Figure 3.2)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for flow ($p=0.0003$) and a significant influence of inspired gas provocation on flow ($p<0.0001$). In addition, the interaction of measurement site and inspired gas provocation was significant ($p<0.0001$).

The *first* hyperoxic provocation resulted in a significant decrease in flow relative to baseline (Tukey HSD, $p<0.0001$) and relative to recovery 1 ($p<0.0001$) for both the narrow and wide measurement sites (see Figure 3.17 & 3.18). Hypercapnia resulted in a significant increase in flow for both the narrow ($p<0.0001$ and $p=0.0328$) and wide ($p<0.0001$ and $p<0.0001$) measurement sites relative to recovery 1 and recovery 2, respectively. The *repeat* hyperoxic provocation resulted in a significant decrease in flow relative to recovery 2 ($p<0.0001$and $p<0.0001$) and relative to recovery 3 ($p=0.0002$ and $p<0.0001$) for both the narrow and wide measurement sites, respectively.

Flow values *following* the first hyperoxic provocation (i.e. at recovery 1) were significantly less for the narrow ($p=0.0459$) and wide ($p=0.0008$) measurement sites compared to baseline (see Figure 3.16 and Table 3.5). Flow values *following* hypercapnia (i.e. at recovery 2) were not different from baseline ($p=0.9097$ and $p=0.7118$) for narrow and wide measurement sites, respectively (see Table 3.6). Flow values *following* the repeat hyperoxic provocation (i.e. at recovery 3) were significantly less for the narrow ($p=0.0120$) and wide ($p<0.0001$) measurement sites compared to baseline.

	Hyperoxia Repeat		Hyperoxia Repeat		Hypercapnia Repeat	
FLOW	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	4.99	4.25	4.99	4.04	4.59	4.09
(SD)	(1.38)	(1.09)	(1.38)	(1.15)	(1.29)	(1.05)
p-value	p=0.0459		p=0.0120		p=0.2101	
Wide						
Mean	11.13	9.84	11.13	9.57	11.53	9.93
(SD)	(3.63)	(3.01)	(3.63)	(3.33)	(3.73)	(2.88)
p-value	p=0.0008		p<0.0001		p=0.0002	

Table 3. 5 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hyperoxic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

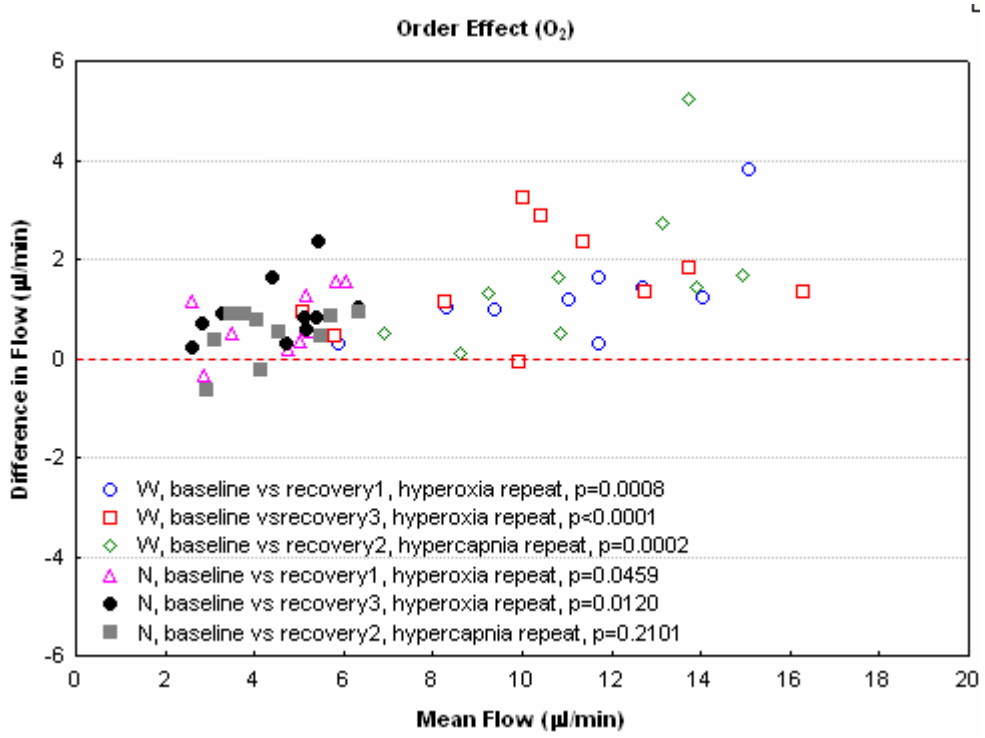


Figure 3. 16 Difference in arteriolar blood flow (i.e. between baseline and recovery following O_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean flow for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following O_2 administration are shown for each situation / plot legend.

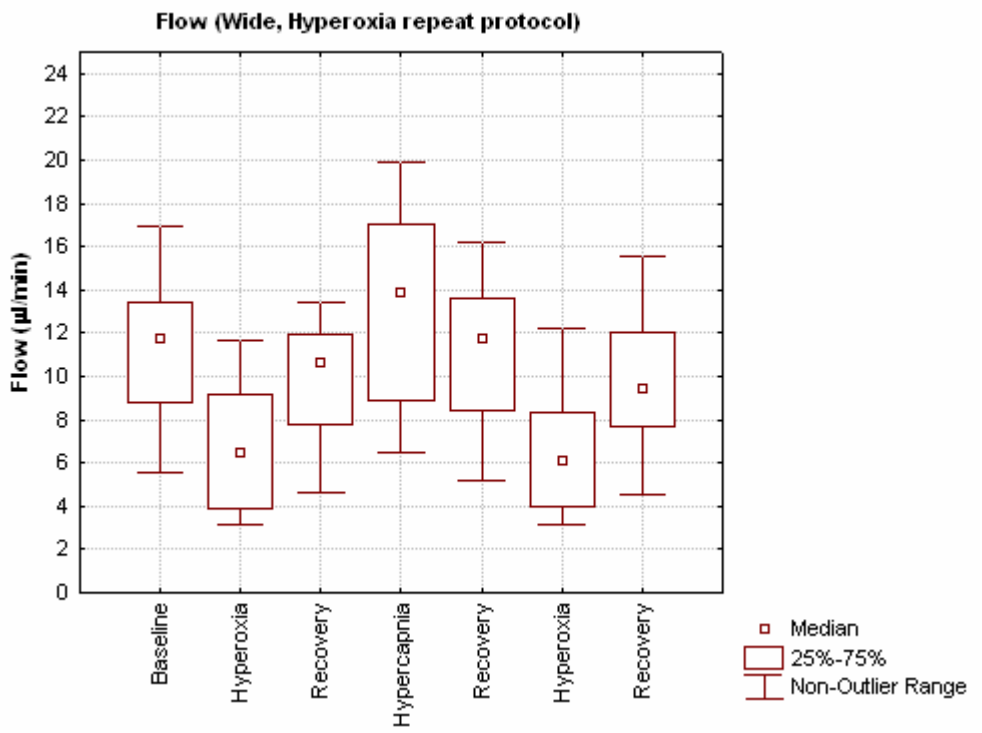


Figure 3. 17 Box plots illustrating group change in retinal arteriolar blood flow during the hyperoxia repeat protocol for the wide measurement site.

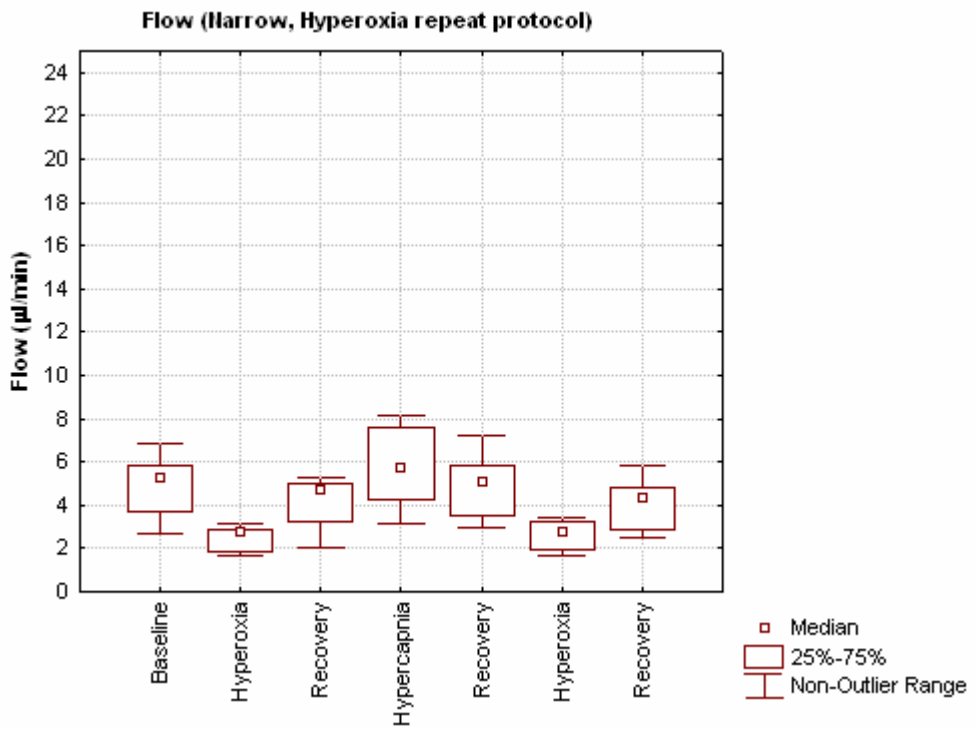


Figure 3. 18 Box plots illustrating group change in retinal arteriolar blood flow during the hyperoxia repeat protocol for the narrow measurement site.

3.3.3.2 Hypercapnia Repeat Gas Provocation Protocol (see Figure 3.3)

The results of the repeated measures ANOVA showed a significant difference between the narrow and wide measurement sites for flow ($p<0.0001$) and a significant influence of inspired gas provocation on flow ($p<0.0001$). In addition, the interaction of measurement site and inspired gas provocation was significant ($p<0.0001$).

The *first* hypercapnic provocation resulted in a significant increase in flow relative to baseline (Tukey HSD, $p=0.0026$ and $p<0.0001$) and relative to recovery 1 ($p=0.0050$ and $p<0.0001$) for the narrow and wide measurement sites, respectively (see Figure 3.20 & 3.21). Hyperoxia resulted in a significant reduction in flow relative to recovery 1 and recovery 2 for the narrow ($p<0.0001$ and $p=0.0004$, respectively) and wide ($p<0.0001$ and $p<0.0001$, respectively) measurement sites. The *repeat* hypercapnic provocation resulted in a significant increase in flow relative to recovery 2 ($p=0.0003$ and $p<0.0001$) and recovery 3 ($p=0.0226$ and $p=0.0039$) for the narrow and wide measurement sites, respectively.

Flow values *following* the first hypercapnic provocation (i.e. at recovery 1) were not significantly different compared to baseline ($p=0.8147$ and $p=0.3524$) for the narrow and wide measurement sites, respectively (see Figure 3.19 and Table 3.6). Flow values *following* hyperoxia (i.e. at recovery 2) were not different compared to baseline for the narrow measurement site ($p=0.2101$) but were significantly less for the wide measurement site ($p=0.0002$) (see Table 3.5). Flow values *following* the repeat hypercapnic provocation (i.e. at recovery 3) were not different compared to baseline ($p=0.7823$ and $p=0.4010$) for the narrow and wide measurement sites, respectively.

	Hypercapnia Repeat		Hypercapnia Repeat		Hyperoxia Repeat	
FLOW	Baseline	Recovery 1	Baseline	Recovery 3	Baseline	Recovery 2
Narrow						
Mean	4.59	4.69	4.59	4.70	4.99	5.03
(SD)	(1.29)	(1.18)	(1.29)	(1.32)	(1.38)	(1.48)
p-value	p=0.8147		p=0.7823		p=0.9097	
Wide						
Mean	11.53	11.16	11.53	11.87	11.13	10.99
(SD)	(3.73)	(3.54)	(3.73)	(3.81)	(3.63)	(3.66)
p-value	p=0.6524		p=0.4010		p=0.7118	

Table 3. 6 Group mean diameter (SD, standard deviation) values for the narrow and wide arteriolar measurement sites at baseline and recovery following *hypercapnic* provocation in both the hyperoxia and hypercapnia repeat protocols. The p-values designate the significance of any change between baseline and recovery.

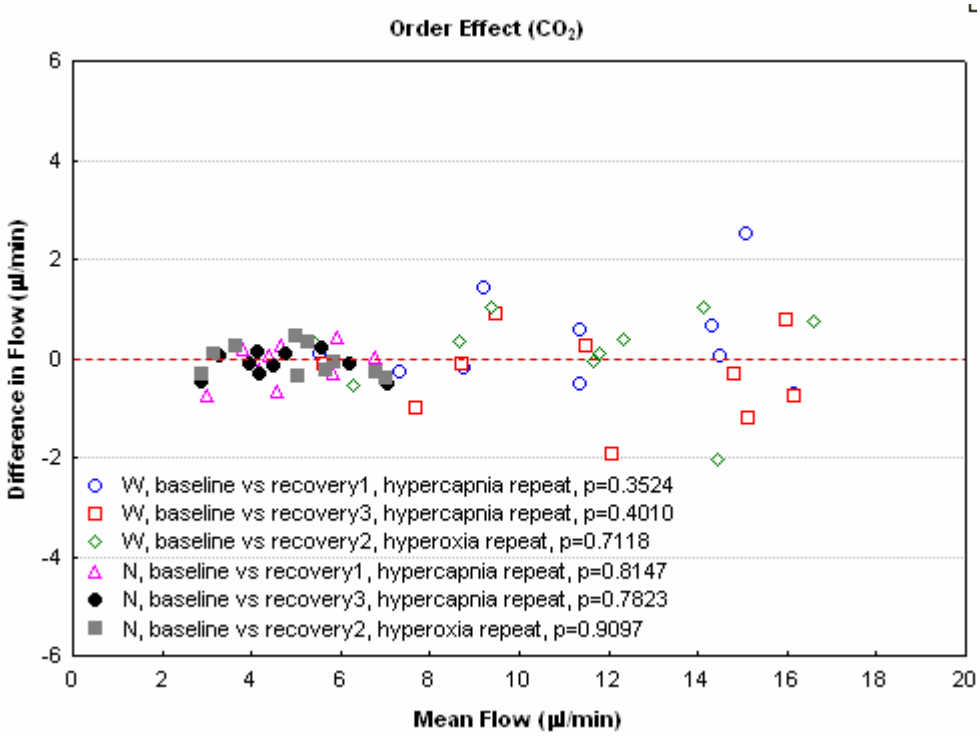


Figure 3. 19 Difference in arteriolar blood flow (i.e. between baseline and recovery following CO_2 provocations for both the hypercapnia and hyperoxia repeat protocols) as a function of mean flow for wide and narrow arteriolar measurement sites. The p-values showing the statistical significance of any change between baseline and recovery following CO_2 administration are shown for each situation / plot legend.

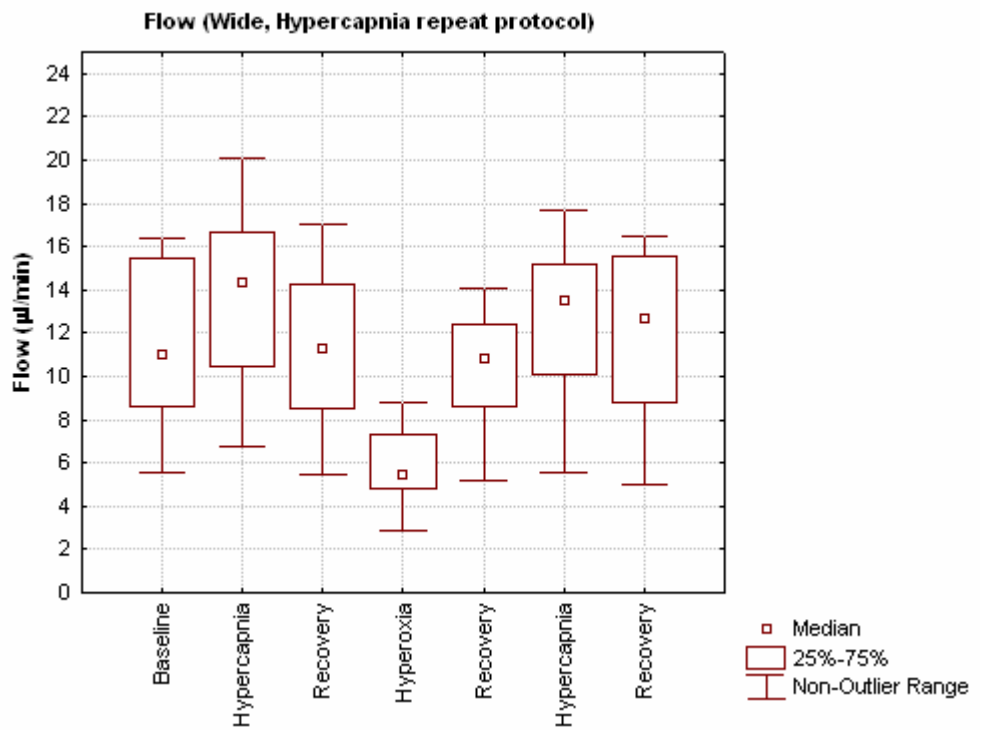


Figure 3. 20 Box plots illustrating group change in retinal arteriolar blood flow during the hypercapnia repeat protocol for the wide measurement site.

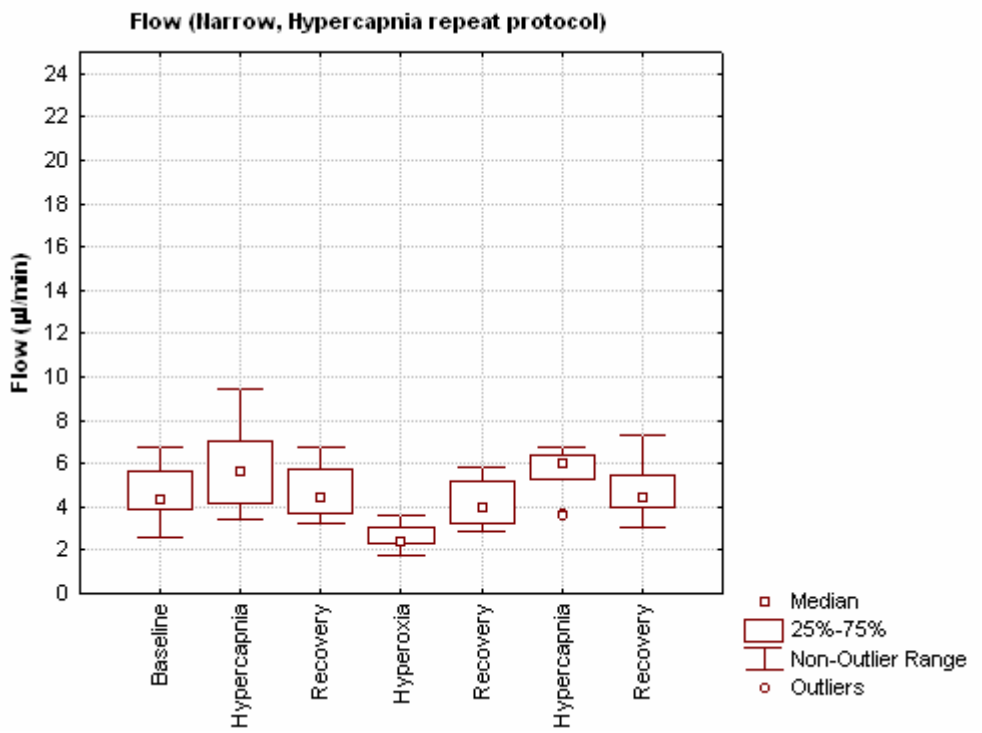


Figure 3. 21 Box plots illustrating group change in retinal arteriolar blood flow during the hypercapnia repeat protocol for the narrow measurement site.

3.3.4 Repeatability of Vascular Reactivity

Since hyperoxia resulted in a prolonged vasoconstriction, the repeatability of retinal vascular reactivity was determined by calculating the COR for blood flow values between baseline and recovery 2 for the hyperoxia repeat protocol (i.e. Figure 3.2; 2a rather than 2b) and between recovery 1 and recovery 3 for the hypercapnia repeat protocol (i.e. Figure 3.3; 2b rather than 2a).

The group mean blood flow COR for the narrow measurement site was 0.74 µl/min (relative to a group mean flow of 4.85 µl/min ± SD 1.31; Figure 3.22) and for the wide measurement site was 1.49 µl/min (relative to a group mean flow of 11.29 µl/min ± SD 3.55; Figure 3.23).

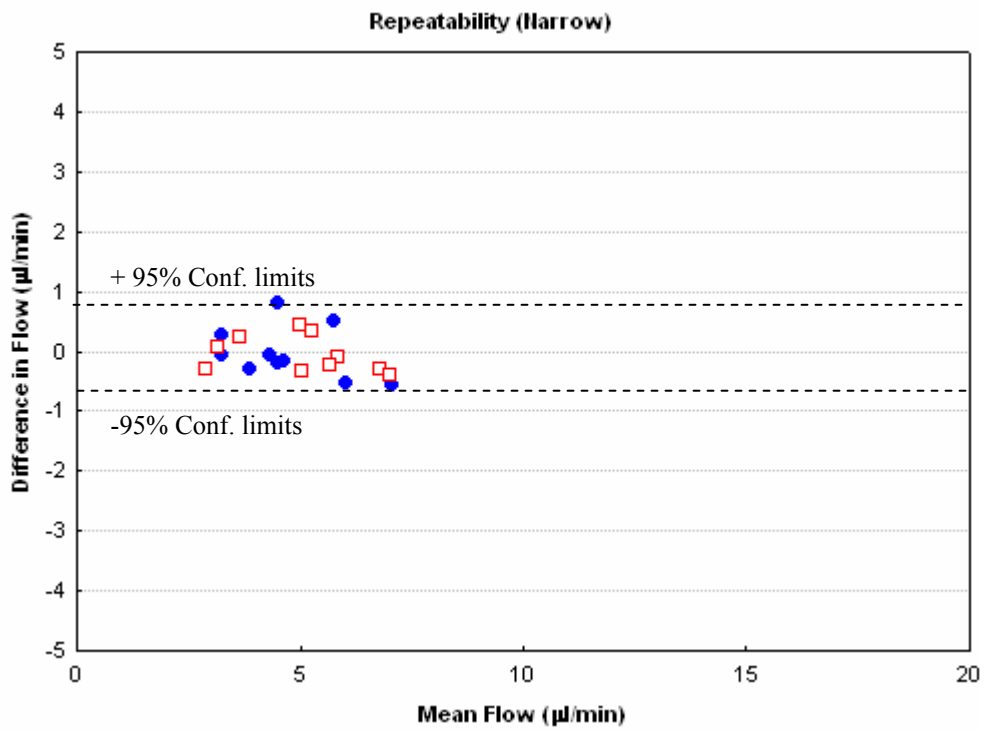


Figure 3. 22 Difference in arteriolar blood flow as a function of mean flow for the narrow arteriolar measurement site. The closed circle represents data from the hyperoxia repeat protocol and the open square represents the hypercapnia repeat protocol.

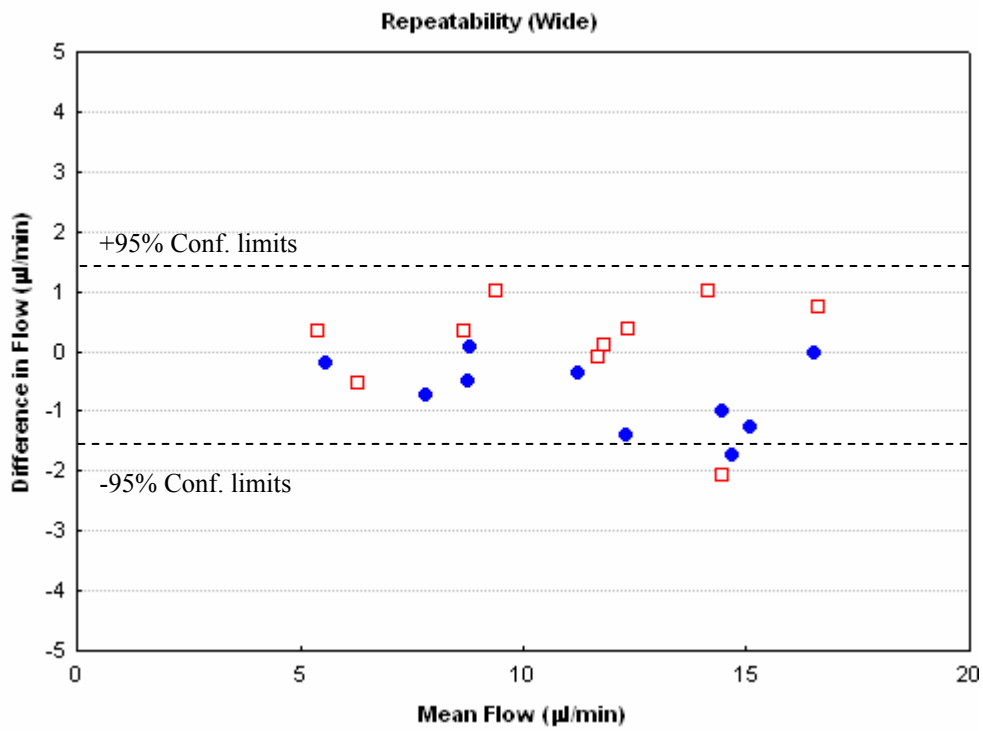


Figure 3. 23 Difference in arteriolar blood flow as a function of mean flow for the wide arteriolar measurement site. The closed circle represents data from the hyperoxia repeat protocol and the open square represents the hypercapnia repeat protocol.

3.3.5 Influence of Vessel Diameter on the Magnitude of Retinal Vascular Reactivity

Since hyperoxia resulted in a prolonged vasoconstriction, the influence of vessel diameter on retinal vascular reactivity was determined by calculating the difference in flow response to hypercapnia and *subsequently* hyperoxia for both protocols (i.e. Figure 3.2; 3b rather than 3a. Figure 3.3; 3a rather than 3b).

The magnitude of retinal vascular reactivity showed a significantly greater blood flow response for the wide measurement site ($p<0.0001$; Figure 3.24). The influence of protocol (i.e. Figure 3.2 & 3.3) was not significant ($p=0.6504$) and the interaction of protocol and measurement site was also not significant ($p=0.6209$).

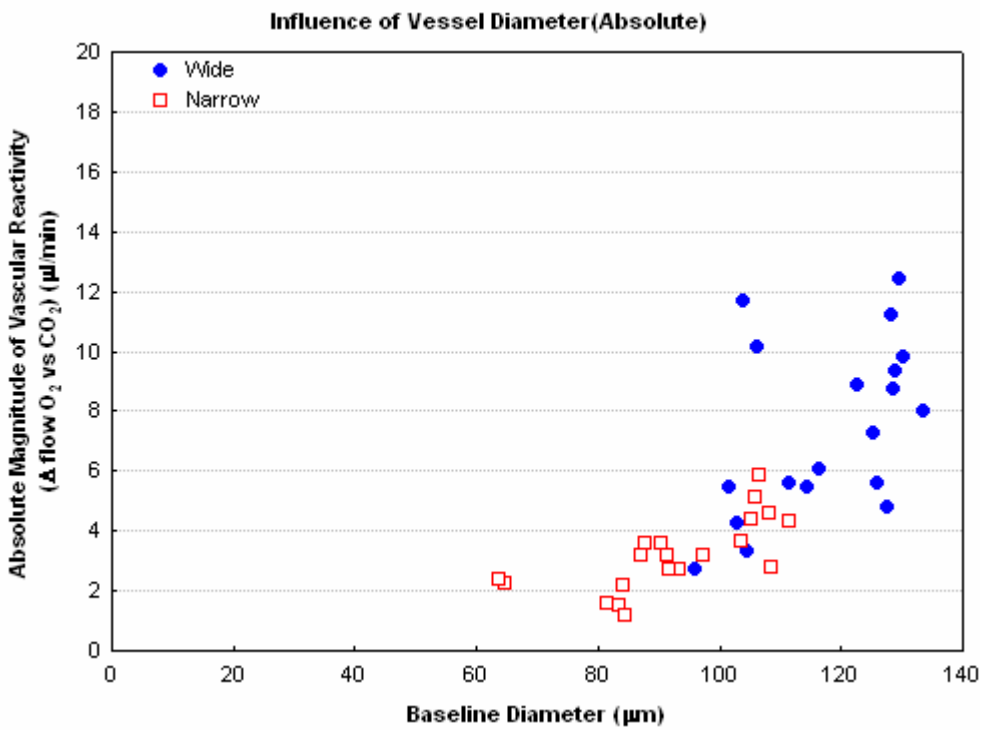


Figure 3. 24 Baseline arteriolar diameters as a function of absolute magnitude of vascular reactivity (i.e. difference in flow between CO_2 and subsequently O_2 provocation) for the narrow and wide arteriolar measurement sites.

3.4 Gas Parameter Results

Group mean FiO_2 (± SD) and $F_{ET}CO_2$ (± SD) values for each gas parameter condition across protocols 2 and 3 are shown in Tables 3.7 and 3.8, respectively.

	Baseline	**O_2**	**Recovery**	**CO_2**	**Recovery**	**O_2**	**Recovery**
$F_{ET}CO_2$ (%)	5.01 (0.37)	5.00 (0.12)	4.90 (0.20)	6.12 (0.40)	5.01 (0.13)	5.01 (0.12)	4.99 (0.15)
FiO_2 (%)	20.02 (0.16)	93.39 (1.14)	19.96 (0.23)	18.90 (0.87)	19.76 (0.32)	92.43 (2.04)	19.89 (0.32)

Table 3. 7 Fractional end-tidal CO_2 and fractional inspired O_2 as a function of gas parameter condition for the hyperoxia repeat protocol. $F_{ET}CO_2$ is the partial pressure or maximal concentration of carbon dioxide (CO_2) at the end of an exhaled breath expressed as a percentage of CO_2. FiO_2 is fractional concentration of inspired O_2 expressed as percentage of O_2.

	Baseline	**CO_2**	**Recovery**	**O_2**	**Recovery**	**CO_2**	**Recovery**
$F_{ET}CO_2$ (%)	4.89 (0.18)	6.11 (0.35)	5.08 (0.15)	5.00 (0.12)	4.92 (0.17)	6.06 (0.34)	5.12 (0.24)
FiO_2 (%)	19.79 (0.45)	18.18 (1.12)	19.58 (0.84)	93.49 (1.30)	19.79 (0.24)	18.19 (1.41)	19.75 (0.41)

Table 3. 8 Fractional end-tidal CO_2 and fractional inspired O_2 as a function of gas parameter condition for the hypercapnia repeat protocol. $F_{ET}CO_2$ is the partial pressure or maximal concentration of carbon dioxide (CO_2) at the end of an exhaled breath expressed as a percentage of CO_2. FiO_2 is fractional concentration of inspired O_2 expressed as percentage of O_2.

FiO_2 showed a group mean (i.e. mean of 3 provocations across protocols 2 and 3) increase from 19.79% to 93.10% (paired Student t-test, $p<0.0001$) during hyperoxic provocation. $F_{ET}CO_2$ showed a group mean (i.e. mean of 3 provocations across protocols 2 and 3) increase of 18.6% relative to baseline (from 37.65 mmHg to 44.67 mmHg; paired Student t-test, $p<0.0001$) during hypercapnic provocation. Importantly, $F_{ET}CO_2$ did not change relative to baseline or recovery during hyperoxic provocation.

3.5 Discussion

It has been generally accepted that smaller diameter vessels demonstrate a greater magnitude of vascular reactivity [107]. The retinal arterioles in part represent the resistance vessels of the inner retina and therefore contribute to the control of overall blood flow to the capillary bed. Some studies have found that smaller diameter retinal venules and arterioles respond to a greater degree than larger vessels [42;62], while others have found no relationship [3;61]. Unlike previous work [3;42;61;62], this study found that measurement sites of wider diameter arterioles show a greater response in terms of the absolute magnitude of change in flow. In other words, the magnitude of vascular reactivity, measured as the difference in flow between O_2 and CO_2 provocation, was greater for arteriolar measurement sites with *wider* baseline diameters. The difference between the results of this study and previous work may be explained by differences in the techniques used to assess hemodynamics and to provoke vascular reactivity.

Kiss and co-workers (2002) [42] showed an inverse relationship between the magnitude of vascular reactivity and vessel diameter. Their study was performed to investigate the impact of 100% O_2 breathing on retinal hemodynamics using blue field entoptic phenomenon and continuous measurement of retinal vessel diameter using the Zeiss retinal vessel analyzer [42]. However, the subjective nature of the blue field entoptic phenomenon used to estimate retinal hemodynamics is widely regarded to have problems pertaining to measurement validity. Nagel and co-workers (2003) [61] did not find any correlation between retinal vascular reactivity and vessel diameter in response to retinal flicker. This study was designed to determine the impact of age, blood pressure and vessel diameter on the retinal

vessels using retinal vessel analyzer [61]. Similarly, Polak and co-workers (2002) [3] noticed no difference to flicker provocation in the hemodynamic response of the peripapillary retinal arterioles. This study aimed to determine the influence of flicker frequency on flicker-induced changes of retinal vessel diameter [3]. More recently, Knudtson and co-workers (2004) [62] noticed a greater magnitude of vessel wall pulsatility as a result of the pulse cycle in smaller compared to larger retinal arterioles. This study was performed to investigate the impact of the pulse cycle on the measurement of retinal vessel diameters in small and large arterioles and venules [62]. In support of Kiss and co-workers (2002) [42], Lamport and co-workers suggested that a reduced vascular reactivity response in larger diameter vessels in the cerebral vasculature might be related to greater stiffness of the larger vessel wall [124]. In addition, a larger magnitude of vascular reactivity response in smaller diameter vessels might be explained by inherent differences in the responsiveness of the vascular smooth muscle [125].

The design of our study was unique in that two different locations along the same arteriole were sought whilst other studies have used different vessels. Also, vascular reactivity was quantified in our study by calculating the change in volumetric blood flow that occurred between standardized isocapnic hyperoxia and hypercapnia inspired gas challenges; a gas challenge was utilized that resulted in extreme vasoconstriction and vasodilation. In support of the results of our study, the number of smooth muscle cells surrounding the retinal vascular endothelium is progressively reduced from the optic disk along the course of the arterioles [126]. It is of interest to note that, in terms of percentage change in blood flow, the

response of the retinal vessels was essentially the same, irrespective of baseline diameter (Figure 3.25).

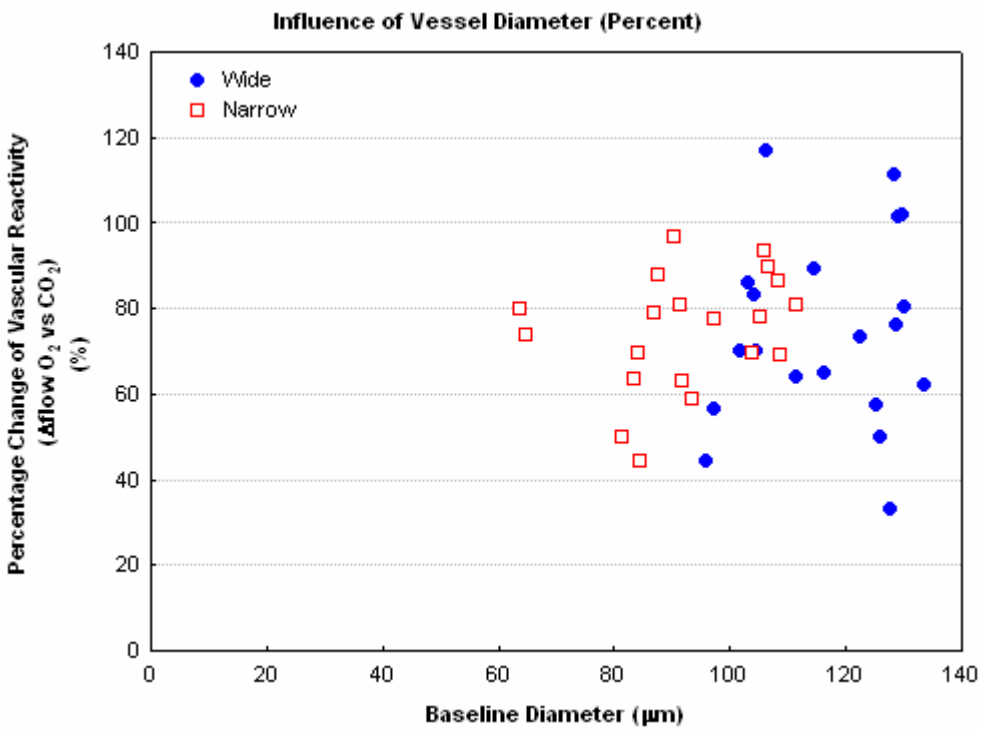


Figure 3. 25 Baseline arteriolar diameters as a function of percent magnitude of vascular reactivity (i.e. difference in flow between CO_2 and subsequently O_2 provocation) for the narrow and wide arteriolar measurement sites.

Hyperoxic provocation resulted in a persistent vasoconstriction that was still present within the period of 3 to 10 minutes after cessation of the stimulus. No such persistent order effect was apparent for the hypercapnic provocation. This surprising result was achieved by comparing the data for recovery after hyperoxia with baseline and similarly for hypercapnia; both diameter and flow values were significantly lower following hyperoxia compared to the preceding baseline, irrespective of gas provocation paradigm. Interestingly, repeat hypercapnic provocation failed to produce an increase of diameter ($p=0.1063$) and velocity

($p \leq 0.0891$) following the intermediate hyperoxic provocation and this also suggests the presence of a persistent vasoconstrictive order effect. The persistent vasoconstriction after hyperoxia may be explained by an effect of tissue oxygenation and the half-life of endothelin-1. It is known that NO has a half-life of approximately 5 seconds or less [127] but the biological half life of endothelin-1 is 1.4 to 3.6 minutes while other studies suggest a half-life of 35 minutes [128]. In addition, the persistent vasoconstriction following pronounced hyperoxia (i.e. approximately 94% inspired O_2) could even reflect oxidative damage effects at the level of the retinal vasculature [129]. Unlike diameter and flow, the velocity values after hyperoxia did not show any difference compared to the baseline and recovery values. This difference in response compared to that of diameter and flow may be explained by the higher variability of velocity measurements. A limitation of our study is that the transition from one condition to the next in terms of arterial gas concentration is not square wave; however, the slope of the arterial gas concentration transition is expected to be equivalent across conditions thereby still suggesting the presence of a persistent vasoconstrictive order effect. Future work will utilize a computer controlled gas sequencer to provide square wave changes in arterial gas concentrations.

The COR of retinal blood flow was greater for arteriolar measurement sites with wider baseline diameters than those with narrower baseline diameters; however, the ratio of group mean blood flow to group mean COR (6.55 versus 7.58 for narrow and wide diameter sites, respectively) was similar across the measurement sites. This data demonstrates that the repeatability of retinal blood flow measurements in absolute terms is lower for smaller diameter vessels but when related to the group mean flow there is little, if any, difference in

repeatability between smaller and larger diameter vessels. These findings need to take into account the range of vessel diameters that were investigated in this study i.e. group mean diameter 92.40 μm (SD 13.64) versus 116.68 μm (SD 12.68) diameter.

In summary, this study demonstrated that the magnitude of retinal vascular reactivity was greater for arteriolar measurement sites with *wider* baseline vessel diameters. The percentage change in vascular reactivity, however, was equivalent between the two measurement sites. In addition, it demonstrated that hyperoxic provocation resulted in a persistent vasoconstriction up to 10 minutes after cessation of the stimulus. Finally, the study demonstrated that the repeatability of retinal blood flow measurements in absolute terms is lower for smaller diameter vessels. However, the ratio of COR to group mean flow was equivalent between the two measurement sites. Future work should examine the effect of aging on the relationship between baseline diameter and retinal vascular reactivity and the effect of hyperbaric hyperoxia treatment on the magnitude of retinal vascular reactivity.

4 The Influence of Healthy Aging on the Relationship between Retinal Vascular Reactivity and Vessel Diameter

4.1 Introduction

There is a growing interest in the area of retinal vascular reactivity since this functional outcome measure may provide a non-invasive index of ocular, and probably systemic, vascular health. Vascular reactivity is defined as the magnitude of change of hemodynamic parameters shown to provocative stimuli, such as that initiated by the manipulation of the partial pressure of oxygen and carbon dioxide in inspired gases. Previous studies have shown that both the retinal and cerebral vessels react by constricting to oxygen and by dilating to carbon dioxide[10;11;130]. Hyperoxia (i.e. elevated partial pressure of O_2 in systemic arterial blood, PaO_2) induces a pronounced decrease in retinal blood flow in both retinal and cerebral vessels as a result of vasoconstriction [10;11;111;112;114;115;130;131]. The vasoconstriction of retinal vessels and the blood flow reduction has been demonstrated using a variety of measurement techniques[10;53-55;60;110-119]. Conversely, hypercapnia (i.e. elevated partial pressure of CO_2 in systemic arterial blood, $PaCO_2$) induces an increase in retinal blood flow as a result of vessel vasodilation[11;110;113;118-121]. In summary, oxygen promotes local vasoconstriction of the arterioles, venules, and capillaries in the retina, whereas carbon dioxide results in vasodilation [10].

Various organs in the human body are influenced by the process of aging. Although it has been suggested that retinal blood flow and autoregulation decreases in healthy people above 40 years of age [63], the age-related change of these two distinct parameters in elderly healthy subjects is still equivocal. Boehm and co-workers (2005) found that there is a correlation

between age and optic nerve head blood flow; the perfusion of the optic nerve head decreased with increasing age [64]. Furthermore, Groh and co-workers (1996) noticed a significant decrease of blood flow of the retina with increasing age and also reported a decrease in blood velocity in the central retinal artery [65]. Conversely, Harris and co-workers (2000) suggested that aging does not affect the central retinal arterial flow velocity; however, they suggested that ophthalmic arterial end-diastolic velocity is age-dependent [66]. They also reported that the flow velocities in the posterior ciliary arteries in men were independent of age; however, the end-diastolic velocity of the posterior ciliary arteries was found to decrease with age in women. Grunwald (1998) reported no significant correlation between choroidal blood velocity and the subjects age [67].

There is also limited evidence to suggest that age may have an impact on the autoregulatory response of the retinal arterioles [68]. Blum and co-workers (2000) found that age had a significant negative effect on the myogenic response, assessed using isometric exercise, of retinal arterioles using the Retinal Vessel Analyzer. In consideration of the potential important role of vascular reactivity and the ambiguity of previous results when considered as a whole, we have undertaken a pilot study to investigate the effect of aging on the relationship between baseline diameter and the magnitude of retinal vascular reactivity.

4.2 Material and Methods

4.2.1 Sample

Before commencement of the study, ethics clearance was obtained from the Office of Research Ethics at the University of Waterloo and University Health Network Research Ethics Board, Toronto. Informed consent was obtained from all participants prior to enrollment in the study. The sample comprised of 4 women and 3 men of average age 55.43 years (range 46 to 61 years; SD 5.41). The data obtained from this group of individuals was compared to the data of the 10 young, healthy subjects described in Chapter 3 i.e. five females and five males of average age 26.2 years (range 21 to 33; SD 3.49). All subjects had a logMAR visual acuity of 0.00, or better, and no personal or immediate family history of glaucoma or diabetes. Subjects with any ocular or systemic disease, cardiovascular or respiratory disorders, obesity (i.e. Body Mass Index of 30 and above), habitual smoking habits and a refractive error greater than ± 6.00 diopters sphere or ± 2.00 diopters cylinder were excluded from the study. In addition, subjects had to manifest a minimum difference of 15 μm between the narrow and wide measurement sites in order to be included in the study.

4.2.2 Visits

Subjects attended for one visit. Eligibility for entry into the study was determined by acquiring baseline information (e.g. health status) and data (e.g. weight, height, waist circumference). A total of 11 older healthy individuals were screened for the study. Eligible

subjects underwent pupil dilation and CLBF measurements at two sites along the supero-temporal arteriole. The necessary safeguards in terms of anterior segment assessment, van Herrick's anterior chamber depth assessment and intra-ocular pressure measurement were undertaken prior to pupil dilation. Intra-ocular pressure measurement was repeated at the end of the visit to ensure no post-dilation increase in intra-ocular pressure.

4.2.3 Quantitative Assessment of Retinal Arteriolar Blood Flow

Non-invasive, quantitative assessment of retinal arteriolar blood flow was achieved using the Canon Laser Blood Flowmeter, model 100 (CLBF). Details of the CLBF are provided in Section 3.2.3.

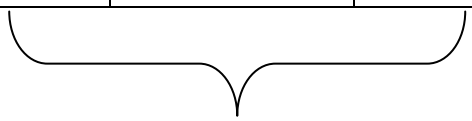
4.2.4 Gas Provocation

The face mask and sequential re-breathing circuit (Hi-Ox80, Viasys Healthcare, Yorba Linda, CA) described in Section 3.2.4 was used to manipulate inspired and expired gases.

The gas provocation protocol used in this study was designed to *minimize* the order effect of hyperoxic provocation revealed in Chapter 3. Volunteers underwent assessment in which either isocapnic hyperoxia (Figure 4.1) or hypercapnia (Figure 4.2) was undertaken first. The order of hyperoxia and hypercapnia was systematically varied between volunteers in order to closely resemble the methodology used in Chapter 3. Depending on the initial gas challenge, a 10 or 20 minute recovery period was included to permit the return of retinal

hemodynamics to baseline values. A minimum of three minutes was allowed following the initiation of hypercapnic stimulation and recovery periods prior to retinal blood flow assessment. However, a minimum of ten minutes was allowed following hyperoxic provocation prior to retinal blood flow assessment in order to reduce any persistent vasoconstrictive effects. To summarize, taking into account the half life of ET-1, we chose a recovery period of 20 minutes following hyperoxia and ensured to undertake data collection in only the final 10 minutes of the 20 minute period. The methods used to achieve hyperoxia and hypercapnia were the same as those used in Section 3.3.5.

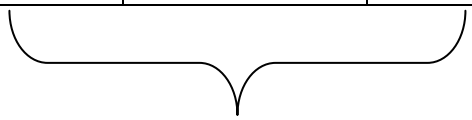
Baseline	**O_2**	**Recovery 1**	**CO_2**	**Recovery 2**
10 minutes	10 minutes	**20 minutes**	10 minutes	10 minutes



Vascular reactivity

Figure 4. 1 Hyperoxia first gas provocation protocol (O_2; isocapnic hyperoxia. CO_2; hypercapnia). A narrow and a relatively wide diameter measurement site along a superior temporal were established for each individual.

Baseline	**CO_2**	**Recovery 1**	**O_2**	**Recovery 2**
10 minutes	10 minutes	10 minutes	10 minutes	**20 minutes**



Vascular reactivity

Figure 4. 2 Hypercapnia first gas provocation protocol (O_2; isocapnic hyperoxia. CO_2; hypercapnia). A narrow and a relatively wide diameter measurement site along a superior temporal were established for each individual.

4.2.5 Procedures

The pupil of the study eye was dilated using Tropicamide 1 % (Alcon, Mississauga, Canada) to achieve an adequate view of the fundus for the retinal blood flow image acquisition. Subjects rested for 10 minutes prior to the start of each study visit to stabilize baseline cardiovascular and respiratory parameters. Two CLBF measurement sites representing different diameter values along the superior temporal arteriole were sought for each subject. Relatively straight segments, distant from vessel branches, of the superior temporal arteriole were selected. Five retinal blood flow measurements were acquired at each location for each condition and the order of measurement location was randomized between subjects with respect to distance from the optic nerve head.

Hyperoxic and hypercapnic stimuli were consecutively administered to assess the magnitude of retinal vascular reactivity but the order of stimuli was systematically varied between subjects (4 undertook the protocol described in Figure 4.1 and 3 undertook Figure 4.2 protocol). Data was not analyzed from the first 10 minutes of the 20 minute recovery period following hyperoxia to minimize any persistent vasoconstriction. The measurements during Recovery 2 (i.e. at the end of the procedure) were acquired without the mask and with the subject breathing room air to minimize fatigue.

4.2.6 CLBF Velocity Waveform Analysis

CLBF analysis software was used to analyze each acquired velocity waveform. A standardized protocol was used to remove aberrant portions due to eye movement and improper vessel tracking. For each acquisition, the maximum number of acceptable pulse cycles was included in the analysis, while a minimum of one complete systolic-diastolic cycle was essential for including a given waveform.

4.2.7 Statistical Analysis

Mean hemodynamic parameters were calculated for each condition (i.e. baseline, O_2, recovery, CO_2, recovery, etc.) of each individual (Figure 4.1 & 4.2). The magnitude of vascular reactivity (i.e. difference in flow between CO_2 and O_2 provocation) was compared between the older and younger age groups for the narrow and wide measurement sites (using two-tailed Student t-test). In order to standardize the results for the effect of differing measurement sites between individuals, a "vascular reactivity gradient index" was derived, which represented the difference in vascular reactivity between wide and narrow measurement sites as a function of the difference in baseline diameter between the two sites.

4.3 Results

4.3.1 Diameter

The baseline diameter for the older age group for the narrow arteriolar measurement site was 98.87 μm (SD=14.34) and for the wide measurement site was 114.39 μm (SD=10.18) (paired Student t-test, p=0.0033).

Comparing to equivalent results of Chapter 3, the younger age group exhibited a baseline diameter of 92.40 μm (SD 13.64) and 116.68 μm (SD 12.68) for the narrow and wide measurement sites, respectively (re-ANOVA $p<0.0001$).

4.3.2 The Effect of Age on the Relationship between Retinal Vascular Reactivity and Vessel Diameter

The relationship between baseline diameter and the magnitude of retinal vascular reactivity for narrow and wide measurement sites as a function of group is shown in Figure 4.3. There was no difference between the young and the older age groups in retinal vascular reactivity for both the narrow (two-tailed Student t-test, p=0.8692) and wide (two-tailed Student t-test, p=0.2795) measurement sites.

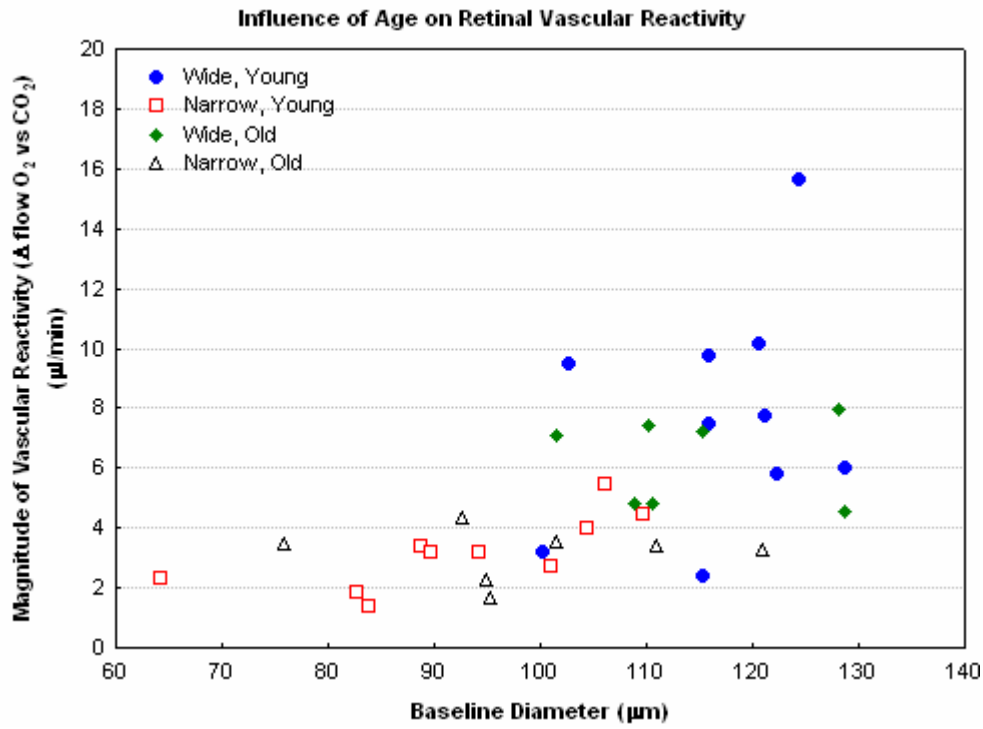


Figure 4. 3 Absolute magnitude of vascular reactivity (i.e. difference in flow between CO_2 and O_2 provocation) as a function of baseline arteriolar diameters for the narrow and wide arteriolar measurement sites for the young and older age groups.

4.3.3 The Effect of Age on Retinal Vascular Reactivity Gradient Index

The relationship between age and retinal vascular reactivity gradient index is shown in Figure 4.4. There was no difference between the young and the older age groups in retinal vascular reactivity gradient index (two-tailed Student t-test, p=0.3561).

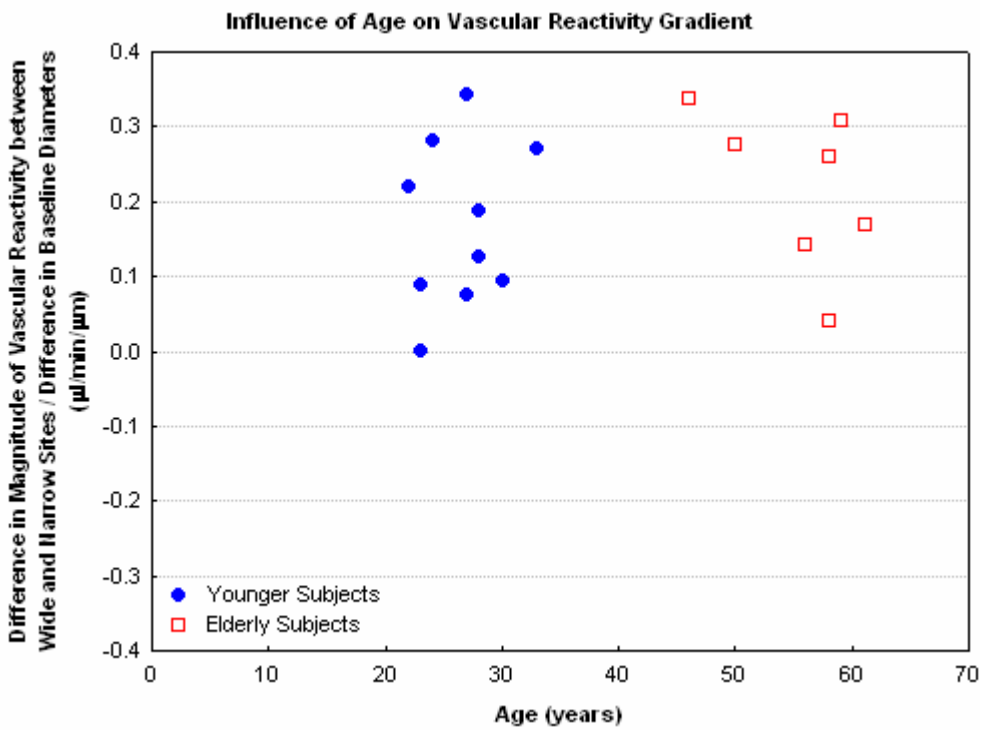


Figure 4. 4 The difference in the magnitude of retinal vascular reactivity gradient index as a function of age.

4.4 Gas Parameters Results

Group mean FiO_2 (± SD) and $F_{ET}CO_2$ (± SD) values for each gas parameter condition are shown in Tables 4.1 and 4.2.

	Baseline	O_2	Recovery	CO_2
$F_{ET}CO_2$ (%)	5.14 (0.46)	5.11 (0.23)	5.16 (0.45)	5.81 (0.40)
FiO_2 (%)	19.97 (0.32)	94.10 (1.46)	20.01 (0.18)	17.84 (1.54)

Table 4. 1 Fractional end-tidal CO_2 and fractional inspired O_2 as a function of gas parameter condition for the hyperoxia first protocol for the older age group (n=4). $F_{ET}CO_2$ is the partial pressure or maximal concentration of carbon dioxide (CO_2) at the end of an exhaled breath expressed as a percentage of CO_2. FiO_2 is the fractional concentration of inspired O_2 expressed as percentage of O_2.

	Baseline	CO_2	Recovery	O_2
$F_{ET}CO_2$ (%)	5.16 (0.01)	5.82 (0.16)	5.10 (0.05)	5.03 (0.01)
FiO_2 (%)	20.07 (0.39)	18.58 (0.28)	20.13 (0.28)	93.47 (0.74)

Table 4. 2 Fractional end-tidal CO_2 and fractional inspired O_2 as a function of gas parameter condition for the hyperoxia first protocol for the older age group (n=3). $F_{ET}CO_2$ is the partial pressure or maximal concentration of carbon dioxide (CO_2) at the end of an exhaled breath expressed as a percentage of CO_2. FiO_2 is the fractional concentration of inspired O_2 expressed as percentage of O_2.

For the older age group, FiO_2 showed a group mean (i.e. mean of 2 provocation across two protocols) increase from 20.04% to 93.83% (paired Student t-test, $p<0.0001$) during hyperoxic provocation. $F_{ET}CO_2$ showed a group mean (i.e. mean of 2 provocations across two protocols) increase of 12.6% relative to baseline (from 39.22 mmHg to 44.16 mmHg; paired Student t-test, $p<0.0001$) during hypercapnic provocation. Importantly, $F_{ET}CO_2$ did not change relative to baseline during hyperoxic provocation.

4.5 Discussion

The results of this pilot study suggest that there is no effect of age on the magnitude of retinal vascular reactivity (Figure 4.3) and on the relationship between the magnitude of retinal vascular reactivity and vessel diameter (Figure 4.4). We might not have observed any influence of age on the relationship between retinal vascular reactivity and vessel diameter due to study limitations. These limitations could include the small sample size, the relatively small difference in age between the two groups (i.e. 55.4yrs for the older group and 26.2yrs for the young group), subject fatigue (i.e. which limited the protocol design for the older age group) and, finally, the gas provocations may overwhelm any vascular reactivity deficit associated with healthy aging. Similar provocations have been shown to be sensitive to effects of diabetes mellitus [132] on retinal vascular reactivity. Future work will investigate the effect of healthy aging on stepped increments of gas stimuli on retinal hemodynamics.

It has been generally accepted that age-induced adaptations of the vasculature contribute to a reduction in muscle blood flow in man [133;134]. Jeppesen suggested that there is an age-related decrease in diameter response of retinal arterioles when the blood pressure is changed [63]. Studies performed by Boehm and co-workers [64], Grunwald and co-workers [135] and Groh and co-workers [65] suggested a negative correlation between retinal blood flow and age, while those studies performed by Harris and co-workers [66] and Grunwald and co-workers [67] essentially suggested no relationship between retinal blood flow and age. The impact of age on retinal vascular reactivity is still ambiguous.

Numerous techniques have been used by other groups to assess retinal blood flow. Jeppesen and co-workers (2004) investigated the age-dependent change in the myogenic response of retinal arterioles using the Retinal Vessel Analyzer (RVA) to evaluate change in vessel diameter [63]. Groh and co-workers (1996) used pulsed Doppler sonography to quantify the influence of age on retinal and optic nerve head blood circulation [65]. Boehm and co-workers (2005) studied the effect of age on optic nerve head blood flow using Laser Doppler Flowmetry [64]. Harris and co-workers (2000) used color Doppler imaging to perform a study on the effects of aging on the retrobulbar circulation [66]. Grunwald and co-workers (1993) examined the effect of age on retinal macular microcirculation using the blue field stimulation technique [135].

In this study, the effect of age on retinal vascular reactivity and on the relationship between the magnitude of retinal vascular reactivity and vessel diameter was investigated. Unlike previous work [63-65;135], this study found that there was no impact of aging on retinal hemodynamics and vascular reactivity. The difference between the results of this study and previous work may be explained by the differences in the technique used to assess hemodynamics and to provoke vascular reactivity. The design of our study was unique in that vascular reactivity was quantified by calculating the change in volumetric blood flow that occurred between standardized isocapnic hyperoxia and hypercapnia inspired gas challenges.

In support of our study, Muller-Delp (2006) [136] has emphasized that studies which have investigated change in vascular function with age were mostly conducted on large conduit arteries rather than on resistance arteries and arterioles [137-143]. An observed effect of aging in large conduit arteries cannot be extended to resistance arteries and arterioles. Several studies have demonstrated vasoconstrictor responses in the foot to be reduced [137;144] , unchanged [137;145] or enhanced [146] with advancing age. These studies show that age-related adaptations of vasoconstrictor responsiveness are heterogenous, are different between conduit arteries and resistance arterioles [138;147-151], are different between vascular beds [136;137;147;149;152;153] and are different between muscles of different fiber type [147].

In summary, this study demonstrated that healthy aging does not have any effect on the relationship between the magnitude of retinal vascular reactivity and vessel diameter.

5 Discussion

It has been generally accepted that smaller diameter vessels demonstrate a greater magnitude of vascular reactivity [107]. Some studies have shown that smaller diameter vessels respond to greater degree than larger vessels [42;62], while others have shown no relationship [3;61]. Unlike previous work [3;42;61;62], this study found that measurement sites of wider diameter arterioles show a greater response in terms of the absolute magnitude of change in flow. In other words, the magnitude of vascular reactivity, measured as the difference in flow between O_2 and CO_2 provocation, was greater for arteriolar measurement sites with *wider* baseline diameters; however, when vascular reactivity is expressed in percentage change, there was no influence of vessel diameter on reactivity. The difference between the results of this study and previous work may be explained by differences in the techniques used to assess hemodynamics and to provoke vascular reactivity. Also, this study was the first to systematically investigate the influence of retinal vessel diameter on the magnitude of retinal vascular reactivity.

The design of our study was unique in that two different locations along the same arteriole were sought whilst other studies have used different vessels. Also, vascular reactivity was quantified in our study by calculating the change in volumetric blood flow that occurred between standardized isocapnic hyperoxia and hypercapnia inspired gas challenges; a gas challenge was utilized that resulted in extreme vasoconstriction and vasodilation. In support of the results of our study, the number of smooth muscle cells surrounding the retinal vascular endothelium is progressively reduced from the optic disk along the course of the

arterioles [126]. It is of interest to note that, in terms of percentage change in blood flow, the response of the retinal vessels was essentially the same, irrespective of baseline diameter.

Hyperoxic provocation resulted in a persistent vasoconstriction that was still present within the period of 3 to 10 minutes after cessation of the stimulus. No such persistent order effect was apparent for the hypercapnic provocation. This surprising result was achieved by comparing the data for recovery after hyperoxia with baseline and similarly for hypercapnia; both diameter and flow values were significantly lower following hyperoxia compared to the preceding baseline, irrespective of gas provocation paradigm. The persistent vasoconstriction after hyperoxia may be explained by an effect of tissue oxygenation and the half-life of endothelin-1. It is known that NO has a half-life of approximately 5 seconds or less [127] but the biological half life of endothelin-1 is 1.4 to 3.6 minutes, while other studies suggest a half-life of 35 minutes [128]. In addition, the persistent vasoconstriction following pronounced hyperoxia (i.e. approximately 94% inspired O_2) could even reflect oxidative damage effects at the level of the retinal vasculature [129]. A limitation of our study is that the transition from one condition to the next in terms of arterial gas concentration is not square wave; however, the slope of the arterial gas concentration transition is expected to be equivalent across conditions (i.e. hyperoxia and hypercapnia) thereby still suggesting the presence of a persistent vasoconstrictive order effect.

The COR of retinal blood flow was greater for arteriolar measurement sites with wider baseline diameters than those with narrower baseline diameters; however, the ratio of group mean COR to group mean blood flow (6.55 versus 7.58 for narrow and wide diameter sites,

respectively) was similar across the measurement sites. These data demonstrate that the repeatability of retinal blood flow measurements in absolute terms is lower for smaller diameter vessels but when related to the group mean flow there is little, if any, difference in repeatability between smaller and larger diameter vessels. These findings need to take into account the range of vessel diameters that were investigated in this study i.e. group mean diameter 92.40 μm (SD 13.64) versus 116.68 μm (SD 12.68) diameter.

Finally, the pilot study data (i.e. Chapter 4) suggested that there is no effect of aging on the relationship between the magnitude of retinal vascular reactivity and vessel diameter. We might not have observed any influence of age on the relationship between retinal vascular reactivity and vessel diameter due to some limitations. These limitations could include the small sample size, the relatively small difference in ages between two groups, subject fatigue which limited the protocol design for the older age group and, finally, the gas provocations utilized may overwhelm any vascular reactivity deficit associated with healthy aging. The difference between the results of this study and previous work may also be explained by the differences in the technique used to assess hemodynamic and to provoke vascular reactivity. Similar provocations have been shown to be sensitive to effects of diabetes mellitus [132] on retinal vascular reactivity. Future work will investigate the effect of healthy aging on stepped increments of gas stimuli on retinal hemodynamics.

In summary, this work demonstrated that the magnitude of retinal vascular reactivity was greater for arteriolar measurement sites with wider baseline diameter. In addition, it demonstrated that the percent magnitude of vascular reactivity was not influenced by

baseline arteriolar measurement site diameter, although the variability of response was greater for the wider measurement sites. It was also demonstrated that hyperoxic provocation resulted in a persistent vasoconstriction up to 10 minutes after cessation of the stimulus. Furthermore, the study demonstrated that the repeatability of retinal blood flow measurements in absolute terms is lower for smaller diameter vessels. Finally, the pilot study demonstrated that there is no effect of healthy aging on the relationship between the magnitude of retinal vascular reactivity and vessel diameter.

6 References

1. Dorner GT, Garhofer G, Kiss B, Polska E, Polak K, Riva CE et al. Nitric oxide regulates retinal vascular tone in humans. *Am.J.Physiol Heart Circ.Physiol* 2003;**285**:H631-H636.

2. Dorner GT, Garhofer G, Huemer KH, Riva CE, Wolzt M, Schmetterer L. Hyperglycemia affects flicker-induced vasodilation in the retina of healthy subjects. *Vision Res.* 2003;**43**:1495-500.

3. Polak K, Schmetterer L, Riva CE. Influence of flicker frequency on flicker-induced changes of retinal vessel diameter. *Invest Ophthalmol.Vis.Sci.* 2002;**43**:2721-6.

4. Michelson G, Patzelt A, Harazny J. Flickering light increases retinal blood flow. *Retina* 2002;**22**:336-43.

5. Buerk DG, Riva CE, Cranstoun SD. Nitric oxide has a vasodilatory role in cat optic nerve head during flicker stimuli. *Microvasc.Res.* 1996;**52**:13-26.

6. Falsini B, Riva CE, Logean E. Flicker-evoked changes in human optic nerve blood flow: relationship with retinal neural activity. *Invest Ophthalmol.Vis.Sci.* 2002;**43**:2309-16.

7. Riva CE, Falsini B, Logean E. Flicker-evoked responses of human optic nerve head blood flow: luminance versus chromatic modulation. *Invest Ophthalmol.Vis.Sci.* 2001;**42**:756-62.

8. Formaz F, Riva CE, Geiser M. Diffuse luminance flicker increases retinal vessel diameter in humans. *Curr.Eye Res.* 1997;**16**:1252-7.

9. Johnson PC. Autoregulation of blood flow. *Circ.Res.* 1986;**59**:483-95.

10. Dorner GT, Garhofer G, Zawinka C, Kiss B, Schmetterer L. Response of retinal blood flow to CO2-breathing in humans. *Eur.J.Ophthalmol.* 2002;**12**:459-66.

11. Kety SS, Schmidt CF. The effects of altered arterial tensions of carbon dioxide and oxygen on cerebral blood flow and cerebral oxygen consumption of normal young men. *J Clin.Invest* 1948;**27**:484-92.

12. Kaufman PL, Alm A. *Adler's Physiology of the Eye*. 10th Edition ed. St. Louis: Mosby, 2003.

13. Haefliger IO, Flammer J, Beny JL, Luscher TF. Endothelium-dependent vasoactive modulation in the ophthalmic circulation. *Prog.Retin.Eye Res.* 2001;**20**:209-25.

14. Levick J.R. *An Introduction to Cardiovascular Physiology*. 4th Edition ed. London: Amember of the Hodder Headline Group, 2003.

15. Remington LA. *Clinical Anatomy of the Visual System*. 2nd Edition ed. St. Louis, Missouri: Elsevier, 2005.

16. Harris A, Jonescu-Cuypers CP, Kagemann L, Ciulla TA, Krieglstein G. *Atlas of Ocular Blood Flow: Vasculr Anatomy, Pathophysiology, and Metabolism. Philadelphia; Elsevier*. 2003.

17. Saint-Geniez M, D'Amore PA. Development and pathology of the hyaloid, choroidal and retinal vasculature. *Int.J.Dev.Biol.* 2004;**48**:1045-58.

18. Levick JR. *Cardivascular Physiology*. London: Arnold, 2000: 15-28.

19. Guyton A.C., Hall J. *Textbook of Medical Physiology*. 10th edition ed. Philadelphia, Penncylvania: W.B. Saunders Company, 2000: 144-50.

20. Feke GT, Tagawa H, Deupree DM, Goger DG, Sebag J, Weiter JJ. Blood flow in the normal human retina. *Invest Ophthalmol.Vis.Sci.* 1989;**30**:58-65.

21. Riva CE, Grunwald JE, Sinclair SH, Petrig BL. Blood velocity and volumetric flow rate in human retinal vessels. *Invest Ophthalmol.Vis.Sci.* 1985;**26**:1124-32.

22. Pournaras Constantin J. In: Kaiser H.J. FJHPh, ed. *Ocular Blood Flow New Insights into the Pathogenesis of Ocular Diseases*. Basel, Switzerland: S. Karger AG, 1996: 40-50.

23. Harris A, Kagemann L, Cioffi GA. Assessment of human ocular hemodynamics. *Surv.Ophthalmol.* 1998;**42**:509-33.

24. Dosne AM, Dupuy E, Bodevin E. Production of a fibrinolytic inhibitor by cultured endothelial cells derived from human umbilical vein. *Thromb.Res.* 1978;**12**:377-87.

25. Orgul S, Gugleta K, Flammer J. Physiology of perfusion as it relates to the optic nerve head. *Surv.Ophthalmol.* 1999;**43 Suppl 1**:S17-S26.

26. Johnson PC. Review of previous studies and current theories of autoregulation. *Circ.Res.* 1964;**15**:SUPPL-9.

27. Harris A, Ciulla TA, Chung HS, Martin B. Regulation of retinal and optic nerve blood flow. *Arch.Ophthalmol.* 1998;**116**:1491-5.

28. Busse R, Luckhoff A, Bassenge E. Endothelium-derived relaxant factor inhibits platelet activation. *Naunyn Schmiedebergs Arch.Pharmacol.* 1987;**336**:566-71.

29. Ignarro L.J., Byrns R.E, Wood K.S. Pharmacological and biochemical properties of endothelium-derived relaxing factor (EDRF): evidence that it is closely related to nitric oxide (NO) radical. *Circulation 74 Suppl II,* 1986;p. S287.

30. Moncada S, Palmer RM, Gryglewski RJ. Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc.Natl.Acad.Sci.U.S.A* 1986;**83**:9164-8.

31. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;**327**:524-6.

32. Radomski MW, Palmer RM, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br.J.Pharmacol.* 1987;**92**:639-46.

33. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* 1987;**2**:1057-8.

34. Baynes J and Dominiczak. In: *Medical Biochemistry*. London: Mosby, 1999: 469-85.

35. Schmetterer L, Polak K. Role of nitric oxide in the control of ocular blood flow. *Prog.Retin.Eye Res.* 2001;**20**:823-47.

36. Venkataraman ST, Hudson C, Fisher JA, Flanagan JG. The impact of hypercapnia on retinal capillary blood flow assessed by scanning laser Doppler flowmetry. *Microvasc.Res.* 2005;**69**:149-55.

37. Schiffrin EL, Touyz RM. Vascular biology of endothelin. *J Cardiovasc.Pharmacol.* 1998;**32 Suppl 3**:S2-13.

38. Mamluk R, Levy N, Rueda B, Davis JS, Meidan R. Characterization and regulation of type A endothelin receptor gene expression in bovine luteal cell types. *Endocrinology* 1999;**140**:2110-6.

39. Novotny H.R., Alvis D.L. A method of photographing fluorescence in circulating blood in the human retina. *Circulation* 1961;**24**:82-6.

40. Preussner PR, Richard G, Darrelmann O, Weber J, Kreissig I. Quantitative measurement of retinal blood flow in human beings by application of digital image-processing methods to television fluorescein angiograms. *Graefes Arch.Clin.Exp.Ophthalmol.* 1983;**221**:110-2.

41. Wolf S, Jung F, Kiesewetter H, Korber N, Reim M. Video fluorescein angiography: method and clinical application. *Graefes Arch.Clin.Exp.Ophthalmol.* 1989;**227**:145-51.

42. Kiss B, Polska E, Dorner G, Polak K, Findl O, Mayrl GF et al. Retinal blood flow during hyperoxia in humans revisited: concerted results using different measurement techniques. *Microvasc.Res.* 2002;**64**:75-85.

43. Dallinger S, Dorner GT, Wenzel R, Graselli U, Findl O, Eichler HG et al. Endothelin-1 contributes to hyperoxia-induced vasoconstriction in the human retina. *Invest Ophthalmol.Vis.Sci.* 2000;**41**:864-9.

44. De Mey JG, Vanhoutte PM. Anoxia and endothelium-dependent reactivity of the canine femoral artery. *J.Physiol* 1983;**335**:65-74.

45. Kim P, Sundt TM, Jr., Vanhoutte PM. Alterations in endothelium-dependent responsiveness of the canine basilar artery subarachnoid hemorrhage. *J.Neurosurg.* 1988;**69**:239-46.

46. Lüscher TF, Vanhoutte PM. *The endothelium: modulator of cardiovascular function*. *CRC Press, Boca Raton, FL* 1990;1-228.

47. Vanhoutte PM, Rubanyi GM, Miller VM, Houston DS. Modulation of vascular smooth muscle contraction by the endothelium. *Annu.Rev.Physiol* 1986;**48**:307-20.

48. D'Amore P, Black CM, Garcia JGN, Haudenschild C, Hechtman HB, Jain RK et al. *Microvascular Research*. 2006.

49. Harder DR. Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circ.Res.* 1987;**60**:102-7.

50. Harder DR, Kauser K, Roman RJ, Lombard JH. Mechanisms of pressure-induced myogenic activation of cerebral and renal arteries: role of the endothelium. *J.Hypertens.Suppl* 1989;**7**:S11-S15.

51. Davies PF, Tripathi SC. Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ.Res.* 1993;**72**:239-45.

52. Levick JR. *Cardiac cycle. Cardiovascular physiology. London; Arnold*. 2000: 107.

53. Strenn K, Menapace R, Rainer G, Findl O, Wolzt M, Schmetterer L. Reproducibility and sensitivity of scanning laser Doppler flowmetry during graded changes in PO2. *Br.J.Ophthalmol.* 1997;**81**:360-4.

54. Riva CE, Grunwald JE, Sinclair SH. Laser Doppler Velocimetry study of the effect of pure oxygen breathing on retinal blood flow. *Invest Ophthalmol.Vis.Sci.* 1983;**24**:47-51.

55. Fallon TJ, Maxwell D, Kohner EM. Retinal vascular autoregulation in conditions of hyperoxia and hypoxia using the blue field entoptic phenomenon. *Ophthalmology* 1985;**92**:701-5.

56. Eperon G, Johnson M, David NJ. The effect of arterial PO2 on relative retinal blood flow in monkeys. *Invest Ophthalmol.* 1975;**14**:342-52.

57. Riva CE, Pournaras CJ, Tsacopoulos M. Regulation of local oxygen tension and blood flow in the inner retina during hyperoxia. *J.Appl.Physiol* 1986;**61**:592-8.

58. Takagi C, King GL, Takagi H, Lin YW, Clermont AC, Bursell SE. Endothelin-1 action via endothelin receptors is a primary mechanism modulating retinal circulatory response to hyperoxia. *Invest Ophthalmol.Vis.Sci.* 1996;**37**:2099-109.

59. Schmetterer L, Wolzt M, Lexer F, Alschinger C, Gouya G, Zanaschka G et al. The effect of hyperoxia and hypercapnia on fundus pulsations in the macular and optic disc region in healthy young men. *Exp.Eye Res.* 1995;**61**:685-90.

60. Langhans M, Michelson G, Groh MJ. Effect of breathing 100% oxygen on retinal and optic nerve head capillary blood flow in smokers and non-smokers. *Br.J.Ophthalmol.* 1997;**81**:365-9.

61. Nagel E, Vilser W, Lanzl I. Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. *Invest Ophthalmol.Vis.Sci.* 2004;**45**:1486-92.

62. Knudtson MD, Klein BE, Klein R, Wong TY, Hubbard LD, Lee KE et al. Variation associated with measurement of retinal vessel diameters at different points in the pulse cycle. *Br.J.Ophthalmol.* 2004;**88**:57-61.

63. Jeppesen P, Gregersen PA, Bek T. The age-dependent decrease in the myogenic response of retinal arterioles as studied with the Retinal Vessel Analyzer. *Graefes Arch.Clin.Exp.Ophthalmol.* 2004;**242**:914-9.

64. Boehm AG, Koeller AU, Pillunat LE. The effect of age on optic nerve head blood flow. *Invest Ophthalmol.Vis.Sci.* 2005;**46**:1291-5.

65. Groh MJ, Michelson G, Langhans MJ, Harazny J. Influence of age on retinal and optic nerve head blood circulation. *Ophthalmology* 1996;**103**:529-34.

66. Harris A, Harris M, Biller J, Garzozi H, Zarfty D, Ciulla TA et al. Aging affects the retrobulbar circulation differently in women and men. *Arch.Ophthalmol.* 2000;**118**:1076-80.

67. Grunwald JE, Hariprasad SM, DuPont J. Effect of aging on foveolar choroidal circulation. *Arch.Ophthalmol.* 1998;**116**:150-4.

68. Blum M, Bachmann K, Strobel J. [Age-correlation of blood pressure induced myogenic autoregulation of human retinal arterioles in 40 volunteers]. *Klin.Monatsbl.Augenheilkd.* 2000;**217**:225-30.

69. Bursell SE, Clermont AC, Shiba T, King GL. Evaluating retinal circulation using video fluorescein angiography in control and diabetic rats. *Curr.Eye Res.* 1992;**11**:287-95.

70. Wolf S, Arend O, Toonen H, Bertram B, Jung F, Reim M. Retinal capillary blood flow measurement with a scanning laser ophthalmoscope. Preliminary results. *Ophthalmology* 1991;**98**:996-1000.

71. Miyamoto K, Ogura Y, Nishiwaki H, Matsuda N, Honda Y, Kato S et al. Evaluation of retinal microcirculatory alterations in the Goto-Kakizaki rat. A spontaneous model of non-insulin-dependent diabetes. *Invest Ophthalmol.Vis.Sci.* 1996;**37**:898-905.

72. Lee ET, Rehkopf PG, Warnicki JW, Friberg T, Finegold DN, Cape EG. A new method for assessment of changes in retinal blood flow. *Med.Eng Phys.* 1997;**19**:125-30.

73. Schmetterer L, Wolzt M. Ocular blood flow and associated functional deviations in diabetic retinopathy. *Diabetologia* 1999;**42**:387-405.

74. Nakahashi K, Asai T, Okubo K, Yamamoto M, Masumi K. [Analysis of the retinal blood flow by means of the blue field entoptic phenomenon and simulation of entoptic leucocytes with a personal-computer system]. *Nippon Ganka Gakkai Zasshi* 1989;**93**:533-9.

75. Kolodjaschna J, Berisha F, Lung S, Schaller G, Polska E, Jilma B et al. LPS-induced microvascular leukocytosis can be assessed by blue-field entoptic phenomenon. *Am.J.Physiol Heart Circ.Physiol* 2004;**287**:H691-H694.

76. Harris A, Kagemann L, Cioffi GA. Assessment of human ocular hemodynamics. *Surv.Ophthalmol.* 1998;**42**:509-33.

77. Harris A, Chung HS, Ciulla TA, Kagemann L. Progress in measurement of ocular blood flow and relevance to our understanding of glaucoma and age-related macular degeneration. *Prog.Retin.Eye Res.* 1999;**18**:669-87.

78. Tamaki Y, Araie M, Kawamoto E, Eguchi S, Fujii H. Noncontact, two-dimensional measurement of retinal microcirculation using laser speckle phenomenon. *Invest Ophthalmol.Vis.Sci.* 1994;**35**:3825-34.

79. Tamaki Y, Araie M, Tomita K, Nagahara M, Tomidokoro A, Fujii H. Real-time measurement of human optic nerve head and choroid circulation, using the laser speckle phenomenon. *Jpn.J Ophthalmol.* 1997;**41**:49-54.

80. Briers JD, Fercher AF. Retinal blood-flow visualization by means of laser speckle photography. *Invest Ophthalmol.Vis.Sci.* 1982;**22**:255-9.

81. Harris A, et.al. *Atlas of Ocular Blood Flow*. Philadelphia, Pennsylvania: Butterworth Heinemann, 2003.

82. Schmetterer L, Dallinger S, Findl O, Strenn K, Graselli U, Eichler HG et al. Noninvasive investigations of the normal ocular circulation in humans. *Invest Ophthalmol.Vis.Sci.* 1998;**39**:1210-20.

83. Boehm AG, Pillunat LE, Koeller U, Katz B, Schicketanz C, Klemm M et al. Regional distribution of optic nerve head blood flow. *Graefes Arch.Clin.Exp.Ophthalmol.* 1999;**237**:484-8.

84. Bebie H. In: Kaiser H.J. FJHPh, ed. *Ocular Blood Flow*. Basel: S. Karger AG, 1996: 93-9.

85. Riva CE, Grunwald JE, Sinclair SH, Okeefe K. Fundus Camera Based Retinal Ldv. *Applied Optics* 1981;**20**:117-20.

86. Riva CE, Feke GT, Eberli B, Benary V. Bidirectional Ldv System for Absolute Measurement of Blood Speed in Retinal-Vessels. *Applied Optics* 1979;**18**:2301-6.

87. Feke GT, Riva CE. Laser Doppler measurements of blood velocity in human retinal vessels. *J Opt Soc.Am.* 1978;**68**:526-31.

88. Riva CE, Feke GT, Ben Sira I. Fluorescein dye-dilution technique and retinal circulation. *Am.J Physiol* 1978;**234**:H315-H322.

89. Harris A, Kagemann L, Cioffi GA. Assessment of human ocular hemodynamics. *Surv.Ophthalmol.* 1998;**42**:509-33.

90. Riva CE, Grunwald JE, Sinclair SH, Petrig BL. Blood velocity and volumetric flow rate in human retinal vessels. *Invest Ophthalmol.Vis.Sci.* 1985;**26**:1124-32.

91. Gilmore ED, Hudson C, Preiss D, Fisher J. Retinal arteriolar diameter, blood velocity and blood flow response to an isocapnic hyperoxic provocation. *Am.J.Physiol Heart Circ.Physiol* 2005.

92. Guan K, Hudson C, Flanagan JG. Variability and repeatability of retinal blood flow measurements using the Canon Laser Blood Flowmeter. *Microvasc.Res.* 2003;**65**:145-51.

93. Riva CE, Logean E, Falsini B. Visually evoked hemodynamical response and assessment of neurovascular coupling in the optic nerve and retina. *Prog.Retin.Eye Res.* 2005;**24**:183-215.

94. Michelson G, Schmauss B. Two dimensional mapping of the perfusion of the retina and optic nerve head. *Br.J.Ophthalmol.* 1995;**79**:1126-32.

95. Feke GT, Yoshida A, Schicketanz C. Laser based instruments for ocular blood flow assessment. *J Biomed Opt 3* 1998;415-22.

96. Delori FC, Fitch KA, Feke GT, Deupree DM, Weiter JJ. Evaluation of micrometric and microdensitometric methods for measuring the width of retinal vessel images on fundus photographs. *Graefes Arch.Clin.Exp.Ophthalmol.* 1988;**226**:393-9.

97. Milbocker MT, Feke GT, Goger DG. Laser Doppler velocimetry stabilized in one dimension. *IEEE Trans.Biomed.Eng* 1991;**38**:928-30.

98. Yoshida A, Feke GT, Mori F, Nagaoka T, Fujio N, Ogasawara H et al. Reproducibility and clinical application of a newly developed stabilized retinal laser Doppler instrument. *Am.J Ophthalmol.* 2003;**135**:356-61.

99. Riva CE et al. *Laser Doppler velocimetry and blue field stimulation technique. In: Masters BR, ed. Noninvasive Diagnostic Techniques in Ophthalmology. New york; Springer*. 1990: 390-409.

100. Dumskyj MJ, Aldington SJ, Dore CJ, Kohner EM. The accurate assessment of changes in retinal vessel diameter using multiple frame electrocardiograph synchronised fundus photography. *Curr.Eye Res.* 1996;**15**:625-32.

101. Michelson G, Schmauss B, Langhans MJ, Harazny J, Groh MJ. Principle, validity, and reliability of scanning laser Doppler flowmetry. *J.Glaucoma.* 1996;**5**:99-105.

102. Butt Z, McKillop G, O'Brien C, Allan P, Aspinall P. Measurement of ocular blood flow velocity using colour Doppler imaging in low tension glaucoma. *Eye* 1995;**9 (Pt 1)**:29-33.

103. Gilmore ED, Hudson C, Venkataraman ST, Preiss D, Fisher J. Comparison of different hyperoxic paradigms to induce vasoconstriction: implications for the investigation of retinal vascular reactivity. *Invest Ophthalmol.Vis.Sci.* 2004;**45**:3207-12.

104. Sommer LZ, Iscoe S, Robicsek A, Kruger J, Silverman J, Rucker J et al. A simple breathing circuit minimizing changes in alveolar ventilation during hyperpnoea. *Eur.Respir.J* 1998;**12**:698-701.

105. Gilmore ED, Hudson C, Preiss D, Fisher J. Retinal arteriolar diameter, blood velocity, and blood flow response to an isocapnic hyperoxic provocation. *Am.J Physiol Heart Circ.Physiol* 2005;**288**:H2912-H2917.

106. Venkataraman ST, Hudson C, Fisher JA, Flanagan JG. Novel methodology to comprehensively assess retinal arteriolar vascular reactivity to hypercapnia. *Microvasc.Res.* 2006.

107. Giller CA, Bowman G, Dyer H, Mootz L, Krippner W. Cerebral arterial diameters during changes in blood pressure and carbon dioxide during craniotomy. *Neurosurgery* 1993;**32**:737-41.

108. MacKenzie ET, Strandgaard S, Graham DI, Jones JV, Harper AM, Farrar JK. Effects of acutely induced hypertension in cats on pial arteriolar caliber, local cerebral blood flow, and the blood-brain barrier. *Circ.Res.* 1976;**39**:33-41.

109. Chilian WM, Layne SM. Coronary microvascular responses to reductions in perfusion pressure. Evidence for persistent arteriolar vasomotor tone during coronary hypoperfusion. *Circ.Res.* 1990;**66**:1227-38.

110. Chung HS, Harris A, Halter PJ, Kagemann L, Roff EJ, Garzozi HJ et al. Regional differences in retinal vascular reactivity. *Invest Ophthalmol.Vis.Sci.* 1999;**40**:2448-53.

111. Grunwald JE, Riva CE, Petrig BL, Sinclair SH, Brucker AJ. Effect of pure O2-breathing on retinal blood flow in normals and in patients with background diabetic retinopathy. *Curr.Eye Res.* 1984;**3**:239-41.

112. Harris A, Arend O, Kopecky K, Caldemeyer K, Wolf S, Sponsel W et al. Physiological perturbation of ocular and cerebral blood flow as measured by scanning laser ophthalmoscopy and color Doppler imaging. *Surv.Ophthalmol.* 1994;**38 Suppl**:S81-S86.

113. Hickam J.B., Sieker H.O., Frayser R. Studies of retinal circulation and A-V oxygen difference in man. *Trans.Am.Clin.Climatol.Assoc.* 1959;**71**:34-44.

114. Luksch A, Garhofer G, Imhof A, Polak K, Polska E, Dorner GT et al. Effect of inhalation of different mixtures of O(2) and CO(2) on retinal blood flow. *Br.J Ophthalmol.* 2002;**86**:1143-7.

115. Pakola SJ, Grunwald JE. Effects of oxygen and carbon dioxide on human retinal circulation. *Invest Ophthalmol.Vis.Sci.* 1993;**34**:2866-70.

116. Polska E, Kircher K, Ehrlich P, Vecsei PV, Schmetterer L. RI in central retinal artery as assessed by CDI does not correspond to retinal vascular resistance. *Am.J Physiol Heart Circ.Physiol* 2001;**280**:H1442-H1447.

117. Rassam SM, Patel V, Chen HC, Kohner EM. Regional retinal blood flow and vascular autoregulation. *Eye* 1996;**10 (Pt 3)**:331-7.

118. Roff EJ, Harris A, Chung HS, Hosking SL, Morrison AM, Halter PJ et al. Comprehensive assessment of retinal, choroidal and retrobulbar haemodynamics during blood gas perturbation. *Graefes Arch.Clin.Exp.Ophthalmol.* 1999;**237**:984-90.

119. Sponsel WE, DePaul KL, Zetlan SR. Retinal hemodynamic effects of carbon dioxide, hyperoxia, and mild hypoxia. *Invest Ophthalmol.Vis.Sci.* 1992;**33**:1864-9.

120. Haefliger IO, Lietz A, Griesser SM, Ulrich A, Schotzau A, Hendrickson P et al. Modulation of Heidelberg Retinal Flowmeter parameter flow at the papilla of healthy subjects: effect of carbogen, oxygen, high intraocular pressure, and beta-blockers. *Surv.Ophthalmol.* 1999;**43 Suppl 1**:S59-S65.

121. Harris A, Arend O, Wolf S, Cantor LB, Martin BJ. CO2 dependence of retinal arterial and capillary blood velocity. *Acta Ophthalmol.Scand.* 1995;**73**:421-4.

122. Dorner GT, Garhoefer G, Zawinka C, Kiss B, Schmetterer L. Response of retinal blood flow to CO2-breathing in humans. *Eur.J.Ophthalmol.* 2002;**12**:459-66.

123. Kida T, Harino S, Sugiyama T, Kitanishi K, Iwahashi Y, Ikeda T. Change in retinal arterial blood flow in the contralateral eye of retinal vein occlusion during glucose tolerance test. *Graefes Arch.Clin.Exp.Ophthalmol.* 2002;**240**:342-7.

124. Lamport H., Baez S. Physical properties of small arterial vessels. *Physiol Rev.Suppl* 1962;**5**:328-52.

125. Kontos HA, Wei EP, Navari RM, Levasseur JE, Rosenblum WI, Patterson JL, Jr. Responses of cerebral arteries and arterioles to acute hypotension and hypertension. *Am.J Physiol* 1978;**234**:H371-H383.

126. In: Shepro D, D'Amore P, Black C.M, Garcia JGN, Granger DN, Haudenschild C et al, eds. Elsevier Academic Press, 2006: 401.

127. Hakim TS, Sugimori K, Camporesi EM, Anderson G. Half-life of nitric oxide in aqueous solutions with and without haemoglobin. *Physiol Meas.* 1996;**17**:267-77.

128. Parker JD, Thiessen JJ, Reilly R, Tong JH, Stewart DJ, Pandey AS. Human endothelin-1 clearance kinetics revealed by a radiotracer technique. *J Pharmacol.Exp.Ther.* 1999;**289**:261-5.

129. Iscoe S, Fisher JA. Hyperoxia-induced hypocapnia: an underappreciated risk. *Chest* 2005;**128**:430-3.

130. Meadows GE, Dunroy HM, Morrell MJ, Corfield DR. Hypercapnic cerebral vascular reactivity is decreased, in humans, during sleep compared with wakefulness. *J Appl.Physiol* 2003;**94**:2197-202.

131. Vucetic M, Jensen PK, Jansen EC. Diameter variations of retinal blood vessels during and after treatment with hyperbaric oxygen. *Br.J Ophthalmol.* 2004;**88**:771-5.

132. Gilmore, E. D. Doctoral thesis. 2006. University of Waterloo.

Ref Type: Thesis/Dissertation

133. Wahren J, Saltin B, Jorfeldt L, Pernow B. Influence of age on the local circulatory adaptation to leg exercise. *Scand.J Clin.Lab Invest* 1974;**33**:79-86.

134. Proctor DN, Shen PH, Dietz NM, Eickhoff TJ, Lawler LA, Ebersold EJ et al. Reduced leg blood flow during dynamic exercise in older endurance-trained men. *J Appl.Physiol* 1998;**85**:68-75.

135. Grunwald JE, Piltz J, Patel N, Bose S, Riva CE. Effect of aging on retinal macular microcirculation: a blue field simulation study. *Invest Ophthalmol.Vis.Sci.* 1993;**34**:3609-13.

136. Muller-Delp JM. Aging-induced adaptations of microvascular reactivity. *Microcirculation.* 2006;**13**:301-14.

137. Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension* 1997;**30**:817-24.

138. Delp MD, Brown M, Laughlin MH, Hasser EM. Rat aortic vasoreactivity is altered by old age and hindlimb unloading. *J Appl.Physiol* 1995;**78**:2079-86.

139. Docherty JR. Cardiovascular responses in ageing: a review. *Pharmacol.Rev.* 1990;**42**:103-25.

140. Kahonen M, Karjala K, Hutri-Kahonen N, Wu X, Jaatinen P, Riihioja P et al. Influence of chronic ethanol consumption on arterial tone in young and aged rats. *Am.J Physiol* 1999;**276**:H464-H471.

141. Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Ageing suppresses endothelium-dependent relaxation and generates contraction mediated by the muscarinic receptors in vascular smooth muscle of normotensive Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens.Suppl* 1988;**6**:S243-S245.

142. Marin J, Rodriguez-Martinez MA. Age-related changes in vascular responses. *Exp.Gerontol.* 1999;**34**:503-12.

143. Hansson L, Berglund G, Andersson O, Holm M. Controlled trial of acebutolol in hypertension. *Eur.J Clin.Pharmacol.* 1977;**12**:89-92.

144. Dohi Y, Luscher TF. Aging differentially affects direct and indirect actions of endothelin-1 in perfused mesenteric arteries of the rat. *Br.J Pharmacol.* 1990;**100**:889-93.

145. Moreau P, d'Uscio LV, Luscher TF. Structure and reactivity of small arteries in aging. *Cardiovasc.Res.* 1998;**37**:247-53.

146. Weinheimer G, Dooley DJ, Cazzonelli M, Osswald H. Aging and endothelin-1 induced vascular contractions. *Experientia* 1990;**46**:1008-9.

147. Donato AJ, Lesniewski LA, Delp MD. The effects of aging and exercise training on endothelin-1 vasoconstrictor responses in rat skeletal muscle arterioles. *Cardiovasc.Res.* 2005;**66**:393-401.

148. Ishihata A, Katano Y, Nakamura M, Doi K, Tasaki K, Ono A. Differential modulation of nitric oxide and prostacyclin release in senescent rat heart stimulated by angiotensin II. *Eur.J Pharmacol.* 1999;**382**:19-26.

149. Korzick DH, Muller-Delp JM, Dougherty P, Heaps CL, Bowles DK, Krick KK. Exaggerated coronary vasoreactivity to endothelin-1 in aged rats: role of protein kinase C. *Cardiovasc.Res.* 2005;**66**:384-92.

150. Muller-Delp J, Spier SA, Ramsey MW, Lesniewski LA, Papadopoulos A, Humphrey JD et al. Effects of aging on vasoconstrictor and mechanical properties of rat skeletal muscle arterioles. *Am.J Physiol Heart Circ.Physiol* 2002;**282**:H1843-H1854.

151. Shipley RD, Muller-Delp JM. Aging decreases vasoconstrictor responses of coronary resistance arterioles through endothelium-dependent mechanisms. *Cardiovasc.Res.* 2005;**66**:374-83.

152. Gerhard M, Roddy MA, Creager SJ, Creager MA. Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension* 1996;**27**:849-53.

153. Tschudi MR, Luscher TF. Age and hypertension differently affect coronary contractions to endothelin-1, serotonin, and angiotensins. *Circulation* 1995;**91**:2415-22.