OCULAR AND SYSTEMIC MARKERS FOR VASCULAR FUNCTION IN THOSE AT RISK OF TYPE 2 DIABETES MELLITUS AND CARDIOVASCULAR DISEASE

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Thesis Summary

The devastating impact of Type 2 Diabetes Mellitus (T2DM) -related morbidity and mortality on global healthcare is escalating with higher prevalences of obesity, poor diet, and sedentary lifestyles. Therefore, the clinical need for early diagnosis and prevention in groups of high-risk individuals is necessary. The purpose of this thesis was to investigate the use of surrogate markers, namely retinal vascular function, to determine future vascular endothelial dysfunction, atherosclerosis, large vessel disease and cardiovascular risk in certain groups. This namely covered normoglycaemic and normotensive South Asians (SAs), those with Impaired-Glucose Tolerance (IGT) and individuals with a familial history (FH) of T2DM. Additionally the effect of overweight and obesity was studied.

The techniques and modified protocols adopted for this thesis involved the investigation of endothelial function by means of vascular reactivity at the ocular and systemic level. Furthermore, the relationships between retinal and systemic function with circulating markers for endothelial cell function and cardiovascular risk markers were explored. The principal studies and findings of the research were:

Vascular Function in Normoglycaemic Individuals with and without a FH of T2DM
WE FH individuals exhibited higher levels of total cholesterol levels that correlated well with the retinal arterial dilation amplitude to flicker light stimulus. However this did not extend to noticeable differences in markers for endothelial cell damage and impaired retinal and systemic function.

Vascular Function in Normoglycaemic South-Asians vs. White-Europeans without a FH and Vascular Disturbances
Compared to healthy WEs (normo -glycaemic and -tensive), SA participants exhibited levels of dyslipidaemia and a state of oxidative stress that extended to impaired vascular function as detected by reduced brachial artery flow-mediated dilation, slower retinal arterial vessel dilation reaction times (Appendix 3) and steeper constriction profiles. Furthermore, gender sub-group analysis presented in a sub-chapter shows that SA males demonstrated 24-hour systemic blood pressure (BP) and heart rate variability (HRV) abnormalities and heightened cardiovascular disease (CVD) risk.

Vascular Function in Individuals Newly Diagnosed with IGT as compared to Normoglycaemic Healthy Controls
Newly-diagnosed WE and SA IGT patients showed a greater risk for CVD and T2DM progression by means of 24-hour BP abnormalities, dyslipidaemia, increased carotid artery intimal-media thickness (c-IMT), Framingham scores and cholesterol ratios. Additionally, pre-clinical markers for oxidative stress and endothelial dysfunction, as evident by significantly lower levels of plasma glutathione and increased levels of von-Willebrand factor in IGT individuals, extended to impaired vascular systemic and retinal function compared to normal controls. This originally shows retinal, systemic and biochemical disturbances in newly-diagnosed IGT not previously reported before.

Vascular Function in Normal, Overweight and Obese Individuals of SA and WE Ethnicity
In addition to the intended study chapters, the thesis also investigated the influence of obesity and overweight on vascular function. Most importantly, it was found for the first time that compared to lean individuals it was overweight and not obese individuals that exhibited signs of vascular systemic and ocular dysfunction that was evident alongside markers of atherosclerosis, CVD risk and endothelial damage.

Keywords: T2DM, CVD, IGT, SAs, Overweight, Retinal Vessel Reactivity, Vascular Endothelial Function

Dedicated to my Parents

Jai Shri Krishna

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List of Abbreviations

2D..........Two-dimensional

ABP..........Ambulatory Blood Pressure

ACE..........Angiotensin-converting Enzyme

Ach..........Acetylcholine

ADA..........American Diabetes Association

AD..........Absolute Diameter

AGEs..........Advanced Glycation End-Products

ANCOVA..........Analysis of Co-Variance

ANOVA..........Analysis of Variance

ANS..........Autonomic Nervous System

ATP..........Adenosine Triphosphate

AU..........arbitrary units

BDF..........Baseline diameter Fluctuation

bFR..........baseline-corrected Flicker Response

BFS..........Blue Field Stimulation

BMI..........Body Mass Index

BP..........Blood Pressure

CAD..........Coronary Artery Disease

CCD..........charged couple device

CCA..........common carotid artery

CDI..........Colour Doppler Imaging

cGKI..........cGMP-dependent protein kinase type I

CHD..........Coronary Heart Disease

c-IMT..........carotid-Intimal media thickness

CVD..........Cardiovascular Disease

DA..........Dilation Amplitude

DBP..........Diastolic Blood Pressure

DM..........Diabetes Mellitus

DR..........Diabetic Retinopathy

DRVA..........Dynamic Retinal Vessel Analyser

DTNB..........Ellman's Reagent

ECG..........Electrocardiogram

EDTA..........Ethylenediaminetetraacetic acid

ELISA..........Enzyme-linked immunosorbent assay

eNOS..........endothelial-derived Nitric Oxide synthase

ET..........Endothelin

ET-1..........Endothelin-1

FA..........Fluorescein Angiography

FADH..........Flavin Adenine Dinucleotide

FH..........Family History

FMD..........Flow Mediated Dilation

GLUT-1..........Glucose Transporter-1

GMP..........Guanosine Monophosphate

GSH..........reduced Glutathione

GTN..........Glyceryl Tri-Nitrate

GSSG..........oxidised Glutathione

GSR..........diphenylamine solution

GTP..........Guanosine Triphosphate

H⁺..........Hydrogen Ion

H₂O..........water

H₂O₂..........hydrogen peroxide

HbA1c..........glycosylated haemoglobin

HDL-C..........High-density Lipoprotein

HF..........High Frequency

HOMA-IR..........Haemostatic Model Assessment-Insulin Resistance

HRF..........Heidelberg Retina Flowmeter

HRV..........Heart Rate Variability

Hs-CRP..........High sensitivity C-reactive Protein

IGA..........Indocyanine green Angiography

IGT..........Impaired Glucose Tolerance

IMT..........Intimal Media Thickness

IOP..........Intraocular Pressure

IR..........Insulin Resistance

K⁺..........Potassium Ion

LDf..........Laser Doppler Flowmetry

LDL-C..........Low-density Lipoprotein

LDv..........Laser Doppler Velocimetry

LF..........Low Frequency

MAP..........Mean Arterial Pressure

MC..........Maximum Constriction

MCRT..........Maximum Constriction Reaction Time

MDA..........Malondialdehyde

MDRT..........Maximum Diameter Reaction Time

MetS..........Metabolic Syndrome

NADH..........Nicotinamide adenine dinucleotide

NADPH.......... Nicotinamide adenine dinucleotide phosphate

NHS..........National Health System

NO..........Nitric Oxide

NO₂..........Nitrogen dioxide

NT-3..........Nitrotyrosine

O₂⁻..........superoxide radical

OGTT..........Oral Glucose Tolerance Test

OH..........hydroxyl radical

ONOO⁻..........peryoxynitrite

p..........probability value

PAI-1..........Plasminogen Activator Inhibitor-1

PBF..........Percentage Body Fat

PCO₂..........Pressure of Carbon Dioxide

pH..........power of Hydrogen

PKG..........Protein Kinase G

PO₂..........Pressure of Oxygen

POBF..........Pulsatile Ocular Blood Flow System

PP..........Perfusion Pressure

PSNS..........Parasympathetic Nervous System

ROS..........Reactive Oxygen Species

RPE..........retinal pigment epithelium

RT..........Reaction Time

RVA..........Retinal Vessel Analyser

SA..........South Asian

SBP..........Systolic Blood Pressure

SDRA..........Sequential and Diameter Response Analysis

s-ICAM..........soluble-Intercellular adhesion molecule

SNS..........Sympathetic Nervous System

T2DM..........Type 2 Diabetes Mellitus

TG..........Triglyceride

TG:HDL-C..........Triglyceride/High-density Lipoprotein Cholesterol Ratio

tGSH..........total Glutathione

Total:HDL-C..........Total Cholesterol/High-density lipoprotein Cholesterol Ratio

v-ICAM..........vascular-Intercellular adhesion molecule

VIP..........Vasoactive Intestinal Peptide

WE..........White European

WHO..........World Health Organisation

WHR..........Waist-to-Hip Ratio

VSMC..........Vascular Smooth Muscle cell

vWF..........von Willebrand Factor

°C..........degrees Celsius

DC..........Dioptre Cylindrical

DS..........Dioptre Spherical

g..........grams

Hz..........Hertz

kg..........kilograms

kg/m^2..........kilograms per metre squared

min..........minute

mL..........millilitre

mm..........millimetre

mmHg..........millimetres of mercury

mmol/L..........millimoles per litre

ms..........milliseconds

MU..........measured units

nm..........nano-meter

NU..........normalised units

%..........percentage

rpm..........revolutions per minute

SD..........Standard Deviation

secs..........seconds

µ/dL..........units per decilitre

µL..........microlitre

1. Introduction and Research Rationale

1.1. Background

The incidence of Type 2 Diabetes Mellitus (T2DM) and associated cardiovascular disease (CVD) is increasing exponentially worldwide, primarily due to the increase in obesity and a sedentary lifestyle. The current worldwide prevalence of DM is estimated to be around 170 million and it is thought that this will increase to a number of more than 360 million by 2030.[1] Therefore, the rising prevalences of T2DM will undoubtedly confer major burdens on the health care system and thus health care and economy costs.

T2DM is a metabolic condition as a result of the interaction between genetic predisposition and environmental as well as behavioural risk factors.[2] The genetic basis behind the aetiology of T2DM is yet to be determined, but there is justifiable evidence to suggest that modifiable risk factors such as diet, obesity and physical inactivity are some of the main non-genetic determinants of T2DM.[3, 4]

T2DM may largely be preventable, but a comprehensive understanding of its aetiology is still needed. The development of atherosclerotic and clinical CVD is the principal complication in T2DM, but can also precede development of diabetes, lending support to the hypothesis that both vascular disorders share common antecedents.[5-7]

A syndrome of insulin resistance (IR) may constitute this common antecedent, and is understood to be an established factor in the pathogenesis of T2DM. IR is a hallmark of T2DM that precedes and predicts the onset for several years, and is related to several cardiovascular risk factors including hyperglycaemia, dyslipidaemia and hypertension.[2, 8] Closely interlinked conditions such as atherosclerosis, CVD and T2DM have a common pathophysiological basis that is underpinned by IR and the Metabolic Syndrome (MetS). Therefore, it is of clinical interest to establish whether an IR state contributes to the onset of vascular disease processes such as atherosclerosis.[9]

Atherosclerosis is a disease of arterial lipid deposition characterised by a complex and dynamic number of biological responses central to which is a preliminary oxidative and inflammatory reaction. Pathological studies have demonstrated a series of changes in the vessel wall and there is further evidence to suggest that before these structural changes, there are important pro-atherosclerotic changes in vascular endothelial cell phenotyping such as a decline in the

bioavailability of the signalling radical nitric oxide (NO).[10] Whether, vascular endothelial cell dysfunction; a systemic pathological imbalance between vaso-dilating and –constricting substances released (or acting on) the vascular endothelium. This in turn promotes several disease processes such as T2DM and atherosclerosis.

Those with T2DM and known atherosclerosis exhibit a number of risk factors for serious vascular complications, and for this reason it is important to also focus on cardio-metabolic risk factors in known susceptible individuals. That is, the combined vascular and metabolic components of risk that may lead to a CVD or a cardiovascular event.

1.2. Risk Factors

The clustering of vascular risk factors such as age, familial history, ethnicity, obesity, hypertension and dyslipidaemia; risk factors known to contribute towards atherosclerosis and ultimately CVD, T2DM and IR have been documented in many urban studies (Table 1.1).[11-13] Furthermore, it has been shown that the combinations of these characteristics can occur more frequently than expected by chance.[14]

Documented Risk Factors of Vascular Disease
Age
Familial History
Metabolic Syndrome/Obesity
Circulation Abnormalities (Hypertension and Stroke)
Ethnicity
Impaired Glucose Tolerance/Impaired Fasting Glycaemia
Gestational Diabetes Mellitus
Mental Health Problems (Schizophrenia)
Sedentary Lifestyle Factors (Exercise and Diet)

Table 1.1. Known risk factors for vascular disease (T2DM and CVD)

The primary aim of this thesis and its studies will be to investigate the influence of vascular endothelial dysfunction on micro- and macro-vascular function alongside biochemical and metabolic function on future vascular risk of T2DM and CVD. Furthermore, the use of retinal vascular function as a surrogate means of providing a 'clinical picture' on macrovascular function and predictor for future risk of systemic and macrovascular disease will be closely evaluated.

Therefore, the studies will focus on ethnicity; namely South Asians (SA) as compared to White Europeans (WE), those of WE origin with a family history (FH) of T2DM in one/both parents compared to those without, and individuals with impaired glucose tolerance (IGT) vs. normoglycaemic individuals.

1.2.1. South Asian Ethnicity (SA)

The SA diaspora comprises mainly of Indians, Sri Lankans, Pakistanis and Bangladeshis. There has been a dramatic increase in the prevalence of T2DM in the SA community in many parts of the world. SAs are undeniably at an increased risk of CVD and IR, and much of this excess risk may be attributed to the increased risk of T2DM (four to six times that of Europeans) developing at about 10 years earlier than in White Europeans (WE).[15] Alarmingly, it has been projected that the number of those diagnosed with T2DM in India alone will escalate from 31.7 to 79.4 million in 2030.[1] More importantly, in the Birmingham area of the UK the Health Survey for England reports increasing levels of T2DM, obesity and sedentary lifestyles that contribute towards an increased vascular risk. Therefore, the investigation of vascular function in SAs, especially of those in the Birmingham area seems valid.

Although genetic factors play an integral role, the increased incidence of T2DM is strongly associated with increasing central obesity and hyperinsulinaemia.[16] For instance, SAs appear to be more IR at a much earlier age and as a result of this, the relation between obesity and IR may occur at lower levels of obesity in SAs as compared with WEs.[17]

Furthermore, the prevalence of major established risk factors amongst SAs when compared to WEs is also present at a younger age.[18] In comparison to WEs, SAs appear to present with higher plasma triglyceride (TG) and lower high-density lipoprotein cholesterol (HDL-C) levels, suggesting underlying IR.[19] Additionally, some reports also show higher plasma total and LDL-C levels in SAs.[20] The higher prevalence of IR, IGT and increased fasting glucose levels in SAs have been shown to increase the risk of T2DM and this could be ultimately explained by the close link with the higher incidence of the Metabolic Syndrome (MetS) amongst this minority.[21]

The MetS is intimately linked with IR and is independently associated with a two-fold risk of CVD in SAs and therefore could provide a means of identifying high-risk individuals for T2DM and CVD.[22-24] It represents an asymptomatic condition that consists of obesity (central intra-abdominal), IR, impaired glucose metabolism, dyslipidaemia of the high TG and low HDL-C type, and elevated blood pressure (BP).

However, the causes behind the increased prevalence of IR and T2DM in SAs still remain unclear. Abdominal obesity contributes to the pathophysiological mechanisms of IR in a number

of ways, and despite SAs being more IR than WEs for every level of body fat; it does not fully explain the excess risk.[25-27]

Other emerging environmental and lifestyle confounders must also be taken into account with SAs. Physical inactivity and sedentary lifestyles combined with a low protein and high carbohydrate, fat, eggs and dairy diet, socioeconomic and cultural factors may also play an important role.[21] Furthermore, a number of studies suggest that established risk factors might not fully explain the excess risk and that established risk calculators such as the Framingham may underestimate vascular risk in SAs.[28] Preliminary studies also show that vascular endothelial function might be more impaired in SAs when compared to WEs, suggesting the influence of other biological and biochemical processes.[26, 29, 30] For instance, some studies have reported higher concentrations of fibrinogen and plasminogen activator inhibitor-1 (PAI-1), commonly found with IR and the MetS.[31] Elevated levels of plasma homocysteine and high-sensitivity C-reactive protein (hs-CRP) in SAs are likely to reflect higher levels of vascular inflammation and central obesity in SAs.[32-35] The emergence of genetic studies, ethnicity and T2DM also raises the possibility that IR and T2DM are genetically determined to a greater extent in SAs than WEs. Nonetheless, these factors help to demonstrate the complicated and multi-faceted nature behind the risks and pathophysiology of T2DM, IR and IGT in SAs.

1.2.2. Family History (FH)

Familial history also appears to play an influential independent risk for T2DM as the condition is known to be strongly genetically determined.[36] Recent studies have reported a higher prevalence of IR and impaired vascular endothelial function in normotensive and normoglycaemic offspring of T2DM (FH), suggesting interplay between genetics and atherosclerosis/endothelial dysfunction that may be inherited.[36-40] In contrast, biological studies investigating normoglycaemic individuals with a FH of T2DM and those with an identical twin with T2DM have found impaired insulin secretion as opposed to IR when correcting for confounding variables suggesting a genetic involvement with beta-cell dysfunction.[41]

Whether FH for T2DM predisposes those at risk to an alteration in vascular endothelial function through a genetic 'susceptibility' or higher prevalence of MetS requires further longitudinal work. The limited amount of research into familial history and T2DM restricts understanding behind the mechanisms of DM and to understand the role of genetics if any, and whether predisposing environmental, lifestyle and cultural factors are causal and effect.

1.2.3. Impaired Glucose Tolerance (IGT)

IGT represents an inter-mediatory between normal glucose tolerance and overt T2DM, and is identified by an oral glucose tolerance test (OGTT). Those with IGT have been found to be at an increased risk of T2DM and form an important group for investigating primary means of intervention.

IGT is largely the result of reduced insulin-stimulated glucose disposal due to decreased insulin secretion and a decreased response of glucose uptake by skeletal muscle to insulin. IGT is commonly diagnosed with the threshold criteria established by the World Health Organisation (WHO) and the American Diabetes Association (ADA).

IGT is not uncommon in patients with coronary atherosclerosis and furthermore, is well associated with abnormal scores for atherosclerosis surrogate markers (carotid intima-media thickness [IMT], flow-mediated brachial artery dilation [FMD] and homeostasis model assessment of insulin resistance [HOMA-IR]).[42] Therefore, IGT has been linked with clinical events other than sole progression to T2DM, suggesting its clinical significance in cardiovascular mortality.

Although IGT has been associated with an increased risk behind the development of T2DM and adverse clinical outcomes, there is limited amount of research on using microvascular functional analysis (especially retinal) for the clinical diagnosis and utility in medical practice. However, there is consistent information about differences amongst different ethnic populations and age groups.[43]

1.3. Anatomy and Physiology

1.3.1. The Vascular Endothelium

The endothelium is made up of an elaborate network of tight junctions and lacks any fenestrations; resembling that of a cobblestone arrangement. Structurally, the endothelium is based on an amorphous basal membrane made up of rare internal, dense external and rare external layers that contains type IV collagen, glycoproteins, laminin and fibronectin.

Its morphology changes accordingly to the required function and its location. At the capillary level the endothelium is a single layer that folds outwards to cover the vessel wall. Additionally at this level, pericyte cells form a contractile layer that helps to maintain vessel diameter and intimal cell turnover. Pericyte cells are known only to be located at the retinal level and central nervous system. In contrast, large vessel endothelium exhibits homogeneity and is

anatomically made up of a basal membrane covered by the tunica media and adventitia (Figure 1.1.).
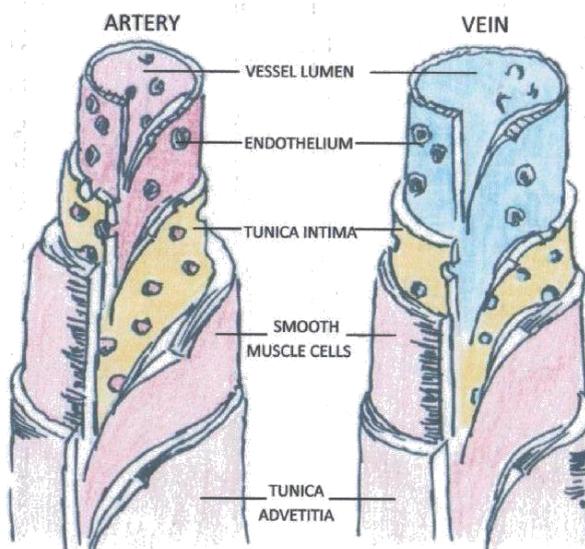
Figure 1.1. An anatomical drawing of the large vessel vascular endothelium, illustrating it as the innermost mechanical vessel lining.

The endothelium is classed as an important organ that is involved very heavily in cardiovascular haemostasis.[44-47] It is known to have a number of important primary regulatory functions;

1. A selective gate permitting the transport of glucose, nutrients, metabolites and hormones. Importantly, the non-insulin-dependent glucose transporter (GLUT1) transports glucose through the endothelial cells.
2. The endothelium synthesises collagen I, fibronectin, laminin and growth factors that aid tissue synthesis and catabolism.
3. It produces intracellular adhesion molecules, vascular cell adhesion molecules, and E-selectin promoting leukocyte recruitment and activation.
4. The presence of lipoprotein lipase in the endothelial layer allows the hydrolysis of low-density lipoprotein triglycerides to aid lipoprotein metabolism.
5. Most importantly, the endothelium produces and releases coagulant and anticoagulant factors as well as vasoconstrictor and vasoconstrictor factors to aid haemostasis.

Thus, under physiological conditions the endothelium contributes to vascular haemostasis by continuously monitoring blood and local stimuli, and modifying itself in response to these stimuli and environmental changes.[48] Anatomically, the endothelium is seen as the

interface between circulating blood and the vascular smooth muscle cells (VSMC), but in addition to serving as a physical barrier, the endothelium facilitates a complex and intimate interaction with the VSMC and a complicated system of chemical mediators. Figure 1.2.

NO, which has been identified as a major endothelial derived relaxing factor is one of the most important substances involved in maintaining the normal function of vessels. It is a vasodilator, inhibits abnormal growth and inflammation and exerts anti-aggregatory effects on platelets. Additionally, NO maintains choroidal vascular tone in humans and plays an important role in flicker-induced retinal vasodilation.[49-51]

It is released by the vascular endothelium in response to a variety of chemical and physical stimuli (platelet derived factors, shear stress, angiotensin II, acetylcholine, and cytokines) which stimulate the production of NO by endothelial nitric oxide synthase (eNOS). eNOS synthesizes NO from the terminal guanidine-nitrogen of L-arginine and oxygen. NO, a highly reactive free radical, then diffuses into the smooth muscle cells of the blood vessel and interacts with soluble guanylate cyclase. Nitric oxide stimulates the soluble guanylate cyclase to generate the second messenger cyclic GMP (3',5' guanosine monophosphate) from guanosine triphosphate (GTP). The soluble cGMP activates cyclic nucleotide dependent protein kinase G (PKG or cGKI). PKG is a kinase that phosphorylates a number of proteins that regulate; calcium concentrations, calcium sensitization, hyperpolarize cell through potassium channels, actin filament and myosin dynamic alterations that result in smooth muscle relaxation.[52]

Impaired endothelial function is synonymous with reduced NO release because of inactivity of the endothelial NO enzyme eNOS, as a result of biochemical imbalances, namely increased endo- and exo-genous inhibitors and/or low availability of enzyme substrate L-arginine.[53]

VASOACTIVE SUBSTANCES & MECHANICAL STIMULI (SHEAR STRESS & PRESSURE)

ENDOTHELIUM REACTS TO THESE STIMULI

RELEASE OF AGENTS:

VASODILATORS - NO & ENDOTHELIUM- DERIVED HYPERPOLARIZING FACTOR

VASOCONSTRICTORS - ET-1

ADEQUATE BLOOD FLOW
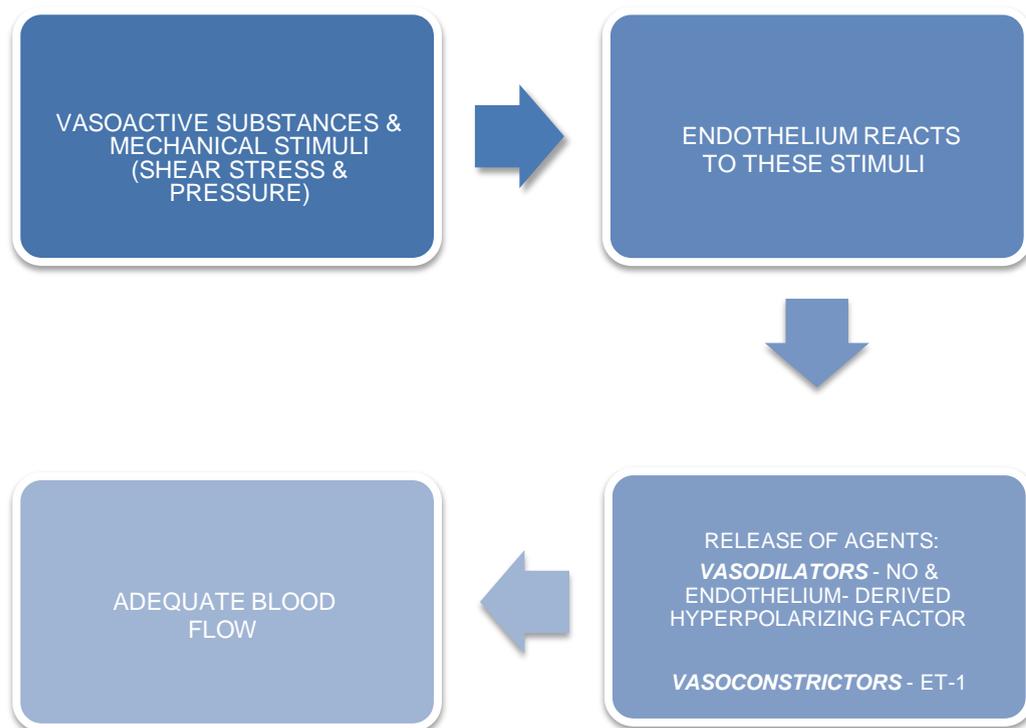
Figure 1.2. Biochemical processes undergone by the endothelium to maintain adequate blood flow.

1.3.2. The Retina

The retinal vascular supply is essentially an end-arterial system; whereby the central retinal artery emerges at the optic nerve into two major branches. These branches divide further into arterioles that supply each retinal quadrant. The venous system is very similar to the arteriolar arrangement explained above; the central retinal vein exits through the optic nerve to drain blood into the cavernous sinus.[54]

Depending on retinal location, retinal arteries have an unusually developed smooth muscle layer and lack internal elastic laminar. The smooth muscle cells that make up this layer lie circularly and longitudinally and are surrounded by a basal lamina with progressively increasing amounts of collagen towards the acellular adventitia.

Towards the optic nerve, the artery wall comprises of 5-7 layers of smooth muscle cells which decreases to 2-3 layers at the equator and furthermore to 1-2 layers at the periphery.[55-57]

Capillary walls consist of an inner layer of endothelial cells that orientate along the axis of the capillary, which is then surrounded by basement lamina within which pericyte cells form a discontinuous layer.

Retinal vasculature possesses a limited supply of autonomic innervation, namely from glial cell signalling, and unlike the choroid lacks full intrinsic innervation. Therefore, the central retinal artery is innervated by sympathetic nerves from the superior cervical ganglion, whilst within and throughout the retinal layers; angiotensin II, α-adrenergic and β- adrenergic receptors are present.[58-61]

Due to their functional structure, the retinal endothelial cells are considered to be a major component of the inner blood-retinal barrier. Retinal endothelial cells release high levels of superoxides and have been found to be more vulnerable to the damaging consequences of oxidative stress associated with diabetes.[62] The endothelium maintains retinal blood flow and vessel tone by releasing vasoactive factors (NO, Endothelin-1) to stimulate mechanisms.[63]

1.3.2.1. Physiology of Retinal Blood Flow and Autoregulation

Maintenance of adequate perfusion is a basic requirement for all tissue beds. The local vascular mechanism that maintains a relatively constant metabolic environment in the tissue,

despite varying conditions that tend to disturb this homeostasis, is called autoregulation. The autoregulation mechanism contributes also to the maintenance of a relatively constant capillary pressure, which is important for the tissue fluid balance.[64, 65]

Additionally, blood flow depends on perfusion pressure (the pressure that drives blood), vascular resistance and blood viscosity. This is further impacted by other local and environmental factors: local haematocrit, shear rate, vessel diameter and length, and interactions of systemic factors that affect the tone of pericytes, smooth muscle cells and endothelium. For instance, autoregulation is accomplished through the ability of the cardiovascular system to adjust the resistance of particular vessels by controlling the diameter of their lumen. The lumen diameter is maintained by vascular smooth muscle or other contractile elements in the vessel wall. This vascular tone is adjusted by vasoactive nerves and circulating hormones, as well as endothelial factors, myogenic and metabolic factors.

Therefore, autoregulation ensures a constant rate of blood flow in order to preserve the function of tissues by a means of different known mechanisms. This can involve changes in precapillary arteriole lumen size, vascular endothelium function, or ocular perfusion pressure above/below the critical autoregulatory range.

A number of studies have shown that ocular and retinal circulation has been found to show autoregulatory capacity by a means of inducing changes in perfusion pressure (increasing Blood Pressure or Intraocular Pressure) or changes in metabolic rate (hypercapnia or hyperoxia).[66-70] Thus, blood flow is regulated by the autonomic nervous system (ANS) and locally to meet local needs by a number of mechanisms (Table 1.2.).

AUTOREGULATORY MECHANISMS	DESCRIPTION
METABOLIC	A change in blood supply to satisfy tissue metabolic demand by release of vasoactive substances that maintains supply of oxygen and removes toxic metabolites
MYOGENIC	Arterial and arteriolar smooth-muscle cell changes proportionate to variations in perfusion pressure
NEUROGENIC	Blood circulation maintained by neural sympathetic subsystems
ENDOTHELIUM-MEDIATED	Vasoactive factors known to interact with the smooth muscle cells allow vaso -constriction or -dilation.

Table 1.2. The mechanisms involved in regulating retinal blood flow.

1.3.2.2. Metabolic Autoregulation

The concept of a metabolic control of the retinal blood flow is that factors on which retinal metabolism is dependent (retinal-tissue PO_2, PCO_2, pH, metabolic products) strive to optimise retinal blood flow according to the metabolic needs of the retinal tissue.

According to the metabolic hypothesis of blood flow regulation, perfusion and tissue metabolism are tightly coupled in such a way that any reduction in arterial inflow causes an increase of vasodilator metabolites in the tissue, due to an insufficient washout or to an increase in the production, or both. Factors involved in determining metabolic autoregulation include hypoxia, vasoactive substances (Table 1.3.), and, tissue metabolites and ions (Table 1.4.).

Current evidence suggests that increased production of vasodilator substances due to hypoxia is the dominating mechanism for metabolic autoregulation.[71] Hypoxia is caused when there is an increased oxygen demand or reduced supply causing a resultant vasodilation in order to meet the increased tissue demand.

	Origin	Action
Nitric Oxide (NO)	Stimulus-derivative (shear stress, growth factors) that activates eNOS	Vessel protection and ***vasodilation***
Endothelin (ET-1, ET-2, ET-3)	Endothelial cells	***Vasoconstriction***
Superoxide Anions	Endothelial cells	Inactivates NO to induce indirect ***vasoconstriction***
Angiotensin-converting enzyme (ACE)	Renal	Inactivates bradykinins to produce Angiotensin-II, which then causes indirect ***vasoconstriction***

Table 1.3. An example of several vasoactive substances involved in the metabolic autoregulation of retinal blood flow.

	Action
Adenosine	A potent ***vasodilator*** during hypoxia
Potassium (K$^+$)	Regulated the NA^+/K^+-ATPase pump to regulate muscle contraction and thus ***vasoconstriction***
Carbon Dioxide (CO$_2$)	***Vasodilator*** in VSMC
Hydrogen Ions (H$^+$)	***Vasodilator***

Table 1.4. An example of several metabolites and ions involved in the metabolic autoregulation of retinal blood flow.

1.3.2.3. Myogenic autoregulation

As blood pressure and flow to a tissue is increased above normal levels, pressure-induced vascular myogenic mechanisms appear to dominate the regulation of the vascular tone. By means of myogenic autoregulation, the retinal blood flow is maintained constantly despite moderate variations in perfusion pressure (PP). The stimulus for a myogenic mechanism is a variation in the transmural pressure difference during moderate variations in PP, and is achieved by changing the vascular resistance. In cases where the pressure difference is reduced, as for instance with increased IOP or reduced arterial pressure, the activity of the pacemaker cells in the arteriolar wall is reduced, which results in reduced arteriolar tone, and consequently, reduced vascular resistance.[72, 73] In addition, a contracting endothelium-derived factor released during increases in transmural pressure may be another component of myogenic autoregulation.[74, 75]

1.3.2.4. Neurogenic Control of Vascular Tone

There are a number of vasoactive nerves influencing local blood flow. The eye is a rich supply of autonomic nerves within the uvea, the posterior ciliary arteries, and the extraocular portion of the central retinal artery.[58, 59] Vessels in the retina and prelaminar portion of the optic nerve have no neural innervation.[76] Consequently, a stimulation of the cervical sympathetic chain produces a vasoconstriction in the uvea but has no effect on retinal or anterior optic nerve blood flow.[77-80]

Neurogenic control is mediated by a multitude of substances such as acetylcholine, noradrenaline, calcitonin, gene-related peptide, cholecystokinin, vasoactive intestinal polypeptide (VIP), nitric oxide, neuropeptide Y, and adenosine triphosphate (ATP).[81-83]

1.3.2.5. Perfusion Pressure and Autoregulation

The driving force of ocular blood flow is PP, which is the difference between the pressure in the arteries entering the eye and the pressure in the veins leaving it. The pressure in the arteries entering the eye cannot be determined directly, and as a rule the mean arterial pressure (MAP) in the brachial artery is used as a substitute.

There is a loss of pressure between the heart, and the pressure in the arteries entering the eye is 35-40 mmHg lower than MAP as determined in the brachial arteries when we stand up. The pressure in the veins leaving the eye is almost the same as the IOP and for the eye.

Blood flow is reduced if MAP is reduced or IOP increased unless there is a concomitant

change in the vascular resistance. In most tissues, such changes in PP are compensated by a change in the vascular resistance, and the blood flow is kept at the same level despite moderate changes in PP. In the eye, blood flow through the retina is autoregulated, and one can estimate that in a healthy eye retinal blood flow is essentially unchanged up to an IOP of about 35 mmHg.[84, 85] A reduction in MAP or an increase in venous pressure has the same effect on the PP, but the effect on local blood flow may vary. A marked reduction in BP will activate the sympathetic nerves. This protective mechanism is aimed to distribute the remaining blood to tissues in need. No such mechanism appears to exist in the eye.

1.3.2.6. Retinal changes with diabetic retinopathy

The damaging effects of chronic hyperglycaemia on the microvasculature and subsequent focal ischemia are primary factors in diabetic retinopathy (DR) progression. Even with no clinically detectable retinopathy, haemodynamic and cellular changes are evident. The endothelial cell supporting cells, known as pericytes are affected early resulting in endothelial damage. Retinal hyper-perfusion caused by dilation of retinal arterioles is an early change involved in the pathogenesis of DR. Under physiological conditions, the retina has the ability to regulate blood flow in response to different metabolic demands by autoregulating retinal vessel diameter. This maintains blood flow at a balanced level despite changes in BP. This autoregulatory mechanism is impaired in diabetes altering the ocular blood flow and promoting retinal perfusion abnormalities; moreover, failure of autoregulation and abnormal vascular reactivity is often an early feature of DR.[86-91] Clinically evident retinopathy begins to appear as the systemic disease and pathophysiological changes progress. Thus, the clinical need to investigate haemodynamic processes associated with DR is valid.

There are a number of methods available in clinical practice for the assessment of ocular haemodynamics, each possess certain advantages and disadvantages depending on the measured variable required (Table 1.5.). For the purpose of this thesis; retinal vessel analysis was adopted to determine retinal vessel diameter and reactivity. This technique is explained in more detail later on in Section 2.1.

Instrument	Assessed physiology	Measurement output
Laser Doppler Flowmetry (LDf)	Optic nerve head and choroidal vessel	Optic nerve head and choroidal capillary blood flow
Laser Doppler Velocimetry (LDv)	Retinal vessels	Retinal vessel blood velocity
Laser interferometry	Optic nerve head and choroidal vessels	Pulsatile ocular blood flow
Heidelberg Retina Flowmeter (HRF)	Optic nerve head and retinal vessels	Optic nerve head and retinal velocity and flow
Colour Doppler Imaging (CDI)	Retrobulbar blood vessels	Retrobulbar vessel blood flow velocity
Pulsatile Ocular Blood Flow system (POBF)	Choroidal vessels	IOP and pulsatile choroidal blood flow
Fluorescein Angiography (FA)	Retinal vessel	Perfusion mapping and blood flow velocity and circulation
Indocyanine green Angiography (IGA)	Choroidal vessels	Choroidal blood flow velocity
Blue-field stimulation (BFS)	Foveal vessels	Foveal circulation and capillary retinal blood flow
Retinal vessel analyser (RVA)	Retinal vessels	Continuous and static diameter

Table 1.5. The different instrumentation used in determining retinal haemodynamics in ocular disease.

1.4. Endothelial Dysfunction

Normal endothelial function is determined by a harmonious equilibrium of both vaso -active and -dilatory substances that allow sufficient permeability, secretion and expression of these substances by the endothelial cells according to local and global tissue demand. Therefore, endothelial dysfunction indicates a generalised alteration in cell function resulting in abnormal vasodilatory responses, impaired endothelial control of inflammation and fibrinolysis and an imbalance in the expression of vascular adhesion molecules.

Endothelial dysfunction is detectable before any angiographic or ultrasound evidence of disease in cardiovascular risk factor groups (dyslipidaemia, hypertension, diabetes mellitus and smoking), as a result of overproduction of reactive oxidative species (ROS) and increased oxidative stress that contribute to reduced bioavailability of vascular NO, promoting cellular damage.[92-94] There are a number of associated alterations to the vascular endothelium which can contribute towards endothelial dysfunction. These include impaired NO release and response, increased expression and plasma levels of ROS, vasoconstrictors (ET-1, angiotension II), adhesion molecules, and enhanced platelet adhesion to the endothelium.[95,

96] Ultimately, the chronic exposure to hyperglycaemia and inflammatory state triggers a set of chain reactions causing biochemical changes that finally lead to structural changes. This biochemical change creates an imbalance of opposing physiological and molecular effects causing vascular inflammation and coagulation.

The mechanisms of endothelial dysfunction in IR states are not yet fully established. Dyslipidaemia, oxidative stress, chronic vascular inflammation and increased low-density lipoprotein cholesterol (LDL-C) transport may be considered as important factors. However, a loss of NO bio- activity and -availability has been found to be the central feature of endothelial dysfunction and is accepted to be a good predictor of atherosclerosis. This in turn promotes a state of vascular inflammation that causes endothelial cell dysfunction.[97, 98]

Previous studies have demonstrated impaired insulin-mediated and endothelial-dependent vasodilation in IR states, including obesity, T2DM and IGT.[99] Therefore, this suggests that endothelial dysfunction may play an integral role into the cause of IR; independent of hyperglycaemia. Therefore, it could then be hypothesised that markers of impaired endothelial function could be caused by a severe state of IR.

The vital roles played by the vascular endothelium and NO to reduce vascular tone, platelet aggregation and adhesion, thrombosis, leukocyte adhesion and smooth-muscle cell proliferation and adhesion, seems to suggest that severely impaired endothelial-dependent vasodilation may be partly responsible for the incidence of macrovascular disease observed in T2DM.[100-102]

1.4.1. Markers of Endothelial (Dys)Function

The assessment of endothelial function refers to the measurement of cell response to stimulation, by substances known to be released by, or interact with, the endothelium.

It is hypothesised that T2DM and IGT are associated with impaired endothelial synthesis of NO and that this may explain the increased cardiovascular disease risk with these conditions.[103-111] Endothelial synthesis of NO can be estimated from vasodilation and/or blood flow increase in response to stimuli (Ach, shear stress, bradykinins). These responses are collectively referred to as endothelium-dependent vasodilation.[112]

Early research has provided the insight and means of understanding the mechanisms (Figure 1.3.) behind thrombosis, vascular inflammation and endothelial dysfunction.[113, 114] With the added knowledge from larger clinical studies, the complex interactions that take place between platelet adherence; aggregation and release, clotting

factor activation, and vascular endothelial damage with thrombogenesis show that the pathogenesis of vascular diseases are multi-factorial.
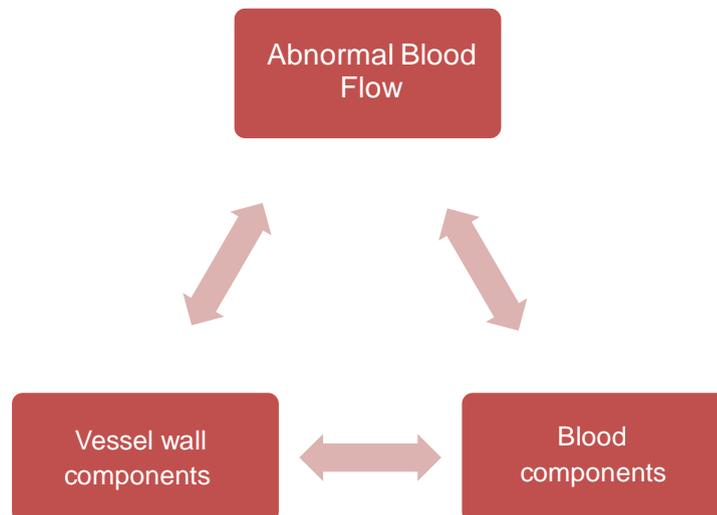
Figure 1.3. The early proposed mechanisms of vascular thrombosis.

1.4.1.3. Circulating Markers

1.4.1.3.1. Endothelin-1 (ET-1)

Endothelin-1 (ET-1) is an amino acid peptide produced mainly by vascular endothelial cells and is a potent endogenous vasoconstrictor implicated in the pathogenesis of ischaemic heart failure, hypertension, vasospasm, stroke and diabetic complications.[115, 116] It has been identified in ocular tissues and ET-1 binding sites are located in the retina and choroid. There are namely three receptor sites; ET_A located on the smooth muscle and therefore plays a key role in vasoconstriction, ET_B found on endothelial cells and mediates vasodilation by the release of NO, and finally ET_{B2} which mediate direct vasoconstriction.[117, 118]

ET-1 has been implicated in risk for T2DM and its complications. For instance a linear relationship between BP, ET-1 and obesity has been found in individuals at risk of diabetes.[119-122]

Furthermore, animal studies have reported an increase in endogenous ET-1 with experimentally induced DR that contributed to the haemodynamic changes found with DR, and that with DR there was a profound alteration in density and localisation of retinal ET-1 receptors.[91, 123] Additionally it is also evident that with the administration of ET-1, there is an increase in DBP along with an expected increase in ocular perfusion pressure (OPP) but surprisingly there is also a reduction of retinal blood flow by 20%.[118] The retinal blood flow, like many other vascular beds, is determined by perfusion pressure and resistance. Therefore, for a reduction of this kind, an autoregulatory response would be the cause, (i.e. by a change in vascular resistance). However, autoregulation would not allow a decrease in OPP beyond baseline, and so it is unknown whether ET-1 causes atherosclerosis, endothelial function and autoregulatory disturbances or if it is damage endothelial cells that release increased levels of ET-1.

1.4.1.3.2. Von-Willebrand Factor (vWF)

Von Willebrand factor (vWF) is an important glycoprotein (produced by, released and stored in the endothelial cells and platelets) involved in cardiovascular physiology as it is associated with platelet aggregation and adhesion. The primary function of vWF is to bind other proteins (factor VIII in its inactive form) to be activated by thrombin so that it can bind to collagen or platelets when exposed to vessel damage and shear stress. Plasma levels of vWF are dependent on factors such as aging via impaired NO production, vascular inflammation, ROS and T2DM. Therefore, it has been found that raised levels of

vWF are associated with endothelial dysfunction and atherosclerosis and have prognostic value in those with heart disease, peripheral vascular disease and inflammatory vascular disease.[124-126] However, it is also important to note that high levels of vWF can sometimes be attributed to an acute phase reactant as it is a very sensitive marker and is easily influenced by many conditions.[127]

There is limited information as to whether increased levels of vWF worsen disease progression and whether therapeutically reducing its levels is beneficial. However, many animal studies have confirmed that vWF deficiency (von Willebrand disease and haemophiliacs) provide protection from spontaneous and diet-induced atherosclerosis and thrombosis along with a lower-than-expected incidence of heart disease.[128-130]

In the case of T2DM, vWF abnormalities have been demonstrated with this disorder with increased urinary albumin levels. This confirms its involvement in the pathogenesis of vasculopathy, namely nephropathy.[131-133] On the other hand, with IR and MetS, some studies have found no correlation with vWF and risk groups. This finding may be attributed to smaller patient groups as compared to control groups.[134, 135]

1.5. Autonomic Nervous System (ANS)

The ANS (or visceral nervous system) is a part of the peripheral nervous system that maintains homeostasis in the body. It can be divided into the parasympathetic nervous system (PSNS) and sympathetic nervous system (SNS) and also functionally, into its sensory and motor systems.

The ANS and endothelium have closely-linked interactions that help to maintain vascular tone by balancing the endothelial release of vasodilating factors and sympathetic nerve terminal release of vasoconstricting factors. These opposing factors act on the smooth muscle cells to maintain tone.[136] To confirm these findings, a study found that by stimulating the SNS there was a resultant reduction in FMD; and this was abolished in those that were given α_2-receptor blockades.[137] This shows that there may be a possible link between SNS activity and reduced endothelial function. Furthermore, there is evidence to show that the endothelium possesses both α_2-adrenoreceptors and β-adrenoreceptors which could explain ANS influence on the endothelium (Figure 1.4.).[138, 139]

SNS EFFERENTS

Activation of receptors on SMC :

β-adregenic causes *vasodilation*
α_1/α_2-adregenic causes *vasoconstriction*

PSNS EFFERENTS

Stimulation of receptors on SMC :

Muscarinic causes *vasoconstriction*

ANS Nerve Terminals   Smooth Muscle Cells   Vascular Endothelium   Vessel Lumen
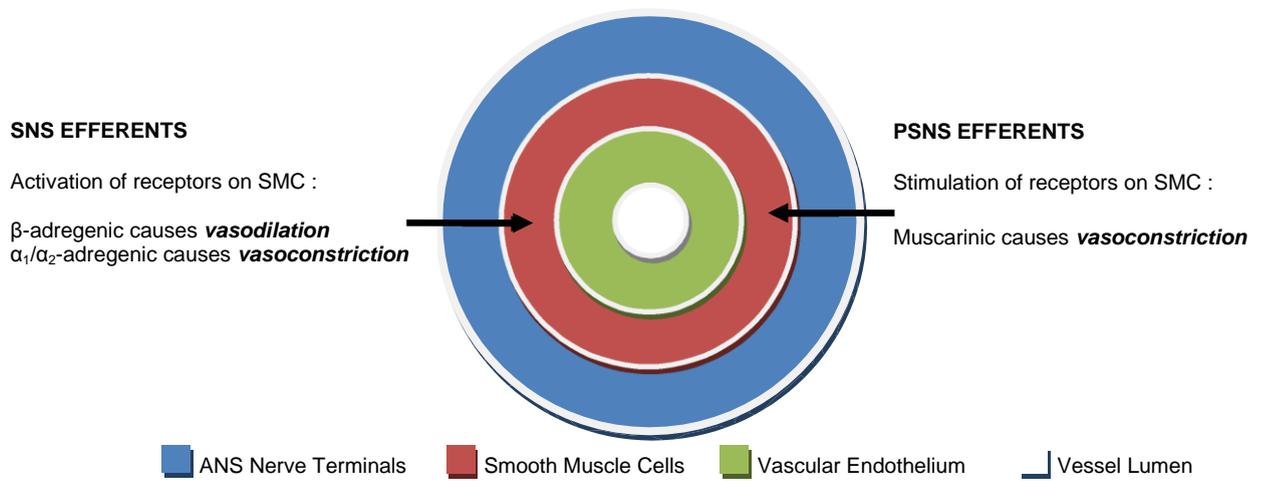
Figure 1.4. The influence of SNS and PSNS receptors on the vascular endothelium.

Certain conditions (DM, CVD, and Hypertension) have been found to reduce PSNS activation as well as enhance SNS activation. This has been found to impair endothelial function and enhance endothelial-mediated atherogenic processes; contributing to endothelial cell dysfunction. However, there are two possibilities for these findings. Firstly, ANS impairment may contribute to abnormal endothelial changes or endothelial dysfunction may lead to maladaptive alterations in the regulation of the ANS.

1.5.1. Links between ANS and Endothelial Function

1.5.1.1. Impaired ANS causes Endothelial Dysfunction

DM, CVD, Hypertension and Coronary Heart Disease (CHD) are associated with ANS regulation abnormalities and endothelial dysfunction and there is an increasing amount of indirect evidence supporting the linknbetween Heart Rate Variability (HRV) and these states.[140-144] For instance, in those with DM, reduced HRV has been found with increased levels of arterial stiffness and vWF, both thought to be indirect markers of endothelial function.[145, 146]

1.5.1.2. Endothelial Dysfunction causes ANS Irregularities

It has been hypothesised that impaired endothelial function may influence the ANS by altering neurotransmitter release, reuptake, or receptor sensitivity due to reduced NO release.[147] This, in turn, increases the contractile effects of SNS stimulation. Additionally, higher levels of vWF found in newly-diagnosed diabetics predict those who developed deficient limb nerve conduction velocity, and so the possibility of vWF predicting future autonomic neuropathy should be considered.[148]

1.5.1.3. Factors affecting the ANS and the Endothelium

1. Oxidative Stress has a negative influence on the endothelium and the ANS by bringing about free-radical production and lipid peroxidation.[96, 149] It exposes the endothelium to oxidants leading to morphological changes that reduces its efficiency in forming a barrier between blood and the sub-endothelial matrix by promoting the release of ET-1 and leukocyte adhesion.[150]

   Peroxynitrite also mediates neuronal cell death (apoptosis of SNS neurons).[151] This has been confirmed with drug-intervention studies that found improved HRV with Vitamin E treatment in diabetics. This finding reflects the upregulation of the PSNS and

downregulation of the SNS with appropriate management, as it attenuates the negative effect of oxidative stress on ANS function.[152]

2. The ANS and endothelium retain a certain degree of plasticity but are prone to age-related changes.[153] Reduced FMD[154] and NO release along with increased endothelin-1 (ET-1) levels with age have a direct effect on the endothelium[155], whereas ANS changes with age (enhanced SNS and suppressed PSNS activity) allow the increased vasoconstrictive forces to overpower endothelial-dependent vasodilation.[155, 156]

3. IR has been associated with impaired HRV and enhanced SNS activity (conversely, SNS activity has been found to increase the state of insulin resistance), and poor endothelial function.[141, 157]

4. Suppressed ANS activity increases platelet activation which in turn enhances platelet aggregation and adhesion to vessel walls.[158] Additionally enhancing SNS activity influences the interplay between the endothelium and circulating platelets allowing platelet aggregation at much lower levels of shear stress.[159]

1.5.2. Ambulatory Blood Pressure (ABP) Measurements

There is an increasing attention of major cardiovascular studies to investigate the 24-hour rhythms of the cardiovascular system. Many of these have consistently found a circadian pattern in the occurrence of cardiovascular events.[160] Twenty-four hour recordings of ambulatory blood pressure (ABP) are widely used in the diagnosis and treatment of hypertension as well as in epidemiological studies. These profiles are better at predicting cardiovascular morbidity and mortality than isolated measurements done in clinics and make it possible to record BP during habitual daily activities and sleep.

The main integrating system for BP control is situated in the brain, namely the medullary cardiovascular centre. This centre maintains adequate blood flow to the brain and heart via sensory inputs from the baroreceptors located in the walls of the aortic arch and carotid artery. After integration and analysis of the input, the medullary cardiovascular centre sends appropriate efferent outputs *via* the sympathetic and parasympathetic divisions of the ANS in order to regulate the heart function and blood circulation throughout the body.

The diurnal variation in systemic blood pressure has been extensively studied.[161-163] Generally, blood pressure rises rapidly in the morning upon awaking and then continues to do so until reaching a plateau during the day (active period). It can often become slightly higher in the early evening, until the late evening where blood pressure starts to decline and shows the

lowest level during sleep (passive period). This circadian rhythm may be influenced by demographic, neurohormonal, and pathophysiological factors. For instance, it has been found that in night shift workers diurnal blood pressure variation is reversed, becoming higher at night and lower during the day.[164-167] Circadian rhythm in BP is defined as active BP minus passive BP for both systolic (SBP) and diastolic (DBP), whereby these periods are determined by a personal time diary.

A normal nocturnal dip in blood pressure at night of around 10% or more occurs in approximately two thirds of the healthy population. Non-dippers, however exhibit a diminished nocturnal BP fall of less than 10%, and are prone to an increased frequency of cerebrovascular damage including haemorrhages, thrombosis and vascular dementia.[168] In turn, extreme dippers have a nocturnal fall in BP of more than 20%, which may occur naturally or due to the use of antihypertensive medications. These patients show a high occurrence of ischaemic phenomena, including cardiac ischaemia, silent cerebrovascular damage[169] and anterior ischaemic optic neuropathy.[170]

1.5.3. Heart Rate Variability (HRV)

In normal conditions, the cardiovascular system is stable due to the autonomic control of the heart rate, BP, and other factors that react rapidly to a range of internal and external stimuli such as ischaemia, metabolic imbalance and changes in physical or mental activity. The analysis of sinus heart rate fluctuations, referred to as heart rate variability (HRV) can be used to indirectly assess the autonomic control of the heart.[171]

Both ANS divisions, sympathetic and parasympathetic, densely innervate the sinus node and heart rate will reflect their modulating effect on the cardiac pacemaker cells. Parasympathetic innervation slows down the heart rate via acetylcholine.[171] Sympathetic activation results in an increase in heart rate and conduction system velocity, together with an increase in contractility. This effect is mediated by synaptic release of noradrenaline. The two subsystems of ANS tend to operate at different frequencies.[172] In normal individuals cyclic changes in heart rate occur in association with respiration[173-175], and this particular type of high-frequency cyclical HRV is mediated by the parasympathetic nervous system. Cyclical variation occurring with changes in baroreceptor activity (due to changes in BP)[176] is of low frequency type and is mediated via the sympathetic nervous system. Simultaneous measurements of high and low frequency HRV can be used to investigate changes in the sympathovagal balance. The analysis of HRV has been extensively applied in the investigation of normal physiology as well as in pathological conditions. HRV analysis has provided useful results

in clarifying the role of the ANS in regulating the cardiovascular response to change in posture, stress and exercise, including:

- Exploring the physiology of normal ageing and identifying those at risk of premature cardiac diseases;[177]

- Assessing the autonomic function in a variety of non-cardiac diseases such as T2DM[178-180], renal diseases[181], chronic liver[182], respiratory[183] and neurological diseases;

- Occupational health, when exploring elevated cardiovascular risk in shift workers;[184-186]

- Investigating cardiovascular diseases such as myocardial infarction[187], chronic cardiac failure[188] and hypertension.[189]

More recently, the investigation of HRV with ocular disease has been proposed.[190] It is hypothesised that a change in blood flow regulation at the heart, cerebral or peripheral level could have a detrimental effect on the retinal vascular supply. Neurovascular coupling, the relationship between local neural activity and subsequent changes in cerebral blood flow, may explain this phenomenon. This is furthered by research demonstrating the homogeneity between cerebral and retinal microvascular structure and the known endothelial influences on cerebral vascular tone. In fact, ocular evidence indicates increased retinal neural activity is mainly attributable to increased ganglion activity and additional HRV abnormalities in ocular vascular disease.[191]

HRV can be interpreted as either time-domain or frequency-domain specific analysis of electrocardiogram (ECG) recordings.[192, 193] For the purpose of this thesis, frequency-domain analysis was selected as it provides a simpler and accurate analysis of global ECG variations. To allow efficient analysis, ECG recordings must be free of noise and then need to be preselected for fourier transform analysis to obtain power spectra of the transform. The power spectrum then provides two frequency ranges: low frequency (LF: 0.04-0.15Hz) and high frequency (HF: 0.15-0.40Hz) allowing for a measure of sympathetic activity with minor influence of the parasympathetic (LF) and sole parasympathetic activity (HF). Furthermore, the calculation of LF:HF allows a measure of sympathovagal balance, i.e. an increased value indicates a predominance in activity of the sympathetic versus parasympathetic nervous system.

1.6. Oxidative Stress

Oxidative stress describes a state of increased oxidant production in animal cells that is characterised by an increased release of free radicals that results in cellular degeneration. It is seen as a major mechanism in the pathogenesis of atherosclerosis and endothelial dysfunction (a prerequisite to atherosclerosis) and may serve as the mechanism behind the effect of risk factors on the endothelium.[194] Oxidative stress has been implicated in a variety of biological and pathological processes, as an excessive production of free radicals can lead to different pathological conditions.[195]

A free radical is a species that has one or more unpaired electrons. Normally more than 95% of the oxygen consumption by the aerobic organisms is the result of enzymatic reduction to H_2O in mitochondria by the terminal oxidase of the respiratory chain. When molecular oxygen is reduced by one electron, the product is a superoxide radical (O_2^-). The addition of a second electron to O_2^- at physiological pH gives rise to hydrogen peroxide (H_2O_2), an oxidizing species that has no unpaired electrons and thus is not a free radical. The one electron reduction of H_2O_2 yields H_2O and an hydroxyl radical (OH), the strongest oxidant produced in biological systems. Together, O_2^-, H_2O_2 and OH are known as the reactive oxygen species (ROS) and are continuously produced by aerobically growing cells.[195]

ROS (superoxide anion and hydrogen peroxide) are important signalling molecules of endothelial cell damage. To combat the cytotoxic action of the reactive oxygen and nitrogen species, cells are equipped with a large variety of antioxidant defences that include:

1. Enzymes which catalyse the dismutation of O_2^- into H_2O_2
2. Hydrogen peroxide scavenging enzymes such as catalase and glutathione peroxidase which convert H_2O_2 into H_2O
3. Hydrophilic radical scavengers such as ascorbate, urate, and glutathione (GSH)
4. Lipophilic radical scavengers such as tocopherols, flavonoids, carotenoids, and ubiquinol
5. Enzymes involved in the reduction of oxidised forms of small molecular antioxidants (GSH reductase)
6. Cellular enzyme systems that maintain an NADH, NADPH, and FADH-dependent reducing environment (i.e., glucose-6-phosphate dehydrogenase).

1.6.1. Markers of Oxidative Stress

1. The excess production of NO is known to be cytotoxic to tissue and can induce cellular damage. The rapid reaction between NO and O_2^- yields peroxynitrite (ONOO$^-$), a potent oxidizing species.[196] Peroxynitrite is assumed to react preferentially with CO_2 in vivo to produce nitrogen dioxide (NO_2) and trioxocarbonate radicals. It also nitrates tyrosine residues of the proteins, stimulating the production of 3-nitrotyrosine, which disrupts the normal function of the proteins. NO is destroyed in a reaction with superoxide anions immediately after it is produced. Laboratory testing of NO and its free radicals (Nitrates and Nitrites) are heavily influenced by exercise, diet, intestinal bacteria and contaminants.[197-199]

2. Reduced glutathione (GSH) is a tripeptide, and is primarily a major intracellular antioxidant that protects cells from toxins and free-radical damage. This has been confirmed by many clinical studies using N-acetyl-L-cysteine treatments that have found a substantial increase in intracellular GSH contributing to reduced levels of plasma vascular adhesion molecules. Glutathione is found in high levels within ocular tissues that are involved in multiple functions, to help serve as an antioxidant and as an electron donor for peroxidases. A major study immunohistochemically localising glutathione reductase in the eye of adult rats found significant levels to be distributed in the corneal and conjunctival epithelium, corneal keratocytes and endothelium, iris and ciliary body, neural retina, and retinal pigment epithelium.[200] In addition, glutathione reductase was highly expressed in the retinal ganglion cells. This finding goes some way to support its pivotal role in the protection of ocular tissues against oxidative stress.

Hyperglycaemia promotes the increased production of free radicals (ROS) and impairs the antioxidant defence system; such as depleting the levels of GSH by glucose auto-oxidation and/or non-enzymatic glycation. This promotes the formation of glycation end products (AGEs) that induces ROS and vascular inflammation.[180, 201-203] Furthermore, oxidative stress may be very important in the pathogenesis of IR as it alters the insulin signalling pathways ("phosphoinositide 3-kinase/Akt/protein kinase B" pathway) that emerge from the insulin receptors causing a marked reduction in eNOS activation.[197]

3. Oxidatively modified tyrosine: Nitrotyrosine (NT-3) has been proposed as a marker of oxidative damage to protein and its levels strongly correlate with the severity of coronary artery disease (CAD), peripheral vascular disease and glycaemia.[204-207]

Prolonged oxidative stress, inactivity of the endothelial-derived Nitric Oxide synthase enzyme (eNOS) and endothelial dysfunction activates the reductase function of the enzyme eNOS. This increases the levels of ROS because eNOS has now been shifted from an oxygenase that produces NO into a reductase that produces ROS. This in turn further exaggerates the levels of oxidative stress and emphasises a cellular proatherogenic role as peroxynitrite (cytotoxic oxidant) mediates low-density lipid (LDL) oxidation.[95, 204, 208]

1.7. Research Rationale

The pathophysiological mechanism underlying an increased risk of CVD, atherosclerosis and T2DM in at risk groups is not yet fully understood. Traditional risk factors such as smoking, hypertension and dyslipidaemia only explain a small proportion of the biological processes. Therefore, identification of new risk markers will lead to earlier and more effective interventions to those at risk of T2DM.

T2DM accounts for six percent of the total global mortality with fifty percent of diabetes-associated deaths being attributed to CVD.[1, 209] Therefore, the need for appropriate management programmes and prevention strategies targeting high-risk individuals could show that much of the morbidity and mortality of T2DM is preventable.

IR, together with other conventional risk factors has been proposed to be an important component for the mechanisms behind the cause of T2DM and cardiovascular disease (CVD). Furthermore, it has been reported that IR is associated with other CVD risk factors including age, body mass index (BMI), TG, cholesterol and BP. Recently, carotid IMT and vascular endothelial function have been frequently used as a surrogate marker of atherosclerosis. Therefore, the relationship between IMT and endothelial function in those at risk of T2DM can be determined as surrogate markers of independent risk factors for CVD and T2DM.[42]

Additionally, insulin is well known for its ability to vasodilate skeletal vasculature as a result of endothelial nitric oxide (NO) production that stimulates endothelial-dependent vasodilation. Those with IR, hypertension and T2DM present with a blunted insulin-mediated endothelial-dependent vasodilation as a result of reduced endothelium-derived NO production.

Subclinical inflammation can also be a precursor to CVD and is associated with IR, therefore, can precede the development of T2DM. Inflammatory mediators may induce systemic endothelial

dysfunction; in large arteries this can lead to clinical CVD, whereas in the capillary and arteriolar endothelium, with a vast surface area in contact with metabolically active and insulin-sensitive tissues, endothelial dysfunction may lead to T2DM.[11, 210, 211] Therefore, the identification of endothelial dysfunction might expand options for diabetes prevention and treatment.

Since endothelial dysfunction is an early phase of atherosclerosis, associated alongside an abnormality in the bioavailability of NO, it could be suggested that there is more insulin resistance in peripheral tissues as well as vascular endothelial dysfunction in subjects thought to be at risk of T2DM.[101]

Traditionally, methods that investigate endothelial function at the macrocirculatory level have been used for risk evaluations. Endothelial dysfunction however, is known to occur much earlier at the microvascular than at the macrovascular level and, consequently, several vascular reactivity tests including retinal vascular function have been developed for the clinical assessment of endothelial function in these type of vessels.[212] Indeed, as the eye is vulnerable to minor changes in perfusion leading to structural and functional abnormalities it is a suitable site to investigate early changes that may predict large vessel disease.[213]. Changes of the retinal arteriolar calibre have been shown to be associated with high risk of hypertension and can be used as a predictive marker for future vascular complications in already diseased patients. More recently, functional retinal vascular impairment using dynamic vessel analysis have been reported in patients with diabetes[214, 215], CVD and smoking individuals[216], showing its utility as an accurate and clinical means of analysing functional changes in vascular disease.

Therefore, a key concept in studying groups at risk of T2DM is to recognise that there is a clustering of risk factors. For instance, the association of hyperglycaemia, hypertension, dyslipidaemia and obesity has been recognised for several years and adopted the clinical definition known as the MetS. More recently, the number of associated characteristics has evolved to include a spectrum of markers that indicate vascular inflammation, endothelial dysfunction, cell adhesion, microalbuminuria, and blood coagulation abnormalities.[43]

Recent publications investigating vascular function in at-risk individuals have provided a foundation in identifying pre-clinical markers for future T2DM and CVD risk.[217-219] However, as shown in Table 1.2., there is limited data available to allow for clinical strategies and screening programmes to be made available. For the purposes of this thesis, Dynamic Retinal Vessel Analysis (DRVA) was used to measure retinal vascular function and is explained later in detail in Section 2.1.

Therefore, the purpose of this thesis was to investigate vascular function in those at risk of T2DM, namely South Asians (SA) and gender differences, those with impaired glucose tolerance (IGT) and normoglycaemic offspring of T2DM, i.e. those with a familial history (FH) in an attempt to further already existing knowledge. Additional to these main chapters, a subsidiary investigation on the effect of overweight and obesity was conducted on vascular function. Furthermore, the investigation of surrogate markers and their use in well known at-risk individuals for vascular screening will provide clinical information for future applications.

	Family History (FH)	South-Asians (SAs)	Impaired Glucose Tolerance (IGT)
Retinal Haemodynamics	DR is associated with incident T2DM[220]	↑Arterial MDRT[221]	IGT not related to retinal fractal dimension[222] IGT is associated with ↑DR[223-226] ↑ Venular calibre associated with presence of DR[227] RPE changes and lesions with IGT[228, 229]
Systemic Endothelial Function	↓FMD[37-39, 230-236] No differences in FMD[237, 238]	↓FMD[26]	↓FMD[236, 239-242] No differences in FMD[243]
Biochemical Markers	↑ (vWF, PAI-1) markers of endothelial dysfunction[244, 245] State (↑ MDA) of oxidative stress [246, 247]	↑ (vWF, hs-CRP) markers of endothelial cell damage and dysfunction[26, 248-251] IR linked with endothelial damage markers[252] Comparable levels[253, 254]	↑ (ET-1, CAM-1) markers of endothelial dysfunction[255] State (↓ GSH, ↑ MDA) of oxidative stress [246, 247, 256, 257]
Autonomic Function	↓HRV[235, 258-260]	↓ANS function in SA men[261] ↓HRV[258]	↓ANS function[262-269] No differences in ANS[270, 271]

Table 1.2. Recent published vascular function data available on groups known to be at risk of T2DM. DR: diabetic retinopathy; ↑: increased; MDRT: maximum diameter reaction time; RPE: retinal pigment epithelium; ↓: reduced; FMD: flow mediated dilation; vWF: von Willebrand factor; PAI-1: plasminogen activator inhibitor-1; MDA: malondialdehyde; hs-CRP: high sensitivity C-reactive protein; IR: insulin resistance; ET-1: endothelin1; CAM-1: cellular adhesion molecule-1; GSH: reduced glutathione; HRV: heart rate variability; ANS: autonomic nervous system.

2. Methodology

This chapter explores the methods used for this thesis and its major studies in greater detail, discussing the mechanisms and limitations as well as the protocols used for the measurement protocols adopted in the Appendix 1.

2.1. Retinal Vessel Analysis

Dynamic retinal vessel analysis (DRVA) is a technique used to assess retinal artery and venous behaviour by means of measuring diameter change. Measurements are obtained with the use of a retinal vessel analyser (RVA, IMEDOS GmbH, Germany) that consists of a high resolution fundus camera (FF450, Carl Zeiss Meditech, Germany) modified to measure retinal vessel diameter continuously. Recordings are stored by video capability that is coupled to the CCD camera imaging the retina (Figure 2.1.).
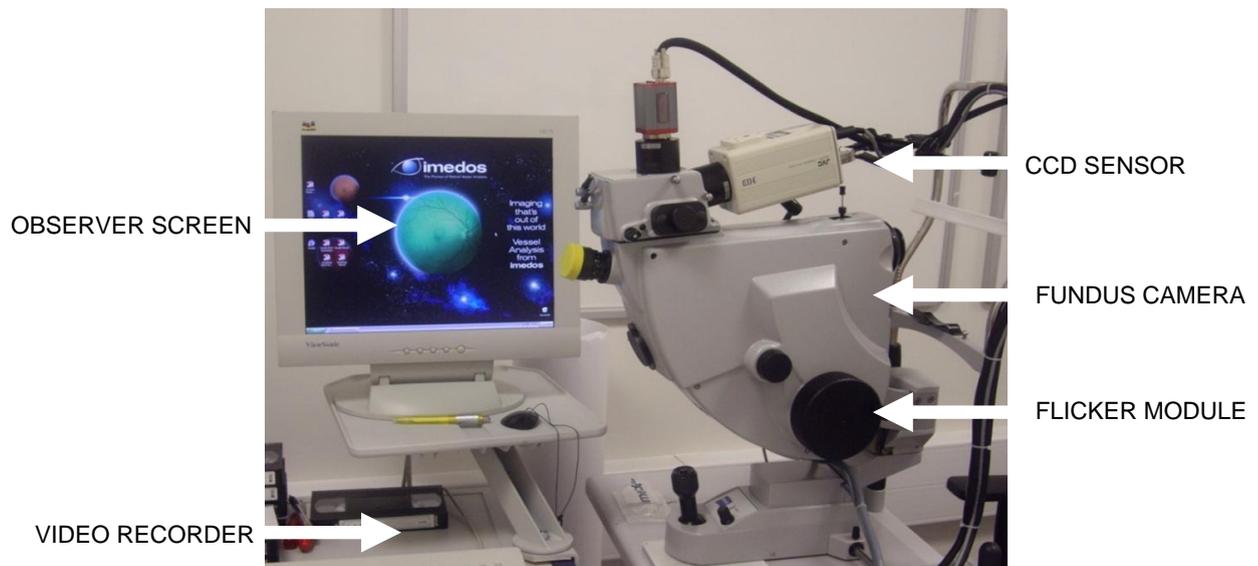
Figure 2.1. The hardware components of the Dynamic Retinal Vessel Analyser [84].

In order to obtain a good quality and evenly contrasted and illuminated fundus image, full pupil dilation is required using a mydriatic drug (typically Tropicamide 1%, Chauvin Pharmaceuticals, UK). The camera is set at an angle to provide 30° field of view with a temporal resolution of 40ms to allow a sampling rate of 25Hz (Figure 2.2.). Frame to frame analysis is used to process the recordings as described elsewhere.[272]
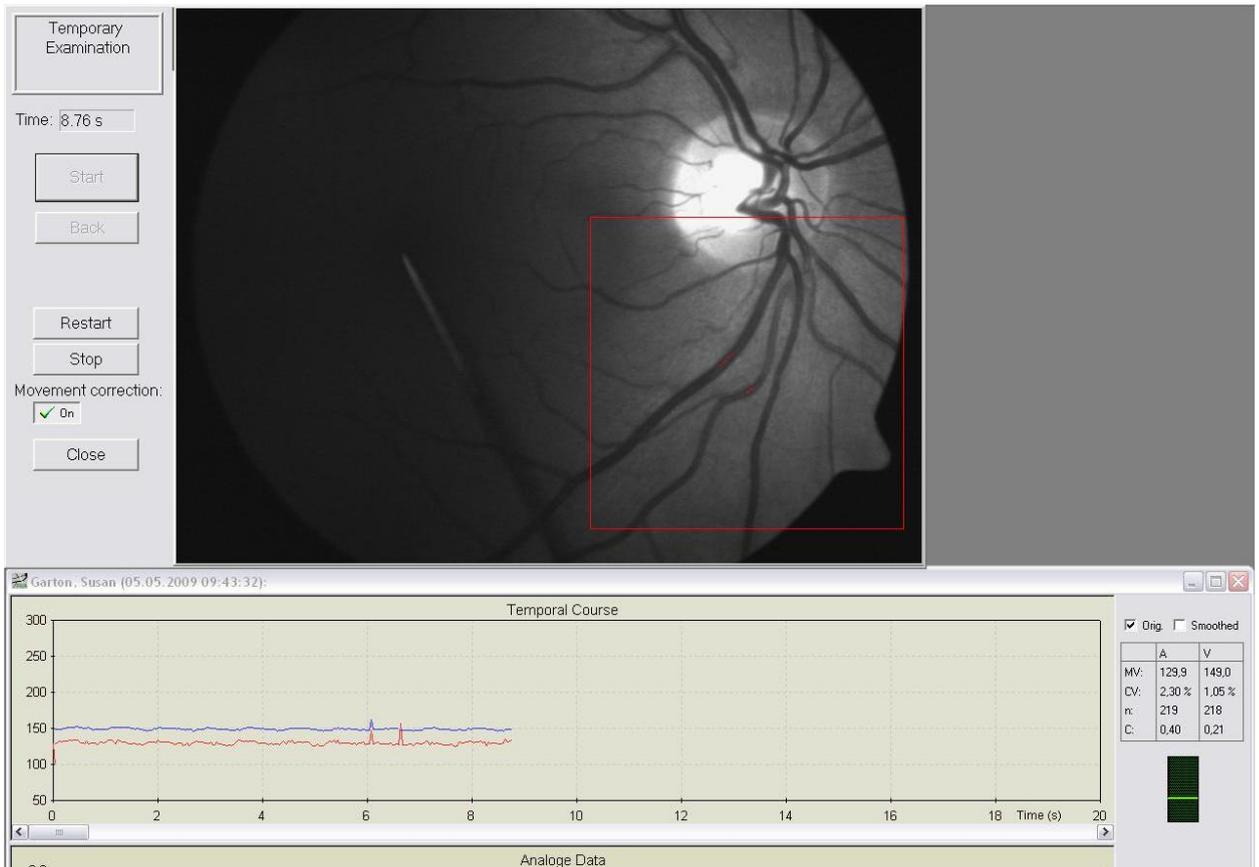
Figure 2.2. The DRVA data acquisition software recording retinal vessel diameter of chosen segments in real time.

The DRVA measures the width of the red blood cell column within the chosen vessel of interest and allows the means of assessing the autoregulatory mechanisms of the retinal vessels.[273] This is achieved by using various stimuli (hypercapnia or increased IOP with a suction cup), or, measuring vessel dilation to flicker light stimulation.[274-278]

However, it is important to note that the instrument has limitations that can affect the overall measurement outcome. Namely, the image quality is strongly dependent on clear optical media and good fixation abilities. Therefore, measurements in subjects with corneal and lenticular opacities, central vision loss or poor visual acuity will result in increased variability.

This thesis adopted flickering light provocation to assess retinal vessel reactivity as a means of a stimulus of the vessels via metabolic increase (NO-induced endothelial-mediated dilation), neural coupling, or a combination of both.

Flickering light is modulated by the DRVA, and due to the natural reactions of the retina to respond to light modulation, flicker provides the most natural stimuli to assess its function.[277-280] To achieve flicker light, an optoelectronic shutter is placed in the optical pathway of the

camera illumination. The shutter generated flicker by interrupting the illumination to provide a bright-to-dark contrast ratio of at least 25:1. As the video frequency is set at 25Hz, the flicker frequency provides a sampling rate of 12.5Hz which has been shown to be in the maximal exciting flicker frequency range.[277]

An increase in the metabolic rate (and thus oxygen consumption) of the photoreceptor cells triggers the release of NO in retinal vascular endothelial cells to meet the metabolic demands causing active vasodilation. Following stimuli cessation, the vessel returns to baseline diameter value with the exception of the artery which has been found to constrict beyond its baseline diameter. This over-constriction is thought to be a regulatory mechanism of the retinal vascular endothelium as a result of an imbalance of vasoactive substances, namely NO and ET-1.

The measurement procedure used for this thesis has been described and validated elsewhere.[281] In short, the whole measurement duration encompasses 350 seconds that consists of 50 seconds baseline followed by three 20-second cycles of flicker and 80 seconds of recovery. This protocol has been shown to be of value in a range of clinical and ocular conditions including glaucoma[274, 282-285], vein occlusions[286-290] and DM[214, 215, 291-301]. (Appendix 2)

The DRVA then provides a measurement profile for both arteries and veins (Figure 2.3.). The algorithm used by the software calculates the maximum diameter response by firstly averaging all three flicker cycles and then calculating the flicker response ±3 seconds flicker cessation.[295, 302] Therefore, for those that reach maximum retinal dilation before 17 seconds or after 23 seconds of flicker light commencing, their dilation response will be underestimated. Furthermore, the averaging of all three cycles does not take into consideration differences in spatial and temporal reaction patterns in each individual cycle. Therefore, taking into account of these limitations, an analysis of individual flicker cycles[275-277, 303] has been incorporated with a different analysis method known as sequential and diameter response analysis (SDRA). It has been tested and validated in volunteers likely to exhibit vascular disturbances and has been shown to allow the analysis of dynamic retinal vessel responses and their time course in serial stimulation.[216] This has also been further extended in a small sample of otherwise healthy SAs. (Appendix 3)

The absolute diameter (AD) is recorded in arbitrary units (AU) as this best corresponds to µm in the ametropic eye; unlike other papers quoting measuring units (MU) as this correspond to µm only in the Gullstrand eye.[273, 304] Three single curves, obtained for each flicker cycle, alongside averaged values, are used for statistical analyses. Essentially, thirty seconds of baseline assessment before twenty seconds of flicker application and thereafter eighty seconds

were used for analysis. Due to inter- and intra-individual vessel diameter variation, the baseline diameter was defined as 100%.[305]
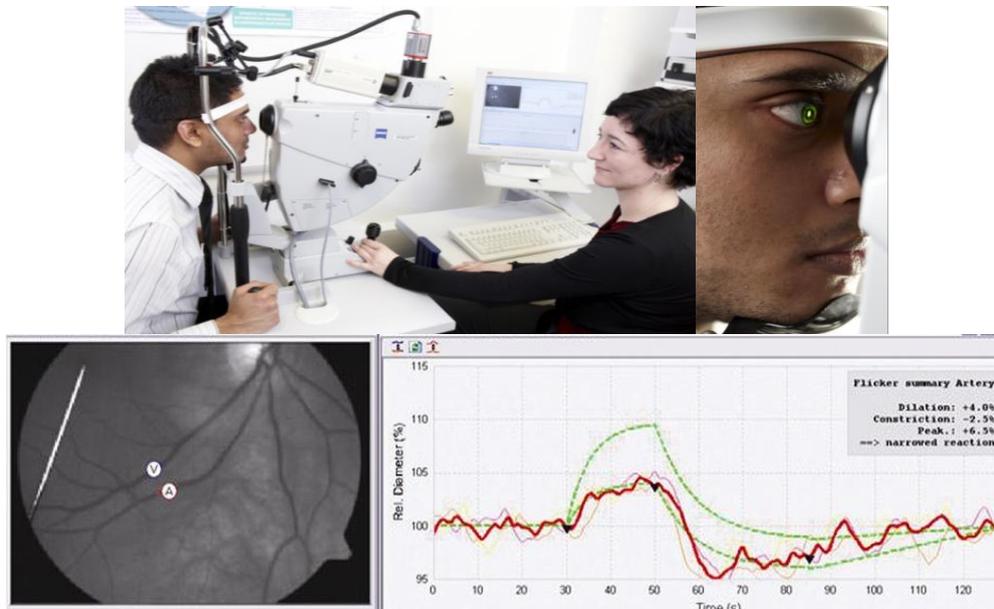
Figure 2.3. The patient set-up on the DRVA along with the pre-defined output profiles provided by the DRVA software.

The following retinal vessel reactivity and time course parameters were then calculated (Figure 2.4.); the differences between maximum and minimum baseline vessel diameter termed as baseline-diameter fluctuation (BDF), the maximum diameter (MD) was used to describe the maximal vessel dilation in response to flicker light stimulation expressed as a percentage from baseline, the time taken (seconds) to reach the maximum vessel diameter during twenty second flicker exposure was termed as MD reaction time (MDRT), the minimal vessel diameter within thirty seconds of the recovery period was calculated as a percentage of baseline and expressed as the maximum constriction (MC) whilst the time taken (seconds) to reach maximal vessel constriction was termed maximum constriction reaction time (MCRT), and finally, the difference between maximal dilation and constriction responses was termed as the dilation amplitude (DA).[117]

The impact of atherosclerosis and thus vessel stiffness must also be taken into account. For instance, depending on a vessel's elasticity, the ability to reach MD can be fast or slow. Therefore, the calculation of reaction time taken to reach maximum dilation (RT) was performed alongside slope analysis for dilation and constriction profiles during and after flicker stimulation respectively. The slope analysis has been furthered to involve both MD and MC in order to take

into consideration endothelial and autoregulation mechanisms as it is proposed that the initial reaction to flicker could be endothelial-mediated until NO reserves are depleted allowing autoregulative mechanisms to continue the dilation response. Therefore, MD and MC slopes were calculated at 50% and 100% time points (Figure 2.5.)

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Figure 2.4. The retinal vessel reactivity and time course components used in SDRA. [117]
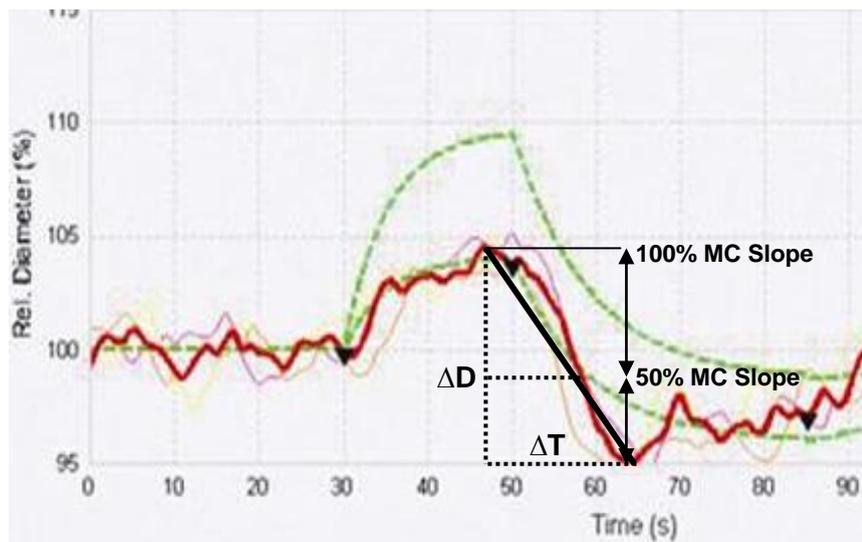
Figure 2.5. Constriction slopes calculated for both 50% and 100% time-points. The green dashed lines represent Gaussian distribution and normal distribution as calculated by the DRVA software using a limited database of healthy controls. The red line represents an average of all three flicker cycles.

2.2. Flow-mediated Dilation of the Brachial Artery (FMD)

The capacity of blood vessels to respond to physical and chemical stimuli in the lumen confers the ability to self-regulate tone and to adjust blood flow and distribution in response to changes in the local environment. Many blood vessels respond to an increase in flow, or more precisely shear stress, by dilating. This phenomenon is designated *FMD*.[112, 117, 306-308]

Therefore, FMD is an endothelium-dependent process that describes the relaxation of a conduit artery when it is exposed to increased blood flow (shear stress). Shear stress is determined by blood flow and the force that it exerts perpendicular to the long axis of the vessel.[309] As the shear stress changes, the endothelium acts as a mechanotransducer and subsequently modifies the expression of vasodilators (Figure 2.6.).
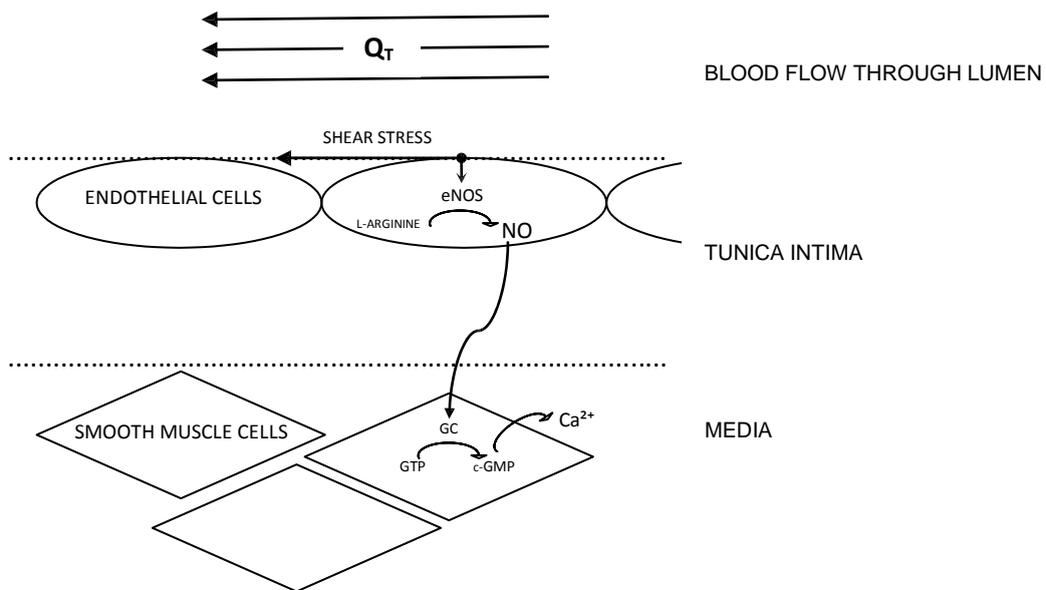
Figure 2.6. The endothelial cell processes from shear-stress

Shear stress is usually induced by increasing local blood flow (reactive hyperaemia), and this is achieved by inflating a pneumatic cuff 50mmHg above systolic blood pressure (SBP). The increase in pressure causes an abrupt decrease in vascular resistance due to an occlusion in arterial inflow. This causes ischemia and consequent dilation of downstream resistance vessels via autoregulatory mechanisms. Subsequent cuff deflation induces a brief high-flow state through the brachial artery (reactive hyperaemia) to accommodate the dilated resistance vessels. The resulting increase in shear stress causes the brachial artery to dilate.[48, 308] This phenomenon was first described by Schretzenmayer and has been demonstrated in a number of conduit arteries (brachial, radial and femoral).[310-315]

The maximum post-hyperaemic diameter is determined and FMD is expressed as the relative change from baseline. An impaired/diminished FMD response reflects endothelial dysfunction.[308]

For the purpose of this thesis FMD was detected by means of colour Doppler imaging (CDI), a technique that detects the Doppler shift induced by moving red blood cells through the use of an ultrasonic signal. CDI combines conventional b-scan (grey-scale imaging of anatomical structures) together with the Doppler shift frequency measurement of blood flow velocities and the colour representation of blood flow based on these frequency shifts. The Doppler equation (Equation 2.1.) explains the relationship (and thus the mechanism of doppler imaging) between the frequency of the ultrasound beam (f), the velocity of the blood (V_{blood}), the velocity of the ultrasound through the blood (V_{sound}), and the angle of incidence between the direction of the blood flow and the approaching sound beam (θ) to give the Doppler shift:

$$\text{Shift} = \frac{2(f.V_{blood}.\text{Cos } \theta)}{V_{sound}}$$

Equation 2.1. The doppler equation.

The ultrasound system (Acuson Sequoia®, Siemens Medical Solutions, US) used for this thesis consists of a screen unit linked to a keyboard where the data and the optimization of the image can be adjusted. In conjunction with the ultrasound system, arterial diameters are continuously measured by wall-detection using specialised artificial neural networking software (VIA® Software, UK) from the anterior to the posterior interface between the media and adventitia on a personal computer (Figure 2.7.). An 8L5

liner-array transducer is used to image macrovasculature. A surgical bed is also used in conjunction with this instrument to keep subjects in a supine position.
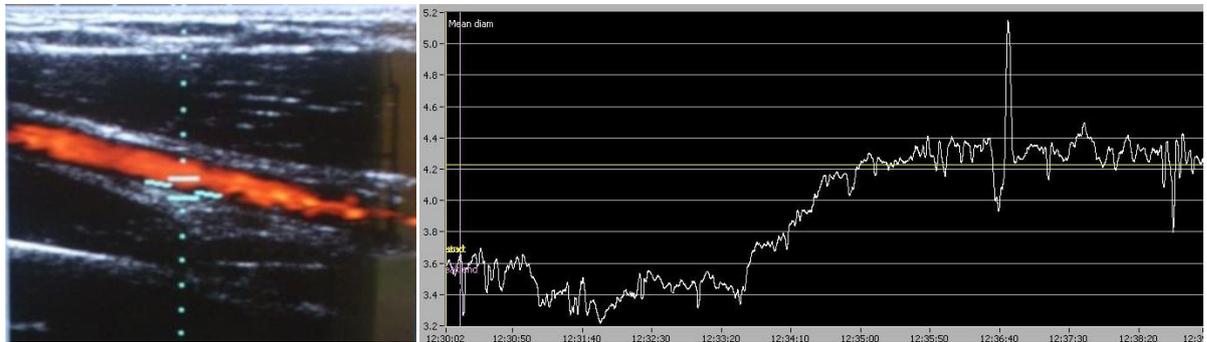
Figure 2.7. The brachial artery image using the Sequoia$^®$ system and FMD profile generated by the VIA$^®$ system.

The Sequoia$^®$ system allows for the manipulation of certain parameters so that an adequate quality of the 2D vessel image representation can be obtained. Additionally, certain influences on the image quality can be controlled and taken account of:

1. *Aliasing*: The Doppler waveform is made up of a series of samples that are in turn made of an inherent maximum frequency. When this frequency is exceeded, the waveform is no longer constructed accurately, giving an overall lower frequency. Therefore, aliasing is the misinterpretation of high frequencies that disobey Shannon's theorem (above half the sampling rate).

2. *Angle and Blood flow errors*: When considering the Doppler shift equation, θ; the angle subtended between the ultrasound beam and the longitudinal axis of the vessel, is an important factor. Large inaccuracies are found when θ is continually changing (influenced by movement, intrinsic spectral broadening). This is because each point along the transducer face can become a point source, i.e. with increased transducer array width; there are an increased number of elements firing off a Doppler signal if θ is not kept constant.

3. *Mirror Images* represent multiple reflections from artefacts; be it resolution artefacts (axial, lateral or speckle), or, acoustic shadowing. Mirror images are affected heavily by path length errors, finite dimensions of arrays and phase quadrature.

4. *Doppler Parameters*: The effect of this variable is minimised if settings are kept constant throughout the study procedure.

The brachial artery is imaged above the antecubital fossa in the longitudinal plane and a segment with clear anterior and posterior intimal interfaces between the lumen and vessel wall is selected for continuous 2D greyscale imaging. A baseline rest image is acquired for two minutes, and thereafter, arterial occlusion is created by cuff inflation to suprasystolic pressure for a standardised five minutes. The longitudinal image of the artery is recorded continuously for two minutes (min) after cuff deflation. At least 10 min of rest is needed (as according to the protocol adopted) after reactive hyperaemia before another image is acquired to reflect the re-established baseline conditions. An exogenous NO donor, such as a sublingual glyceryl trinitrate tablet is given to determine the maximum obtainable vasodilator response, and to serve as a measure of endothelium-independent vasodilation reflecting vascular smooth muscle function.[308]

In order to achieve optimisation of parameters all known factors that can affect FMD have to be limited. For instance, the participant should fast, including taking no caffeine for 12 hours before the study, and not have exercised before the test. FMD should be measured in the morning in a quiet, temperature-controlled room (22°C). In addition participants shouldn't smoke and all vasoactive medications should be withheld for 4 half-lives. Furthermore, the investigator should be cognisant of sleep deprivation, mental stress and the phase of the menstrual cycle.[112, 316, 317]

2.3. Endothelin-1 (ET-1) Levels

ET-1 levels were determined by the author and an experienced lab technician, in fasted venous plasma samples collected in EDTA tubes that have been centrifuged at 3000rpm for fifteen minutes and then aliqouted and stored in a -80°C freezer. Once all EDTA plasma samples have been collected, they are thawed and ET-1 levels are determined using a standardised and commercially available ELISA kit which involves:

1. Initially, 100µl of assay buffer, eight standardised solutions and plasma sample are pipetted into each microwell.
2. The plates are sealed and incubated overnight at 4°C.
3. The microwells are washed seven times using 400µl of wash buffer solution and 100µl of labelled antibody is added, sealed and incubated at 37°C for thirty minutes.
4. Each well is then washed again using 400µl of wash buffer solution for a total of nine times before 100µl of substrate solution is added and incubated for thirty minutes at room temperature in the dark.
5. The enzymatic reaction is then stopped using 100µl of composed stop solution and the

plates read using a plate reader set at an optical density of 450nm.

2.4. von-Willebrand Factor (vWF) Levels

In order to evaluate vWF levels, fasted venous blood samples have to be collected in citrate tubes. After spinning the blood in a centrifuge at 3000rpm for fifteen minutes, the supernatant is aliqouted and stored in a -80°C freezer. The citrated plasma is then thawed and analysed for vWF levels using a standardised ELISA kit which was optimised according to previously established methods.[318, 319] The procedure carried out by the author and an experienced lab technician briefly involves:

1. A microtitre plate is coated with 100µl of dilated primary antiserum solution (30µl in 20.5ml coating buffer at pH 9.6) at room temperature and then refrigerated for a minimum of 60 minutes to overnight.
2. The microplate is washed four times with 250µl of wash buffer per well before 100µl of substrate is added with working strength detection antibody dilutant and incubated for sixty minutes at room temperature.
3. The microplate is then washed three times with wash buffer before 100µl of secondary antiserum is added and incubated at room temperature for forty-five minutes.
4. The microplate is washed again for a final three times and 100µl of substrate is added and incubated at room temperature for twenty minutes.
5. The enzymatic reaction is then stopped by adding 50µl of hydrosulphuric acid.
6. The absorbance of the solution is then immediately read on a microwell plate reader set at 492nm.

2.5. Glutathione Levels

The ratio of reduced (GSH) and oxidised (GSSG) glutathione within cells is thought to be a valid measure of cellular toxicity. For the purpose of this thesis, this marker of oxidative stress was determined by centrifuging (15000rpm for five minutes) 30µl of fasted venous blood collected in EDTA tubes with 33.3µl of sulfosalicylic acid and 936.7µl of sodium phosphate stock buffer solution within ten minutes of blood collection. 150µl of the supernatant is aliqouted and cooled at -80°C for later analysis. The adopted protocols have been previously optimised in-house according to previously reported and validated methods. [320] Glutathione levels were then determined by the author and an experienced lab technician using an enzymatic reaction created by:

2.1. Firstly, 150µl of daily buffer is added to 50µl of DTNB solution in each microwell.

2.2. 25µl of the prepared plasma sample is added to each well and incubated at 37°C for three minutes.

2.3. 25µl of GSR solution is added to the previous mixture and read using a microplate reader set at 410nm.

2.6. ABPM and HRV

Both ABP and ECG recordings for HRV analysis can be obtained with a computer-operated monitor. The device used in this thesis (Cardiotens-01, PMS Instruments, UK) indirectly obtains BP using the oscillometric technique whereby the magnitude of the pressure oscillation is compared to the pressure of the cuff. The ECG is measured by using a validated electrode and lead set-up. The monitor is programmed via a fibre-optic cable connection to a personal computer programmed with the BP monitoring software (Cardiovisions 1.7.2., PMS Instruments, UK). This allows for customised protocols set at specific measurement intervals alongside graphical and statistical packages for detailed BP and ECG analysis. The validated protocol adopted for this device involves 15-minute interval BP measurements during the active period and 30-minute interval measurements during the passive period (Figure 2.8.).[321]
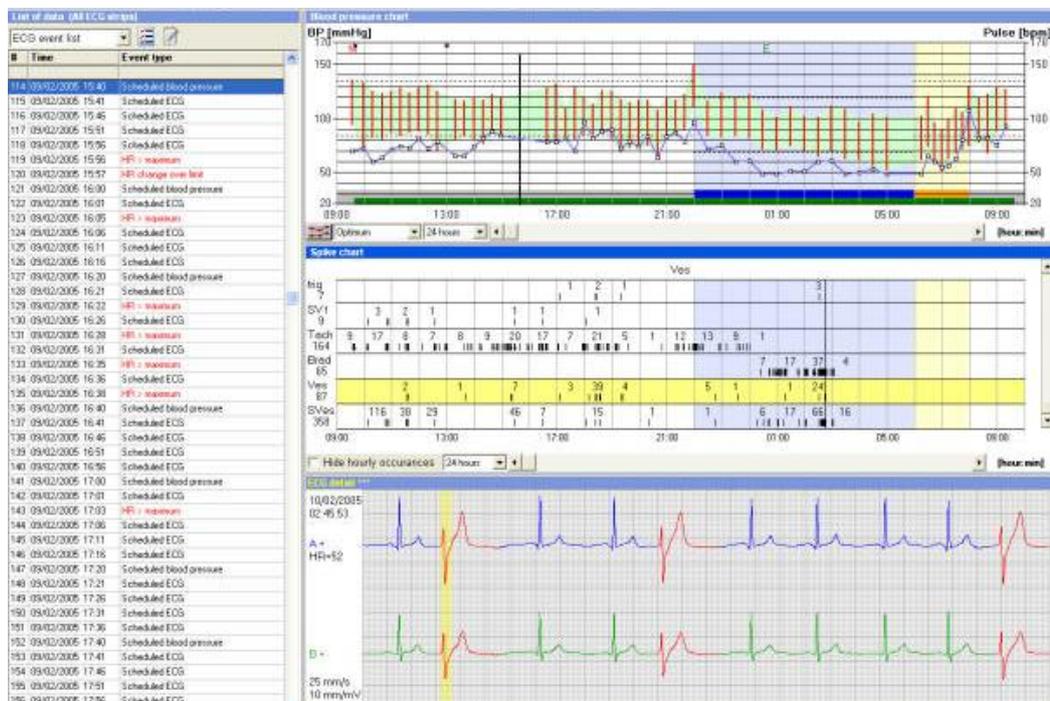
Figure 2.8. The 24-hour blood pressure and ECG profiles provided by Cardiotens system and software.

Mean arterial pressure (MAP), as a means of describing cardiac output function in relation to arteriolar resistance was calculated according to Equation 2.2.

$$MAP \approx (\tfrac{2}{3} \times DBP) + (\tfrac{1}{3} \times SBP)$$

Equation 2.2. Mean arterial pressure equation

To allow sufficient BP analysis, a minimum of 80% programmed recordings were required and furthermore, any suspected outliers based on pulse pressure (PP) determination (i.e. PP <10mmHg with SBP <100mmHg) were rejected on the assumption that the data was non-physiological.

HRV analysis was performed for each individual using the Cardiovision software (PMS Instruments Ltd., Maidenhead, UK) to obtain twenty-four hour, active and passive period LF, HF and LF:HF values. From, this circadian BP and HRV changes were calculated according to Equations 2.3. and 2.4. respectively.

$$Circadian\ BP = Active\ Period\ BP - Passive\ Period\ BP$$

Equation 2.3. Circadian BP equation

$$Circadian\ HRV = Active\ Period\ HRV - Passive\ Period\ HRV$$

Equation 2.4. Circadian HRV equation

3. Vascular Function in Normoglycaemic WE Individuals with and Without a Family History of T2DM

3.1. Abstract

3.1.1. Background/Aims: To investigate ocular and systemic vascular reactivity to stress in normoglycaemic and normotensive individuals with a parental FH of T2DM.

3.1.2. Methods: Healthy WE participants aged between 25-55 years with (n=55) and without (n=55) a FH of T2DM were recruited. Fasted venous samples were taken to analyse for glucose, TG and cholesterol (HDL, LDL and total). Retinal vessel reactivity was assessed by using the Dynamic Retinal Vessel Analyser (DRVA, Imedos GmbH, Jena). In addition, systemic endothelial function was assessed by using the flow mediated dilation (FMD) technique.

3.1.3. Results: Those with a FH, including gender sub-groups showed higher levels of dyslipidaemia (p<0.05) but no differences in retinal artery reactivity or FMD (p>0.05). Those with a FH did exhibit negative correlations between cholesterol values (total and LDL) and retinal arterial dilation (r=-0.90, p=0.003), maximum dilation (MD; r=-0.90, p=0.03) and dilation amplitude (DA; r=-0.94, p=0.019).

3.1.4. Conclusion: Subjects with a FH of T2DM show similar vascular responses at both macro- and microcirculation levels to that of healthy controls. Whether the correlations show that, in certain conditions, FH could contribute to a higher risk of developing widespread systemic vascular disease before the development of overt diabetes needs further investigation.

3.2. Introduction

In healthy individuals, the vascular endothelium plays an important role in vascular homeostasis by regulating vascular tone. In addition, it also acts in preventing inflammation, platelet aggregation and leukocytes adhesion. Ageing, smoking, dyslipidemia, hypertension, obesity, DM and chronic inflammatory diseases result in the so-called endothelial dysfunction, a condition in which NO is reduced and the reactive oxygen species levels are increased. Moreover, in addition to vasoconstriction and tissue ischaemia, endothelial dysfunction is also characterised by an increase in inflammation and cells adhesion leading to atherosclerosis.[48]

It has already been documented that endothelial dysfunction contributes to the occurrence and progression of both DM and its cardiovascular complications.[322, 323] Moreover, signs of endothelial dysfunction have been found in those with a FH of parents suffering from T2DM.[38, 39, 234] Consequently, as endothelial dysfunction contributes to both impaired insulin action and occurrence of atherosclerosis, those with a FH of diabetic patients could be at an increased risk of early development of a large variety of pathologies, from metabolic to cardiovascular disorders.[244]

As emphasised previously in Section 1.4., it is important to detect changes in vascular function that precede cardiovascular events; this is possible through studying endothelial function. Indeed, decreased endothelial function is a factor that predicts independently from other risk factors the occurrence of cardiovascular disease.[93, 94, 324] Endothelial function can be assessed using a large variety of techniques, from laboratory markers, such as inflammatory cytokines, adhesion molecules, NO and markers of endothelial damage and repair, to vascular reactivity tests such as FMD of the brachial artery by ultrasound (Section 2.2.), forearm venous pletysmography, digital pulse amplitude tonometry and laser Doppler measurements of the peripheral circulation.[212] These methods are successful in demonstrating the presence of endothelial dysfunction in both diabetic patients and their offspring, however, their complicated nature and reduced reproducibility in medical practice have proven to be of limited use in routine clinical screening.[212] Therefore, the need for reproducible, non-invasive and less laborious techniques has never been greater. In this respect, the measurement of vascular reactivity at both macro- and microvascular levels seems to be the best available option.

Microvascular assessments such as retinal vessel reactivity can be performed quickly using relatively affordable techniques and offers reliable information on vascular function in patients suffering from various systemic diseases including DM. It is thought that the microvascular complications of DM are a direct result of increased vascular permeability and impaired autoregulation of blood flow and vascular tone. Clinical studies using the DRVA in patients suffering from DM, hypertension or hypercholesteraemia have shown a reduced retinal vessel response to flickering light, demonstrating that endothelial dysfunction may be measured at the ocular level in various systemic vascular pathologies.[49]

Therefore, by using direct visualisation of the vascular function at the retinal vessel level, we may be able to diagnose earlier stages of vascular disease in populations at risk. Indeed, changes in retinal vessel reactivity in those with T2DM has been extensively studied. Little is known about when the first signs of vascular dysfunction occur in both patients with, and subjects at risk of this disease.

This study aims to investigate the retinal vascular function and its relationship to systemic reactivity in normoglycaemic subjects with and without a positive FH of T2DM, and whether this helps to further understand and predict the vascular risk.

3.3. Materials and Methods

The study cohort consisted of healthy WE normotensive participants aged 25-55 years that were screened and recruited from the Health Clinics at Aston University, Birmingham, UK. FH was confirmed through questionnaire and/or evidence of parental diagnosis. The author carried out recruitment, data and blood collection, as well as biochemical and statistical analysis on site (Aston University Day Hospital).

Exclusion criteria were: a positive diagnosis of cardio- or cerebro-vascular disease, (coronary artery disease, heart failure, arrhythmia, stroke, transient ischaemic attacks), peripheral vascular disease, severe dyslipidaemia (defined as plasma TG>6.00mmol/L or cholesterol levels>7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment.

Furthermore, subjects were also excluded if they had a refractive error of more than ±3DS and more than ±1DC equivalent, IOP >24 mmHg, cataract or any other media opacities, as well as if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system. This excluded refractive error and known vascular variables and their effects on retinal imaging so that reliable results and retinal analysis could be achieved.

Written informed consent was obtained from all participants and ethical approval was sought by the author from local and NHS ethical committees (08/H1202/112). The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki.

An OGTT was performed according to the WHO protocols on all participants a week prior to all other measurements and only participants with normal results were asked to come back for the subsequent tests.

The OGTT was carried out by analysing baseline (12-hour fasted) and 2-hour post consumption capillary blood samples. The solution was made to a standardised 75g glucose drink, whereby 113mL of neutral glucose (Polycal Liquid, Nutricia, Wiltshire, UK) was diluted with 87mL of water. The subject was acclimatised to 22°C and was not allowed to undertake physical activity, smoke or consume any food or drinks during the test.

According to an already established procedure when examining endothelial function, female participants were asked to fill in a validated menstrual cycle questionnaire and their investigations were carried out during the first week of the menstrual cycle (follicular phase) thereby controlling for hormonal influences on endothelial function.[112, 317]

3.3.1. Investigations
3.3.1.1. Blood sampling and analyses

All participants were asked to fast and refrain from caffeine, alcohol, chocolate and carbonated drinks and to not exercise for 12 hours prior to the date of the study. All blood samples were obtained by a qualified phlebotomist in the morning, between 9am and 10am. Fasting plasma glucose, TG, total and HDL-C were measured using standard routine laboratory techniques using the Reflotron Desktop Analyser (Roche Diagnostics, UK).

The Reflotron Analyser is a microprocessor-controlled reflectance photometer that incorporates an Ulbricht's sphere to automatically measure reflectance, i.e. the intensity of the light directed onto the reagent strip and reflected back according to its depth of colour. The depth of colour is determined by the concentration of analyte in the sample undergoing investigation. A 30µL measured sample (capillary, fasted venous or plasma) is applied to a reagent strip with a laboratory pipette, and then placed in the photometer for testing. The analyser provides self-calibrated results for metabolic markers (glucose, TG, total and HDL-C) using internationally applied reference methods.

The TG/HDL cholesterol ratio (TG:HDL-C), a measure of endothelial function,[325], and Framingham score as a means of cardiovascular risk (including myocardial infarction and coronary death) were also determined from the above values. Framingham scores were obtained using an automated electronic risk calculator using the Framingham Heart Study equation whereby basic medical data was inputted according to the Adult Treatment Panel III criteria (National Cholesterol Treatment Panel, USA). The risk factors included in the Framingham calculation are age, total cholesterol, HDL-C, SBP, treatment for hypertension, and cigarette smoking. Because of a larger database, Framingham estimates are more robust for total cholesterol than for LDL-C.

3.3.1.2. ABPM and HRV analysis

Systemic BP was measured using a 24-hour computer-operated ambulatory BP and electrocardiography (ECG) monitor (Cardiotens-01, Meditech Ltd., Hungary) for each subject (described in detail in Section 2.6.). Measurements were performed in ambulatory conditions and programmed to measure BP oscillometrically every 15 minutes during the subject's active period and every 30 minutes in the passive period.

3.3.1.3. Body Composition Analysis

Body composition analysis is a physical test that allows for the measurement of various components, and thus their proportion to one another, in the human body. It is achieved by bioelectrical impedance, whereby the electrical impedance (the opposition to an electrical current flow through bodily tissues) is used to calculate an estimate of total body water. This in turn can then be used to estimate fat free body mass and, by difference with body weight the actual percentage body fat.[326, 327]

Anthropometric measures including height and weight were measured using standard procedures. Body composition was measured using bioelectrical impedance (Biostat 220, Biospace, UK) to determine Body Mass Index (BMI), Percentage Body Fat (PBF), Waist-to-Hip ratio (WHR), total fat and fat free mass.

3.3.1.4. Intima-media thickness

IMT, is a measurement of the thickness of arterial walls, in this case; the carotid artery, by external ultrasound, to detect the presence and track the progression of atherosclerotic disease in vivo. Many studies have documented the relationship between carotid artery IMT and the presence and severity of atherosclerosis in CVD.[328-330] However, secondary to atherosclerosis, local haemodynamics, higher blood pressure and changes in shear stress also play an important role as being potential causes of intimal thickening. It is thought that these local factors may cause a local delay in lumen transportation of potentially atherogenic particles, which in turn favours the penetration of particles into the arterial wall and consequent plaque formation.

For the purpose of this thesis, high-resolution B-mode ultrasound system (Acuson Sequoia, 5 MHz linear transducer, Siemens, USA) was used to obtain longitudinal images of the right and left extracranial far wall of the common carotid artery (CCA). During image acquisition

participants were placed in a supine position with the head rotated approximately 45° to the opposite side of the body to that being imaged. The jugular vein and carotid artery were first imaged transversely and the transducer then rotated 90° around the central line of the stacked jugular vein–carotid artery to obtain a longitudinal image. The common carotid artery was interrogated from all three angles (anterolateral, lateral or posterolateral). The images were evaluated using ultrasonagraphic frames of the CCA as recommended.[331] In each carotid segment, far-wall IMT was measured bilaterally for a single CCA view. Each IMT measure, also used for statistical analysis, represented an average of three to five measurement points.

3.3.1.5. Vascular function studies

Retinal vessel reactivity was measured using the DRVA (Imedos; GmbH, Jena, Germany). All measurements were performed in one unselected eye for each subject, between 8:00 and 11:00 AM in a quiet, temperature-controlled room (22°C). Following full pupil dilation with Tropicamide 1% (Minims; Chauvin Pharmaceuticals Ltd, UK) a region of interest encompassing vessel segments of approximately 500 µm was chosen. Retinal diameters were assessed continuously over 350 seconds according to an accepted and widely used protocol.[277, 332]

Brachial artery FMD was measured using high-resolution CDI ultrasonography, with a 7 mm 8MHz linear-array (Siemens; Acuson Sequoia, UK). The arterial diameter of the measured brachial artery was determined in real time by edge-detection neural networking software (VIA Software®, UK). The technique has already been explained in detail in Section 2.2. According to a published protocol,[308], a baseline image was acquired for 2 minutes, and thereafter, arterial occlusion was created by inflation of a sphygmomanometer cuff on the lower arm to suprasystolic pressure (50 mmHg above systolic pressure) for 5 minutes. Following this, the cuff was deflated, and the artery diameter recorded for a subsequent 2 minutes during the hyperaemic response (endothelial-dependent vasodilation). After 10 minutes of recovery another image was acquired to reflect the re-established baseline conditions. An exogenous NO donor (sublingual 0.3 mg glyceryl trinitrate tablet- GTN) was given to determine the maximum obtainable vasodilator response. FMD was expressed as the maximal artery dilation during hyperaemia from baseline (%).

3.4. Power calculation and statistical analysis

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessel reactivity was shown to be significant.[214, 304] Additionally FMD studies have shown a 33% reduction with a SD of 5.2% in FMD response in those with a FH of diabetes.[39] As the study design was multi-factorial in nature it was calculated (using SISA; a web-based sample calculator approved by the NHS ethics committee) that n=36 subjects per group was sufficient to provide 90% power with an alpha of 0.05. Furthermore, the sensitivity and reproducibility of the techniques in healthy subjects has been reported previously.[112, 333]

All analyses were performed using Statistica$^®$ software (StatSoft Inc.; Version 9, USA). Prior to any analysis, all data was tested for normal distribution and thus a suitable test was adopted. Differences in mean values for each of the measured variables were compared by independent samples t-test (or Wilcoxon) for continuous variables. A multivariate analysis was performed to test the influence of age, gender, BP, and circulating markers on the measured variables. Comparison of retinal vessel reactivity for each flicker period was made by repeated measures (or Friedman) analysis of variance (ANOVA) following within-group analysis. Differences between groups in retinal and systemic vascular function were computed by analysis of covariance (ANCOVA). Univariate linear regression analysis was carried out using appropriate correlation analysis. A p value of <0.05 was considered statistically significant, unless stricter criteria were adopted for within-group and multiple comparisons (p≤0.01 to account for multiple comparisons and thereby minimise bias towards Type II errors).

3.5. Results

A total of 120 subjects were screened for eligibility. Following OGTT, 10 subjects were excluded (1 diagnosed with T2DM and 9 with IGT) and the remaining 55 healthy FH subjects (30 men and 25 women) and 55 healthy controls (30 men and 25 women) were recruited (in excess to the calculated sample calculation) for the final protocol (Appendix 1).

The baseline characteristics of both groups are presented in Table 3.1. There were no statistically significant differences in age, systemic BP, IOP, TG, LDL cholesterol, carotid-IMT and Framingham risk scores (p>0.05). Compared to healthy normo -glycaemic and –tensive controls, those with a positive FH of T2DM had higher, albeit normal fasting glucose levels, lower HDL and only slightly higher total cholesterol values (p=0.035, p=0.013 and p=0.004 respectively).

Furthermore, the body composition variables as outlined in Table 3.2. were comparable amongst both groups (p>0.05). This was also the case for plasma markers for oxidative stress and endothelial function (p>0.05) in Table 3.3.

Both groups also exhibited similar twenty-four hour BP and HRV profiles showing non-statistically significant differences in circadian rhythm or ANS function (p>0.05).

Additionally, no such differences (anthropometric, biochemical or vascular reactivity) were detected when comparing normoglycaemic offspring with one parent or both (n=15) parents with T2DM (p>0.05). This is also extended to SA individuals with and without (n=35) a FH of T2DM (p>0.05).

	Control [n=55]	FH [n=55]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	43.9 ± 12	43.8 ± 11	0.670
SBP (mmHg)	117 ± 11	120 ± 11	0.178
DBP (mmHg)	71 ± 8	72 ± 8	0.469
MAP (mmHg)	86 ± 8	88 ± 9	0.303
IOP (mmHg)	13 ± 3	14 ± 3	0.077
METABOLIC DATA			
Glucose (mmol/L)	5.37 ± 0.37	5.60 ± 0.55*	0.036
2 hour GTT (mmol/L)	5.95 ± 1.13	6.16 ± 0.94	0.368
TG (mmol/L)	1.23 ± 0.50	1.16 ± 0.53	0.483
HDL Cholesterol (mmol/L)	1.29 ± 0.34	1.11 ± 0.41*	0.013
LDL Cholesterol (mmol/L)	2.77 ± 0.70	2.76 ± 0.83	0.095
Total Cholesterol (mmol/L)	4.16 ± 0.84	4.61 ± 0.74*	**0.004**
TG:HDL-C (mmol/L)	2.45 ± 1.52	2.76 ± 1.74	0.336
CVD RISK DATA			
R-IMT (mm)	0.060 ± 0.018	0.054 ± 0.013	0.171
L-IMT (mm)	0.060 ± 0.019	0.057 ± 0.012	0.365
Framingham Score (%)	2.3 ± 3.3	1.7 ± 2.1	0.067

Table 3.1. Baseline data of both groups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

	Control [n=55]	FH [n=55]	T-test p-value
Weight (kg)	74.6 ± 14.6	78.0 ± 14.7	0.193
BMI (kg/m^2)	25.7 ± 4.9	26.8 ± 3.8	0.143
WHR (AU)	0.95 ± 0.13	0.92 ± 0.06	0.205
PBF (%)	30.5 ± 8.8	30.3 ± 7.7	0.845
Fat Mass (kg)	23.0 ± 8.8	23.7 ± 7.9	0.639
Fat Free Mass (kg)	51.6 ± 11.2	54.3 ± 11.2	0.169

Table 3.2. Obesity correlates and body composition (by means of bioelectrical impedance) of both groups. Values quoted in mean ± SD. BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat.

	Control [n=55]	FH [n=55]	Wilcoxon p-value
BIOCHEMICAL DATA			
GSH (µM)	878.1 ± 563.5	987.4 ± 755.9	0.366
GSSG (µM)	105.4 ± 96.5	90.9 ± 86.6	0.379
GSH:GSSG (µM)	14.3 ± 11.4	16.7 ± 13.2	0.292
tGSH (µM)	1088.9 ± 576.2	1169.3 ± 783.5	0.519
vWF (µ/dL)	120.1 ± 56.8	128.7 ± 61.1	0.453

Table 3.3. Plasma markers for oxidative stress and endothelial function in both groups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. GSH: reduced Glutathione; GSSG: oxidised glutathione; tGSH: total glutathione; vWF: von willebrand factor.

The vascular reactivity values for brachial artery and retinal vessels are presented in tables 3.4. and 3.5. respectively. Both FH individuals and controls showed comparable values in response to brachial artery hyperaemia and retinal vessel flicker light (p>0.05).

The characteristics for male and female individuals with a positive FH of T2DM were also investigated and shown in Table 3.6. Male subjects showed significant levels of dyslipidaemia (higher TG, lower HDL causing higher TG:HDL-C ratio) and poorer glucose regulation (greater fasting and 2-hour GTT glucose values) when compared to female subjects. Furthermore, the BP profiles were elevated in FH males (p<0.001), contributing to a greater overall Framingham score (p<0.001). The obesity correlates in Table 3.7 show that female FH subjects had higher fat free mass values that contributed to a greater percentage body fat despite weighing significantly less than their male counterparts. No significant differences were found in oxidative stress or endothelial function markers between both groups (p>0.05). Retinal and brachial vessel responses were also comparable between both genders (p>0.05).

	Control [n=55]	FH [n=55]	Wilcoxon p-value
BRACHIAL ARTERY			
AD (mm)	4.00 (3.37-4.59)	4.14 (3.41-4.66)	0.305
BDF (mm)	0.39 (0.22-0.45)	0.33 (0.17-0.42)	0.207
MD (mm)	4.44 (3.84-4.98)	4.50 (3.69-5.09)	0.741
RT (secs)	23.0 (1-53)	21.6 (3.0-38.0)	0.814
FMD (%)	7.55 (2.73-11.3)	6.61 (3.7-9.51)	0.400
GTN			
GTN-MD (mm)	5.04 (4.42-5.64)	5.12 (4.37-5.60)	0.723
RT (secs)	303 (257-395)	315 (267-350)	0.618
GID (%)	22.66 (16.58-29.43)	23.36 (15.10-30.73)	0.816
FMD/GID (%)	0.16 (0.09-0.52)	0.28 (0.07-0.52)	0.542

Table 3.4. Brachial artery reactivity as means of systemic vascular endothelial function between both groups. Values quoted in mean (IQR). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.

	Control [n=55]	FH [n=55]	Friedman p-value
ARTERY			
AD (AU)	123.29 (90.50-161.04)	122.53 (87.70-170.88)	0.789
BDF (AU)	5.11 (0.97-1.55)	5.56 (1.77-17.49)	0.352
MD (%)	4.90 (1.01-13.54)	5.38 (0.19-12.92)	0.309
MDRT (secs)	19.9 (9.0-37.0)	18.1 (6.7-37.5)	0.117
MC (%)	2.80 (3.53-6.92)	3.55 (0.68-13.33)	0.054
MCRT (secs)	20.8 (7.7-29.0)	19.5 (7.0-29.3)	0.117
DA (%)	7.68 (1.67-16.29)	8.93 (2.10-20.58)	**0.041**
bFR (%)	2.57 (-3.35-8.36)	3.36 (-5.57-14.15)	0.084
VEIN			
AD (AU)	155.13 (111.74-132.50)	158.19 (112.82-133.77)	0.462
BDF (AU)	4.15 (3.33-6.79)	4.16 (3.78-7.09)	0.970
MD (%)	5.43 (3.49-5.94)	6.14 (2.95-7.19)	0.133
MDRT (secs)	20.3 (14.7-24.5)	20.0 (13.3-22.3)	0.766
MC (%)	1.80 (4.13-4.43)	1.37 (2.10-4.49)	0.181
MCRT (secs)	21.7 (18.3-23.7)	21.3 (17.0-23.0)	0.679
DA (%)	7.23 (6.02-9.19)	7.51 (5.92-11.57)	0.615
bFR (%)	3.13 (0.83-4.38)	3.35 (1.72-4.75)	0.611

Table 3.5. Retinal arterial and venous measures for both groups. Values quoted in mean (IQR). *Significant values in bold ($p < 0.05$). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum dilation; MDRT: reaction time to reach maximum diameter to flicker stimulation; MC: maximum constriction; MCRT: reaction time to maximum constriction post flicker; DA: dilation amplitude; bFR: baseline-corrected flicker response.

	FH Male [n=30]	FH Female [n=25]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	45.0 ± 10	41.9 ± 13.2	0.309
SBP (mmHg)	123 ± 11*	114 ± 8	**<0.001**
DBP (mmHg)	75 ± 8*	67 ± 6	**<0.001**
MAP (mmHg)	91 ± 8*	83 ± 6	**<0.001**
IOP (mmHg)	14 ± 3	13 ± 3	0.110
METABOLIC DATA			
Glucose (mmol/L)	5.15 ± 0.61*	4.64 ± 0.78	**0.003**
2 hour GTT (mmol/L)	7.07 ± 1.69*	5.95 ± 1.31	**0.014**
TG (mmol/L)	1.52 ± 0.79*	1.12 ± 0.40	**0.009**
HDL Cholesterol (mmol/L)	1.15 ± 0.40*	1.37 ± 0.30	**0.010**
LDL Cholesterol (mmol/L)	2.81 ± 0.68	2.72 ± 0.81	0.644
Total Cholesterol (mmol/L)	4.64 ± 0.79	4.61 ± 0.83	0.853
TG:HDL-C (mmol/L)	3.63 ± 3.23*	2.00 ± 1.00	**0.006**
CVD RISK DATA			
R-IMT (mm)	0.063 ± 0.019	0.054 ± 0.014	0.177
L-IMT (mm)	0.062 ± 0.020	0.058 ± 0.016	0.570
Framingham Score (%)	4.6 ± 5.1*	0.8 ± 0.5	**<0.001**

Table 3.6. Baseline data of positive FH groups of male and female gender. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

	FH Male [n=30]	FH Female [n=25]	T-test p-value
BMI (kg/m^2)	26.2 ± 5.2	25.6 ± 5.1	0.634
WHR (AU)	0.96 ± 0.16	0.93 ± 0.09	0.291
PBF (%)	25.9 ± 7.0	35.8 ± 8.4*	**<0.001**
Fat Mass (kg)	21.6 ± 8.8	24.9 ± 9.2	0.137
Fat Free Mass (kg)	59.8 ± 8.4	43.1 ± 6.3*	**<0.001**

Table 3.7. Obesity correlates and body composition (by means of bioelectrical impedance) of positive FH groups of male and female gender. Values quoted in mean ± SD. *Significant differences indicated in bold as p<0.05.BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat.

Those with a FH of T2DM showed levels of impaired lipid function by means of raised total cholesterol and lower HDL-C values. Furthermore the levels of total cholesterol and LDL-C correlated negatively with selected retinal arterial dilation values as shown in Figure 3.1.

FH individuals exhibited significantly greater DA values and fasted plasma cholesterol levels that both correlated negatively with each other in Figure 3.2. (spearmans r= -0.9374; p= 0.019). This also extended to negative correlations between DA and LDL-C (r= -0.9718; p= 0.006) and also bFR with total cholesterol (r= -0.8801; p= 0.049).
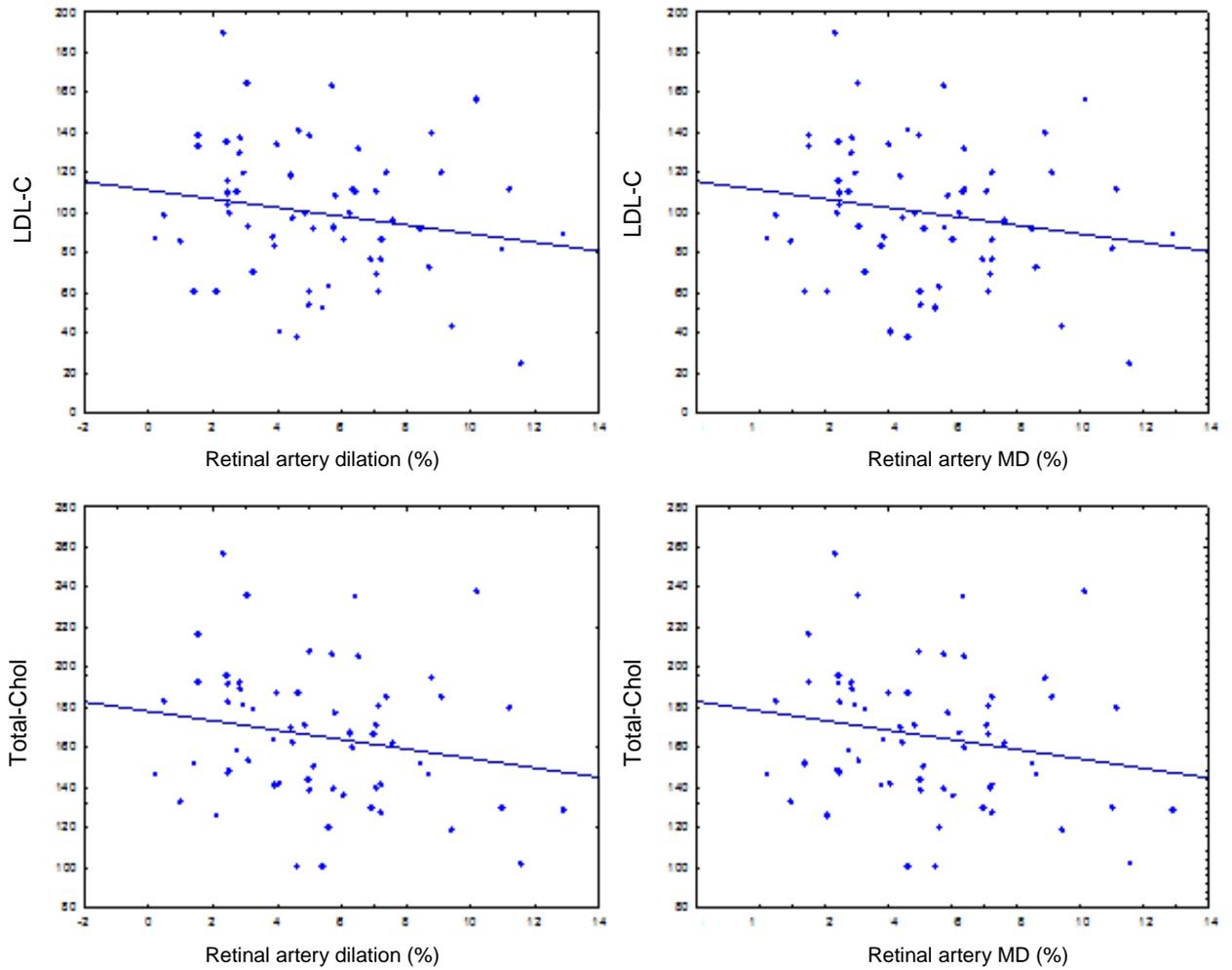
Figure 3.1. Cholesterol and Retinal arterial dilation correlations in those with a positive FH. From top-left to clockwise. Negative Spearmans correlations for arterial dilation against LDL-C (r= -0.9824; p= 0.003), arterial MD against LDL-C (r= -0.9826; p=0.003), arterial dilation against total cholesterol (r= -0.9874 ;p= 0.002) and arterial MD against total cholesterol (r= -0.9845; p= 0.002).
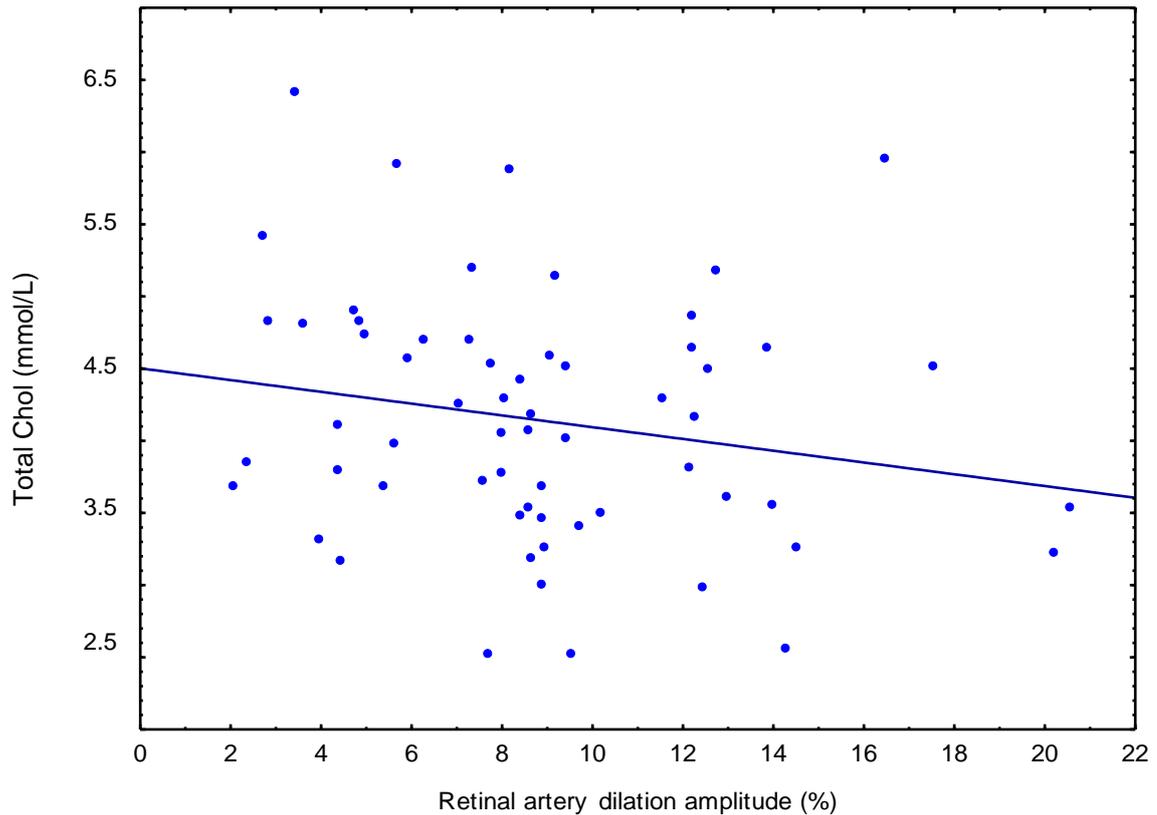
Figure 3.2. Cholesterol and Retinal arterial dilation amplitude correlation in those with a positive FH (r= -0.9374; p= 0.019).

3.6. Discussion

In this present study, it has been shown that those with a known FH of T2DM have no detectable differences in endothelial and vascular function at the micro- and macro-vascular level. Therefore, it could be suggested that in young FH individuals there are no clinical signs of impaired vascular function or endothelial cell damage at the retinal and systemic level. Furthermore, akin to that of the findings reported in this study, several other authors have also shown comparable levels in vWF as well as other inflammatory markers (s-ICAM, s-VCAM and C-reactive protein) in young FH individuals.[334-336] These results are further supported by comparable findings between individuals with one or both parents and SAs with and without a FH of T2DM. Therefore, this suggests that even within a SA sample; a minority with well-documented CVD and T2DM risk, FH may not be an important vascular risk factor when considering otherwise healthy individuals.

The importance of detecting when the first signs of vascular dysfunction occur in at-risk individuals including FH subjects is well established. Other studies have reported peripheral

microvascular changes, such as reduced skin hyperaemia in subjects with impaired fasting glucose (IFG), possibly due to an IR [337], showing that microvascular abnormalities are present and detectable in individuals at risk for diabetes.[338] Furthermore, several vascular abnormalities, including increased IR; beta-cell dysfunction, obesity, ET-1, IMT and reduced FMD, aortic distensibility and sensitivity to activated protein C, have been reported in normoglycaemic FH individuals.[37, 234, 238, 339-344] However, whether this is due to a genetic determinant or possibly due to the presence of IR is not fully understood. The results of this chapter would suggest that in healthy offspring of diabetics, FH may be a different determinant of future vascular risk when compared to genetic and lifestyle factors.

Furthermore, the prevalence, incidence and mortality rates of T2DM are known to increase with age; therefore, in this regard FH of T2DM may have an impact on age of diagnosis, onset and duration of the disease. Some have reported that those with a FH are diagnosed with T2DM at earlier ages, on average around forty-three years of age.[40] Although the sample presented in this study are of similar age, the presence of other well-established vascular risk factors, namely MetS components, were absent; thus going some way to explain the lack of detectable differences in FMD and retinal reactivity. The findings of greater prevalences of arterial stiffness with IR also goes some way in showing that in an otherwise healthy cohort of FH individuals with metabolic alterations, no such vascular disturbances would be expected. Furthermore, there are results supporting positive associations between insulin action and endothelial-dependent vasodilation in young FH individuals.[335]

However it is important to note that vascular inflammation is not necessarily associated with early IR in FH individuals. Despite studies describing T2DM as an inflammatory condition, by means of increased C-reactive protein, white blood cell count and fibrinogen levels, there are some results showing that the IR state per se is not characterised by inflammation.[36] Therefore, if vascular inflammation and endothelial dysfunction are thought to be simultaneous events for T2DM pathophysiology and risk, it could be suggested that that the FH individuals in this cohort were equally insulin sensitive as their healthy counterparts, which led to similar (brachial and retinal) vascular responses.

This can also be said for the current cohort when comparing FMD values. It has been found that when comparing FH individuals with controls, FMD has been found to be similar. However it is only when sedentary and IR (and reduced insulin sensitivity) individuals with a FH are compared to controls and diabetics that endothelial dysfunction, by means of reduced

FMD and increased levels of inflammatory markers are reported.[231, 233] This is also true of systemic BP, obesity correlates and metabolic plasma markers.[37, 39, 238]

As compared to FMD, retinal vascular function (as a surrogate measure of systemic and ocular endothelial function) can be examined quickly and non-invasively and, therefore, can become an important investigative tool in everyday clinical practice for early detection of vascular risk and complications in subjects at risk. In addition to macrovascular dysfunction, hyperglycaemia can also result in a high level of oxidative stress, inflammation and vascular dysfunction that occur at the microvascular level, such as the retinal vascular bed.[345] For example, it has already been demonstrated that in patients suffering from T2DM there is a measurable reduction in retinal vascular response to flicker, possibly suggesting local endothelial dysfunction that exists in addition or as a consequence to systemic vascular changes.[214, 295, 300]   However, it is possible that the retinal vessel analysis performed in this study could not identify any changes that would otherwise help to suggest a reduced vascular function in those with a FH of T2DM. Nevertheless, the microcirculation still represents the first vascular area to be affected by pre-clinical signs of endothelial dysfunction.[346, 347] This is supported by findings showing increased carotid-radial pulse-wave analysis and hence muscular-artery stiffness in FH individuals known to be predisposed to IR prior to the development of the MetS.[334]

The presence of a functional correlation between brachial (FMD) and retinal arterial function (arterial MD) has also been described previously in healthy participants.[144] However, no such associations, (concordant to that also reported in patients with T2DM and hypercholesteraemia) were found in the FH group presented in this study (r=0.3302, p>0.05). Whether the weak correlation between these two separate vascular beds provides evidence that similar functional vascular changes occur at both a macro- and microvascular circulatory level in T2DM and possibly in those with a FH, remains to be seen.[300] Further work to investigate correlations between these two distinctly different vascular beds would help to see whether a 'vascular breakdown' corresponds to the high risk reported for developing cardiovascular complications before overt diabetes occurs in FH individuals.[38, 244, 348]

The negative correlations with several retinal arterial dilation responses and fasting plasma cholesterol in the current FH group, however, is consistent to that previously reported.[112] This has been suggested to further the argument that a reduced flicker response is endothelial cell-mediated as cholesterol and LDL-C are known to be associated with reduced NO bioavailability.[349] Furthermore, statin therapy used to reduce cholesterol levels have been shown to improve endothelial function by means of increased FMD.[350] Therefore,

understanding the complex relationship between vascular dysfunctions at multiple levels and when they occur, as well as their interaction with other various metabolic and genetic factors that play a role in the onset of T2DM, could result in early interventions for prevention of vascular disease in subjects at risk for this disease.

When compared to other FH studies the sample size adopted for this study appears sufficient. Furthermore, the male-to-female ratio adopted is more balanced in order to account for gender differences (despite no detectable differences found in reactivity between both gender groups).[38] However, post-hoc power calculation analysis suggests that n=94 would be sufficient to detect a difference between normoglycaemic, normotensive and otherwise healthy FH individuals and controls. This could be as a result of using a healthy cohort as opposed to individuals with known vascular disturbances or disease. This follows that of large-scale prospective studies and would therefore allow a more robust study design to be incorporated.

Despite findings inconsistent to the proposed hypothesis and to that of some studies, the preliminary results show that in healthy FH individuals without metabolic alterations, obvious signs of vascular function impairment are absent. Therefore, the vascular risk noted in those with FH of T2DM may need to be revised when considering otherwise healthy individuals. However, there is opportunity for further work in IR FH individuals. The concept behind the necessity for early screening for diabetes in FH risk groups and more active prevention in CVD risk may not be clinically valid or necessary, but can only be validated with larger study samples.

4. Vascular Function in Normoglycaemic South-Asians vs. Normoglycaemic White-Europeans without FH and Vascular Disturbances

4.1. Abstract

4.1.1. Background/Aims: To determine whether differences in retinal and brachial vascular function and their relationship to traditional vascular disease risk and endothelial dysfunction indicators exist in SAs (Indian, Sri-Lankan, Bangladeshi and Pakistani) when compared to WEs.

4.1.2. Methods: A total of 130 normoglycaemic subjects (65 SA and 65 age- and gender-matched WE) were recruited for the present study. Retinal vessel reactivity was assessed using the Dynamic Retinal Vessel Analyser (DRVA) according to a modified protocol. Brachial artery reactivity, by means of flow-mediated dilation (FMD) was assessed by CDI according to an established procedure. Fasting plasma glucose, triglycerides (TG), total, LDL and HDL cholesterol were also measured in all individuals.

4.1.3. Results: SA individuals showed higher fasting triglyceride (p=0.001) and lower HDL levels (p=0.007), leading to a higher TG:HDL-C ratio (p=0.001) than age-matched WE subjects. Additionally, SAs presented with higher levels of vWF and lower levels of GSH (p=0.036) and GSSG (p=0.013) than in WEs. Furthermore, SAs showed reduced FMD values (p=0.012), and despite comparable retinal vessel dilation to flicker-light (p>0.05), differences in arterial constriction profiles were significant.

4.1.4. Conclusion: In otherwise healthy SAs there are potential signs of biochemical and metabolic imbalances that could result in future endothelial cell damage/dysfunction and a permanent state of oxidative stress. Further SDRA analysis in SAs suggests a complex interaction with autoregulatory and metabolic mechanisms that cause retinal vascular "over-compensation".

4.2. Introduction

The SA population (Indian, Pakistani, Bangladeshi and Sri-Lankan) represents approximately 20% of the world's populace and is one of the fastest growing populations. There are over two million South-Asians in the USA, over one million in Canada and over six million in the UK. It is well documented that when compared to WEs, migrant SAs are at an increased risk of CVD and vascular disease due to a higher prevalence of established vascular risk factors causing higher rates of mortality than in any other minority.[351] Furthermore, SAs appear to

present with CVD at a much younger age when compared to WEs. Despite this, there are very few studies that determine vascular risk for CVD and diseases such as T2DM and stroke in this minority.

Findings from studies in urban populations also further extend known CVD risk in SAs, where average cholesterol readings have increased from 4.1 to 5.5mmol/L leading to an accompanied three-fold increase in the prevalence of CVD. It has therefore been shown that SAs present with a specific risk profile that represents that of the MetS ultimately causing a state of IR.[352]

Various factors such as abnormal levels of plasma glucose, fasting insulin, TG, C-reactive protein and glycosylated haemoglobin (HbA1c), as well as higher central adiposity have been implicated in the increased risk for both IR and T2DM in this population.[27] Although the exact mechanism behind a higher risk for vascular disease in SA populations is still unclear, IR and its consequences on the vascular health could explain it, at least in part.[17] Moreover, signs of possible risk for future vascular disease including T2DM, such as subclinical inflammation and endothelial dysfunction are present from early childhood in SAs.[353, 354] Consequently, early detection of risk for vascular disease could allow earlier primary preventive measures that might reduce the occurrence and/or slow progression of disease in populations at risk. Indeed, it has been proposed that more aggressive preventive measure and treatments should be applied to SA populations to prevent CVD.[355]

The traditional approach to CVD risk assessment is based on identifying and quantifying few established cardiovascular risk factors, such as HDL cholesterol, high blood pressure, and smoking status. As a result, various scores have been generated. Among those, the Framingham Risk Assessment Model is one of the most used and accepted in cardiovascular medicine.[356, 357] Nevertheless, this score is based on data collected in a White US population and has limited use in other ethnic groups. In addition, the factors included in the Framingham score fail to explain up to 50% of cardiovascular morbidity and mortality.[358] Therefore, more accurate measures that are tailored to various populations should be used. In addition, when looking at risk stratification, pre-clinical modifications in vascular function are a better option; they are subtler, precede structural changes and signal danger at an earlier stage.[359] This approach would allow more effective, evidence-based prevention tailored to apparently healthy individuals and groups but with a higher risk factor.

The vascular endothelium plays an important role in vascular homeostasis by preserving the vascular tone and preventing inflammation, platelet aggregation and leukocytes adhesion. Under certain conditions, these functions are affected and the so-called endothelial dysfunction occurs. It has been demonstrated that endothelial dysfunction contributes to IR,

hypertension, dyslipidaemia, proteinuria and atherosclerosis, most of which are associated either with the onset of T2DM or its complications. Moreover, it has been proved that in addition to other biochemical or clinical markers, early signs of endothelial dysfunction are also present in healthy individuals with various risk factors for T2DM, including SA ethnicity.[37, 234, 238, 360] Therefore, the assessment of endothelial function could provide a better indicator of individual risk for vascular disease.

As already mentioned, the SA population is at a higher risk of developing T2DM and premature CVD.[361] Moreover, diabetes-related morbidity and mortality, as well as the incidence of clinically significant DR are higher amongst this ethnic group than in an age-matched WE population.[362, 363] Therefore, early identification of pre-clinical markers for vascular disease could be very important in this population and, possibly contribute to an early implementation of prevention and/or disease-modifying interventions.[364] Retinal vessel analysis may provide a means for identifying pre-clinical risk for CVD and vascular disease, as shown by increased MDRT in SAs (Appendix 3).

The purpose of this study was to determine whether healthy (first- and second-generation) migrant SAs present with a similar risk profile and evidence of impaired endothelial/vascular function that has been shown previously in the older South-Asian generation when compared to age- and sex-matched WEs. Furthermore, differences in vascular function with gender sub-groups in and between SA and WE samples were also investigated.

4.3. Materials and Methods

Healthy subjects aged 25-55 years were recruited by advertisement and screened by questionnaire to exclude those with a FH of diabetes or existing vascular conditions. SA ethnicity (India, Pakistani, Sri Lankan, and Bangladeshi) was defined by a self-reported questionnaire.

Exclusion criteria were: a positive family history for DM, a positive diagnosis of cardio- or cerebro-vascular disease, (coronary artery disease, heart failure, arrhythmia, stroke, transient ischaemic attacks), peripheral vascular disease, severe dyslipidaemia (defined as plasma TG>6.00mmol/L or cholesterol levels>7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment.

Furthermore, subjects were excluded if they had a refractive error of more than ±3DS and more than ±1DC equivalent, IOP >24 mmHg, cataract or any other media opacities, as well as if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system.

Written informed consent was obtained from all participants and ethical approval was sought by the author from local and NHS ethical committees (08/H1202/112). The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki.

An OGTT was performed according to the WHO protocols on all participants a week prior to all other measurements (Section 3.3.) and only participants with normal results were asked to come back for the subsequent tests.

According to an already established procedure when examining endothelial function, female participants were asked to fill in a validated menstrual cycle questionnaire and their investigations were carried out during the first week of the menstrual cycle (follicular phase), thereby controlling for hormonal influences on endothelial function.[112, 317]

4.3.1. Investigations
4.3.1.1. Blood sampling and analyses

All participants were asked to fast and refrain from caffeine, alcohol, chocolate and carbonated drinks and to not exercise for 12 hours prior to the date of the study. All blood samples were obtained by a qualified phlebotomist in the morning, between 9am and 10am. Fasting plasma glucose, TG, total and HDL-C were measured using standard routine laboratory techniques (outlined in Section 3.3.1.1.) using the Reflotron Desktop Analyser (Roche Diagnostics, UK). The TG/HDL cholesterol ratio,[325], a measure of endothelial function and Total/HDL cholesterol ratio alongside Framingham score as a means of cardiovascular risk (Section 2.3.1.1.) were also determined from the above values.[365]

Further analysis extended to laboratory and ELISA-test sampling for fasting plasma vWF and blood gluthathione to investigate possible signs for endothelial damage and oxidative stress respectively. Validated protocols were used for in-house testing as described in Sections 2.4. and 2.5.

4.3.1.2. ABPM and HRV analysis

Systemic BP was measured using a 24-hour computer-operated ambulatory BP and electrocardiography (ECG monitor (Cardiotens-01, Meditech Ltd., Hungary) for each subject. Measurements were performed in ambulatory conditions and programmed to measure BP oscillometrically every 15 minutes during the subject's active period and every 30 minutes in the passive period (explained previously in Section 2.6.). Mean arterial pressure (MAP), as a means of describing cardiac output function in relation to arteriolar resistance was calculated

according to Equation 2.2. The impact of pulse pressure (PP) as a risk factor for CVD was also calculated as the below equation:

$$PP = SBP - DBP$$

Equation 4.1. Calculation of Pulse Pressure

HRV analysis was calculated for each individual using the Cardiovision software (PMS Instruments Ltd., Maidenhead, UK) to obtain twenty-four hour, active and passive period LF, HF and LF:HF values. From, this circadian BP and HRV changes were calculated according to Formula 2.3. and 2.4. respectively.

4.3.1.3. Body Composition Analysis

Anthropometric measures including height and weight were measured using standard procedures as explained in section 3.3.1.3. Body composition was measured using bioelectrical impedance (Biostat 220, Biospace, UK) to determine BMI, PBF, WHR, total fat and fat free mass.

4.3.1.4. Intima-media thickness

High-resolution B-mode ultrasound system (Acuson Sequoia, 5 MHz linear transducer, Siemens, USA) was used to obtain longitudinal images of the right and left extracranial far wall of the CCA as described in section 3.3.1.4. Each IMT measure, also used for statistical analysis, represented an average of three to five measurement points.

4.3.1.5. Vascular function studies

Retinal vessel reactivity was measured using the DVRA (Imedos; GmbH, Jena, Germany). All measurements were performed in one unselected eye for each subject, between 8:00 and 11:00 AM in a quiet, temperature-controlled room (22˚C). Following full pupil dilation with Tropicamide 1% (Minims; Chauvin Pharmaceuticals Ltd, UK) a region of interest encompassing vessel segments of approximately 500 µm was chosen. Retinal diameters were assessed continuously over 350 seconds according to an accepted and widely used protocol.[277, 332]

Retinal vessel reactivity and time course parameters, as explained in Section 2.1., were then calculated. Previous work investigating retinal arterial MD and MDRT in SAs has been reported previously (Appendix 3). Therefore to further the application of SDRA in this ethnic minority, constriction analyses was performed as shown in Section 2.1.

The effect of atherosclerosis on vessel stiffness is well known, and therefore the clinical significance of assessing changes in retinal reactivity is of importance. Depending on a vessel's "stiffness", the reaction to flicker can be slow or fast.[118] Thus, the rate at which retinal vessel diameter increases to stress seems an ideal approach. This can be ascertained by calculating the dilation slope for diameter against time. Furthermore, studies have documented the autoregulation response of retinal vessels to stress [65], and so to advance the understanding on the mechanisms of retinal flicker response, the slope following flicker cessation was also determined. This was named constriction slope.(Section 2.1.) The constriction response is known to occur approximately 10-40 seconds post flicker,[333] but the time-course and reasons for the constriction still remain unclear. Therefore, to gain further insight, the maximum constriction diameter was calculated within a period of 30 seconds after flicker and the slope calculated against the maximum dilation to flicker.

Brachial artery FMD was measured using high-resolution CDI ultrasonography, with a 7 mm 8MHz linear-array (Siemens; Acuson Sequoia, UK). According to a published protocol,[308], as described in section 2.2., arterial occlusion was created by inflation of a sphygmomanometer cuff on the lower arm to suprasystolic pressure and an exogenous NO donor (sublingual 0.3 mg GTN tablet) was given to determine the maximum obtainable vasodilator response. FMD was then expressed as the maximal artery dilation during hyperaemia from baseline (%).

4.4. Power calculation and statistical analysis

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessel reactivity was shown to be significant.[214] The reproducibility for repeated measures using the RVA has also been investigated and validated for East-Asians.[366] Additionally FMD studies have shown typical values of around 6.9% with a SD of 0.3% in FMD response in SAs.[26] As the study design was multi-factorial in nature it was calculated (using SISA; a web-based sample calculator approved by the NHS ethics committee) that n=30 per group was sufficient to provide 90% power with an alpha of 0.05. Furthermore, the sensitivity and reproducibility of the techniques in healthy subjects has been reported previously.[112, 333]

All analyses were performed using Statistica$^®$ software (StatSoft Inc.; Version 9, USA). Prior to any analysis, all data was tested for normal distribution and thus a suitable test was adopted. Differences in mean values for each of the measured variables were compared by independent samples t-test (or Wilcoxon) for continuous variables. A multivariate analysis was performed to test the influence of age, gender, BP, and circulating markers on the measured variables. Comparison of retinal vessel reactivity for each flicker period was made by repeated measures (or Friedman) analysis of variance (ANOVA) following within-group analysis. Differences between groups in retinal and systemic vascular function were computed by analysis of covariance (ANCOVA). Univariate linear regression analysis was carried out using appropriate correlation analysis. A p value of <0.05 was considered statistically significant, unless stricter criteria were adopted for within-group and multiple comparisons (p≤0.01 to account for multiple comparisons and thereby minimise bias towards Type II errors).

4.5. Results

A total of 155 subjects were screened for eligibility. Following OGTT, 25 subjects were excluded (3 diagnosed with T2DM and 22 with IGT) and the remaining 65 healthy SAs subjects (33 men and 32 women) and 65 healthy WE (33 men and 32 women) were recruited in excess of the intended sample size for the final protocol (Appendix 1).

The baseline characteristics of both groups are presented in Table 4.1. There were no statistically significant differences in age, SBP, MAP and PP, IOP, fasting plasma glucose, LDL and total cholesterol and carotid-IMT. Compared to WE, SAs had raised average DBP values (p=0.019), 2-hour glucose tolerance test and TG values (p<0.001), and lower HDL levels (p=0.040). This led to the TG:HDL cholesterol ratio (TG:HDL-C) also being significantly higher in SAs than in WEs (p<0.001) as well as a higher Total:HDL cholesterol (Total:HDL-C) ratio (p=0.001). No such differences were found in HRV components, suggesting similar ANS outputs (p>0.05).

Traditional Framingham risk scores as shown in Table 4.2, were similar between both groups (p>0.05), however, when modifying the equations for ethnicity, i.e. adding 10 years to the inputted age [296]; SAs presented with a higher CVD percentage risk than WEs (p=0.021). This was evident despite any differences in weight, body mass index (BMI), waist-to-hip ratio (WHR), percentage body fat (PBF), fat mass and fat free mass indices (p>0.05). Table 4.3.

When investigating plasma markers of oxidative stress, SAs presented with statistically significant levels of reduced GSH, GSSG, tGSH and GSH:GSSG (p=0.019, p=0.016,

p=0.013 and p=0.014 respectively). Whilst this minority also showed markedly higher levels of von-Willebrand factor (vWF) when compared to WEs, it did fail to reach statistical significance. Table 4.4.

The FMD results for SAs against WEs are presented in Table 4.5./Figure 4.1. and show reduced maximal obtainable brachial artery dilation and thus FMD(%). This did not extend to any obvious differences in retinal arterial MD (Table 4.6.). There were significant differences however; in BDF, arterial constriction post flicker light cessation (MC) and time taken to reach maximal constriction; MCRT (p=0.002, p=0.041 and p=0.001 respectively). Further slope analysis was performed and presented in tables 4.7. and 4.8. showing increased arterial constriction slopes in SAs. This also applied to venous reactions, whereby SAs showed increased venous BDF, but also increased venous MD and DA values. Within-group repeated measures analysis was performed on these variables and are presented in tables 4.9. – 4.13 showing no statistically significant differences in SDRA varaibles. MDRT results (Table 4.14.) are shown as those reported in a previous publication (Appendix 3).

	White European [n=65]	South Asian [n=65]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	39.0 ± 10.6	39.0 ± 10.6	-
SBP (mmHg)	117 ± 10	120 ± 11	0.129
DBP (mmHg)	70 ± 8	73 ± 8*	**0.010**
MAP (mmHg)	86 ± 8	89 ± 9	0.053
PP (mmHg)	43 ± 8	44 ± 11	0.680
IOP (mmHg)	14 ± 3	14 ± 3	0.909
METABOLIC DATA			
Glucose (mmol/L)	4.86 ± 0.75	5.06 ± 0.77	0.121
2 hour GTT (mmol/L)	6.20 ± 1.44	7.77 ± 2.40*	**<0.001**
TG (mmol/L)	1.08 ± 0.36	1.49 ± 0.82*	**<0.001**
HDL Cholesterol (mmol/L)	1.28 ± 1.36	1.07 ± 1.12*	**0.001**
LDL Cholesterol (mmol/L)	2.61 ± 0.85	2.68 ± 0.80	0.590
Total Cholesterol (mmol/L)	4.35 ± 0.88	4.49 ± 0.86	0.345
TG:HDL-C (mmol/L)	2.12 ± 1.03	3.76 ± 3.15*	**<0.001**
CVD RISK DATA			
Total:HDL-C (mmol/L)	3.65 ± 1.05	4.52 ± 1.78*	**0.001**
R-IMT (mm)	0.056 ± 0.016	0.059 ± 0.016	0.435
L-IMT (mm)	0.055 ± 0.018	0.063 ± 0.017	0.050

Table 4.1. Baseline data of both groups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; PP: pulse pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; Total:HDL-C: Total-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

	White European [n=65]	South Asian [n=65]	T-test p-value
CVD RISK DATA			
Framingham Score (%)	2.3 ± 2.7	2.5 ± 4.4	0.787
M1 Framingham (%)	2.4 ± 2.8	3.8 ± 6.6	0.138
M2 Framingham (%)	2.5 ± 3.1	5.2 ± 8.6*	**0.021**

Table 4.2. Framingham (and modified) risk scores for both ethnic groups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. M1 Framingham: Modified-1 Framingham for ethnicity by adding 50% of original score; M2 Framingham: Modified-2 Framingham for ethnicity by increasing age by 10 years.[367]

	White European [n=65]	South Asian [n=65]	T-test p-value
Weight (kg)	76.9 ± 16.2	76.3 ± 13.7	0.818
BMI (kg/m^2)	26.0 ± 5.0	26.7 ± 4.1	0.365
WHR (AU)	0.92 ± 0.07	0.95 ± 0.12	0.219
PBF (%)	29.5 ± 8.4	30.8 ± 8.4	0.356
Fat Mass (kg)	23.0 ± 9.0	23.6 ± 8.0	0.671
Fat Free Mass (kg)	53.9 ± 11.7	52.7 ± 10.8	0.541

Table 4.3. Obesity correlates and body composition (by means of bioelectrical impedance) of both groups. Values quoted in mean ± SD. BMI: body mass index; WHR: waist-to-rip ratio; PBF: percentage body fat.

	White European [n=65]	South Asian [n=65]	Wilcoxon p-value
BIOCHEMICAL DATA			
GSH (µM)	1015.9 (445.6-1416.0)	710.7 (350.3-974.9)*	**0.019**
GSSG (µM)	96.7 (32.1-107.6)	76.4 (39.3-96.4)*	**0.013**
GSH:GSSG (µM)	17.7 (8.0-22.3)	12.8 (5.1-18.8)*	**0.014**
tGSH (µM)	1168.7 (616.1-1713.2)	904.0 (510.3-1147.5)*	**0.019**
vWF (µ/dL)	121.1 ± 54.0	135.2 ± 58.9	0.154

Table 4.4. Plasma markers for oxidative stress and endothelial function in both groups. Values quoted in IQR or mean ± SD.*Significant differences indicated in bold as p<0.05. GSH: reduced Glutathione; GSSG: oxidised glutathione; tGSH: total glutathione; vWF: von Willebrand factor.

	White European [n=65]	South Asian [n=65]	Wilcoxon p-value
BRACHIAL ARTERY			
AD (mm)	4.04 (3.44-4.62)	4.10 (3.39-4.64)	0.674
BDF (mm)	0.38 (0.17-0.44)	0.34 (0.23-0.44)	0.311
MD (mm)	4.64 (4.01-5.17)	4.25 (3.63-4.85)	**0.018**
RT (secs)	19.8 (0.5-50.5)	24.1 (3.0-38.0)	0.465
FMD (%)	8.23 (3.43-11.13)	5.49 (2.44-8.83)	**0.012**
GTN			
GTN-MD (mm)	5.24 (4.63-5.78)	4.95 (4.31-5.55)	0.154
RT (secs)	285 (257-345)	325 (273-401)	0.084
GID (%)	25.58 (16.21-29.83)	21.50 (13.29-29.43)	0.180
FMD/GID (%)	0.40 (0.13-0.55)	0.07 (0.05-0.46)	0.075

Table 4.5. Brachial artery reactivity as means of systemic vascular endothelial function between both groups. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.
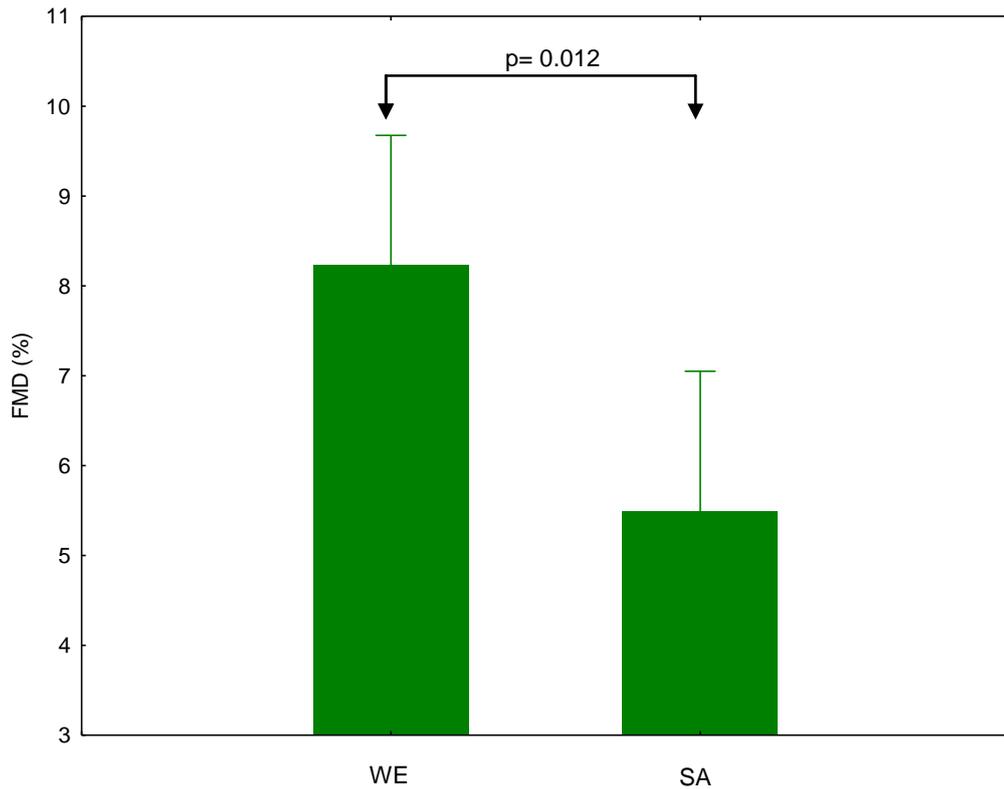
Figure 4.1. Difference in FMD (%) of the brachial artery in response to reactive hyperaemia for both ethnic groups.
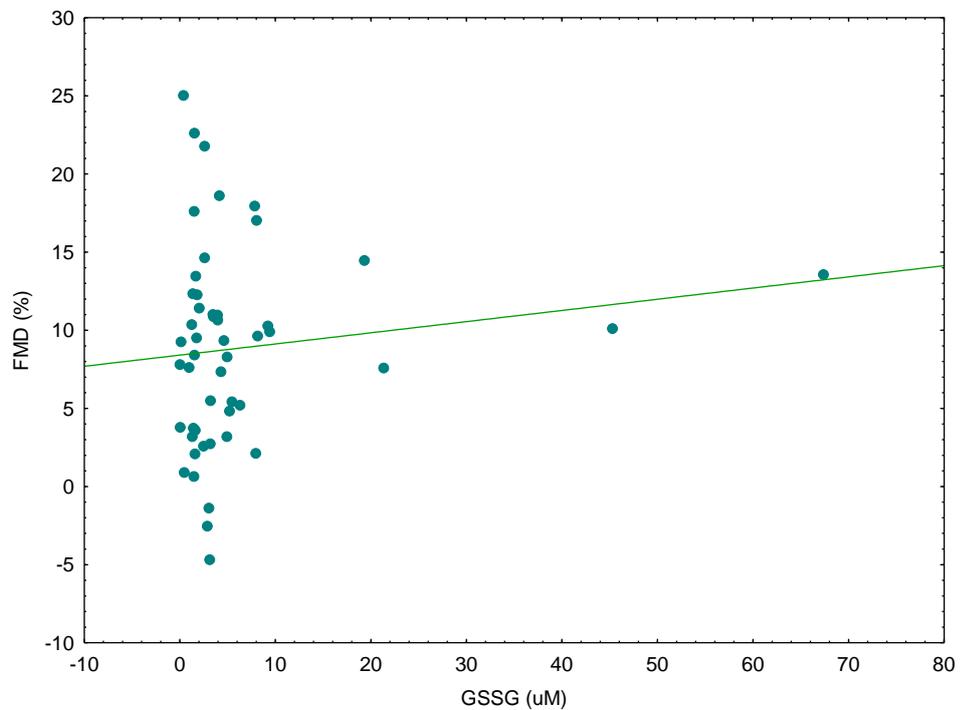
Figure 4.2. Correlation between brachial artery FMD and plasma levels of oxidised-form glutathione in SAs. Spearmans correlations showing a moderate correlation between FMD and GSSG (SAs: r=0.3248, p=0.013; WEs: r=0.287, p=0.048)

	White European [n=65]	South Asian [n=65]	Friedman p-value
ARTERY			
AD (AU)	121.76 (112.72-131.75)	124.71 (87.70-174.28)	0.305
BDF (AU)	4.56 (3.10-5.46)	6.07 (1.57-17.49)	**0.002**
MC (%)	2.73 (1.35-3.40)	3.51 (3.53-10.06)	**0.041**
MCRT (secs)	21.5 (19.0-23.7)	18.8 (7.0-29.3)	**0.001**
DA (%)	7.65 (5.81-8.91)	8.81 (2.10-20.24)	0.057
bFR (%)	3.09 (1.68-4.38)	2.74 (-5.57-9.45)	0.462
VEIN			
AD (AU)	153.98 (141.0-164.34)	158.43 (111.74-134.58)	0.271
BDF (AU)	3.55 (2.32-4.43)	4.65 (3.84-7.64)	**0.002**
MC (%)	1.36 (0.32-1.80)	1.70 (2.01-4.82)	0.259
MCRT (secs)	21.7 (19.5-25.0)	21.5 (15.7-22.3)	0.792
DA (%)	6.57 (4.62-7.52)	7.84 (6.02-10.96)	**0.018**
bFR (%)	3.02 (1.49-4.31)	3.20 (0.85-5.01)	0.692

Table 4.6. Retinal arterial and venous measures for both groups. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MC: maximum constriction; MCRT: reaction time to maximum constriction post flicker; DA: dilation amplitude; bFR: baseline-corrected flicker response.

	White European [n=65]	South Asian [n=65]	Friedman p-value
ARTERY DILATION SLOPE			
Flicker cycle 1	0.34 (0.15-0.4)	0.37 (0.15-0.43)	0.574
Flicker cycle 2	0.29 (0.17-0.42)	0.36 (0.18-0.46)	0.124
Flicker cycle 3	0.31 (0.15-0.46)	0.34 (0.15-0.43)	0.554
Average	0.31 (0.19-0.40)	0.36 (0.22-0.46)	0.142
VEIN DILATION SLOPE			
Flicker cycle 1	0.32 (0.14-0.41)	0.34 (0.20-0.39)	0.653
Flicker cycle 2	0.32 (0.19-0.40)	0.37 (0.21-0.50)	0.475
Flicker cycle 3	0.29 (0.18-0.32)	0.34 (0.18-0.46)	0.160
Average	0.31 (0.21-0.38)	0.37 (0.24-0.47)	**0.029**

Table 4.7. Retinal arterial and venous dilation slope values for each individual flicker cycle. Values quoted in mean (IQR).

	White European [n=65]	South Asian [n=65]	Friedman p-value
ARTERY CONSTRICTION SLOPE			
Flicker cycle 1	-0.38 (-0.19/-0.45)	-0.47 (-0.25/-0.64)	0.089
Flicker cycle 2	-0.46 (-0.23/-0.48)	-0.55 (-0.28/-0.60)	0.280
Flicker cycle 3	-0.33 (-0.19/-0.39)	-0.55 (-0.22/-0.59)	**0.026**
Average	-0.39 (-0.26/-0.45)	-0.52 (-0.28/-0.64)	**0.015**
VEIN CONSTRICTION SLOPE			
Flicker cycle 1	-0.34 (-0.18/-0.44)	-0.55 (-0.20/-0.56)	0.080
Flicker cycle 2	-0.37 (-0.16/-0.56)	-0.50 (-0.23/-0.55)	0.134
Flicker cycle 3	-0.36 (-0.18/-0.41)	-0.43 (-0.22/-0.55)	0.255
Average	-0.36 (-0.21/-0.48)	-0.50 (-0.26/-0.55)	**0.022**

Table 4.8. Retinal arterial and venous constriction slope values for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05).

	White European [n=65]	South Asian [n=65]	Post-hoc p-value
ARTERY BDF (AU)			
Flicker cycle 1	4.48 (2.97-5.26)	6.06 (3.41-8.20)	**0.002**
Flicker cycle 2	4.73 (2.53-5.25)	6.23 (3.36-8.08)	**0.017**
Flicker cycle 3	4.35 (2.77-5.52)	5.79 (3.61-7.33)	**0.006**
Within-group ANOVA	0.194	0.454	
VEIN BDF (AU)			
Flicker cycle 1	3.55 (2.09-4.50)	4.34 (2.61-4.59)	0.083
Flicker cycle 2	3.84 (2.32-4.70)	4.51 (2.42-5.96)	0.173
Flicker cycle 3	3.56 (2.24-4.38)	4.72 (2.97-5.43)	**0.007**
Within-group ANOVA	0.986	0.596	

Table 4.9. Retinal arterial and venous BDF for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). BDF: baseline diameter fluctuation.
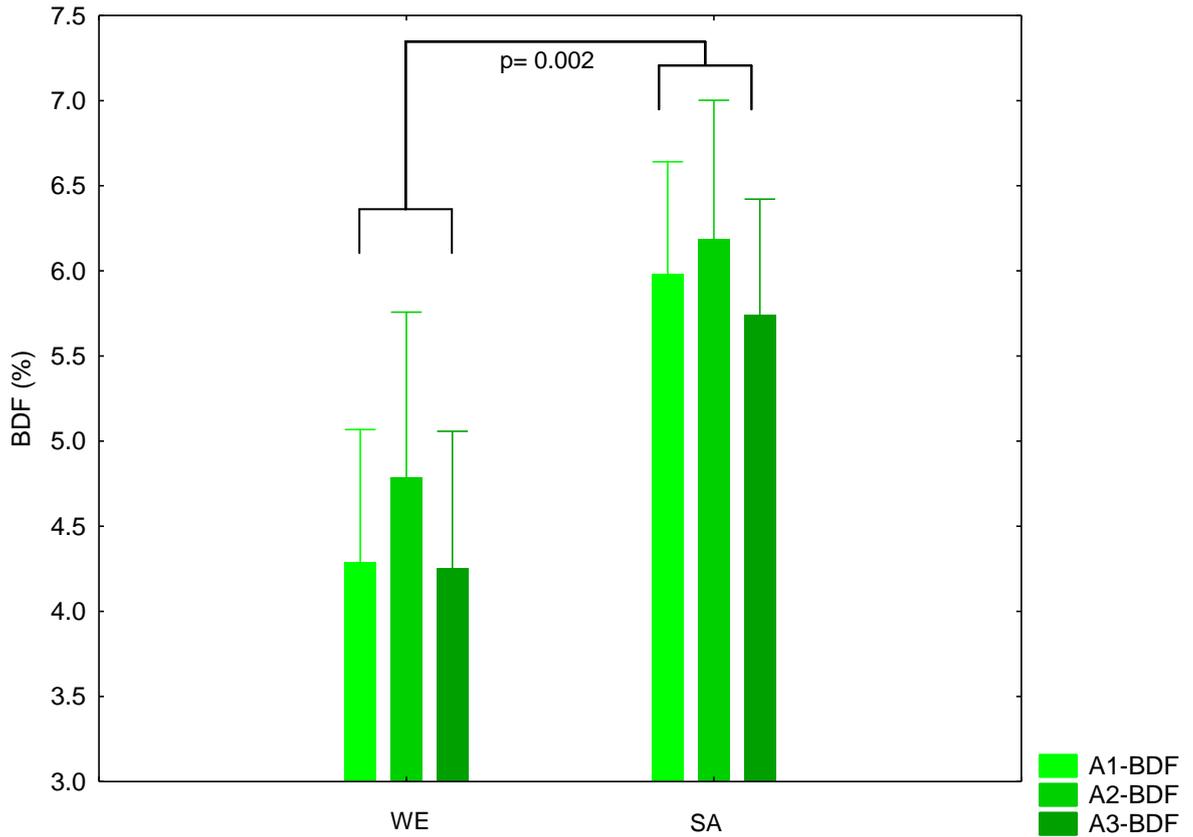
Figure 4.3. Repeated-measure values for retinal arterial baseline-diameter fluctuations in each flicker cycle for both ethnic groups

	White European [n=65]	South Asian [n=65]	Post-hoc p-value
ARTERY MC (%)			
Flicker cycle 1	2.77 (1.13-3.87)	3.53 (1.26-4.90)	0.111
Flicker cycle 2	2.81 (1.80-4.21)	3.73 (2.01-5.16)	0.063
Flicker cycle 3	2.67 (0.65-3.64)	3.27 (1.57-4.17)	0.215
Within-group ANOVA	0.909	0.261	

Table 4.10. Retinal arterial MC for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). MC: maximum constriction as a percentage from initial baseline diameter.

	White European [n=65]	South Asian [n=65]	Post-hoc p-value
ARTERY MCRT (secs)			
Flicker cycle 1	22.8 (19.0-28.0)	19.0 (13.5-24.0)	**0.002**
Flicker cycle 2	20.6 (14.0-27.0)	17.5 (11.0-24.0)	**0.003**
Flicker cycle 3	21.2 (17.0-26.0)	19.7 (13.0-27.0)	0.242
Within-group ANOVA	0.237	0.297	

Table 4.11. Retinal arterial MCRT for each individual flicker cycle. Values quoted in mean ± SD. *Significant differences indicated in bold (p<0.05). MCRT: maximum constriction reaction time post-flicker.
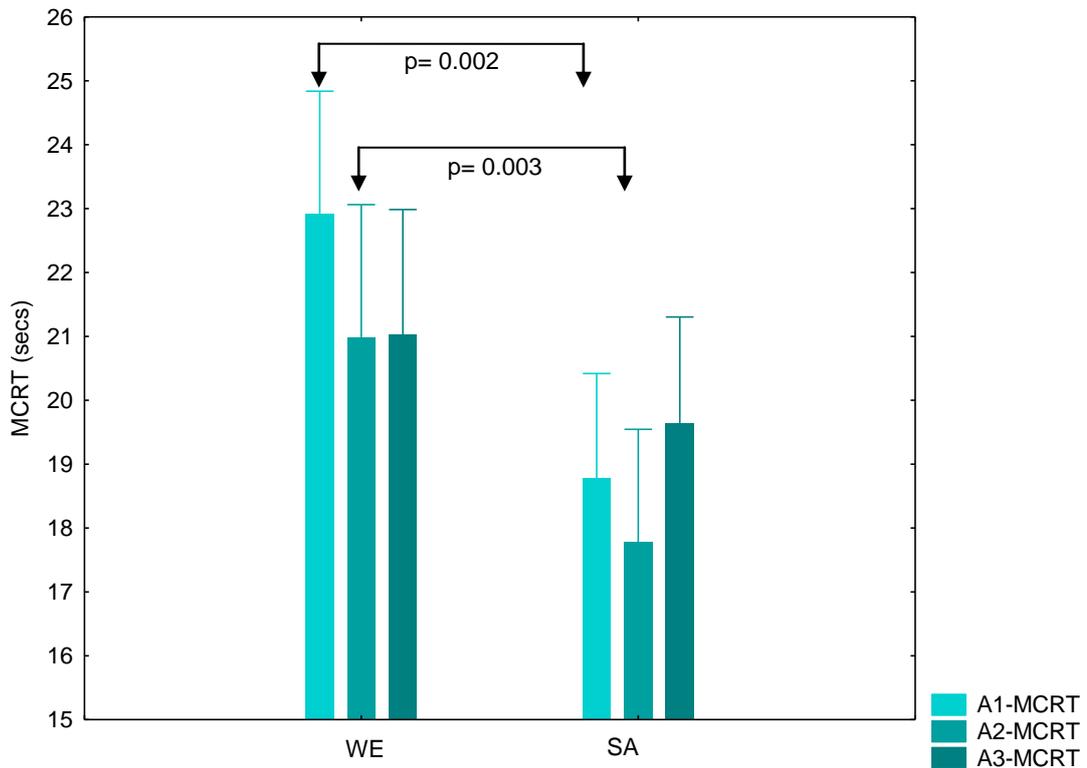
Figure 4.4. Differences in arterial RT to reach maximal constriction diameter post-flicker for both ethnic groups

	White European [n=65]	South Asian [n=65]	Post-hoc p-value
VEIN MD (%)			
Flicker cycle 1	5.18 (2.86-6.36)	5.66 (4.18-7.57)	0.385
Flicker cycle 2	5.30 (3.55-6.34)	7.03 (4.28-8.34)	**0.008**
Flicker cycle 3	5.22 (3.40-6.11)	5.79 (3.83-7.59)	0.312
Within-group ANOVA	0.928	0.055	

Table 4.12. Retinal venous MD for each individual flicker cycle. Values quoted in mean ± SD. *Significant differences indicated in bold (p<0.05). MD: maximum dilation.

	White European [n=65]	South Asian [n=65]	Post-hoc p-value
VEIN DA (%)			
Flicker cycle 1	18.1 (4.28-7.68)	22.8 (5.15-9.72)	**0.011**
Flicker cycle 2	19.5 (4.60-9.03)	19.4 (5.50-10.17)	0.947
Flicker cycle 3	16.9 (4.42-7.51))	21.6 (4.89-9.36)	**0.022**
Within-group ANOVA	0.166	0.067	

Table 4.13. Retinal venous DA for each individual flicker cycle. Values quoted in mean and IQR. *Significant differences indicated in bold (p<0.05). DA: dilation amplitude.

	White European	South Asian	Post-hoc p-value
ARTERY MDRT (secs)			
Flicker cycle 1	18.7 ± 10.6	19.5 ± 10.7	0.694
Flicker cycle 2	21.3 ± 11.7	19.8 ± 11.8	0.527
Flicker cycle 3	16.2 ± 6.9	20.0 ± 10.9*	**0.039**
VEIN MDRT (secs)			
Flicker cycle 1	20.4 ± 9.3	19.7 ± 7.7	0.705
Flicker cycle 2	20.6 ± 12.1	19.8 ± 7.6	0.689
Flicker cycle 3	21.7 ± 12.7	21.7 ± 9.6	0.989

Table 4.14. Retinal arterial MDRT for each individual flicker cycle. Values quoted in mean and IQR. *Significant differences indicated in bold (p<0.05). MDRT: maximum dilation reaction time.

Correlation analysis between distinct biomarkers and vascular tests yielded weak-to-moderate correlations between oxidative stress markers, namely GSSG and FMD (Figure 4.2.) but not with any retinal vessel components. The time taken for SAs (but not WEs) to reach maximal retinal arterial constriction, termed MCRT, did correlate modestly with GSH (r=0.372, p=0.009); tGSH (r=0.376, p=0.009); TG (r=-0.330, p=0.012) and TG:HDL-C ratio (r=-0.361, p=0.006). (Figure 4.5). Finally, arterial baseline-diameter fluctuations (BDF) correlated, but again somewhat moderately, to TG:HDL-C (r=0.277, p=0.004) and Total:HDL-C (r=0.322, p=0.014) in SAs.
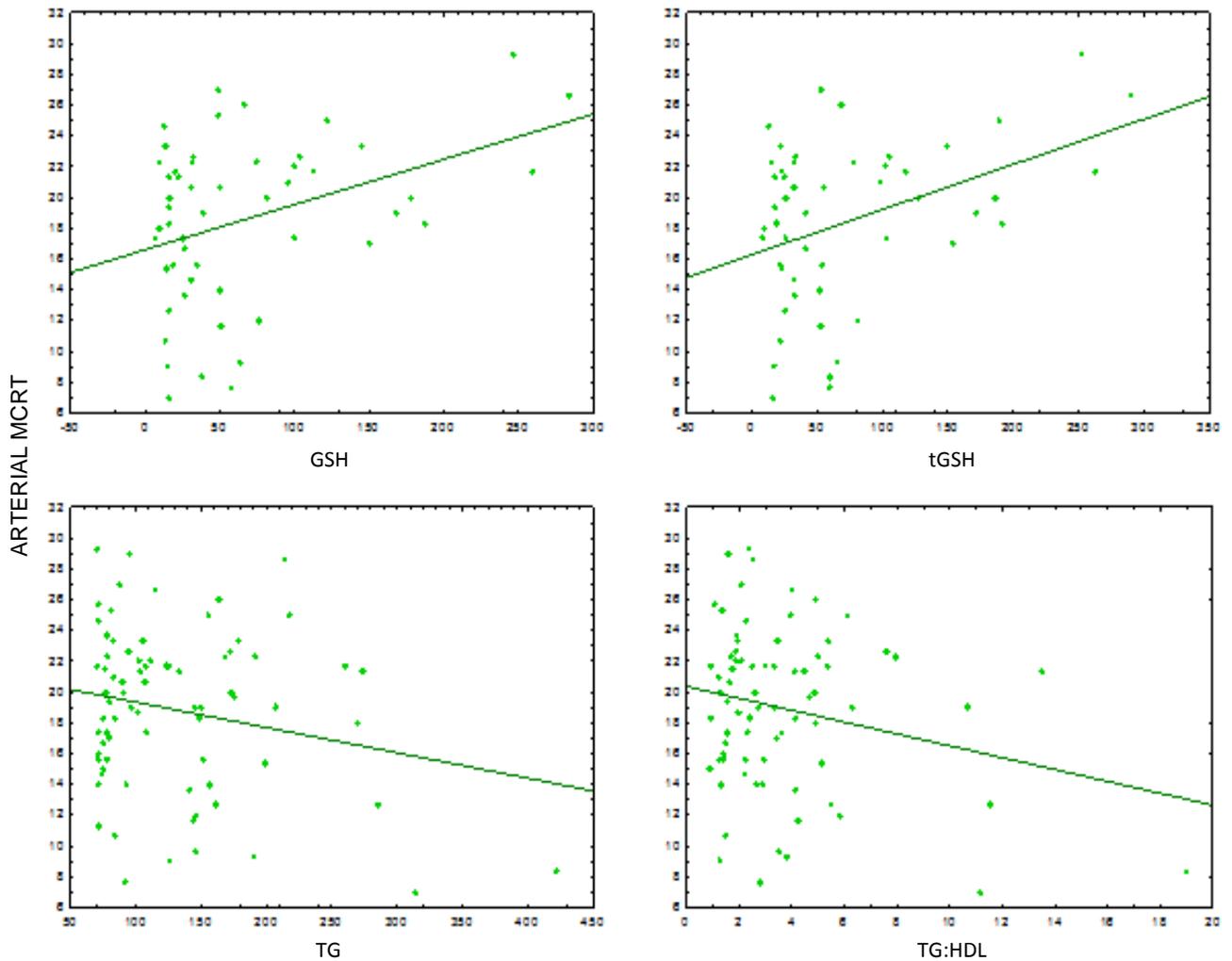
Figure 4.5. Biochemical and MCRT correlations in SAs. From top-left to clockwise. Positive Spearmans correlations for MCRT against GSH (r= 0.372; p= 0.013) and tGSH (r= 0.376; p=0.009), and negative correlations between MCRT and TG (r= -0.330 ;p= 0.012) and TG:HDL (r= -0.361; p= 0.006).

4.5.1. Influence of Gender on ANS and Vascular Function Differences in SAs

Further analysis involved comparisons between male (n=33) and female (n=32) groups in both cohorts. The demographic data for SA males is presented in Table 4.15, and showed similar distributions for age, systemic BP, MAP, IOP, fasting levels of glucose; HDL, LDL and total cholesterol, and carotid-IMT. When compared to their WE counterparts, SA males did however show abnormally higher (borderline IGT values) 2-hour post consumption glucose values (p=0.007), and raised TG (p=0.022) and cholesterol ratios; evident by higher TG:HDL-C (p=0.011) and Total:HDL-C (p=0.013) levels.

The demographic values for the female subgroups in Table 4.16 showed comparable profiles with only significant differences in 2-hour GTT levels, plasma TGs and left-side common carotid artery intima media thickness (p=0.018, p=0.036, and p=0.030 respectively).

When comparing Framingham scores for CVD risk (Table 4.17), however, there were no differences between both genders in either group (p>0.05). This was also true for plasma markers for oxidative stress and endothelial function. Nor were there any significant differences in micro- and macro-vascular function (ANOVA p>0.05).

	WE Male [n=33]	SA Male [n=33]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	40.0 ± 9.4	39.3 ± 10.3	0.739
SBP (mmHg)	122 ± 10	124 ± 11	0.435
DBP (mmHg)	74 ± 7	75 ± 8	0.764
MAP (mmHg)	93 ± 8	94 ± 12	0.672
IOP (mmHg)	14 ± 3	14 ± 3	0.659
METABOLIC DATA			
Glucose (mmol/L)	5.14 ± 8.67	5.10 ± 14.63	0.800
2 hour GTT (mmol/L)	6.53 ± 1.30	7.60 ± 2.63*	**0.007**
TG (mmol/L)	1.18 ± 1.01	1.57 ± 0.83*	**0.010**
HDL Cholesterol (mmol/L)	1.16 ± 0.29	1.01 ± 0.39	0.084
LDL Cholesterol (mmol/L)	2.64 ± 0.78	2.65 ± 0.81	0.951
Total Cholesterol (mmol/L)	4.32 ± 0.87	4.37 ± 0.81	0.782
TG:HDL-C (mmol/L)	2.51 ± 1.32	4.28 ± 3.40*	**0.011**
CVD RISK DATA			
Total:HDL-C (mmol/L)	3.91 ± 1.02	4.91 ± 1.91*	**0.013**
R-IMT (mm)	0.063 ± 0.018	0.060 ± 0.016	0.567
L-IMT (mm)	0.061 ± 0.021	0.063 ± 0.016	0.722

Table 4.15. Baseline data for male subgroups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; Total:HDL-C: Total-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

	WE Female [n=32]	SA Female [n=32]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	39.5 ± 10.0	38.2 ± 11.5	0.521
SBP (mmHg)	114 ± 8	111 ± 5	0.209
DBP (mmHg)	66 ± 6	69 ± 5	0.123
MAP (mmHg)	82 ± 6	82 ± 4	0.968
IOP (mmHg)	14 ± 2	13 ± 3	0.375
METABOLIC DATA			
Glucose (mmol/L)	4.61 ± 0.86	4.97 ± 0.67	0.090
2 hour GTT (mmol/L)	5.88 ± 1.53	7.07 ± 1.67*	**0.018**
TG (mmol/L)	1.00 ± 0.25	1.31 ± 0.77*	**0.036**
HDL Cholesterol (mmol/L)	1.34 ± 0.31	1.40 ± 0.39	0.540
LDL Cholesterol (mmol/L)	2.58 ± 0.85	2.78 ± 0.80	0.426
Total Cholesterol (mmol/L)	4.37 ± 0.91	4.76 ± 0.91	0.120
TG:HDL-C (mmol/L)	1.78 ± 0.55	2.44 ± 1.89	0.070
CVD RISK DATA			
Total:HDL-C (mmol/L)	3.43 ± 1.05	3.56 ± 0.86	0.621
R-IMT (mm)	0.049 ± 0.012	0.056 ± 0.015	0.227
L-IMT (mm)	0.050 ± 0.013	0.064 ± 0.018*	**0.030**

Table 4.16. Baseline data for female subgroups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; Total:HDL-C: Total-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

CVD RISK DATA	Framingham Score (%)	M1 Framingham (%)	M2 Framingham (%)
WE Male	3.9 ± 3.2	4.1 ± 3.4	4.3 ± 3.7
SA Male	3.2 ± 5.1	4.9 ± 7.6	6.9 ± 7.6
T-test p-value	0.500	0.604	0.185
WE Female	0.9 ± 0.7	0.9 ± 0.7	0.9 ± 0.7
SA Female	0.7 ± 0.2	1.0 ± 0.4	1.1 ± 0.8
T-test p-value	0.140	0.632	0.384

Table 4.17. Framingham (and modified) risk scores for gender subgroups. Values quoted in mean ± SD. M1 Framingham: Modified-1 Framingham for ethnicity by adding 50% of original score; M2 Framingham: Modified-2 Framingham for ethnicity by increasing age by 10 years.[367]

Additional analysis extended to comparing gender groups within the SA cohort as presented in Table 4.18. This yielded comparative differences in systemic BP profiles, with SA men showing higher SBP, DBP, MAP and PP values ($p<0.001$, $p=0.006$, $p<0.001$ and $p<0.001$ respectively) than SA women. Despite higher fasting glucose values ($p=0.002$), SA males gave similar 2-hour GTT results as SA women ($p>0.05$). Both groups showed similar levels in plasma markers of metabolic function, with only statistically significant differences in HDL cholesterol ($p<0.001$) and TG:HDL-C ($p=0.019$) and Total:HDL-C ($p=0.002$). However, it is important to note that SA males presented with lower total cholesterol values that was close to, but didn't reach, statistical significance. This impacted CVD risk scores, with SA men showing significantly higher percentages for eventual CVD in 10 years ($p=0.020$, $p=0.020$ and $p=0.007$). (Table 4.19/Figure 4.6.)

ANS functional analysis by means of twenty-four hour BP and HRV measurements are presented in Table 4.20 and 4.21 respectively. Unlike the similar findings found between SA and WE groups, when compared to SA women, SA men exhibited statistically significant signs of possible ANS dysfunction by means of increased systemic BP profiles and sympathetic innervation (raised LF readings $p=<0.05$) and reduced parasympathetic stimulation (lower HF readings $p=<0.05$). Despite these consistent findings in SA men, there were no significant differences found in circadian parameters ($p>0.05$). Correlations between glucose levels and sympathovagal imbalances (LF:HF values) were evident in SA men in both active ($r=0.376$, $p=0.017$) and passive phases ($p=0.421$, $p=0.007$). (Figure 4.7.)

Although SA men weighed more than their female counterparts ($p<0.001$), both groups had similar BMI and WHR values. Despite this, SA females had greater levels of body fat as evident by a higher PBF value, and lower fat free mass, total body water, protein, mineral and skeletal muscle mass scores ($p<0.001$) when compared to SA males. Table 4.22

Despite apparent differences in obesity indices, there were no significant disparities in plasma markers for oxidative stress and endothelial function ($p>0.05$).

Additionally, SA men and women presented with similar retinal vessel reactivity and SRDA components showing no differences in retinal vascular function. However, as shown in Table 4.23, SA men did exhibited reduced, albeit the lower side of normal, brachial artery MD as induced by reactive hyperaemia ($p=0.002$) and GTN ($p=0.035$).

	SA Male [n=33]	SA Female [n=32]	Unpaired T-test p-value
DEMOGRAPHIC DATA			
Age (years)	39.3 ± 10.3	38.2 ± 11.5	0.670
SBP (mmHg)	124 ± 11*	110 ± 5	**<0.001**
DBP (mmHg)	75 ± 8*	69 ± 5	**0.006**
MAP (mmHg)	91 ± 9*	82 ± 4	**<0.001**
PP (mmHg)	47 ± 9*	38 ± 13	**<0.001**
IOP (mmHg)	14 ± 3	13 ± 3	0.367
METABOLIC DATA			
Glucose (mmol/L)	5.69 ± 0.61*	5.16 ± 0.42	**0.002**
2 hour GTT (mmol/L)	7.60 ± 2.63	7.07 ± 1.66	0.135
TG (mmol/L)	1.57 ± 0.83	1.30 ± 0.77	0.191
HDL Cholesterol (mmol/L)	1.01 ± 0.39*	1.40 ± 0.39	**<0.001**
LDL Cholesterol (mmol/L)	2.65 ± 0.81	2.78 ± 0.80	0.523
Total Cholesterol (mmol/L)	4.37 ± 0.81	4.76 ± 0.91	0.065
TG:HDL-C (mmol/L)	4.28 ± 3.40*	2.44 ± 1.89	**0.019**
CVD RISK DATA			
Total:HDL-C (mmol/L)	4.91 ± 1.91*	3.56 ± 0.86	**0.002**
R-IMT (mm)	0.060 ± 0.016	0.056 ± 0.015	0.429
L-IMT (mm)	0.063 ± 0.016	0.064 ± 0.018	0.911

Table 4.18. **Baseline data for SA gender subgroups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; PP: pulse pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; Total:HDL-C: Total-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.**

	Framingham Score (%)	M1 Framingham (%)	M2 Framingham (%)
SA Male	3.2 ± 5.1*	4.9 ± 7.6*	6.9 ± 7.6*
SA Female	0.7 ± 0.2	1.0 ± 0.4	1.1 ± 0.8
Unpaired T-test	**0.020**	**0.020**	**0.007**

Table 4.19. Framingham (and modified) risk scores for SA gender subgroups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. M1 Framingham: Modified-1 Framingham for ethnicity by adding 50% of original score; M2 Framingham: Modified-2 Framingham for ethnicity by increasing age by 10 years.[367]
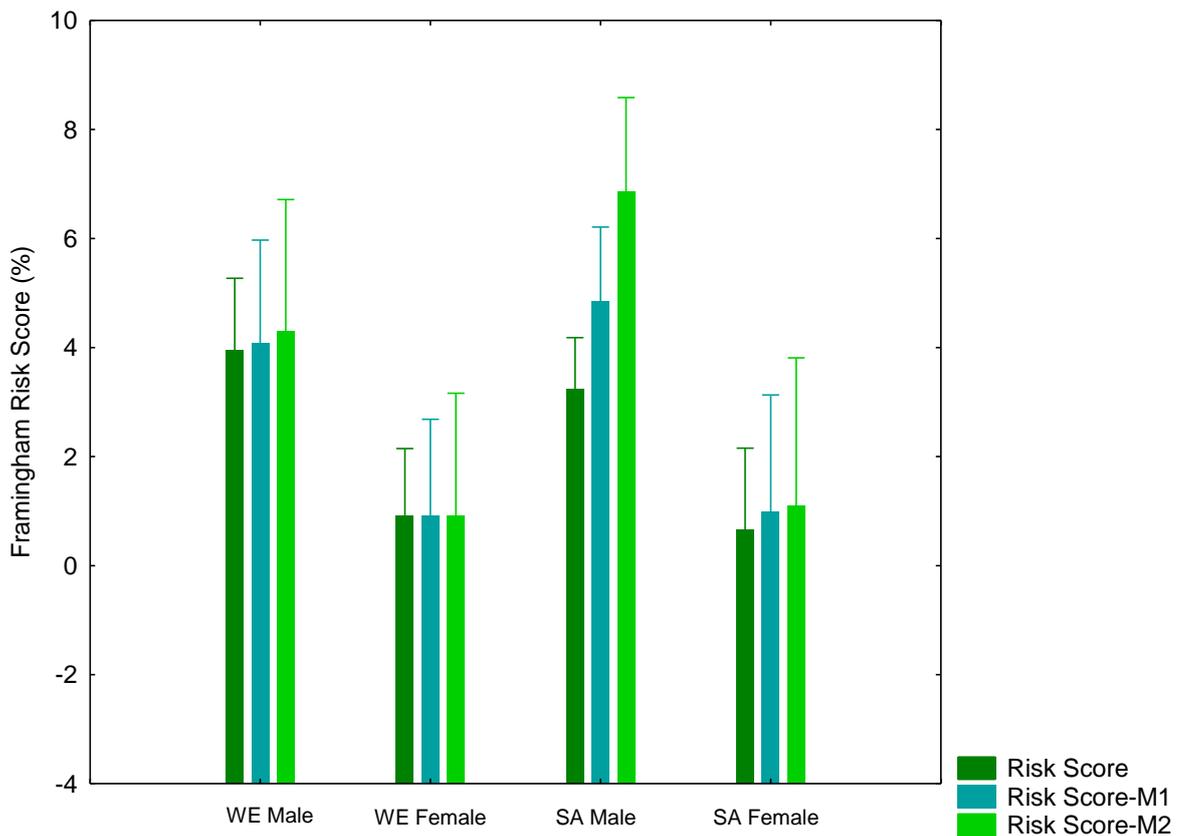
Figure 4.6. Framingham risk score differences for different gender sub-groups in WE and SA categories. Risk Score: traditional Framingham %, Risk Score-M1: modified risk score to account for ethnicity by increasing traditional score by 50%, Risk Score-M2: modified risk score to account for ethnicity by increasing the inputted age by 10 years.

BP PROFILE DATA	SA Male [n=33]	SA Female [n=32]	Unpaired T-test p-value
24 hr-SBP (mmHg)	124 ± 11*	110 ± 5	**<0.001**
24 hr-DBP (mmHg)	75 ± 8*	69 ± 5	**0.006**
24 hr-MAP (mmHg)	91 ± 9*	82 ± 4	**<0.001**
Active-SBP (mmHg)	129 ± 11*	114 ± 5	**<0.001**
Active-DBP (mmHg)	80 ± 8*	72 ± 4	**<0.001**
Active-MAP (mmHg)	95 ± 11*	85 ± 4	**<0.001**
Passive-SBP (mmHg)	115 ± 12*	103 ± 4	**<0.001**
Passive-DBP (mmHg)	66 ± 9*	61 ± 8	**0.033**
Passive-MAP (mmHg)	66 ± 11	65 ± 10	0.684
Circadian-SBP (mmHg)	14 ± 11	13 ± 5	0.759
Circadian-DBP (mmHg)	15 ± 12	12 ± 8	0.117
Circadian-MAP (mmHg)	29 ± 12	20 ± 10	0.883

Table 4.20. Systemic BP data for SA gender subgroups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure.

ANS PROFILE DATA	SA Male [n=33]	SA Female [n=32]	Unpaired T-test p-value
24 hr-LF (NU)	68 ± 10*	62 ± 11	**0.043**
24 hr-HF(NU)	30 ± 10	34 ± 10	0.077
24 hr-LF/HF (NU)	2.7 ± 1.3	2.2 ± 1.2	0.155
Active-LF (NU)	72 ± 9*	63 ± 11	**<0.001**
Active-HF (NU)	26 ± 9*	34 ± 10	**<0.001**
Active-LF/HF (NU)	3.1 ± 1.4*	2.1 ± 1.2	**0.006**
Passive-LF (NU)	66 ± 12*	58 ± 10	**0.008**
Passive-HF (NU)	32 ± 12*	39 ± 10	**0.020**
Passive-LF/HF (NU)	2.6 ± 1.7*	1.6 ± 0.7	**0.015**
Circadian-LF (NU)	6.7 ± 21.5	7.4 ± 13.7	0.892
Circadian-HF (NU)	-6.0 ± 12.7	-4.8 ± 9.3	0.717
Circadian-LF/HF (NU)	0.5 ± 1.6	0.4 ± 0.9	0.768

Table 4.21. HRV data for SA gender subgroups. Values quoted in NU (normalised units) mean ± SD.*Significant differences indicated in bold as p<0.05. LF: low frequency component of the HRV, HF: high frequency component of the HRV.
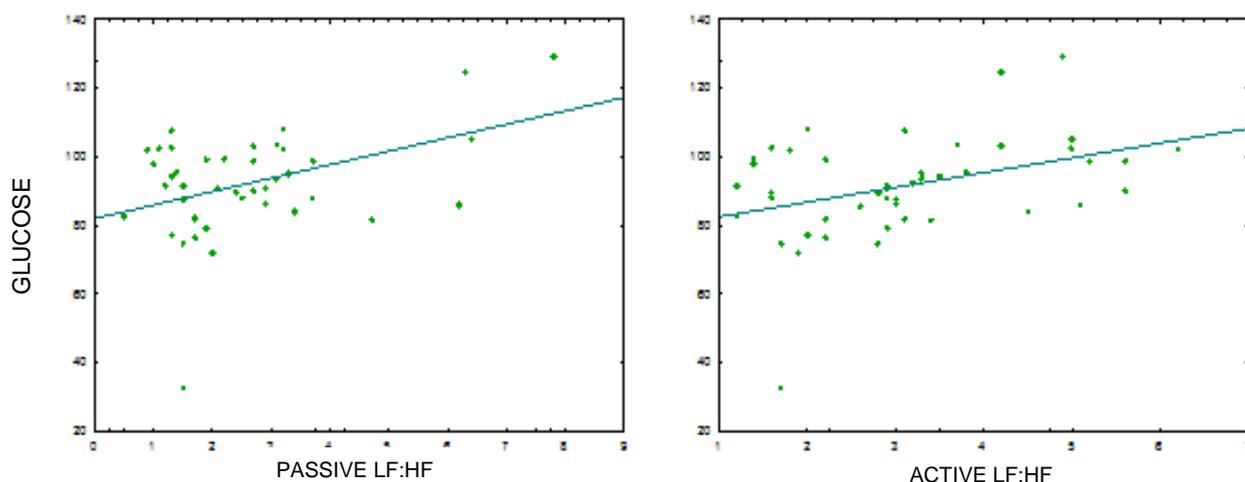
Figure 4.7. Correlation between glucose levels and sympathovagal imbalances in SA men. Spearmans correlations showing a moderate correlation between hyperglycaemia and passive LF:HF (r=0.421, p=0.007) and a weaker correlation on the right side for active LF:HF (r=0.376, p=0.017)

	SA Male [n=33]	SA Female [n=32]	Unpaired T-test p-value
Weight (kg)	81.3 ± 12.2*	65.4 ± 9.9	**<0.001**
BMI (kg/m^2)	26.8 ± 3.8	26.3 ± 4.5	0.603
WHR (AU)	0.95 ± 0.13	0.94 ± 0.08	0.649
PBF (%)	27.2 ± 6.4	38.8 ± 6.9*	**<0.001**
Fat Mass (kg)	22.6 ± 8.1	25.6 ± 7.7	0.107
Fat Free Mass (kg)	58.7 ± 6.8	39.6 ± 4.6*	**<0.001**
SMM (kg)	33.1 ± 4.2	21.5 ± 2.8*	**<0.001**
TBW (l)	43.1 ± 5.0	29.0 ± 3.4*	**<0.001**
Protein (kg)	11.6 ± 1.4	7.8 ± 0.9*	**<0.001**
Mineral (kg)	4.0 ± 0.5	2.8 ± 0.3*	**<0.001**

Table 4.22. Obesity correlates and body composition (by means of bioelectrical impedance) of SA gender subgroups. Values quoted in mean ± SD. *Significant differences indicated in bold as p<0.05. BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat; SMM: skeletal muscle mass; TBW: total body water.

	SA Male [n=33]	SA Female [n=32]	Mann-Whitney p-value
BRACHIAL ARTERY			
AD (mm)	4.42 (4.09-4.91)	3.32 (3.06-3.48)	**<0.001**
BDF (mm)	0.33 (0.17-0.44)	0.34 (0.17-0.44)	0.919
MD (mm)	4.89 (4.32-5.37)	4.03 (3.37-4.10)	**0.002**
RT (secs)	24.3 (1.0-55.0)	23.6 (6.0-37.0)	0.941
FMD (%)	8.30 (3.18-11.33)	8.08 (5.47-10.86)	0.886
GTN			
GTN-MD (mm)	5.35 (4.63-5.88)	4.12 (3.27-5.03)	**0.035**
RT (secs)	281 (255-346)	328 (321-333)	0.529
GID (%)	24.97 (16.21-29.69)	31.89 (8.45-36.48)	0.431
FMD/GID (%)	0.40 (0.13-0.55)	0.39 (0.04-0.91)	0.980

Table 4.23. Brachial artery reactivity as means of systemic vascular endothelial function between both groups. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.

4.6. Discussion

The findings, in this chapter, suggest that an otherwise healthy second-generation SA immigrant population present with an early risk for vascular disease, as shown by increased Framingham scores, reduced brachial artery FMD and retinal arterial over-constriction. Additionally, when compared to WEs, SAs presented with a pattern of dyslipidaemia (increased TG, lower HDL concentrations and higher cholesterol ratios) characteristically present in their parents and older generations that is also found with T2DM and is an important feature found in the MetS.[368] Furthermore, gender influences in SAs showed differences in lipid and systemic BP profiles and ANS function in SA men.

Dyslipidaemia and CVD risk

Even though the total cholesterol values were comparable amongst SAs and WEs, this is not an uncommon finding.[369, 370] Interestingly, albeit normal, some studies have reported cholesterol values higher than their SA counterparts in the Indian subcontinent.[361] However, total cholesterol as a means of predicting vascular risk, tends to underestimate the risk in SAs when using CVD risk predictors. The Total:HDL Cholesterol ratio (Total:HDL-C)

has instead been proven to be a better predictor for CVD in this ethnic minority.[365] This is supported by the findings of this thesis with significantly higher ratios in the SA cohort despite similar total cholesterol findings.

Very similar to other vascular risk studies, the current results show that with migration, lipid profiles are becoming more adverse; illustrated by falling HDL and rising TG and cholesterol ratio values.[371, 372] Furthermore, a majority of studies have reported classic lipid profile anomalies in the form of lower HDL concentrations and increased prevalence of atherogenic small particle-sized LDL cholesterol levels.[373] These findings alongside those reported in this thesis strongly suggest that abnormal lipid profiles are a contributing factor to excess vascular risk in SAs.[374, 375]

Interestingly, when accounting for vascular risk, the widely used Framingham risk calculator shows little differences when comparing SAs with WEs. This is despite definite differences in systemic BP, HDL-C and TG. It is only when the traditional risk calculator is modified for ethnicity, i.e. by adding 10 years to the Framingham equation, that SAs show a definite increased risk for CVD. This suggests, and has indeed been proposed on a number of occasions, that thresholds should be significantly lowered for SAs when accounting for CVD risk.[28, 367] This is true too, for SA men who showed a greater sensitivity and considerable difference in risk percentages when modified for ethnicity as compared to SA women. Whether this could allow for reduced thresholds in vascular function studies; i.e. reduced FMD and RVA values to account for ethnicity, opens up avenues for further work and a clinical need for normative databases in different ethnic groups.

The relevance of obesity and central adiposity within the SA community has also been highlighted by many large population-based studies.[376] Abdominal obesity significantly contributes to the pathogenesis of IR through a number of mechanisms.[377-380] Unlike that of the results reported in this chapter, these studies have shown increased WHR values in SAs than in WEs.[381-384] Although obesity can contribute, only in part, towards the excess vascular risk, it has been suggested that for every level of body fat SAs appear more IR than WEs.[25, 26, 384, 385] This could possibly be the case for SA women as shown by their significantly different obesity components when compared to SA men. This is supported by data suggesting that skeletal muscle mass and distribution is important in many physiological and disease processes, including ageing.[386, 387] However, to investigate this fully, further body composition studies would need to be conducted.

SAs are also known to present with BP profiles indicative of future hypertensive and CVD risk, by means of higher SBP, DBP and PP recordings, which was only evident with increased DBP values in this cohort.[388, 389] The influence of dyslipidaemia and hyperglycaemia in SAs on increased heart rate and abnormal cardiac sympathovagal balance is also well recorded. However, consistent with other studies, further 24-hour systemic BP and ECG analysis yielded no differences in ANS and HRV function between SAs and WEs.[390]

Gender Differences

It is only when considering SA men alone that increased BP profiles are consistently seen and early signs of HRV and ANS dysfunction (increased LF and reduced HF components) are registered. These results show that cardiac burden and future risk, by means of autonomic dysfunction is evident in otherwise healthy SA men, but was not found to be a relevant factor in SA females (independent of height ANCOVA $p > 0.05$). Although this is consistent to previously reported data, only moderate influences were found between hyperglycaemia and ANS dysfunction.[261] Unlike previous findings, SA men in this cohort presented with higher FMD results (8.30% versus 6.9%) and furthermore this was comparable to the WE sample. This would suggest endothelial dysfunction may occur independently to ANS dysfunction in SA men.[26, 383] To validate these findings though, extensive longitudinal data are required. This would also establish whether the impact of CVD risk in men is mediated through metabolic and/or autonomic processes.

Interestingly, SA women showed reduced FMD and GID percentages when compared to their male counterparts, suggesting a slightly blunted response in both endothelial and smooth-muscle cell response when compared to SA men. However, this cannot be explained by smaller initial brachial artery diameters ($p < 0.001$) as multiple regressional analysis yielded no influence (ANCOVA $p > 0.05$) and no such correlations existed between obesity parameters and brachial artery dilation ($p > 0.05$). Therefore, further work would need to be conducted to understand the influence on gender and ethnicity on endothelial function.

The findings of the present study can be applied to healthy males, and future studies would need to examine whether ethnic differences are demonstrable in those with overt vascular risk factors.

Systemic Endothelial Function

FMD allows for the assessment of the shear-stress mediated NO production mechanism and brachial artery FMD is closely related with coronary vasomotor response.[391] Therefore, a reduction in FMD is thought to represent an early functional disturbance in the development of CVD. The SA sample in this thesis demonstrated significantly lower FMD than WEs, indicating reduced NO bioavailability. Furthermore, the average FMD of 5.49% in SAs falls short of healthy FMD values of 7-10% of the baseline diameter and instead follows that of impaired FMD values found in CVD.[392-397] Whether this relates to endothelial-cell dysfunction remains to be seen. The increased but not significant levels of vWF in the SA sample though, could help to suggest so (no correlations were found between these two variables). However, contradictory reports showing comparable indices in angiogenesis and endothelial-cell dysfunction in SAs suggest that their pathophysiological role in CVD is minimal.[253] The results of this thesis would suggest otherwise but could only be substantiated with further research.

The findings in SA diabetics that oxidative stress pathway genes are important predictors for the development of diabetic complications also help to show the importance of determining oxidative stress levels in SAs.[398] The findings of lower levels in GSH and GSSG and its correlations with reactivity/SDRA components in SAs but not the WE group support this thinking (Figure 4.2. and 4.5.).

Retinal Vascular Function

Despite evidence for endothelial cell damage/dysfunction (reduced FMD, lower GSH/GSSG levels and increased vWF) in SAs, this did not relate to impaired retinal vessel reactivity. Previous reports have shown that SDRA may be more sensitive in detecting retinal vascular function, and this may be the case with SAs (Appendix 3) Although there were no differences detected in the rate of retinal vessel diameter increase with flicker stimulation, i.e. the dilatory slopes, SAs exhibited interesting constriction profiles post-flicker cessation. The increased retinal arterial constriction percentages (MC), which suggest a possible over-constriction when compared to WEs, follow that found in chronic smokers.[399] Furthermore, the increased arterial (Figure 4.3.) and venous baseline fluctuations (BDF) as well as higher dilation and dilation amplitude responses are not surprising results when considering other

vascular haemodynamics findings. Several studies have shown a shifted vasomotion in chronic smokers that leans towards greater vaso-constriction as a result of interacting mechanisms. Additionally, these subjects have also shown to suffer from a "chronic vasodilation" due to an alteration in vaso-active substances. [216, 399] This is somewhat supported by the correlation analysis of this chapter, albeit moderate, between arterial constriction components and GSH, tGSH, TG and TG:HDL-C as well as BDF and TG:HDL-C and Total:HDL-C ratios (Figure 4.5.). This could, therefore, suggest a biochemical involvement, not just autoregulative, in quicker MCRT (Figure 4.4.) and possible steeper constriction slopes in the SA group.

Conclusions

Compared to previous studies, this thesis is presenting newly-found systemic and retinal vascular function data in SAs. The data as a whole suggests quantitatively for the first time that the increased risk of future vascular disease presents at a much earlier age than in WEs. The interplay between systemic, retinal and biochemical markers for endothelial function/damage, metabolic control and oxidative stress also illustrates the complex nature of vascular risk in SAs. Furthermore, SA men, compared with corresponding SA women of similar age and glycaemic status, present with greater levels of dyslipidaemia, Framingham scores and abnormal BP and HRV independent of height (ANCOVA $p>0.05$), which may contribute to increased CVD risk in SA men.

It is not clear whether the observed metabolic and vascular abnormalities in SA migrants are genetic in origin or whether environmental factors might contribute to expression of the abnormalities. One obvious limitation to these results is indeed the small sample size. However, as the data presented in this chapter is preliminary, further work would need to be performed in order to validate the use of SDRA in ethnic vascular screening and whether these functional tests would also be beneficial in diagnostic and therapeutic approaches to those with established vascular disease; namely SAs with T2DM, stroke or CVD.

5. Vascular Function in Individuals Newly Diagnosed with IGT as compared to Normoglycaemic Healthy Controls

5.1. Abstract

5.1.1. Background/Aims: To investigate and demonstrate whether endothelial dysfunction/damage at an ocular and systemic level exists in newly-diagnosed IGT patients when compared to age-matched healthy controls.

5.1.2. Methods: A total of 80 normotensive (SA and WE) subjects (40 IGT and 40 age- and gender- matched controls) were recruited for the present study. Baseline measurements including body composition, c-IMT, 24-hour systemic BP and HRV, fasting plasma glucose, TG, total, LDL-C, HDL-C were measured in all individuals. Retinal vessel reactivity to flickering light was assessed by means of the Dynamic Retinal Vessel Analyser (DRVA) and brachial artery reactivity to reactive hyperaemia (FMD) by means of ultrasound according to a modified protocol. Blood glutathione and plasma vWF were also measured in all individuals.

5.1.3. Results: IGT individuals showed signs of dyslipidaemia as indicated by higher fasting triglyceride (p<0.001) and TG:HDL-C ratio (p<0.001) than age-matched normoglycaemic subjects. Additionally, in IGT subjects, an increase in carotid artery wall thickening as determined by means of ultrasound IMT measurement, was evident (p=0.010 and p=0.005). Impaired systemic vasomotor function as shown by blunted FMD (p=0.026) and retinal vessel reactivity as represented by greater fluctuations in arterial diameter (p=0.026), longer retinal arterial reaction time in response to flicker stimulation (p=0.032) and a reduced flicker response (p=0.045) was present only in subjects with IGT. Furthermore, regression analysis demonstrated correlations between arterial-BDF and several lipid markers as well as arterial-RT and FMD (r=-0.4546, p=0.017).

5.1.4. Conclusion: In newly-diagnosed IGT subjects free of other systemic vascular pathologies, there are potential signs of retinal and systemic vascular function impairment that highlights the importance for early vascular screening in pre-diabetes.

5.2. Introduction

Isolated IGT refers to an intermediate metabolic state between normal and diabetic glucose homeostasis and is characterised by IR and impairments in insulin secretion, involving more

severe muscle IR than hepatic IR. The prevalences of IGT have been likened to that of a pandemic, with expected numbers to rise from 200 to 400 million people worldwide.[400]

Very little is known about the effect of IGT on vascular function. The recognition however, of IGT and its associated heightened risk of CVD, along with its role as a risk factor for T2DM and the emergence of new treatment options have resulted in IGT being recognised clinically as a disease state.[401]

With IGT being linked with clinical events other than progression to T2DM, establishing it as a condition with clinical significance, illustrates the impact of the condition on healthcare. Its relationship with an increased risk of CVD and increased mortality risk when compared to those with normal glucose tolerance also suggest that the threshold of risk may vary amongst different populations.

A proportion (5-12%) of those diagnosed with IGT develop T2DM each year, and alarmingly, associated microvascular complications during this pre-diabetic phase are encountered more commonly.[402]

Identifying populations at risk of T2DM and developing atherosclerosis and then determining their glucose metabolism and vascular function could allow for an improved understanding behind the pathophysiology of IR and help further the characterisation of IGT and its associations with other disease processes.

Recent evidence showing that endothelial dysfunction can be detected early on in IR, even before the detection of distinct carbohydrate intolerance or clinical manifestations of MetS, suggest that the investigation of endothelial function in IGT groups could be of clinical relevance in the primary management of vascular risk.[403]

Therefore, the aim of this study was to investigate vascular risk for CVD and T2DM progression by means of systemic and ocular vascular function in newly-diagnosed (within six months of official diagnosis made by OGTT by a Diabetologist) individuals with IGT.

5.3. Materials and Methods

IGT subjects (SA and WE; with and without a FH) aged 25-55 years were recruited and screened through a nurse- led community OGTT clinic in the Birmingham vicinity. The OGTT was carried out under standardised protocols and diagnosis made under WHO criteria (Section 3.3.).

Exclusion criteria were: a positive diagnosis of cardio- or cerebro-vascular disease, (coronary artery disease, heart failure, arrhythmia, stroke, transient ischaemic attacks), peripheral vascular disease, severe dyslipidaemia (defined as plasma TG>6.00mmol/L or cholesterol levels>7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment.

Furthermore, subjects were excluded if they had a refractive error of more than ±3DS and more than ±1DC equivalent, IOP >24 mmHg, cataract or any other media opacities, as well as if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system.

Written informed consent was obtained from all participants and ethical approval was sought by the author from local and NHS ethical committees (08/H1202/112). The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki.

According to an already established procedure when examining endothelial function, female participants were asked to fill in a validated menstrual cycle questionnaire and their investigations were carried out during the first week of the menstrual cycle (follicular phase) thereby controlling for hormonal influences on endothelial function.[112, 317]

5.3.1. Investigations
5.3.1.1. Blood sampling and analyses

All participants were asked to fast and refrain from caffeine, alcohol, chocolate and carbonated drinks and to not exercise for 12 hours prior to the date of the study. All blood samples were obtained by a qualified phlebotomist in the morning, between 9am and 10am. Fasting plasma glucose, TG, total and HDL-C were measured using standard routine laboratory techniques using the Reflotron Desktop Analyser (Roche Diagnostics, UK) as described in Section 3.3.1.1. The TG/HDL cholesterol ratio, a measure of endothelial function, [244], and Framingham score as a means of cardiovascular risk (Section 3.3.1.1.) was also determined from the above values.

Further analysis extended to laboratory and ELISA-test sampling for blood glutathione and plasma vWF to investigate possible signs for endothelial damage. Validated protocols were used for in-house testing as described in Sections 2.4. and 2.5.

5.3.1.2. ABPM and HRV analysis

Systemic BP was measured using a 24-hour computer-operated ambulatory BP and electrocardiography (ECG) monitor (Cardiotens-01, Meditech Ltd., Hungary) for each subject. Measurements were performed in ambulatory conditions and programmed to measure BP oscillometrically every 15 minutes during the subject's active period and every 30 minutes in the passive period (as explained in Sections 2.6.). Mean arterial pressure (MAP), as a means of describing cardiac output function in relation to arteriolar resistance was calculated according to Equation 2.2. The impact of pulse pressure (PP), as a risk factor for CVD was also calculated by using Equation 4.1.

HRV analysis was calculated for each individual using the Cardiovision software (PMS Instruments Ltd., Maidenhead, UK) to obtain twenty-four hour, active and passive period LF, HF and LF:HF values. From, this circadian BP and HRV changes were calculated according to Equations 2.3. and 2.4. respectively.

5.3.1.3. Body Composition Analysis

Anthropometric measures including height and weight were measured using standard procedures as outlined in Section 3.3.1.3. Body composition was measured using bioelectrical impedance (Biostat 220, Biospace, UK) to determine BMI, PBF, WHR, total fat and fat free mass.

5.3.1.4. Intima-media thickness

High-resolution B-mode ultrasound system (Acuson Sequoia, 5 MHz linear transducer, Siemens, USA) was used to obtain longitudinal images (described in Section 3.3.1.4.) of the right and left extracranial far wall of the CCA. Each IMT measure, also used for statistical analysis, represented an average of three to five measurement points.

5.3.1.5. Vascular function studies

Retinal vessel reactivity was measured using the DRVA (Imedos; GmbH, Jena, Germany). All measurements were performed in one unselected eye for each subject, between 8:00 and 11:00 AM in a quiet, temperature-controlled room (22°C). Following full pupil dilation with Tropicamide 1% (Minims; Chauvin Pharmaceuticals Ltd, UK) a region of interest

encompassing vessel segments of approximately 500 μm was chosen. Retinal diameters were assessed continuously over 350 seconds according to an accepted and widely used protocol.[277, 332]

Brachial artery FMD was measured using high-resolution CDI ultrasonography, with a 7 mm 8MHz linear-array (Siemens; Acuson Sequoia, UK). According to a published protocol,[308], as described in Section 2.2., arterial occlusion was created by inflation of a sphygmomanometer cuff on the lower arm to suprasystolic pressure and an exogenous NO donor (sublingual 0.3 mg GTN tablet) was given to determine the maximum obtainable vasodilator response. FMD was then expressed as the maximal artery dilation during hyperaemia from baseline (%).

5.4. Power calculation and statistical analysis

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessel reactivity was shown to be significant.[214, 301] Additionally FMD studies have shown a 20% reduction with a SD of 1.85% in FMD response in those with IGT.[242] As the study design was multi-factorial in nature it was calculated (using SISA; a web-based sample calculator approved by the NHS ethics committee) that n=25 per group was sufficient to provide 90% power with an alpha of 0.05. Furthermore, the sensitivity and reproducibility of the techniques in healthy subjects has been reported previously.[112, 333]

All analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). Prior to any analysis, all data was tested for normal distribution and thus a suitable test was adopted. Differences in mean values for each of the measured variables were compared by independent samples t-test (or Wilcoxon) for continuous variables. A multivariate analysis was performed to test the influence of age, gender, BP, and circulating markers on the measured variables. Comparison of retinal vessel reactivity for each flicker period was made by repeated measures (or Friedman) analysis of variance (ANOVA) following within-group analysis. Differences between groups in retinal and systemic vascular function were computed by analysis of covariance (ANCOVA). Univariate linear regression analysis was carried out using appropriate correlation analysis. A p value of <0.05 was considered statistically significant, unless stricter criteria were adopted for within-group and multivariate (p≤0.01 to account for multiple comparisons and thereby minimise bias towards Type II errors).

5.5. Results

Following OGTT diagnosis and recruitment, 40 IGT subjects (20 men and 20 women) and 40 healthy controls (20 men and 20 women) were recruited in excess to the calculated sample size for the final protocol. Following close examination of the data acquired, a total of 10 patients were excluded (due to poor fixation, poor image quality and missing data sets) and therefore only 30 individuals were included for the SDRA element of the study.

The baseline characteristics showing several demographic differences of both groups are presented in Table 5.1. There were no significant dissimilarities in age, IOP, and cholesterol levels (HDL, LDL and Total). Compared to healthy age- and sex- matched controls, the IGT group had significantly higher SBP, DBP, MAP and PP values (p=0.001, p=0.017, p=0.004 p=0.023 respectively), alongside raised fasting levels of glucose, TG and 2-hour post-consumption glucose (p<0.001). Although cholesterol values were alike amongst both groups, the cholesterol ratios and Framingham score were still significantly higher in those with IGT (p<0.001). Furthermore, those with IGT showed greater right (p=0.017) and left-side (p=0.005) carotid artery IMT.

When comparing obesity components between both groups, those with IGT showed similar weight, BMI, WHR, PBF, and fat mass values to their healthy equivalent (p>0.05). (Table 5.2.)

Markers for oxidative stress, as outlined in Table 5.3., showed reduced levels of plasma GSH (p<0.001), GSSG (p=0.039) and t-GSH (p<0.001), with obvious differences also in vWF plasma levels (p=0.014).

	Control [n=40]	IGT [n=40]	T-test p-value
DEMOGRAPHIC DATA			
Age (years)	47.7 ± 10	47.7 ± 10.2	-
SBP (mmHg)	118 ± 12	127 ± 18*	**0.001**
DBP (mmHg)	75 ± 8	80 ± 11*	**0.010**
MAP (mmHg)	89 ± 10	96 ± 13*	**0.004**
PP (mmHg)	43 ± 10	47 ± 10*	**0.010**
IOP (mmHg)	14 ± 3	15 ± 2	0.090
METABOLIC DATA			
Glucose (mmol/L)	4.84 ± 0.69	5.46 ± 0.84*	**<0.001**
2 hour GTT (mmol/L)	6.06 ± 1.03	9.81 ± 2.09*	**<0.001**
TG (mmol/L)	1.20 ± 0.51	1.76 ± 1.02*	**<0.001**
HDL Cholesterol (mmol/L)	1.21 ± 0.38	1.08 ± 0.40	0.120
LDL Cholesterol (mmol/L)	2.65 ± 0.77	2.67 ± 1.03	0.883
Total Cholesterol (mmol/L)	4.39 ± 0.82	4.56 ± 1.06	0.372
TG:HDL-C (mmol/L)	2.59 ± 1.62	4.81 ± 4.36*	**<0.001**
CVD RISK DATA			
Total:HDL-C (mmol/L)	3.98 ± 1.33	4.78 ± 2.17*	**0.010**
R-IMT (mm)	0.055 ± 0.015	0.065 ± 0.015*	**0.010**
L-IMT (mm)	0.057 ± 0.017	0.069 ± 0.016*	**0.005**
Framingham Score (%)	1.9 ± 2.8	4.5 ± 5.8*	**<0.001**

Table 5.1. Baseline data of both groups. Values quoted in mean ± SD.*Significant differences indicated in bold as p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; PP: pulse pressure; IOP: intraocular pressure; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; Total:HDL-C: Total-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness.

	Control [n=40]	IGT [n=40]	T-test p-value
Weight (kg)	75.6 ± 14.7	80.2 ± 14.6	0.127
BMI (kg/m^2)	26.0 ± 4.22	27.6 ± 5.33	0.092
WHR (AU)	0.93 ± 0.11	0.97 ± 0.05	0.075
PBF (%)	30.0 ± 8.2	31.1 ± 9.2	0.564
Fat Mass (kg)	22.9 ± 8.4	25.0 ± 8.5	0.238
Fat Free Mass (kg)	52.7 ± 10.9	55.3 ± 12.2	0.250

Table 5.2. Obesity correlates and body composition (by means of bioelectrical impedance) of both groups. Values quoted in mean ± SD. BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat.

	Control [n=40]	IGT [n=40]	Wilcoxon p-value
GSH (µM)	934.3 (407.2-1293.8)	536.1 (300.2-688.5)	**<0.001**
GSSG (µM)	98.1 (40.9-117.3)	64.5 (26.8-68.4)	**0.039**
GSH:GSSG (µM)	15.3 (5.4-21.9)	13.1 (6.1-18.8)	0.311
tGSH (µM)	1130.5 (592.7-1634.9)	665.2 (399.5-761.2)	**<0.001**
vWF (µ/dL)	122.3 ± 54.9	149.8 ± 59.2*	**0.014**

Table 5.3. Plasma markers for oxidative stress and endothelial function in both groups. Values quoted in IQR/mean ± SD.*Significant differences indicated in bold as p<0.05. GSH: reduced glutathione; GSSG: oxidised glutathione; tGSH: total glutathione; vWF: von willebrand factor.

Table 5.4. represents the FMD values for the IGT versus control samples showing an increased brachial artery diameter (p=0.015) and reduced FMD (%) (p=0.026). Furthermore, the glyceryl-trinitrate induced dilation, known to represent endothelial-independent brachial artery dilation, was also reduced in the IGT group (p=0.012) resulting in a greater FMD:GID ratio (p=0.034). (Figure 5.1.)

This extended to differences in retinal vessel reactivity as represented in Table 5.5. which shows greater fluctuations in arterial diameter; termed BDF (p=0.026), longer arterial dilation time to flicker light; known as MDRT (p=0.032) and also a reduced flicker response, i.e. an attenuated bFR (p=0.045). Additionally, those with IGT showed increased venous MDRT that was close to reaching statistical significance (p-0.053). Although there were no obvious correlations between retinal and brachial parameters with known biomarkers, arterial-BDF [positive spearmans correlations with Total:HDL (r= 0.924; p= 0.008), TG (r= 0.985; p=<0.001), TG:HDL (r= 0.979 ;p= 0.001) and a negative correlation with HDL-C (r= -0.962; p= 0.002)] and arterial-RT (spearmans r= -0.4546, p=0.017) did show correlations with lipid markers and FMD respectively (Figure 5.2. and 5.4. respectively).

	Control [n=40]	IGT [n=40]	Wilcoxon p-value
BRACHIAL ARTERY			
AD (mm)	4.04 (3.34-4.61)	4.53 (3.94-4.91)	**0.015**
BDF (mm)	0.35 (0.19-0.43)	0.37 (0.20-0.44)	0.690
MD (mm)	4.40 (3.63-5.000)	4.72 (4.26-5.26)	0.119
RT (secs)	21.7 (0-40.0)	23.9 (0-54.0)	0.763
FMD (%)	8.61 (2.98-10.65)	5.50 (2.16-8.51)	**0.026**
GTN			
GTN-MD (mm)	5.02 (4.31-5.56)	5.27 (4.67-5.94)	0.307
RT (secs)	314.5 (263-373)	288.5 (267-401)	0.346
GID (%)	25.20 (17.35-31.69)	16.28 (14.16-19.27)	**0.012**
FMD/GID (%)	0.11 (0.08-0.40)	0.57 (0.32-0.60)	**0.034**

Table 5.4. Brachial artery reactivity as means of systemic vascular endothelial function between both groups. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.

	Control [n=30]	IGT [n=30]	Friedman p-value
ARTERY			
AD (AU)	123.94 (112.85-133.77)	121.61 (96.91-174.28)	0.501
BDF (AU)	5.14 (3.47-6.44)	6.42 (1.57-13.16)	**0.026**
MD (%)	5.05 (3.18-6.37)	5.48 (1.17-10.3)	0.433
MDRT (secs)	18.4 (13.3-22.7)	21.4 (10.7-37.0)	**0.032**
MC (%)	3.24 (1.84-4.29)	2.96 (0.45-6.48)	0.546
MCRT (secs)	20.1 (17.3-23.3)	19.7 (8.3-29.0)	0.691
DA (%)	8.27 (5.99-9.73)	8.44 (2.19-16.47)	0.820
bFR (%)	3.13 (1.48-4.75)	2.01 (-5.57-7.17)	**0.045**
VEIN			
AD (AU)	155.75 (142.16-170.29)	159.29 (124.70-202.27)	0.468
BDF (AU)	4.03 (2.54-4.96)	4.66 (2.04-9.89)	0.150
MD (%)	5.71 (4.09-6.54)	5.92 (1.61-12.08)	0.704
MDRT (secs)	19.5 (17.0-22.0)	21.7 (12.0-31.7)	0.053
MC (%)	1.43 (0.40-1.84)	2.01 (0.95-10.15)	0.114
MCRT (secs)	21.9 (19.3-25.3)	20.3 (7.0-29.3)	0.149
DA (%)	7.14 (4.90-8.54)	7.83 (3.12-13.09)	0.290
bFR (%)	3.12 (1.57-4.30)	3.14 (-1.92-10.76)	0.975

Table 5.5. Retinal arterial and venous measures for both groups Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum dilation; MDRT: reaction time to reach maximum diameter to flicker stimulation; MC: maximum constriction; MCRT: reaction time to maximum constriction post flicker; DA: dilation amplitude; bFR: baseline-corrected flicker response.
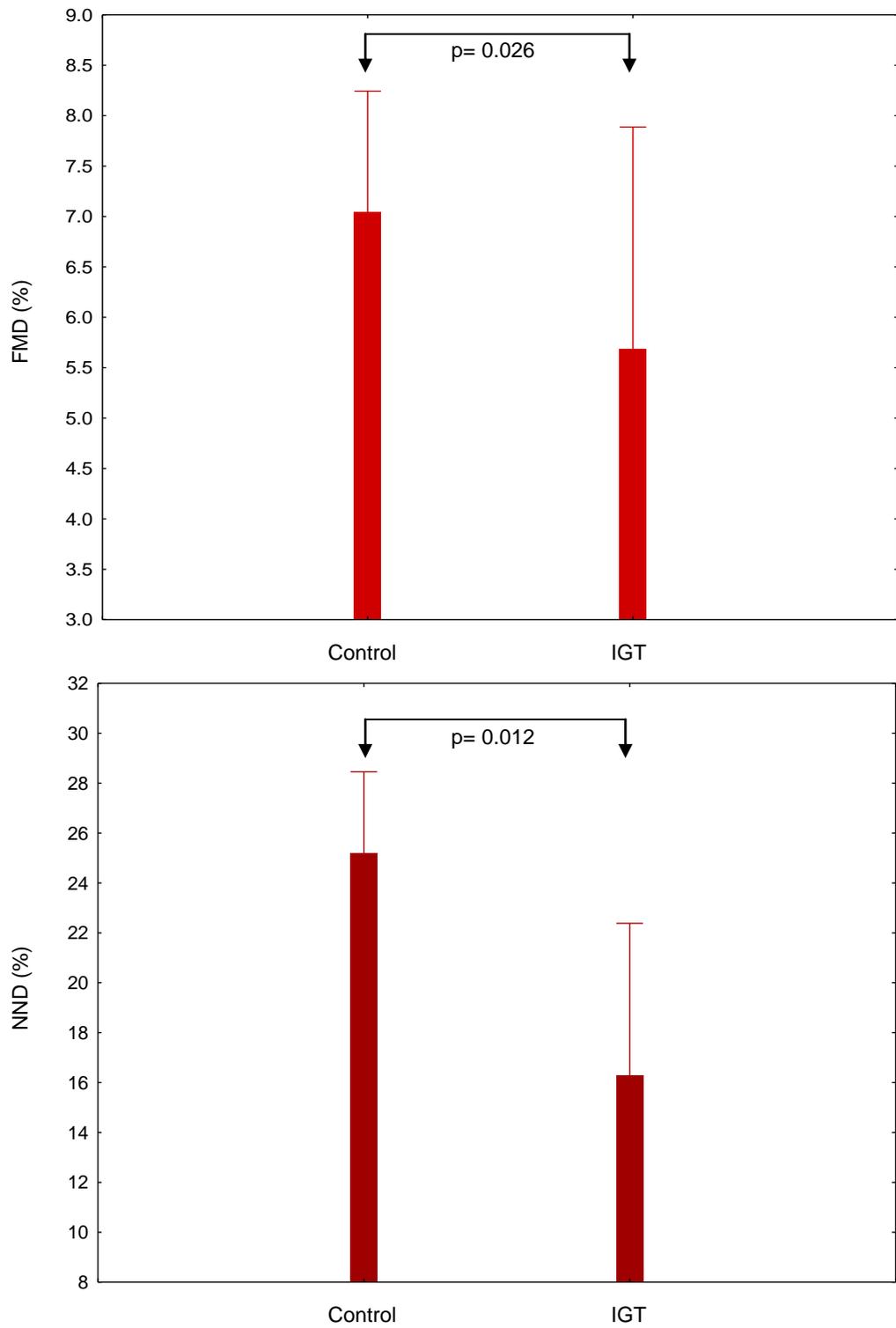
Figure 5.1. FMD and NND percentage differences in control and IGT groups.

	Control [n=30]	IGT [n=30]	Post-hoc p-value
ARTERY BDF (AU)			
Flicker cycle 1	5.19 (1.16-16.33)	6.12 (1.22-16.00)	0.141
Flicker cycle 2	5.14 (0.59-19.40)	7.25 (1.81-18.74)	**0.005**
Flicker cycle 3	5.00 (1.15-23.62)	5.85 (1.54-13.39)	0.188
Within-group ANOVA	0.673	0.097	
VEIN BDF (AU)			
Flicker cycle 1	3.79 (0.96-15.44)	4.78 (1.79-12.10)	0.070
Flicker cycle 2	3.97 (1.20-19.61)	5.14 (1.53-18.03)	**0.045**
Flicker cycle 3	4.17 (0.99-15.75)	4.42 (1.78-14.65)	0.637
Within-group ANOVA	0.397	0.748	

Table 5.6. Retinal arterial and venous BDF for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). BDF: baseline diameter fluctuation.
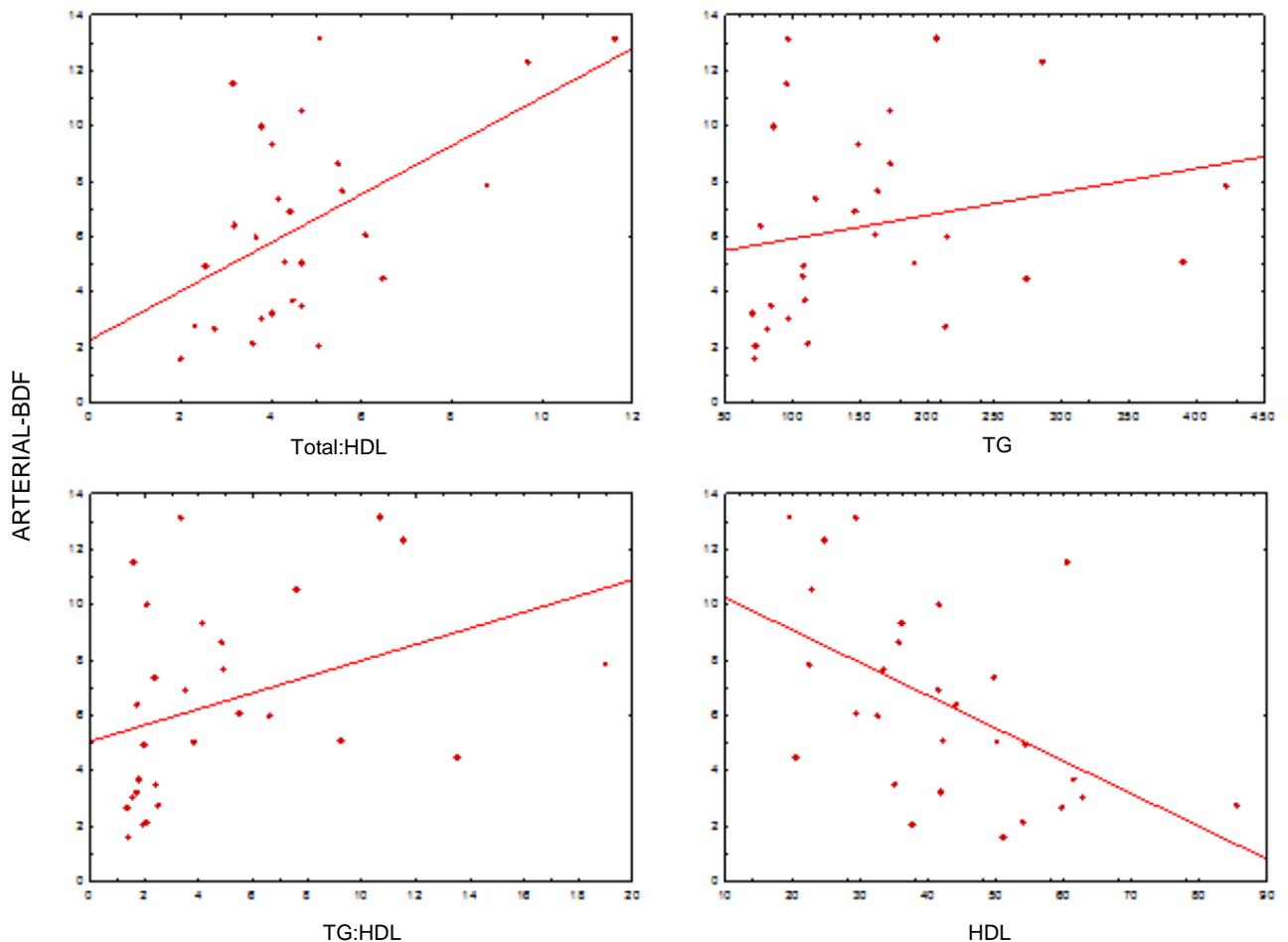
Figure 5.2. Lipid level and arterial baseline-diameter fluctuation correlations in IGTs. From top-left to clockwise. Positive Spearmans correlations for BDF against Total:HDL (r= 0.924; p= 0.008), arterial BDF against TG (r= 0.985; p=<0.001), and BDF against TG:HDL (r= 0.979 ;p= 0.001) and a negative correlation with arterial BDF against HDL-C (r= -0.962; p= 0.002).

	Control [n=30]	IGT [n=30]	Post-hoc p-value
ARTERY MDRT (secs)			
Flicker cycle 1	18.1 (2.0-39.0)	22.8 (2.0-50.0)	**0.011**
Flicker cycle 2	19.5 (1.0-51.0)	19.4 (4.0-50.0)	0.947
Flicker cycle 3	16.9 (3.0-50.0)	21.6 (4.0-50.0)	**0.022**
Within-group ANOVA	0.157	0.511	
VEIN MDRT (secs)			
Flicker cycle 1	20.2 (1.0-50.0)	22.1 (12.0-48.0)	0.287
Flicker cycle 2	19.4 (3.0-48.0)	21.1 (3.0-50.0)	0.313
Flicker cycle 3	19.9 (5.0-47.0)	22.0 (9.0-49.0)	0.233

Table 5.7. Retinal arterial and venous RT for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). MDRT: maximum dilation reaction time.
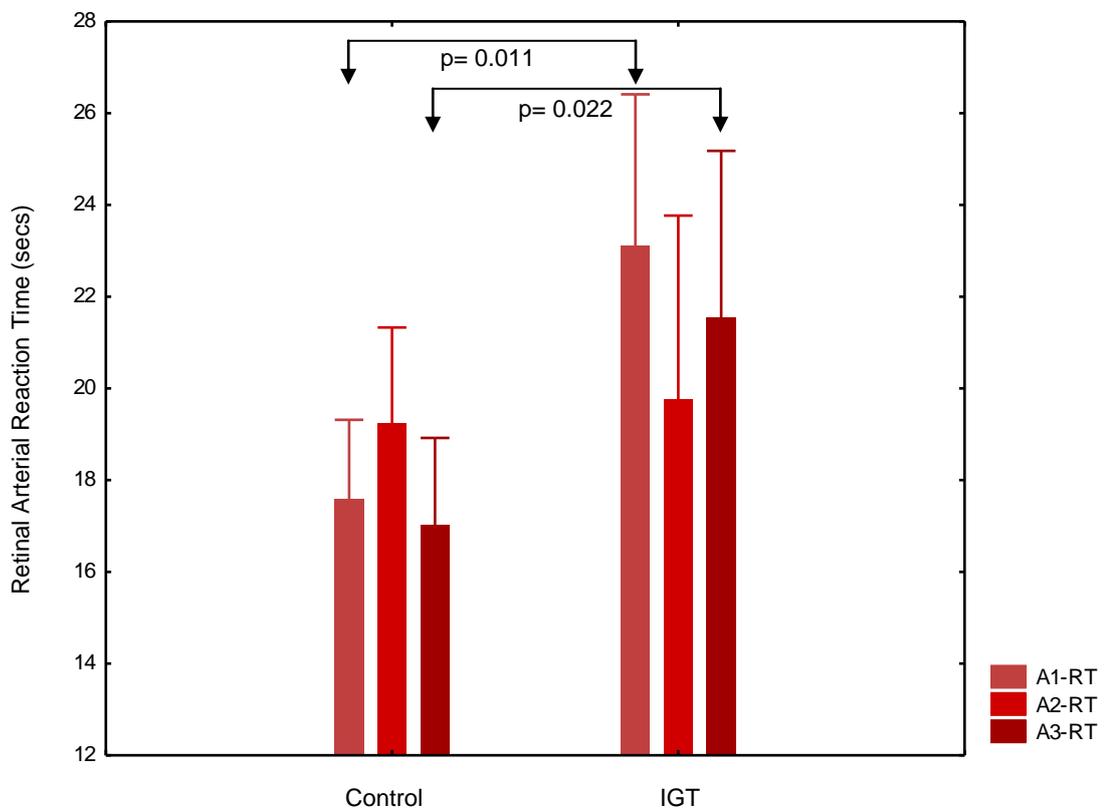
Figure 5.3. Arterial MDRT for each individual flicker cycle for the IGT group as compared to controls.
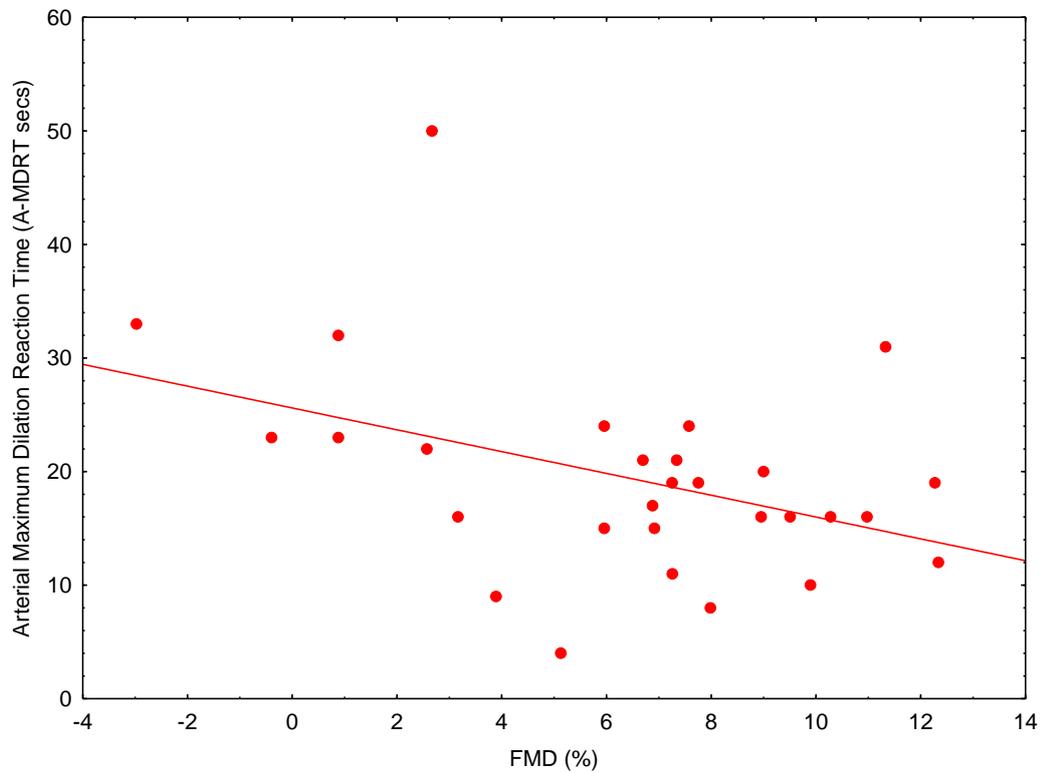
Figure 5.4. Negative average arterial MDRT correlation with brachial artery FMD in the IGT group (Spearmans r= -0.4546, p=0.017).

	Control [n=30]	IGT [n=30]	Post-hoc p-value
ARTERY bFR (%)			
Flicker cycle 1	3.17 (-9.11-13.70)	2.08 (-5.11-12.22)	0.173
Flicker cycle 2	3.38 (-5.13-13.42)	1.55 (-7.89-8.18)	**0.014**
Flicker cycle 3	2.94 (-10.12-16.64)	2.31 (-4.90-8.13)	0.415
Within-group ANOVA	0.417	0.820	
VEIN bFR (%)			
Flicker cycle 1	3.09 (-7.11-14.19)	3.05 (-3.98-12.27)	0.957
Flicker cycle 2	3.74 (-6.37-15.66)	3.19 (-6.88-14.53)	0.472
Flicker cycle 3	2.73 (-2.61-9.36)	2.95 (-4.21-11.28)	0.716

Table 5.8. Retinal arterial and venous bFR for each individual flicker cycle. Values quoted in mean (IQR). *Significant differences indicated in bold (p<0.05). bFR: baseline-corrected flicker response.
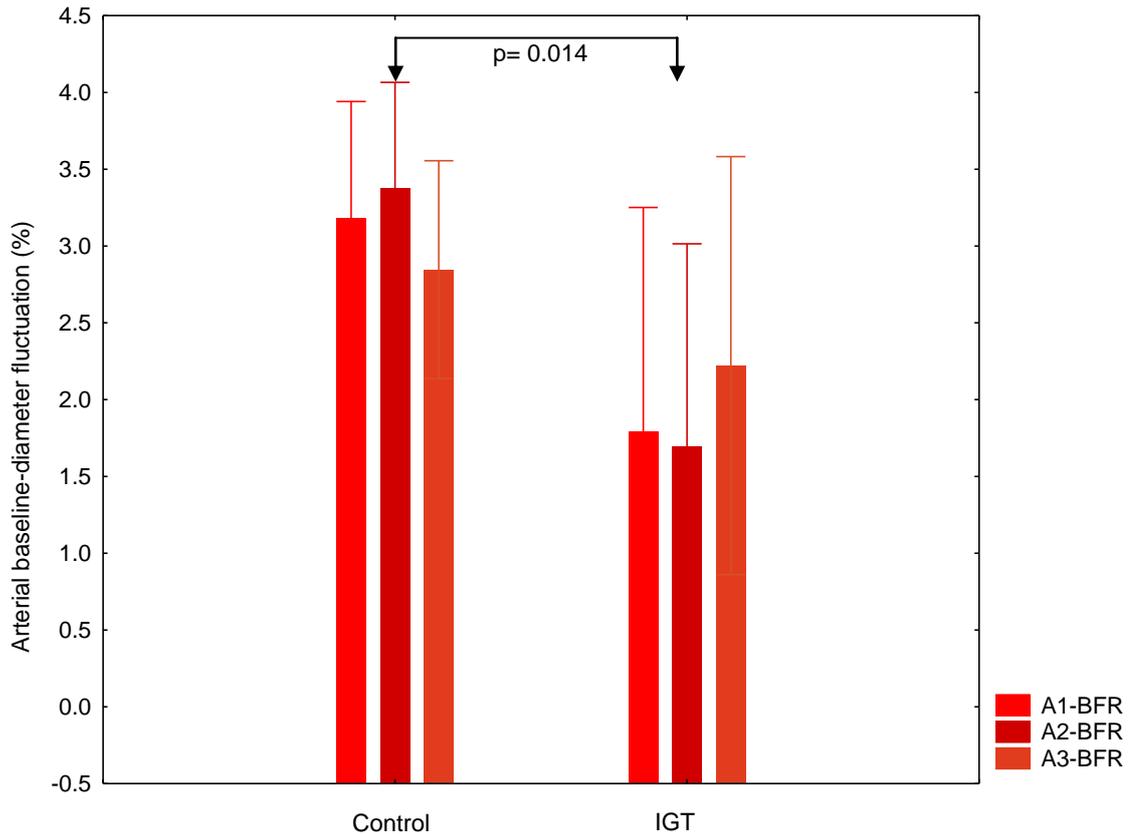
Figure 5.5. Arterial baseline-corrected retinal vessel response for each individual flicker cycle for the IGT group as compared to controls.

5.6. Discussion

It is thought that atherosclerotic processes already develop in a 'pre-diabetic' stage, i.e. during stages of IGT or when abnormal glucose haemostasis begins.[404] The results of this chapter furthers this suggestion, by illustrating that newly-diagnosed IGT patients present with levels of dyslipidaemia and higher, albeit within the normal range; systemic BP values that may contribute to future vascular risk for T2DM and CVD progression. These characteristic levels of higher TG and cholesterol ratios are indicative of the traits found with the MetS. Furthermore, the presence of impaired endothelial function detected by FMD and DVA shows that newly-diagnosed IGT individuals have blunted ocular and endothelial reactivity alongside reduced blood glutathione, increased vWF plasma levels and greater c-IMT than healthy controls.

The IGT individuals in this study exhibited associations with features of IR and MetS, including endothelial dysfunction (reduced FMD), markers of vascular inflammation

(increased vWF), hypertension, and dyslipidaemia (higher TG levels and cholesterol ratios).[405-408] Secondary analyses from large, international, randomised clinical trials have provided further supporting evidence, consistent with this study, that there is a clustering of traits of the MetS in those with IGT prior to progression into T2DM.[409-411] Although IGT is associated with IR and increased insulin secretion, it has been suggested that the IGT condition alone is not equivalent in predicting the development of T2DM or CVD.[412]

Therefore, the importance of assessing known markers of vascular and endothelial dysfunction by means of functional tests is well established and clinically necessary. The current IGT cohort showed evidence of impaired systemic endothelial function by means of blunted FMD responses. Abnormal vasomotion by means of FMD has also been found in first-degree relatives of those with T2DM; however, this observation is true mainly in those with IR. Therefore, IR itself appears to be associated with endothelial dysfunction, thus implicating the links between vascular insulin signalling alterations, endothelial dysfunction and insulin itself contributing to vascular damage and the cause of IR.[413]

Furthermore, the impaired vascular smooth muscle cell function, as indicated by a lower GID, alongside reduced FMD is also important to note in the current IGT group. Especially so as hyperglycaemia is known to elicit an activation of protein kinase-C, elevation in advanced glycation end (AGE) products and oxidative stress. These changes can then activate proliferation and migration of vascular smooth muscle cell inducing atherogenesis. Furthermore, impaired endothelial-independent brachial artery dilation in DM has been reported as a possible implication of increased oxidative stress.[414]

The findings of FMD abnormalities in IGT individuals also extends further to biochemical imbalances in the blood redox system as shown by differences in glutathione levels, and the endothelial-cell system by increased levels of vWF. Although this is supported by previous publications showing differences in biochemical markers (Table 5.6.), the current data is the first to our knowledge to report findings of imbalances in newly-diagnosed IGT individuals.[256] However as there were no correlations found, whether this indicates early oxidative stress and endothelial damage with future implications on vascular function and T2DM/CVD risk still requires further exploration.

In addition to systemic differences, the IGT sample also exhibited signs of impaired retinal vessel reactivity as assessed by the DRVA. It has been shown that the DRVA allows for quick and non-invasive assessment in a number of systemic conditions with ocular consequences. However, the data presented in this chapter shows that for the first time, DRVA and the newly-adopted SDRA can detect retinal vasomotion abnormalities in pre-

diabetes. This was evident in the current IGT individuals by means of greater fluctuations in arterial BDF that also extended to increased MDRT and a reduced bFR.

As suggested previously (Appendix 3), the increased arterial reaction time; MDRT, could be a useful measure in asymptomatic individuals and represent atherosclerotic vessel wall changes or increased arterial stiffness.[227, 415-419] Furthermore, the possibility of reduced NO bio-availability to peripheral tissues in IGT could also have an influence. Indeed, an increased MDRT could also characterise a combination of one or both factors.[216] This would be supported by the data of the current chapter showing increased c-IMT thickness and reduced FMD values in the normotensive IGT sample when compared to their age- and sex-matched counterparts. Whether a greater fluctuation in arterial diameter (BDF) has a similar aetiology remains to be seen. However, as shown previously in smokers, the greater BDF in those with IGT could suggest a greater propensity to impaired retinal vasomotion.[399]

The lack of association between these two retinal components, i.e. MDRT and BDF (r=0.341, p=0.065), could also suggest subtle differences in autoregulation within the retinal vascular architecture and the influence of these measures on retinal vascular function. The link with arterial BDF and dyslipidaemia furthers the argument that abnormal lipid metabolism is implicated in the causation of IR which, in turn, is linked to both endothelial dysfunction and vascular disease.[420] Furthermore, the correlation between FMD and SDRA components, namely brachial artery percentage dilation (FMD%) and retinal arterial dilation time (MDRT), suggests that pre-diabetes and possibly certain other vascular disorders, such as T2DM, can affect different vascular beds with diverse functions similarly. Therefore, the results of this chapter as shown by increased MDRT and reduced flicker response by means of bFR help to support the hypothesis that alterations in retinal vasculature are present in those with IGT. Furthermore, the correlations with SDRA and systemic markers help to support the theory of flicker-induced vasodilation as NO-mediated and thus reflecting endothelial function.[49] Whether this finding would extend to larger samples and support the use of SDRA as a surrogate marker to future vascular risk needs further work.

This increase in c-IMT also conforms to that generally observed with IGT subjects as a third of that usually seen in T2DM.[421] Therefore, the data in this chapter suggests that endothelial cell dysfunction can indeed be detected very early in the onset of IR in IGT subjects and that early structural change, indicated by a thickening of the c-IMT layer, and increased brachial diameter and larger fluctuations in retinal arterial diameter are found. Thus, IGT subjects show high risk for accelerated atherosclerosis and vascular disease as indicated by FMD, c-IMT and lipid results. However, as it has not been reported previously,

the study also suggests that for the first time, there are obvious changes at the retinal level in newly-diagnosed IGT individuals. Whether this would apply to larger samples, as the current sample was quite small, remains to be seen.

The signs of endothelial dysfunction with IGT also suggest that vascular damage, potentially associated with oxidative stress, vascular inflammation and thrombosis may also be present. Therefore, early recognition and treatment of IR are critical in the prevention of atherosclerosis and T2DM. Whether therapeutic or lifestyle interventions improve endothelial function in IR and also decrease CVD risk remain to be researched. Therefore, to justify pharmacological treatments for IGT, identification of high-risk individuals is necessary. Alongside this, a greater understanding of the underlying mechanisms and differences, if any, in vascular and endothelial function in IGT patients may help to improve the prediction of T2DM and enhance targeted therapeutics.

6. Vascular Function in Normal, Overweight and Obese Individuals of SA and WE ethnicity

6.1. Abstract

6.1.1. Background/Aims: To investigate ocular and systemic vascular reactivity to stress in otherwise healthy overweight and obese individuals and whether this would extend to differences in known markers of atherosclerosis and cardiovascular risk.

6.1.2. Methods: In this cross-sectional study, healthy participants (SA and WE) aged between 25-45 years classed as overweight (n=50) or obese (n=50) and lean age- and sex-matched controls (n=50) were recruited. Baseline measurements including body composition, carotid intimal-media thickness (c-IMT), 24-hour systemic blood pressure (BP), fasting plasma glucose, triglycerides (TG) and cholesterol were measured in all individuals. Retinal vessel reactivity to flickering light was assessed by means of the Dynamic Retinal Vessel Analyser (DRVA, Imedos GmbH, Jena) to a modified protocol. Additionally brachial artery reactivity to reactive hyperaemia by means of ultrasound was measured alongside fasting plasma von Willebrand factor (vWF) levels.

6.1.3. Results: Overweight and Obese individuals presented with levels of dyslipidaemia and CVD risk by means of increased c-IMT and Framingham scores. Moreover, it is only overweight individuals that showed levels of endothelial damage (increased vWF levels p=0.004), impaired coronary vasomotion (blunted brachial MD p=0.002), and reduced retinal artery reactivity to flicker (lower arterial MD p=0.039 and bFR p=0.022 alongside prolonged MDRT p=0.047).

6.1.4. Conclusion: Overweight individuals may have an increased risk for vascular disease when compared to lean individuals. Furthermore, the retinal functional abnormalities alongside classic cardiovascular and endothelial damage markers warrants further investigation into vascular disease risk in this group.

6.2. Introduction

Obesity is defined as a preventable condition characterised by abnormal or excessive fat accumulation and with prevalences of obesity doubling over the past three decades, it has also been identified as a major risk factor for non-communicable diseases such as T2DM and CVD.[422]   Furthermore, obesity is now ranked as the fifth leading cause for global

deaths, and in addition, a high proportion of co-morbidity and mortality related burdens (economical, social and healthcare) are attributable to obesity.

Obesity is a major risk factor for CVD and can induce several other major risk factors that are evident by increased arterial stiffness, carotid intimal media thickness and microvascular dysfunction.[423-425] Therefore, it is important to detect changes in vascular function that precede cardiovascular events; possible through studying endothelial function. Indeed, decreased endothelial function is a factor that predicts, independently from other risk factors, the occurrence of CVD.[324] Endothelial function can be assessed using a large variety of techniques, from laboratory markers, such as inflammatory cytokines, adhesion molecules, NO and markers of endothelial damage and repair, to vascular reactivity tests such as FMD of the brachial artery by ultrasound, forearm venous pletysmography, digital pulse amplitude tonometry and laser Doppler measurements of the peripheral circulation.[212] Microvascular assessments such as retinal vessel reactivity, however, can be performed quickly using relatively affordable techniques and offers reliable information on vascular function in patients suffering from various systemic diseases including DM, hypertension and hypercholesterolaemia.[214, 300] More recently DRVA and its proposed analysis methods have been proven useful in asymptomatic individuals and in those with established coronary disease.[221, 426]

As reported recently, retinal microvascular data on obese individuals has been distributed as a control group with BMI of normal-moderate weight (BMI 19-27.5 kg/m^2) and an obese group (BMI >27.5-29.9 kg/m^2).[304] Further to this, grouping overweight and obese individuals together (BMI >25 kg/m^2) also seems a plausible approach to investigate the effect of obesity on vascular function.

Therefore the aims of this study was to further microvascular data on the groups suggested previously (normal-moderate and obese), by incorporating sub-analysis of an overweight-obese group and the three groups (normal, overweight and obese) as recommended definitions by the WHO. Furthermore, the study aimed to investigate whether signs of impaired systemic (FMD) and biochemical (vWF) endothelial function was evident alongside previously reported retinal abnormalities in obese and overweight individuals.

6.3. Materials and Methods

The study cohort consisted of healthy normotensive participants aged 30-45 years (SA and WE with and without a FH) that were screened and recruited from the Health Clinics at Aston University, Birmingham, UK. Obesity classifications were determined according to WHO definitions, whereby normal weight was classed as a BMI of 18.5–24.9 kg/m^2; overweight individuals as a BMI of 25–29.9 kg/m^2; and obese subjects with a BMI of 30 kg/m^2 or greater.

Exclusion criteria were: a positive diagnosis of cardio- or cerebro-vascular disease, (coronary artery disease, heart failure, arrhythmia, stroke, transient ischaemic attacks), peripheral vascular disease, severe dyslipidaemia (defined as plasma TG>6.00mmol/L or cholesterol levels>7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment.

Furthermore, subjects were excluded if they had a refractive error of more than ±3DS and more than ±1DC equivalent, IOP >24 mmHg, cataract or any other media opacities, as well as if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system.

Written informed consent was obtained from all participants and ethical approval was sought by the author from local and NHS ethical committees (08/H1202/112). The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki.

According to an already established procedure when examining endothelial function, female participants were asked to fill in a validated menstrual cycle questionnaire and their investigations were carried out during the first week of the menstrual cycle (follicular phase) thereby controlling for hormonal influences on endothelial function.[112, 317]

6.3.1. Investigations
6.3.1.1. Blood sampling and analyses

All participants were asked to fast and refrain from caffeine, alcohol, chocolate and carbonated drinks and to not exercise for 12 hours prior to the date of the study. All blood samples were obtained by a qualified phlebotomist in the morning, between 9am and 10am. Fasting plasma glucose, TG, total and HDL-C were measured using standard routine laboratory techniques using the Reflotron Desktop Analyser (Roche Diagnostics, UK) as described in Section 3.3.1.1. The TG/HDL cholesterol ratio, a measure of endothelial function, [244], and Framingham score as a means of cardiovascular risk (Section 3.3.1.1.) was also determined from the above values.

Further analysis extended to laboratory and ELISA-test plasma sampling for vWF to investigate possible signs for endothelial damage. Validated protocols were used for in-house testing as described in Section 2.4.

6.3.1.2. ABPM and HRV analysis

Systemic BP was measured using a 24-hour computer-operated ambulatory BP and electrocardiography (ECG) monitor (Cardiotens-01, Meditech Ltd., Hungary) for each subject. Measurements were performed in ambulatory conditions and programmed to measure BP oscillometrically every 15 minutes during the subject's active period and every 30 minutes in the passive period (as explained in Sections 2.6.). Mean arterial pressure (MAP), as a means of describing cardiac output function in relation to arteriolar resistance was calculated according to Equation 2.2. The impact of pulse pressure (PP), as a risk factor for CVD was also calculated by using Equation 4.1.

HRV analysis was calculated for each individual using the Cardiovision software (PMS Instruments Ltd., Maidenhead, UK) to obtain twenty-four hour, active and passive period LF, HF and LF:HF values. From, this circadian BP and HRV changes were calculated according to Formula 2.3. and 2.4. respectively.

6.3.1.3. Body Composition Analysis

Anthropometric measures including height and weight were measured using standard procedures as outlined in Section 3.3.1.3. Body composition was measured using bioelectrical impedance (Biostat 220, Biospace, UK) to determine BMI, PBF, WHR, total fat and fat free mass.

6.3.1.4. Intima-media thickness

High-resolution B-mode ultrasound system (Acuson Sequoia, 5 MHz linear transducer, Siemens, USA) was used to obtain longitudinal images (described in Section 3.3.1.4.) of the right and left extracranial far wall of the CCA. Each IMT measure, also used for statistical analysis, represented an average of three to five measurement points.

6.3.1.5. Vascular function studies

Retinal vessel reactivity was measured using the DRVA (Imedos; GmbH, Jena, Germany). All measurements were performed in one unselected eye for each subject, between 8:00 and 11:00 AM in a quiet, temperature-controlled room (22°C). Following full pupil dilation with Tropicamide 1% (Minims; Chauvin Pharmaceuticals Ltd, UK) a region of interest encompassing vessel segments of approximately 500 μm was chosen. Retinal diameters were assessed continuously over 350 seconds according to an accepted and widely used protocol.[277, 332]

Brachial artery FMD was measured using high-resolution CDI ultrasonography, with a 7 mm 8MHz linear-array (Siemens; Acuson Sequoia, UK). According to a published protocol,[308], as described in Section 2.2., arterial occlusion was created by inflation of a sphygmomanometer cuff on the lower arm to suprasystolic pressure and an exogenous NO donor (sublingual 0.3 mg GTN tablet) was given to determine the maximum obtainable vasodilator response. FMD was then expressed as the maximal artery dilation during hyperaemia from baseline (%).

6.4. Power calculation and statistical analysis

Based on previous studies, a change of 28% with a SD of 1.8% in retinal vessel reactivity was shown to be significant.[304] Additionally FMD studies have shown a 15-45% reduction with a SD of 2.7-4.0% in FMD response in young obese individuals.[233, 427] As the study design was multi-factorial in nature it was calculated (using SISA; a web-based sample calculator approved by the NHS ethics committee) that n=40 per group was sufficient to provide 90% power with an alpha of 0.05. Furthermore, the sensitivity and reproducibility of the techniques in healthy subjects has been reported previously.[112, 333]

All analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). Prior to any analysis, all data was tested for normal distribution and thus a suitable test was adopted. Differences in mean values for each of the measured variables were compared by independent samples t-test (or Wilcoxon) for continuous variables. A multivariate analysis was performed to test the influence of age, gender, BP, and circulating markers on the measured variables. Comparison of retinal vessel reactivity for each flicker period was made by repeated measures (or Friedman) analysis of variance (ANOVA) following within-group analysis. Differences between groups in retinal and systemic vascular function were

computed by analysis of covariance (ANCOVA). Univariate linear regression analysis was carried out using appropriate correlation analysis. A p value of <0.05 was considered statistically significant, unless stricter criteria were adopted for within-group and multivariate (p≤0.01 to account for multiple comparisons and thereby minimise bias towards Type II errors).

6.5. Results

A total of 160 subjects were screened for eligibility. Following OGTT, 10 subjects were excluded (1 diagnosed with T2DM and 9 with IGT) and the remaining 150 healthy were recruited and allocated to normal (n=50), overweight (n=50) or obese (n=50) groups for the final protocol (Appendix 1).

The baseline characteristics of normal-moderate and obese groups are presented in Table 6.1. There were no significant differences in age, IOP, Total Cholesterol, HDL cholesterol (albeit close with p=0.05), LDL cholesterol, and cholesterol ratios (p>0.05). Compared to healthy normo -glycaemic and –tensive normal-moderate subjects, obese individuals had higher, albeit normal fasting glucose levels and greater TG levels (p=0.048 and p=0.047 respectively). Furthermore, obese individuals presented with greater systemic BP values (p<0.001), c-IMT measurements (p=0.005 and p=0.006) as well as greater CVD risk as indicated by higher Framingham scores (p=0.007).

There were no evident signs of endothelial damage or dysfunction as found by comparable vWF levels amongst both groups (p=0.452). Unlike that reported previously the obese group also showed no significant differences in retinal vessel reactivity or SDRA components when compared to normal-moderates (p>0.05). This also did not extend to apparent differences in FMD between both groups (p>0.05).

	Normal-Moderate [n=100]	Obese [n=50]	p-value
DEMOGRAPHIC DATA			
Age (years)	41.9 ± 10.8	43.2 ± 10.2	0.075
SBP (mmHg)	117 ± 10	125 ± 12	**<0.001**
DBP (mmHg)	75 ± 10	80 ± 9	**<0.001**
MAP (mmHg)	89 ± 11	94 ± 11	**0.010**
IOP (mmHg)	13 ± 2	14 ± 4	0.077
BODY COMPOSITION DATA			
Weight (kg)	71.9 ± 11.2	95.8 ± 15.5	**<0.001**
BMI (kg/m^2)	24.6 ± 3.2	33.2 ± 3.5	**<0.001**
WHR (AU)	0.92 ± 0.10	1.01 ± 0.05	**<0.001**
PBF (%)	28.8 ± 7.7	33.2 ± 7.4	**<0.001**
Fat Mass (kg)	20.6 ± 5.9	36.1 ± 8.5	**<0.001**
Fat Free Mass (kg)	51.3 ± 10.3	59.6 ± 12.8	**<0.001**
METABOLIC DATA			
Glucose (mmol/L)	5.49 ± 0.58	5.76 ± 0.47	0.048*
2 hour GTT (mmol/L)	7.04 ± 2.22	7.54 ± 1.54	0.368
TG (mmol/L)	1.25 ± 0.63	1.52 ± 0.79	0.047*
HDL Cholesterol (mmol/L)	1.21 ± 0.40	1.05 ± 0.28	0.050
LDL Cholesterol (mmol/L)	2.63 ± 0.84	2.56 ± 0.81	0.672
Total Cholesterol (mmol/L)	4.40 ± 0.91	4.30 ± 0.79	0.571
TG:HDL-C (mmol/L)	2.86 ± 2.59	3.68 ± 2.30	0.115
CVD RISK DATA			
R-IMT (mm)	0.055 ± 0.014	0.067 ± 0.016	**0.005**
L-IMT (mm)	0.059 ± 0.016	0.072 ± 0.020	**0.006**
Framingham Score (%)	1.9 ± 2.9	4.0 ± 5.4	**0.007**
Total:HDL (mmol/L)	4.01 ± 1.48	4.42 ± 1.77	0.204
BIOCHEMICAL DATA			
v-WF (µ/dL)	127.3 ± 50.6	136.7 ± 50.4	0.452

Table 6.1. Baseline data of both groups. Values quoted in mean ± SD. Significant differences indicated in bold as p<0.01. *Significant differences if p<0.05. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness; vWF: von-willebrand factor.

The data presented in Table 6.2. shows the demographic result of groups when organised into a normal weight and an overweight-obese sample. When compared to healthy lean individuals, the overweight-obese group showed greater systemic BP values as indicated by higher SBP, DBP and MAP (p<0.001). Furthermore, there were significant differences in glucose (p=0.010), TG (<0.001), HDL (p=0.010) and cholesterol ratios (p=0.008 and

p=0.009). This ultimately led to greater c-IMT measurements (p=0.010 and p<0.001) but not higher Framingham scores (p=0.093).

Alongside obvious differences in body composition and obesity indices, the overweight-obese group also showed greater levels of blood vWF (p=0.004) that also extended to differences in FMD as shown in Table 6.3. and a reduced retinal arterial dilatory response (as well as near statistical differences in arterial DA) in Table 6.4.

	Normal [n=50]	Overweight-Obese [n=100]	p-value
DEMOGRAPHIC DATA			
Age (years)	41.7 ± 11.1	42.6 ± 10.3	0.832
SBP (mmHg)	114 ± 14	124 ± 13	**<0.001**
DBP (mmHg)	72 ± 10	79 ± 9	**<0.001**
MAP (mmHg)	86 ± 10	93 ± 10	**<0.001**
IOP (mmHg)	13 ± 3	14 ± 3	0.166
BODY COMPOSITION DATA			
Weight (kg)	65.4 ± 9.4	84.2 ± 14.3	**<0.001**
BMI (kg/m^2)	22.4 ± 3.1	29.2 ± 3.5	**<0.001**
WHR (AU)	0.90 ± 0.14	0.97 ± 0.05	**<0.001**
PBF (%)	25.8 ± 7.1	33.9 ± 7.9	**<0.001**
Fat Mass (kg)	16.8 ± 5.3	28.4 ± 8.0	**<0.001**
Fat Free Mass (kg)	48.7 ± 9.2	55.8 ± 11.7	**<0.001**
METABOLIC DATA			
Glucose (mmol/L)	4.78 ± 0.75	5.09 ± 0.77	**0.010**
2 hour GTT (mmol/L)	6.67 ± 2.29	7.39 ± 2.00	0.077
TG (mmol/L)	1.08 ± 0.41	1.46 ± 0.77	**<0.001**
HDL Cholesterol (mmol/L)	1.27 ± 0.41	1.11 ± 0.35	**0.010**
LDL Cholesterol (mmol/L)	2.53 ± 0.79	2.67 ± 0.86	0.343
Total Cholesterol (mmol/L)	4.30 ± 0.83	4.44 ± 0.91	0.341
TG:HDL-C (mmol/L)	2.36 ± 2.06	3.49 ± 2.75	**0.008**
CVD RISK DATA			
R-IMT (mm)	0.052 ± 0.015	0.061 ± 0.015	**0.010**
L-IMT (mm)	0.053 ± 0.016	0.067 ± 0.017	**<0.001**
Framingham Score (%)	1.7 ± 2.4	2.8 ± 4.3	0.093
Total:HDL (mmol/L)	3.70 ± 1.32	4.38 ± 1.64	**0.009**
BIOCHEMICAL DATA			
v-WF (µ/dL)	112.4 ± 58.7	140.7 ± 53.3	**0.004**

Table 6.2. Baseline data of both groups. Values quoted in mean ± SD. Significant differences indicated in bold as p<0.01. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness; vWF: von-willebrand factor.

	Normal [n=50]	Overweight-Obese [n=100]	p-value
BRACHIAL ARTERY			
AD (mm)	4.28 (3.75-4.77)	3.76 (3.20-4.31)*	**<0.001**
BDF (mm)	0.35 (0.18-0.44)	0.36 (0.21-1.42)	0.808
MD (mm)	4.67 (4.04-5.18)	4.11 (3.53-4.69)*	**<0.001**
RT (secs)	22.0 (3.0-46.0)	23.0 (2.0-42.0)	0.811
FMD (%)	7.83 (2.67-9.48)	5.98 (3.66-11.94)	0.083
GTN			
GTN-MD (mm)	5.27 (4.75-5.61)	4.75 (4.13-5.64)*	**0.012**
RT (secs)	313 (265-400)	327 (277-383)	0.546
GID (%)	21.32 (14.16-27.25)	26.27 (18.59-31.00)	0.109
FMD/GID (%)	0.27 (0.12-0.55)	0.13 (0.05-0.44)	0.430

Table 6.3. Brachial artery reactivity as means of systemic vascular endothelial function between both groups. *Significant values in bold (p<0.05). Values quoted in mean (IQR). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.

	Normal [n=50]	Overweight-Obese [n=100]	p-value
ARTERY			
AD (AU)	121.59 (112.40-132.60)	126.09 (112.87-133.84)	0.120
BDF (AU)	5.71 (3.75-7.09)	5.00 (2.67-6.44)	0.146
MD (%)	5.55 (3.66-7.12)	4.55 (2.82-5.74)*	**0.029**
MDRT (secs)	17.2 (12.3-20.7)	19.1 (14.3-24.3)	0.803
MC (%)	3.24 (1.66-4.57)	3.09 (1.49-3.96)	0.686
MCRT (secs)	19.7 (17.3-23.3)	20.4 (17.3-23.3)	0.444
DA (%)	8.78 (6.78-10.18)	7.63 (5.09-8.93)	0.061
bFR (%)	3.07 (1.02-4.83)	2.63 (0.89-3.95)	0.357
VEIN			
AD (AU)	156.73 (139.18-173.78)	156.20 (142.48-167.16)	0.896
BDF (AU)	4.13 (2.56-5.12)	4.23 (2.67-5.27)	0.770
MD (%)	5.59 (4.44-6.54)	5.87 (3.84-6.70)	0.540
MDRT (secs)	20.1 (17.3-22.7)	29.9 (17.0-23.0)	0.870
MC (%)	1.44 (0.33-4.84)	1.72 (0.62-2.10)	0.375
MCRT (secs)	21.8 (19.7-25.3)	21.2 (18.7-23.7)	0.517
DA (%)	7.27 (5.33-9.00)	7.31 (4.76-9.27)	0.942
bFR (%)	3.12 (1.74-4.40)	3.13 (1.19-4.72)	0.990

Table 6.4. Retinal arterial and venous measures for both groups. Values quoted in mean (IQR). *Significant values in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum dilation; MDRT: reaction time to reach maximum diameter to flicker stimulation; MC: maximum constriction; MCRT: reaction time to maximum constriction post flicker; DA: dilation amplitude; bFR: baseline-corrected flicker response.

The characteristics for normal, overweight and obese individuals were also investigated and shown in Table 6.5. Obese subjects showed significant levels of dyslipidaemia (higher TG, lower HDL causing higher TG:HDL-C and Total:HDL ratios) and greater fasting glucose values when compared to normal weight subjects. Furthermore, the BP profiles were elevated with obesity (p<0.001), contributing to a greater overall Framingham score (p<0.001) and c-IMT.

The overweight group presented with similar results showing greater TG and glucose levels alongside higher cholesterol ratios and systemic BP values. This also extended to significant differences in left-side c-IMT, weight indices and vWF levels (p<0.01).

Tables 6.6. and 6.7. shows the systemic (brachial artery) and the ocular (retinal artery and vein) reactivity to shear stress and flickering light respectively. Obese individuals showed no apparent differences in reactivity at a systemic or ocular level presenting with similar values. It is only when investigating the overweight group that significant disparities are found. This is shown by reduced brachial artery diameter and maximal obtainable diameter (Figure 6.1.) following reactive hyperaemia (MD), increased retinal artery MDRT and reduced arterial MD (Figure 6.2.) and bFR. However this did not extend into venous reactions (p>0.05).

Correlational analysis in obese individuals showed no significant associations between retinal and brachial reactivity and anthropometric measures.

Overweight individuals showed strong correlations between macrovascular function (brachial MD) and obesity correlates (fat free mass and PBF) as shown in Figure 6.3 (MD and fat free mass (r= 0.620, p<0.001), MD and PBF (r=-0.589, p<0.001). However this did not extend to known correlations between SDRA components and obesity correlates (p>0.05)

	Normal [n=50]	Overweight [n=50]	Obese [n=50]
DEMOGRAPHIC DATA			
Age (years)	41.7 ± 11.1	42.2 ± 10.6	43.2 ± 10.2
SBP (mmHg)	114 ± 14	123 ± 12*	125 ± 12*
DBP (mmHg)	72 ± 10	11 ± 9*	80 ± 9*
MAP (mmHg)	86 ± 10	92 ± 9*	94 ± 11*
IOP (mmHg)	13 ± 3	14 ± 2	14 ± 4
BODY COMPOSITION DATA			
Weight (kg)	65.4 ± 9.4	78.5 ± 9.3*	95.8 ± 15.5*†
BMI (kg/m^2)	22.4 ± 3.1	27.4 ± 1.3*	33.2 ± 3.5*†
WHR (AU)	0.90 ± 0.14	0.95 ± 0.03*	1.01 ± 0.05*†
PBF (%)	25.8 ± 7.1	32.1 ± 7.6*	33.2 ± 7.4*†
Fat Mass (kg)	16.8 ± 5.3	24.8 ± 4.7*	36.1 ± 8.5*†
Fat Free Mass (kg)	48.7 ± 9.2	53.7 ± 10.7*	59.6 ± 12.8*†
METABOLIC DATA			
Glucose (mmol/L)	4.78 ± 0.75	5.12 ± 0.64*	5.76 ± 0.47
2 hour GTT (mmol/L)	6.67 ± 2.29	7.33 ± 2.14	7.54 ± 1.54
TG (mmol/L)	1.08 ± 0.41	1.42 ± 0.79*	1.52 ± 0.79*
HDL Cholesterol (mmol/L)	1.27 ± 0.41	1.14 ± 0.38	1.05 ± 0.28*
LDL Cholesterol (mmol/L)	2.53 ± 0.79	2.74 ± 0.88	2.56 ± 0.81
Total Cholesterol (mmol/L)	4.30 ± 0.83	4.53 ± 0.97	4.30 ± 0.79
TG:HDL-C (mmol/L)	2.36 ± 2.06	3.37 ± 2.95*	3.68 ± 2.30*
CVD RISK DATA			
R-IMT (mm)	0.052 ± 0.015	0.058 ± 0.014	0.067 ± 0.016*†
L-IMT (mm)	0.053 ± 0.016	0.064 ± 0.015*	0.072 ± 0.020*
Framingham Score (%)	1.7 ± 2.4	2.1 ± 3.4	4.0 ± 5.4*
Total:HDL (mmol/L)	3.70 ± 1.32	4.36 ± 1.57*	4.42 ± 1.77*
BIOCHEMICAL DATA			
v-WF (μ/dL)	112.37 ± 58.67	141.24 ± 55.36*	136.73 ± 50.38

Table 6.5. Baseline data of all groups. Values quoted in mean ± SD. *Significant differences when compared to normal weight controls (p<0.01). †Significant differences when overweight and obese groups compared (p<0.01). SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; IOP: intraocular pressure; BMI: body mass index; WHR: waist-to-hip ratio; PBF: percentage body fat; GTT: glucose tolerance test; TG: triglyceride; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG:HDL-C: TG-to-HDL ratio; R-IMT: right intima media thickness L-IMT: left intima media thickness; vWF: von-willebrand factor.

	Normal [n=50]	Overweight [n=50]	Obese [n=50]
BRACHIAL ARTERY			
AD (mm)	4.28 (3.75-4.77)	**3.77** (3.20-4.32)*	4.39 (3.94-4.93)
BDF (mm)	0.35 (0.18-0.44)	0.36 (0.20-0.43)	0.34 (0.21-0.44)
MD (mm)	4.67 (4.04-5.18)	**4.11** (3.53-4.72)*	4.68 (3.98-5.17)
RT (secs)	22.0 (3.0-46.0)	21.9 (3.0-43.0)	25.0 (9.0-32.0)
FMD (%)	7.83 (2.67-9.48)	7.78 (3.59-12.07)	5.15 (2.98-9.64)
GTN			
GTN-MD (mm)	5.27 (4.75-5.61)	4.77 (4.13-5.64)	5.52 (5.41-5.94)
RT (secs)	313 (265-400)	330 (285-383)	341 (316-400)
GID (%)	21.32 (14.16-27.25)	26.45 (18.59-31.00)	23.96 (17.76-29.43)
FMD/GID (%)	0.27 (0.12-0.55)	0.11 (0.05-0.44)	0.17 (0.10-0.51)

Table 6.6. FMD data between all groups. Values quoted in mean (IQR). *Significant values in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum diameter response; RT: Reaction time; FMD: flow-mediation dilation response; GID: GTN-induced dilation; FMD/GID: FMD/GID ratio.
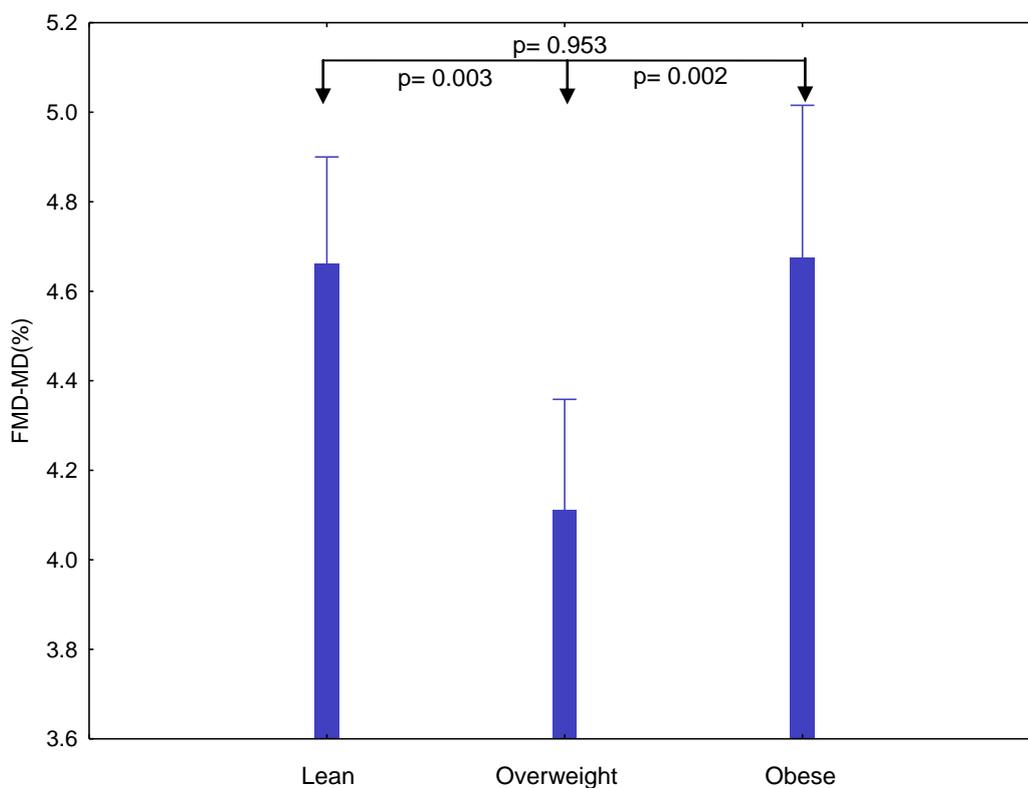

Figure 6.1. Brachial artery MD for all groups

	Normal [n=50]	Overweight [n=50]	Obese [n=50]
ARTERY			
AD (AU)	121.59 (112.40-132.60)	120.96 (109.19-131.22)	122.30 (113.20-133.84)
BDF (AU)	5.71 (3.75-7.09)	5.04 (2.77-6.67)	5.24 (3.32-6.90)
MD (%)	5.55 (3.66-7.12)	**4.54** (2.80-5.98)*	5.44 (3.59-7.18)
MDRT (secs)	17.2 (12.3-20.7)	**20.5** (14.0-24.0)*	18.1 (13.5-21.0)
MC (%)	3.24 (1.66-4.57)	3.14(1.58-4.59)	2.78 (1.51-3.57)
MCRT (secs)	19.7 (17.3-23.3)	20.3 (17.2-23.2)	21.2 (19.0-24.7)
DA (%)	8.78 (6.78-10.18)	7.68 (5.24-9.00)	8.15 (5.39-10.18)
bFR (%)	3.07 (1.02-4.83)	**2.12** (0.43-4.05)*	2.91 (0.85-4.77)
VEIN			
AD (AU)	156.73 (139.18-173.78)	156.00 (142.47-166.35)	151.92 (137.24-167.56)
BDF (AU)	4.13 (2.56-5.12)	4.29 (2.68-5.31)	4.31 (1.56-5.25)
MD (%)	5.59 (4.44-6.54)	5.64 (3.87-6.71)	6.94 (5.18-7.67)
MDRT (secs)	20.1 (17.3-22.7)	19.9 (17.0-23.2)	19.8 (17.0-22.7)
MC (%)	1.44 (0.33-4.84)	1.75 (0.63-2.16)	1.07 (0.22-1.79)
MCRT (secs)	21.8 (19.7-25.3)	21.1 (18.6-23.7)	22.2 (19.5-26.0)
DA (%)	7.27 (5.33-9.00)	7.39 (4.83-9.31)	7.99 (6.04-10.12)
bFR (%)	3.12 (1.74-4.40)	3.16 (1.13-4.91)	3.73 (2.06-5.09)

Table 6.7. Retinal arterial and venous measures for all groups. Values quoted in mean (IQR).   *Significant values in bold (p<0.05). AD: absolute diameter; BDF: baseline diameter fluctuation; MD: maximum dilation; MDRT: reaction time to reach maximum diameter to flicker stimulation; MC: maximum constriction; MCRT: reaction time to maximum constriction post flicker; DA: dilation amplitude; bFR: baseline-corrected flicker response.
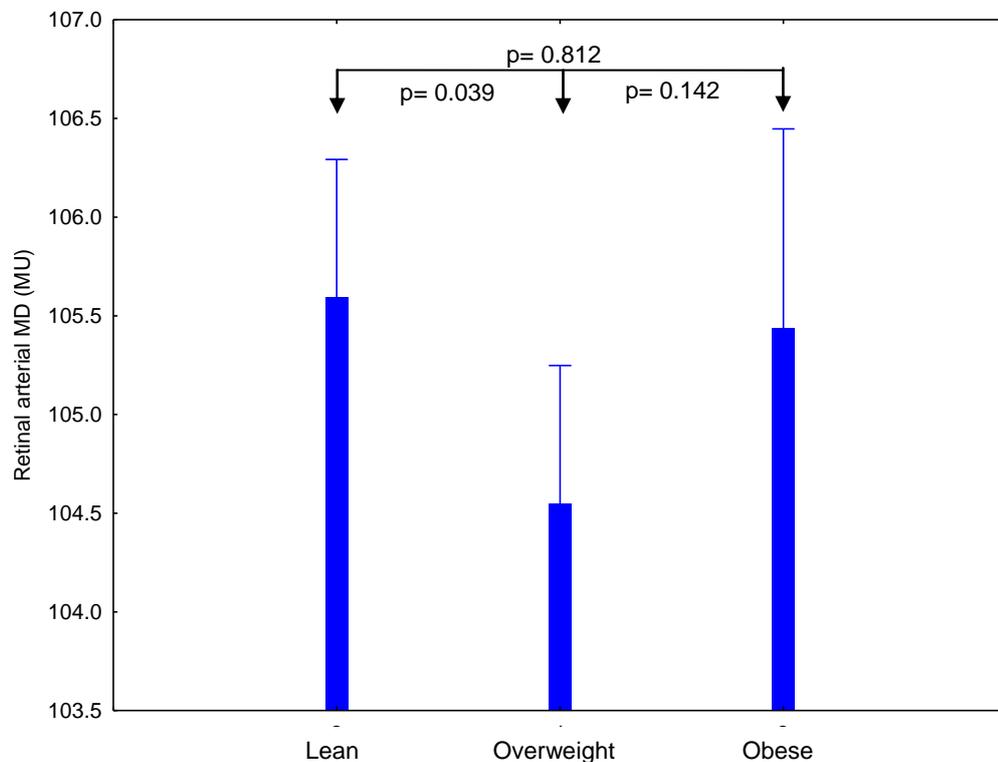

**Figure 6.2. Retinal arterial MD differences between all groups.**

129
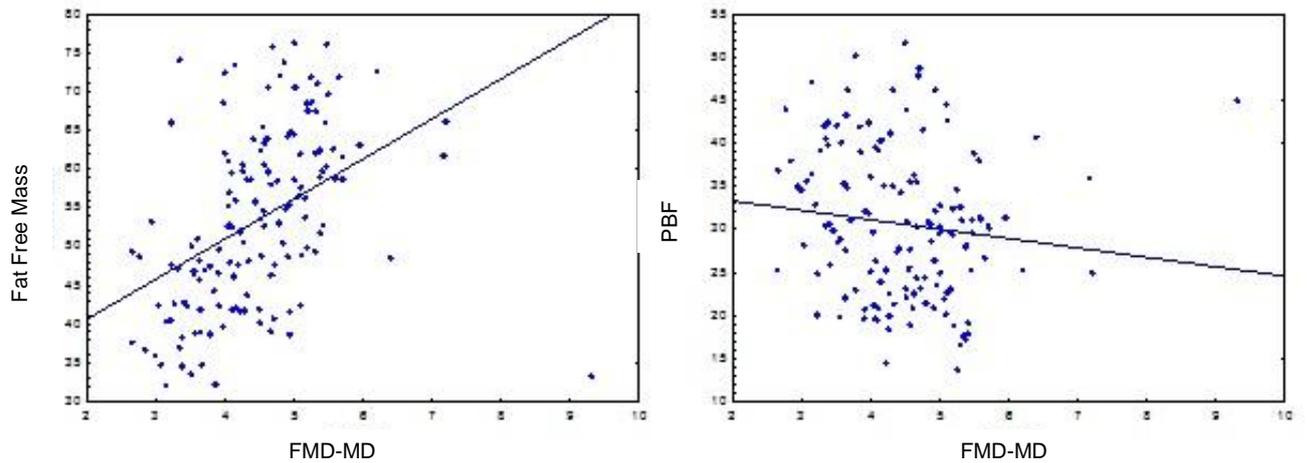
Figure 6.3. Brachial artery MD and obesity correlate analyses in the overweight sample. Left-hand side: spearmans correlation between MD and fat free mass (r= 0.620, p<0.001). Right-hand side: Spearmans correlation between MD and PBF (r=-0.589, p<0.001).

The overweight group also showed a moderate correlation (Figure 6.4) between brachial artery MD and retinal artery MD (spearmans r=0.31, p=0.021) that was not evident in lean (r=0.11, p=0.412) or obese (r=0.29, p=0.140) individuals.
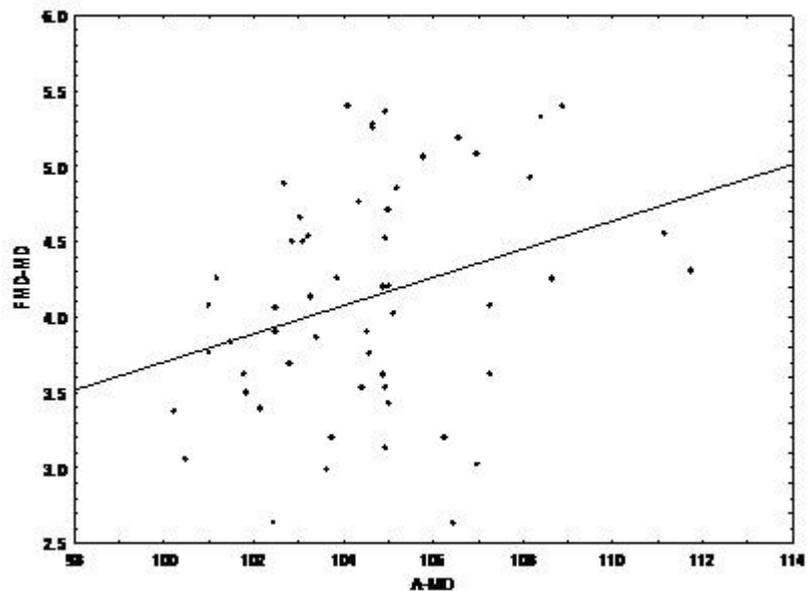

Figure 6.4. Brachial artery MD and retinal artery MD correlation in the overweight sample. Spearmans correlation (r=0.31, p=0.021).

6.6. Discussion

This study examined the effects of obesity and overweight on vascular function at the micro- and macrovascular level. Additionally, the study aimed to further current human microcirculation data by investigating other markers of atherosclerosis and endothelial damage alongside Framingham risk scores and body composition analyses.

The risk of obesity has been shown to be associated with retinal arteriolar attenuation and venous dilation as well as heightened CVD risk and mortality rates.[428-430] Moreover, there is extremely limited data on retinal vascular function by means of retinal vessel reactivity in obesity.[298, 304] The results presented in this paper shows work for the first time investigating early markers of endothelial dysfunction (brachial artery FMD), morphological arterial changes (c-IMT) and biochemical markers of inflammation (vWF) together with retinal vessel reactivity and CVD risk scores in obese and overweight individuals.

By doing this, the results show that those that are obese, as well as those that are overweight present with patterns of dyslipidaemia, irregular systemic BP and abnormal body composition. More importantly, the results in this thesis show for the first time that overweight individuals may present with greater CVD risk, future endothelial cell damage and impaired vascular function as found by increased c-IMT and vWF levels as well as attenuated brachial artery FMD (blunted MD) and retinal arterial dilation responses (reduced MD and bFR and prolonged MDRT).

Many clinical trials have also found a variety of vascular alterations in overweight and obese subjects demonstrating not only an increased c-IMT, elevated BP values, higher TG, cholesterol and fasting blood glucose values, but also increased CRP and fasting insulin concentrations compared with normal weight subjects.[431] Furthermore, there is data to suggest that these alterations affect the structural and functional properties of macro- and microvasculature. Therefore, the data provided by this study supports previous clear evidence that being overweight or obese, are associated with dyslipidaemia, atherosclerosis, and alterations of major and minor blood vessels factors that are determinants of increased risk for cardiovascular diseases.

Obesity has also been suggested to impair brachial artery FMD but no obvious associations have been found with BMI in overweight and obese individuals. Although visceral obesity more strongly predicts endothelial function than BMI, obesity might not be a strong predictor of endothelial function in all populations. This is also confirmed by our own analyses showing

a non-significant correlation between BMI and FMD (r=0.041 and p=0.771 respectively). The present data however, does suggest that impairment in endothelial function by means of reduced brachial artery MD may represent a pathophysiological mechanism linking being overweight to CVD risk.[432] This is supported by correlations (Figure 6.3.) between brachial artery MD and fat free mass (spearmans r=0.620, p<0.001) and PBF (spearmans r=-0.589, p<0.001), but it is important to note that only a weak, but significant, correlation was found between brachial MD and vWF (spearmans r=0.283, p=0.049).

Associations between increased BMI with pro-thrombotic factors, such as vWF, have also been reported previously, suggesting that obesity is indeed a risk factor whose effect is mediated partly by a pro-thrombotic state.[433] Despite significant differences in blood vWF levels in the current overweight group, no other correlations were found with anthropometric features to suggest similar vascular aetiology.

Obesity may also extend to profound retinal effects, causing changes in retinal vasculature. For instance, an association between retinal arteriolar narrowing with obesity has been reported in different populations suggesting a possible role in microvascular structural change with the pathogenesis of weight gain.[428] The results of this paper show that using the newly-developed SDRA, as reported previously, allows for a valid means of screening in at risk groups, in particular overweight individuals.[216, 221] Although no differences were found in retinal vessel reactivity for obesity. Interestingly, overweight individuals showed early signs of impaired retinal vascular function by means of reduced arterial MD, bFR and delayed MDRT. As suggested previously, SDRA could be a useful measure in asymptomatic individuals and the reduced arterial MD and bFR may represent the possibility of reduced NO bio-availability to peripheral tissues in overweight individuals. Atherosclerotic vessel wall changes or increased arterial stiffness may also play an influence in impaired retinal reactivity.[415, 417] Indeed, an increased MDRT could also characterise a combination of one or both factors.[216] This would be supported by the data of the current work showing increased c-IMT thickness and reduced FMD values in the overweight sample when compared to their age- and sex-matched lean counterparts. Despite a weak-moderate correlation between retinal and brachial artery MD (Figure 6.4.), there were no further correlations with body composition, atherosclerosis measures or biochemical markers of endothelial function.

Despite the newly reported applications of SDRA and validated markers of endothelial cell function and CVD risk in an overweight sample the relatively small sample size in this study only allows for generalised assumptions relating specifically to this sample to be made.

Larger studies would need to be conducted to investigate the vascular differences between overweight and obese individuals more closely. Furthermore, the use of SDRA in "at-risk" individuals including obesity and overweight needs further longitudinal and cross-sectional work. Additionally, adiposity has been found to be associated with insulin resistance (IR) and dyslipidemia, and as we did not identify insulin sensitivity in our groups, the results cannot be judged to be due to IR in the current overweight cohort.[432]

The preliminary findings of this study suggest that the adverse effect of obesity on the long term risk of CVD may begin in those that are overweight. This is evident in apparent differences in cardiovascular markers (dyslipidaemia and c-IMT), biochemical analysis (vWF) and ocular (MD, bFR and MDRT) and systemic (brachial MD) vascular function. Whether these differences lead to a greater propensity for future CVD and vascular disease risk remains to be seen. Future work would, therefore, need to investigate the pathophysiological mechanisms behind obesity/overweight and vascular function.

7. Summary of Findings on Vascular Function in those at Risk of T2DM and CVD

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7.1. Aims

The effect of atherosclerosis, vascular inflammation and endothelial dysfunction on the pathophysiology of T2DM and CVD has been extensively reported. The impact of endothelial dysfunction as a precursor to vascular atherogenesis in known individuals at risk of T2DM however, has been scarcely investigated. Furthermore, the presence of haemodynamic relationships between macro- and micro-vasculature, if any, has also not been fully explored.

The studies outlined in this thesis aimed to explore the use of surrogate markers for endothelial/vascular function and CVD risk by means of retinal vessel reactivity and SDRA against already available and validated measures (FMD, c-IMT, Framingham, vWF and ET-1). Thus, any outcomes showing the relevance of retinal vascular function against these measures could impact new avenues in screening, diagnostic and therapeutic for T2DM and CVD.

The aims of this thesis, therefore, were to investigate vascular function; by means of systemic, ocular and circulating plasma markers for endothelial function in relation to the risk of T2DM and CVD. The findings, including any established differences and associations between ocular and systemic function, are summarised below.

7.2. Vascular Function in Normoglycaemic Individuals with and without a FH of T2DM

Many studies have reported metabolic and vascular abnormalities in normoglycaemic individuals with a FH of T2DM. Vascular dysfunction has been investigated by brachial artery and skin microcirculation reactivity. Furthermore, elevated markers in plasma levels of vasoconstrictors, coagulators and inflammation have been found alongside increased c-IMT and decreased aortic distensibility in FH groups.[37, 234, 238, 340-343] However, the results of this thesis do not follow that reported previously. This furthers the argument about whether multiple vascular abnormalities are related to the presence of IR and/or are genetically determined. Therefore, further investigations would deserve evaluations of insulin sensitivity/resistance by means of a euglycaemic clamp.

7.3. Vascular Function in Normoglycaemic South Asians vs. White Europeans

SAs originating from the Indian sub-continent represent an increasing population worldwide since 2001. This ethnic minority are at increased risk of developing vascular disorders; namely T2DM, CVD and stroke when compared to WEs.[21, 361] Therefore, this increased vascular predisposition towards the development of T2DM and CVD necessitates early identification of risk factors for the purposes of primary prevention.

The increased levels of dyslipidaemia and blunted FMD response with disparities in retinal vessel reactivity components, found in this study, illustrate the need for tailored screening programmes in different ethnic minorities. The additional differences in SA men and women also illustrate the complexity in nature of the differences in vascular risk. Therefore, despite the lack of relevant data in screening and retinal haemodynamics, there is a definite need for more trials in SAs.[434]

7.4. Vascular Function in Individuals Newly-diagnosed with IGT as compared to Healthy Controls

Those with IGT have been identified to have increased levels of C-reactive protein, fibrinogen and plasminogen activators. Furthermore, impaired endothelium-dependent vasodilation in the brachial artery has been reported with elevated levels of ET-1 and adhesion molecules.[435] Therefore, it has been suggested that acute hyperglycaemia can contribute to vascular dysfunction by means of an oxidative stress mechanism in IGT individuals.[436]

Alongside reduced plasma glutathione levels, IGT individuals showed greater levels of vWF and impaired brachial and retinal artery reactivity that suggests early risk for prolonged oxidative stress and endothelial cell dysfunction/damage that is implicated by certain cardio-metabolic factors thus increasing the future risk for vascular disease.

7.5. Vascular Function in Normal, Overweight and Obese Individuals

As previously suggested the prevalence of vascular diseases such as T2DM, CVD and stroke and existing co-morbidity and mortality rates have been put down to the increasing levels of obesity and overweight worldwide. Therefore, the need to investigate the influence

of obesity and overweight on vascular function and preceding future vascular risk is indeed valid.

This additional study aimed to further the understanding of vascular function in known risk factors and demonstrated the validity of the differently used techniques in other at-risk individuals. More importantly, this chapter shows that overweight individuals may carry risk for vascular disease as shown by impaired FMD, retinal artery MD, MDRT and bFR that existed alongside well-known markers for atherosclerosis (increased c-IMT and dyslipidaemia) and endothelial damage (higher plasma vWF levels).

7.6. Future Directions

The preliminary results reported in the major chapters of this thesis illustrate the use of SDRA as a surrogate marker for vascular function alongside clinically proven and validated markers for endothelial dysfunction. The limitations demonstrated from the studies also help to show future directions that can be adopted to further our understanding behind the biochemical, vascular and metabolic pathophysiological causes of vascular disease.

7.6.1. Population Studies

The sample sizes used in the studies of this thesis provide preliminary pilot study data. Therefore, to determine the role of vascular function screening and novel surrogate markers in ethnic minorities, larger cohort studies of SAs (essentially second-generation migrants) need to be established. This should involve adequate follow-up intervals including the development of efficient media to self-report dietary and exercise behaviours. This could also incorporate qualitative studies to investigate cultural attitudes and beliefs as well as literacy and language issues to govern the design of screening programmes where traditional methods have been deemed inappropriate.

Further work should also investigate vascular and endothelial function in individuals with T2DM and DR. This would help determine the relationship of well-established vascular risk factors in T2DM and DR within the SA community and further investigate the difference, if any, in the pathogenesis and severity of the condition and its complications within this minority. This could also extend into those of SA origin with stroke or CVD.

7.6.2. Genetics Studies

Genetic factors, albeit varied, are known to play a significant role into the pathogenesis of T2DM. This can involve monogenic (single gene defects) or polygenic (several gene polymorphisms) forms of T2DM. To this date, there is very limited genetic data on SA communities and at-risk individuals, and so to better understand the pathophysiology of T2DM further gene characterisation and genome-wide association studies are needed. This can also expand to genes associated with obesity, IR and dyslipidaemia in SAs.

7.6.3. Biomedical Studies

The logistical and financial issues of the studies encountered in this thesis limited the degree and extent of analysis undertaken for plasma markers of endothelial function and vascular inflammation. Namely, the investigation of fasted insulin levels and glycosylated haemoglobin are known to be of clinical use in T2DM screening and diagnosis programmes. Furthermore, the study of renal function, by means of creatinine and albuminuria levels would provide insight into vascular risk for microvascular disease in T2DM.

Furthermore, the constraints of finances limited the resources to achieve available data on ET-1 levels in at-risk individuals. Additional work will need to investigate endothelial cell damage and vascular function.

7.6.4. Intervention Studies

The classic lipid profile of SAs, namely higher plasma TG and lower HDL-C levels is widely acknowledged and also documented in the studies of this thesis. Therefore, future ethnicity studies may focus on lipid-lowering therapies in this minority, researching the relative efficacy and thresholds for intervention of statins in SAs as compared to WEs. Furthermore, the emergence of new HDL-C raising agents into the pharmaceutical arena will be advantageous to SA individuals and therefore would require longitudinal clinical trials.

This can further extend to evaluating therapeutic agents in those with IGT, in particular the investigation into the efficacy of IR agents with angiotensin-converting enzyme (ACE) inhibitors and angiotensin-receptor blockers (ARBs) in improving glucose regulation.

7.6.5. Data Analytical Studies

Further work is necessary in compiling a normative database of values for a range of age and ethnicity categories across multiple sites to assess the application of SDRA and slope analysis. This can then help to further extend the development of complex algorithms and data-mining applications for larger samples in screening programmes for a range of ocular and vascular diseases.

Current FMD analysis is restricted, and its use as a correlate with retinal vascular parameters is extremely limited as it is yet to be fully investigated. Future FMD analysis should incorporate more complex analyses, for example including BDF as a factor for FMD%, or slope calculations, and involve cluster-analysis with larger samples.

7.6.6. Psychosocial Studies

The effects of confounding factors other than vascular risk factors in at-risk individuals, for example, in SAs and obese individuals, include diet; lifestyle and other social issues have not been extensively researched.[21] Furthermore, the religious and hostility barriers evident in certain groups are known to impact the risk for future vascular burden.[261] Future work investigating the implications of these factors, namely sedentary lifestyles (restricted exercise and poor diet) on vascular health would thus be extremely useful in gearing treatment plans for those with, and at risk of vascular diseases like T2DM, CVD and stroke.

7.7. Clinical Implications

The findings of this thesis present preliminary work on the clinical applications of surrogate markers, namely the DRVA and its SDRA, alongside well known markers for atherosclerosis, metabolic function and systemic endothelial function in groups known to be at risk of future vascular disease. Therefore, the use of SDRA is valid and quite possibly clinically necessary with more research. The results of this thesis show that SDRA could be used in a number other applications, such as; mass population screening for vascular disease, therapeutic efficacy in overt disease, therapeutic (i.e. nutritional, exercise) prevention of vascular disease, assessment of vascular function in other vascular/ocular disorders (glaucoma, hypertensive retinopathy, age-related macular degeneration), vascular function in other at-risk individuals (Afro-Caribbean origin) and demonstrating the use of the DRVA (retinal vessel reactivity, oximetry, static analysis and fundus photography) in patient diagnostic plans.

Appendices

1. Experimental Protocol

2. Retinal Vessel Analysis: A novel approach to understanding vascular ocular disease?
Optometry Today, New Techniques, 26 Nov 2011; 42-46

3. Abnormal Retinal Vascular Function and Lipid Levels in a Sample of Healthy UK South-Asians
British Journal of Ophthalmology, Mar 2011;

4. Ocular and Systemic Endothelial Function in the Offspring of Diabetics: A Pilot Study
Acta Ophthalmologica: Suppl 2009

5. Endothelial Function and Vascular Risk in South Asians
Investigative Ophthalmology and Visual Science: Suppl 2010

Experimental Protocol

Suitable participant identified and approached. Information pack given to outline study and procedures. Menstrual cycle questionnaire given to female participants to determine phase of menstrual cycle.

1. Letter sent to GP to notify of participation.
2. Procedures and risks explained (Information sheet); consent form to be read, understood, completed and signed.
3. Preliminary measurements to be taken: BCVA, spectacle prescription and IOP (of selected eye), height; body composition measurements, and baseline BP measurements.
4. OGTT ADMINISTERED A WEEK BEFORE THE MAIN STUDY
5. Collection of blood sample by venepuncture.
6. Temperature of the room should be noted.
7. BP measurements taken.
8. Participant fitted with 24-hour blood pressure monitor.
9. Flow-mediated dilation measurements to be taken.
10. BP measurements taken.
11. Retinal vessel analyser measurements to be taken.
12. Drug side effects leaflet given.
13. Clean all areas and equipment.
14. Participant to return monitor 24 hours later.

PROTOCOL FOR THE COLLECTION OF BLOOD SAMPLES BY VENEPUNCTURE

1. Position the patient either sitting/supine and support the preferred arm.
2. Assemble equipment.
3. Check that the seal on the needle is intact and the expiry date has not been exceeded and attach to the holder.
4. Apply the tourniquet 3-4 inches above the recommended site (antecubital fossa), for a maximum of 1 minute only, tight enough to slow the blood flow in the veins but not too tightly to prevent the blood flow of the arteries.
5. Select a vein by palpation and trace its path.
6. Swab the area thoroughly with 70% alcohol using increasing concentric movements starting at the puncture site. Allow the area to air dry for 30 seconds/wipe dry.
7. Holding the vacutainer barrel in the correct manner, uncap the needle. Position with bevel side up and holding the patient's skin down to anchor the vein, insert the needle smoothly through the skin at an angle of 15°-30° along the direction of the selected vein.
8. Using the flanges at the base of the barrel, fill the bottle in the correct order of draw, gently inverting the bottle (as often as indicated) as it is removed from the barrel.
9. Release the tourniquet.
10. Apply a clean swab over the puncture site, withdraw the needle and press firmly over the site for a minimum of 1 minute.

11. Dispose of needle and holder intact into a sharps box.
12. Complete bottle inversions.
13. Label tubes clearly by hand in the presence of the patient documenting any pre-analytical variables. Check the venepuncture site and dress the wound.
14. Remove gloves and dispose of all contaminated material.
15. Wash hands/use antiseptic gel.
16. Sample to be centrifuged (for plasma analysis) and stored (frozen).

PROTOCOL FOR ORAL GLUCOSE TOLERANCE TEST

1. A baseline fasted capillary blood sample is drawn.
2. The participant is then given a glucose solution to drink. The WHO protocol for OGTT is a 75g glucose solution. It should be drunk within 5 minutes.
3. Capillary blood sample drawn at 2 hour post glucose solution consumption.

PROTOCOL FOR FLOW-MEDIATED DILATION

1. BP measured.
2. Procedure and risks explained.
3. System, software and pre-decided settings set-up for artery examination.
4. Patient to lie supine with arm on support/side table comfortably.
5. 2 minute baseline measurements to be taken (CDI with vascular software).
6. Wrist compression (50mmHg above systolic) with cuff/wrist sphygmomanometer.
7. Measurements to be taken for 5 minutes.
8. Cease wrist compression.
9. 2 minute post cuff-release measurements to be taken.
10. Patient to rest (supine) for 10 minutes.
11. Another 2 minute baseline measurement to be taken.
12. Administer glyceryl trinitrate 0.3mg sublingual tablet and begin wrist compression (50mmHg above systolic) simultaneously.
13. Take further measurements for 5 minutes.
14. Cease wrist compression.
15. 2 minute post cuff-release measurements to be taken.
16. Patient to rest (supine) once all measurements taken.
17. Drug side effects leaflet to be given.

PROTOCOL FOR RETINAL VESSEL ANALYSER

1. BP measured.
2. Procedure explained.
3. IOPs of the selected eye(randomised) to be taken
4. Tropicamide 1% instilled (post explanation) to the selected eye.
5. Wait 15-20 minutes for drugs to begin acting.
6. Start program and fill in patient data, examination type, and location.
7. Sit patient comfortably at the retinal camera.
8. Ask patient to look at inner fixation target.
9. Adjust focus of camera.
10. Allow 1 minute for pupil adaptation to retinal camera illumination.
11. Optimise illumination and arrange illumination to enter directly through pupil.
12. Select retinal location (two chosen segments of the major temporal inferior branch of the arteriole and venule).
13. Set eye tracking template.
14. Mark the major artery and vein.
15. Start automatic measurements: baseline measurement of 50 seconds and three flicker period measurements (20 seconds each), interrupted with 80 seconds of still illumination.
16. Take IOPs (post-dilation) of the selected eye.
17. Drug side effects leaflet to be given.

Selected Publications and Presentations

Ocular and Systemic Endothelial Function in the Normoglycaemic Offspring of Type II Diabetics: A Pilot Study

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Purpose   To investigate ocular and systemic correlates of endothelial function in the normoglycaemic offspring of Type 2 Diabetics (T2DM).

Methods   Healthy participants aged between 25-65 years with (n=30) and without (n=39) a family history of Type II diabetes (T2DM) were recruited. Retinal vessel reactivity was assessed by using the Dynamic Retinal Vessel Analyser (DRVA, Imedos GmBH, Jena). In addition, systemic endothelial function was assessed by using the flow mediated dilation (FMD) technique.

Results   Statistical analysis showed no significant differences in anthropometric, blood assay or ocular and systemic function between both groups (p>0.05). The average maximum dilation (MD) in the measured retinal artery correlated significantly with the maximum dilation of the measured brachial artery (p=0.002 R=0.55) in healthy controls; however, this was not true for subjects with family history of T2DM.

Conclusions   Subjects with family history of T2DM show possibly early signs of endothelial dysfunction that, in certain conditions, could contribute to the higher risk of this group of developing similar pathology to their parents. Additionally this preliminary risk could be determined at an ocular level.

Abnormal Retinal Vascular Function and Lipid Levels in a Sample of Healthy UK South-Asians

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Background/Aims: To investigate ethnic differences in retinal vascular function and their relationship to traditional risk indicators for cardiovascular disease (CVD).

Methods: A total of 90 normoglycaemic subjects (45 South-Asian (SA) and 45 age- and gender- matched White Europeans (WE) were recruited for the present study. Retinal vessel reactivity to flickering light was assessed by means of the Dynamic Retinal Vessel Analyser (DRVA) according to a modified protocol. Fasting plasma glucose, triglycerides (TG), total, LDL and HDL cholesterol were also measured in all individuals.

Results: SA individuals showed higher fasting triglyceride (p=0.001) and lower HDL levels (p=0.007), leading to a higher TG:HDL-C ratio (p=0.001) than age-matched WE subjects. Additionally, in SAs, the retinal arterial reaction time in response to flicker stimulation was significantly longer in the last flicker cycle than in the WEs (p=0.039), and this change correlated positively with measured plasma TG levels (r=0.60; p=0.01). No such relationship was observed in the WEs (p>0.05).

Conclusion: Even in absence of overt vascular disease, in otherwise healthy SAs there are potential signs of retinal vascular function impairment that correlates with established plasma markers for CVD risk.

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