Retinal Blood Flow and Markers of Vascular Inflammation and Endothelial Dysfunction in Type 2 Diabetes

By

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AUTHOR'S DECLARATION

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

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Abstract

Abnormal leukocyte adhesion (i.e. leukostasis) to retinal vascular endothelial cells occurs in early diabetes [(2)6]. The processes of leukostasis have been clearly demonstrated in the vascular endothelium of patients with diabetes. In non-proliferative DR, clinical outcomes are manifested by excessive permeability from inflammatory progression leading to iBRB disruption [(6)], endothelial cell damage and widespread capillary nonperfusion [(2, 172)]. Diabetes promotes vascular leakage in DR by upregulation of adhesion molecules. Moreover, many of the pathological changes in NPDR are related to abnormalities in retinal blood flow [(39, 97, 99)]. Studies have shown that specific circulating markers of inflammatory activity and endothelial dysfunction are associated with clinical signs of diabetic retinopathy [(121, 172)]. However, few have found an association between circulating levels of inflammatory and endothelial dysfunctional markers and abnormal retinal hemodynamics in patients with non-proliferative DR.

The specific aims of this thesis are as follows:

Chapter 3: To correlate baseline levels of inflammatory and endothelial dysfunction markers and 1) baseline retinal arteriolar hemodynamics and 2) any disturbance in retinal hemodynamics over 6-month time in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and volumetric flow.

Chapter 4: To correlate circulating levels of inflammatory and endothelial dysfunction markers and 1) baseline vascular reactivity and 2) any disturbance in vascular reactivity after 6-month time in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and volumetric flow in patients with increasing non-proliferative diabetic retinopathy (NPDR) severity.

Methods

Chapter 3: Diabetes subjects were stratified into either mild-to-moderate (Group 2) or moderate-to-severe (Group 3) NPDR based on their retinopathy status. Age-matched non-diabetics were recruited as controls (Group 1). Forearm blood sample was collected to determine baseline levels of inflammatory and endothelial dysfunctional markers. At visit 1, baseline retinal hemodynamics was acquired using Canon Laser Blood Flowmeter. Patients returned for a visit 2 (6 month follow-up visit) and retinal hemodynamics was reassessed. Baseline levels of inflammatory and endothelial dysfunctional markers compared between groups and correlated with both baseline and change in retinal hemodynamic parameters over 6-month time.

Chapter 4: Diabetes subjects were stratified into either mild-to-moderate NPDR or moderate-to-severe NPDR based on their retinopathy status. Age-matched non-diabetics were recruited as controls. At visit 1, forearm blood sample was collected to determine levels of inflammatory and endothelial dysfunctional markers and baseline vascular reactivity response was acquired. Retinal blood flow data was acquired while subjects breathed air. Retinal blood flow measurements were then acquired after exposure to isocapnic hyperoxic stimuli. At visit 2 (6 month follow-up), retinal vascular reactivity was reassessed. Baseline levels of inflammatory and endothelial dysfunctional markers compared between groups and correlated with both magnitude of baseline and change in vascular reactivity in terms of retinal hemodynamics.

Results

Chapter 3: Maximum-to-minimum velocity ratio (max: min) was found to be significantly elevated in the group 3 compared to group 1 at baseline (0.72 vs. 0.49, after Bonferroni correction $P<0.01$).

Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA p=0.02 and p=0.04). A post hoc Bonferroni test showed that Group 3 had significantly higher in both sICAM-1 and sE-selectin levels compared to Group 1 (234.0 vs. 151.5 ng/ml, P=0.02 and 53.4 vs. 27.6 ng/ml, P<0.01, respectively). Hemoglobin A1c was significantly elevated across the groups (ANOVA p<0.01). A post hoc Bonferroni test showed that Group 3 had significantly higher hemoglobin A1c level compared to Group 1 (7.9 vs. 5.6 % , P<0.01). There were no significant associations found between baseline markers of inflammation and baseline retinal hemodynamics across all groups. The Δ velocity was correlated with the baseline sICAM-1 (r=0.42, p=0.02) and A1c levels (r=0.37, p=0.04) in patients with NPDR. After adjustment for all other variables (A1c, hsCRP and vWF), Δ velocity, sICAM-1 and A1c were found not to be reliable predictors of baseline retinal hemodynamics.

Chapter 4: There were no significant differences in magnitude of retinal vascular reactivity in hemodynamic parameters between groups at visit 1 or visit 2. Over 6 months time, compliance was found to be significantly reduced in patients of Group 3 compared to Group 2 (-0.4 vs. 0.1, t-test p<0.01). Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA p=0.02 and p<0.01). A post hoc Bonferroni test showed that Group 3 had significantly higher in both sICAM-1 and sE-selectin levels compared to Group 1 (243.4 vs. 157.3ngml, P<0.01 and 57.0 vs. 29.3 ng/ml, P<0.01, respectively). Hemoglobin A1c was significantly elevated across the groups (ANOVA p<0.01). A post hoc Bonferroni test showed that Group 3 had significantly higher hemoglobin A1c level compared to Group 1 (8.8 vs. 5.6 % , P<0.01). Baseline VR in blood velocity weakly correlates with sE-selectin (r=0.31, p=0.04) across all groups while sVCAM-1 was associated with VR in terms of blood flow (r=-0.62, p<0.01) in patients with mild-to-moderate NPDR. TheΔ blood flow after 6 months was found to be weakly associated with sE-selectin (r=0.46, p=0.03) across

all groups. Finally, theΔ blood velocity after 6 month time was found to be moderately correlated with baseline vWF Ag level (r=-0.78, p=0.02). Multiple regression analysis found that vascular inflammatory and endothelial function markers had weak predictive power for Δ hemodynamic parameters.

Conclusions

Chapter 3: We found weak associations between circulating markers and baseline or the disturbance in retinal hemodynamics after 6 months time. Overall, we found both an increase in rigidity of the arteriolar circulation and elevated inflammatory adhesion markers (sICAM-1 and sE-selectin) within the same population sample. Change in velocity over the follow-up period was correlated with sICAM-1 and A1c levels in patients with NPDR but the level of association was such that neither sICAM-1 nor A1c proved to reliably predict retinal hemodynamics.

Chapter 4: We demonstrated two important characteristics in early NPDR; 1) a disturbance in vascular reactivity in terms of compliance and 2) an increase in systemic markers of inflammation were found in patients with NPDR. Although systemic markers of vascular inflammation and endothelial dysfunction are not predictive of hemodynamic parameters, our study found moderate associations between baseline and disturbances in VR after 6 months time. Therefore, there is evidence that inflammation and vascular function may be related with respect to their development in NPDR.

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Finally, I would like to thank my family and friends for their faith and encouragement throughout this endeavour. I am fortunate for such an excellent support network of people whom I can rely upon.

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Dedication

I dedicate my work to my brilliant husband Tyson, who has encouraged me to persevere and has always inspired me to chase my dreams.

To my parents Ken and Anne Khuu, the strongest, most selfless, hard working people a child is blessed to have. Thank you for believing in me.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Advanced glycation end-products (AGE); Average velocity (V_{mean}); Basement membrane (BM); Blood flow (Q); Blood pressure (BP); Blood retinal barrier (BRB); Branch retinal vein occlusion (BRVO); Calcium ion concentration (Ca^{2+}); Canon laser blood flowmeter (CLBF); High sensitivity C-reactive protein (hs CRP); Carbon dioxide (CO_2); Central retinal artery (CRA); Clinically significant DME (CSDME); Diabetes control and complications trial (DCCT); Diabetic macular edema (DME); Diabetic retinopathy (DR); Diameter (D); Early Treatment Diabetic Retinopathy Study (ETDRS); Endothelial-derived contracting factors (EDCF); Endothelial-derived relaxing factors (EDRF); Endothelin (ET); Fractional inspired oxygen (FiO_2); Frequency (f); Glycosylated hemoglobin (A1c); Internal limiting membrane (ILM); Intra-ocular pressure (IOP); Length (L); Lens Opacity Classification System III (LOCS III); Maximum frequency shift (Δf_{max}); Maximum to minimum (max:min); Microaneurysms (MAs); Monocyte chemoattractant protein type 1 (MCP-1); Nitric oxide (NO); Oxygen (O_2); Partial pressure of arterial carbon dioxide (PCO_2); Partial pressure of arterial oxygen (PO_2); Positive end-expiratory pressure (PEEP); Pressure gradient (ΔP); Protein kinase C (PKC); Proliferative diabetic retinopathy (PDR); Resistance (R); Retinal pigment epithelium (RPE); Smooth muscle cells (SMCs); Soluble vascular cell adhesion molecule type 1 (sVCAM-1); soluble intercellular adhesion molecule type 1 (sICAM-1); soluble E-selectin (sE-selectin); Tumour necrosis factor- alpha (TNFα); United Kingdom Prospective Study (UKPDS); Vascular endothelial growth factor (VEGF); Visual acuity (VA); von Willebrand factor antigen (vWF ag); von Willebrand factor activity (vWF act); Wall shear rate (WSR).

List of Equations

Chapter 1
Literature Review: Retinal Blood Flow

1.1 The Anatomy of Retinal Blood Supply

The delivery of oxygen and metabolic substrates to the retina, and the removal of waste products, is primarily accomplished by the inner retinal and choroidal circulatory systems. Although the inner retinal and choroidal systems are both branches of the ophthalmic artery, they differ anatomically and functionally.

1.1.1 Retinal Microvascular Arrangement

Unlike most other organs, the inner retinal circulatory system is a unique end-artery microvascular system. Oxygenated blood travels to the eye from the aortic artery of the heart. The first two arteries branching from the aorta are the brachiocephalic artery, which supplies the right arm and right common carotid artery, and the left common carotid artery. Each of the common carotid arteries branch into an internal, and an external, carotid artery. The main blood supply to the orbit appears from the internal carotid via the ophthalmic artery (OA). Smaller vascular contributions are from the external carotid artery via infraorbital artery and orbital branch of the maxillary artery. The maxillary artery supplies the soft tissue of the orbital floor, lacrimal sac, nasolacrimal duct, and give a minor supply to the inferior rectus, inferior oblique, and orbicularis muscles [(81)]. The OA runs inferior to the optic nerve and into the optic canal and branches into the central retinal artery (CRA), long- and short-posterior ciliary arteries, and the anterior ciliary arteries that also receive supply from facial artery [(78)].

The central retinal artery (CRA), along side the central retina vein (CRV), supplies the inner two thirds of the retina [(78)]. The CRA enters the optic nerve about 10mm behind the eyeball and appears at

the junction of the optic disc and the surface of the retina through the *lamina cribrosa*. The first bifurcation of the CRA occurs at this point and it branches into four major retinal vessels, each of which supplies one quadrant of the retina [(1)78)]. Each of the arterioles continue to bifurcate within the transparent never fiber layer, supplying blood to each sector of the retina. The superior and inferior temporal artereioles arch above and below the *macula region* creating a *foveal avascular zone* (FAZ) approximately 0.4mm in diameter. This vessel free region facilitates the maximum absorption of light by the central photoreceptors without the disruption of a single blood vessel [(43)]. Overall, retinal capillaries run parallel to the retinal nerve fiber layer. Layers of retinal capillaries are greater in number in the posterior pole compared to the more peripheral retina. The terminal ends of the arterioles and venules are linked through the retinal capillary beds. To complete the circulatory cycle, the central retinal venule is the site for retinal drainage via the vortex venules. This vein exits the eye through the optic nerve and drains into the cavernous sinus[i] or into the superior ophthalmic vein.

The outer retina includes photoreceptors and bipolar cells which are avascular and primarily receive metabolic energy and O_2 from the highly vascularized choroidal circulation [(133)]. This tissue is by far the most metabolically active in the body and is therefore highly demanding of nutrients. This layer is separated from the inner retinal layer by the retinal pigment epithelium (RPE). With an apical-basal polarity, the RPE is a highly active inter-face in the transport of waste and [(85)] metabolites to and from the retina to the choroid [(4)].

1.1.2 The Choroidal Microvascular Arrangement

The choroidal circulation exhibits one of the highest rates of blood flow in the body. At any one time, more than 70 % of all the blood in the globe can be found in the capillary network of the choroid, the *choriocapillaris.* Anatomically, the choroid is a highly vascularized and pigmented to form the

posterior part of the uveal tract. It extends from the *ora serrata* to the optic nerve posteriorly. The choroid is attached to the sclera by strands of connective tissue and is tightly adherent to the optic nerve and retinal pigment epithelium. The unique structure of the choroid is important in: 1) heat dissipation as a result of the high flow rate generated by the high metabolic activity associated with phototransduction of the photoreceptors; 2) nourishment of the retinal pigment epithelium and a part of the retina, up to the outer aspect of the inner nuclear layer; and 3) in the establishment of pigmentation of the fundus [(75)].

Blood supply to the choroidal vasculature stems from the ophthalmic artery (OA), the first branch of the internal carotid artery (CRA), via the medial and lateral posterior ciliary arteries. Before piercing the sclera, the posterior ciliary divides into a long posterior artery (LPCA). A variable number of short posterior arteries (SPCAs) arise separately from the OA. Together a total of 2 LPCAs and 15-20 SPCAs are produced. The LPCAs pierce the sclera approximately 3-4mm from the optic nerve and course anteriorly through the *suprachoroidal space* between the choroid and sclera, to a branch point near the ora serrata. The LPCAs supply the anterior choriocapillaries and at this point, each of the LPCA branches three to five times to extend posteriorly to supply the choriocapillaries near the equator. The SPCAs on the other hand, enter the sclera in close proximity to the ONH and continue only a short distance from the optic nerve. These vessels branch immediately to supply the posterior portion of the choriocapillaries up to the equator [(68)]. Unlike most vascular networks in the body, the choroidal arteries and veins do not run parallel. Venous drainage of the choriocapillaries is mainly through the vortex vein system. Minor drainage occurs through the ciliary body through the anterior ciliary veins.

Finally, the capillary walls in the choroid are quite different from those of the capillary walls of the retina. In the choriocapillaries, the walls have a thin endothelial covering with large, numerous fenestrations. Such fenestrations allow for high permeability to low molecular weight substances. This degree of permeability is necessary to maintain a high concentration of glucose at the retinal pigment epithelium and to permit passage of proteins involved in the supply of the retina [(4)]. Whereas in the inner ret require tight endothelium that creates the inner BRB.

1.1.3 Retinal Barriers

Optimal cell function is achieved when the biological environment is tightly regulated. Just as the brain neurons depend stringently on controlled homeostasis, the retina although structurally different, relies on the barriers that prevent adverse changes affecting the composition of the nurturing blood and the neural architecture [(4)]. In both cases, this regulation is established by the cellular barriers, which separate functional compartments and control the transport between them; the inner and outer blood retinal barriers. The main structures involved, for the inner blood-retinal barrier, are the endothelial membrane, and for the outer blood-retinal barrier, is the retinal pigment epithelium [(4)].

1.1.3.1 Inner Blood-Retinal Barrier (iBRB)

The endothelial membrane of the inner retinal vessels is an elaborate network that separates the blood from the retina at the level of the vessels, termed the *inner blood retinal barrier* (iBRB). The structure and pattern of the retinal blood vessels present marked differences from those of other organs. It was in 1966, Shakib and Cunha-Vaz described the fundamental difference between the endothelial membrane of vessels with and without a barrier [(157)]. The endothelial cells form a continuous, non-fenestrated, “tight” monolayer of cells that line the inner retinal vessel lumen. The most outstanding feature of the inner retinal endothelial cells is the presence of junctional inter-endothelial structures,

zonulae occludens or tight junctions, areas of complete fusion of the outer leaf of the neighboring membrane [25]. The presence of these tight junctions is to maintain an inner blood-retina barrier. The fusion of the endothelial membrane creates a barrier around the inner retinal vessels that is resistant to the leakage of blood-borne substances. Retinal capillaries do not leak usually flourescein, glucose or amino acids but are highly permeable to water and lipid soluble substances such as oxygen and carbon dioxide [133] and small metabolic weight compounds. Interactions with glial cells such as astrocytes are required for the endothelial cells to develop tight junctions. Disruption of the iBRB, as in some pathological situations (diabetes, hypertension), can compromise the endothelial cells and result in lost tight junction integrity.

1.1.3.2 Outer Blood- Retinal Barrier (oBRB)

Within the outer retina, the *retinal pigment epithelium* and *Bruch's membrane* complete the array of *outer blood retinal barrier* (oBRB) tissues. Together, these structures show increasing restriction to size and polarity of blood borne solutes, with the most permissive being the fenestrated endothelium of the *choriocapillaris* to the most stringent *retinal pigment epithelium* (RPE), a consequence of tight junctions [133].

The RPE plays a central role in the oBRB and supports the function of the photoreceptors. The RPE is a highly polarized monolayer with an elaborate transcellular pathway system. Considered a "tight" epithelium, the RPE's integrity and complexity is attributed to the partially overlapping intercellular junctions, the *zonula adherens*, tight junctions and gap junction. The RPE's apical-basal polarity allows the transport of; 1) nutrients including essential amino acids and vitamin A from the blood to the photoreceptors and 2) removal of metabolites, extracellular fluid and ions from the subretinal space towards the choriocapillary bed. Trans-epithelial ion transport is responsible for the transport of lactic acid, the major end product of neuronal function. The accumulation of water from

intensive metabolic turnover requires constant removal by the glial cells from the inner retina and RPE from the subretinal space [133]. Although the RPE is capable of site specific functions, it nevertheless possesses many similar functions with endothelial barriers [166].

Bruch's membrane (BM) carefully regulates the passage of molecules across the oBRB. The BM is an elastin and collagen rich extracellular matrix that acts as a molecular sieve. This membrane has five distinct layers; 1) the basal lamina of the RPE 2) the inner and 3) outer collagenous layers and 4) the central discontinuous elastic layer and 5) the endothelium of the choriocapillaris. This pentilaminar BM structure forms a single function unit / complex with juxtaposition with the RPE and choriocapillaris and regulates the exchange of products between the retina and the general circulation [16]. More specifically, the BM serves three primary roles; 1) regulating the diffusion of biomolecules between the choroid and the RPE 2) providing physical support for the RPE cell adhesion, migration and perhaps differentiation and 3) acting as a division barrier, restricting choroidal and retinal cellular migration. Given its unique location, the BM plays a critical role in cell-cell communication, cellular differentiation, proliferation or migration, tissue remodeling and in shaping pathological processes [16][17]. Accumulating evidence suggests that properties of the BM are dependent on age, genetic constitution, environmental influences and disease state. Disruption to the BM can lead to the onset and progression of diseases like retinitis pigmentosa and age related macular degeneration [16]. As a result, properties of the BM are unique to each human individual and can uniquely affect the development of normal vision and ocular state.

Summary

There is evidence that a failure of either the iBRB or oBRB can lead to vision threatening diseases. A compromised blood retina barrier occurs in diseases such as diabetic retinopathy / macular edema, hypertensive retinopathy, sickle cell retinopathy and acquired immunodeficiency syndrome (AIDS).

1.2 Structure and Function of Retinal Vessels

1.2.1 General Structure of the Blood Vessels

Blood vessels carry blood to and from the heart and regardless of their exact role, blood vessels have a comparable structural organization. Characteristically, the walls of larger blood vessels are composed of three layers; tunica intima, tunica media, and tunica adventitia. The *tunica intima* (*or tunica interna*) is the innermost layer and lines the inside of the lumen, consisting of a single layer of squamous endothelial cells. The endothelial layer is in direct contact with the actively moving erythrocytes and reduces the friction between the vessel wall and blood. The *tunica media* is a region that is largely composed of circularly arranged smooth muscle cells and sheets of elastin. Finally, the outermost layer is the *tunica externa (or tunica adventitia)* is made up of collagen fibers which protects, reinforces and anchors the vessel to the surrounding tissues [(114)].

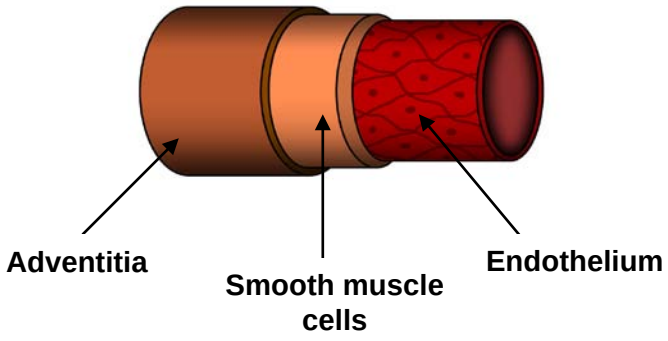

Figure 1-1 Structure of a blood vessel

1.2.1.1 The Arterial System

The walls of the aorta and other large diameter arteries have relatively more elastin than smooth muscle layers. This allows the vessel to resist high blood pressure fluctuations by stretching during systole and recoiling on the blood during diastole [115]. From the aorta, as the artery divides, each subsequent branch has a proportionately greater amount of smooth muscle to elastin. Although larger arterioles possess all three tunics, smaller arterioles can contain as little as a single layer of smooth muscle around an epithelial lining. Generally, smooth muscle is innervated by noradrenergic fibers that function as constrictors and in some instances, cholinergic fibers that can dilate the vessels. Specifically, muscular endowed and highly flexible arterioles deliver oxygenated blood to specific organs and are specifically active in vasoconstriction. Whilst arterioles are the smallest of all arteries, they determine and regulate blood flow into the capillary bed by both vasoconstriction and vasodilation.

1.2.1.2 The Capillary Bed

Arteries divide into muscle walled arterioles (resistance vessels) and feed into capillaries (exchange vessels). Capillaries are the smallest blood vessel and are the location for the exchange of substances between the blood and the interstitial fluid. In the simplest form, the capillary wall is made up of only a *tunica interna* consisting of a layer of endothelium and a basement membrane. The narrow lumen allows for a single file line of red blood cells to pass through. Generally, there are three main types of capillaries; *continuous, fenestrated* and *discontinuous*. In the inner retina, capillary beds are made up of two types; continuous and non-fenestrated capillaries. Continuous capillaries are the least permeable type but allow the passage of fluids, small solutes and molecules. Capillary beds are an interweaving network of true capillaries and meta-arterioles; channels which connect the arterioles and the venules at both ends of a bed. True capillaries are 5μm at the arteriole end and about 9 μm at the venous end. These convoluted arrays of capillaries function as exchange vessels for oxygen,

hormones and nutrients. To locally modulate blood flow into the capillary bed, a cuff of smooth muscle, called the *precapillary sphincter*, surrounds each capillary at the meta-arteriole [7]. When the sphincter is dilated, the diameter of the vessel is permits red blood cells to just "squeeze" through capillaries in a single file. As a result, red blood cells fold into a parachute shape as a consequence of flow pushing at the center [115] to form a parabolic velocity profile. Overall, the structure and permeability of capillaries vary can differ depending on the needs of the organ.

1.2.1.3 The Venous System

Blood collecting from the capillary bed exits into the venular system that is very porous to allow for absorption of white blood cells and other fluids. The walls of the venules are only slightly thicker than that of capillaries and consist mainly of a layer of endothelium. Overall, veins have a thinner wall, very few layers of smooth muscle, thin layer of tunica externa, and have a larger lumen than equivalent arteries. The blood pressure in veins is low because of the relative absence of a ventricular contraction. Although smooth muscles are nearly non existent, considerable vasoconstriction is produced as a result of noradrenergic nerve fibers to the vein and also circulating vasoconstrictors such as endothelin-1 [127, 187]. One way valves, present as folds in the tunica interna, prevent back flow. The presence of valves within the venous system is especially important in the extremities since the flow of blood is often opposed by gravity.

1.2.2 The Retinal Microvasculature

Hierarchal arteriolar networks differ in function and in structure. Not unlike the general network, the retinal microvascular circulatory system is also made up of arterioles, capillaries and venules; each with a unique structure and function. The arterioles are well invested in smooth muscle cells and are responsible for the delivery of blood to localized tissue and also regulate the flow of blood by responding to myogenic, metabolic and hormonal stimuli. Capillaries have thin walls to facilitate the

exchange of nutrients between tissue and blood. The venules drain blood from the capillaries and parallel the organization of the arterioles. Structural differences allow the supply of nutrients to meet the specific metabolic demands of an organ under various stress conditions. The specialized blood vessels of the retina are unique since it is a complex system that differs anatomically and physiologically.

1.2.2.1 Retinal Arterioles

The structure of the retinal vasculature is highly specialized since it is dependent on diameter in order to maintain consistent blood flow. This is primarily accomplished because of its unique ability to respond to a variety of stimuli. Structurally, when compared to the same size arteries of other organs in the human body, arteriole branches of the central retinal artery lack an internal elastic lamina and gradually contain decreased layers of smooth muscle cells from the optic disk to the periphery [(106)]. Direct branch arterioles of the central retinal artery are approximately ~100 to150 μm in diameter close to the ONH. A retinal pre-capillary arteriole has capillary dimensions (~10μm in diameter) but is enveloped by a single continuous layer of smooth muscle. These vessels merge seamlessly into a vast capillary network. It is apparent that arterioles are uniquely suitable for flow regulation as they are capable of larger changes in diameter as a percentage; greater than any other vessel in the circulatory system.

1.2.2.2 Retinal Capillaries

With the advent of the electron microscope, classifying capillaries based on morphological features lead to the formation of functional concepts. When the flow of blood is modulated by the upstream retinal arterioles, flow in the retinal capillary bed also changes. The retinal capillaries are distributed into two distinct layers; 1) the superficial layer that resides in the nerve fiber and ganglion cell layer and 2) the inner layer that is located in the inner portion of the inner nuclear layer between the inner

and outer plexiform layers [106]. Additionally, there is a network of capillaries located superficially in the nerve fiber layer called the *peripapillary capillaries* and run along the major superotemporal and inferotemporal vessels. The periparillary capillaries anastomose with each other and with the deeper capillaries. The two distinct capillary layers run in parallel until they reach the macular region where they join as a single layered ring; *the perimacular ring*. Within the macula area, there is a blood vessel and capillary free zone known denoting the fovea that is 400μm in diameter [4].

Histological evidence shows that the retinal capillary walls are composed of endothelial cells, intramural pericytes and a basement lamina [133, 134]. Each capillary unit is approximately ~5-6 μm in diameter [4]. The endothelial cells form a continuous, non-fenestrated, monolayer that is coupled to neighboring endothelial cells by tight junctions, *zona occludentes.* The endothelial cells are oriented along the axis of the capillary, with cytoplasmic extensions encircling the lumen [106]. Retinal pericytes surround the endothelium and occur in a 1:1 ratio with the endothelial cells and communicate via gap junctions [7].

Experimental evidence has shown that pericytes possess contractile properties [90]. This contractile property allows pericytes in the capillary [90] wall to further "fine tune" local flow of blood. Therefore, capillary perfusion is primarily regulated by the upstream arterioles but then also further modulated by the contractile pericytes that are located on the luminal wall of capillaries.

Summary

Overall, it is apparent that the structure the arteriole specifically dictates its function within the retinal microcirculation. The primary role of blood vessels is to regulate the flow of blood and intravascular

pressure. This is accomplished by changes in upstream arteriolar diameter as a result of several mechanisms that potentiate these changes. While arterioles regulate total blood flow to the retina, the capillary network, which independently contracts and further modulates the local distribution within the tissue. Retinal blood flow is determined by a combination of myogenic mechanisms, the local demand for metabolites, the resting vascular resistance and capillary perfusion pressure in the capillary bed.

1.3 Blood Flow Dynamics

Blood flow is the volume of blood that flows through a certain point in the cardiovascular system during a specified amount of time. The underlying principles that govern the flow of fluid within a cylindrical tube are applicable to the movement of blood cells within a blood vessel. Specifically, the flow of fluid within a tube is dependent entirely on two factors; 1) the perfusion pressure between the two ends of the vessel and 2) the impediment of flow through a vessel, known as *vascular resistance*. *Ohm's law* describes the same physical factors that govern the flow of any fluid, and are based on the fundamental law of physics [10].

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

Equation 1-1 Ohm's law

Flow (Q) is directly related to the difference in pressure (ΔP) between the two ends of the tube and inversely related to the resistance (R). Within a vessel segment, there is a difference in pressure between two points along a given length. This will result in a movement of blood from a high to a lower pressure zone. According to Ohm's equation, the blood flow between two points is directly

proportional to the difference in pressure between the two points (ΔP), also known as the *perfusion pressure* (assuming constant vascular resistance and blood viscosity). This force perpetuates the movement of blood throughout the entire body. In order to accomplish this forward movement, there must be a difference in blood pressure within our circulatory system.

Resistance is dependent on the properties of the fluid and the tube to which it is flowing through. Blood pressure is the product of two separate forces. The first is created by the heart as it pumps blood into the arteries through the circulatory system and the other is the force that resists the movement of blood. Resistance to blood flow within a vascular system is dependent on the size of the individual vessels (length and diameter), the arrangement of the vessel bed (series or parallel), physical characteristics of the blood (viscosity, laminar vs. turbulent flow) and vasoactive substances acting upon the vasculature. Within a tube, Ohm's law describes the relationship between flow and resistance; the higher the resistance to the flow, the lower the blood flow through a vessel.

1.3.1 Poiseuille's Principal of Laminar Flow

Of the mentioned factors that contribute to resistance, a change in vessel diameter is the most important factor in quantifying blood flow within an organ, as well as regulating arterial pressure [(10)]. It was not until 1846 that the physiologist J.L.B Poiseuille combined the study of flow of liquids with properties of a tube and established an equation that described the relationship between pressure (P), flow (Q), radius (r), visocity(η) and dimensions (L) of the tube.

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

Equation 1-2 Poiseuille's Equation

From this, Poiseuille's law was derived; the rate of blood flow is directly proportional to the forth power of the radius (r) of the vessel[10]. This illustrates that diameter plays a great role in determining the rate of blood flow through the vessel.

1.3.1.1 Poiseuille's Law of Laminar Blood Flow

Laminar flow is the normal condition of blood flow through most of the circulatory system. This movement is described as concentric layers of blood moving in parallel throughout the length of a blood vessel. The outcome of this arrangement is that the highest velocity occurs in the center of the vessel, while the lowest velocity occurs along the vessel wall, often termed a parabolic velocity profile. Alternatively, plug flow is the simplest type of flow where all velocities are constant. When there is a disruption in laminar flow, the flow of blood is no longer smooth but chaotic and is known to be *turbulent*.

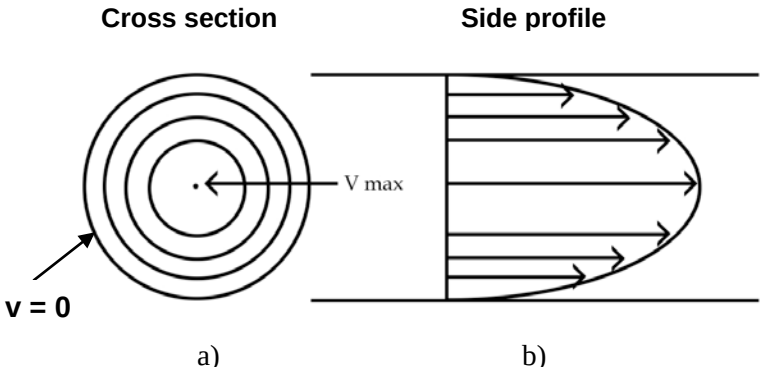

Figure 1-2 A schematic diagram of laminar flow within a vessel.

Cross section profile of laminar flow. Concentric rings represent changing velocity, from the outside having zero velocity and increases as we move towards the center. b) The side profile of laminar flow. The highest velocity for both profiles is located in the center.

Laminar flow reduces the amount of energy that is lost to the vicious interactions between the layers of the blood and the walls of the vessel [10]. One method of quantifying the parabolic velocity profile is using SDOCT Doppler. The peak or cut-off frequency shift of the scattered light produced by the moving erythrocytes is proportional to the maximum velocity found in the centerline flow. In small vessels (<40 to 60 μm in diameter), essentially all of the blood is very near to the wall and the central stream of rapid flowing blood does not exist.

Overall, *Poiseuille's law* describes the relationship between these variables based on several major assumptions; 1) the tube is stiff, straight, and uniform and 2) the blood behaves in a Newtonian manner (i.e. viscosity is non-existent such that the fluid flows freely and does not resist flow). That is with regard to the flow through cylindrical tubes, it will vary as a function of the nature of the fluid itself. This flow determining property is viscosity and is defined by Newton as the ratio of shear stress to the shear rate of the fluid. Lastly, 3) the flow of blood is laminar and steady and the velocity at the wall is zero (*law of Poiseuille*). However, the cardiovascular system differs from the rigid tubes studied by Poiseuille in that 1) the vessels of the human body are elastic and 2) rhythmic contractions of the heart produce pulsatile blood flow. Despite these limitations, Poiseuille's equation is a useful approximation for the calculation of blood flow and is believed to exist in the retinal blood vessels that are 60 to 80μm in diameter or larger [10].

1.3.2 Blood Flow Regulatory Mechanisms

Each organ within the body has an intrinsic ability to regulate the flow of blood. Mechanisms to regulate the blood flow are unique to each organ where some originate within the blood vessels and others are regulated by the central nervous system.

Specialization of function within the walls of blood vessels allow for the regulation of blood flow. Two types of methods derived from walls of the blood vessels include the *myogenic mechanism* and *endothelial factors*. The myogenic mechanism is the intrinsic ability of the smooth muscle cells to respond to changes in blood pressure to maintain the demand for nutrients, particularly in small arteries and arterioles that supply organs. If there is a pressure increase, the smooth muscle layer within the vessel wall responds by vasoconstricting. If there is a decrease in pressure, the smooth muscles responds by vasodilation. The vascular endothelium plays an important role in the regulation of smooth muscle condition. Endothelial derived factors lead to vasoconstriction [by endothelin (ET-1)] or vasodilation [by nitric oxide (NO) or prostacylcin (PGI_2)], acting on vascular smooth muscle cells. These vasoactive agents originate via blood and exert on the terminals of adrenergic nerves that reinforces action on the smooth muscle cells ([89].

Summary

Overall, the relationship pressure waveform and total blood flow can be explained by fluid mechanics. Normal arterial blood flow is laminar with secondary flows generated at curves and branches. Arteries are organs that can adopt to change with the varying hemodynamic conditions, especially in abnormal biological response. Atherosclerosis for example, can result in localized narrowing of the artery lumen, known as stenosis. This can cause turbulence, reduce flow and create high shear stresses on the vessel wall. The study of arterial blood flow will lead to the prediction of individual abnormal hemodynamic flows and the development of diagnostic tools to quantify vascular

diseases such as diabetes. Specific local blood flow mechanisms also exist that are independent of extrinsic regulatory control mechanisms such as sympathetic innervation or circulating hormones that reinforce a constant flow of blood to an organ. Examples of local control mechanism include autoregulation, active hyperemia and reactive hyperemia [89]. Retinal circulation is tightly controlled *autoregulation*. Ultimately, the balance between local regulatory mechanisms and extrinsic factors in vivo determines the vascular tone and therefore the blood flow within a tissue.

1.4 Blood Flow Regulation in the Retina

In the peripheral circulation, blood flow is controlled either by the central nervous system or locally by the environmental conditions in the immediate vicinity. Unlike the extra-ocular and choroidal vessels that are innervated by the sympathetic and parasympathetic pathways, neural regulation plays no known role in the regulation of the inner retinal vasculature [107]. Retinal vessels do not have any known functioning nerve supply. Since the retina lacks CNS innervations, blood flow is regulated by autoregulatory mechanisms via a combination of myogenic driven negative feedback mechanism and by metabolic local tissue demands. To ensure adequate oxygen supply, the retina relies heavily for matching metabolism on the local vascular control mechanisms termed *autoregulation* [70].

1.4.1 Autoregulation in Retinal Blood Flow

Autoregulation is the ability of a tissue to maintain a constant flow of blood despite variations in perfusion pressure [32, 69, 138, 159] and/or metabolic stimuli. The intrinsic ability to autoregulate is vital to normal functioning of the retina since retinal vessels do not have a functioning sympathetic nerve innervation [4]. Mechanisms of autoregulation have been investigated through experimental isolation of tissue from neural and hormonal influences. It has been found that a failure or impairment of autoregulation has been implicated in various retinal diseases including diabetic retinopathy [138, 159]. Guyton and co-workers (1964) expanded the original definition of autoregulation to include tissue

responses to changes in blood gas concentrations, often referred to as metabolic autoregulation [70]. *Vascular reactivity* is the change in hemodynamic parameters in response to physiological provocations including carbon dioxide (CO_2) [30, 173, 174, 176], oxygen (O_2) [26, 53, 95], cold stress [44, 117, 120]or light flicker [46, 47, 128]. Endothelial dysfunction is an important determinant of altered vascular reactivity and plays a major role in the genesis of micro- and macrovascular complications in diabetes.

1.4.1.1 Mechanisms of Autoregulation: Myogenic Mechanism

The vascular response to acute changes in local blood flow produced by altered perfusion pressure occurs via the direct stimulation of smooth muscle stretch receptors within the arteriolar wall [156]. Dynamic muscular activity was first described in the work of *Bayliss (1902)*, who suggested that much like other areas of our body, the smooth muscle layer of arteries respond to a stretching force by contraction and to a reduction of tension by relaxation (*the Bayliss Effect*)[15]. In the retina, an increase in transmural pressure has been shown to elicit a myogenic response of isolated retinal arterioles [29] . The retinal pre-capillary bed is autoregulated in order to ensure a stable and sufficient blood supply despite changes in perfusion pressure [147]. An increase in blood pressure would consequently lead to an increase in the blood flow through an arteriole in a non-reactive system. However, smooth muscle fibers in the arteriole wall are able to sense this distension and the arteriole responds by constricting. This results in an increase in the resistance of the arteriole, thereby stabilizing blood flow. The structure of the pre-capillary arterioles with a highly organized smooth muscle covering facilitates sensitive control of luminal diameter and effective maintenance of constant retinal blood flow.

1.4.1.1.1 The Loss of Smooth Muscle Cells and Pericytes in Blood Flow Regulation

The smooth muscle layers are made up of small, mononucleated and spindle shaped cells [10]. Similar to other smooth muscle in the body, this cell type contracts slowly, develops high force and can sustain this force for long periods with low ATP use. The interaction of actin and myosin filaments are controlled by myoplasmic Ca++ through voltage gated calcium channels (electromechanical coupling) and through receptor-operated calcium channels (pharmacomechanical coupling). In diabetic retinopathy, loss of vascular smooth muscle cells (VSMC) is an important factor to the downstream capillary bed with a major impact on endothelial cell survival [178].

The ability of these retinal arterioles to regulate flow is important to control blood flow through the retinal capillaries. Retinal capillaries lack smooth muscle cells and its endothelium is surrounded by *pericytes* that also exhibit some contractile properties. Studies have shown that the regulation of blood flow occurs, not only in the pre-capillary arterioles, but is also fine tuned within the capillary / pericyte complex [90, 133, 135]. Nevertheless, the role of pericytes in retinal blood flow regulation is much less than that of the arterioles. In early exploration of the pathogenesis of diabetic retinopathy, one of the earliest abnormalities that predisposes to the formation of microaneurysms are is the loss of pericytes [72, 178, 182]. Moreover, Hammes and colleagues found that retinal capillary coverage of pericytes is crucial in the survival of endothelial cells, particularly under high stress conditions such as the elevated glucose environment of diabetes [72]. Overall, it is clear that the loss vascular smooth muscle cells and pericytes contribute to the development of vascular disorders in the retina.

1.4.1.1.2 Mechanism of Autoregulation: Variations in Systemic Blood Pressure

Various methods have been applied to test the efficacy of autoregulation to systemic blood pressure changes induced by static exercise, posture change and cold pressure test [32, 117, 130]. Static and isometric exercises notably cause an increase in blood pressure, heart rate and sympathetic nerve

activity. Earlier studies that have measured blood velocity changes in the retinal veins following an isometric exercise stimulus have found evidence of autoregulation [145]. The effectiveness of autoregulation, however, was limited to blood pressure changes of up to 40% from baseline [139]. Above this point, autoregulation appears to breakdown and velocity increases in a linear manner with further increases in blood pressure [33]. Intuitively the *myogenic mechanism* of autoregulation likely accounts for the response to perfusion pressure changes caused by an increase in blood pressure. .

1.4.1.2 Mechanisms of Autoregulation: Metabolic Autoregulation

Later in 1967, Guyton and colleagues extended Johnson's previous notion of retinal autoregulation; as the "ability of a tissue to adapt blood flow to metabolic needs" referred to as *metabolic autoregulation* [70]. Unlike myogenic autoregulation that stabilizes retinal blood flow, metabolic autoregulation changes blood flow to stabilize metabolic requirements. Metabolic blood flow regulation alters vascular resistance so that the blood supply maintains the concentrations of certain metabolites and waste-products within narrow limits, principally the partial pressure of O_2 and CO_2. We are able to induce a metabolic response and quantify the hemodynamic response, i.e. vascular reactivity. Retinal blood flow is regulated by local factors that modulate vessel resistance. These factors are released by the endothelium or by neural tissue surrounding the vessel, i.e. neurons, glial and endothelial cells [133, 138]. They can be ionic, molecular and/or related to arterial gas modification.

1.4.1.2.1 Metabolic Autoregulation by Ionic/Metabolic Substances

The release of local ionic/metabolic substances by neurons, glial and endothelial cells can alter vascular tone influencing vessel resistance. Endothelial specific derived relaxing factors (EDRF) are released by the endothelial cells and elicit a vasodilatory response to increase blood flow [28]. Among

the various EDRFs, many studies have shown that nitric oxide (NO) plays a major role in the dilation of inner retinal vessels [31, 118, 155]. The NO isoform, NOS-3, is expressed in the vascular endothelial cells of the retinal vessels, capillaries and pericytes. The existence of a pre-NO gradient from the vitro-retinal surface to the vitreous indicates a continuous production of NO to maintain tone. As well, oxygen sensitive red blood cells (RBC) can induce an NO-mediated vasodilatory response. There are two basic pathways in which NO production leading to a vasodilatory response; flow dependent and receptor stimulated NO formation. Flow dependent NO formation is generated by the shear stress of blood acting on the vascular endothelium, which causes release of calcium and subsequently NO production. In receptor mediated NO formation, endothelial receptors for a variety of ligands stimulate calcium release and subsequent NO production. In either situation, vasodilation will improve blood flow to tissue with low oxygen affinity. Potent vasodilating factors are subclass of prostaglandins; prostacyclin (PGI_2). Prostoglandins are produced by the retina and choroid and play a role in the physiological regulatory response to hypercapnia and induce dilation [131, 171] and changes in ocular perfusion [74]. Lastly, adenosine is a product of adenosine triphosphate and a potent vasodilator in many vascular beds. Adenosine also plays a role in hypoxia-induced vasodilation and retinal autoregulation by potentiating the action of endogenous extracellular adenosine [49, 108].

Endothelial derived constricting factors (EDCFs) elicit a vasoconstricting response. EDCFs include; endothelin-1 (ET-1), angiotensin II, and cyclooxygenase (COX) products, such as thromboxane-A2 (TXA2) and prostaglandin H2 (PGH2) [133]. Among the various EDCFs, ET-1 is a major player responsible for modulation of blood flow in the eye and is expressed by retinal neurons and astrocytes. ET-1 has a large influence on retinal arterioles under both normoxic and hyperoxic conditions [127].

NO and ET-1 are continuously secreted by the surrounding endothelial cells. A fine balance between the two opposing factors is critical for proper regulation of the retinal vasculature. A possible disturbance of this balance between NO and/or ET-1 has been proposed in diseases such as glaucoma [20, 71] and diabetic retinopathy [24, 154]. Further, this disturbance has been suggested to trigger a series of events that lead to vascular dysfunction found in diabetes [125].

1.4.1.2.2 Metabolic Autoregulation by Arterial Gases

Oxygen (O_2) is important to retinal function and arterial oxygen tension (pO_2) is an important determinant of retinal blood flow [56]. Arterial oxygen tension is the amount of oxygen dissolved in the blood. In healthy individuals, arterial blood entering the capillaries has a pO_2 of 100mmHg. The inner retinal microcirculation readily adapts to changes in physiological variation in oxygen saturation to maintain adequate oxygenation to the neuropile [7]. For this to occur, some mechanism must be coupled to determine oxygen utilization within the tissue bed. One possibility is that the vascular endothelium serves to control the smooth muscle cell to a variety of circulating vasoactive stimuli. Also, within the past decade evidence has suggested that red blood cells themselves respond to low O_2 environment and respond to the metabolic need of the tissue [34]. The demand for adenosine triphosphate (ATP) results in an unusually high rate of glycolysis and lactate production. ATP is needed for the active neuronal transport processes maintaining the ionic gradients necessary for electrical activity and visual transduction [132]. As evidenced in human and animal studies, the uniqueness of the retinal autoregulation mechanism is its high dependence on arterial oxygen tension, unlike for example the cerebral circulation [94].

When oxygenation reaches extreme levels this can have a profound impact on the retina and the microcirculation. Exaggerated hyperoxia in certain cases can lead to damaging effect on retinal cells.

For example, various oxidants and oxygen free radicals that are associated with ischemic reperfusion injury appear to be retinotoxic. Likewise, prolonged oxygen deprivation can also lead to permanent damage and trigger retinal pathologies. A prolonged hypoxic retina by circulatory failure or obstruction can stimulate neovascularization by stimulating the production of the growth factor VEGF . Recently it has been suggested that vascular tissue stretching increased thymidine uptake and VEGF production in capillaries, leading to neovascularization [164, 169]. Chronic hypoxia induces endothelial cell proliferation to re-vascularize the deprived retina. The growth of these new, fragile and unsupportive vessels can have perilous ramifications on the pre-retinal and vitreous space. Improved tissue : oxygenation ratio by laser treatment has been shown to lower VEGF production and capillary stretching, reducing new vessel growth through these two mechanisms [3, 8]. It is clear that oxygen demand must be precise and dynamically balanced through metabolic autoregulation to ensure the health of the retina. Efficient autoregulation of blood flow maintains retinal homeostasis over a considerable range of blood oxygen levels as evidenced in the active retina of both anaesthetized animal and human models [185].

Analogous to its effect on the cerebral arteries, carbon dioxide also has a potent effect on retinal vessel tone. Hypercapnia (elevated carbon dioxide arterial tension) leads to vasodilation of the retinal vessels. Studies that have investigated the normal retinal response to hypercapnia have found consistent increases in inner retinal blood flow to CO_2 [30, 173, 174, 176, 177] and a relatively exaggerated effect on the cerebral vasculature [94].

1.4.1.2.2.1 Vascular Response to Alterations in Arterial Gas

The oxygen reactivity of the retinal vascular bed is of major importance to the functional integrity of the retina. Moreover, alterations in this tightly regulated system may play a pathogenic role in

diabetes mellitus. Impaired vascular reactivity has also been demonstrated in the pathogenesis of other retinal diseases, including that of age-related macular degeneration [153, 186], glaucoma [57, 175] and diabetic retinopathy[35, 51, 52, 62, 120, 123, 170]. Studies that investigate the vessel response to a variation in arterial gas may be a useful quantitative evaluation of vascular health.

Decades of studies have described in both animal and human models the hemodynamic response of the retina to acute hyperoxia [143], characterized by vasoconstriction and decreased blood velocity. Inhaled hyperoxia leads to an increase in arterial partial pressure of O_2. Normal inhalation of 100% (hyperoxia) oxygen for 5 minutes cause a 12% and 15% decrease in vessel diameter in the large retinal arterials and veins, respectively [62, 143]. Hyperoxic induced vasoconstriction reduces blood flow of the main retinal artery by up to ~60% and less oxygen penetrates the retina, but the retina receives additional oxygen from the less responsive choroidal circulation. This observation suggests that autoregulation occurs mainly in the inner retina; compensating for the variation in oxygen tension by adaptation of the inner retinal circulation only. The underlying mechanism mediating the hyperoxia induced vasoconstriction in the retinal bed is still unclear. However, results from animal studies found that the vasoconstricting response may be mediated by the increase in ET-1 production [26]. Biochemical mechanisms mediating vasoconstriction involve the interaction between retinal PO_2 and L-arginine/nitric oxide (NO) pathways [155]. Patients with diabetes alone and diabetes with concomitant hypertension have demonstrated an impairment of vascular response to an isocapnic hyperoxia stimulus [50, 51].

Hypoxia, on the other hand, is the decrease in arterial pressure O_2. In 1940 Cusick, Benson, and Boothby found that inhalation of 10% oxygen (hypoxia) by normal healthy subjects elicits a

vasodilatory response of the retinal vessels [105, 163]. Relevant mediators responsible for the physiological vasodilation of the retinal vessels include prostacyclin and nitric oxide (NO). ET-1 can interact with three types of endothelin receptors; ET_A, ET_{B1} and ET_{B2}. Unlike its interaction with ET_A and ET_{B1} receptors which leads to vasoconstriction, ET_{B2}- receptor is present on endothelial cells and mediates vasodilation via the release of NO [127, 129, 150]. Recent studies have found a reduction in ET-1 levels in patients with NPDR compared to controls, suggesting that this could account for the increase in retinal blood flow found in patients with PDR [121]. More recent work also strongly suggests that retinal arterial vasodilation involves the activation of plasma membrane Ca^{2+}- ATPase [18, 28]. Low retinal oxygenation has damaging effects on photoreceptors [132] and it is now known to play a role in the pathogenesis of various vascular diseases, including diabetic retinopathy, vascular occlusive diseases, and glaucoma.

Summary

Retinal blood flow is regulated by several mechanisms including myogenic and metabolic autoregulation. In order to maintain flow homeostasis and adequate metabolism, these mechanisms are unlikely to operate independently of one another. Blood flow regulation occurs under the influence of vasoactive molecules released by endothelial cells. All these autoregulatory mechanisms have been shown be disturbed in diabetes mellitus facilitating the breakdown of the blood retinal barrier [50, 66, 99, 138, 159].

1.5 Quantification of Retinal Blood Flow

The development of imaging modalities has allowed us to characterize the vascular pathology of various ocular and systemic diseases, although the outcomes are often contradictory depending upon

a number of factors, not least the method of assessment. These modalities and analysis techniques facilitate very precise and comprehensive study of retinal, choroidal, and retrobulbar circulations.

1.5.1 The Doppler Principle

Discovered in 1842 by Christian Doppler, the Doppler Effect is the change in frequency of a wave perceived by a moving observer relative to the frequency of the source of the wave. Laser Doppler devices are based on the Doppler principle and are used to assess blood velocity in retinal hemodynamics. Since moving red blood cells are surrounded by stationary tissue, light of non-shifted frequency acts as a reference frequency (f). Light reflect by the moving blood cells is Doppler shifted and returns with an altered "Doppler shifted" frequency (f'). The velocity of a moving blood cell is proportional to the frequency difference ($\Delta f = f - f'$) of shifted and non-shifted frequencies. The Doppler shift is highly dependent upon the angle (α) between the beam of light transmitted from the detector and the moving red blood cells.

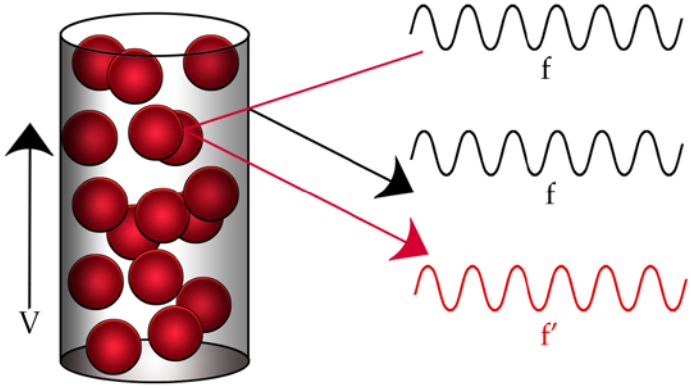

Figure 1-3 The Doppler principle

Two frequencies are reflected back from the original beam; 1) light scattered from the stationary tissue with frequency f and 2) is the scatter from the red blood cells (moving at velocity V) resulting in a frequency of f'.

1.5.2 Laser Doppler Velocimetry

One approach to measuring retinal blood velocity in retinal arterioles or veins is laser Doppler velocimetry (LDV). This approach is based on the principle that the Doppler shift of light is proportional to the blood velocity when the vessel is illuminated with a single high coherent laser beam. The beam is scattered across the vessel and the moving erythrocytes. The back scatter consists of light at the frequency of the light source and the frequency of shifted light. The Doppler shift corresponds to a spectrum of all flow velocities. The maximum frequency shift corresponds to the maximum centerline velocity within the vessel.

To acquire information of volumetric blood flow, measurement of the vessel diameter is necessary. This is accomplished by a fundus camera based technique using photography or continuous video recording of the vessel. With the diameter estimation, the cross sectional area of the vessel can be found and this is multiplied with the mean flow velocity. The product of blood velocity and vessel diameter is an estimation of the volumetric blood flow in a retinal vessel. This approach is currently limited, however, to larger vessels and only the maximum center velocity can be determined. Quantitative information of the velocity profiles cannot be extracted from the power spectrum. Therefore, calculation of flow is based on a theoretical relation between the maximum centerline velocity and all other unmeasured velocities within the vessel. The previous optical set-up described by Feke and Riva (1978) [41, 142] did not measure absolute velocity due to the difficulty in determining the angles between the incident laser light and the moving blood cells. Therefore, the largest problem with any Doppler technique that employs a single photodetector is that the measured velocity is highly dependent upon the angle between the beam of light transmitted from the detector and the moving red blood cells.

1.5.3 Bidirectional Laser Velocimetry

In a complicated system, the reflected Doppler signal contains a large number of velocities. Using bidirectional LDV, an absolute determination of maximum centerline blood velocity is possible. To quantify the absolute centerline blood velocity, two photo-detectors separated by a known angle are used to assess the difference in frequency shift detected between the detectors [41, 141]. The two photomultiplier tubes are used to sample the Doppler shifted light. Since the magnitude of Doppler shift is highly dependent on the angle between the detector and the motion of the erythrocytes and angle (α) between the reflective source and two detectors is known, each detector observes a different magnitude of Doppler shift. The velocity is determined by the difference in the frequency shift from the two directions in which the detectors are orientated, represented as K1 and K2 (Figure 1-4).

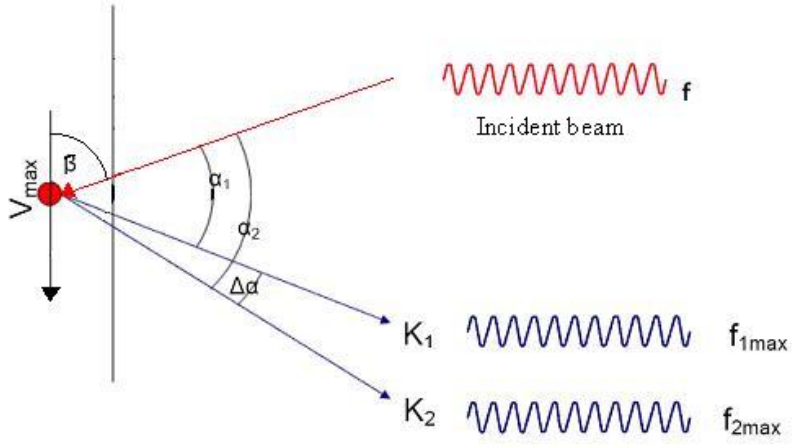

Figure 1-4 Diagrammatic representation of bidirectional laser Doppler velocimetry.

The incident beam with frequency (f) strikes to the vessel with red blood cells moving with centre-line velocity Vmax. The maximum frequency shift is detected at K1 and K2 that are separated by a known angle (adapted from Gilmore et al, 2007).

When an incident beam strikes the moving red blood cells (RBC) it is known that a vessel that exhibits Poiseuille flow will result in a frequency shift of up to a maximum f_{max} [40]. This shift Δf_{max} corresponds to the maximum velocity at the centre of the vessel lumen.

$$\Delta f_{max} = f_{2\,max} - f_{1\,max}$$

Where f1 max and f2 max are the maximum frequency shifts of K1 and K2 are the cut-off frequencies recorded from the two directions of scattered light, respectively. First described by Riva et al [126], V_{max} can be calculated from the difference in f1 and f2 max.

$$V_{max} = k\,(f_{2\,max} - f_{1\,max})$$

Where V_{max} is the maximum centerline velocity of blood and k is a constant dependent on the wavelength of the laser, the axial length of the eye, the refractive index of the medium and the geometry of the path of the laser light in the fundus camera [126].

Volumetric flow rate is determined from Vmax and vessel diameter.

$$\text{Flow} = 1/2.\pi.D^{2}/4.\text{Vmax}.60$$

where D= vessel diameter and V= mean velocity across a cardiac cycle [40, 184].

1.5.4 Canon Laser Blood Flowmeter

The Canon Laser Blood Flowmeter (CLBF) is the only instrument that applies bidirectional quantification of blood velocity while simultaneously measuring vessel diameter to derive retinal blood flow in real units. More importantly, since centerline velocity values depend highly on the centering of the incident beam on a retinal vessel, the CLBF has a built in eye tracking device that eliminates errors caused by significant horizontal saccades. Using this method, reliable results can be compared within different areas of one retina or between the retinas of different individuals.

Figure 1-5 Photograph of the Canon laser blood flowmeter (model 100).

Fundus camera like laser head mounted on a table and attached to a chin rest (left). The measurements acquired by the CLBF are displayed on a computer (right) and analyzed using custom software provided by the manufacturer.

This CLBF displays a 30 degree image of the retina. The instrument is equipped with two lasers; a green (543nm, 1500 µm × 150µm rectangle) HeNe laser used for diameter measurements and eye tracking and a red (675 nm, 80 µm × 50 µm ellipse) diode laser used for the velocity measurement. The green HeNe laser tracking strip is rotated so that it is

perpendicular to the axis of the vessel. The position of the tracking laser on the photodetector array is updated every 4 ms and centering is achieved, via a negative feedback loop that controls a galvanometer to stabilize the relative position of the green laser, to a precision of ± 5µm under normal eye movements [112, 113]. The red ellipse diode laser is centered in the rectangular green HeNe laser so that it is also centered onto the target vessel [41]. The red laser captures velocity measurements every 0.02 sec throughout a 2 sec measuring window. Analysis of the vessel image provides a means for vessel diameter measurement. The diameter is determined by automatic computer analysis of the signal produced by the image of the vessel; using half the [41]height of the transmittance profile to define the vessel column edge. The diameter is measured every 4 ms during the first 60ms and during the last 60 ms of each 2sec measurement window. Two separate 2 sec measurement windows are required for accurate measurement of a target site (path 1 and path 2). The additional measurement with a different incident beam angle eliminates ambiguity in the orientation of the incident and scatter beam [38].

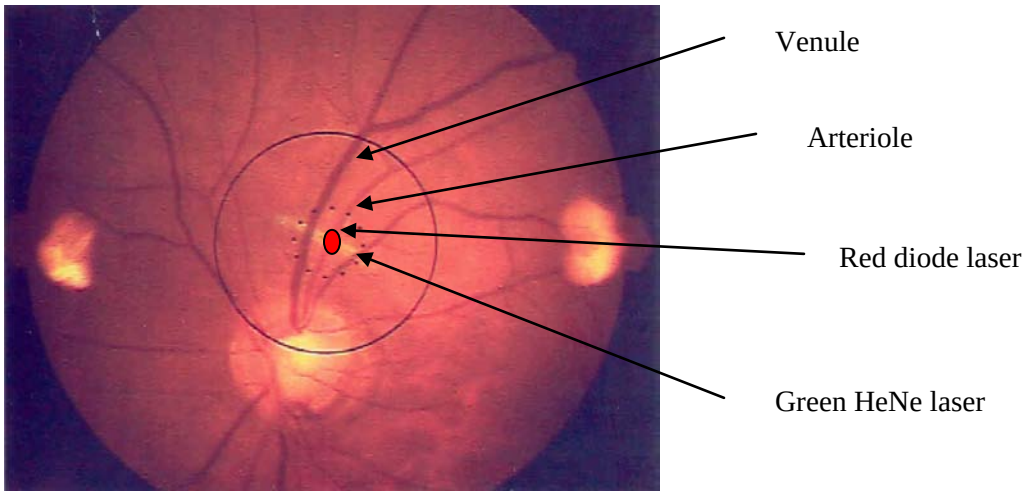

Figure 1-6 Photograph of the fundus acquired using the Canon laser blood flowmeter

Photograph shows fundus including the - optic nerve head, superior temporal arteriole and venule. A HeNe laser of 543nm wavelength is centered on the arteriole of interest and oriented perpendicular to

the vessel segment for measurement of diameter. The red diode laser is centered within the green laser for velocity measurement.

The CLBF is the only device that can calculate volumetric flow in the retina in absolute units and has been described in detail elsewhere [40]. Older versions of laser technology were unable to automatically track the center of a vessel [41, 142]. Unlike previous devices that require two separate imaging tools [15], the CLBF is the only instrument that simultaneously measures velocity and diameter.

The reproducibility of the CLBF has been reported for diameter, average velocity and blood flow in separate studies [45, 67, 93, 117, 184]. For diameter, coefficient of variation for repeated measurements (COV) ranged from 1.9% to 4.9%; for average velocity, COV ranged from 4.7% to 19.9% and for blood flow, COV ranged from 6.7% to 19.3%.

Summary

To date, the CLBF has already proven to be useful in elucidating aspects of physiology and pathophysiology in health and disease. In previous investigations of baseline retinal circulation found that changes retinal arteriolar pulsatility were related to the risk of developing diabetic macular edema (DME) [66]. Subsequently, the retinal vascular response to systemic hyperoxia was found to be impaired in patients with diabetes and early DR compared to patient with non-diabetes [50]. Moreover, the investigation of retinal circulatory abnormalities using the CLBF have also been investigated in other ocular diseases such as glaucoma [9, 175], age related macular degeneration [153]

and blood pressure changes [149] Overall, the CLBF has proven to be an effective tool in the investigation of retinal hemodynamics in both health and disease [9, 45, 52, 153, 153]

1.6 Retinal Hemodynamic Disturbance in Diabetic Retinopathy

1.6.1 Diabetic Retinopathy

Diabetic retinopathy (DR) is the leading cause of new vision loss and blindness among working age people in the Western world [6]. Although the exact mechanism of DR is yet to be elucidated, chronic hyperglycemia initiates the process leading to microvascular disruption [59, 63]. Two landmark studies, the Diabetic Retinopathy Study (DRS) and the Early Treatment Diabetic Retinopathy Study (ETDRS), have demonstrated the effectiveness of laser treatment by reducing severe vision loss by 90%. Further, The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) established the value of intensive glycemic control in reducing the risk of onset and progression of DR and other microvascular abnormalities.

1.6.2 Clinical Presentation of Diabetic Retinopathy

Although the pathogenic mechanism of DR has not been clearly defined, the clinical progression of DR is well understood. Diabetic retinopathy is classified into two stages; background or nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). Changes in NPDR stage occur in the early phase of the disease and may or may not be followed by PDR, which is characterized by neovascularization and vitreous involvement.

Early NPDR results from primarily lesions of the capillary vessels. This later extends to larger vessels; venules and arterioles. However, preceding these clinical features of DR, histological changes take place that include thickening of the basement membrane, loss of pericytes and smooth muscle cells and eventually the formation of microaneurysms. Microvascular changes that are

characteristics of NPDR include microaneurysms (MA), blot haemorrhages, cotton wool spots, intraretinal microvascular abnormalities (IRMA) and/or macular edema.

Proliferative Diabetic Retinopathy (PDR) is characterized by new vessel growth either on the optic disc or on the periphery of the retina. New vessel formation arises when there is a large avascular area. This uncontrolled vessel growth develops a fibrous covering which has the potential to break through the internal limiting membrane and attach to the posterior surface of the vitreous[(96)].

Diabetic macular edema (DME) is the most frequent cause of vision loss in patients with diabetic retinopathy. The pathogenesis of DME results from the breakdown of the blood-retinal barrier (BRB) which leads to the accumulation of fluid within the intraretinal layers of the macula region [(12)]. Local factors that influence DME development include angiogenic factors such as vascular endothelial growth factor (VEGF), protein kinase C, protaglandins, growth hormones, and the local anatomy of the posterior hyaloid face inducing to vitreomacular separation [(82)]. At any direction during the progression of DR, patients with diabetes can develop DME.

Overall, several factors are important in the evolution of diabetic retinopathy. One possible mechanism in the pathophysiology of DR is abnormal retinal blood flow. Retinal blood flow has been investigated and a number of studies have shown vascular dysregulation in patients with diabetes [(51, 58, 66, 98, 122, 159)].

1.6.3 Retinal Hemodynamics in Patients with Diabetes

Retinal blood flow abnormalities have been investigated in detail in patients with and without diabetes. Whether or not, retinal blood flow of patients with diabetes is different from healthy

individuals is still controversial since there are conflicting evidence between studies. For instance, patients with diabetes with no signs of retinopathy have shown to; increase [60], decrease [19, 23] and show no difference [39, 50, 65, 66, 122] in changes in blood flow. Studies of patients with background retinopathy have also demonstrated an increase [61, 122, 183], decrease [11]and no difference [14, 23, 50, 65, 66] in retinal blood flow. Thus, there is evidence that vascular abnormalities occur both before and during the development of clinically evident diabetic retinopathy.

1.6.4 Loss of Compliance in patients with increasing severity of NPDR

Previously published studies from our lab have found that in homeostatic blood flow measurements, there is a loss of compliance, i.e. an increase in rigidity, in early DR including patients with increasing risk for diabetic macular edema (DME) development [66]. Moreover, arterial stiffness is enhanced in patients with diabetes [102] and atheroschlerosis [179]. The outcome of arterial stiffness is an increase in pulsatile blood pressure from a higher systolic pressure and a lowered diastolic pressure. The retinal maximum-to-minimum (max:min) velocity ratio reflects vascular compliance where an elevation of max:min ratio indicates increased vascular rigidity [66]. The effects of arterial rigidity from diabetes, hypertension and age [116] have been found to be detrimental to the retinal microvasculature [103, 140, 181].

1.6.5 Vascular Reactivity in Patients with Diabetes

Vascular reactivity refers to the response of the blood vessels as a result of a provocation. Non-invasive and quantitative measurements of retinal blood flow in combination with standardized vaso-active stimuli have enabled in vivo hemodynamic studies of human retinal vascular bed regulation in health and disease. Administration of oxygen (O_2) has been used to provoke a response in previous studies [50, 51, 53, 54, 62, 64, 95, 123, 143, 163]. Overall, retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant level.

Although healthy human studies have found a reduction in retinal blood flow to 100% oxygen breathing, the exact amount is a uncertain; a 60% reduction in retinal blood flow by laser Doppler velocimetry [62, 143] whereas others reported a 30% reduction in retinal blood flow [37, 163]by blue field entoptic technique. Discrepancies between results can be partly attributed to differences in hemodynamic measuring and gas delivery techniques.

Previously published retinal vascular reactivity studies have detailed methods used to deliver the hyperoxic stimulus while controlling the arterial partial pressure of carbon dioxide (PCO_2) [36, 37, 50, 53, 148]. This is necessary since an increase in $PETO_2$ in hyperoxia tends to result in a concomitant reduction in PET CO_2. Past studies have used a non-standardized closed loop system while manually adding small amounts of CO_2 to counteract the $PETCO_2$ change [76, 77, 79]. However, this method results in high inter-subject variability [22, 146]. Recognizing the need to eliminate the combined effects of elevated PO_2 and reduced PCO_2, a sequential *re-breathing system* circuit was designed composed of a fresh gas reservoir, an expiratory gas reservoir and a face-mask (Hi-Ox^{SR}, ViasysHealthcare, Yorba Linda, CA). The inspiratory and expiratory limbs are interconnected by a single positive end-expiratory pressure (PEEP) valve, allowing exhaled gas to be re-breathed when the gas in the inspiratory limb was depleted [54]. This system allows for the maintenance of homeostatic PCO_2, termed *isocapnia*. This system provides a feedback loop to compensate for any hyperventilation induced by a reduction in PCO_2. Therefore, eliminating the exaggerated affects that are caused by PCO_2.

In the physiological situation, compliance is expected to reduce and rigidity increase during hyperoxia due to increased tonus of the vessel wall in response to a vasoconstrictive stimulus. Results

from Gilmore et al [50] indicate an inability to increase vessel tonus and reduce shear stress (as indicated by the absence of change in max:min velocity ratio and WSR, respectively) with respect to an isocapnic hyperoxic provocation in patients with increase risk for DME development. Impaired responses to hyperoxia suggest damage in the diabetic vasculature and possibly reflect mechanisms of atherosclerosis. This impairment precedes change in homeostatic blood flow parameters. Overall, altered vascular reactivity to hyperoxia is a sensitive marker of functional changes in early diabetic retinopathy.

Summary

Retinal arteriolar vasoconstriction caused by an isocapnic hyperoxic stimulus is a useful method to test the efficacy of the retina to regulate blood flow. Thus far, studies have indicated that retinal vascular dysregulation is an early process in diabetic retinopathy.

1.7 Inflammation and Endothelial Dysfunction in NPDR

1.7.1 Vascular Inflammation in Diabetes

Inflammation has been strongly implicated in both diabetes and atherosclerosis [13, 73, 158]. Monocytes from poorly controlled diabetes patients have shown to induction of inflammatory mediators such as protein kinase C and nuclear factor- κβ [27]. Particularly, vascular inflammation promotes expression of interleukin-6 (IL-6), vascular cellular adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein (MCP-1). Additionally, studies have shown a marked decrease in NO bioavailability, leading to impaired endothelial function [80]. Inflammation contributes to the pathogenesis of diabetic retinopathy [85, 86, 91, 172], nephropathy [110, 144, 151] and neuropathy [84, 101, 136].

1.7.2 Pathogenesis of DR Mechanisms; Retinal Leukostasis

Although the exact pathogenesis of diabetic retinopathy remains unclear, results from two large studies, Diabetes Control and Complications (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), have confirmed that hyperglycemia is a major factor in the development. Prevailing biochemical theories that support the role of glucose in the development of retinopathy include; the polyol (sorbitol/aldose reductase) pathway flux, hexosamine pathway [137], accelerated formation of advanced glycation end-products (AGEs) [167], hemodynamics changes[97], oxidative stress [104], and activation of diacylglycerol and protein kinase C beta (PKCβ) isoforms [180]. In 2005, Krady and colleagues found that inflammation and the activation of microglia, which are immune cells in the central nervous system (CNS) and the retina, play a role in the pathogenesis of DR. Evidence in this study suggest an antibiotic with anti-inflammatory properties has the potential to block activation of microglia and prevent DR. It is this and other accumulating evidence that suggests that increased leukocyte adhesion to the endothelial wall and entrapment (leukostasis) is an early event in DR development. NPDR manifests as permeability secondary to inflammatory processes leading to iBRB disruption [6], basement membrane (BM) thickening [168], pericytes and smooth muscle loss which eventually results in widespread nonperfusion, hypoxia, neovascularization and PDR [91].

1.7.3 Leukocyte-Endothelial Interaction

As demonstrated in fluorescein angiography, a sign of diabetic retinopathy (DR) is decreased local perfusion, a product of capillary occlusion [100]. The exact pathological mechanisms remain unclear. Mechanisms that contribute to the degeneration of retinal capillaries in diabetes include 1) occlusion of vascular lumen by white blood cells or platelets 2) death of capillaries secondary to biochemical abnormalities within the vascular cells themselves 3) death of capillaries secondary to products generated by nearby cells (such as neurons or glia) and 4) increased blood viscosity. Recent studies have suggested that leukocytes play a key role in DR development. There is now a general acceptance

that DR represents a low grade chronic inflammatory disease [2]. This inflammatory condition is associated with increased leukocyte entrapment (leukostasis) in the retinal capillaries, areas of capillary nonperfusion and areas of endothelial damage [87]. In the investigation between inflammation and retinopathy, the Hoorn study found a close association between inflammatory activity and progression of retinopathy [172]. Further supporting the involvement of leukocytes, Meleth and colleagues [111] also showed a close relationship between serum inflammatory markers, cell adhesion molecules, and the severity of diabetic retinopathy.

The pathogenic mechanisms mediating the leukocyte-endothelial cell adhesion include increased expression of cell adhesion molecules on the surface of endothelial cells and changes on the leukocyte surface. Leukocyte adhesion to the vascular endothelium, a key step to in the inflammatory process, involves three steps. The adhesion of leukocytes is largely dependent upon the interactions between the endothelial cells and the leukocyte expressed adhesion molecules [55]. The initial and key step to the recruitment of leukocytes is the “low affinity” attachment to adhesion molecules by three member of the selectin family; E-, P- and L- selectin. E-selectin is expressed by the endothelial cells, P-selectin is expressed by cytosolic storage granules and L-selectins are expressed on leukocyte surface [161]. The interactions between the selectins and their counter parts cause the “tethered” leukocyte and resist detachment caused by physiological shear forces secondary to blood flow. The leukocyte rolls along the vascular endothelium drawing it into close proximity facilitating the chemokine-induced integrin avidity to the vessel wall [5]. The firm adhesion step, resistant to physiologic shear stress, is mainly by endothelial ligands of the CAM family; ICAM-1, ICAM-2, VCAM-1 and MadCAM [161].

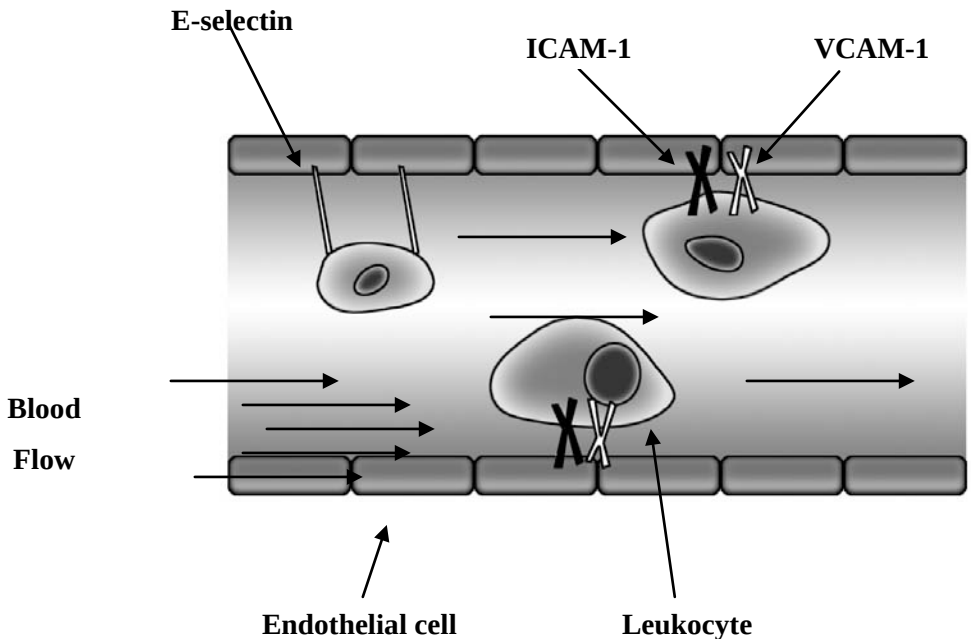

Figure 1-6 A schematic diagram of increased leukocyte-endothelial cell adhesion and entrapment (leukostasis) in diabetic retinopathy.

Diabetes-induced expression of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule type 1 (VCAM-1) and E-selectin, causes increased leukocyte-endothelial cell adhesion and lead to capillary nonperfusion and vascular damage in diabetic retinopathy (adapted from Chibber et al, 2007).

1.7.4 Circulating Markers of Inflammation

Early in the inflammatory process, release of chemokines such as monocyte chemoattractant protein (MCP-1) and others are released from stressed tissues. In diabetes this has been clearly demonstrated in the vascular endothelium and adipose tissue. These chemokines cause an increase in expression of interstitial and vascular cell adhesion molecules. Several plasma markers of inflammation have been evaluated as potential tools for prediction of microvascular outcomes in diseases such as DR [(172)]. Among the markers of systemic inflammation are high sensitivity C-reactive protein (hsCRP) and cellular adhesion molecules (CAMs). Adhesion molecules are endothelial cell surface glycoproteins

that facilitate the attachment of leukocytes in inflammation and include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [21, 85, 152].

1.7.4.1 C-reactive Protein (CRP)

C-reactive protein (CRP) is a prototypic marker of underlying systemic inflammation. CRP is synthesized by the liver and responds to factors released by the adipose cells. These factors increase the expression of vascular cell adhesion molecules and attract monocytes that gain access to stressed sites. At the site of inflammation, chemokine induced proliferation and pro-inflammatory gene activation produces cytokines, like members of the interleukin (IL-) family. Interleukin-6 (IL-6) appears to be an important stimulator of CRP production. Therefore, CRP is a clinically relevant marker of inflammation. These underlying factors stimulate endothelial induced expression of genes that include: reactive oxygen species (ROS), oxidized lipids, reduced nitric oxide (NO), increase angiotensin II and advanced glycation end products (AGE). The combination of endothelial derived factors and macrophage migration contribute to the generation of altered vaso-reactivity through the establishment of a procoagulant state via expression of a series of platelet activating factors such as factor VIII. Many of these enter the blood circulation at levels that have been found to correlate with the degree of inflammatory activity. Studies have demonstrated the association between CRP and the incidence of diabetes development [172]

1.7.4.2 Intercellular Adhesion Molecule-1 (ICAM-1)

Intercellular adhesion molecule type 1 (ICAM-1), also known as CD54, is a ubiquitous trans-membrane glycoprotein that is a key mediator in leukocyte migration and activation. ICAM-1 functions as a ligand for LFA-1 (CD11a/CD18) which is leukocyte expressed. At the site of inflammation, ICAM-1 is up-regulated and mediates adhesion of leukocytes expressing LFA-1. This protein is basally expressed on a limited number of endothelial cells and monocytes. It is widely

inducible or up-regulated by endothelial cells, lymphocyte and macrophage [160]. Estimate of the mean level sICAM-1 range from 102-450 ng/ml in normal individuals [48]. sICAM-1 has been reported in inflammation, allergy, autoimmune diseases, infections and cancers. Specifically, elevated levels of sICAM-1 are associated with cardiovascular disease, type 2 diabetes, hypertension, organ transplant dysfunction, oxidant stress, liver disease and certain malignancies [160]. Investigative animal studies found that mice deficient in CD18 and ICAM-1 had reduced adherent leukocyte, less endothelial cell damage and lesser vascular leakage, which are key in the early development of diabetic retinopathy [85]. Studies have found that the prevalence of DR is positively associated with measured levels of soluble ICAM-1 [172]. This highlights the important role of ICAM-1 in retinal leukostasis and in the pathogenesis of DR.

1.7.4.3 Vascular Cell Adhesion Molecule-1 (VCAM-1)

Vascular cell adhesion molecule type 1 is also a glycoprotein belonging to the immunoglobulin super family. The counterpart for VCAM-1 is the VLA-4, an integrin expressed on the family of leukocytes: monocytes, lymphocytes, basophils and eosinophils. VCAM-1 is expressed on cytokine activated endothelial cells, dendritic cells, macrophages and epithelium. Unlike ICAM-1, VCAM-1 is not significantly expressed on un-stimulated endothelial cells. Estimates of the mean level of sVCAM-1 range from 431-504 ng/ml in normal individuals [48]. Increased sVCAM-1 has been found to be associated with inflammation, infectious diseases, vasculitus and diabetes mellitus [160].

1.7.4.4 E-selectin

E-selectin is considered a specific marker of activated endothelium exclusively expressed by endothelial cells. Unlike ICAM-1 and VCAM-1 which are expressed by different types of cells, the origin of circulating E-selectin is easily identified. The restriction of E-selectin expression to activated endothelium makes this adhesion receptor an ideal marker of endothelial cell activation. The

normal levels of E-selectin range from 1-50 ng/ml [160]. Marked elevation of E-selectin occurs in plasma of patients with septic shock, a condition of acutely decreased tissue perfusion and oxygen delivery, an example in which this marker serves as a sensitive parameter for widespread endothelial cell injury. Specifically, E-selectin has been found to be elevated in patients with hypertension. The absence of correlation between E-selectin and von Willebrand factor, a marker of endothelial injury, suggests that elevated levels of E-selectin in hypertension do not reflect endothelial injury but rather an altered endothelial cell activity. Previous studies found that baseline sE-selectin levels were associated with progression of retinopathy [162].

Soluble forms of these both adhesion molecules ICAM-1, VCAM-1 and E-selectin have been described and detected in the plasma and other bodily fluids. In the investigation between inflammation and retinopathy, the Hoorn study measured levels of C-reactive protein (CRP), soluble intercellular adhesion molecule (sICAM-1), von Willebrand factor and soluble vascular cell adhesion molecule (sVCAM-1) [172]. The increased levels of circulating adhesion molecules, thus, may reflect local expression of adhesion molecules in an immuno-pathologic process. Studies of immuno-pathologic diseases have identified different patterns of expression which are dependent on the disease stimulus. Although many results that support diabetes induced changes come from large vessels, studies do suggest that high glucose promotes upregulation of cell surface adhesion molecules such as ICAM-1, E-selectin, P-selectin [88] in both human and animal models. In vitro studies in cultured human retinal capillary endothelial cells (HREC), confirmed that high glucose enhances leukocyte-endothelial interaction through increased ICAM-1 expression [83]. Similarly in human studies, soluble forms of ICAM-1 and VCAM-1 were found to be up-regulated and correlated with DR severity [92, 119]. The close association between high expression of adhesion molecules and the severity of DR suggests an important role in the development DR.

1.7.5 Endothelial Cell Dysfunction in Type 2 Diabetes

Endothelial dysfunction is defined as changes to the endothelial properties, as a result of injury, that can lead to its abnormal function. In terms of blood serum markers, this can be clinically assessed by von Willebrand factor production; a marker of endothelial cell injury. Amongst the widely distributed circulating adhesion molecules that potentially reflect inflammation, von Willebrand factor (vWF) released specifically by the endothelial cells reflects vascular endothelial injury and dysfunction. This marker has been shown to be important in diabetic retinopathy development.

1.7.5.1 von Willebrand Factor

Endothelial cells normally present a non-thrombogenic and anticoagulant surface for the flow of blood. However, following vessel damage and damage to the endothelial cells, their properties are modulated to ensure effective platelet aggregation and haemostatic plug formation. One of the main endothelial mediators for platelet formation is von Willebrand factor (vWF). vWF is a multi-dimeric glycoprotein that is secreted by exocytosis from granular stores [(124)]. It assists factor VIII in the formation of fibrin at the site of injury. Since vWF release is increased when endothelial cells are damaged, vWF levels have been a proposed indicator for endothelial dysfunction. Abnormalities of vWF have been demonstrated with patients with diabetes and may be involved in diabetic vasculopathy [(165)]. In diabetic retinopathy, Feng and colleagues found that increased vWF was associated with prolonged retinal circulation time and reduced retinal blood flow in patients with minimal or no DR [(42)]. However, the results of other studies with respect to vWf as a predictor of abnormal endothelial secretory function were less convincing [(109)]. Despite the discrepancies in the contributions of vWF to DR development, vWF is a clear common denominator in endothelial cell injury.

Summary

In recent years, increasing evidence suggests DR is a low-grade chronic inflammatory condition associated with leukocyte adhesion to the diabetic vasculature. Abnormal leukocyte-endothelial interactions are the result of increased expression of surface adhesion molecules (ICAM-1 and VCAM-1) that attach to specific integrin counterparts, leading to leukostasis. Moreover, endothelial dysfunction has been found to be marked by high expressions of endothelial specific proteins E-selectin and vWF in patients with DR. With the accumulation of leukocytes and endothelial cell changes, this is thought to lead to an increase in vessel permeability, ultimately influencing retinal blood flow. Overall, leukostasis and endothelial dysfunction has been found to be involved in the development of DR.

Chapter 2
Rationale

Many of the clinical changes in DR are thought to be related to abnormalities in retinal blood flow [9, 16, 23, 24], although direct proof of such a relationship is lacking. Work from our laboratory indicates that disturbance of retinal hemodynamics may provide a useful surrogate marker of the clinical progression of diabetic retinopathy [10]. Guan and co-workers (2006) found an increase of velocity pulse wave amplitude with increasing severity of mild-to-moderate NPDR indicating a loss of vessel compliance in early DR and the development of DME. The same study found no difference in arteriolar diameter, velocity and blood flow in the identical cohort.

There is also increasing evidence for the loss of retinal vascular regulation / reactivity in the diabetic vasculature [17, 24]. The retinal vasculature regulates local blood flow using intrinsic mechanisms, rather than being under central nervous system control. The blue field entoptic technique [24], flicker stimulus[6] and gas provocation [5] are a few methods applied to evaluate vascular regulation in patients with diabetes. Earlier studies found that the retinal vascular reactivity response to a non-standardized 100% oxygen breathing was reduced in diabetic patients with a wide range of DR including PDR. Our laboratory has recently developed an isocapnic hyperoxic stimulus to evaluate the vascular reactivity of retinal arterioles and found an impaired response in patients with *early* DR and increasing potential for development of diabetic macular edema [7]. The response of smooth muscle cells and endothelial cells to local metabolic factors is thought to be altered in diabetes, disrupting retinal vascular regulation. The standardized isocapnic hyperoxic provocation has proven to be an effective and reliable method to assess the magnitude of retinal vascular reactivity in *early* DR.

Abnormal leukocyte adhesion (i.e. leukostasis) to retinal vascular endothelial cells also occurs relatively *early* in diabetes [19]. Vascular damage is shown to be temporally and spatially associated with retinal leukostasis in experimental diabetes [12]. In non-proliferative DR, clinical outcomes are manifested by endothelial cell damage [1, 25], excessive permeability due to BRB disruption and subsequent dropout of retinal capillaries resulting in nonperfusion [2]. Hyperglycemia is suggested to promote vascular damage in DR by signaling the release of inflammatory cytokines which triggers the upregulation of vascular adhesion molecules [20]. Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that can stimulate vascular endothelial growth factor (VEGF) expression by the retinal pigment epithelium. Besides its angiogenic properties, VEGF up regulates intercellular adhesion molecule type 1 (ICAM-1) expression resulting in leukostasis and increased vascular permeability [4, 4, 11]. Other adhesion molecules such as soluble vascular cell adhesion molecule type 1 (VCAM-1) and E-selectin have also been known to participate in the attachment of leukocytes in the diabetic retinal vasculature [3, 15, 21].

Earlier studies have shown abnormalities of blood flow parameters along with increased leukocyte adhesion in the retinal capillaries in animal models, suggesting that these changes may represent a precursor to any clinical findings [18, 19]. Further, the Hoorn study has shown that circulating markers of inflammation and endothelial dysfunction are positively associated with clinical signs of DR [25]. However, the temporal relationship between abnormal retinal hemodynamics and increasing inflammatory activity or increasing endothelial dysfunction markers in NPDR in humans is unknown. Since blood flow into the capillary bed is largely determined by the upstream arterioles, it is essential to determine the relationship between leukostasis and retinal hemodynamics since one change may be

predictive of the other, thereby providing the possibility to develop surrogate markers. Excessive perfusion pressure may lead to capillary leakage effectively via hyperfiltration (Starling's Law), protein leakage, edema formation and damage to the capillaries and surrounding tissue. We hypothesized that an increase in circulating soluble adhesion molecules, signaling increased inflammatory activity, and an increase in endothelial dysfunction markers are associated with retinal hemodynamic disturbance in patients with early diabetes. The potential changes in retinal blood flow may be secondary to increased expression of cytokines or vice versa. This study would potentially lead to a further understanding of DR on a biochemical / cytokine-functional level and will ultimately lead to earlier and more effective therapeutic intervention. Overall, the results of such study may have important health implications.

The global purpose of this work is to assess the relationship between levels of circulating markers of inflammation and endothelial dysfunction and retinal hemodynamic parameters in patients with increasing severity of early NPDR. In patients with diabetic retinopathy, the following candidate molecules have been found to be elevated; intracellular adhesion molecule-1 (ICAM-1) [(13)], vascular adhesion molecule-1 (VCAM-1), E-selectin [(14)] and high sensitivity C-reactive protein (hs CRP) [(8)]. A marker of endothelial dysfunction in DR is von Willebrand factor antigen (vWf ag) [(22)]. Using standardized techniques developed in by our group, retinal blood flow and vascular reactivity to isocapnic hyperoxic provocation will be assessed. The association of inflammatory activity, endothelial dysfunction and arteriolar retinal hemodynamics will be investigated.

2.1 Aims

The first aim of the study is to determine the associations between markers of inflammation, endothelial dysfunction and retinal hemodynamic disturbance in type 2 diabetes; retinal

hemodynamic disturbance will be assessed in terms of baseline values and change over a 6-month period (Chapter 3). Inflammatory and endothelial dysfunctional makers (sICAM-1, sVCAM-1, sE-selectin, hs CRP and vWF) will be quantified and compared at baseline and then correlated with retinal hemodynamic parameters at baseline and in terms of change in hemodynamics over a 6-month period. Retinal hemodynamic parameters in this study include retinal arteriolar vessel diameter, centerline blood velocity, maximum-to-minimum velocity ratio and volumetric blood flow. We hypothesize that: 1) baseline arteriolar hemodynamics and 2) the magnitude of retinal hemodynamic disturbance over 6-month time are positively correlated with systemic markers of inflammation and endothelial dysfunction in type 2 diabetes.

The second aim of the study is to determine the associations between markers of inflammation, endothelial dysfunction and the magnitude of retinal vascular reactivity in type 2 diabetes; retinal vascular reactivity will be assessed in terms of the response of the vessels to isocapnic hyperoxic provocation at baseline and in terms of change over a 6-month period (Chapter 4). Inflammatory and endothelial dysfunctional makers (sICAM-1, sVCAM-1, sE-selectin, hs CRP and vWF) will be quantified and correlated with retinal hemodynamic parameters at baseline and in terms of change in hemodynamics over a 6-month period. Hemodynamic parameters in this study include retinal arteriolar vascular reactivity to an isocapnic hyperoxic provocation in terms of the measured response in vessel diameter, centerline blood velocity, maximum-to-minimum velocity ratio and volumetric blood flow. We hypothesize that; 1) the magnitude of retinal vascular reactivity at baseline 2) the disturbance of retinal vascular reactivity over 6 month time and are positively correlated with systemic markers of vascular inflammation and endothelial dysfunction in type 2 diabetes.

2.2 Relevance

Overall, the results of this study may have important health implications. This study aims to associate molecular (circulating markers of inflammation and endothelial dysfunction) and functional biomarkers (vascular function, retinal hemodynamics and arterial stiffness) that are thought to be relevant to the pathogenesis of *early* DR. If a relationship exists between circulating markers of inflammation and/or endothelial dysfunction and retinal hemodynamics then perhaps one or the other of these assays depending upon application, can be used as surrogate markers of vascular health. The discovery of such a relationship would significantly improve our understanding of the pathogenesis of early DR. Moreover, this study could indicate that standardized imaging techniques may provide tangible ways of predicating risk or monitoring progression of DR in patients. This study would potentially lead to a further understanding of DR on a molecular / cytokine-functional level and could ultimately lead to earlier and more effective therapeutic intervention.

Chapter 3

Baseline Retinal Hemodynamics, Systemic Markers of Vascular Inflammation and Endothelial Dysfunction in Type 2 Diabetes

3.1 Overview

Purpose: The purpose of this study was to investigate in patients with *type 2* diabetes and *non-proliferative* DR (NPDR): 1) The concomitant disturbance of retinal blood flow and systemic markers of vascular inflammation and endothelial dysfunction and 2) the associations between retinal blood flow and systemic markers of vascular inflammation and endothelial dysfunction.

Methods: This study consisted of 12 *Group 1* control subjects, 25 *Group 2* patients with mild-to-moderate NPDR (mean age 54 ± 11 y.o) and 13 *Group 3* patients with moderate-to-severe NPDR (mean age 60 ± 11 y.o). At the first visit, baseline retinal arteriolar diameter, blood velocity, maximum-to-minimum velocity ratio and flow were assessed non-invasively using the Canon Laser Blood Flowmeter (CLBF). From forearm blood, plasma concentrations of vascular inflammatory markers (sICAM-1, sVCAM-1 and sE-selectin) and the endothelial dysfunction marker (vWF antigen) were assessed by enzyme-linked immuno-sorbent assay methods. Hs CRP and A1c were also assessed at this visit. After 6 months, retinal hemodynamics was re-evaluated. The baseline systemic markers of vascular inflammation and endothelial dysfunction were correlated with baseline and change (Δ) in retinal hemodynamic parameters over a 6 month follow-up period and compared between and within groups.

Results: Maximum-to-minimum velocity ratio was found to be significantly elevated in the group 3 compared to group 1 at baseline (0.72 vs. 0.49, ANOVA $p<0.01$) and at the follow-up visit (0.77 vs. 0.55, t-test $p=0.02$). However, there were no significant differences in vessel diameter, blood velocity and flow between groups at baseline (visit 1) or follow-up visit (visit 2). There were no significant differences found within or between Group 2 and Group 3 in terms Δ in retinal hemodynamic parameters over the follow-up period.

Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA $p=0.02$ and $p=0.04$). A post hoc Bonferroni test showed that Group 3 had significantly higher sICAM-1 and sE-selectin levels compared to Group 1 (234.0 vs. 151.5 ng/ml, $P=0.02$ and 53.4 vs. 27.6 ng/ml, $P<0.01$, respectively). VCAM-1 and vWF were not statistically different (ANOVA $p=0.25$ & $p=0.69$, respectively) between groups. Hemoglobin A1c was significantly elevated across the groups (ANOVA $p<0.01$), with Group 3 having significantly higher hemoglobin A1c levels compared to Group 1 (7.9 vs. 5.6 %, $P<0.01$).

There were no significant associations found between baseline markers of inflammation and baseline retinal hemodynamics across all groups.

Δ velocity was correlated with the baseline sICAM-1 ($r=0.42$, $p=0.02$) and A1c levels ($r=0.37$, $p=0.04$) in patients with NPDR. After adjustment for all other variables (A1c, hsCRP and vWF), Δ velocity, sICAM-1 and A1c were found not to be reliable predictors of baseline retinal hemodynamics.

Conclusion: Overall, we found both an increase rigidity of the arteriolar circulation at baseline and follow-up and an increase vascular adhesion markers sICAM-1 and sE-selectin within the same population sample. Change in velocity over the follow-up period was correlated with sICAM-1 and A1c levels in patients with NPDR but the level of association was such that neither sICAM-1 and A1c proved to reliably predict retinal hemodynamics.

3.2 Introduction

Many of the pathological changes in non-proliferative diabetic retinopathy (NPDR) are thought to be related to abnormalities in retinal blood flow [11, 25, 42, 43, 62], although proof of a cause and effect relationship is still lacking. Previously reported retinal blood flow results in patients with NPDR are conflicting [61]. A variety of techniques have been utilized for the measurement of blood velocity and flow and this may contribute to the heterogeneity of the results. However, a disturbance in retinal vessel pulsatility has been an accepted marker of change in early diabetic retinopathy (DR) [27]. Using laser Doppler velocimetry and simultaneous vessel densitometry, our group has shown an increase of retinal arteriolar velocity pulse wave amplitude with increasing severity of mild-to-moderate NPDR in type 2 diabetes, indicating a loss of vessel compliance in relatively early DR that includes the development of DME [27]. There is also increasing evidence for a loss of retinal vascular regulation / reactivity to isocapnic hyperoxia in the vasculature of patients with early DR [44, 62]. A number of papers have been published using the quantitative laser Doppler velocimetry and simultaneous vessel densitometry technique to quantify the magnitude of volumetric blood flow perturbation as result of some form of provocation and due to the patho-physiological influence of disease [18-22, 26, 28, 39, 59, 67, 68].

Leukocyte-endothelial interaction plays an important role in the early development of DR [32, 48, 50, 55, 66]. A component of DR is a low-grade chronic vascular inflammation associated with increased leukocyte entrapment in the retinal vasculature [5]. The inflammatory process, termed leukostasis, is secondary to the enhanced expression of soluble adhesion molecules [5, 32, 56, 66]. These inflammatory mediators include soluble intercellular adhesion molecule (sICAM-1)[45, 47][37, 54, 66], vascular cell adhesion molecule (sVCAM-1) [37, 54, 66] and sE-selectin [41]. In turn, endothelial cell injury results in the expression of endothelial dysfunction marker von Willebrand factor antigen (vWF), the up-regulation of which has also been found to be related to the severity of DR [6, 16].

The purpose of this study was to investigate in patients with *type 2* diabetes and *non-proliferative* DR (NPDR): 1) The concomitant disturbance of retinal blood flow and systemic markers of vascular inflammation and endothelial dysfunction and 2) the associations between retinal blood flow and systemic markers of vascular inflammation and endothelial dysfunction. Earlier studies have shown that specific circulating markers of vascular inflammation and endothelial dysfunction are associated with clinical signs of DR [66]. However, it is not known if markers of vascular inflammatory activity or endothelial dysfunction relate to retinal arteriolar hemodynamics in patients with *early* NPDR. If a relationship exists between circulating markers of inflammation and/or endothelial dysfunction and retinal hemodynamics in early NPDR then perhaps quantitative measurement of retinal hemodynamics can be a surrogate marker of vascular health and ultimately it can be used as a monitoring tool for patients with diabetes. Moreover, this study will lead to a further understanding of early DR through the definition of the relationship between a biochemical markers and functional measurements of vascular health.

3.3 Materials and Methods

3.3.1 Sample

The sample comprised 12 non-diabetes control subjects (*Group 1*, 6M: 6F, mean age of 54 ± 11.6 yrs), 25 mild-to-moderate NPDR patients with type 2 diabetes (*Group 2*, 13M: 12F, mean age of 64 ± 8.3 yrs) and 13 moderate-to-severe NPDR patients with type 2 diabetes (*Group 3*, 6M: 7F, mean age of 60 ± 11.1 yrs) (see Table 3-1). All volunteers were between the ages of 35 and 75 years and had a logarithm of the minimum angle of resolution (log MAR) visual acuity of 0.30, or better. Subjects were excluded from the study if they had a refractive error greater than ±6.00 DS and/or ±2.5 DC, ocular disease or disorder (other than evidence of DR), and a history of ocular surgery. Participants with nuclear opalescence, nuclear colour and posterior subcapsular cataract greater than 3, and cortical cataract greater than 2, as defined by the Lens Opacity Classification System III were also excluded [(10)]. Subjects taking medications with known affects on blood flow (except for well controlled systemic hypertension) or have known rheumatologic diseases were excluded from the study. To eliminate the confounding effect of vasoactive medications that could affect blood flow (e.g. beta-blockers), all hypertensive medications were kept constant throughout the study. Control subjects were excluded from the study if there was an existing, or history of, diabetes and/or glaucoma in a first degree relative. The glaucoma exclusion criterion was also applied to the diabetic patient groups.

This study was approved by the University of Waterloo Office Of Research Ethics, Waterloo, and by the University Health Network Research Ethics Board, Toronto. Informed consent was obtained from each participant after a thorough explanation of the nature of the study and its possible consequences, according to the tenets of the Declaration of Helsinki.

Group	n	Group mean age (SD, yrs)	Group mean duration (SD, yrs))	# of patients using insulin	Male : Female	Group mean A1c % (SD)
1	12	54 (11.6)	n/a	n/a	6:6	5.6 (0.4)
2	25	64 (8.3)	9.8 (10.6)	5	13:12	7.0 (1.7)
3	13	60 (11.1)	16.0 (10.1)	2	6:7	7.9 (2.0)

Table 3-1: Demographic details of the study sample.

Sample size per group (n), group mean age (standard deviation, SD), reported duration of diabetes (years), number (#) using insulin treatment, male: female, and glycosylated hemoglobin (A1c) as a function of group.

3.3.2 Methodology

Each volunteer underwent assessment to establish the overall DR status and a general health profile screening to determine study eligibility. This assessment included contact lens stereo fundus biomicroscopy, binocular indirect ophthalmoscopy and digital stereo fundus photography (Canon CR-DGi with a 12.8 megapixel DSLR back; Canon, Tokyo Japan). One eye of each eligible patient was randomly assigned to the study. Prior to dilation with 1% tropicamide (Alcon Canada Inc), refraction, logarithm of the minimum angle of resolution (log MAR) visual acuity, intraocular pressure (IOP) and resting blood pressure were assessed. All subjects were given a topical anesthesia (Alcaine; Alcon, Mississauga, Canada), prior to undertaking Goldmann applanation tonometry to IOP. Following pupil dilation and fundus photography, assessment of the presence and severity of lens opacity was undertaken. Volunteers were allocated into groups according to their retinal status defined using dilated stereo fundus biomicroscopy 1991}. Participants were categorized into two

groups based on their risk factors for progression of proliferative diabetic retinopathy: mild-to-moderate (Group 2) or moderate-to-severe NPDR (Group 3).

3.3.2.1 Quantitative Assessment of Retinal Arteriolar Vessel Diameter, Centerline Velocity and Blood Flow

Non-invasive retinal arteriolar blood flow was quantified using the laser Doppler velocimetry and simultaneous vessel densitometry technique as incorporated into the Canon Laser Blood Flowmeter model 100 (CLBF: Canon, Tokyo, Japan). The technique and instrument has been described in detail in Chapter 1. Briefly, this instrument is comprised of a fundus camera with two lasers and a pair of photo-detectors incorporated into the unit. The principal underlying the CLBF is based on the Doppler effect. A red diode laser (675 nm, 80 x 50 µm oval) is used to measure velocity and a second green diode laser (543nm, 1,500x150µm rectangle) tracks eye movement stability and measures vessel diameter. A vessel that obeys Poiseuille's flow produces a range of velocities up to a maximum frequency shift [15, 58]. The maximum shifted frequency, Δf_{max}, are the red blood cells (RBCs) flowing through the center of the retinal vessel and corresponds to the maximum velocity (V_{max}) of moving particles. The combination of the average velocity (V_{max}) over a pulse cycle and diameter (D), we can calculate flow.

$$\text{Flow} = \tfrac{1}{2} \times (\Pi/4) \times V_{max} \times 60 \times D^2.$$

In summary, absolute measurements of vessel diameter (µm), velocity (mm/s) and flow (µl/min) are obtained (For technical summary see Kida et al., 2002 [38]; Canon, 1997). The method has been described extensively in detail in previous publications [17, 18, 21, 27, 28].

3.3.3 Procedures

At visit 1, volunteers underwent eligibility screening, group assignment, baseline homeostatic retinal blood flow measurements and blood work. At this visit, at least six retinal hemodynamic readings were taken of the supero-temporal arteriole. Each acquired measurement assessed the arteriolar diameter, blood velocity, max:min velocity ratio and blood flow. The site of CLBF measurement was approximately 1 disc diameter from the edge of the optic nerve head along a relatively straight segment of the supero-temporal arteriole and distant from any bifurcations. Forearm blood samples were collected for the quantification of plasma sICAM-1, sVCAM-1, sE-selectin, vWF Ag, A1c and hs CRP levels. Further, blood pressure was also measured at this visit. At visit 2, at least 6 additional retinal hemodynamic measurements were taken using the CLBF at the identical arteriolar site as in visit 1. "Δ" was defined as the difference between each retinal hemodynamic parameter at visit 1 and visit 2 in terms of arteriolar diameter, blood velocity, max: min velocity ratio and blood flow.

3.3.3.1 Molecular Markers of Vascular Inflammation and Endothelial Dysfunction

Forearm blood sample was collected prior to retinal hemodynamic assessment for each patient. For vascular inflammatory markers sICAM-1, sVCAM-1 and sE-selectin, blood was collected into a 7 ml heparin vacutainer and plasma was obtained by centrifugation. The plasma sample was then separated into polystyrene tubes in 4 equal amounts (one sample tube for each marker plus an additional sample if a test was to be repeated) and stored at -80^{o}C until assayed. Plasma samples were determined using standard, validated techniques via enzyme-linked immunosorbent assay methods (R&D Systems, Minneapolis, MN). The assay is the standard ELISA sandwich enzyme immunoassay technique and was performed by an experienced lab technician (see Appendix A).

Blood samples were also collected at baseline from all patients with diabetes and controls for the quantitative determination of von Willebrand factor antigen (vWF Ag), a marker of endothelial

dysfunction, in plasma and assessed with an immuno-turbidimetric assay (STA-Liatest vWF, Diagnostica Stago, France) (see Appendix A).

In terms of the manufacturers reported reproducibility, the coefficient of variation (COV) of sICAM-1, sVCAM-1 and sE-selectin was found to be 2.3-6.6% for intra-assay precision (tested twenty times) and COV of 4.4-7.8% for inter-assay precision (tested forty times) (R&D Systems, Minneapolis, MN).

3.3.3.2 Additional measurements of diabetes related complications

Additionally, blood pressure, hemoglobin A1c and hs CRP were also measured. Hemoglobin A1c was quantified using ion exchange high performance liquid chromatography (HPLC) (Bio-Rad Variant, Bio-Rad Laboratories, Montreal, Canada), While high sensitivity C-reactive protein was quantified by Immuno Nephelometry (NII nephelometer, Siemens Healthcare Diagnostics (previously Dade Behring), Deerfield, Illinois). The assay was completed by a trained lab technician.

3.4 Analysis

3.4.1 Blood Flow Analysis

Axial length and refractive error data of each study eye was used to correct CLBF measurements for magnification effects. A post-acquisition analysis of the velocity waveforms were performed using a standardized laboratory protocol to remove aberrant waveforms affected by eye movements, tear film breakup, or improper tracking of the measurement laser. Velocity waveforms were accepted if there was a minimum of one complete cardiac cycle which was not adversely affected by major eye movements, i.e. more than 25 μm in each direction.

The data was analyzed using Statistica 9.0 software for windows (Statsoft Inc, Tulsa, OK-USA). The normality of data of each hemodynamic parameter and systemic blood markers were checked prior to the use of parametric statistics. The maximum-to-minimum velocity ratio was log transformed to satisfy statistical normality. Differences between groups for each inflammatory (sICAM-1, sVCAM-1, and E-selectin), endothelial dysfunction markers (vWF), A1c and hs CRP were analyzed using One-way ANOVA. Similarly, differences in retinal hemodynamic parameters (blood vessel diameter, blood velocity, and blood flow) between groups were also analyzed using One-way ANOVA. For all significant One-way ANOVAs, Bonferroni post hoc tests were used in order to reduce Type I experimental errors. "Δ" was defined as the difference between each retinal hemodynamic parameter at visit 1 and visit 2 in terms of arteriolar diameter, blood velocity, max: min velocity ratio and blood flow. Change (Δ) in each hemodynamic parameter *between* Groups 2 and 3 were tested using paired t-tests. Significant correlations between markers of inflammation, endothelial dysfunction, A1c, hs CRP and retinal hemodynamic parameters at baseline and Δ were analyzed using bivariate Pearson correlation. All significant associations are entered into a multiple regression model to assess whether the dependent variable (a given hemodynamic parameter) could be predicted from markers of vascular inflammation and endothelial dysfunction (independent variables).

3.5 Results

3.5.1 Baseline, follow-up and change (Δ) over 6 months time in retinal hemodynamic parameters

Baseline (visit 1). Group mean values for the retinal hemodynamic parameters vessel diameter, blood velocity, flow and log max:min velocity ratio at baseline are found in Table 3-2 and are illustrated in Figure 3-1. Maximum-to-minimum velocity ratio was found to be significantly elevated in the group

3 compared to group 1 at baseline (0.72 vs. 0.49, ANOVA p<0.01) and at the follow-up visit (0.77 vs. 0.55, t-test p=0.02). However, there were no significant differences in vessel diameter, blood velocity and flow between groups at baseline (visit 1) or follow-up visit (visit 2).

Baseline Retinal Hemodynamics	**Group 1** **n=12**	**Group 2** **n=25**	**Group 3** **n=13**	**ANOVA p-value**
Diameter (µm)	101.1 (13.6)	107.5 (15.7)	106.7 (11.8)	0.42
Velocity (mm/sec)	33.7 (7.6)	33.6 (6.7)	34.1 (6.1)	0.97
Blood Flow (µL/min)	8.2 (2.6)	9.4 (3.4)	9.4 (3.1)	0.50
Log max:min velocity ratio	0.49 (0.07)	0.58 (0.13)	0.72* (0.22)	**<0.01***

Table 3-2: Group mean retinal hemodynamic parameters at baseline visit.

Vessel diameter, blood velocity, log maximum-to-minimum ratio and blood flow (standard deviation) as a function of group. A p< 0.05 is considered statistically significant. (* indicates significantly different from group 1). Group 1; age matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

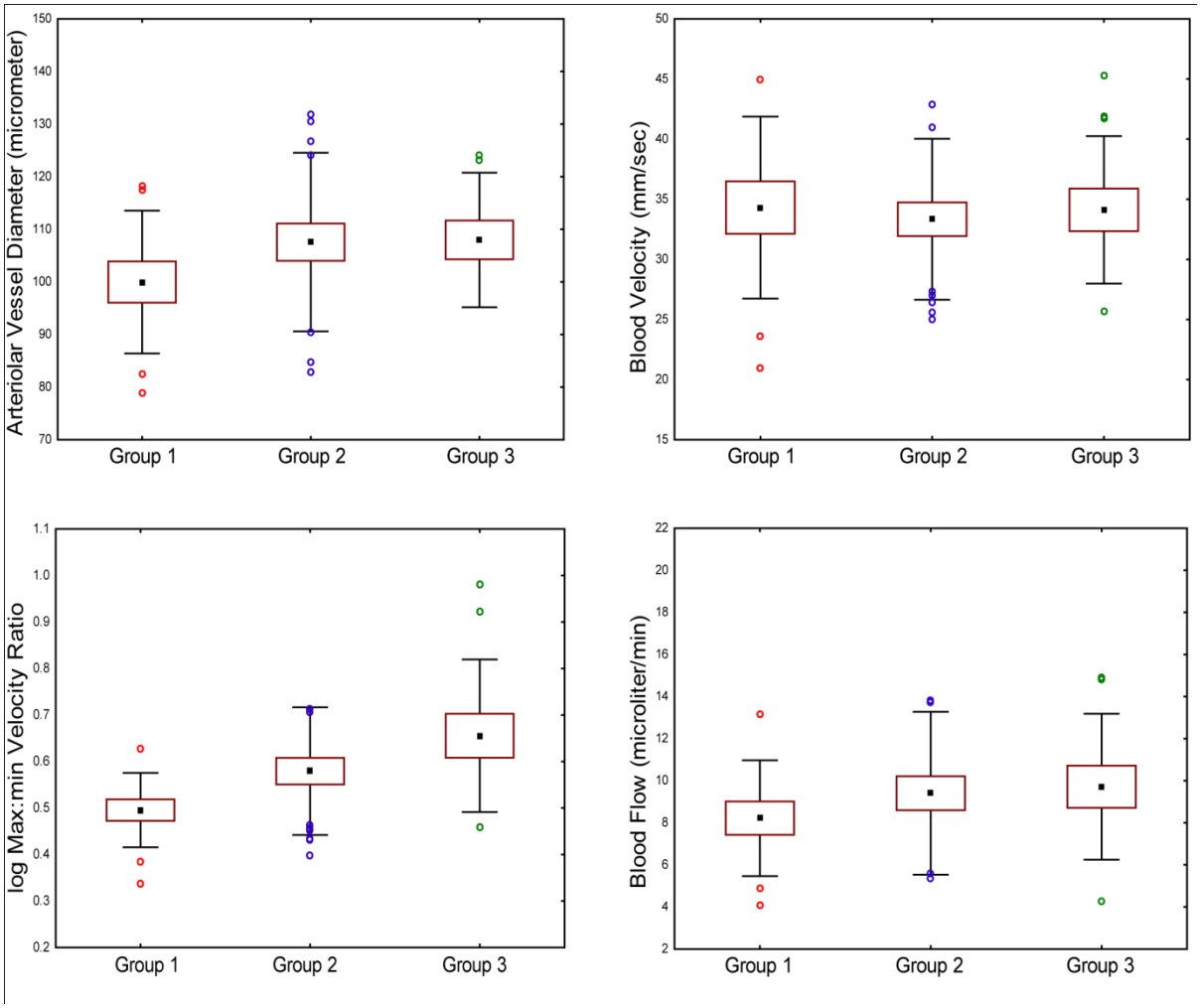

Figure 3-1: Baseline retinal hemodynamic parameters as a function of group.

Vessel diameter (upper left), blood velocity (upper right), blood flow (bottom left) and log maximum-to-minimum velocity ratio as a function of group. For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. An open circle represents outlier values. Group 1; age matched controls, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

Retinal hemodynamics at visit 2 (follow-up)	Group 2 n=25	Group 3 n=13	t-test p-value
Diameter (µm)	110.9 (11.6)	110.3 (14.7)	0.90
Velocity (mm/sec)	34.5 (32.7)	32.7 (10.1)	0.55
Blood Flow (µL/min)	10.2 (2.3)	9.5 (4.1)	0.62
Log max:min velocity ratio	0.55 (0.15)	0.77 (0.32)	**0.02***

Table 3-3:Group mean retinal hemodynamic parameters at follow-up visit.

Vessel diameter, blood velocity, log maximum-to-minimum ratio and blood flow as a function of group. A $p< 0.05$ is considered statistically significant. (* indicates significantly different from group 1). Group 1; age matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

There were no significant differences found within or between Group 2 or Group 3 in terms Δ in retinal hemodynamic parameters.

Change (Δ) in terms of	**Group 2** **n=18**	**Group 3** **n=10**	**t-test** **p-value**
ΔDiameter **(μm)**	9.3 (11.0)	5.3 (4.8)	0.29
ΔVelocity **(mm/sec)**	0.5 (4.8)	2.9 (5.7)	0.26
ΔBlood Flow **(μL/min)**	1.7 (2.2)	1.8 (1.5)	0.89
ΔLog max:min velocity ratio	1.1 (1.7)	1.4 (1.8)	0.69

Table 3-4: Magnitude of change in retinal hemodynamics from baseline to follow-up visit in patients with NPDR.

The variable called "Δ" was defined as the difference between each retinal hemodynamic parameter at visit 1 and visit 2 in terms of arteriolar diameter, blood velocity, max: min velocity ratio and blood flow. Group 1; age matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

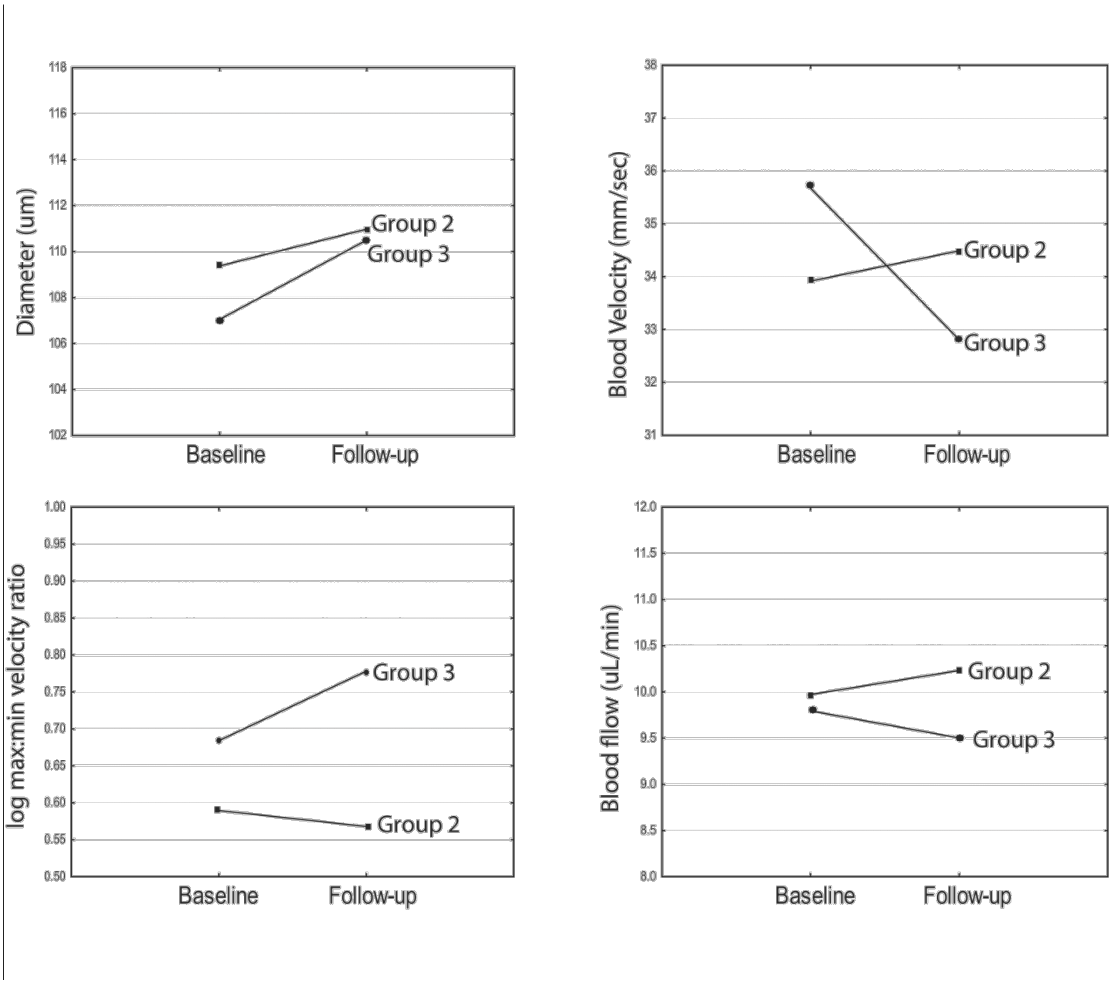

Figure 3-2:Group means at baseline and follow-up as a function of group.

Change over time between baseline and follow-up of retinal hemodynamic parameters (diameter, blood velocity, max:min velocity ratio and blood flow) as a function of group. Group 2; patients with mild-to-moderate NPDR, Group 3: patients with moderate-to-severe NPDR.

3.5.2 Baseline inflammatory and endothelial dysfunction marker levels

Group mean baseline values for soluble markers of inflammation and endothelial dysfunction are described in Table 3-3 and illustrated in Figure 3-2. Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA p=0.02 and p=0.04, respectively). A post hoc Bonferroni test showed that Group 3 had significantly higher sICAM-1 and sE-selectin levels compared to Group 1 (234.0 vs. 151.5 ng/ml, P=0.02 and 53.4 vs. 27.6 ng/ml, P<0.01, respectively). VCAM-1 and vWF antigen were not statistically different (ANOVA p=0.25) between groups. Hemoglobin A1c was significantly elevated across the groups (ANOVA p<0.01), with Group 3 having significantly higher hemoglobin A1c levels compared to Group 1 (7.9 vs. 5.6 % , P<0.01).

	Group 1 **n=12**	**Group 2** **n=25**	**Group 3** **n=13**	**ANOVA p-value**
sICAM-1 (ng/ml)	151.5 (50.4)	205.2 (65.8)	234.0* (102.2)	**0.02***
sVCAM-1 (ng/ml)	371.8 (78.5)	482.5 (247.8)	454.4 (127.2)	0.25
sE-selectin (ng/ml)	27.6 (7.8)	45.3 (27.7)	53.4* (32.5)	**0.04***
vWF antigen (U/ml)	1.2 (0.3)	1.3 (0.5)	1.3 (0.4)	0.69

Table 3-5: Group mean and standard deviation of markers inflammation and endothelial dysfunction as a function of group.

sICAM-1, sVCAM-1 and sE-selectin are markers of inflammatory activity, while vWF Ag is a marker of endothelial dysfunction. ICAM-1: Intercellular adhesion molecule type 1, VCAM-1: vascular cell adhesion molecule type 1, and vWF; von Willebrand factor antigen. (* indicates significantly different from group 1). Group 1; age matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

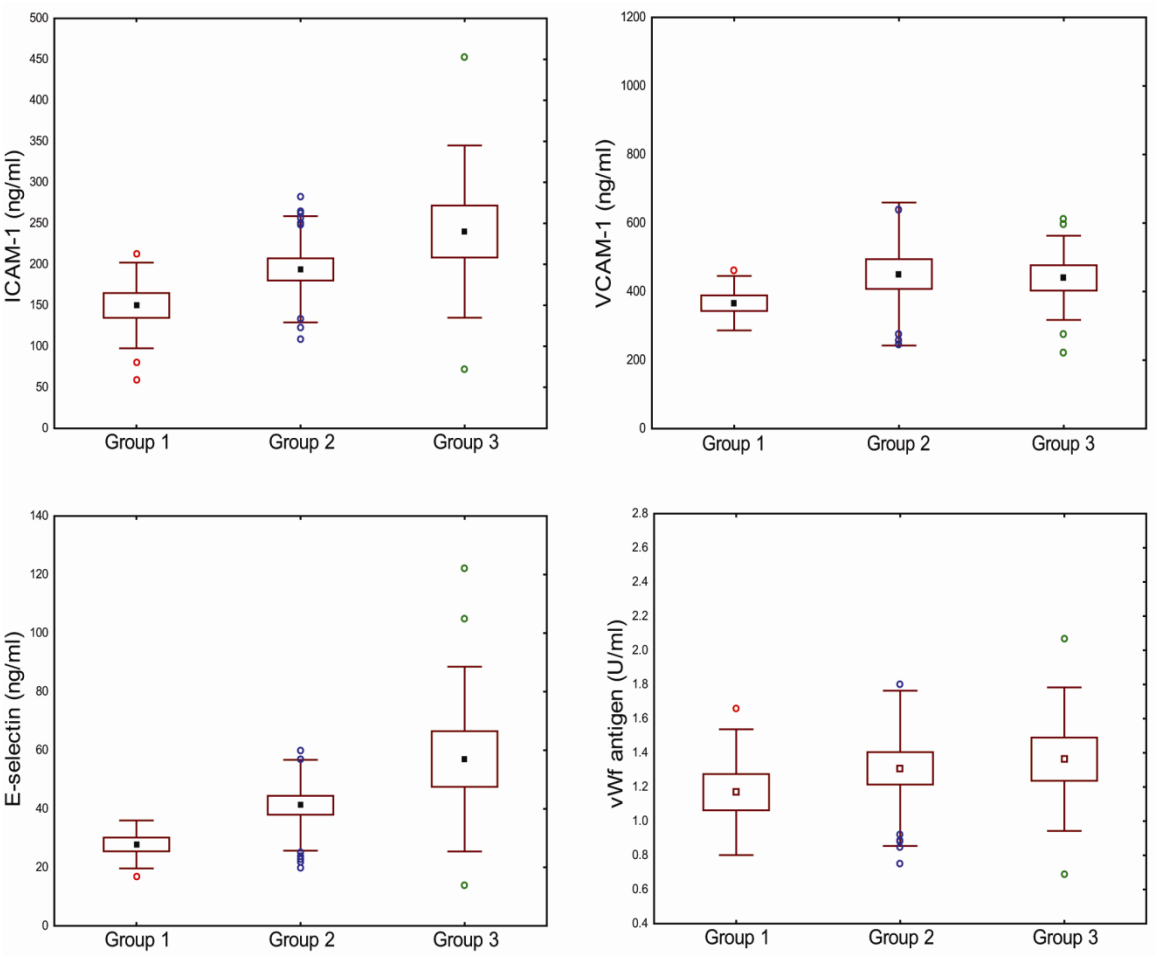

Figure 3-3: Box plots of inflammatory and endothelial dysfunction, markers as a function of group.

The inflammatory markers are; soluble intracellular adhesion molecule type 1 (sICAM-1) (upper left), soluble vascular cell adhesion molecule type 1 (sVCAM-1) (upper right) and soluble E-selectin (bottom left). The endothelial dysfunctional marker is von Willebrand factor antigen (vWF) (bottom right). For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. An open circle represents outlier values. Group 1: age matched controls, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

	Group 1 n=12	Group 2 n=25	Group 3 n=13	ANOVA p-value
Hemoglobin A1c (%)	5.6 (0.4)	7.0 (1.6)	7.9* (2.0)	**<0.01***
hs CRP (mg/L)	1.3 (0.8)	2.5 (3.0)	2.4 (3.1)	0.43

Table 3-6: Group mean and standard deviation of A1c and hs CRP as a function of group.

hsCRP: high sensitivity C-reactive protein and A1c; glycosylated hemoglobin (* indicates significantly different from group 1). Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

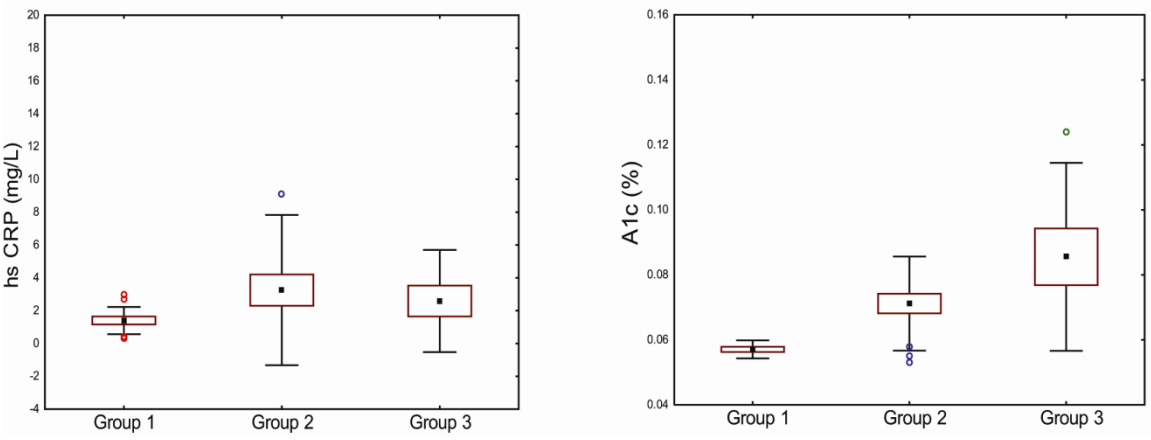

Figure 3-4: Box plots of hs CRP and A1c markers as a function of group.

Hs CRP; high sensitivity C-reactive protein and A1c; glycosylated hemoglobin. For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. An open circle represents outlier values. Group 1: age matched controls, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

3.5.3 Bivariate Pearson correlations between baseline markers of inflammation/endothelial dysfunction and baseline retinal hemodynamics measurements

Baseline (Visit 1). The relationships between each hemodynamic parameter and systemic markers of inflammation and endothelial dysfunction were examined using a bivariate Pearson correlation. There were no significant associations found between baseline markers of inflammation and baseline retinal hemodynamics across all groups. Pearson correlation coefficients between baseline blood markers and baseline retinal hemodynamics are found in Table 3-7.

Type of Markers	**Inflammatory**			**Endothelial Dysfunction**		
	ICAM-1 (ng/ml)	**VCAM-1 (ng/ml)**	**E-selectin (ng/ml)**	**vWF Ag (U/ml)**	**A1c (%)**	**hs CRP (mg/L)**
Diameter (µm)	0.16 (0.28)	0.19 (0.19)	0.19 (0.19)	0.24 (0.10)	0.18 (0.21)	0.14 (0.31)
Velocity (mm/sec)	-0.02 (0.89)	-0.14 (0.33)	-0.01 (0.92)	0.06 (0.68)	-0.78 (0.59)	0.06 (0.68)
Blood Flow (µL/min)	0.10 (0.48)	0.06 (0.66)	0.17 (0.23)	0.23 (0.11)	0.21 (0.16)	0.21 (0.16)
Log max:min velocity ratio	0.07 (0.61)	0.27 (0.06)	-0.19 (0.18)	0.08 (0.54)	-0.18 (0.22)	-0.17 (0.22)

Table 3-7: Pearson correlation values (r) and statistical significance (p) between baseline levels of markers of inflammation/ endothelial dysfunction and baseline retinal hemodynamic parameters across the pooled group.

Retinal hemodynamic parameters as a function of vessel diameter, blood velocity, blood flow and log maximum-to-minimum velocity ratio in the pooled group. Systemic markers are; soluble intracellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), soluble E-selectin and von Willebrand factor antigen (vWF). A significant correlation was taken at $r > \pm 0.30$ and $p < 0.05$ after Bonferroni correction. Maximum-to-minimum velocity ratio was log transformed to satisfy normality. (*indicates statistical significance).

3.5.4 Pearson correlation between baseline levels of inflammatory/endothelial dysfunction markers and change (Δ) in retinal hemodynamics over 6 month time

Six months post baseline visit. Analysis of Δ (difference between visit 1 and visit 2) in retinal hemodynamic parameters was limited to type 2 diabetes patients with NPDR. Pearson coefficients between Δ in terms of retinal hemodynamics and baseline levels of inflammation/endothelial dysfunction markers across Groups 2 and 3 are in Table 3-8. Δ velocity was correlated with the baseline sICAM-1 (r=0.42, p=0.02) and A1c levels (r=0.37, p=0.04) in patients with NPDR. Additional correlations between baseline blood markers and change in retinal hemodynamics from baseline in Groups 2 and 3 are found in Table 3-8.

Type of Marker	Inflammatory			Endothelial Dysfunction		
	ICAM-1 (ng/ml)	**VCAM-1 (ng/ml)**	**E-selectin (ng/ml)**	**vWF Ag (U/ml)**	**A1c (%)**	**hsCRP (mg/L)**
ΔDiameter (μm)	-0.16 (0.39)	-0.24 (0.20)	-0.16 (0.42)	-0.05 (0.79)	-0.25 (0.18)	-0.01 (0.94)
ΔVelocity (mm/sec)	**0.42 (0.02)***	0.21 (0.26)	0.35 (0.06)	0.10 (0.57)	**0.37 (0.04)***	0.08 (0.67)
ΔBlood Flow (μL/min)	0.21 (0.26)	-0.01 (0.94)	0.20 (0.28)	0.11 (0.55)	0.11 (0.54)	0.13 (0.49)
ΔLog max:min velocity ratio	-0.16 (0.40)	0.04 (0.82)	-0.25 (0.18)	-0.15 (0.43)	-0.06 (0.74)	-0.04 (0.80)

Table 3-8: Pearson correlation values (with associated p-values) between baseline levels of inflammatory/endothelial dysfunction markers and Δ in retinal hemodynamic parameters in 6 months time among patients with NPDR in Study 1.

Retinal hemodynamic parameters are taken as vessel diameter, blood velocity, blood flow and log maximum-to-minimum velocity ratio) in patients with NPDR. Systemic markers are; soluble intracellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), soluble E-selectin and von Willebrand factor antigen (vWF). A significant correlation was taken at $r \geq \pm 0.30$ and $p<0.05$. (*indicates statistical significance). Patients with NPDR include groups 2 and 3. Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

The multiple regression analysis indicated that none of the independent variables was significant predictors of change in Δ velocity. Based on these results we conclude that sICAM-1 and A1c have weak predictive power for Δ velocity groups with NPDR. The relationship between Δ velocity and sICAM-1 was plotted in Figure 6.

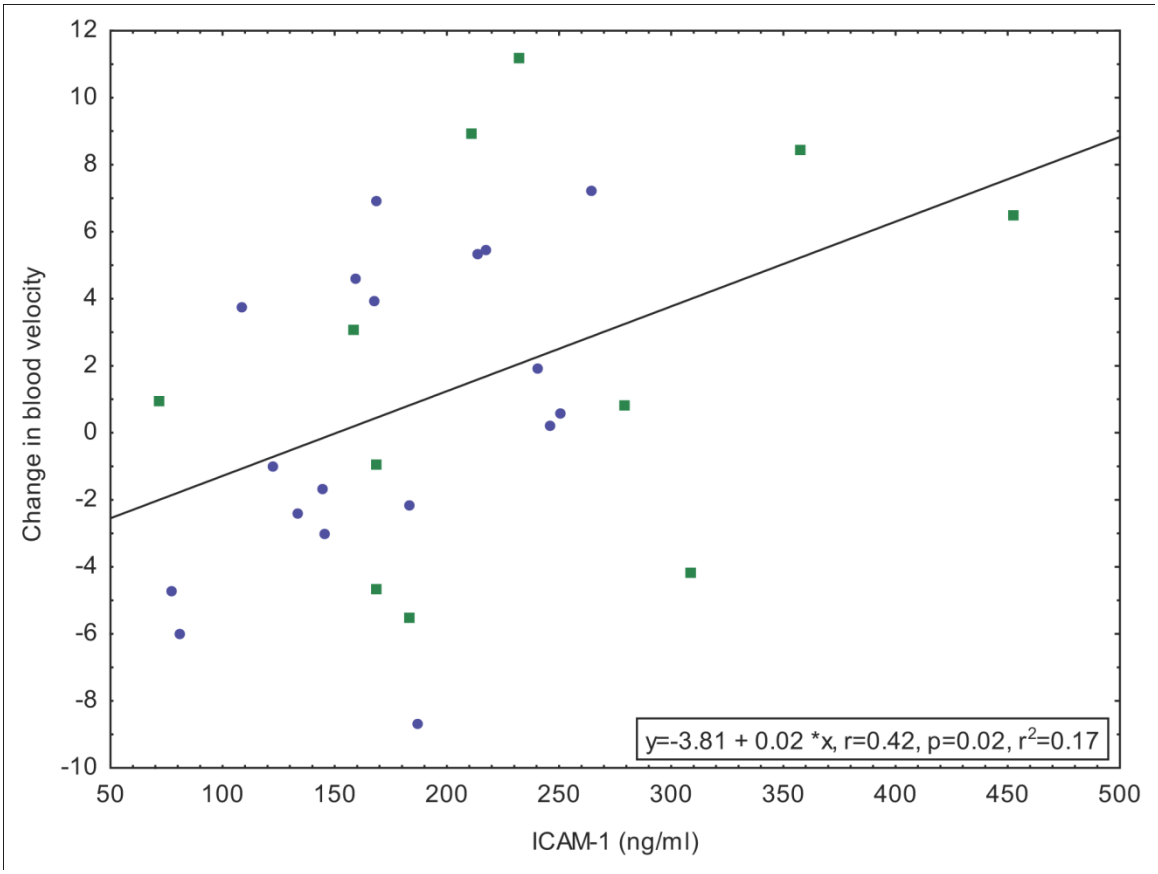

Figure 3-5: Scatter plot of baseline sICAM-1 levels and change (Δ) velocity in 6 months time across patients with NPDR.

Each circle indicates a subject of group 2 (mild-to-moderate NPDR) while each square indicates subjects of group 3 (moderate-to-severe NPDR). sICAM-1; Soluble intercellular adhesion molecule type 1.

3.6 Discussion

To the best of our knowledge, this is the first study to investigate the relationship between arteriolar retinal blood flow parameters and circulating markers of vascular inflammation and endothelial dysfunction in patients with increasing severity of NPDR. In our sample, we had characterized two changes that are related to early NPDR: 1) an increase in velocity pulse wave amplitude representing a loss of vessel compliance at the baseline and at the follow-up visit and 2) elevated levels of soluble adhesion markers (sICAM-1 and sE-selectin) indicating inflammatory activity. This study suggests that a decrease in vessel compliance may occur along with an increase in inflammatory activity in patients with increasing NPDR severity. Although sICAM-1 and A1c have weak predictive power for Δ hemodynamic parameters, there was a bi-variate association found between these markers and Δ velocity after 6-month time in patients with NPDR.

The max:min velocity ratio has previously been shown to be sensitive to alterations in retinal hemodynamics in patients with diabetes [27]. At visit 1 and visit 2, we found an increase in baseline maximum-to-minimum velocity ratio, indicating a loss in vessel compliance with increasing severity of NPDR. Previous reports from our lab also suggest that arteriosclerotic changes occur in subjects with increasing risk for DME development [17, 27]. An increase in max:min velocity ratio was correlated with age, duration of diabetes and blood pressure but not with blood glucose. With decreased compliance in the retinal circulation, an increase in pulsatility is expected due to a lack of dampening of the pulse wave. Recent work from our laboratory has also demonstrated a reduced vascular reactivity in retinal arterioles to isocapnic hyperoxic provocation in type 2 patients with NPDR and DME [17]. Loss of vascular reactivity and a reduced compliance of the retinal arterioles would support the findings of the current study.

Moreover, arteriosclerotic features may relate to the increase in inflammatory activity. Leukocyte mediated basement membrane (BM) thickening is a morphological sign of diabetic atherogenesis [60]. Overall, our observations support increased vessel rigidity in the retinal vascular bed in patients with increasing NPDR severity.

Our results also demonstrated elevated vascular adhesion levels. We found that inflammatory activity was increased with increasing severity of NPDR. Vascular inflammatory markers sICAM-1 and sE-selectin were significantly higher in the moderate-to-severe group compared to healthy controls. Other systemic markers sVCAM-1, hsCRP and vWF were not statistically different in patients with diabetes from those without ($p=0.24$, $p=0.43$ and $p=0.69$). Our results were consistent with earlier studies that show that sICAM-1 is elevated in patients with increasing severity of NPDR and plays a crucial role in leukocyte adherence to the endothelium [31, 51, 66]. The up-regulation of sICAM-1 in the diabetic vasculature links leukostasis to early stages of DR [31, 34, 36, 66]. This is thought to be enhanced by several stimuli including vascular endothelial growth factor (VEGF) [9, 30, 33], advanced glycosylated end products (AGEs) [46, 51, 64] and oxidative stress [12, 14, 29] via the release of pro-inflammatory cytokines like Tumor Necrosis Factor-alpha (TNF-α). Moreover, we found that the expression of sE-selectin was significantly higher in patients with moderate-to-severe NPDR compared to the control group. Previous studies did not find a difference in serum sE-selectin levels in patients with NPDR compared to healthy controls [7, 35]. However, sE-selectin is consistently elevated in the vitreous and serum samples of patients with PDR compared to healthy controls [3, 4]. Although our results show that endothelial dysfunction marker vWF Ag was not different between groups, one earlier study reported an elevated vitreous concentration of vWF in type 2 diabetes subjects with DR [52]. One of the reasons for these differences in findings could be a large biological

variation of circulating adhesion molecules and vWF. Since we are measuring systemic serum vWF, its expression is unlikely isolated to retinal endothelial cells. The expression of the vascular adhesion molecules may be derived from cells outside of the retinal cells, which may explain the discrepancy in reports. For this and previous studies, only one measurement is performed. Further investigation of serial measurements of inflammatory and endothelial dysfunction markers over time might help predict vascular inflammatory activity more accurately. Our results suggest that there were elevated vascular inflammatory levels of sICAM-1 and sE-selectin in patients with DR compared to controls. Elevated vascular inflammatory activity could play a role in the development arteriosclerotic characteristics in the vessels of patients with NPDR.

Several past reports suggest that increases in leukocyte adhesion are a critical factor in early DR and leads to stagnation in retinal blood flow [33, 49]. We did not find a significant correlation between baseline retinal hemodynamics and inflammatory or endothelial dysfunction markers across all groups. However, recent reports of retinal vein occlusion (RVO) patients with macular edema have found that vitreous ICAM-1 level is inversely related to retinal blood flow velocity in the perifoveal capillaries [53] and likely contributes to the development of RVO. In our study, we found no such correlation. Our results found blood velocity (Δ velocity) to be worsened after 6 months time with an early elevation in baseline sICAM-1 levels ($r=0.42$, $p=0.02$) and A1c levels ($r=0.37$, $p=0.04$) in patients with NPDR. After adjustment for all other variables (A1c, hsCRP and vWF), the relationship between Δ velocity, sICAM-1 and A1c disappeared. Firstly, this trend suggests that high levels of sICAM-1 were associated with greater disturbance in blood velocity from baseline. The up-regulation of sICAM-1 may induce hemodynamic disturbance in DR by accelerating change in retinal blood velocity. Leukostasis and the subsequent extravasations of leukocytes may relate to abnormal release of endothelial derived factors that modulate blood velocity. The control of vascular tone is a

balance between local endothelial cell released vasoconstrictive and vasodilatory factors. For instance, endothelin-1 (ET-1) plays an important role in retinal vascular autoregulation via regulating vessel vasoconstriction [13, 57]. A recent study shows that endothelin-1 (ET-1) levels are low in patients with NPDR compared to controls and increase in proliferative retinopathy [56]. Endothelin-1 can bind to either ET_A stimulating smooth muscle vasoconstriction, while ET-3 binds to ET_B, which stimulates vasodilation. The increased change in blood velocity may relate to altered ET-1 levels that interact with ET_A receptors minimizing the vasoconstrictive tone. Low ET-1 levels relate to vessel vasodilation that is a clinical feature of DR [40, 63]. Secondly and not surprisingly, the progression of DR is observed following the institution of strict diabetic glycemic control [1, 2, 8]. The analysis of several published studies show consistent results, suggesting that progression is likely associated with hemodynamic changes in the retinal circulation [23, 24]. Overall, further work is required to investigate whether inhibition of sICAM-1 could be a target for future treatment and a possible surrogate end-point is the preservation of retinal blood velocity.

Alternatively, it is also likely that linear relationships do not exist. Since we are examining markers of inflammation and endothelial dysfunction systemically and hemodynamics in the retinal arterioles only represents a small portion of the entire body's vasculature, it is can be argued that we would not find a strong relationship. Overall, it is important to identify that in our study both retinal arterial stiffness and inflammatory markers increased in the moderate-to-severe NPDR group compared to the controls. The presence of these two important DR determining factors within the same sample group warrants further investigation. In addition, future work will aim to use aqueous samples to assay ICAM-1, VCAM-1 and E-selectin. This may provide further evidence to support vascular inflammation related to DR complications [65].

In conclusion, we found a significant bi-variate relationship between Δvelocity after 6-month time and sICAM-1 and A1c levels. Although both sICAM-1 and A1c were of no predictive value for hemodynamic parameters, there is evidence that these events are related with respect to their development in NPDR. This study is the first to characterize two important events which occur simultaneously in patients with increasing NPDR; 1) increased pulsatility of the velocity wave profile in the retinal arteriolar vasculature and 2) elevated vascular adhesion molecule sICAM-1 and sE-selectin levels. Blood flow dynamics, such as blood velocity, pulsatility and vascular regulation have been shown to change throughout the progression of DR. Therefore, there is value in further investigating any possible association between increased rigidity and leukostasis. The process of leukostasis and its affiliation to retinal hemodynamics is clearly complex and warrants further investigation. Understanding whether adhesion molecules interplay with a disturbance in hemodynamics may lead to the generation of strategies that could target early abnormalities in DR.

Chapter 4

Retinal Vascular Reactivity and Systemic Markers of Vascular Inflammation, Endothelial Dysfunction in Type 2 Diabetes

4.1 Overview

Purpose: The purpose of this study was to investigate in patients with *type 2* diabetes and *non-proliferative* DR (NPDR); 1) The concomitant disturbance of retinal vascular reactivity and systemic markers of vascular inflammation and endothelial dysfunction and 2) the associations between retinal vascular reactivity and systemic markers of vascular inflammation and endothelial dysfunction. Retinal vascular reactivity (VR) was assessed both in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and volumetric flow at baseline and in terms of change in VR over 6 months time in patients with non-proliferative diabetic retinopathy (NPDR).

Method: The sample comprised of 36 NPDR patients with type 2 diabetes and 10 age matched control subjects (mean age 55.4±11.9 y.o; 6M: 4F). Patients were divided into mild-to-moderate (Group 2) (n=22, mean age 61.1±8.8 y.o, 12M: 10F) or moderate-to-severe (Group 3) (n=14, mean age 61.7±11.2 y.o, 7M:7F) NPDR according to ETDRS criteria. Retinal VR was assessed in the supero-temporal arteriole using the Canon Laser Blood Flowmeter. At baseline (visit 1), a minimum of 6 measurements were taken to determine hemodynamic parameters. Then 6 additional measurements were taken during exposure to isocapnic hyperoxia. The difference between baseline and hyperoxic provocation was taken as a measure of retinal VR. These measurements were repeated after 6 months follow-up. Baseline blood samples were collected to derive levels of vascular inflammation and endothelial dysfunction; soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), sE-selectin, C-reactive protein (hsCRP) and

von Willebrand factor antigen (vWF Ag). The baseline systemic markers of vascular inflammation and endothelial dysfunction were correlated with baseline and change in retinal VR over 6 month time and compared between and within groups.

Results: Of the total enrolled subjects, 14 mild-to-moderate NPDR (Group 2) (mean age 64.1±8.1 y.o, 8M: 6F) and 7 moderate-to-severe NPDR (Group 3) (mean age 58.7±11.3 y.o, 3M: 4F) returned for vascular reactivity assessment after 6 months. VR at visit 1 and visit 2 showed no significant differences in change in terms of vessel diameter, blood velocity, log max:min velocity ratio and blood flow between groups. There were no significant differences in the Δ VR in retinal hemodynamic parameters over 6 months time between Groups 2 and 3. However, compliance was found to be significantly reduced in patients of Group 3 compared to Group 2 (-0.4 vs. 0.1, t-test $p<0.01$).

Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA $p=0.02$ and $p<0.01$, respectively). A post hoc Bonferroni test showed that Group 3 had significantly higher sICAM-1 and sE-selectin levels compared to Group 1 (243.4 vs. 157.3ngml, $P<0.01$ and 57.0 vs. 29.3 ng/ml, $P<0.01$, respectively). A1c was significantly elevated across the groups (ANOVA $p<0.01$), with Group 3 having significantly than Group 1 (8.8 vs. 5.6 %, $P<0.01$).

Multiple regression analysis found that vascular inflammatory and endothelial function markers had weak predictive power for Δ hemodynamic parameters. However, weak to moderate correlations were found in both baseline and in the disturbance of VR after 6-month time and markers of vascular inflammation and endothelial dysfunction across all groups. Specifically, baseline VR in blood

velocity weakly correlates with sE-selectin (r=0.31, p=0.04) across all groups while sVCAM-1 was associated with VR in terms of blood flow (r=-0.62, p<0.01) in patients with mild-to-moderate NPDR. The Δ blood flow after 6 months was found to be weakly associated with sE-selectin (r=0.46, p=0.03) across all groups. Finally, the Δ blood velocity after 6 month time was found to be moderately correlated with baseline vWF Ag level (r=-0.78, p=0.02).

Conclusion: Overall, we demonstrated two important characteristics in early NPDR; 1) a disturbance in vascular reactivity in terms of compliance and 2) an increase in systemic markers of inflammation were found in patients with NPDR. Although systemic markers of vascular inflammation and endothelial dysfunction are not predictive of hemodynamic parameters, our study found moderate associations between baseline and disturbances in VR after 6 months time. Therefore, there is evidence that inflammation and vascular function may be related with respect to their development in NPDR.

4.2 Introduction

The retinal microcirculation is unique since it is able to regulate blood flow to meet the metabolic demand of the surrounding tissue. *Vascular reactivity* (VR) is defined as the magnitude of change of hemodynamic parameters (vessel diameter, velocity, or flow) in response to provocative stimuli such as oxygen (O_2) [12, 21, 38], cold stress [15, 52, 56] or light flicker [16, 17, 58]. The retinal VR response is a non-invasive measure of vascular regulation. Not only is a disturbance of homeostatic retinal blood flow is an accepted marker of early diabetic retinopathy (DR) [24] but there is also evidence of impaired VR in patients with non-proliferative diabetic retinopathy (NPDR). Impaired vascular function has been shown to occur in patients with an increasing risk for diabetic macular edema (DME) development [19,

[23, 24, 40]. With the aid of respiratory physiology experts, our laboratory has developed a standardized isocapnic hyperoxic stimulus to evaluate the VR of retinal vessels. This technique has proven to be a reliable sensitive method to assess retinal VR [21]. Although the exact mechanism of response to elevated arterial oxygen (pO_2) is unclear, endothelium derived vasoactive factors such as nitric oxide (NO) and endothelin-1 (ET-1) may be primary in modulating retinal vascular reactivity. Endothelial cell dysfunction is an important determinant of altered VR and plays a major role in the genesis of both micro- and macrovascular complications in diabetes [4, 27, 50].

Leukocyte-endothelial interaction plays an important role in the early development of DR [30, 46, 47, 55, 60]. A component of DR is a low-grade chronic inflammation associated with increased leukocyte entrapment in the retinal vasculature [3]. The vascular inflammatory process, termed leukostasis, is secondary to the enhanced expression of soluble adhesion molecules [3, 30, 57, 60]. These inflammatory mediators include soluble intercellular adhesion molecule (sICAM-1) [41, 44], vascular cell adhesion molecule (sVCAM-1) [36, 54, 60] and sE-selectin [39]. Endothelial cell injury results in the expression of endothelial dysfunction marker von Willebrand factor antigen (vWF Ag), the up-regulation of which has also been found to be related to the severity of DR [6, 14].

The purpose of this study was to investigate in patients with *type 2* diabetes and *non-proliferative* DR (NPDR): 1) The concomitant disturbance of retinal vascular reactivity and systemic markers of vascular inflammation and endothelial dysfunction; and 2) The associations between retinal vascular reactivity and systemic markers of vascular inflammation and endothelial dysfunction. Retinal VR was assessed both in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and volumetric flow at baseline and in terms of change in VR over 6 months time in patients with non-proliferative diabetic retinopathy (NPDR).

Earlier studies have shown that circulating markers of vascular inflammation and endothelial dysfunction are associated with clinically visible signs of DR [61]. However, the association between vascular inflammatory activity and retinal vascular function in early DR has not been investigated. Increased inflammatory activity may have modulating effects on VR. Therefore, we hypothesize that baseline parameters of arteriolar VR are negatively correlated with systemic markers of vascular inflammation and endothelial dysfunction in type 2 diabetes. If a relationship can be shown to exist between circulating markers of inflammation and/or endothelial dysfunction and retinal vascular reactivity then the measurement of retinal vascular function can be utilized as a surrogate marker of vascular health.

4.3 Materials and Methods

4.3.1 Sample

The sample comprised of 10 non-diabetes control subjects (*Group 1*) (6 M: 4 F, mean age of 55.4 ± 11.9 years), 22 mild-to-moderate NPDR subjects (*Group 2*) (12M:10F, mean age of 61.1± 8.8 years), 14 moderate-to-severe NPDR subjects (*Group 3*) (7 M: 7 F , mean age of 61.7 ± 11.2 years) (see Table 4-1).

All volunteers were between the ages of 35 and 75 years and had a logarithm of the minimum angle of resolution (log MAR) visual acuity of 0.30, or better. Subjects were excluded from the study if they had a refractive error greater than ±6.00 DS and/or ±2.5 DC, ocular disease or disorder (other than evidence of DR), and a history of ocular surgery. Participants with nuclear opalescence, nuclear colour and posterior subcapsular cataract greater than 3, and cortical cataract greater than 2, as defined by the Lens Opacity Classification System III, were also

excluded [9]. Subjects taking medications with known affects on blood flow (except for well-controlled systemic hypertension) or who had known rheumatologic diseases were excluded from the study. To eliminate the confounding effect of vasoactive medications that affect blood flow (e.g. beta-blockers), all hypertensive medications were kept constant throughout the study. Control subjects were excluded from the study if there was an existing or history of diabetes and/or glaucoma in a first-degree relative. The glaucoma exclusion criterion was also applied to the diabetic patient groups.

This study was approved by the University of Waterloo Office Of Research Ethics and the University Health Network Research Ethics Board, Toronto. Informed consent was obtained from each participant after thorough explanation of the nature of the study and its possible consequences, according to the tenets of the Declaration of Helsinki.

Group	NPDR Severity	n	Group mean age (SD,years)	Group mean duration (SD,years)	# of patients using insulin	Male:Female	Group mean A1c (%)
1	n/a	10	55.4 (11.9)	n/a	n/a	6:4	5.6 (0.4)
2	Mild-to-moderate	22	61.1 (8.8)	9.0 (8.8)	6	12:10	7.7 (2.0)
3	Moderate-to-severe	14	61.7 (11.1)	16.7 (12.4)	3	7:7	8.8 (3.4)

Table 4-1: Demographic details of the baseline study sample.

Sample size per group (n), group mean age (standard deviation, SD), known duration of diabetes (years), number (#) using insulin treatment, male: female, and glycosylated hemoglobin (A1c) as a function of group.

4.3.2 Methodology

Each volunteer underwent assessment to establish the overall DR status and a general health profile screening to determine study eligibility. This assessment included contact lens stereo fundus biomicroscopy, binocular indirect ophthalmoscopy and digital stereo fundus photography (Canon CR-DGi with a 12.8 megapixel DSLR back; Canon, Tokyo Japan). One eye of each eligible patient was randomly assigned to the study. Prior to dilation with 1% tropicamide (Alcon Canada Inc), refraction, log Mar visual acuity, intraocular pressure and resting blood pressure (Omron HEM-907, Intellisense^{TM}) were all assessed. All volunteers underwent topic anesthesia (Alcaine; Alcon, Mississauga, Canada) prior to intraocular pressure (IOP) measurement using Goldmann applanation tonometry. Following pupil dilation and fundus photography, assessment of the presence and severity of lens opacity was undertaken. Volunteers were allocated into groups according to their retinal status defined using dilated stereo fundus biomicroscopy. Participants were categorized into two groups based on their risk factors for progression of DR: mild-to-moderate (Group 2) or moderate-to-severe NPDR (Group 3). Axial length data of the study eye was acquired using the I^3 System ABD A-scan ultrasound (I^3 Innovative Imaging Inc, Sacramento, CA).

4.3.2.1 Quantitative Assessment of Retinal Arteriolar Vessel Diameter, Centerline Velocity and Blood Flow

Non-invasive retinal arteriolar blood flow was quantified using the laser Doppler velocimetry and simultaneous vessel densitometry technique as incorporated into the Canon Laser Blood Flowmeter model 100 (CLBF: Canon, Tokyo, Japan). The technique and instrument has been described in detail in Chapter 1 and in Chapter 3. Using the CLBF, absolute measurements of vessel diameter (μm),

velocity (mm/s) and flow (µl/min) are obtained (For technical summary see Kida et al., 2002 [37]; Canon, 1997). The method has been described extensively in detail in previous publications [18, 19, 22, 24, 26].

4.3.2.2 Isocapnic Hyperoxic Gas Delivery System

In order to establish an isocapnic hyperoxic provocation, subjects were fitted to a silicon facemask that was made up of a fresh gas reservoir and an expiratory gas reservoir (Hi-OxSR, ViasysHealthcare, Yorba Linda, CA). The mask was designed such that the two separate inspiratory and expiratory limbs were connected by a single positive end-expiratory pressure (PEEP valve), allowing exhaled gas to be re-breathed as inhaled limb was depleted. The sequential re-breathing circuit allows for the manual manipulation of inspired and, consequently, expired gases. The rates of each air and oxygen flow were controlled using standard rotometers as flowmeters (Flowmeter 56073-11-01-000, Controls corp. of America, Virginia beach, Virginia). This method is described in previous publications from our lab [22] in the investigation of vascular reactivity in both healthy and diseased eyes [18, 20-22, 62-64].

4.3.3 Procedures

At study visit 1, all volunteers had undergone an eligibility screening, group assignment, baseline retinal VR assessment and blood work. Prior to the VR assessment, each subject until heart rate and blood pressure were stabilized and then measurements were acquired. Subjects were fitted to the sequential re-breathing circuit (Hi-Ox SR, Viasys Healthcare, Yorba Linda, Ca). The circuit was comprised of a non-rebreathing valve, a fresh gas and a rebreathing as reservoir, which are connected to the gas supply. In the administration of air for the first 10 minutes, subjects were instructed to breath at a steady rate until a stable resting concentration of CO_2 in the expired breath ($P_{ET}CO_2$) of

38mmHg (± 2 mmHg) and a concentration of O_2 in the expired breath $P_{ET}O_2$ of 16% was reached. Tidal gas concentrations were measured continuously. Blood pressure was monitored every 2.5 minutes while pulse rate and oxygen saturation was measured every minute during the breathing period using a rapid response critical care gas analyzer (Cardiocap 5, Datex-Ohmeda, Helsinki, Finland). Once stabilized, 6 good quality CLBF measurements were acquired to determine vessel diameter, blood velocity and blood flow at a chosen site of the study eye. The site of CLBF measurement was approximately 1 disc diameter distant from the edge of the optic nerve head along a relatively straight segment of the supero-temporal arteriole and distant from any bifurcations. In the following isocapnic hyperoxic breathing stage, $P_{ET}O_2$ was increased to 100% while maintaining a stable resting $P_{ET}CO_2$ of 38mmHg (±2mmHg). During this next phase, 6 additional CLBF measurements were acquired. VR was quantified as the magnitude of change from baseline (air) to the isocapnic hyperoxia in terms of retinal arteriolar diameter, blood velocity, maximum: minimum velocity ratio and blood flow. At visit 2, VR measurements were taken using the CLBF at the identical arteriole site as in visit 1.

4.3.3.1 Isolating Soluble Markers of Inflammation and Endothelial Dysfunction

Forearm blood sample was collected at visit 1 after retinal VR assessment for each patient. For vascular inflammatory markers sICAM-1, sVCAM-1 and sE-selectin, blood was collected into a 7 ml heparin vacutainer and plasma was obtained by centrifugation. The plasma sample was then separated into polystyrene tubes in 4 equal amounts (one sample tube for each marker plus an additional sample if a test was to be repeated) and stored at -80^{o}C until assayed. Plasma samples were determined using standard, validated techniques via enzyme-linked immuno-sorbent assay methods (R&D Systems, Minneapolis, MN). Standard ELISA sandwich enzyme immuno-assay technique was applied and performed by a experienced lab technician (see Appendix A).

Blood samples were also collected at baseline from all patients with diabetes and controls for the quantitative determination of von Willebrand factor antigen (vWF Ag), a marker of endothelial dysfunction, in plasma and assessed with an immuno-turbidimetric assay (STA-Liatest vWF, Diagnostica Stago, France). (see Appendix A).

In terms of the manufacturers reported reproducibility, the coefficient of variation (COV) of sICAM-1, sVCAM-1 and sE-selectin was found to be between 2.3-6.6% for intra-assay precision (tested twenty times) and COV of 4.4-7.8% for inter-assay precision (tested forty times) (R&D Systems, Minneapolis, MN).

4.4 Analysis

4.4.1 Blood Flow Data

Axial length and refractive error data of each study eye was used to correct CLBF measurements for magnification effects. A post-acquisition analysis of the velocity waveforms were performed using a standardized laboratory protocol to remove aberrant waveforms affected by eye movements, tear film breakup, or improper tracking of the measurement laser. Velocity waveforms were accepted if there was a minimum of one complete cardiac cycle which was not adversely affected by major eye movements, i.e. more than 25 μm in each direction.

4.4.2 Evaluation of disturbance in vascular reactivity over 6 months

"Δ" was defined as the difference between vascular reactivity at visit 1 and visit 2 in terms of arteriolar diameter, blood velocity, max: min velocity ratio and blood flow. A worsening of vascular reactivity response in visit 2 compared to visit 1 was assigned a negative value (-).

4.4.3 Statistical Analysis

The normality for all variables as a function of group was confirmed to satisfy the use of parametric statistics. All parameters followed a normal distribution, except for the maximum-to-minimum velocity ratio that was then log transformed in order to satisfy normality. A One-way ANOVA was used to determine differences in VR between groups at baseline and at visit 2. For all significant one-way ANOVAs, Bonferroni corrections were used in order to reduce Type I experimental errors. The Δ VR parameters between Group 2 and Group 3 were tested using paired t-tests. The dependant variables were diameter, velocity maximum-to-minimum velocity ratio and blood flow. The within subject factor was isocapnic hyperoxia and the between subject factor was group. The results of sICAM-1, sVCAM-1, sE-selectin and vWF were represented using box-plots. The magnitude of VR at baseline and ΔVR over 6 months time for each of the hemodynamic parameters in each group was correlated with baseline sICAM-1, sVCAM-1, sE-selectin, vWF Ag, hs CRP and A1c values using bivariate Pearson correlation. A Pearson correlation coefficient value of $r>0.3$ and $p<0.05$ was considered statistically significant. All significant correlations were then entered into a multiple regression model to determine if any of the significant factors were predictive of the dependent variable (a given hemodynamic parameter).

4.5 Results

Of the total subjects enrolled in the study, 21 patients with diabetes returned for follow-up assessment 6 months later, comprising 14 mild-to-moderate NPDR subjects (*Group 2*) (8M: 6F, mean age of 64.1 ± 8.1years) and 7 moderate-to-severe NPDR subjects (*Group 3*) (3M: 4F, mean age of 58.7 ± 11.3 years).

4.5.1 Baseline (visit 1) and follow-up (visit 2) vascular reactivity response

VR at visit 1 showed no significant differences in mean values in terms of vessel diameter, blood velocity, log maximum-to-minimum velocity ratio and blood flow between groups (see Table 4-2). Figure 4-1 illustrates the VR response to the standardized isocapnic hyperoxic stimulus at visit 1 for all retinal hemodynamic parameters.

Mean VR response at visit 1 in terms of	**Group 1** **n=10**	**Group 2** **n=22**	**Group 3** **n=14**	**ANOVA p-value**
Diameter (µm)	-3.9 (4.9)	-2.4 (5.8)	-0.7 (6.2)	0.41
Velocity (mm/sec)	-9.7 (3.0)	-10.4 (6.6)	-9.9 (7.3)	0.96
Flow (µL/min)	-2.4 (2.0)	-3.4 (2.3)	-2.8 (2.8)	0.54
Log Max:Min Ratio	0.05 (0.2)	0.07 (0.2)	0.09 (0.1)	0.79

Table 4-2: Group mean vascular reactivity in terms of retinal hemodynamic parameters to a standardized isocapnic hyperoxic stimulus at visit 1.

Retinal hemodynamics in terms of vessel diameter, blood velocity, log max:min velocity ratio and blood flow. Statistical significance are assumed when $p<0.05$. Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

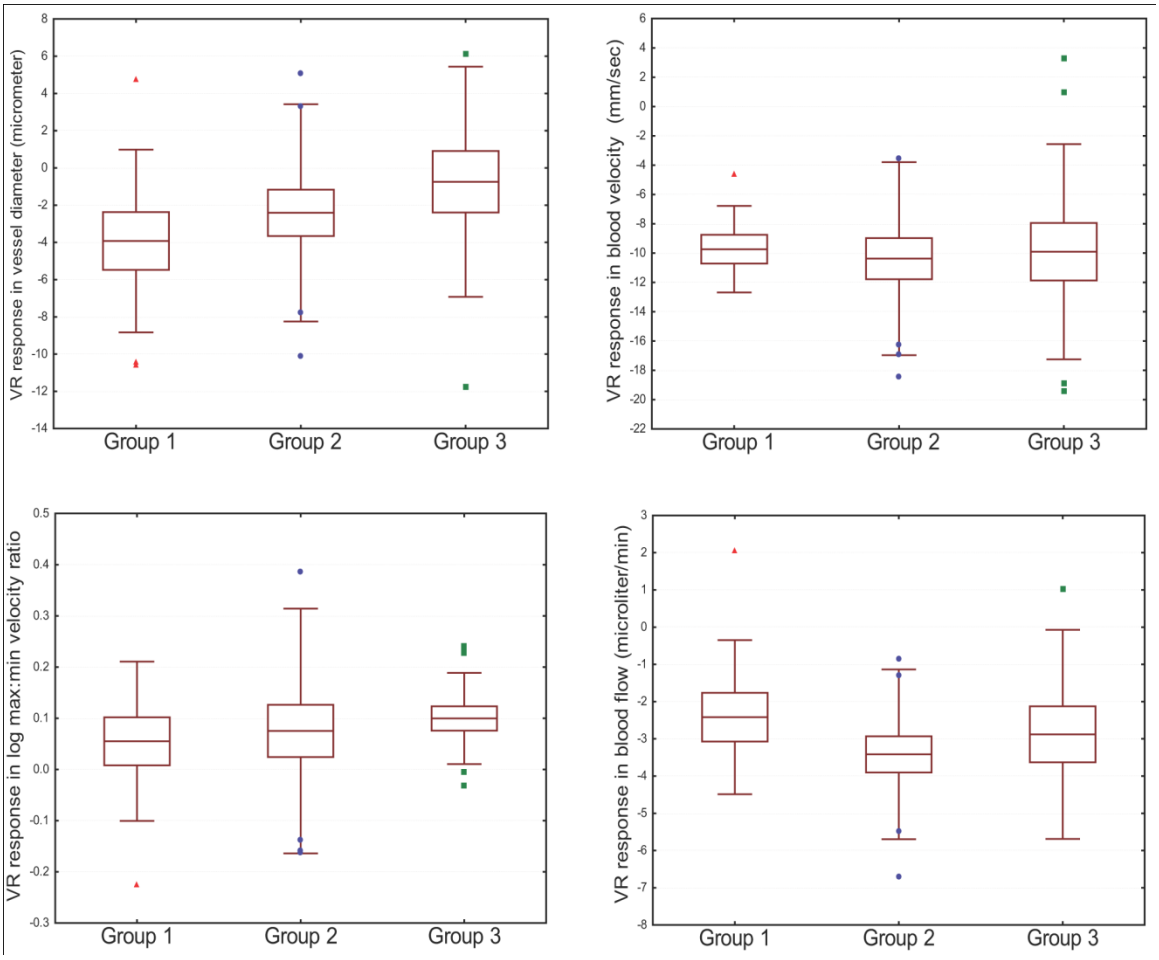

Figure 4-1: Box plots of vascular reactivity in retinal hemodynamic parameters in response to an isocapnic hyperoxic stimulus a function of group at visit 1.

Arteriolar vessel diameter (upper left), blood velocity (upper right), blood flow (bottom left) and log maximum-to-minimum velocity ratio (bottom right). For each graph, the center of the box represents the group mean, the limits of the box represent ±1 SE and the whiskers represent ±1 standard deviation. Each point represents an outlier. Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

VR at follow-up (visit 2) also showed no significant differences in change in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and blood flow between Groups 2 and 3 (see Table 4-3).

Mean VR response at visit 2 in terms of	Group 1 N=10	Group 2 n=22	Group 3 n=14	t-test p-value
Diameter (µm)	n/a	-3.0 (3.2)	-3.0 (4.2)	0.99
Velocity (mm/sec)	n/a	-11.7 (9.1)	-7.5 (4.1)	0.23
Flow (µL/min)	n/a	-3.5 (2.5)	-2.8 (2.0)	0.55
Log Max:Min Ratio	n/a	0.04 (0.7)	0.11 (0.6)	0.60

Table 4-3: Group mean vascular reactivity (VR) in terms of retinal hemodynamic parameters to a standardized isocapnic hyperoxic stimulus at visit 2.

Retinal hemodynamics in terms of vessel diameter, blood velocity, log max:min velocity ratio and blood flow. Statistical significance are assumed when $p<0.05$. Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

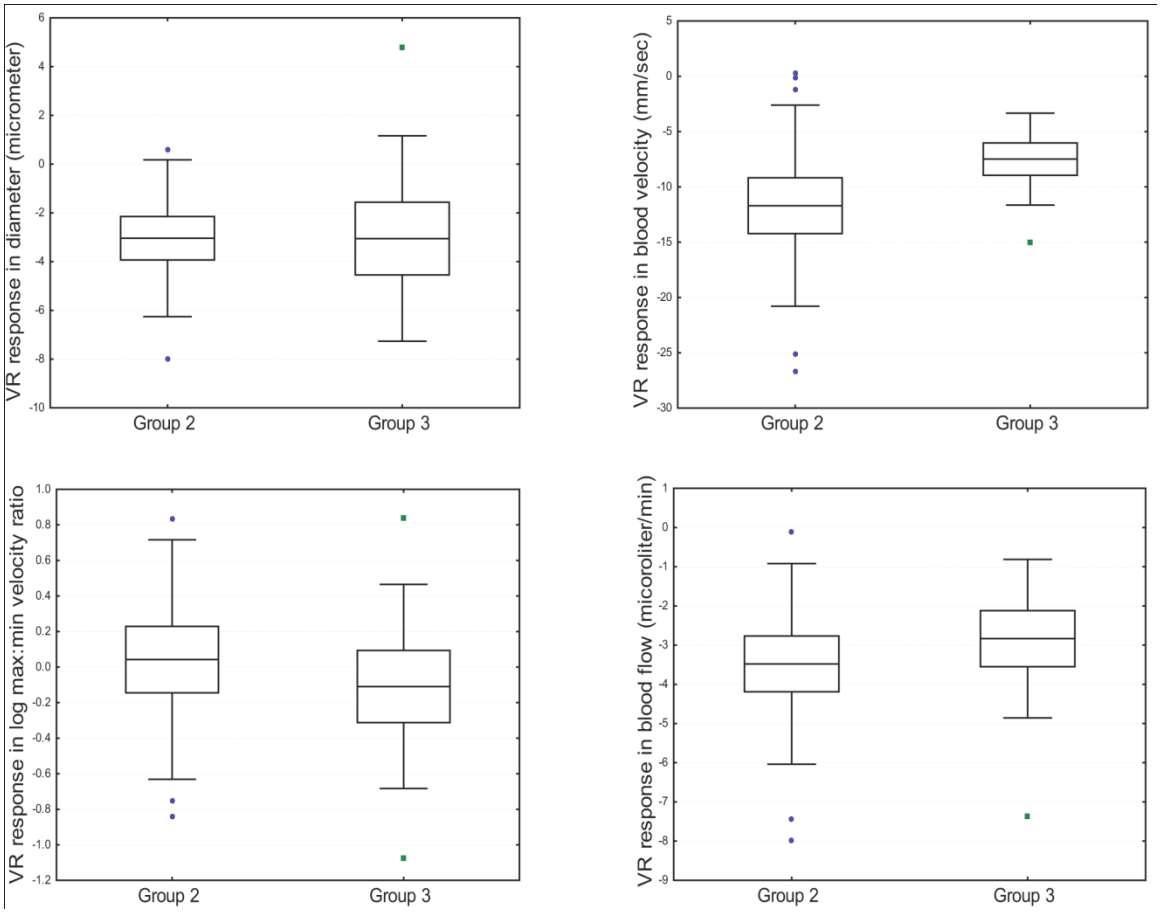

Figure 4-2: Box plots of vascular reactivity in retinal hemodynamic parameters in response to an isocapnic hyperoxic stimulus a function of group at visit 2.

Arteriolar vessel diameter (upper left), blood velocity (upper right), blood flow (bottom left) and log maximum-to-minimum velocity ratio (bottom right). For each graph, the center of the box represents the group mean, the limits of the box represent ±1 SE and the whiskers represent ±1 standard deviation. Each point represents an outlier. Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

4.5.2 Change in vascular reactivity response (ΔVR) over 6 month time

Δ max:min velocity ratio (compliance) was found to be significantly reduced in Group 3 patients compared to Group 2 (-0.4 vs. 0.1, t-test p<0.01) (See Figure 4-3). There were no significant differences in the mean ΔVR (VR difference between visit 1 and visit 2) in terms of arteriolar diameter, blood velocity and flow between Groups 2 and 3 (see Table 4-4).

Mean ΔVR in terms of	**Group 2** **n=14**	**Group 3** **n=7**	**t-test** **p-value**
ΔDiameter (μm)	1.6 (6.7)	-0.9 (4.0)	0.33
ΔVelocity (mm/sec)	-0.4 (5.8)	1.6 (7.5)	0.49
ΔBlood Flow (μL/min)	-0.2 (1.8)	1.2 (2.3)	0.13
ΔLog max:min velocity ratio	0.1 (0.4)	-0.4 (0.4)	<0.01*

Table 4-4: Mean Δ vascular reactivity in terms of retinal hemodynamic parameters as a function of group.

Retinal hemodynamics in terms of vessel diameter, blood velocity, log max:min velocity ratio and blood flow. An impaired ΔVR response in visit 2 compared to visit 1 was assigned a negative value (-), indicating direction of change. Statistical significance are assumed when p<0.05. Group 2; subjects with mild-to-moderate NPDR, Group 3: subjects with moderate-to-severe NPDR.

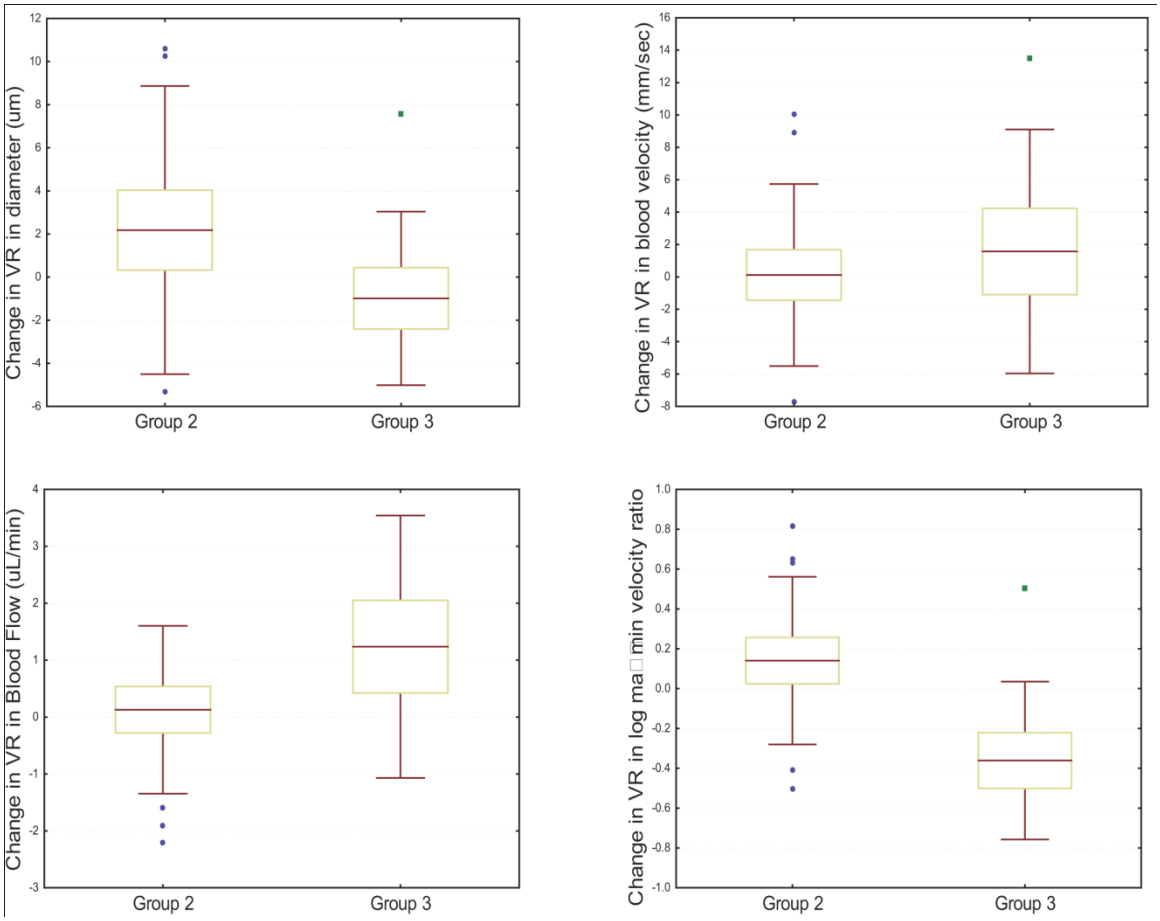

Figure 4-3: Box plots of change in vascular reactivity (ΔVR) from visit 1 to visit 2 (6 month follow-up) in terms of retinal hemodynamic parameters.

Retinal hemodynamic parameters include arteriolar vessel diameter (upper left), blood velocity (upper right), blood flow (bottom left) and log maximum-to-minimum velocity ratio (bottom right). For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. Each point indicates outliers. Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR. A negative value represents worsening of vascular reactivity response.

4.5.3 Baseline levels of inflammation and endothelial dysfunction markers

Baseline group mean levels of inflammation and endothelial dysfunction are defined below (see Table 4-5), and are illustrated in terms of box-plot distributions in Figure 4-4. Both sICAM-1 and sE-selectin were significantly elevated as a function of group (ANOVA p=0.02 and p<0.01). A post hoc Bonferroni test showed that Group 3 had significantly higher sICAM-1 and sE-selectin levels compared to Group 1 (243.4 vs. 157.3ngml, P<0.01 and 57.0 vs. 29.3 ng/ml, P<0.01, respectively). sVCAM-1 and vWF Ag was not significantly different between groups (ANOVA p=0.11 and p=0.49, respectively). A1c was significantly elevated in across the groups (ANOVA p=0.013) (see Table 4-6 and Figure 4-5), with Group 3 having higher A1c levels compared to Group 1 (8.8 vs. 5.6 %, P<0.01).

	Group 1 **n=10**	**Group 2** **n=22**	**Group 3** **n=14**	**ANOVA p-value**
sICAM-1 (ng/ml)	157.3 (53.9)	198.4 (68.3)	243.4* (82.1)	**0.01***
sVCAM-1 (ng/ml)	346.8 (57.7)	473.9 (218.6)	492.4 (158.4)	0.11
sE-selectin (ng/ml)	29.3 (8.2)	44.8 (15.9)	57.0* (28.9)	**<0.01***
vWF Ag (U/ml)	1.1 (0.4)	1.2 (0.4)	1.1 (0.4)	0.49

Table 4-5: Group mean and standard deviation (SD) of markers inflammation and endothelial dysfunction as a function of group.

sICAM-1, sVCAM-1 and sE-selectin are markers of inflammatory activity, while vWF Ag is a marker of endothelial dysfunction. sICAM-1: soluble intercellular adhesion molecule type 1, sVCAM-1: soluble vascular cell adhesion molecule type 1, and vWF Ag; von Willebrand factor antigen. (* indicates significantly different from group 1). Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

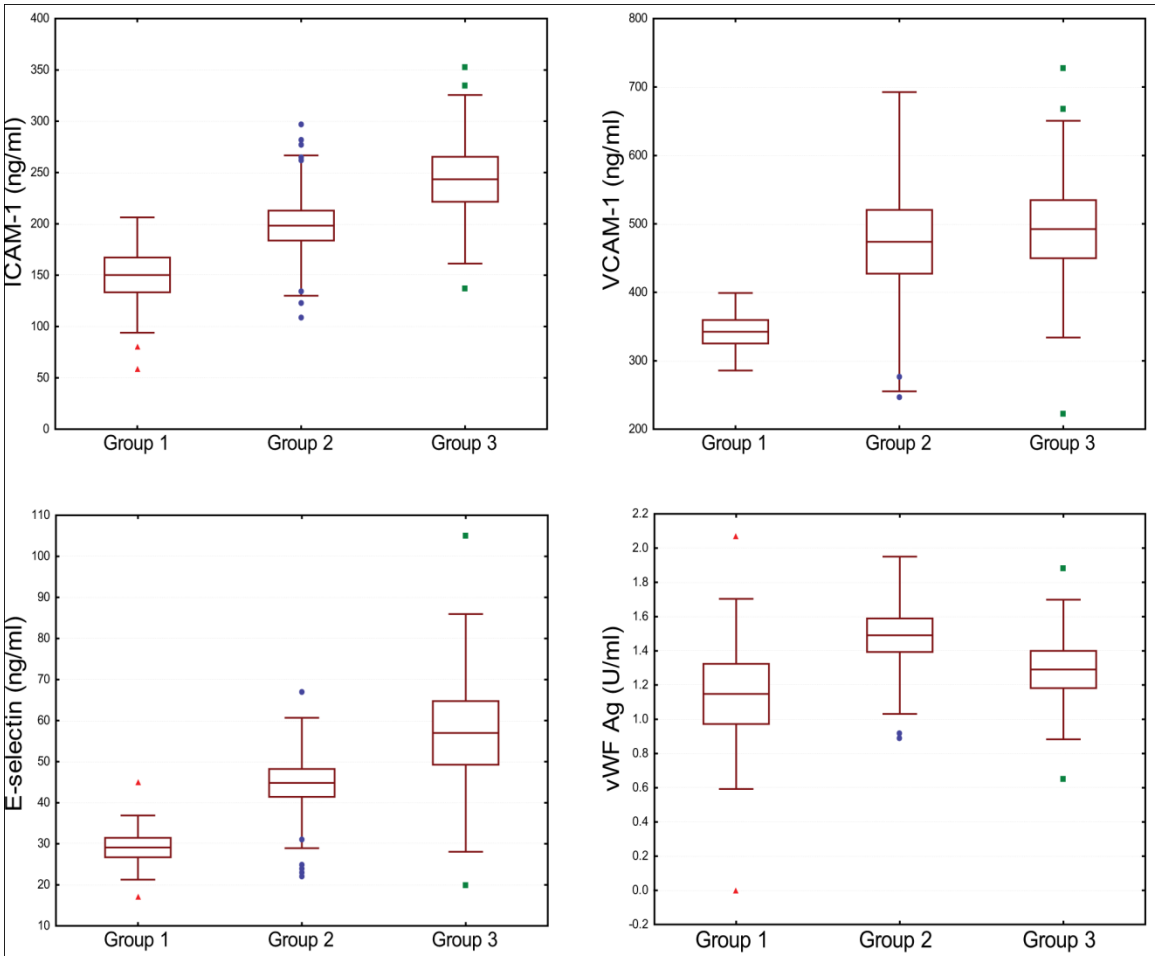

Figure 4-4: Box plots of baseline levels of inflammation and endothelial dysfunction markers as a function of group.

The inflammatory markers are; soluble intracellular adhesion molecule type 1 (sICAM-1) (upper left), soluble vascular cell adhesion molecule type 1 (sVCAM-1) (upper right) and soluble E-selectin (bottom left). The endothelial dysfunctional marker is von Willebrand factor antigen (vWF) (bottom right). For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. Each point represents an outlier values. Group 1: age matched controls, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

	Group 1 n=10	Group 2 n=22	Group 3 n=14	ANOVA p-value
Hemoglobin A1c (%)	5.6 (0.4)	7.7 (2.3)	8.8* (3.4)	**0.01***
hsCRP (mg/L)	1.3 (0.9)	2.8 (3.3)	2.2 (2.8)	0.35

Table 4-6: Group mean and standard deviation of A1c and hs CRP as a function of group.

hsCRP: high sensitivity C-reactive protein and A1c; glycosylated hemoglobin (* indicates significantly different from group 1). Group 1; age-matched control, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

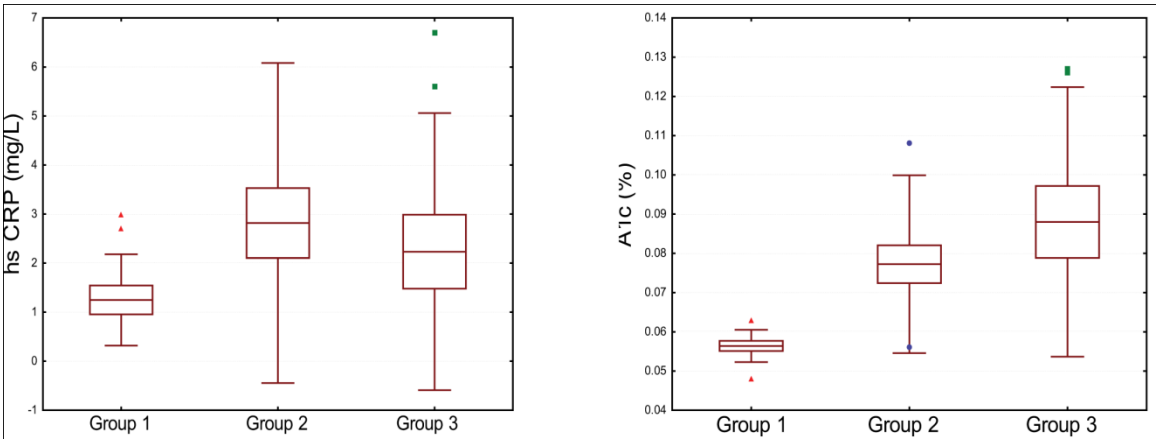

Figure 4-5: Box plots of hs CRP and A1c markers as a function of group.

Hs CRP; high sensitivity C-reactive protein and A1c; glycosylated hemoglobin. For each graph, the center of the box represents the group mean level, the limits of the box represent ±1 SE and the whiskers represent ±1 SD. An open circle represents outlier values. Group 1: age matched controls, Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR.

4.5.4 Relationship between baseline (visit 1) VR and baseline levels of vascular inflammation and endothelial dysfunction

Baseline (Visit 1). The relationships between each hemodynamic aspect of baseline VR and systemic markers of vascular inflammation and endothelial dysfunction were examined using a bivariate Pearson correlation. Results showed that VR in terms of velocity was weakly associated with sE-selectin (r=0.31, p=0.04). Otherwise, there were no significant associations found between baseline VR in terms of diameter, blood flow and max:min velocity ratio and baseline levels of vascular inflammation and endothelial dysfunction across all group (Table 4-7).

Type of marker		**Inflammation**		**Endothelial dysfunction**		
VR in terms of:	**sICAM-1 (ng/ml)**	**sVCAM-1 (ng/ml)**	**sE-selectin (ng/ml)**	**vWF Ag (U/ml)**	**A1c (%)**	**hs CRP (mg/L)**
Diameter (µm)	0.04 (0.76)	-0.11 (0.48)	0.01 (0.96)	0.16 (0.58)	0.09 (0.55)	-0.15 (0.32)
Velocity (mm/sec)	0.18 (0.24)	-0.19 (0.19)	**0.31 (0.04)***	0.28 (0.32)	0.14 (0.36)	0.06 (0.65)
Blood Flow (µL/min)	0.14 (0.36)	-0.27 (0.08)	0.23 (0.14)	0.25 (0.39)	0.15 (0.34)	0.15 (0.32)
Log max:min velocity ratio	0.04 (0.77)	0.15 (0.31)	0.11 (0.47)	0.19 (0.51)	-0.05 (0.73)	0.07 (0.65)

Table 4-7: Pearson correlation coefficients (r) and statistical significance (p) between markers of inflammation/endothelial dysfunction and baseline vascular reactivity (VR) in retinal hemodynamics ratio across all groups.

Retinal hemodynamic parameters as a function of vessel diameter, blood velocity, blood flow and log maximum-to-minimum velocity ratio in the pooled group. Systemic markers are; soluble intracellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), soluble E-selectin and von Willebrand factor antigen (vWF). A significant correlation was taken at r>±

0.30 and $p<0.05$. Maximum-to-minimum velocity ratio was log transformed to satisfy normality. (*indicates statistical significance).

Multiple regression analysis found that none of the independent variables were significant predictors of change in VR in velocity. Based on these results we conclude that sE-selectin was weakly correlated with VR in terms of velocity across all groups (Figure 4-6).

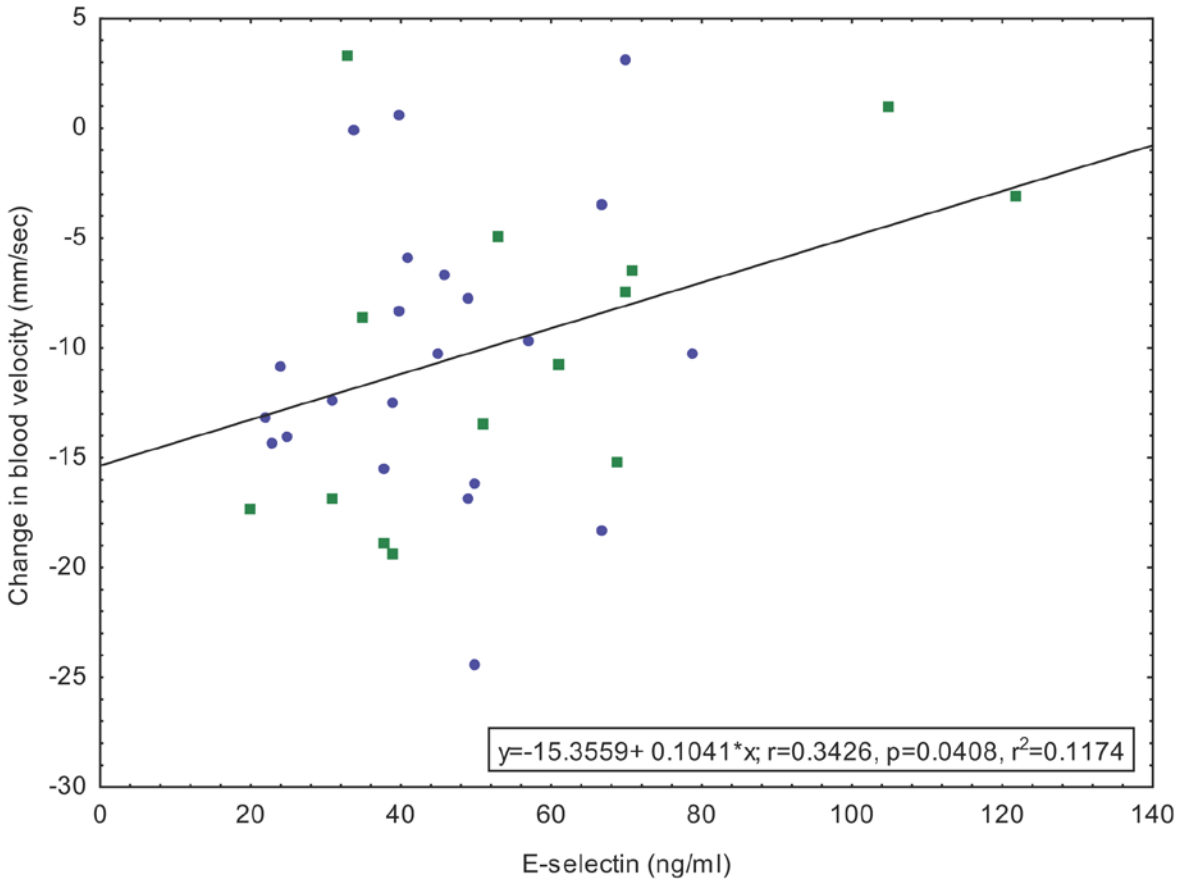

Figure 4-6: Scatter-plot of baseline vascular reactivity in blood velocity and baseline sE-selectin across all groups.

Each triangle represents subjects from Group 1 (healthy controls), circle represents subjects from Group 2 (mild-to-moderate NPDR), while each square represents subjects from Group 3 (moderate-to-severe NPDR).

Considering the likelihood of differing levels of inflammatory and endothelial dysfunction activity specific to each stage of DR, we explored possible correlations within each of Groups 2 and 3. There were no significant correlations in Group 3. A moderate inverse association was revealed between the VR in blood flow and sVCAM-1 ($r=-0.62$, $p<0.01$) in subjects of Group 2 (Table 4-8).

VR in terms of:	**sICAM-1 (ng/ml)**	**sVCAM-1 (ng/ml)**	**sE-selectin (ng/ml)**	**vWF Ag (U/ml)**	**A1c (%)**	**hs CRP (mg/L)**
Blood Flow (µL/min)	-.11 (0.64)	**-.62 (<0.01)***	.15 (0.51)	.1934 (0.40)	.0667 (0.77)	-.37 (0.09)

Table 4-8: Pearson correlation coefficients (r) and statistical significance (p) between markers of vascular inflammation/endothelial dysfunction and baseline vascular reactivity in blood flow in group 2

Multiple regression analysis indicated that none of the independent variables were significant predictors of change in VR in terms of blood flow. Based on the results we conclude that sVCAM-1 has weak predictive power for VR in blood flow in patients with mild-to-moderate NPDR but still shows moderate correlation.

4.5.5 Bivariate Pearson correlations between change in vascular reactivity (ΔVR) and baseline levels of vascular inflammation and endothelial dysfunction

Pearson coefficients between ΔVR in terms of retinal hemodynamics and change in levels of vascular inflammation and endothelial dysfunction markers across Groups 2 and 3 are in Table 4-9. Our results show that Δ blood flow was weakly associated with sE-selectin levels ($r=0.46$, $p=0.03$).

Type of marker	Inflammation			Endothelial dysfunction		
	sICAM-1 (ng/ml)	**sVCAM-1 (ng/ml)**	**sE-selectin (ng/ml)**	**vWF (U/ml)**	**A1c (%)**	**hs CRP (mg/L)**
ΔDiameter (μm)	0.05 (0.83)	-0.08 (0.71)	-0.19 (0.39)	-0.18 (0.42)	-0.25 (0.28)	-0.16 (0.49)
ΔVelocity (mm/sec)	-0.06 (0.79)	0.09 (0.68)	0.25 (0.28)	-0.16 (0.49)	0.27 (0.24)	0.04 (0.85)
ΔBlood Flow (μL/min)	0.29 (0.19)	0.11 (0.63)	**0.46 (0.03)***	-0.21 (0.35)	0.42 (0.05)	-0.07 (0.75)
ΔLog max:min velocity ratio	0.14 (0.52)	0.05 (0.81)	0.07 (0.73)	-0.09 (0.69)	0.05 (0.82)	-0.26 (0.24)

Table 4-9: Pearson correlation coefficients (r) and statistical significance (p) between markers of inflammation/endothelial dysfunction and Δ VR in hemodynamic parameters in patients with NPDR.

Retinal hemodynamic parameters are taken as vessel diameter, blood velocity, blood flow and log maximum-to-minimum velocity ratio) in patients with NPDR. Systemic markers are; soluble intracellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), soluble E-selectin and von Willebrand factor antigen (vWF). A significant correlation was taken at $r>\pm 0.30$ and $p<0.05$. Group 2; mild-to-moderate NPDR, Group 3: moderate-to-severe NPDR (*indicates statistical significance).

Multiple regression analysis showed that none of the independent variables were significant predictors of change in blood blow. Based on these results, we conclude that sE-selectin has weak predictive power for Δ blood flow across all groups but is weakly correlated.

Similar to the previous section we considered the likelihood of differing levels of inflammatory and endothelial dysfunction activity specific to each stage of DR, we explored possible correlations

within each Groups 2 and 3. There were no significant correlations in Group 2. A moderate inverse association was revealed between the Δ velocity and vWF (r=-0.78, p=0.02) in subjects of Group 3 (Table 4-10) (Figure 4-7).

Type of marker	**Inflammation**			**Endothelial dysfunction**		
	sICAM-1 (ng/ml)	**sVCAM-1 (ng/ml)**	**sE-selectin (ng/ml)**	**vWF (U/ml)**	**A1c (%)**	**hs CRP (mg/L)**
ΔVelocity (mm/sec)	-0.14 (0.74)	0.16 (0.71)	0.05 (0.91)Δ	**-0.78 (0.02)***	0.05 (0.90)	0.16 (0.69)

Table 4-10: Pearson correlation coefficients (r) and statistical significance (p) between Δ velocity and markers of inflammation/endothelial dysfunction in patients with moderate-to-severe NPDR (Group 3).

Retinal hemodynamic parameters are taken as vessel diameter, blood velocity, blood flow and log maximum-to-minimum velocity ratio) in patients with NPDR. Systemic markers are; soluble intracellular adhesion molecule type 1 (sICAM-1), soluble vascular cell adhesion molecule type 1 (sVCAM-1), soluble E-selectin and von Willebrand factor antigen (vWF). A significant correlation was taken at $r>\pm 0.30$ and $p<0.05$. Group 3: moderate-to-severe NPDR (*indicates statistical significance).

Multiple regression analysis showed that none of the independent variables were significant predictors of Δ blood velocity. Based on these results we conclude that vWF has weak predictive power for Δ velocity in patients with moderate-to-severe NPDR but are moderately correlated. The relationship between Δ velocity and vWF is plotted in Figure 4-7.

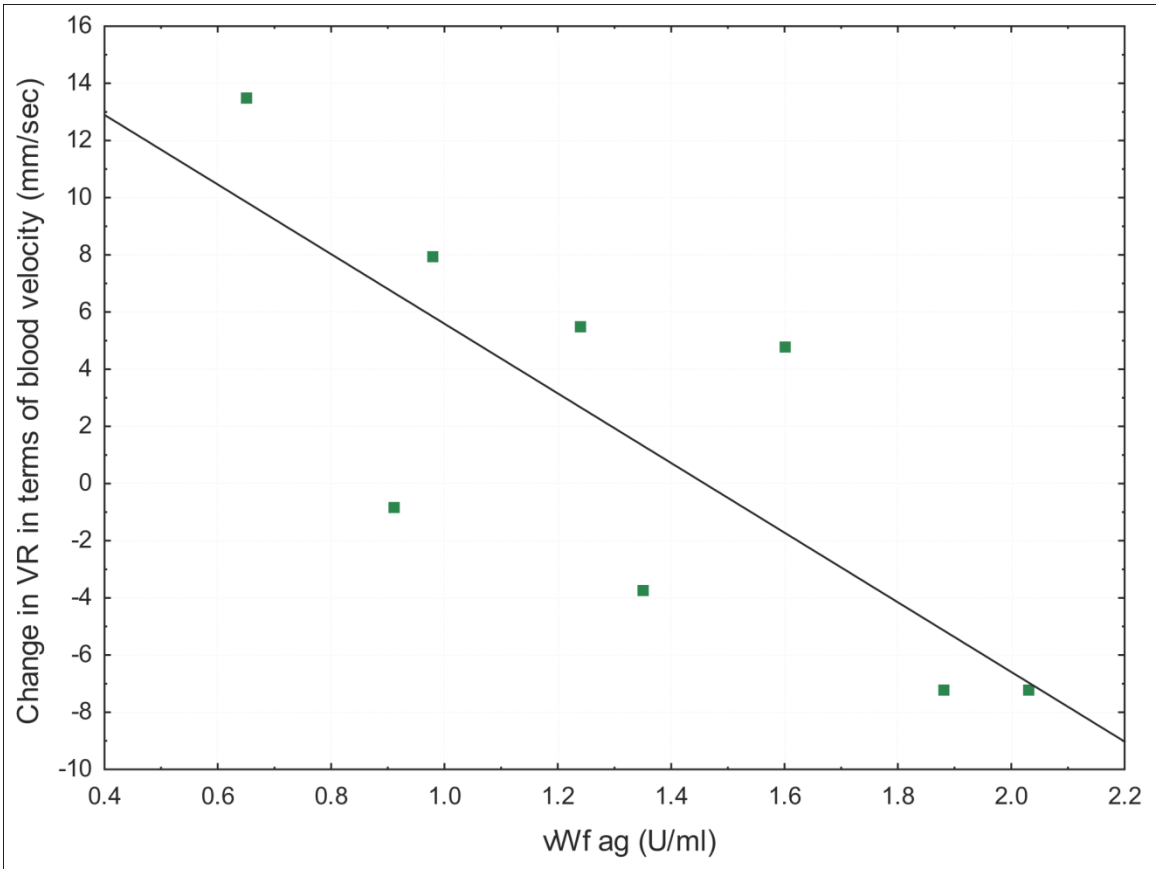

Figure 4-7: Scatter plot of the change in vascular reactivity from visit 1 to visit 2 in terms of blood velocity and von Willebrand factor antigen in patients moderate-to-severe NPDR (Group 3).

Overall, multiple regression analysis showed that none of the independent variables were significant predictors of Δ vessel diameter, blood velocity, max:min velocity ratio or blood flow. Based on these results, we conclude that the vascular inflammatory and endothelial function markers have weak predictive power for Δ hemodynamic parameters in patients with mild-to-moderate and moderate-to-severe NPDR, despite the presence of weak and moderate correlations.

4.6 Discussion

This is the first study to investigate the association between vascular inflammatory and endothelial dysfunction markers and retinal vascular reactivity in patients with increasing severity of NPDR. In our sample, we had characterized two changes that are related to early NPDR: 1) a greater increase in vascular rigidity and reduced vascular reactivity after 6 months follow-up; and 2) elevated levels of soluble adhesion markers (sICAM-1 and sE-selectin) indicating inflammatory activity. This study suggests that vessel compliance may occur along with elevated inflammatory levels in patients with increasing NPDR severity. Although vascular inflammatory and endothelial function markers have weak predictive power for Δ hemodynamic parameters, weak to moderate significant correlations were found in both baseline and in the disturbance of vascular reactivity (ΔVR) after 6 month time and markers of inflammation and endothelial dysfunction across all groups. Specifically, baseline VR in blood velocity weakly correlates with sE-selectin ($r=0.31$, $p=0.04$) across all groups while sVCAM-1 is associated with VR in terms of blood flow ($r=-0.62$, $p<0.01$) in patients with mild-to-moderate NPDR. TheΔ in blood flow after 6 months was found to be weakly associated with sE-selectin ($r=0.46$, $p=0.03$) across all groups. Finally, the Δ velocity after 6 month time was found to be moderately correlated with baseline vWF level ($r=-0.78$, $p=0.02$). Therefore, both inflammatory activity and endothelial dysfunction may be related to abnormal retinal vascular function.

Abnormal macro and microvascular vascular reactivity response in patients with DR are well established [5, 18, 42]. There was no significant difference in VR in visit 1 or 2 across all groups. In a recent study using the standardized isocapnic hyperoxic stimulus, VR in patients with early sight threatening DR was found to be significantly reduced with increasing severity of early DR and sight threatening DME [18]. Physiologically, retinal blood flow varies inversely with the partial pressure of arterial oxygen (PO_2) to maintain retinal oxygenation at a relatively constant

level. In our study, although the magnitude of response in vessel diameter, velocity and flow were not significantly different between groups, the response tended to be impaired with increasing severity of NPDR. The lack of significance is likely because our sample was not sufficient but this never was an aim of the study.

Although functional hemodynamic indices such as maximum-to-minimum velocity ratio, and wall shear rate have been previously studied in DR [24, 51, 53], few other studies have examined the change in retinal vascular reactivity response over time. Results from this study show that patients with moderate-to-severe NPDR exhibited a greater increase in vascular rigidity and reduced vascular reactivity over time than those with mild-to-moderate NPDR. Recent work has also demonstrated a reduced compliance in type 2 patients with NPDR and DME [25]. Loss of vascular reactivity and a reduced compliance of the retinal arterioles with increasing severity of DR would explain these results and support the findings of the current study. In support of this, some conditions including aggravation of vascular risk factors and progression of arteriosclerosis, lead to vessel wall remodeling and greater vessel rigidity over time. Overall, this indicates an inability to increase vessel tonus in response to isocapnic hyperoxic provocation in patients with moderate-to-severe NPDR.

Our results also demonstrated elevated vascular adhesion levels in our cohort. We found that inflammatory activity was increased with increasing severity of NPDR. Inflammatory markers sICAM-1 and sE-selectin were significantly higher in the moderate-to-severe group compared to healthy controls. Other systemic markers sVCAM-1, hsCRP and vWF were not statistically

different in patients with diabetes from those without (ANOVA p=0.11, p=0.35 and p=0.49). Our results were consistent with early studies, which show that sICAM-1 is elevated in patients with increasing severity of NPDR and plays a crucial role in leukocyte adherence to the endothelium [3, 30, 57, 60]. The up-regulation of sICAM-1 in the diabetic vasculature links leukostasis to early stages of DR [30, 32, 33, 60]. This is thought to be enhanced by several stimuli including vascular endothelial growth factor (VEGF) [8, 29, 31], advanced glycosylated end products (AGEs) [43, 48, 59] and oxidative stress [10, 13, 28] via the release of pro-inflammatory cytokines like Tumor Necrosis Factor-alpha (TNF-α) [34]. Moreover, we found that the expression of sE-selectin was significantly higher in patients with moderate-to-severe NPDR compared to the control group. The majority of previous studies did not find a difference in serum E-selectin levels in patients with NPDR compared to healthy controls [7, 32]. However, E-selectin is consistently elevated in the vitreous and serum samples of patients with PDR compared to healthy controls [1, 2].

We found that endothelial dysfunction marker vWF Ag was not different between groups, however, there was one earlier study that reported an elevated vitreous concentration of vWF in type 2 diabetes subjects with DR [49]. One of the reasons for these differences in findings could be a large biological variation of circulating adhesion molecules and vWF. Since we are measuring serum vWF Ag, its expression is unlikely isolated to retinal endothelial cells. The expression of the vascular adhesion molecules may be derived from cells outside of the retinal cells, which may contribute to the discrepancy in reports. For this and previous studies, only one measurement is performed. Further investigation of serial measurements of inflammatory and endothelial dysfunction markers over time might help predict inflammatory activity more accurately. Our

results suggest that there were elevated inflammatory levels of sICAM-1 and sE-selectin in patients with diabetic retinopathy compared to controls. Elevated inflammatory activity could play a role in the development arteriosclerotic characteristics in the vessels of patients with NPDR.

We found significant relationships between baseline VR and systemic markers of vascular inflammation and endothelial dysfunction. Results indicate that VR in terms of velocity was weakly associated with sE-selectin ($r=0.31$, $p=0.04$) across all patients and low VR in terms of blood flow is correlated to high sVCAM-1 levels ($r=-0.62$, $p<0.01$) in mild-to-moderate NPDR. This finding suggests a possible pro-inflammatory role of sE-selectin and sVCAM-1 in compromising retinal endothelial cell integrity. After adjustment for all other variables (A1c, hsCRP and vWF), the predictive power of this correlation was found to be negligible. At the cellular level, both sE-selectin and sVCAM-1 have been shown to exert pro-inflammatory properties by promoting leukocyte adhesion, likely via the NFk-B pathway [45, 48]. Considering the expression of these markers by vascular endothelial cells throughout the body and assuming that DR is a state of chronic vascular inflammation, sE-selectin and sVCAM-1 are not likely to represent a molecular link between metabolic signals, vascular inflammation and vascular dysfunction specifically to DR.

Our study report a correlation between disturbance of VR in terms of blood velocity (Δ velocity) over 6-months follow-up and baseline vWF level in patients with moderate-to-severe NPDR ($r=-0.78$, $p=0.02$). This suggests that a disturbance in blood velocity is associated with

lower levels of vWF. After adjustment for all other variables (A1c, hsCRP and vWF), the predictive power of this correlation was found to be negligible. The analysis of several published studies show consistent results, thus, the presence of impaired endothelial function does not demonstrate elevated vWF levels in diabetic retinopathy [42]. As retinal blood flow represents only a small part of the total blood flow in the body, it is also unlikely that high systemic vWF levels could result from increased production in the retinal endothelium at a single measurement. Although we only found a bi-variate correlation between vWF and VR, there are many studies that suggest that vWF favourably affects endothelial function and vascular homeostasis [6, 11, 49]. Elevations in plasma vWF in persons with diabetes are believed to reflect functional and structural changes in the vascular endothelium [35]. Further studies are needed to address whether intervention that decreases vWF levels can improve endothelial function and reduce clinical signs of NPDR.

Overall, although we only found weak to moderate bi-variate correlations between endothelial dysfunction, vascular inflammation and retinal vascular function in early NPDR, a common denominator of microvascular complications is thought to involve impaired nitric oxide synthesis and the generation of inflammatory state. A recent study by Patel and colleagues found that hemodynamic related marker endothelin-1 (ET-1) and anti-inflammatory cytokine IL-1 Ra are low in patients with NPDR [57]. In this environment, ET-1's vasoconstrictive effect would be minimal. This potential relationship supports the hypothesis, set up by other authors [10] that vascular inflammation is the primary process in endothelial dysfunction resulting in reduced vasoconstrictive response to an isocapnic hyperoxic stimulus.

In conclusion, we found significant bi-variate relationships between baseline VR and in the disturbance of VR after 6 month time and markers of inflammation and endothelial dysfunction across all groups. However, these were of no predictive value for hemodynamic parameters. Overall, this study is the first to characterize two important features that are present in patients with increasing NPDR; 1) tendency for impaired vascular reactivity and reduced compliance in the retinal arteriolar vessel; and 2) increased levels of sICAM-1 and sE-selectin. Elevated inflammatory adhesion molecules in early NPDR may disturb vascular function, possibly mediated through smooth muscle cell dysfunction as well as endothelial dysfunction. The mechanism of leukostasis and its affiliation to a loss of vascular reactivity in the retinal microcirculation is clearly complex and deserves further investigation. Understanding whether adhesion molecules interplay with endothelial dysfunction in producing vascular dysregulation may lead to the generation of strategies that could target early abnormalities before clinically visible signs of DR are apparent.

Chapter 5
General Discussion

Studies have shown that altered retinal blood flow and vascular regulation and elevated vascular inflammatory activity are both involved in the pathogenesis of early diabetic retinopathy (DR)[5-7, 9, 10, 16-18]. A loss of vasoconstrictive capacity of the retinal vasculature to a hyperoxic stimulus has been established [2-4, 8]. Increased levels of systemic vascular inflammatory markers are found in persons with type 2 DR. Endothelial dysfunction is also found to be strongly associated with early DR in both type 1 and 2 diabetes[1, 11, 12, 15]. Earlier studies have shown abnormalities of blood flow parameters along with increased leukocyte adhesion in the retinal capillaries in animal models, suggesting that these changes may represent a precursor to any clinical findings [13, 14]. Prior to this work, however, it was unknown whether abnormal retinal hemodynamics related to increasing vascular inflammatory activity or endothelial dysfunction in human NPDR. We have investigated the concomitant disturbance of retinal hemodynamics and retinal VR with systemic markers of vascular inflammation and endothelial dysfunction.

This investigation of patients with type 2 diabetes with NPDR had two objectives:

1) To investigate the concomitant disturbance of retinal blood flow hemodynamics and its association with systemic markers of vascular inflammation and endothelial dysfunction.
2) To investigate the concomitant disturbance of retinal VR and its association with systemic markers of vascular inflammation and endothelial dysfunction.

For the two objectives, we hypothesized:

1) Baseline arteriolar hemodynamics and the magnitude of retinal hemodynamic disturbance over 6-month follow-up are positively correlated with systemic markers of vascular inflammation and endothelial dysfunction in type 2 diabetes.

2) The magnitude of retinal VR at baseline and the disturbance of VR over 6-month follow-up are positively correlated with systemic markers of vascular inflammation and endothelial dysfunction in type 2 diabetes.

In answering the first objective (Chapter 3), retinal hemodynamics was assessed both in terms of vessel diameter, blood velocity, maximum-to-minimum velocity ratio and volumetric flow at baseline and in terms of change in hemodynamic parameters over 6 months time in patients with NPDR. Our results also show an increase rigidity of the arteriolar circulation at baseline and follow-up and an increase vascular adhesion markers sICAM-1 and sE-selectin within the same population sample. We also found that the Δ velocity over the follow-up period was correlated with sICAM-1 and A1c levels in patients with NPDR but the level of association was such that neither sICAM-1 nor A1c proved to reliably predict retinal hemodynamics. Results from this study support reduced retinal arteriolar compliance in conjunction with increased vascular inflammatory activity in patients with moderate-to-severe NPDR compared to healthy controls, suggesting a possible link between vascular inflammation and prospective increase in retinal vascular rigidity in early NPDR.

In chapter 4, we investigated retinal vascular regulation and circulating systemic markers of inflammation and endothelial dysfunction. Retinal VR was assessed both in terms of vessel diameter,

blood velocity, maximum-to-minimum velocity ratio and volumetric flow at baseline and in terms of change in VR over 6 months time in patients with NPDR. Overall, we found reduced compliance after 6 months follow–up in the moderate-to-severe group compared to the mild-to-moderate group and concurrent elevated baseline levels of sICAM-1 and sE-selectin. Moreover, results indicate a bi-variate association between baseline VR in blood velocity with sE-selectin ($r=0.31$, $p=0.04$) across all groups while sVCAM-1 was associated with VR in terms of blood flow ($r=-0.62$, $p<0.01$) in patients with mild-to-moderate NPDR. The Δ blood flow after 6 months was found to be weakly associate d with sE-selectin ($r=0.46$, $p=0.03$) across all groups. Finally, the Δ blood velocity after 6 month time was found to be moderately correlated with baseline vWF Ag level ($r=-0.78$, $p=0.02$). However, after being entered into a multiple regression model, these vascular inflammatory and endothelial dysfunction markers were of no predictive value for any hemodynamic parameters. These results suggest a possible pro-inflammatory role that might be involved in retinal endothelial integrity. Elevated inflammatory adhesion molecules in early NPDR may disturb vascular function, possibly mediated through smooth muscle cell dysfunction as well as endothelial dysfunction. Although these markers show no predictive value in VR, results indicate that retinal vascular function might alter concomitantly with inflammatory activity in early NPDR.

In conclusion, our study found 1) increased levels of sICAM-1 and sE-selectin along with 2) increased pulsatility or vessel rigidity in the retinal arteriolar vasculature at baseline and follow-up and 3) a tendency for impaired vascular reactivity and reduced compliance in the retinal arteriolar vessel in early DR. These findings support early accelerated arteriosclerosis type changes and altered endothelial cell activity in mediating abnormal in RBF in early development of DR. The mechanism of leukostasis and its possible relationship to a loss of VR in the retinal microcirculation is clearly complex and deserves further investigation. Understanding whether

adhesion molecules interplay with endothelial dysfunction in producing vascular dysregulation may lead to the development of strategies that could target early abnormalities before clinically visible signs of DR are apparent.

Future Studies

Future work should investigate the concomitant disturbance in VR and vascular inflammatory activity over an extended longitudinal study design. It is of significance to understand whether prolonged exposure to hyperglycemia might have a different chronic effect on retinal vascular reactivity and inflammatory and endothelial activity. This question is particularly important in the light of previous studies showing an impaired vascular reactivity following administration of high doses of glucose [(4)]. Intervention and longer longitudinal studies that track changes in vascular inflammatory and endothelial function levels and VR are necessary to better determine their association. A fuller understanding of biochemical influences to the vascular reactivity response can be obtained by measuring certain derived endothelium derived vasoactive factors such as nitric oxide (NO) and endothelin-1 (ET-1). We suspect that vascular inflammatory activity may influence endothelium derived vasoactive factors, thereby affecting the VR response. Recently, it has been shown that vascular adhesion protein-1 (VAP-1) is involved in leukocyte trafficking in inflammatory conditions such as endotoxin-induced uveitis. This molecule expressed on the endothelium of retinal vessels is known to contribute to inflammatory leukocyte recruitment and angiogenesis in experimental models of ocular diseases. However, further studies are needed to address the expression and function of VAP-1 and its role in the pathogenesis of DR.

Appendix A

Methodology

Isolating Markers of Inflammation (sICAM-1, sVCAM-1, and E-selectin)

The assays are based on the standard ELISA sandwich enzyme immunoassay technique. For each type of assay, monoclonal antibodies (sICAM-1, sVCAM-1 or sE-selectin) are pre-coated onto the microplates. Standards, samples, conjugates and controls are pipetted into the wells and then incubated for 1.5 hrs at room temperature. Any adhesion molecules present (sICAM-1, sVCAM-1 or sE-selectin) is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for that adhesion molecule. The microplates are then aspirated and then washed with a buffer solution (buffered surfactants with preservatives) four times so that any unbound antibody-enzyme conjugates are removed. The microplate is then inverted to remove any excess buffer solution. Immediately following the buffer wash, the substrate solution (a prepared solution of hydrogen peroxide and chromogen) are added to the wells and incubated for 20 minutes, away from light. Chromogen changes colour when cleaved by the enzyme attached to the second antibody. A stop solution of sulfuric acid is then added to each well to terminate further changes in colour. Optical density (OD) is determined for each well using a microplate set to 450nm using Spectral Max Plus 384 (*Softmaxpro*).

Isolating a marker of endothelial dysfunction, vWF antigen

The STA Liatest vWF kit involves the measurement of monochromatic light traversing a suspension of microlatex particles to which polyclonal rabbit antihuman vWF antibodies have been attached by covalent bonding. Where the wavelength of the light is much greater than the diameter of the latex

particles, the light is only slightly absorbed. In the presence of the antigen under test, the antibody-coated latex particles agglutinate to form aggregates of diameters greater than the wavelength of the light; more of the latter is absorbed, and is proportional to the antigen level present in the test sample.

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Chapter 5

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