RETINAL VASCULAR FUNCTION & CARDIOVASCULAR RISK FACTORS

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Thesis Summary

The current platform of conventional cardiovascular risk assessments tends to forsake the importance of endothelial function - a key biological mechanism by which cardiovascular risk factors exert their propensity for adverse vascular events. Moreover, the presence and severity of endothelial dysfunction in 'low-risk' individuals suggests considerable variability in pre-clinical risk that could potentially be detected well before the onset of disease. The aim of the present thesis was to investigate the presence and impact of retinal vascular dysfunction, as a barometer of endothelial function, in otherwise healthy individuals with one or more cardiovascular risk factors, but low to moderate cardiovascular risk. Systemic circulatory influences on retinal vascular function were also evaluated. The principle sections and findings of this work are:

1. **Ageing effect on retinal vascular function**
   - In low-risk individuals, there are age differences in retinal vascular function throughout the entire functional response curve for arteries and veins. Gender differences mainly affect the dilatory phase and are only present in young individuals.

2. **Retinal vascular function in healthy individuals with a family history of cardiovascular disease**
   - In low-risk individuals with a family history of cardiovascular disease, impairments in microvascular function at the retinal level correlate with established plasma markers for cardiovascular risk.

3. **Ethnic differences in retinal vascular function**
   - When compared to age-matched White Europeans, in low-risk middle-aged South Asians, there are impairments in retinal vascular function that correlate with established cardiovascular risk indicators.

4. **Systemic circulatory influences on retinal microvascular function**
   - Systemic antioxidant capacity (redox index) and plasma markers for cardiovascular risk (lipids) influence retinal microvascular function at both arterial and venous levels.

5. **Retinal vascular function in individuals with obstructive sleep apnoea: a preliminary study**
   - Patients with moderate to severe sleep apnoea exhibit attenuated retinal vascular function.

Keywords: endothelial function, dynamic retinal vessel analysis, cardiovascular risk, ageing, family history, ethnicity, sleep apnoea

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Abbreviations

AD	Average Diameter
AIx	Augmentation Index
AMD	Age-related Macular Degeneration
ANS	Autonomic Nervous System
AU	Arbitrary Units
BCFR	Baseline Corrected Flicker Response
BDF	Baseline Diameter Fluctuation
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CHOL	total Cholesterol
CO_2	Carbon dioxide
pCO_2	partial pressure of Carbon dioxide
CRA	Central Retinal Artery
hsCRP	high-sensitivity C-reactive Protein
CRV	Central Retinal Vein
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
CV	Coefficient of Variation
DA	Dilation Amplitude
DBP	Diastolic Blood Pressure
DR	Diabetic Retinopathy
DVA	Dynamic retinal Vessel Analysis
ECG	Electrocardiography
EEG	Electroencephalography
EMG	Electromyography
EOG	Electrooculography
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ET-1	Endothelin-1
ETHRISK	Ethnicity-adjusted Framingham Risk Score
DTNB	Dithiobis-2-nitrobenzoic acid
FH	Family History
FRS	Framingham Risk Score
FMD	Flow-Mediated Dilation
FMD_{ED}	Endothelium-Dependent Flow-Mediated Dilation
GLUC	Glucose
GPx	Glutathione Peroxidase
GSH	Glutathione, reduced
GSSG	Glutathione, oxidised
GSR	Glutathione Reductase
GST	Glutathione Transferase
HbA1c	Glycated Haemoglobin
HDL-c	High-Density Lipoprotein cholesterol
HF	High Frequency
HR	Heart Rate
HRV	Heart Rate Variability
ICA	Internal Carotid Artery
c-IMT	carotid Intima-Media Thickness

IOP	Intraocular Pressure
LDL-c	Low-Density Lipoprotein cholesterol
LF	Low Frequency
MAP	Mean Arterial Pressure
MC	Maximum Constriction diameter
tMC	time to Maximum Constriction
MC%	Percent constriction
MD	Maximum Dilation diameter
tMD	time to Maximum Dilation
MD%	Percent dilation
MI	Myocardial Infarction
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
eNOS	endothelial Nitric Oxide Synthase
NU	Normalized Units
O_2	Oxygen
pO_2	partial pressure of Oxygen
OA	Ophthalmic Artery
OBF	Ocular Blood Flow
OPP	Ocular Perfusion Pressure
ONH	Optic Nerve Head
OSA	Obstructive Sleep Apnoea
PCA	Posterior Ciliary Artery
PNS	Parasympathetic Nervous System
PSG	Polysomnography
PWA	Pulse-Wave Analysis
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
RPC	Radial Peripapillary Capillaries
RPE	Retinal Pigment Epithelium
RVA	Retinal Vessel Analyser
SA	South Asians
SBP	Systolic Blood Pressure
SD	Standard Deviation
SDRA	Sequential Diameter Response Analysis
$Slope_{AD}$	Arterial dilation slope
$Slope_{AC}$	Arterial constriction slope
$Slope_{VD}$	Venous dilation slope
$Slope_{VC}$	Venous constriction slope
vSMC	Smooth Muscle Cell
SNS	Sympathetic Nervous System
TEA	Triethanolamine
TG	Triglycerides
2-VP	2-Vinyl Pyridine
WBC	White Blood Cells
WE	White European

1. Introduction

"Longevity is a vascular question, which has been well expressed in the axiom that man is only as old as his arteries…The onset of what may be called physiological arterio-sclerosis depends, in the first place, on the quality of arterial tissue which the individual has inherited, and secondarily on the amount of wear and tear to which he has subjected it."

(Sir William Osler, 1892 [1])

Over a century later, the concept of longevity being a vascular question still holds true as we continue to battle what is known to be the number one cause of morbidity and mortality in the world – cardiovascular disease (CVD).

The process of atherosclerosis is best described as a reparative response to mechanical strain on blood vessel walls, and is often characterized by the appearance of 'fatty streaks' in the intima of mid- and large-size arteries [2]. Clusters of cells attracted from the blood stream become trapped in the vascular wall and vascular smooth muscle cells (vSMC) produce collagen fibres to replace defective elastin fibres, further propagating plaque formation. If the plaque ruptures, then the toxic influence of molecular mediators in the bloodstream can further initiate thrombosis and clot formation, resulting in irreparable damage due to vessel occlusion. With the present preoccupation with obstructive vascular disease, however, wider non-atherosclerotic phenomena that can also signal the early advent of disease, such as dysfunctions of the endothelium (inner lining of all blood vessels), are often overlooked in conventional cardiovascular risk assessments.

It is important to underline that by virtue of its location in the inner vessel wall the endothelium may actively contribute to disease initiation and progression. Typically, endothelial dysfunction arises from otherwise adaptive vasomotive responses that are now excessive, sustained, and/or spatially and temporally misplaced [3]. Endothelial dysfunction is also regarded as a key biological mechanism through which modifiable and non-modifiable cardiovascular risk factors exert their propensity for vascular pathology. For instance, endothelial dysfunction has been observed in ageing [4], in hypertensives [5, 6], normotensives with a family history of hypertension [7], in patients with diabetes [8, 9], dyslipidaemia [10], obesity [11], as well as, active [12, 13] and passive smokers [14, 15]; and, in view of the numerous systemic associations, endothelial dysfunction is now regarded by vascular scientists as a surrogate marker of an individual's inherent atherosclerotic risk [16]. It can, however, be challenging to distinguish between endothelial functions and dysfunctions, particularly in otherwise healthy individuals with none, or one or more cardinal risk factors, and; the temporal relationship between risk factors and the

development and progression of disease also appears to be dependent, in large part, on intrinsic inter-individual variations in endothelial responses. Fortunately, endothelial function can be fine-tuned or re-calibrated with treatment, and endothelial assessments can aid in identifying non-responders to endothelial therapies who would remain at high-risk for future clinical events [17].

The correct analysis of vascular alterations, therefore, is a crucial aspect of preventative medicine, and currently available assessments are directed at examinations of larger peripheral vessels such as the carotid, brachial, radial, or femoral arteries. Microvessels are thought to be more susceptible to endothelial irregularities and one of the central tenets of this thesis is the important role of the retinal microvessels' reflection of systemic vascular health. Indeed, the measurement of retinal vessel diameters is useful for cardiovascular risk stratification [18] however; the focus has largely been on retinal photographs, which can only provide a snapshot of the microcirculatory state. Instead, an assessment of the dynamic properties of the microvessels (dilation and constriction in response to a physiological stressor) could provide additional information about endothelial dysfunction, and facilitate the early diagnosis of related diseases. With recent technical advancements, the retinal vessel analyser system, can detect early changes in retinal microvessel function in response to a stimulus, and comparative studies continue to validate clinical applications, as well as, significant correlations between structural and functional vascular patterns in retinal and systemic vessels. As studies using this instrument also continue to confirm the extent and severity of endothelial dysfunction in patients with existing pathologies, a key question that remains is whether similar retinal microcirculatory changes are also measurable and obvious at an earlier stage of impaired endothelial function in apparently healthy but 'at risk' individuals.

This thesis aims to explore this possibility, and to additionally address the question of whether functional retinal assessments could prove effective as an indirect measure of vascular health. Of particular interest is the influence of select modifiable (sleep disturbances, lipid levels, and oxidative stress markers) and non-modifiable (age, family history, ethnicity) cardiovascular risk factors on retinal vascular function, and though their association with endothelial dysfunction has been increasingly recognized, the nature of this involvement is still uncertain, and many questions remain to be answered. As such, this work aims to explore the influence of these cardiovascular risk factors on retinal vascular function. To provide a basis for the studies described in this thesis, the following chapter outlines the current aetiological thinking for the development of CVD, with a

particular emphasis on the role of vascular factors. To better understand vascular insufficiency occurring in at-risk individuals, the relevant aspects of cardiovascular and ocular anatomy and physiology will be discussed in Chapter 3.

2. Background & Theoretical Framework

2.1. Conventional cardiovascular risk assessments

Currently, the established clinical approach for a cardiovascular risk assessment endorses the use of risk scores, based on a number of cardiovascular risk factors/variables, to estimate an individual's lifetime risk for developing CVD. The most important risk variables incorporated in these scoring algorithms are age, sex, blood pressure (BP), cholesterol, diabetes, and smoking – historically derived from large observational cohort studies such as the Framingham Heart Study [19], and the Systemic Coronary Risk Evaluation study [20]. Deviations in these risk variables typically account for the majority of cases that go on to develop CVD [21]. More recently, ethnicity and family history (FH) [22, 23] have been recognized as additional, important non-modifiable risk factors; though, they are not as frequently incorporated into cardiovascular risk scoring systems. A comparison of the more commonly used cardiovascular risk scores is summarized in Table 2.1.

2.2. Screening for risk in the asymptomatic patient

In many instances, there is a clustering of risk factors; however, the predictive algorithms used in various risk scores indicate that the presence or absence of even one of the risk factor variables would substantially alter an individual's risk score. For illustrative purposes, the following presents a hypothetical case of a patient who wants to know what her risk of developing CVD is. Based on the growing number of cardiovascular risk scores available, her risk estimate could vary according to the assessment model used (see Table 2.1).

> ***Case vignette***[1]
>
> *A 59-year-old White European woman who is currently a non-smoker, but with a history of smoking, has no cardiac symptoms or family history of cardiovascular disease, and reports infrequent exercise and alcohol consumption (1 to 2 times a week). She wants to know what her risk of a future cardiovascular event is? Her body mass index (BMI) is 34.93, resting blood pressure (BP) is 138/76 mmHg, fasting glucose is 6.05 mmol/L, triglyceride level (TG) is 2.30 mmol/L, total cholesterol (CHOL) is 5.40 mmol/L, high-density lipoprotein cholesterol (HDL-c) is 1.11 mmol/L, and high-sensitivity C-reactive (hsCRP) protein is 3.00 mg/L.*

[1] Based on hypothetical values derived from the dataset of participants used for the studies in this thesis.

Table 2.1. Comparison of cardiovascular risk scores

Risk Equation	Variables	Outcomes	Population	Baseline	Advantages	Disadvantages
FRS [24-26]	Age, sex, SBP, TC, HDL-c, smoking, DM, HTN medication	CVD, stroke, CHD (angina, MI, sudden death)	3,969 men and 4,522 women; ages 30-75 years; general population from Framingham, MA, USA	1968 – 1971, 1971 – 1975, 1984 – 1987	Predicts absolute 10-year risk of CVD. Validated in men, women, Europeans, Asians, and Africans.	Under-prediction is likely in those with DM and FH. Emerging risk factors are also not included. Validated only for ages<30yrs, >75 years.
ETHRISK [27]	Age, sex, SBP, ethnicity TC, HDL-c, smoking status	CVD, CHD	3,778 men and 4,544 women; ages 30-70yrs; two community-based surveys; general UK population	1998-1999	Was developed to avoid over- or under- definition of 'at risk' status common to other risk scores. Specific to ethnic minorities in the UK.	Remains to be validated.
QRISK2 [28-30]	Age, sex, SBP, TC/HDL-c ratio, smoking, HTN medication, FH, SD, BMI, ethnicity, RA, kidney disease, AF	MI, CHD, stroke, TIA	1.28 (QRISK1) 2.29 million (QRISK2); ages 35-74 yrs; health records of general practice attendees in the UK	1993-2008	Includes ethnicity and SD, as well as an improved quantification of risk for DM. Accounts for HTN medication and differing effects of risk factors associated with ageing.	Data validated from sample population it was originally derived. Missing data may currently undermine predictive capacity, although further use will improve accuracy.
REYNOLDS [31, 32]	Age, SBP, TC, HDL-c, smoking, hsCRP, FH, HbA1c if DM	MI, stroke, CVD death	10,000 men and 25, 000 women; ages 45-80yrs; those without DM; general USA population	1992-2004 1995-2008	Independently validated in men (>50yrs) and women (>45yrs), includes hsCRP.	Mainly in WE population with SBP, weight and FH by self-report. HTN not used for women and HbA1c not used for men.
ASSIGN [33]	Age, sex, SBP, TC, HDL-c, smoking, FH, SD	CVD, CHD, CABG, PTCA	117,098 men and 88,080 women; ages 30-74 yrs; general Scottish population	1984-1987	Includes FH and SD, theoretically abolishing the effects of social gradient on CVD risk.	Yet to be validated in an independent cohort and is confined to a relatively limited geographical area.
SCORE [20]	Age, sex, TC or TC/HDL-c ratio, smoking, high or low-risk region of Europe	Fatal cardiovascular events	6,540 men and 6,757 women; ages 40-65yrs; general European population	1972-1991	Derived from a relatively diverse cohort that is potentially highly representative of a contemporary British population.	At present predicts only the risk of fatal events with a tendency to over-estimate death rates.
UKPDS Risk engine [34]	Age, sex, SBP, TC, HDL-c, smoking, ethnicity, AF, HbA1c	Life expectancy for those with DM	5,102 people with DM; recruited to UK Prospective Diabetes Study	1977-1997	Differs from other risk scores in that DM is not simply coded as a dichotomous variable but accounts for age at diagnosis and HbA1c.	Tendency to over-estimate CVD and CHD risk. Marginally better for patients diagnosed 10 years ago versus those diagnosed more recently.
PROCAM	Age, SBP, LDL-c, HDL-c, smoking, FH of MI, DM, TG	CVD, stroke, mortality	18,460 men, 8,515 women; ages 20-75; volunteers; general German population	1978-1995	Incorporates broader range of parameters than FRS which over-estimates risk in the German population.	Maybe considered underpowered for risk estimation in women.

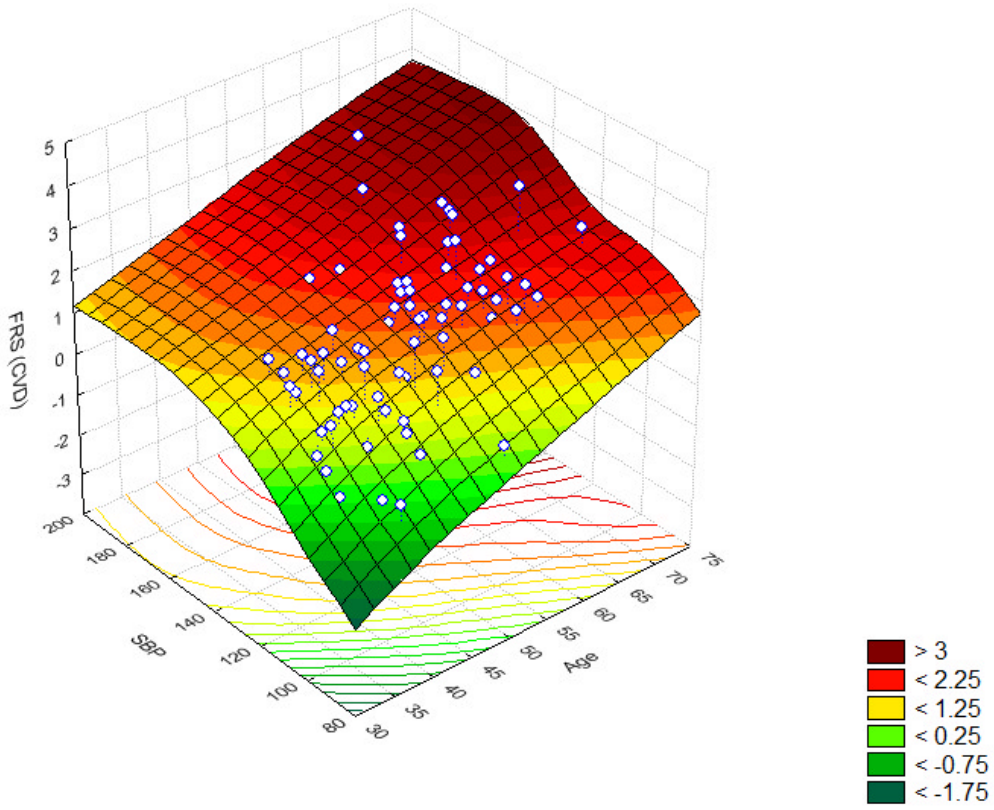
Abbreviations: ASSIGN, Assessing Cardiovascular Risk to Scottish Intercollegiate Guidelines Network/SIGN to Assign Preventative Treatment; AF, atrial fibrillation; BMI, body mass index; CABG, coronary artery bypass graft surgery; CHD, coronary heart disease; CVD, cardiovascular disease; ETHRISK, ethnicity based modification of FRS; DM, diabetes mellitus; FH, family history; FRS, Framingham risk score; HDL-c, high-density lipoprotein cholesterol; HbA1c, glycated haemoglobin; hsCRP, high-sensitivity C-reactive protein; HTN, hypertension; LDL-c, low-density lipoprotein cholesterol; MI, myocardial infarction; PROCAM, Prospective Cardiovascular Mϋnster study; PTCA, percutaneous transluminal coronary angiography; QRISK, QRESEARCH Cardiovascular Risk Algorithm; SCORE, Systematic Coronary Risk Evaluation; SBP, systolic blood pressure; SD; social deprivation; TC, total cholesterol; TG, triglycerides; TIA, transient ischaemic attack; UK, United Kingdom; UKPDS, United Kingdom Prospective Diabetes Study; USA, United States of America; WE, White European.

Using online calculators of each model for the patient described in the case vignette; her 10-year risk of CVD based on the Framingham risk score (FRS) was estimated to be 10% and risk of a fatal event (myocardial infarction, MI) was estimated at 3%. According to ETHRISK [27], an ethnicity based modification of the FRS specific to the UK population; the patient's 10-year risk of CVD was 11%. Additionally, her QRISK (QRESEARCH Cardiovascular Risk Algorithm), Reynolds risk, ASSIGN (Assessing Cardiovascular Risk to Scottish Intercollegiate Guidelines Network/SIGN to Assign Preventative Treatment) and SCORE (Systematic Coronary Risk Evaluation) were estimated at 7%, 4%, 10%, and 2% (1% if residing is a low risk region), respectively. Finally, her PROCAM (Prospective Cardiovascular Mȕnster study) score for risk of an MI was estimated at 8.96%, which is 1.91-fold increase compared to the average person of the same age (4.7% in 10 years).

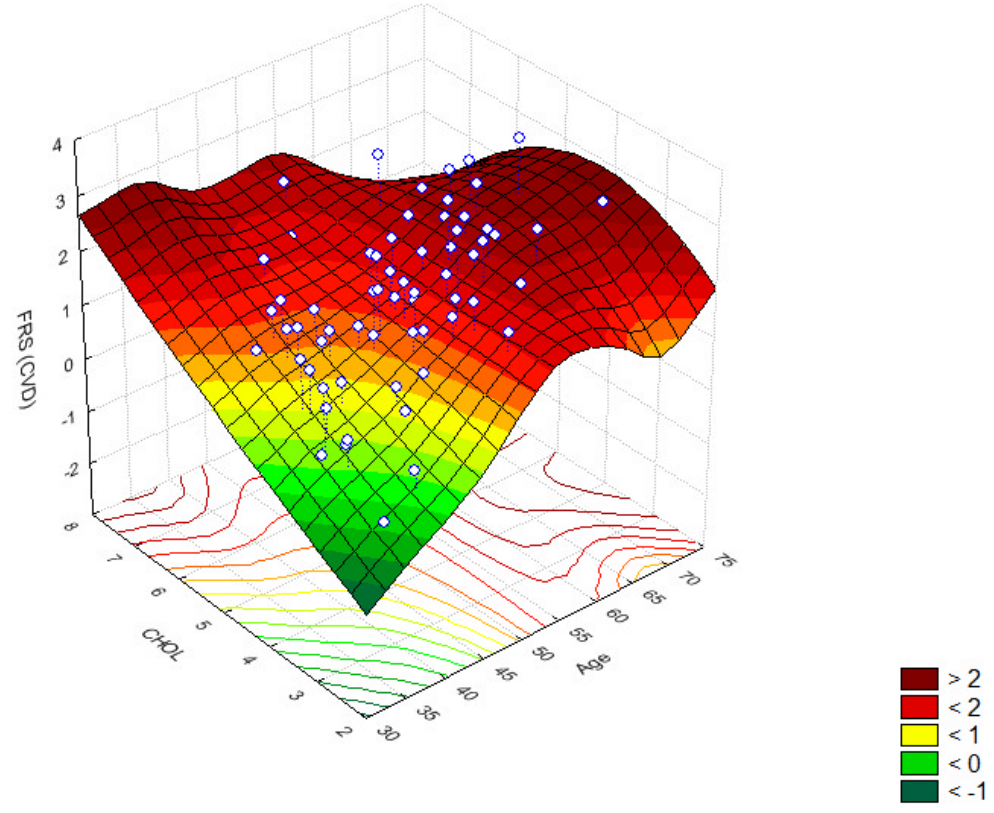
Based on these estimates, it would appear that single risk factor approaches tend to classify patients in a dichotomous way; either as low risk or high risk (in this patient's case low risk), and such emphasis on arbitrary cut off points tends to overlook the large population of asymptomatic individuals who are at risk but not at optimal levels for preventative care. Population surveys indicate that at least 10% to 15% of the population is at high risk; an estimated 5% to 10% is at low risk, while 75% to 80% of asymptomatic middle-aged and elderly individuals are at low, moderate, high, or very high risk [35]. On the other hand, 60% of patients with manifest CVD have also been known to display only one, or in some instances, none of the common cardiovascular risk factors included in such risk scoring systems [36].

From a public health perspective, risk scores are statistically useful, although, from a clinical risk management perspective, they provide at best only a crude assessment of individualized risk and can fail to accurately categorize up to a quarter of CVD risk [37, 38]. Furthermore, the continuous nature of the association between risk factor variables such as age, BP, and cholesterol suggests that risk is not merely present or absent, but is present to a lesser or greater extent (Figure 2.1A-C).

A


FRS (CVD)
5
4
3
2
1
0
-1
-2
-3
SBP
200
180
160
140
120
100
80
Age
30
35
40
45
50
55
60
65
70
75
> 3
< 2.25
< 1.25
< 0.25
< -0.75
< -1.75

B


FRS (CVD)
4
3
2
1
0
-1
-2
CHOL
8
7
6
5
4
3
2
Age
30
35
40
45
50
55
60
65
70
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> 2
< 2
< 1
< 0
< -1

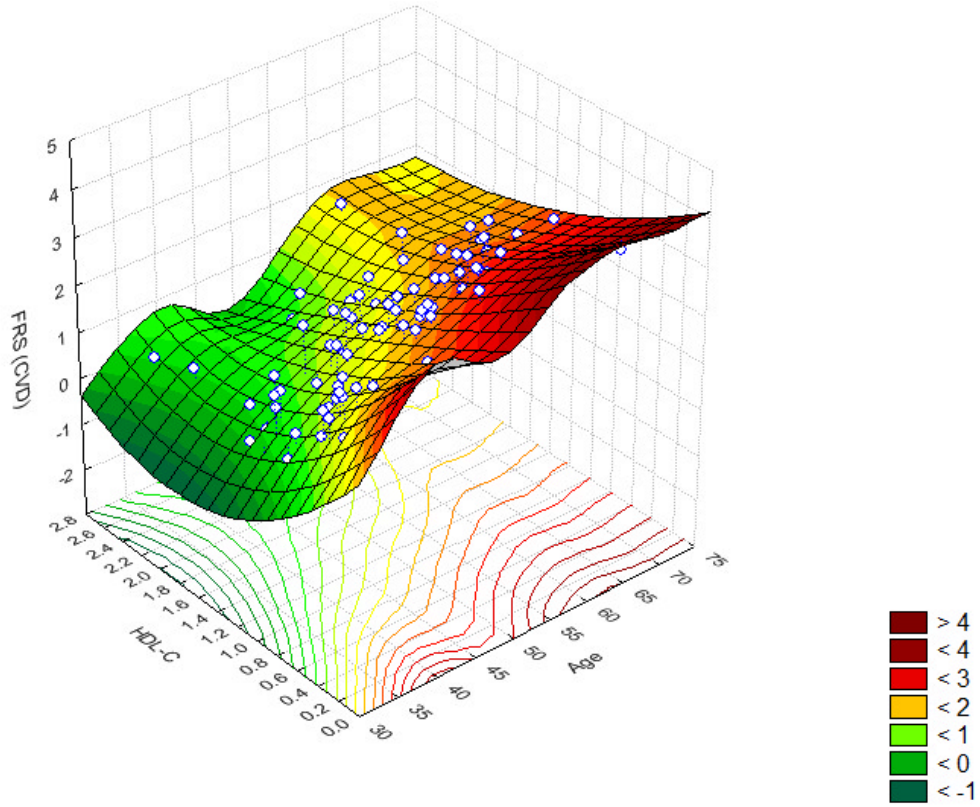


Figure 2.1. Association between continuous cardiovascular risk factor variables
(A) Relationship between FRS, SBP (mmHg), & age (years); (B) Relationship between FRS, CHOL (mmol/L), & age (years); (C) Relationship between FRS, HDL-c (mmol/L), & age (years). The association between continuous risk factor variables indicates that the presence or absence of one of variables can substantial alter the risk score calculation (as indicated by coloured rectangles) suggesting that risk is not merely present or absent, but present to a lesser or greater extent. Abbreviations: CVD, cardiovascular disease; CHOL, total cholesterol; FRS, Framingham risk score; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure. Three-dimensional plots generated using Statistica software (Statsoft, Inc., Version 9, USA) are based on the dataset of participants used for the experimental studies in this thesis.

Moreover, cardiovascular prevention strategies predicated on population-based risk and response appear to have remarkably little statistical impact from a risk reduction perspective [39]; furthering the concept of a certain degree of individualized risk that may re-define what is 'optimal' or 'normal' on an individual basis. At present, however, there is no risk prediction model that perfectly predicts risk at an individual level, and by relying solely on conventional risk factors for symptomatic disease, the focus tends to be on variables that are statistically rather than biologically associated with the disease.

2.3. The importance of vascular factors

In recent years, our understanding of the pathogenesis of CVD has progressed to the level of molecular mechanisms that affect the vascular endothelium – the first line of defence. While it is increasingly common to use intermediate endpoints, such as BP, cholesterol levels, and more recently inflammatory markers; parallel assessments of blood vessel health, endothelial dysfunction, loss of arterial elasticity, and or thickening of the intimal-medial surface of vessels, provide an alternative approach to better identify individuals with overt disease and/or those at risk.

Complexities in the ability of endothelial cells to regulate a multitude of physiological functions have dispelled the fallacy that they act as a simple interface between the blood and vessel wall. Instead, the vascular endothelium is now recognized as a dynamic organ system that is indispensable for vascular homeostasis [40]. By virtue of its location, the endothelium responds rapidly and sensitively to the mechanical conditions generated by the cardiac cycle and maintains blood vessel tone by appropriating the release of vasoactive factors, such as nitric oxide (NO) and endothelin-1 (ET-1) [41, 42]. These mediators affect the tensile properties of vSMCs along the adjacent regions of the originating vessel thereby eliciting vascular responses such as dilation and constriction. Among the most potent vasodilating agents, NO is important for the regulation of basal vessel tone [43]. This effect has important implications for sustained vessel constrictions observed in individuals with conditions that affect NO synthesis and release. A functional endothelium is therefore often characterized by exposure to a physiological stressor that temporarily alters blood flow (occlusion or ischaemia) in a particular vascular region, and observing how the blood vessels in that region respond. Typically, the greater the vasodilation observed, the better the endothelial function [44]. Structural abnormalities due to underlying disease (atherosclerosis) can result in a dysfunctional regulation of vascular tone (endothelial dysfunction). However, endothelial dysfunction can also be present in anatomically normal vessels [45]. For example, patients who suffer from angina-like chest pain, but exhibit no signs of atherosclerotic plaque, are thought to be suffering from local dysregulations of the microcirculatory coronary vessels [46].

2.4. Endothelial function & cardiovascular risk

As such, endothelial dysfunction can be present even in otherwise asymptomatic individuals and is now considered to reflect a vascular phenotype that is prone to atherogenesis (plaque formation). The intimate association between endothelial function

and cardiovascular risk factors also indicates that endothelial dysfunction is not only a consequence of manifest vascular disease, but that it is also the biological mechanism by which cardiovascular risk factors exert their propensity for vascular complications. The relationship between endothelial function and known, as well as, lesser-known cardiovascular risk factors could, therefore, represent an important concept in the early identification of cardiovascular risk; and, one of the main aims of this thesis, as mentioned previously, is to further clarify the importance of endothelial function and dysfunctions in the development and progression of CVD in individuals who would otherwise be classified as having low to moderate cardiovascular risk by conventional standards.

For further contextualization of the experimental studies in this thesis, the following sections highlight the relationship between endothelial function and cardiovascular risk factors of particular relevance to the studies. Currently available assessments that enable a quantification of the relationship between endothelial dysfunction and the presence of risk factors are also of interest and, therefore, the subsequent sections discuss available endothelial assessments that can be carried to examine the effects of risk factor exposures on small (micro) and large (macro) blood vessels. Additionally, the incremental value of microvascular assessments in comparison to macrovascular assessments is addressed in favour of the retinal vascular assessment technique selected to evaluate endothelial function in the experimental studies.

2.4.1. Major cardiovascular risk factors

Age: is one of the most important determinants of vascular health as the ageing cardiovascular system becomes more susceptible to diseases such as atherosclerosis and hypertension [47], and the incidence and prevalence of CVD tends to increase exponentially with age [48-50]. Ageing cardiovascular abnormalities are exemplified by changes in cardiac left ventricular diastolic filling and hypertrophy, as well as, increased heart rate variability and arterial stiffness (arteriosclerosis) [51]. A key change also observed in ageing vessels the presence of a dysfunctional endothelium, although; circulatory derangements due to vascular senescence are often indistinguishable from the early phases of arteriosclerosis [52, 53], and ageing and endothelial dysfunction can progress in parallel. This possibility features endothelial dysfunction as both a potential causative or consequential factor in vascular ageing. Endothelial senescence can also lead to an imbalance in vasoactive substances [54], favouring the development of oxidative stress and inflammation – which are additional important contributors to the development of vasculopathies [55].

Male sex: has predominantly been considered a greater risk factor for CVD, with lifetime risk at age 40 estimated at 50% for men and 33% for women [56]. While cardiovascular risk patterns were previously considered to be favourable in women [57], risk is now thought to be underappreciated in this group [58]. In fact, CVD is still the leading cause of death in women even though a vast majority perceive the chance of dying from breast cancer as far more likely [59]. Interestingly, suboptimal dilation in the coronary microvasculature has been identified as a contributing factor to poor prognosis in women with no signs of obstructive vascular disease [60]. Indeed, sex hormones appear to have an influence on vascular health and could amplify differences in cardiovascular risk patterns that are associated with endothelial function. Even more important is whether endothelial dysfunctions can signal the clinical debut of CVD in both men and women, and in either case, the early identification of risk is paramount.

Hypercholesterolemia: and, in particular, a higher fraction of low-density lipoprotein cholesterol (LDL-c) levels has a considerable effect on CVD risk in both men and women [61, 62]. Based on data from clinical studies the risk management focus, with regards to optimal cholesterol levels, has shifted from treatment based on lipid levels to treatment based on actual risk scores – suggesting a possible interplay between conventional risk factors and lipid levels. A close association between hypercholesterolemia and endothelial function also exists and is evidenced by increases in LDL-c levels that can result in the decreased bioavailability of NO [63], and down-regulation of vascular tone. Since, aggregate risk factors in the presence of abnormal lipids can coalesce to amplify the development and progression of CVD, an understanding of the relationship between endothelial function and lipid levels may be an important step towards the early identification of asymptomatic at-risk individuals that could benefit from targeted therapies.

Hypertension: is a decidedly prevalent risk factor in the elderly population and risk factors such as age and cholesterol can amplify cardiovascular risk in the presence of elevated BP. Unsurprisingly, chronic elevations in BP can have a profound influence on vascular health that can lead to coping mechanisms in blood vessels such as structural and functional adaptations (endothelial dysfunction). Clinical studies also show a significant association between the severity of hypertension and degree of endothelial impairment [64]. Moreover, antihypertensive therapies aimed at improving cardiovascular risk appear to be more effective when they concomitantly improve endothelial function [65, 66]. While age and hypertension can independently or synergistically impair endothelial function, they share

similar cascades for the development of endothelial dysfunction including oxidative stress [67].

2.4.2. Other cardiovascular risk factors

***Oxidative stress*:** is a common underlying mechanism that associates multiple cardiovascular risk factors, supporting its central role in the development of CVD [68]. The conflicting evidence with regards to the efficacy of antioxidants in reducing cardiovascular morbidity and mortality, however, still raises the question as to the importance of oxidative stress as a risk factor. The prime argument to explain mixed outcomes is the lack of biomarker specificity to assess oxidative stress phenotypes. Moreover, most studies tend to focus on the role of oxidative stress in established rather than developing disease. Nevertheless, a significant body of evidence links increased oxidative stress with endothelial dysfunction [69]. As less information is available on risk and prognosis, the associations between vascular factors and oxidative stress are likely avenues for future research in identifying at risk individuals, and may be crucial in defining preventative strategies that include therapies to improve both oxidative stress status and vascular or endothelial function.

Family history (FH): is an important yet frequently underappreciated cardiovascular risk factor; particularly since the aggregation of familial CVD is more commonly associated with the presence of comorbidities such as hypertension and lipid disorders within the family [70-72]. However, while some studies wholly attribute familial risk to the presence of these factors [73], others leaning towards a genetic predisposition propose FH in itself to be an independent cardiovascular risk factor [74-78], and poor endothelial function is thought to be a requisite inherited phenotype for cardiovascular risk in patients with FH of CVD [79].

Ethnicity: also represents another frequently underappreciated risk factor, especially in traditional risk scoring systems that are only validated in certain ethnic populations. The aggregation of conventional risk factors are likely contributors to ethnic variations in disease, however, the presence or absence these factors still does not adequately explain excess cardiovascular risk in certain ethnic groups [80]. For example, cardiovascular-related morbidity and mortality rates are higher in migrant UK South Asians (SAs) [81] in comparison to White Europeans (WEs), despite there being a lower prevalence of hypercholesterolemia, hypertension, and smoking in SAs of the UK. An interesting possibility is the existence of lowered threshold levels for cardiovascular risk factors that

could increase susceptibility in certain ethnic groups. For instance, SAs are susceptible to the adverse effects of lipids on endothelial and vascular health at lesser threshold values than the conventionally accepted definitions of 'optimal' or 'normal' lipid levels [82], and there is additional evidence to suggest that even otherwise apparently healthy (low-risk) SAs can exhibit features indicative of attenuated endothelial function [83].

Sleep disturbances: are likely the least recognized of risk factors that can also have a profound influence on vascular health and associative cardiovascular risk. Clinical recognition rates for conditions such as sleep apnoea are influenced by the other comorbidities (obesity) and can even more frequently be under recognized by patients. Besides the potential hazards associated with daytime sleepiness, sleep apnoea patients are increasingly susceptible to altered blood flow regulation during the disrupted sleep-wake cycle and as a result are at increased risk for endothelial injury and cardiovascular complications. Adverse consequences associated with endothelial dysfunction in sleep apnoea include oxidative stress, hypertension, and CVD [84, 85].

2.4.3. Endothelial assessments

Chronic exposure to cardiovascular risk factors can exhaust the protective effects of the endothelium and contribute to vascular insults, which then culminate into CVD; however, a prolonged latent period separating risk factor exposures from the occurrence of adverse events provides an important opportunity for primary preventions. The temporal relationship between risk factors and the development of disease also appears to be dependent, in large part, on intrinsic inter-individual variations in endothelial responses. Therefore, vascular or endothelial markers provide a means for objectively evaluating the synergistic or independent effects of risk factors on arterial health. Several tools have been developed for this purpose; among these are assessments of vascular structure and function in various vascular beds that may serve as surrogate markers of cardiovascular risk.

2.4.3.1. Macrovessels

Large artery structure and function is more commonly assessed, and vascular markers that have perhaps garnered the most interest as a useful approach to study subclinical atherosclerosis burden include coronary artery calcification (CAC) scores [86] and carotid-intima media thickness (c-IMT) scores [87] (the latter of which is of particular relevance to this thesis). Studies have shown that c-IMT scores can improve upon traditional risk prediction models [88] and risk re-classification [89]. In a previous meta-analysis study, each

0.1 mm increment in c-IMT corresponded with a 10 to 15% increase in the risk for MI [90]. Nevertheless, CAC and c-IMT scores provide more information about vascular structure and established disease, rather than function, and are less affected by transient abnormalities if endothelial function is to be appreciated as a dynamic process.

There are, however, few widely used methods to measure endothelial function with brachial flow-mediated dilation (FMD) presently regarded as the gold-standard technique [91]. With this technique, brachial endothelial function is characterized as an augmented dilation response (reactive hyperaemia) following temporary cuff occlusion of the forearm (ischaemia) or after oral administration of nitroglycerin (a potent NO donor / vasodilating agent). There are, however, conflicting data regarding the prognostic value of this measure in low-risk patients [92]. Protocols for FMD also tend to vary across research settings and being a highly operator-dependent technique [16, 93], there can be wide variability when characterizing a 'normal' FMD response [94].

Conversely, measures of impaired arterial elasticity by means of radial pulse-wave analysis (PWA) or applanation tonometry tend to have better prognostic value in patients with no known CVD [95]. Measurements of c-IMT, FMD, and PWA were included in some of the experimental studies comprised in this thesis and further details on each technique are provided in the methods section 6.3.5.

2.4.3.2. Microvessels

The microcirculation comprises the bulk of the circulatory system and since a large proportion of the endothelium lies within the microcirculation, it plays an important role in regulating haemodynamics of the body. From a clinical standpoint assessments of smaller vessels, such as the coronary microvessels, are important since relatively small changes in microvessel diameter along the vascular network would be expected to have considerable impact on arterial blood flow (see section 3.1.3), resistance, and venous return to the heart. It therefore comes as no surprise that microcirculatory disturbances tend to predate clinically apparent end organ damage [22]. The coronary microvessels are, however, notoriously difficult to examine non-invasively and, as a result, there is increased interest in studying more accessible regions of the microcirculation. In this regard, the retinal microvessels facilitate a more practical approach for the evaluation of early microvascular changes since, the retina is particularly amenable to non-invasive study, and it is known that the cumulative effects of cardiovascular risk factors [96, 97], genetic factors [98, 99], and ageing [100] are reflected in the calibre of retinal vessels.

2.5. The assessment of ocular vasculature for cardiovascular risk screening

2.5.1. Static retinal assessment

Photographic imaging techniques have facilitated the development of methods to quantify subtle changes in the architecture of retinal vessels over time that can convey important information regarding future risk for systemic pathology [101-109]. As such, temporal retinal vessel assessments can provide important information regarding the cumulative effects of risk factor exposures during an individual's lifetime. At present, static retinal assessments are used in broad areas of cardiovascular research including: (i) as a research tool to explore the development of CVD; (ii) as a risk stratification tool in clinical settings to aid physicians in identifying microvascular signs that may signal future risk of adverse cardio- or cerebrovascular events, and; (iii) as a surrogate measure for assessing the microvascular benefits of targeted vascular therapies. Nevertheless, one of the key questions regarding microvascular derangements in CVD addresses the temporal sequence of alterations, and whether they contribute mechanistically to the development of disease, or are early markers secondary to the disease process itself. For instance, one of the unresolved issues in the association between narrowed retinal arterioles and hypertension is whether arteriolar narrowing is antecedent to the development of hypertension by altering vascular haemodynamics, which results in a re-setting of BP or; whether changes in retinal vessel calibres reflect physiological adaptations to increases in BP [110]. Nevertheless, the retinal microcirculation appears to represent a useful vascular marker of sub-clinical, and possibly reversible physiologic deviations of the systemic circulation associated with unfavourable exposures.

With regards to CVD screening, there are indeed, strong indications that retinal vessels share homology with coronary and cerebral vessels. Focal retinal arteriolar narrowing and arterio-venous nicking are structural vascular signs that reflect cardiovascular-related pathological damage [96, 111-122], as well as, cerebrovascular pathology [123-125]. Retinopathy is also associated with parallel pathology in coronary micro- and macrocirculations [126], as well as, a three-fold increased risk for future congestive heart failure [127]. There is also evidence to suggest that irregular vascular signs in retinal arterioles versus retinal venules may be associated with distinctive cardiovascular phenomena. For instance, a consistent association between narrower retinal arteries and elevated BP is frequently reported [108, 128-131], while wider retinal veins are more commonly associated with an increased risk for stroke [132-135]. Moreover, studies have linked arteriolar narrowing [128] with BP and

endothelial dysfunction, and venular widening with endothelial dysfunction, hyperglycaemia and inflammation [108, 128, 136, 137]. It could be suggested that specific vascular signs reflect the cumulative effects of specific risk factor profiles; that is, arterial signs may more commonly reflect hypertensive profiles while venular signs may more commonly reflect metabolic abnormalities. In either case, the prospect that retinal arteriolar and venular changes could represent a microvascular phenotype of endothelial dysfunction warrants further study to validate this phenotype.

Despite the wealth of information pertaining to structural retinal assessments and data regarding deviations that signal future cardiovascular risk, there is still a caveat associated with the static nature of retinal photography; which only provides a snapshot of an individual's circulatory state and conveys more information on established rather than developing phenomena. Accordingly, tools for functional retinal vascular assessments are highly desirable and would be more relevant for the prediction of cardiovascular risk.

2.5.2. Dynamic retinal assessment

The quantification of retinal vessel diameters were first derived from studies examining the passage of dyes through the retinal vascular system [138]. Significant technical advances in the field of optics have since led to the development of a variety of non-invasive techniques that enable the evaluation of ocular haemodynamic parameters including techniques such as scanning laser Doppler flowmetry and scanning laser ophthalmoscopy [139]. In recent years, the retinal vessel analyser (RVA) system (IMEDOS, GmbH, Jena, Germany) has markedly simplified continual retinal diameter measurements. This system now enables real-time and simultaneous quantification of dilatory and constrictory reactions in retinal arterioles and venules [140, 141]. The RVA has since become increasingly recognized as an alternate tool for the evaluation of retinal vascular tone [142] and functional microvascular endothelial assessment at the retinal level [143]; or more specifically, vascular responses to various physiological and pharmacological stimuli – analogous to measuring post-ischaemic dilation responses in the brachial artery via FMD.

Nevertheless, few studies using the RVA have examined whether functional retinal vessel evaluations can be used to identify individuals who may be at risk for CVD. Further details on the technique and a review of existing relevant literature are provided in Chapter 4 as in-depth context for the experimental studies described in Chapters 7 through 11, which explore the effects of ageing, FH, ethnicity, lipid and oxidative stress markers, and sleep

disturbances on retinal vascular function. But first, as a majority of cardiovascular complications are associated with some form of vascular abnormality, and in order to enable a better appreciation of the vascular insufficiency that may be present in at-risk individuals, the following sections (Chapter 3) discuss fluid mechanics in blood vessels, and focus on principles of blood flow regulation in the systemic and retinal vasculature.

3. Concepts in Physiology

3.1. Blood flow regulation

The cardiovascular system is a functionally perfected organ system equipped with complex regulatory feedback mechanisms to ensure moment-to-moment adjustment of blood flow based on the metabolic needs of various tissues. The essential components of the cardiovascular system comprise of the heart (Figure 3.1) the critical pump of the system; blood vessels that link the heart to the various organ systems and tissues; and circulating blood to facilitate the adequate delivery and exchange of nutrients and waste products.

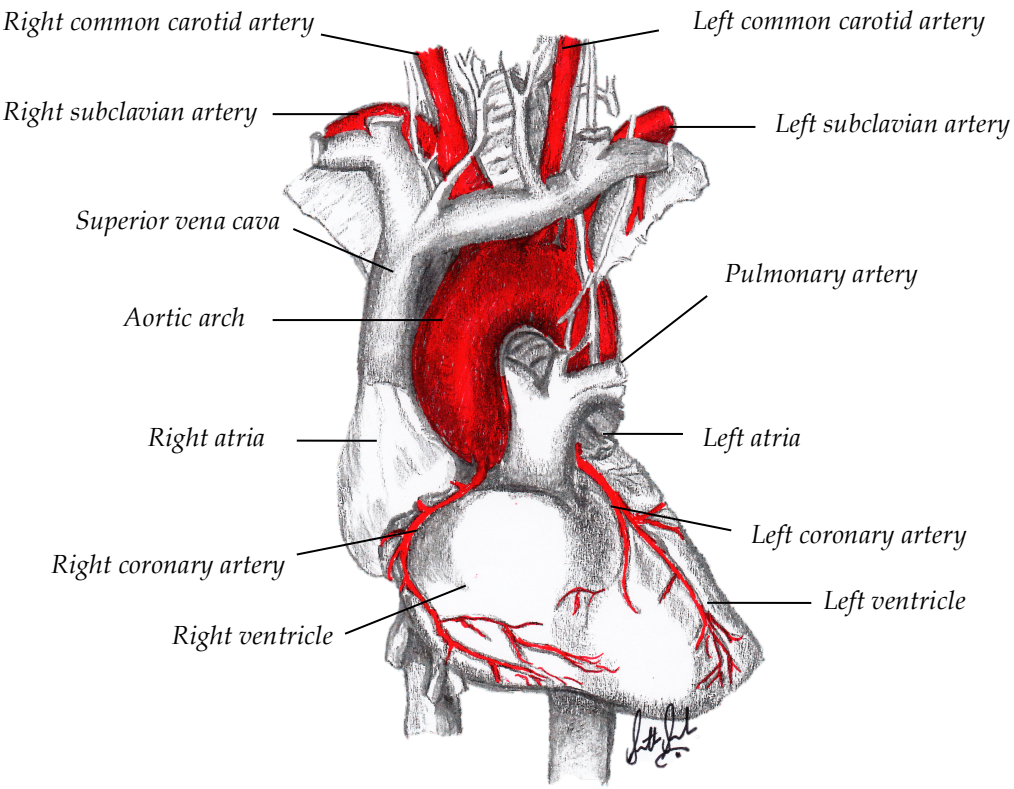


Figure 3.1. Illustration of the human heart
Almost entirely comprised of myocardium, the human heart is a marvel of timing and coordination as it relies on a precise series of contractions to mediate blood flow through its two upper (right and left atria), and two lower (right and left ventricles) chambers. The atria function as reservoirs while ventricles serve as pumps for oxygenated and de-oxygenated blood pathways through arteries and veins, respectively. Oxygen-rich blood vessels are coloured red except for the pulmonary arteries, which carry de-oxygenated blood to the lungs. Illustration sketched by author based on Gray's anatomy [144].

Optimal cardiac activity requires a highly integrated series of electrical, mechanical, and metabolic events that culminate in repetitive contraction and relaxation of the myocardium. Impulses generated in the sinoatrial node synchronously spread across the heart muscle ultimately causing millions of traversed cells to contract in near unison. With each re-setting of the cardiac cycle, the ensuing pressure differential generated in the vasculature (BP) governs the flow of blood [145]. Since blood vessels also possess the intrinsic ability to dilate and constrict in response to changes in the intravascular milieu, gradual pathological impedances (atherosclerosis) in the vasculature may cause the heart to adapt a higher workload by increasing the size of myocardial cells. Longstanding increases in peripheral resistance (arteriosclerosis) can eventually lead to ventricular remodelling and hypertrophy [146]. A condition where cardiac muscle tissue fed by the coronary arteries is damaged due to arterial blockage is referred to as myocardial infarction (MI) or a heart attack.

As blood traverses from a large pressure reservoir in the heart through the sophisticated network of blood vessels, the architectural arrangement of arteries and veins are important in the distribution of blood to and from the capillaries, where the major work of the vascular system is accomplished (Figure 3.2). Arteries have a common basic structure comprising of an inner layer of endothelial cells (endothelium), sub-endothelial connective tissue and elastic lamina (tunica intima), a medial layer of SMCs (tunica media), and an outer layer of fibrous connective tissue (tunica adventitia) [147]. Since vascular impedances such as atherosclerotic changes tend generally to be confined to the arterial side, the structural and functional properties of the main types of arteries of particular relevance to this thesis are further discussed in section 3.1.4.

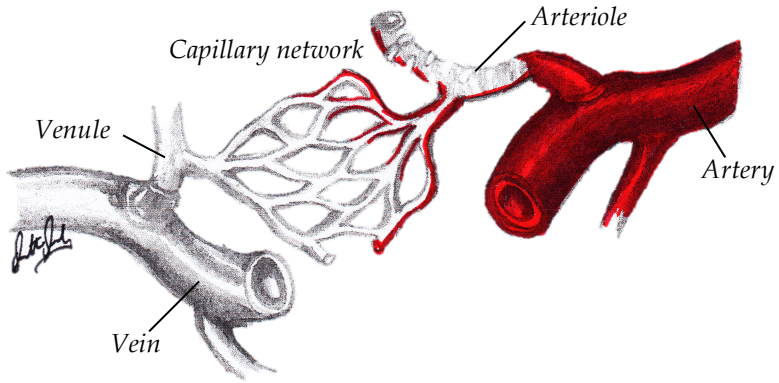


Figure 3.2. Illustration of the main blood vessel types
There are generally five classes of blood vessels in the cardiovascular system: arteries, arterioles, capillaries, venules, and veins. By definition, the arteries deliver blood to the capillaries while veins return it to the heart. Illustration sketched by author.

3.1.1. Autonomic control

Two efferent arms of autonomic nervous system (ANS) – the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) – reconcile the involuntary control of the heart and blood vessels, and most visceral organs. While the SNS and PNS share functional antagonism through a number of differentially regulated neural networks, a common fallacy is that they exert equal and opposing effects to maintain cardiovascular homeostasis. On the contrary, PNS innervations extend to the heart and a limited number of blood vessels such that its influence is largely associated with the modulation of cardiac function, while SNS nerve terminals originating from the brain stem and third and fourth segments of the sacral spinal cord innervate the heart and extensive systemic vasculature; thereby eliciting widespread direct and indirect control of the cardiovascular system [148].

In normal physiology, autonomic control of the heart shifts to vagal (parasympathetic) dominance and increased sympathetic activity results in increased heart rate (HR), peripheral resistance, and stroke volume. Like autonomic nerves in the heart, tonic SNS activity in the vasculature sets a background level of vasoconstriction and decreases in sympathetic outflow result in vasodilation. The regulation of the cardiovascular system, therefore, involves a precise sympathoinhibitory reflex. The sensory monitoring for this critical homeostatic process involves mechanoreceptors (or baroreceptors) located in the heart and major blood vessels, and chemoreceptors located primarily in the carotid arteries and aorta. Baroreceptors respond to mechanical stretch or expansion and contraction of the elastic elements in the vessel wall, while chemoreceptors respond to changes in oxygen and carbon dioxide tensions in the blood. The arterial baroreflex, as such, represents a negative feedback system that is effective for buffering BP fluctuations as changes in arterial pressure evoke changes in sympathetic relays to the heart and blood vessels, thereby adjusting vascular resistance and cardiac output back to baseline values [149].

3.1.2. Interactions between the ANS and endothelium

The vascular endothelium and the sympathetic division of the ANS share functional antagonism to maintain blood vessel tone by appropriating the release of various vasoactive factors [150]. In addition to the vasoconstrictory and dilatory responses stimulated by sympathetic activation of α-adrenergic and β-adrenergic receptors on vSMCs, and the compensatory release of endothelial NO, the ANS may also directly influence endothelial

cells that possess α and β-adrenergic receptors [151, 152]. Sympathetic nerve activity has also been shown to stimulate the release of vasoconstrictive factors such as ET-1 [150].

Currently, much of the clinical evidence linking impaired ANS activity with endothelial dysfunction stems from pathological conditions with signs of alterations in both systems. Vascular diseases such as diabetes and hypertension have independently been associated with alterations in ANS [153-155] and endothelial function [156, 157]. Nevertheless, it remains unclear whether dysregulation in one system elicits maladaptive changes in the other, or whether dysregulation is endured in both systems because of pathology [44]. Indeed, ANS activity can be influenced by impaired endothelial function [158], and there is evidence that is indicative of a potential association between heart rate variability (HRV) and endothelial function; where ET-1 levels have been negatively correlated with HRV parameters [159] and decreased HRV has, in turn, been associated with arterial stiffness [160].

In general, the endothelium may be more responsive to local factors, while autonomic control is regulated by efferent and afferent sensory signals, and peripheral reflexes such as the arterial baroreflex, which modulates feedback regulation of autonomic outflow for BP homeostasis. This perspective alludes to the more compelling evidence for ANS-related mechanisms of endothelial dysfunction and is important for understanding associative mediators between ANS and endothelial impairments, such as oxidative stress and ageing. Ambulatory HR and BP recording devices are inexpensive, non-invasive diagnostic tools that can be used for the assessment of autonomic function. A pre-set program sequence enables the device to store continuous real-time HRV and BP recordings over a period of up to 24 hours and HRV can then be evaluated using time and frequency domain analysis. The use of one such ambulatory BP and HR monitoring device is included in the experimental study described in Chapter 11, where further details on the technique and data analysis are provided.

3.1.3. Blood flow in arteries

The conceptual framework of the arterial system as a single elastic chamber is sometimes used in the determination of cardiac output; although, this simplified context forsakes the phenomenon of pulse-wave propagation throughout the arterial tree as intermittent flow from the ventricles is transformed into steadier outflow. With alternating phases of systole and diastole, pulsatile pressure and flow characteristics are contrasted in different parts of arterial vascular network. The relationship between pressure waveforms and total blood

flow has been simplified using one-dimensional models of fluid dynamics and the most well accepted model is the *Windkessel* model [161]. During systole, compliant arteries, such as the aorta, act as a capacitor to store blood, which is then discharged during diastole through smaller resistive branches that infuse organs and tissues. As blood flows across the vascular endothelium it causes friction, also denoted as 'shear stress', to retard flow. At the luminal surface, shear stress is detected as a mechanical force on vascular endothelial cells and triggers the release of NO [162, 163]. The wall shear stress is proportional to the velocity gradient and viscosity of blood, and inversely related to vessel diameter. As such, vessels with smaller diameters are expected to exhibit an augmented dilatory response due to a higher shear stress stimulus [164] and, basal NO release is likely to be larger in the microcirculation to ensure increased vessel tonus than that in conduit vessels [165].

In linear areas of the vasculature, blood flow patterns occur within a steady rate along the vessel, and flow is streamlined with each layer of blood remaining equidistant from the vessel wall. This type of flow pattern is referred to as laminar flow (Figure 3.3). Shear stress (τ_{wall}) for a laminar steady flow, as a reasonable estimate of mean wall shear stress in arteries, is expressed as:

$$\tau_{wall} = 32\frac{Q}{\pi D^3} \qquad \textbf{(Equation 3.1)}$$

Where, Q, refers to the mean volumetric flow and, D, refers to the vessel diameter. With this type of blood flow pattern in conduit vessels, blood in the centre of the artery has a higher velocity, as it is less affected by friction than the outer concurrent layers of fluid, and a parabolic pattern begins to develop between the layers of blood.

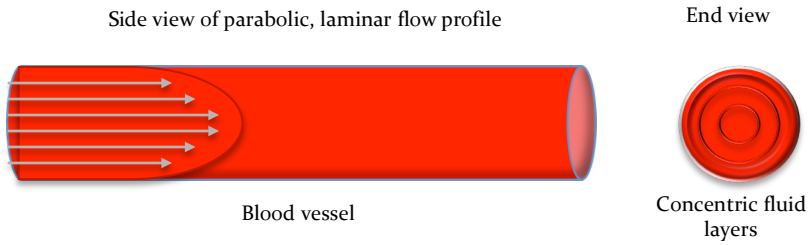


Figure 3.3. Schematic representation of laminar flow
Laminar flow is the normal condition for blood flow throughout most of the circulatory system. It is characterized by concentric layers of blood moving in parallel down the length of a blood vessel. The flow profile is parabolic once laminar flow is fully developed.

In areas with branch points or bifurcations in the vessel, shear stress can vary widely. When the parabolic pattern is disturbed this typically results in turbulent flow [166], and adaptive responses in arteries work maintain a wall shear stress of approximately 15 dynes cm^{-2} [167]. Since this stress value also modulates adaptive responses to pathological impedances such as intimal-medial thickening and thrombosis, shear stress is central to vascular haemodynamics [168].

At the systemic level, flow adaption is mediated by HR control and the arterial baroreflex. Within each vascular bed, local factors influence blood flow regulation in keeping with metabolic needs of the tissue. As such, the rate of blood flow through any vascular bed is considered to be directly proportional to the pressure gradient and inversely proportional to vascular resistance [169].

3.1.4. Main arteries in the systemic circulation

All of the arteries comprised in the systemic circulation branch from the aorta which extends from the left ventricle, arches over the heart, and descends anteriorly to the left of the vertebral column. Three main arteries originating across the aortic arch (the brachiocephalic trunk, left subclavian artery, and the left common carotid artery) are responsible for delivering blood to the upper limbs, shoulders, neck, and head. The following sections highlight features of the main systemic arteries, which serve as common sites for non-invasive vascular assessments and are of relevance to this thesis (Figure 3.4).

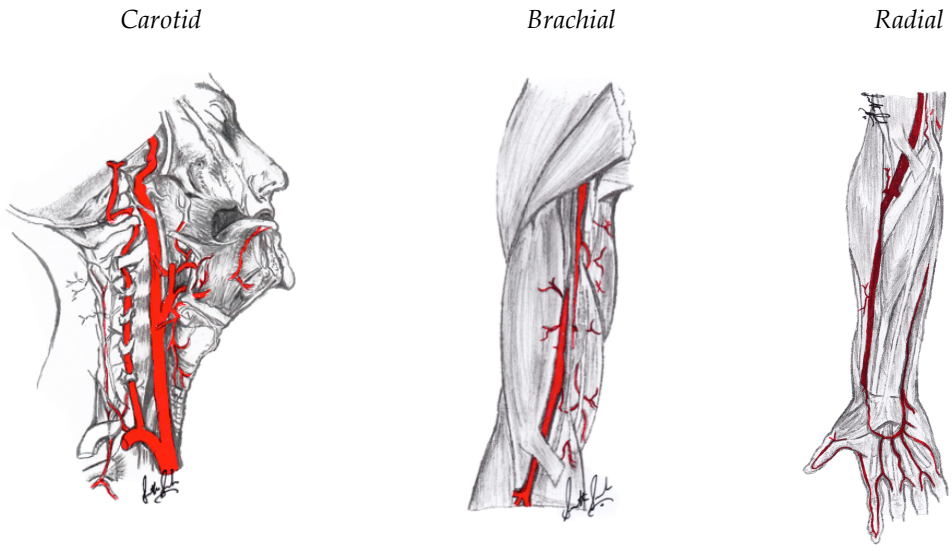


Figure 3.4. Main systemic arteries of interest

3.1.4.1. Carotid artery

Of particular interest are the right and left common carotid arteries (Figure 3.5), which extend superiorly within the corresponding sections of the neck before branching to form the internal and external carotid arteries supplying the vascular networks of the eye and brain.

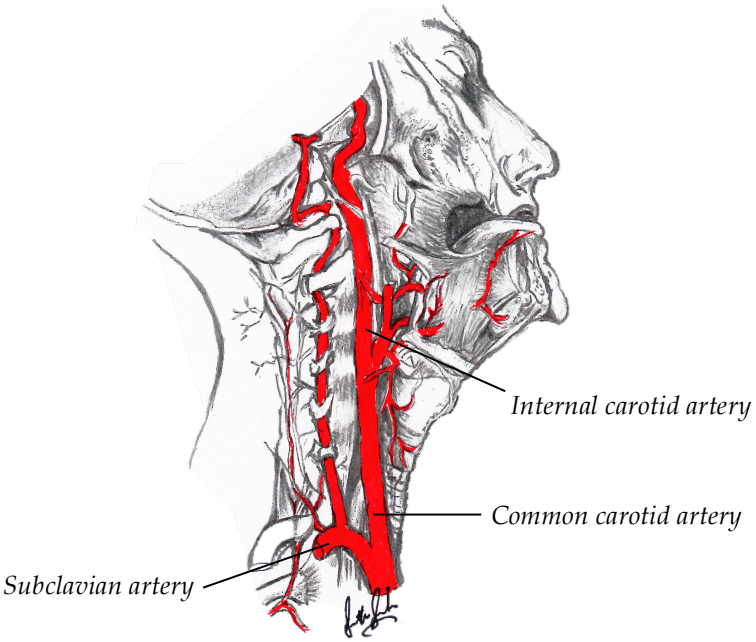


Figure 3.5. Illustration of the common carotid artery
The brachiocephalic trunk, the first and largest branch of the aortic arch divides into the right common carotid artery and right subclavian artery. The left common carotid artery arises directly from the arch of the aorta. The internal carotid artery is more of a small bifurcation of the common carotid artery that enters the carotid canal in the skull and becomes one of the main arterial blood supplies to the brain and eyes while the external carotid artery diverges off the common carotid with its various branches supplying part of the face and head before it terminates into the maxillary artery and superficial temporal artery. Illustration sketched by author based on Gray's anatomy [144].

Owing to the vessel's close proximity to the skin, changes in carotid intimal-medial thickness (c-IMT) can easily be detected via ultrasonography and a description of this technique is later provided in section 6.3.5.2. Typically, an increase in c-IMT scores is regarded as one of the first anatomical signs of atherosclerosis and an obligatory precursor of atheroma formation [170], although; given the somewhat subjective nature of this assessment, at present there is no clinical standard to address inter-operator or inter-reader variability [171]. Nevertheless, this type of assessment provides useful information about vascular structure and progressive disease.

3.1.4.2. Brachial artery

The brachial arteries, located in the upper arms, are formed from the subclavian artery – a branch of the brachiocephalic artery. Owing to a similar proximity to the surface of the skin as that of the carotid, the brachial artery also possesses attributes that render it particularly amenable to non-invasive study. For example, its location facilitates uniform compression or occlusion and the artery can be easily visualized with ultrasonography for measurements of reactive hyperaemic responses or endothelial function with the FMD technique [91]. In addition, BP measurements taken at the brachial artery correlate well with aortic pressures and the upper arm is a comfortable site for routine assessment (Figure 3.6).

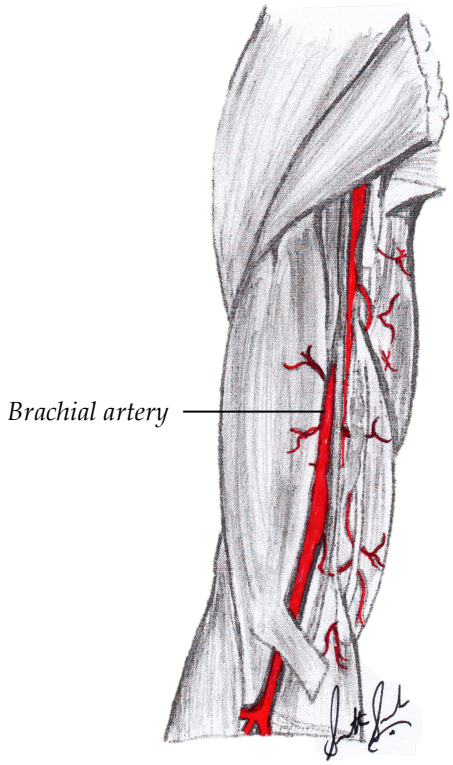


Figure 3.6. Illustration of the brachial artery
The brachial artery moves along the humerus (upper arm bone) down to the elbow. It gives rise to the deep brachial artery, which curves around the back of the humerus to supply blood to the triceps muscles. Shorter branches bifurcate and pierce various other muscles on the front of the upper arm, and others descend on either side of the elbow to join arteries in the forearms. Illustration sketched by author based on Gray's anatomy [144].

A detailed description of the FMD technique is provided in section 6.3.5.2. Briefly, the principle of this technique involves an induced increase in blood flow through the brachial artery based on the principle that shear stress exerted on the endothelium would result in vasodilatation by stimulating the release of NO. An impaired vasodilatation response is interpreted as a decrease in the bioavailability of NO [172].

3.1.4.3. *Radial artery*

As the brachial arteries branch at the elbow they give rise to the radial arteries that traverse along the radial side of the forearm to the wrist providing a site for pulse measurements as they approach the skin surface [145] (Figure 3.7).

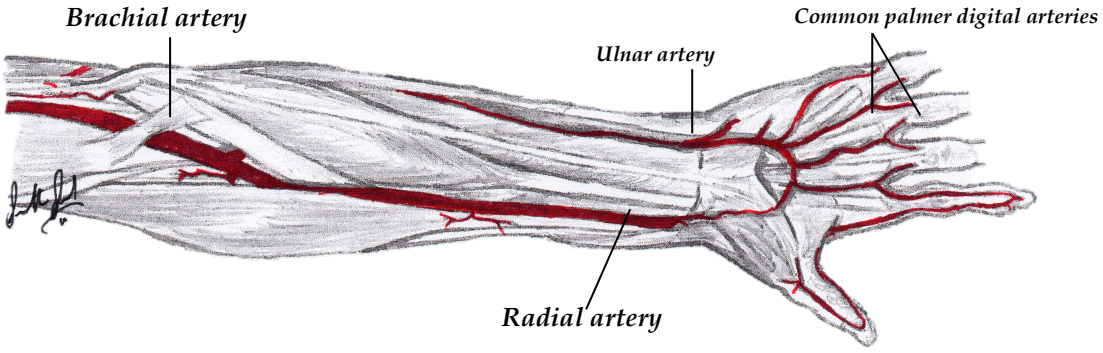


Figure 3.7. Illustration of the radial artery
The radial artery travels down the radial side of the forearm towards the wrist. As it nears the wrist, it rises close to the surface and is therefore a convenient vessel for measuring pulse rate. At the wrist, branches of the ulnar and radial arteries join to form a network of vessels, which supply the structures in the wrist, hand, and fingers. Illustration sketched by author based on Gray's anatomy [144].

In comparison to c-IMT and FMD, measures of impaired arterial elasticity by means of radial PWA (pulse-wave analysis) or applanation tonometry have incremental prognostic value in patients with no known CVD [95]. A detailed description of the PWA technique is provided in section 6.3.5.1. Briefly, the principle of this technique relies on pulsatile pressure waves that travel through the vasculature during cardiac contractions. Typically, thicker arterial walls facilitate the dampening of pressure oscillations so that blood flows smoothly from the aorta to target tissues [173]. At inflection points in the vessel, pressure waves are reflected back to the heart to regulate the filling of coronary vessels during diastole. In individuals with impaired arterial elasticity, pressure waves return to the heart at a much quicker rate and this augments the pressure that the heart has to overcome in order to open the aortic valve. Stiffening of the arterial wall reduces elasticity [174], which has been attributed to degeneration of elastin fibres, deposition of collagen [175], decreased production of NO, and loss of smooth muscle tone [176].

As sophisticated networks of blood vessels evolve from a single continuous structure, a number of systemic vascular disorders can be attended by changes in retinal vessels and vice versa; this is unsurprising when taking into consideration that essentially all blood vessels are directly or indirectly exposed to the same intrinsic influences. The following

sections, therefore, discuss the relevant anatomy and physiology of retinal blood vessels and local control mechanisms that are involved in regulation of retinal blood flow and basal retinal vessel tone.

3.1.5. Anatomy & physiology of the retinal vessels

The main arterial blood supply to the ocular tissues is derived from the internal carotid artery (ICA) (Figure 3.8), which secedes, into several branches of the ophthalmic artery (OA), including the central retinal artery (CRA) and the ciliary arteries, also collectively known as the retrobulbar vessels.

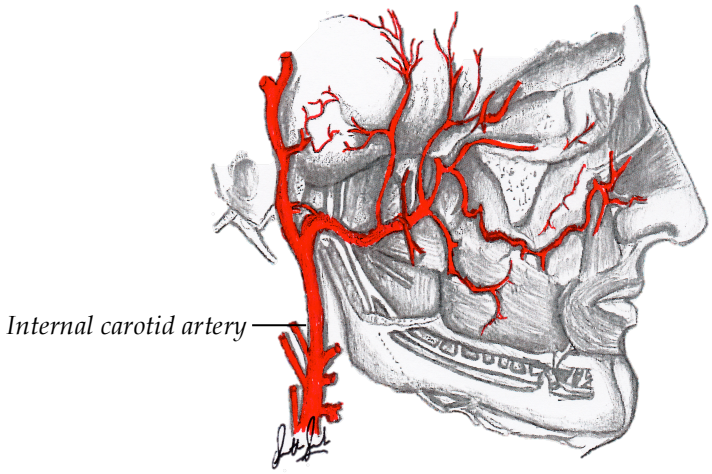


Figure 3.8. Illustration of main arteries in the head and neck
The internal carotid artery is a terminal branch of the common carotid artery. In considering the course and relations of this vessel, it may be divided into four portions: cervical, petrous, cavernous, and cerebral. The ophthalmic artery represents a branch of the internal carotid artery distal to the cavernous sinus. Illustration sketched by author based on Gray's anatomy [144].

Ophthalmic artery (OA): represents a branch of the ICA distal to the cavernous sinus (Figure 3.9) [177]. Anatomical studies collectively reveal inter-individual variations in the site of origin of the OA itself, as well as, in its branches and the path it traverses. As such, it is common practice to discuss its anatomy with respect to what is most frequently observed. The branches of the OA can be divided into an orbital group comprising the lacrimal, supraorbital, anterior and posterior ethmoidal, internal palpebral, supratrochlear, and dorsal nasal arteries (that irrigate the orbit and surrounding structures), and; an ocular

group including the anterior ciliary, short and long posterior ciliary arteries (PCAs), and CRA, which service the muscles and bulb of the eye.

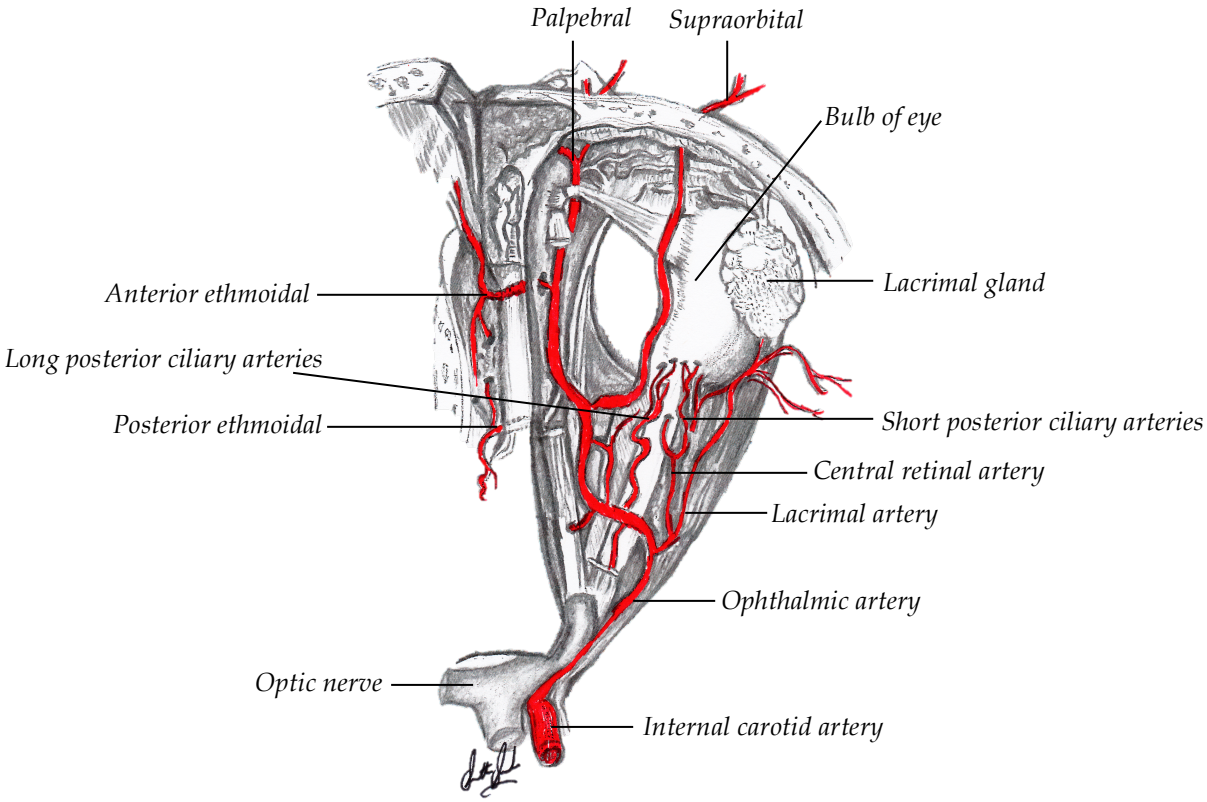


Figure 3.9. Illustration of the retrobulbar vessels
The ophthalmic artery arises from the internal carotid and enters the orbital cavity through the optic foramen, below and lateral to the optic nerve. Branches of the ophthalmic artery may be divided into an orbital group, distributed to the orbit and surrounding parts; and an ocular group, to service the muscles and bulb of the eye. Illustration sketched by author based on Gray's anatomy [144].

Central retinal artery (CRA): represents a branch of the OA that pierces the optic nerve; travels along the centre of the optic nerve; appears at the optic disc through the lamina cribrosa, and; ultimately branches off into four principle intra-retinal arteries that each supply one quadrant of the retina.

Ciliary arteries: can be categorized into three main groups namely the short posterior, long posterior (PCAs), and anterior ciliary arteries. The PCAs arising from the OA, situated on either side of the optic nerve, pierce the sclera laterally, medially, or more infrequently superiorly, and serve as the main source of blood supply to the ocular structures and the optic nerve head (ONH) [178]. The short PCAs, extending to the posterior part of the eyeball, constitute the main source of blood supply to the ONH, and the distal short PCAs supply

the corresponding sectors of the choroid. The long PCAs, one on the medial side and one on the lateral side, traverse towards the iris, while the anterior ciliary arteries extend towards the arterial circle situated around the circumference of the iris.

Based on the structural anatomy of the eye (Figure 3.10), arteries in the middle layer of the eye (uveal vessels) supply the iris, choroid, ciliary body, and outer retinal layers while the retinal vessels originating from the OA supply the inner retinal layers.

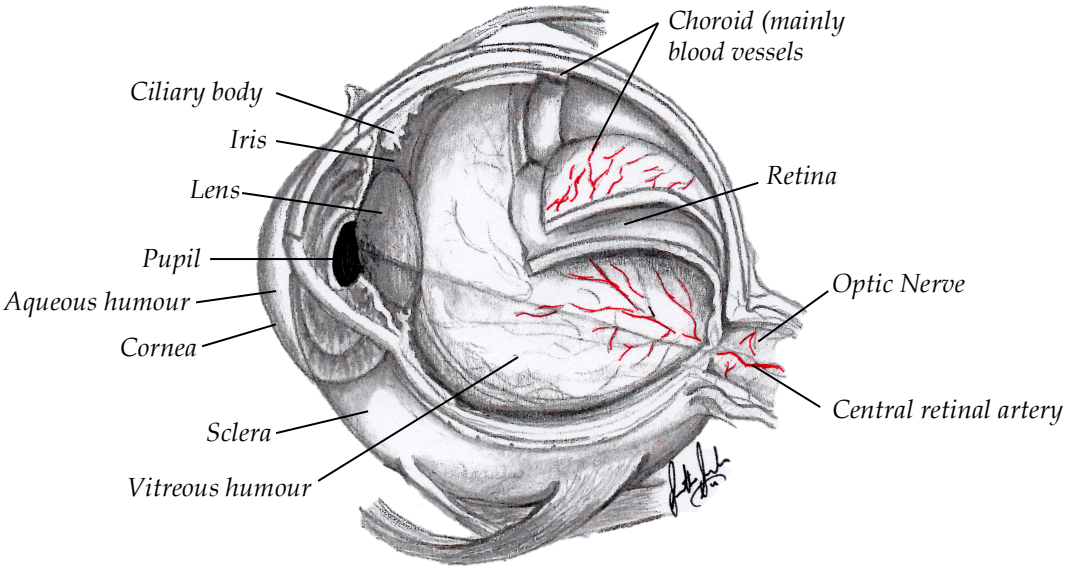


Figure 3.10. Illustration of the human eye
The eye is a fluid-filled sphere enclosed by three layers of tissue. The opaque layer (sclera), mainly comprised of fibrous tissue, transforms into a specialized transparent tissue in the front of the eye (cornea), which the permits entry of light rays. The middle layer comprises three distinct but continuous structures: the iris, the ciliary body, and the choroid. The iris forms the coloured portion of the eye, and contains muscles that allow the size of the pupil to be adjusted. The ciliary body encircles the lens and includes a muscular component for adjusting the refractive power of the lens, and a vascular component (the ciliary processes) that produces the fluid that fills the front of the eye. The choroid comprises a rich capillary bed that serves as the main source of blood supply for the photoreceptors of the retina. Only the innermost layer of the eye, the retina, contains neurons that are sensitive to light and are capable of transmitting visual signals [179]. Illustration sketched by author based on Khurana [180].

3.1.5.1. Retinal vascular supply and drainage

The evolution of two main vascular supplies ensures that blood supply demand is met with minimal optical interference: mainly the inherent intra-retinal vessels and the choroidal circulation, which nourishes the photoreceptors in the outer retinal layer.

Emerging from the nasal side of the optic disc, retinal vessels are arranged in a radial pattern temporally, and an arching pattern nasally. The outer portion of the retina receives

its blood supply from the short PCAs via the choriocapillaris (a network of choroidal capillaries located beneath the retinal pigment epithelium) while the CRA is the major source of blood supply to the inner retina [181]. As sympathetic nerve terminals are localized in the vSMCs of choroidal vessels, retinal vessels do not receive autonomic innervation [182].

The four main intra-retinal branches of the CRA are responsible for blood supply to the whole inner retina. Intra-retinal arteries, or if accurate terminology were to be used, arterioles form smaller terminal arterioles that contribute to a network of capillaries as they extend towards the periphery. The retinal capillary network is organized into three main sections namely the radial peripapillary capillaries (RPCs), an inner capillary network and an outer capillary network. The RPCs form the most superficial layer [183]; the inner capillaries underlying the RPCs form a complex capillary inner (superficial) plexus, and; the outer capillaries form the deeper plexus [184]. Both the superficial and deep plexus layers extend towards the edge of the retina, with the exception of a capillary free zone in the central macula region [185]. Drainage of the retinal circulation occurs in a similar arrangement, as venous outflow occurs primarily via vortex veins and the central retinal vein (CRV), which merge with the ophthalmic vein to drain into the cavernous sinus [186].

3.1.5.2. Physiology of ocular blood flow

The regulation of ocular blood flow (OBF) is intricate, as in most tissues, due to various factors influencing vascular resistance, such as autonomic innervation and contractile elements in the vessel wall and intravascular milieu. The general strategy is that autonomic and circulatory factors mediate the moment-to-moment adjustment of cardiac output while local mediators strive to optimize conditions in response to factors such as CO_2 and O_2 tensions, and pH.

OBF maintains synchronous satisfaction of oxygen and nutrient demand for tissue homeostasis during visual function without interfering with the visual pathway [187, 188]. Blood flow, Q, inside a vessel is directly proportional to perfusion pressure, ΔP, which is the difference in pressure between two ends of a vessel; and inversely proportional to vascular resistance, R, which occurs due to friction between the vascular endothelium and moving blood. This relationship is given by:

$$Q = \frac{\Delta P}{R} \qquad \textbf{(Equation 3.2)}$$

Resistance is determined by the properties of the fluid and is directly proportional to the vessel length, L, blood viscosity, η; and inversely proportional to vessel radius r. This relationship is given by:

$$R = \frac{8\eta \mathrm{L}}{\pi r^4} \qquad \textbf{(Equation 3.3)}$$

The viscosity term, η, depends on the red blood cell (RBC) or haematocrit concentration and is shear rate dependent. Viscosity decreases as shear rate increases as per:

$$\eta = \frac{Shear\ stress\ (dynes/cm^2)}{Shear\ rate^{s-1}} \qquad \textbf{(Equation 3.4)}$$

The *Hagen-Poiseuille* law describes the relationship between all factors influencing blood flow through a vessel:

$$Q = \frac{\Delta P \pi r^4}{8\eta L} \qquad \textbf{(Equation 3.5)}$$

As large arteries secede into smaller arteries, shear rate gradually decreases and η increases, and is greatest in capillaries. This phenomenon is known as the *Fahraeus-Lindquist* effect, and is attributed to 'plasma skimming' or the passage of RBCs through capillaries single-file.

Based on these principles of blood flow, in intraocular tissues flow, Q, is similarly dependent on arterial pressure, P_a, local venous pressure and resistance to flow, R (Equation 3.6). For practical purposes, and based on the drop in BP between the heart and the OA, arterial pressure can be estimated as 2/3 of the mean arterial pressure, MAP (Equation 3.7) and venous pressure is assumed to equal the intraocular pressure (IOP), except in instances where IOP is very low [189]. IOP is defined as the force per unit area exerted on the ocular tissues by the fluids they contain, and is mainly determined by the coupling of aqueous humour production and drainage through the trabecular meshwork of the anterior chamber. The anterior chamber is the fluid-filled space between the iris and the innermost endothelial surface of the cornea. Ocular perfusion pressure (OPP) is influenced by IOP and is based on the principle that pulsatile variations in IOP are due to blood

surging in the eye. The pulsatile component is thought to be that delivered during systole, while diastolic flow accounts for two thirds of ocular flow [190]. By definition, OPP is regarded as the arterial and venous pressure differential, and since this cannot be measured directly in the retinal circulation, it is based on the difference between MAP and IOP (Equation 3.8).

$$Q = \frac{P_a - IOP}{R} \quad \textbf{(Equation 3.6)}$$

$$MAP = \frac{2}{3}\,DBP + \frac{1}{3}\,SBP \quad \textbf{(Equation 3.7)}$$

$$OPP = \frac{2}{3}\,MAP - IOP \quad \textbf{(Equation 3.8)}$$

Given the relationship between BP and OPP, a reduction in OPP can be caused by a rise in IOP or a reduction in systemic BP.

3.1.5.3. Local control mechanisms and autoregulation

In many organs and tissues, blood flow is relatively stable over a range of arterial pressures in the absence of any neurohumoral input – a flow behaviour known as autoregulation [191, 192]. Stated differently, in autoregulated circulations, flow in that region occurs independently of perfusion pressure. The ocular circulation is a classic example of how autoregulatory mechanisms take over to buffer hydrostatic fluctuations in pressure and preserve tissue homeostasis when blood supply originates from regions of high pressure. Probable local control mechanisms in the eye include vascular responses linked to tissue metabolism (reactive hyperaemia, functional hyperaemia, and autoregulation), transmural pressure (myogenic response), and shear stress (flow-mediated vasodilation) [192, 193] – which further discussed in the subsequent sections. In the absence of autonomic innervation, metabolic and myogenic stimuli are more involved in retinal autoregulation. The contribution of flow-mediated vasodilation is, however, less well-understood since the response varies with location in the arterial tree and is likely counterbalanced by metabolic and myogenic local control.

3.1.5.4. *Myogenic control*

The myogenic mechanism, or myogenic response, occurs in vessels from diverse tissues and organs, including the eye [186]. The myogenic mechanism responds to stretch or vascular wall tension and, as such, is considered mechanically independent of the endothelium and intrinsic to vSMCs. As blood flow increases, stretching of the vSMCs in the vessel leads to depolarisation of the vSMC membrane with the influx of calcium ions (Ca^{2+}) into the cytosol inducing vasoconstriction. The myogenic response is thus characterized as vessel diameter decreases induced by increases in transmural pressure [194]. At the level of systemic vasculature, the primary function of the myogenic response is to facilitate capillary fluid exchange. Whether this is also the case in the eye remains unclear [195]. Nevertheless, myogenic regulation has been demonstrated in the retina and ONH [196]. With regards to homeostasis, the mechanism is better suited to regulate capillary hydrostatic pressure than flow, that is, if arterial or venous pressure rise, the myogenic response would act to preserve capillary hydrostatic pressure. Although, this suggests that in some situations the flow response may be exacerbated. For example, when venous pressure is increased, the upstream pressures also increase and elicit a myogenic response whereby the fall in blood flow due to decreased perfusion can be worsened by the myogenic vasoconstriction [197].

There is considerable evidence to support a myogenic role in autoregulation [198]. During variations in perfusion pressure, the myogenic model of autoregulation assumes that resistance vessels have a sensor coupled in series with the contractile elements that respond to changes in wall tension as defined by the *Law of LaPlace* (tension equals the transmural pressure times the vessel radius). This model suggests that if the arterial wall is stretched by an increase in pressure, the muscle cannot simply contract back to its original length, since that would return the vessel to its original radius, resistance would be unchanged, and flow would increase. As such, if arterial pressure increases, the arterial contraction must decrease the radius below typical baseline levels to maintain blood flow at a constant.

3.1.5.5. *Metabolic control*

The underlying assumption of metabolic local control is that tissues regulate blood flow to maintain adequate nutrient delivery and waste removal. Since most tissues use aerobic metabolism, oxygen delivery to the tissue is the regulated variable often considered in metabolic local control. Increased oxygen demand induces a vasodilatory signal that increases blood flow and capillary perfusion. Moreover, physiological phenomena such as reactive hyperaemia (transient increases in blood flow following a period of ischaemia)

and functional hyperaemia (increases in blood flow in response to tissue activity) support the metabolic hypothesis of local control.

In the ocular circulation the metabolic hypothesis of autoregulation is based on a tight coupling mechanism between tissue metabolite concentrations and ocular perfusion [192]. The term 'metabolic autoregulation' can also refer to 'vascular reactivity'. Metabolic factors that can alter vascular tone include the osmolarity of extracellular fluid and its concentrations of O_2, CO_2 [199, 200], potassium (K^+), hydrogen (H^+) [201] and adenosine [202]. Metabolic regulation in the retina also involves factors released by endothelial cells, glial cells, and neurons that affect retinal arteriolar tone. These factors can include NO, prostacyclin, ET-1, cyclooxygenase products, and angiotensin-II [193].

3.1.5.6. Neurogenic control of vascular tone

The ANS supplies a large network of vasomotor nerves in the eye that extend to the uvea, PCAs and the extraocular portion of the CRA, and exclude the retina and the pre-laminar portion of the ONH [203, 204]. Sympathetic and parasympathetic regulatory mechanisms are therefore implicated in the regulation of choroidal blood flow, but have little effect on retinal or ONH blood flow [205]. Studies that are more recent have also shown that even high levels of noradrenaline have little impact on retinal vascular tone [206]. It has been suggested, however, that the probable role of sympathetic innervation is in preventing over perfusion during an increase in systemic BP, thereby assisting myogenic autoregulatory mechanisms [207].

3.1.5.7. Evaluation of autoregulatory responses

The most common method of evaluating autoregulatory function in the ocular and systemic vasculature is by assessing the vessel responses to physiological provocation. Common provocation techniques include: postural changes, vessel compression or occlusion, artificial lowering or enhancement of IOP, cold provocation, hand grip exercises, flicker-light stimulation of the retina, or induced hypoxia or hypercapnia.

Ideally, ocular vascular assessments are said to be of clinical value when the spatial resolution of the imaging device permits assessments in localized sites such as the retina, choroid, or optic nerve head; and when the temporal resolution allows for detailed evaluation of regulatory vascular responses to provocative stimuli [208]. Techniques for assessing vascular function, as opposed to ocular blood flow, were therefore of more interest, and the retinal vessel analyser (RVA) system (IMEDOS GmbH, Jena, Germany)

was chosen for this purpose in this thesis. The role of local control mechanisms in the regulation of basal retinal tone, based on existing RVA studies, is further discussed in section 4.1.1.3 of the following literature review (Chapter 4).

4. Retinal vessel analysis

In particular, two aspects of retinal vessel analysis underscore its importance: (i) retinal vessel diameter is a major determinant of retinal blood flow, and; (ii) since blood flow depends on the square of the vessel radius, vessel diameter largely determines vascular resistance. On the other hand, changes in the calibres of retinal vessels have been linked to various vascular-related pathologies suggesting that vascular changes at the retinal level could convey additional prognostic information [107, 209-212]. Thus, the ability to obtain exact retinal vessel diameter measurements is of crucial importance to our understanding of retinal blood flow and regulation.

The commercially available RVA system enables continual measurements of retinal vessel diameters. While the RVA was designed for scientific purposes, dynamic retinal vessel analysis (DVA) refers to the capacity to provide a visual stimulus (flickering light) while measurements are being taken and is intended for clinical use [213]. Flicker-light stimulation is considered advantageous as a more natural provocation method that stimulates the retina exclusively without the involvement of other vascular beds, and retinal vasodilatory reactions in response to flicker are suggested to reflect endothelial function – analogous to measuring post-ischaemic dilation responses in the brachial artery. However, the mechanisms involved can be complex, and several important issues need to be considered before DVA can be used as a surrogate measure of endothelial function in the clinical setting.

The aims of this chapter are to (i) provide an overview of the instrument and technique (further detailed in the methods section); (ii) discuss the potential mechanisms involved in regulation of basal retinal tone and flicker-induced reactive hyperaemia; (iii) summarise the clinical findings from recent DVA studies using flicker provocation, and (iv) discuss the implications and potential for further research in this area as context for the experimental studies in this thesis.

4.1. Methods of literature review

Articles published from 1990 to 2014 citing endothelial dysfunction, CVD, and use of RVA/DVA were identified using PubMed searches. Keywords for relevant articles included "retinal", "blood flow", "flicker", "retinal vessel analyser, RVA", "dynamic retinal vessel analysis, DVA", "vascular", "endothelial", and "cardiovascular disease, CVD".

4.1.1. Retinal vessel analyser

The RVA system consists of a fundus camera, a high-resolution video recorder, a real-time monitor, and a dedicated personal computer with analysis software. The instrument requires full pupil dilation, which is typically achieved using a muscarinic antagonist such as Tropicamide. Similar to standard fundus photography, a uniformly illuminated image of the fundus is acquired through the dilated pupil. The investigator can then choose a region of interest that encompasses appropriate vessel segments, and continual diameter recordings along the selected segments can be achieved over a specified time-period. Typically, an arterial segment and a venous segment between 0.5 and 1 mm in length and approximately 1 to 2 disc diameters from the ONH are selected (Figure 4.1).

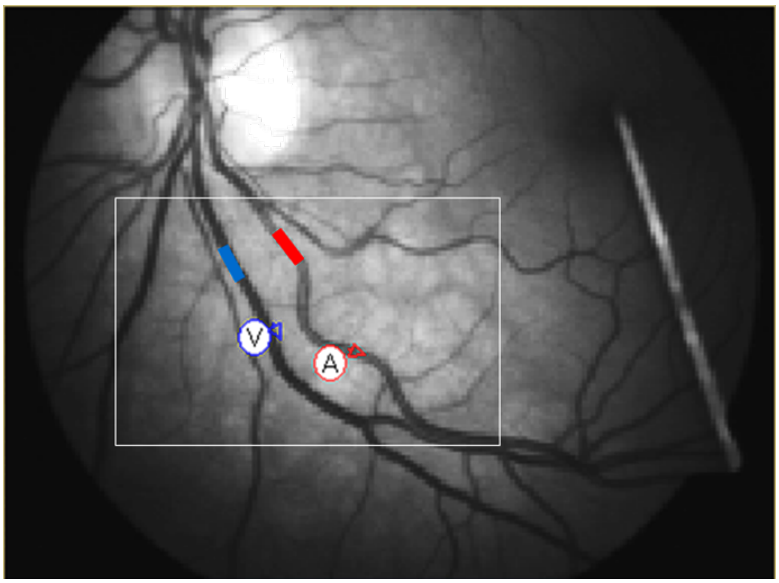


Figure 4.1. Retinal vessel selection for functional assessment
To ensure optimal alignment and patient set-up, an image of the fundus is displayed on the real-time monitor and a green filter inserted into the illumination pathway of the fundus camera is adjusted for optimal contrast sensitivity. Eye movements are controlled for with the use of a visual fixation target (top left) and positioned so that the region of interest is in the centre of the fundus image. Within this region segments along the inferior temporal retinal artery (A) and inferior temporal retinal vein (V), approximately 0.5-1 mm in length and approximately 1 to 2 disc diameters from the optic disc margin, are selected to begin the examination.

4.1.1.1. Recording principle

The general device recording principle involves an analysis of the brightness profile of the retinal vessels, which is based on the absorbing properties of erythrocytes or RBCs within the vessel. As such, in the strictest sense, the RVA measures the width of the RBC column within the selected vessel. To achieve optimal contrast sensitivity, a green filter is inserted into the illumination pathway. Measurements of the width of the RBC column are then achieved when the illumination light of the fundus camera enters the eye through a dilated pupil, is reflected by the different layers of the retina and retinal vessels, and then delivered via the observation pathway to the camera. Retinal vessel diameter is measured in real-time at a maximum frequency of 50 Hz enabling a maximum of 25 readings per second of the vessel diameter as a function of time. A series of adaptive algorithms compensate for any disruption of the vessel brightness profile brought about by the presence of anomalous reflections from the background or vessel surface. The software also has the capability to correct for slight eye movements during the assessment, and can continuously monitor image quality according to image contrast, and automatically remove any inadequate measurements from the analysis [141, 214].

4.1.1.2. Clinical protocol

Adequate pupil dilation prior to measurement is crucial and the use of a muscarinic antagonist is recommended to achieve this [213]. A brief patient acclimatization period before the measurement process begins is also recommended to achieve stable haemodynamic conditions, which can be verified with repeat BP and pulse rate recordings. The use of vasoactive substances that may influence retinal vessel diameter measurements, such as nicotine and caffeine, should also be noted.

4.1.1.3. Applicable stimuli during diameter measurements

Provocation of the vascular system can be achieved with a number of stimulation techniques that can be applied during retinal vessel diameter measurements, including: isometric exercise, artificial changes in IOP using a suction cup, CO_2 (hypoxia/hypercapnia) or O_2 inhalation (hyperoxia), intravenous or oral vasoactive substance administration, and flicker-light stimulation. Although the precise mechanisms involved in retinal autoregulation remain to be investigated, prevailing RVA studies using varying provocative stimuli indicate that influences on retinal blood flow and vascular resistance rely on contributions from both metabolic and myogenic factors [215] (Table 4.1).

Table 4.1. Overview of relevant studies using the RVA system

		Author	Year	Participants	Method	Purpose / Summary	Main Findings
Physiology – Metabolic factors	Oral / Infusion studies	Polak [216]	2000	9 healthy subjects	Infusion	Reproducibility and sensitivity study with administration of vasoconstrictor and vasodilator agents	RVA is an accurate system for the assessment of retinal diameters. NO appears to have a strong influence on retinal vascular tone.
		Polak [217]	2003	18 healthy subjects	Infusion	To assess the effects of intravenous ET-1	ET-1 exerts vasoconstrictor effects in retinal vessels.
		Garhofer [218]	2003	12 healthy subjects	Infusion	To assess the effects of intravenous sodium-lactate	Sodium lactate increases retinal blood flow.
		Zawinka [219]	2004	14 healthy subjects	Infusion	To assess the effects of intravenous histamine	Retinal arterial and venous vessel diameters are significantly increased but retinal blood flow is unaltered.
		Garhofer [220]	2005	12 healthy subjects	Infusion	To assess the effects of intravenous l-arginine	Retinal venous diameters are decreased, whereas red blood cell velocity is significantly increased.
		Noonan [221]	2014	22 healthy subjects	Oral	To assess the effects of epoxyeicosatrienoic acids (EETs) and prostaglandins (PGs) inhibition	EETs and PGs are unlikely mediators of the flicker response, though EETs may play a role in the regulation of retinal vascular tone.
	Hyperoxia/ Hypoxia/ Hypercapnia	Lanzl [222]	2000	10 healthy subjects	Hyperoxia	To assess whether functional changes due to 100% O2 breathing can be assessed by RVA	All subjects demonstrated vasoconstriction. The mean diameter reduction for the group was 6.5% for arteries and 15% for veins.
		Dorner [223]	2002	10 healthy subjects	Hypercapnia	To assess retinal blood flow response with a breathing mixture of normal air and 5% CO_2 for 13 minutes	CO_2 induces vasodilation in retinal arteries and retinal veins by 4.2% and 3.2%, respectively.
		Jean-Louis [224]	2005	20 healthy subjects	Hyperoxia	To assess the influence of systemic hyperoxia on retinal vasomotor response	Retinal vessels change in calibre uniformly across retinal quadrants in healthy young adults.
		Kolodjaschna [225]	2008	18 healthy subjects	Hyperoxia / Infusion	To assess retinal blood flow in response to 100% oxygen breathing after LPS administration	All retinal haemodynamic parameters showed a decrease during 100% oxygen breathing, and LPS-induced inflammation induced vascular dysregulation.
		Palkovits [226]	2014	41 healthy subjects	Hyperoxia	To assess retinal oxygen metabolism during normoxia and hyperoxia	Systemic hyperoxia caused a significant decrease in retinal venous (-13.0% ± 4.5%) and arterial diameters (-12.1% ± 4.0%).
		Petersen [227]	2014	20 healthy subjects	Hypoxia	To assess the effects of a NOS-inhibitor and PG inhibitor	Acute hypoxia-induced vasodilations are modified by NO and PG synthesis inhibition.

Table 4.1. Overview of relevant studies using the RVA system (contd.)

		Author	Year	Participants	Method	Purpose / Summary	Main Findings
Physiology – Myogenic factors	Isometric exercise	Blum [228]	2000	40 healthy subjects	Isometric exercise	Measurement of retinal artery diameters over a 9-min period	Significant differences in BP rise were followed by myogenic response of 1% up to 10% vasoconstriction.
		Jeppesen [229]	2004	51 healthy subjects	Isometric exercise	To assess the age-dependency of myogenic response	An age-related decrease in diameter response of retinal arteries is observed when BP is changed.
		Garhofer [230]	2004	12 healthy subjects	Exercise using euglyceamic / hyperglycaemic clamp	To assess the influence of exercise induced hyperlactataemia on retinal blood flow	Both lactate and glucose induce an increase in retinal blood flow.
		Blum [231]	2005	12 healthy subjects	Isometric exercise	Measurement of retinal artery diameters over a 9-min period (3 min baseline; 3 min isometric exercise; 3 minutes of recovery) – repeated with glucose administration	The myogenic response of the retinal arteries was significantly reduced during an acute rise in blood glucose levels.
		Blum [215]	2005	20 healthy subjects	Isometric exercise / hyperoxia	Comparison of isometric exercise vs. hyperoxia – induced responses	Isometric exercise vs. 100% O_2 breathing resulted in comparable arterial vasoconstrictory responses in the same individuals.
		Jeppesen [232]	2007	10 healthy subjects	Isometric exercise	To assess response of retinal arteries as a function of vessel diameter	BP-induced diameter response of retinal arteries increased with decreasing diameter of the vessels.
	Suction cup	Nagel [233]	2004	13 healthy subjects	Suction cup	To assess the autoregulative retinal response to artificial reductions in perfusion pressure	Artificially elevated IOP increased arterial diameter and venous vessel diameter.
		Garhofer [234]	2005	15 healthy subjects	Suction cup / Flicker	To assess the influence of short-term increases in IOP on flicker-induced changes in retinal vessel diameters	Short-term increase of IOP does not alter retinal blood flow in response to flicker stimulation.

Abbreviations: BP, blood pressure; CO_2, carbon dioxide; COX, cyclooxygenase; EET, epoxyeicosatrienoic acid; ET-1, endothelin-1; IOP, intraocular pressure; l-NMMA; N5-[imino(methylamino)methyl]-L-ornithine, citrate; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; O_2, oxygen; PG, prostaglandin; RVA, retinal vessel analyser.

4.1.1.3.1. Isometric exercise

Provocation of the vascular system by way of isometric exercise is usually achieved with the use of weights, which induce an increase in systemic BP. This technique has been used to examine the *Bayliss* effect [235] (response to mechanical stretch), and to evaluate regional differences in vascular tone [236]. Increases in BP elicit a myogenic vasoconstrictory response in retinal arterioles [228], which is age-dependent [229] and reduced during acute rises in blood glucose levels [231]. Retinal blood flow, however, is increased in response to exercise-induced increases in glucose and lactate [230]. In a more recent study, vasoconstrictory responses in retinal vessels were increased with caffeine consumption, which was associated with increases in BP [237].

4.1.1.3.2. Suction cup IOP enhancement

Artificial elevations in IOP can be induced by means of the episcleral suction cup technique [233]. Typically, a topical anaesthetic is applied before the suction cup is applied to the sclera. The negative pressure that it produces is then transferred onto the globe of the eye, which causes a subsequent increase in IOP. Studies examining the autoregulative behaviour of retinal vessels in response to increases in IOP show opposing autoregulative phenomena; increases in retinal arterial diameters and decreases in retinal venous diameters. These opposing effects have been attributed to physiological variations in the regulative functions of arteries (active) and veins (passive) [233].

4.1.1.3.3. Inhalation & Infusion studies

Since intraocular vessels are very sensitive to changes in oxygen, the partial pressure of oxygen (pO_2) has been identified as one of the main driving forces of metabolic autoregulation [238]. A number of studies have evaluated metabolic regulation in the retina in response to hyperoxia [222, 224, 225, 239-241]. Typically, hyperoxic conditions induce vasoconstrictive responses in retinal vessels [199, 222, 239, 240, 242], while hypoxia [227, 243] and hypercapnia [223] induce vasodilation. It has been reported that the degree of vasoconstriction during 100% O_2 inhalation is comparable in all quadrants of the fundus [224]. Hyperoxia studies tend to focus on diabetic patients, and the vasoconstriction responses in this group of patients has been shown to lessen with progressive stages of the disease [244]. In pathological conditions, the dynamic regulation of vasoactive mediators, such as NO, has furthered our understanding of the mechanisms underlying hyperoxia-induced vasoconstrictive responses in retinal vessels [245]. Hyperoxia-induced retinal responses are mediated by ET-1 [246], which is consistent with other studies confirming the vasoconstrictor effects of ET-1 [217]. Hyperoxic-induced responses are also attenuated with induced

inflammation, supporting a link between inflammation and vascular dysregulation in the retina [225]. However, there is still the possibility of other mechanisms that could contribute to these processes, which remain to be investigated.

Most systemic hyperoxia studies use a one-way valve for O_2 delivery. This may, however, lead to a reduction in the partial pressure of carbon dioxide (pCO_2) [247]. More recently, a technique to maintain isocapnia during O_2 inhalation was introduced [248] and it is recommended that both pO_2 and pCO_2 be monitored in the exhalate or arterial blood. While the exact mechanisms underlying vasodilatory responses under hypoxic and hypercapnic conditions remain elusive, interactions between NO and endothelium-derived prostaglandins have previously been described when pCO_2 is increased [249], and via endothelium-derived prostaglandin and/or adenosine mediated mechanisms [250-252]. Retinal vasodilatory responses during acute hypoxia are modified with NO and prostaglandin synthesis inhibition [227], and NO is considered to be one of the most important contributors to retinal tone [220, 253, 254] . The administration of a non-specific nitric oxide synthase (NOS) inhibitor that induces segmental vasoconstriction also indicates that the continual release of NO is necessary for the maintenance of retinal arterial tone. Other factors that are also involved in the regulation of retinal tone include lactate [218, 230] and glucose [230].

4.1.1.3.4. Flicker-light stimulation

There is compelling evidence that visual stimulation with flickering light increases retinal vessel diameters, and retinal and ONH blood flow [255]. As mentioned previously, DVA refers to the investigation of flicker-induced changes in retinal vessel diameters. It uses the same principle for diameter measurements as the RVA system but includes an integrated flicker-light simulator. Flicker-light stimulation, which stimulates the retina exclusively without the involvement of other vascular beds, was the chosen provocation method for the experimental studies in this thesis. Further details on this technique are provided in the following sections and in the methods (section 6.3.4.1).

4.1.2. Dynamic retinal vessel analysis (DVA)

The flicker stimulation system is based on an optoelectronic shutter placed in the illumination pathway of the fundus camera (section 4.1.1). This device interrupts the device illumination light at a rate of 12.5 Hz. The maximum sensitivity of the human visual system to flicker stimulation, defined as alterations in brightness or colour, is thought to be elicited with a flicker frequency between 10-20 Hz [256], and in retinal vessels vasodilatory responses are observed at all flicker frequencies between 2 and 64 Hz, with a

less-pronounced effect at either end of the range [257]. At present, there is a lack of standardisation with regards to the evaluation and analysis of DVA data resulting in a growing number of vessel response parameters to characterise dynamic vessel behaviour. More recent emphasis on the need for standardised protocols and data evaluations, for comparison purposes across studies, has fortunately seen the use of only two measurement protocols.

4.1.2.1. Flicker Protocol 1

Flicker protocol 1, also referred to as the standard protocol, is widely used due to its incorporation in the device software. This protocol involves a 350-second continuous diameter measurement of selected retinal vessel segments (see Figure 4.1), automatically recorded by the device software. Baseline vessel diameter is first measured for 50 seconds then stimulation begins with 20 seconds of flickering light followed by a still illumination period of 80 seconds. The flicker cycle is repeated twice more, lasting a total of 350 seconds (Figure 4.2). Consequently, during flicker, recording only takes place during half of the frames resulting in a sampling rate of 12.5 Hz [140].

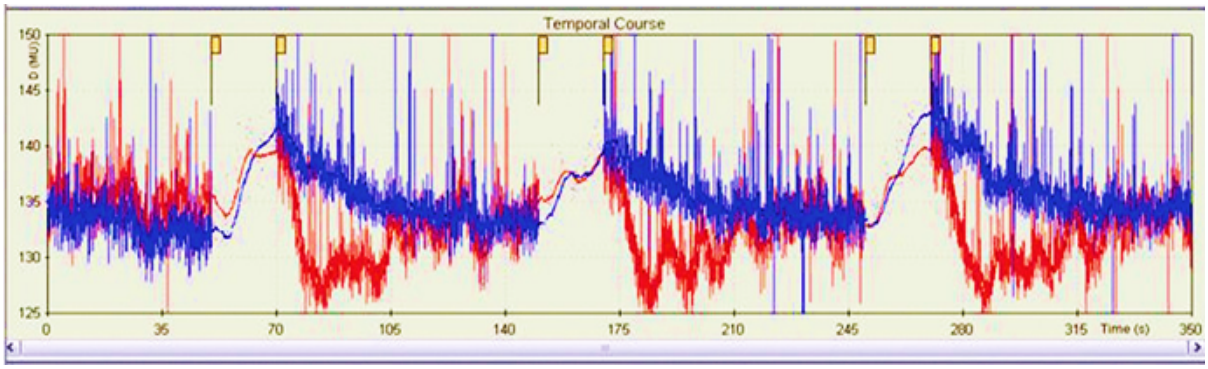


Figure 4.2. Temporal course of completed dynamic retinal vessel examination
Coloured lines reflect arterial (red) and venous (blue) diameter changes over time. The examination begins with a 50-second baseline recording following by three consecutive flicker cycles comprising a 20-second flicker period (between orange flags) and an 80-second recovery period.

4.1.2.2. Flicker protocol 2

Flicker protocol 2 uses a flicker frequency of 8 Hz, which is generated by an additional light source and thereby distinct from the fundus illumination. This set-up also enables uninterrupted data acquisition [229]. Though the recording length of this protocol is similar to that of protocol 1, reports of studies carried out using protocol 2 tend to vary with regards to the durations of the baseline measurement, flicker periods, and recovery periods.

4.1.2.3. Data analysis

Retinal vessel diameters are typically reported in relative units of measurement (UM), as recorded by the device software, which equate to micrometres (μm) in the normal emmetropic eye [213]. Given the volume of data generated from each recording session – multiple data points for the length and width of the selected vessel segments per video frame for the entire recording duration – most researchers consider averaged measurements of the width and length of the vessel profile, flicker cycles, and at different stages of the protocol (spine-point analysis [258]). More recently, Fourier analysis has also been applied to evaluate the longitudinal vessel profile over time [259].

4.1.2.3.1. Spine-point analysis

Diameter recordings are typically averaged over the vessel length and then averaged over 1-second intervals [260]. The average vessel diameter during the 20 to 30 seconds prior to the first flicker is determined as baseline and the time-series data is normalised such that diameter readings are expressed as a percentage of baseline. The main parameters of interest include the maximum dilation diameter (MD) during flicker, and the maximal constriction diameter (MC) post-flicker, expressed as percentage change relative to baseline (percent dilation, MD%; percent constriction, MC%) and the time points at which these occur (tMD and tMC) [258, 261]. Further attempts to characterise the vessel's elastic behaviour have introduced dilation amplitude (DA), a parameter calculated as the difference between MD and MC (Equation 4.1). In order to take into consideration spontaneous variations in vessel diameter or baseline diameter fluctuations (BDF), that can occur under normal resting conditions due to vascular tone and arterial pulsation, the concept of a baseline corrected flicker response (BCFR) was introduced by Nagel and others [258] where BDF, mathematically defined as the range of baseline vessel diameters (baseline maximum – baseline minimum), is subtracted from the DA (Equation 4.2).

$$DA = MD - MC \qquad \textbf{(Equation 4.1)}$$

$$BCFR = DA - BDF \qquad \textbf{(Equation 4.2)}$$

4.1.2.3.2. Sequential diameter response analysis (SDRA)

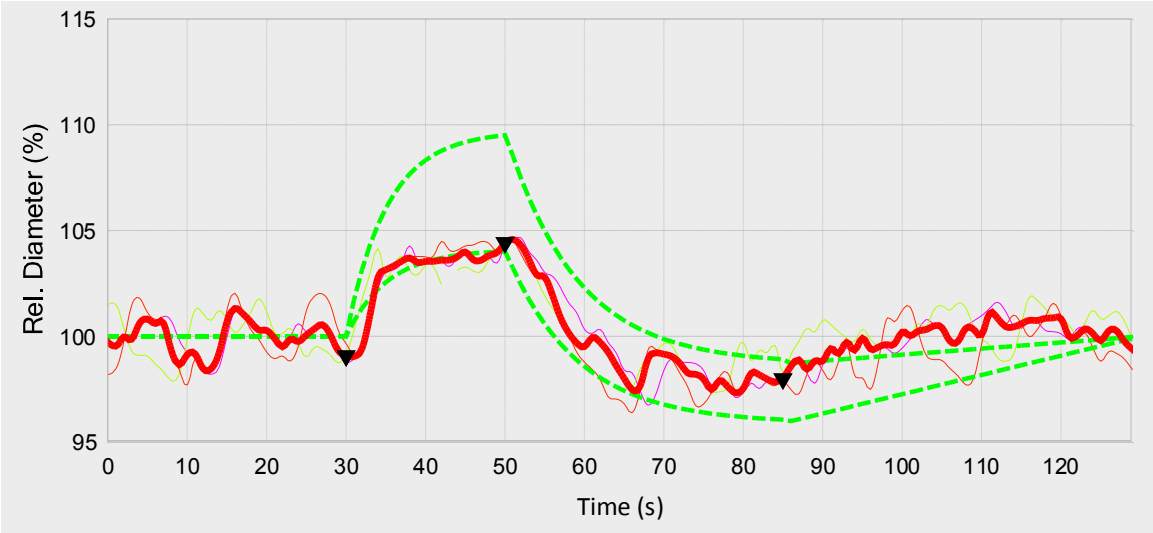
While the in-built device software has the capability to compute MD% and MC%, a number of shortfalls have been recognized in previous studies [214, 260]. The shortfalls of the in-built software analysis method largely arise due the computation of one averaged value across flicker cycles for either MD% or MC%. The software also considers the average diameter from the last +/- 3 seconds of flicker stimulation as the maximum diameter response to flicker. This provides a relatively narrow window (between 17-23 seconds from start of flicker) within which the average diameter is considered as the maximum diameter response, and this would underestimate any individual's response if their maximal dilatory response were to occur outside this window (Figure 4.3). In order to enable assessments of each individual flicker cycle, which can be regarded separately for the artery and vein, sequential diameter response analysis (SDRA) was later introduced and validated as a more sensitive measure of the vascular response to flicker light with good coefficients of variation [260].

SDRA has the advantage of using the raw response data generated by the device software and enables the inclusion of BDF, BCFR, and DA in addition to MD, MC, tMC, and tMD for individual flicker cycles. Another recent, additional parameter is the area under the curve (AUC) for various phases of the measurement cycle i.e., baseline, flicker, and recovery [262, 263]. The AUC, when combined with other spine point measures (MD and tMD), is a parameter that indicates the speed and longevity of the vessel's reaction. Primary vessel response parameters of interest to this thesis are detailed in section 6.3.4.7. Further expansions of the data visualization and response parameter analysis specific to this thesis are described in section 6.3.4.8.

4.1.2.3.1. Fourier analysis

In brief, Fourier analysis is used to delineate signals into a series of sine waves of varying amplitude and frequency. The energy ($amplitude^2$) is represented in the power spectrum and the spectral edge frequency (SEF) of the power spectrum of the longitudinal vessel profile has been used to characterise the microstructure of the vessel wall [259, 264]. Typically, increasing SEF is interpreted as a reflection of the degree of roughness of the vessel wall and has been determined for longitudinal vessel profiles associated with retinal arterial baseline, dilation, constriction, and recovery in response to flicker stimulation in healthy subjects [259] and hypertensives [264].

A



B

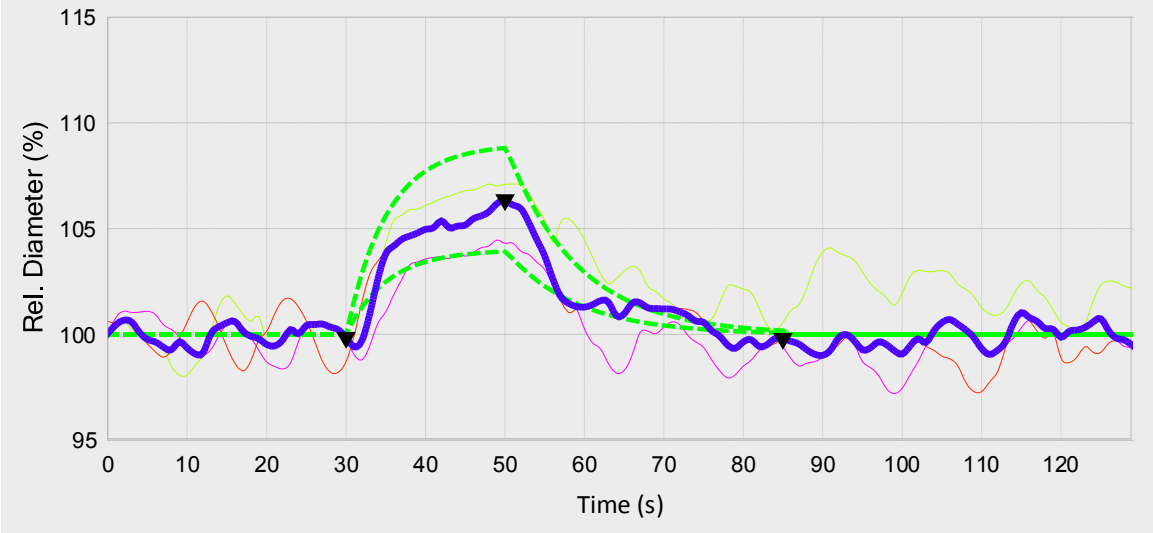


Figure 4.3. Retinal vessel response profiles
The device software displays a retinal vessel response profile summary for the (A) artery (red) and (B) vein (blue). Each vessel profile shows the percentage change in vessel diameter relative to baseline and the reference area of a normal reaction curve (green lines).

4.1.2.4. Reproducibility and repeatability

Measurements with the RVA system, without any stimulation, are reproducible under optimal conditions and appear to be highly repeatable in healthy subjects [141, 216, 265, 266]. Coefficients of variation (CV) for retinal arteries and veins, short-term (12 minutes apart), have been reported at 1.3% and 2.6%, respectively. For day-to-day variability, for arteries and veins, CVs have been reported at 5.2% and 4.4%, respectively [216]. In another study, short-term reproducibility (2 hours) and long-term (1 month) reproducibility of repeated measures of retinal veins were 1.5% and 2.8%, respectively [141]. Further studies assessing short-term and long-term reproducibility report comparable results [265, 266]. It could be

deduced, based on CVs taken over three or four measurements [225], that CV values in the range of 1-5% are typical [216, 260, 265, 266], and that reproducibility may be higher for retinal veins. Whether this reflects phenomena associated with image resolution or due to differences in the absorption properties of arteries and veins (retinal veins being larger than arteries), remains to be clarified. However, with only three to four measurements in each subject, and with relatively large 95% confidence interval values, it is difficult to make inferences about repeatability. Moreover, the exact reproducibility of RVA measurements in patients with overt diseases is yet to be published in the literature. However, Table 4.2 provides an overview of relevant reproducibility and repeatability studies using flicker stimulation (DVA) in healthy subjects.

Retinal blood flow increases in response to flicker [267] and an increase in retinal vasodilatory responses is observed at all flicker frequencies, with a less-pronounced effect at 64 Hz. In retinal veins, all flicker frequencies except 2 and 64 Hz induce vasodilation [257]. DVA studies show high reproducibility [256, 266] and over a short period of time (30 minutes and 60 minutes) [269]. Although, short-term re-testing indicates that at least 30 minutes should be allowed between consecutive tests to minimize suppression of the flicker response [270]. Since a significant correlation between two eyes of the same individual has been identified [265], measurements can be obtained from both eyes or one unselected eye. In vessel comparison studies, there are similarities in dilative amplitudes but differences between arteries and veins in the abatement of the flicker-induced dilation response [268].

A more useful measure of reproducibility is the intraclass coefficient or κ, which has been reported for short-term (<1 day) and long-term (>3 days) [225]. Based on relatively high short-term values ($\kappa_{arteries} = 0.96$; $\kappa_{veins} = 0.98$) and long-term values ($\kappa_{arteries} = 0.87$; $\kappa_{veins} = 0.90$), the estimation of retinal vessel diameters before flicker provocation appears to be highly reproducible in the short-term. However, κ has yet to be computed for spine points and there is limited data on CVs from a single recording session for a limited number of vessel response parameters. In a study of healthy, non-smokers [260], MD ($CV_{arteries} = 1.3\%$; $CV_{veins} = 1.0\%$) and MC ($CV_{arteries} = 1.2\%$; $CV_{veins} = 0.6\%$) appear to be least variable, while tMD ($CV_{arteries} = 30.6\%$; $CV_{veins} = 18.6\%$) is highly variable. Reproducibility data on other measures such as AUC or Fourier analysis are yet to be published. Further details on the characterisation of normal and attenuated flicker-evoked responses are discussed in section 4.1.4.1.

Table 4.2. Overview of reproducibility and repeatability studies

	Author	Year	Participants	Method	Purpose / Summary	Findings
Reproducibility / Repeatability	Seifertl [141]	2002	12 healthy subjects	-	Reproducibility study: describe design and function of RVA	RVA is a suitable device for retinal vessel analysis and continual vessel diameter recordings.
	Polak [257]	2002	9 healthy subjects	Flicker*	To assess retinal vasodilation in response to short wavelength flicker with frequencies between 2 and 64 Hz	Increases were observed at all flicker frequencies, with a less-pronounced effect at 64 Hz. In retinal veins, all flicker frequencies except 2 and 64 Hz induced vasodilation.
	Pache [266]	2002	20 healthy subjects	Flicker*	Reproducibility study: response measured at baseline, 2 hours, 2 weeks	High reproducibility observed. RVA appears to be a useful for both analysis and follow-up of retinal vessel diameters.
	Garhofer [267]	2004	11 healthy subjects	Flicker*	To assess retinal blood flow in response to flicker (60-sec baseline; 60-sec flicker; 60-sec recovery)	Diffuse luminance flicker increases retinal blood flow.
	Kotliar [256]	2004	11 healthy subjects	Flicker*	Reproducibility study: red-green and blue-green flicker stimulation duration of 10 and 30 seconds	Retinal vessel diameter dilation is a reproducible response to the applied flicker stimuli.
	Nagel [268]	2005	26 healthy subjects	Flicker*	Comparison of artery vs. vein (100-sec baseline; five 20-sec flickers; 80-sec recovery)	Flicker-evoked response of retinal arteries and veins do not differ in dilative amplitude but differ in the temporal course of dilation abatement.
	Nagel [265]	2006	28 healthy subjects	Flicker	Comparison of right vs. left eye (50-sec baseline; three 20-sec flickers; 80-sec recovery)	Flicker response parameters between the right and left eyes are significantly correlated.
	Kotliar [259]	2008	33 healthy subjects	Flicker*	To assess the influence of age vessel profile (100-sec baseline; two 60-sec flickers; 150-sec recovery)	In healthy elderly subjects, retinal arteries assume a significantly less regular longitudinal vessel profile than those of young subjects.
	Nguyen [269]	2009	33 healthy subjects	Flicker	Reproducibility study: repeated measures taken 30-60 min	High reproducibility observed for repeated measures over a short period.
	Noonan [270]	2013	20 healthy subjects	Flicker	To assess the effect of short-term testing and re-testing (50-sec baseline; three 20-sec flickers; 80-sec recovery)	Retinal arterial dilation during flicker is reduced on short-term re-testing, but without a significant change in baseline vessel diameter. At least 30 minutes should be allowed between consecutive tests to minimize suppression of the flicker response.

Abbreviations: RVA, retinal vessel analyser. * different flicker stimulation duration, frequency, or protocol to thesis.

4.1.2.5. *Influential factors*

Other factors that have also been shown to have an influence on flicker-induced responses include baseline retinal diameters[232, 258, 271], ethanol [272], smoking [260, 273], and caffeine [237]. Short-term increases in IOP, however, do not appear to have an effect on flicker-evoked retinal responses [234]. More recently, reduced retinal responses to flicker have been reported with higher ambient lighting conditions. As such, it is recommended that ambient lighting levels be consistent to ensure that comparisons across studies are valid [274].

4.1.2.6. *Potential mechanisms underlying flicker-evoked retinal responses*

As discussed in an earlier chapter, in the macrocirculation, brachial FMD represents the gold standard technique for measuring endothelial function and FMD responses are linked with alterations in NO levels [172]. Microvessels are, however, thought to be affected much earlier in the course of disease progression, and this has warranted the assessment of microcirculatory changes for more effective prevention [275].

Previous studies demonstrate that variations in luminance levels alter the metabolic demand in the retina and consequently trigger a cascade of reactions that seek to increase retinal blood flow [267]. A key reaction here also involves the release of NO, which induces a dilatory response in retinal vessels [276, 277]. The role of NO has further been evaluated in response to altered metabolic signals such as oxygen [262, 278], lactate [218, 279] and glucose [280], and a blunted retinal vasodilatory response in response to flicker is observed with the intravenous administration of a NOS inhibitor, namely N-monomethyl-L-arginine [254].

Collectively, these studies reserve a central role for NO in the regulation of retinal vessel tone and demonstrate that flicker-induced retinal vasodilation is dependent on NO. The mediating effect of NO has been further corroborated by studies showing the flicker response to be attenuated in select patient groups with known endothelial dysfunction, such as diabetes and diabetic retinopathy [281].

When considering autoregulation, the determination of dynamic retinal vascular behaviour appears to involve more than just the functional state of the endothelium, and it has become increasingly recognized that a number of feedback mechanisms are involved in the control of retinal blood flow. The contribution of neurovascular coupling mechanisms [282] involving cross talk between neurons, glial cells, and the retinal vascular supply [283, 284] are implicated in the retina, and functional hyperaemia in response to flicker is thought to be mediated by signalling between retinal blood vessels and neurons [256]. Flicker-light stimulates neuronal activity, which has been demonstrated in animal studies to selectively

enhance metabolic activity of the inner retinal layers. The energy reserves of active retinal neurons are restored by metabolic signals (lactate and adenosine) that dilate nearby blood vessels [285].

An alternative to this negative feedback mechanism is a feed forward mechanism [286], whereby neurons release signalling molecules (NO and prostaglandins) that induce vasodilation [287] and augment glucose and oxygen supplies to restore neuronal energy reserves. When retinal activity is increased during flicker stimulation, oxygen diffusion rates from blood vessels must be higher in keeping with increased tissue oxygen consumption rates. An increase in venous oxygen saturation that is linked to pO_2 is thought to reflect a higher oxygen concentration in capillaries, which is necessary for optimal oxygenation of the active inner retinal tissue [278] and explains the phenomenon of increased ONH blood flow. The role of magno- and parvo-cellular pathways is also considered [257, 288]. An overview of these potential mechanisms is provided in Table 4.3.

Flicker stimulation, however, also causes an increase in Ca^{2+} in glial cells, and glial-evoked vasodilations are modulated by oxygen and arachidonic acid metabolites (prostaglandins) [283, 290]. The role of prostaglandins is less clear since flicker-induced retinal vasodilation was more recently shown to be unaffected by prostaglandin inhibitors [221]. Activity-dependent Ca^{2+} increases lead to an enhanced neuronal NO synthesis [291] and vasodilation. Lactate is also implicated in retinal neurovascular coupling mechanisms, as reduced flicker-evoked arterial dilations are augmented in the presence of increased serum lactate concentrations [292], however; definitive evidence as to the exact role of lactate is still lacking owing to the systemic route of compound administration in the aforementioned study.

Neurons and glial cells also release adenosine, which has a vasodilatory influence and is an additional metabolic factor that could mediate flicker-induced functional hyperaemia in the retina. Although, intravenous administration of adenosine enhances blood flow in the cat retina, while the involvement of adenosine in neurovascular coupling mechanisms is yet to be defined for the human retina [293]. Finally, an additional effect of dopamine has also been described in the modulation of flicker-evoked retinal responses [289].

Table 4.3. Overview of potential mechanisms involved in the flicker response

		Author	Year	Participants	Method	Purpose / Summary	Main Findings
Physiology – Functional hyperaemia	Clinical studies	Polak [257]	2002	9 healthy subjects	Flicker*	Investigate the effect of diffuse luminance flicker of different frequencies on retinal vessel diameter	Vasodilation of retinal arteries was observed in response to short wavelength flicker with frequencies between 2 and 64 Hz, indicating that the parvo- and magno-cellular neural pathways are activated with this stimulation.
		Dorner [254]	2003	12 healthy subjects	Flicker* / Infusion	Assess the effect of l-arginine administration. Flicker 16, 32, 64 seconds (8Hz)	NO contributes to basal retinal vascular tone in humans and appears to play a role in flicker-induced vasodilation of the retinal vessels.
		Dorner [280]	2003	12 healthy subjects	Flicker* / Infusion	Investigate the effect of high blood glucose levels on the flicker-induced neurovascular mechanism	Retinal vessel response to flicker-light stimulation is significantly reduced during hyperglycaemia.
		Huemer [289]	2003	12 healthy subjects	Flicker*	Assess the effect of dopamine (60-sec baseline; 60-sec flicker; 60-sec recovery)	The response to 8-Hz flicker light was significantly reduced by dopamine administration. In addition, dopamine slightly but significantly increased retinal vessel diameters.
		Garhofer [279]	2003	12 healthy subjects	Flicker* / Infusion	Assess the effect of lactate and MAP with lactate and tyramine infusion (60-sec baseline; 60-sec flicker; 60-sec recovery)	The signalling between neuronal activity and flow response in the human retina is sensitive to changes in blood lactate levels, whereas changes in systemic blood pressure had no major effect.
		Garhofer [267]	2004	11 healthy subjects	Flicker / LDV	Quantify changes in retinal blood flow during flicker stimulation (60-sec baseline; 60-sec flicker; 60-sec recovery)	Flicker stimulation increased retinal blood flow by +59 ± 20% in arteries and by +53 ± 25% in retinal veins; demonstrating that diffuse luminance flicker increases retinal blood flow in the human retina.
		Kotliar [256]	2004	11 healthy subjects	Flicker*	Reproducibility study: red-green and blue-green flicker stimulation duration of 10 and 30 seconds	Retinal vessel diameter dilation is a reproducible response to the applied flicker stimuli. This finding supports the existence of neurovascular coupling in the human retina.
		Hammer [278]	2011	19 healthy subjects	Flicker	Assessment of vessel oxygen saturation levels (50-sec baseline; three 20-sec flickers; 80-sec recovery)	Retinal venous oxygen saturation was increased by flicker light stimulation.

Abbreviations: LDV, laser Doppler velocimetry; MAP, mean arterial pressure; NO, nitric oxide.

* different flicker stimulation duration, frequency, or protocol to thesis.

While neuronal degeneration could theoretically result in impaired vasodilatory responses in particular conditions, impaired neurovascular coupling may also be a consequence of endothelial dysfunction. For instance, in patients with diabetes, although neural dysfunctions tend to precede clinically apparent diabetic retinopathy [294], neurovascular dysfunction can precede neural dysfunction [295] – implying a vascular cause. On the other hand, the response of retinal vessels to a direct NO-donor has also been shown to be preserved in subsets of diabetic patients [296], and the exogenous administration of NO, therefore, raises the question as to whether the sources of baseline NO secretion and that on demand of neurovascular coupling are the same. Nevertheless, taken together, these studies offer support for the clinical evaluation of flicker-evoked responses in the retina as a useful indicator of endothelial dysfunction and microvascular dysregulation.

4.1.3. Associations with other endothelial assessments

Significant associations have been found between static retinal vessel diameter measurements and circulating plasma markers for endothelial function [103, 297], as well as, markers of atherosclerosis risk such as c-IMT [298] (section 6.3.5.2), but not FMD [298] (section 6.3.5.3). In diabetics, however, static analysis has revealed a significant association between attenuated FMD responses and wider retinal veins [299]. Differences in the results between arteries and veins were attributed to the varied characteristics of the macro- and microcirculations, and particularly the uniqueness of the retinal circulation. However, the lack of an association could also be related to the comparison between methods that capture dynamic properties of the endothelium (FMD) versus one that captures static properties (retinal photography).

Interestingly, DVA studies show weak, but significant associations between attenuated retinal arterial (but not venous) dilation and attenuated FMD responses in controls, diabetics, and hypertensives [300]. Nevertheless, firm conclusions regarding the inter-relationships between measures of systemic arteries and retinal endothelial measurements cannot yet be drawn. The few existing studies do, however, suggest that DVA is capturing functional properties of the vascular system. Although, micro- and macrovascular functions are governed by varying physiological mechanisms, BP is a common underlying regulator, and an imbalance in one system could therefore incite alterations in the other. Moreover, since the microvessels can represent an early target in the onset of vascular

disease, there is the possibility that microvessel dysfunctions precede the appearance of macrovessel dysfunctions.

4.1.4. Clinical studies

Indeed measurements of retinal vessel calibre are important, and several large epidemiological studies consistently report the existence of a correlation between systemic disease factors and retinal vessel calibre. In particular, increased systemic BP is reflected as generalized retinal arteriolar narrowing [128], whereas systemic inflammation or obesity is associated with wider venous diameters [275] (see section 2.5.1). It is also evident, that changes in vessel diameters can predict CHD and stroke mortality [107, 109, 209, 301], and the introduction of new and sophisticated technology such as the RVA/DVA system now allows for a more precise quantification of these changes.

The following sections discuss normal and attenuated flicker-evoked retinal responses, and review DVA studies in relation to overt CVD, cardiovascular-related pathologies (hypertension, diabetes, hyperlipidemia, obesity), and cardiovascular risk factors such as age/ethnicity. A timeline of influential studies leading to the development of DVA and the application of its use in clinical research is also provided in Figure 4.4.

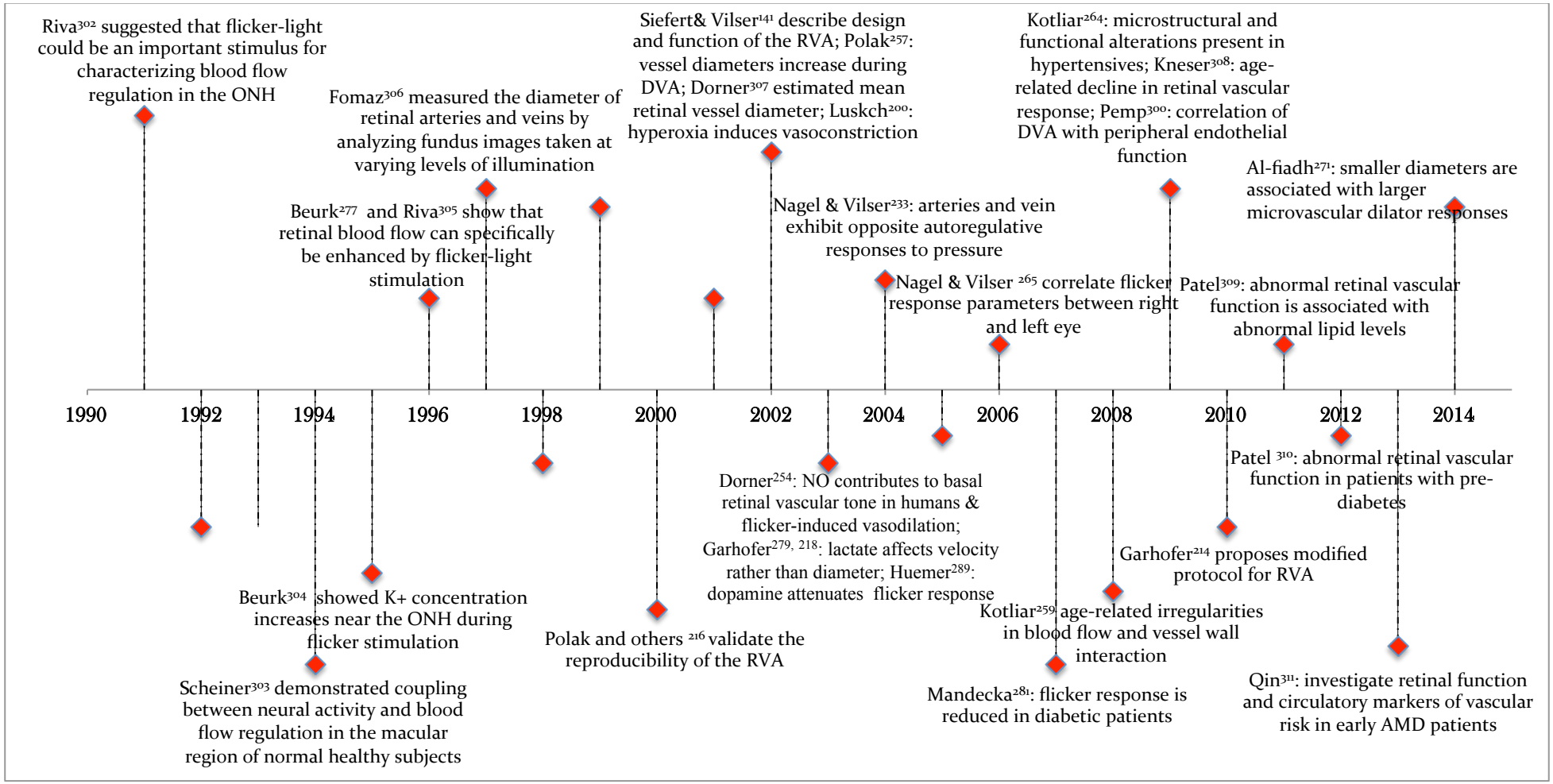


Figure 4.4. Timeline of influential DVA studies
Abbreviations: AMD, age-related macular degeneration, DVA, dynamic retinal vessel analysis; K^+, potassium ion; ONH, optic nerve head; RVA, retinal vessel analyser.

4.1.4.1. Normal and attenuated retinal responses

Comparison studies suggest that flicker stimulation of the retina exhibits a peak dilatory response in the vasculature within 20 seconds [257], after which only small increases in diameter tend to occur. Upon flicker cessation, a steady a decrease in vessel diameters can be observed with overshoots to below baseline levels, or an augmented vasoconstrictive response prior to equilibrium [256]. The diameter declines are thought to occur within 6 to 10 seconds of flicker cessation reaching minimum diameters approximately 10 to 40 seconds after the end of flicker [257, 312]. Although both retinal arteries and veins respond to flicker, there remain some inconsistencies in the literature with one study reporting a less pronounced venous dilation [257], and another study reporting no significant differences between arterial and venous responses [294]. In comparison to arteries, venous responses have also shown a 5-second delay and the absence of an overshoot during diameter returns to baseline [267, 312]. In studies using an assessment protocol and recording duration (protocol 1, section 4.1.2.1) similar to that used in this thesis, healthy subjects exhibit a normal maximum vessel dilation (MD) during flicker of anywhere between 3 and 7% relative to baseline [261, 281, 300, 309, 313, 314].

It should be noted that most studies tend to focus on dilatory responses in retinal vessels while vessel behaviour following the cessation flicker is not extensively studied and, even fewer studies have examined the temporal relationship between retinal vascular functions and the onset and progression of CVD. Nevertheless, DVA has been applied to study endothelial function in patients with overt CAD [313], cardiovascular-related pathologies including hypertension [258, 261, 300, 315], obesity [262], diabetes [281, 300, 316-318], and ocular/systemic vascular dysregulation [311, 319-324], as well as, in relation to a number of cardiovascular risk factors such as age [259, 260, 308], ethnicity [309], and smoking [260, 273].

In patients with overt CVD retinal arterial and venous dilation responses can range between 3.1 to 5.7% and 3.6 to 5%, respectively [313, 325]; in patients with diabetes between 0.1 to 2.9% and 0.5 to 4.6% [236, 281, 314, 318] respectively, and; in patients with hypertension between 3.6 to 4.3% and 3.6 to 6% [261, 300], respectively. Typically, deviations from this normal vascular response are proposed to be indicative of vascular abnormalities or endothelial dysfunction. Less well understood are the influences of other determinants of flicker-induced responses such as age, gender, ethnicity, familial risk, and systemic circulatory mediators.

Indeed, there appears to be close associations between the major risk factors for CVD and abnormal vascular response profiles as assessed by DVA that are further underscored by the presence of endothelial dysfunction. There remain, however, several gaps in the existing literature that together with the lack of reproducibility data in patient subsets [213] still preclude the adequate translation of DVA as an early risk detection tool in clinical practice. The following sections provide a review of DVA assessments carried out in patients with overt CVD, with risk factors for CVD, or with cardiovascular-related pathology to include: hypertension, diabetes, hyperlipidaemia, obesity, and sleep-disordered breathing.

4.1.4.2. Cardiovascular associations of DVA

The various DVA studies relevant to assessing endothelial function in overt systemic disease are summarised in Table 4.4.

4.1.4.2.1. Hypertension

Retinal arteriolar narrowing has historically been recognized as vascular feature in hypertensive individuals during clinical ophthalmic examination [108], and the association between hypertension and retinal arteriolar narrowing has quantitatively been established [326]. There is now evidence that the flicker-evoked retinal arterial response (MD% and BCFR) is reduced in older (> 40 years) untreated hypertensives [258]. In another study, changes in post-therapy BP correlated with changes in baseline arterial diameter rather than vasodilation [261]; indicating that baseline diameter may be more useful in determining vessel reactivity in hypertension. It is likely that determinants of vessel reactivity may differ according to varied disease processes as the underlying cascade of pathologic processes resulting in retinal vascular effects vary. Nevertheless, it is clear from existing DVA studies that retinal vascular behaviour is altered in hypertension; substantiating the hypothesis that endothelial dysfunction is involved in the pathogenesis of hypertension and its related ocular complications.

4.1.4.2.2. Diabetes

The endothelium is a major target in the diabetic milieu and the appearance of endothelial dysfunction signals the risk of developing diabetes-related macro- and microvascular complications [327, 328]. Flicker-stimulation studies confirm the presence of endothelial dysfunction (reduced retinal vasodilation) in diabetes [281, 300, 316]. Moreover, signs of endothelial dysfunction are also present in patients with impaired glucose tolerance (IGT) or pre-diabetes [310], independently of other conventional diabetic risk factors [318].

Table 4.4. Relevant DVA studies in individuals with overt disease

	Author	Year	Measurement	Subjects	Main Outcomes
Diabetes	Garhofer [316]	2004	Flicker protocol 1	26 DM patients (type 1) 26 healthy subjects	Flicker responses of retinal arteries and veins are abnormally reduced in patients with insulin-dependent DM with no or mild non-proliferative retinopathy.
	Mandecka [281]	2007	Flicker protocol 1	172 DM patients (type 2) 172 DM patients (type 1) 53 healthy subjects	Retinal arterial dilation is reduced in response to flicker provocation in type 1 and type 2 diabetics. Flicker response in retinal vessels diminished with increasing severity of retinopathy.
	Mandecka [314]	2009	Flicker protocol 1	18 DM patients (type 1) 19 healthy subjects	Retinal arterial and venous dilation is reduced in response to flicker provocation in diabetics (with no retinopathy) despite comparable static parameters.
	Nguyen [318]	2009	Flicker protocol 1	139 DM patients (type 2) 85 DM patients (type 1) 103 healthy subjects	Retinal arterial and venous dilation is reduced in response to flicker provocation in diabetics.
	Jensen [236]	2011	Isometric exercise / flicker / isometric exercise + flicker	17 DM patients with maculopathy 17 DM patients with retinopathy 17 healthy subjects	During simultaneous isometric exercise and flicker stimulation, there was no difference between the diameter responses of macular arterioles in the three groups.
	Lott [329]	2013	Flicker protocol 1	22 pre-diabetic patients 25 DM patients (type 2) 19 healthy subjects	Reductions in retinal arterial and venous dilation in response to flicker provocation parallel reductions in insulin sensitivity.
	Lim [330]	2014	Flicker protocol 1	279 DM patients with varying stages of retinopathy	Retinal arterial and venous dilation responses are reduced in diabetes and progressively decrease with increasing severity of retinopathy.
HT	Nagel [261]	2006	Flicker protocol 1	9 HT patients (1.5 – 2.5 year follow-up) 11 healthy subjects	Baseline arterial diameter is negatively correlated with changes in MAP during follow-up visits in HT patients.
CVD	Heitmar [313]	2011	Flicker protocol 1	24 CAD patients 30 healthy subjects	Patients with CAD exhibit delays in the arterial dilation (tMD) and constriction (tMC) responses to flicker.
	Al-fiadh [271]	2014	Flicker protocol 1	259 patients with atherosclerotic risk factors and CAD	Retinal microvascular function is correlated with retinal microvascular structure and signs, independently of atherosclerotic risk factors (age, gender and other risk factors).

Abbreviations: DM, diabetes mellitus; CAD, coronary artery disease, CVD, cardiovascular disease; HT; hypertension; MAP, mean arterial pressure; tMD, time to maximum dilation diameter; tMC, time to maximum constriction diameter.

Changes in retinal blood flow are also implicated as one of the key players in the development of diabetic retinopathy (DR) [331, 332]. A reduced capacity for retinal vasodilation has been reported in persons with no clinically detectable signs of retinopathy. This response is further attenuated as the stage of DR worsens [281]. In static retinal analysis studies, larger retinal arterioles are associated with the risk of DR [333-335], whereas wider retinal venules are associated with progression [336]. These alterations have further been shown to be correlated with reduced flicker-induced vasodilation [337], and it has been suggested that altered retinal vascular tone in diabetes and early DR is possibly related to changes in BP or metabolic factors regulating blood flow [317]. Indeed, static analysis shows reduced oxygen saturation in non-proliferative DR [338], signifying pathological disruptions to retinal blood flow. A recent comparison of flicker-evoked dilation and constriction responses, in combination with hyperoxia to simulate vessel narrowing, highlights NO as a possible factor affecting retinal autoregulation in diabetes [339]. Nonetheless, the hypothesis that that there is a reduction in NO reserves in patients with diabetes and DR remains to be confirmed, and further studies are required to investigate the possible mechanisms underlying these structural and functional retinal vessel alterations.

Nevertheless, the prospect that both static and dynamic retinal studies reflect vascular changes associated with endothelial dysfunction and could provide novel vascular markers, which can be detected early and non-invasively, supports the clinical application of DVA with regards to monitoring the effects of vasoprotective agents in conditions with known endothelial dysfunction, such as diabetes and DR.

4.1.4.2.3. Hyperlipidaemia

In individuals with familial hypercholesterolemia, LDL-c apheresis may be a recommended treatment option and, there is evidence that LDL-c apheresis affects a number of clinical parameters including endothelial function and overall cardiovascular outcomes [340]. Studies also show that these effects can be monitored in the microcirculation [341]. Using DVA, a reduced flicker-evoked response has been reported in patients with hypercholesterolemia [325], and improved retinal venular dilation was observed post LDL-c apheresis (related to improved LDL-c and HDL-c fractions, but not total cholesterol and TG), although changes in retinal arteriolar responses remained non-significant. Static retinal studies also show that elevated TG and total cholesterol levels, and lowered HDL-c levels are associated with changes in retinal venular caliber [342]. However, it is not clear from these studies why venular rather than arteriolar changes are more prominent. Nevertheless, these data indicate that DVA is able to detect systemic vascular conditions

with microvascular effects, signalling the potential to monitor the microvascular effects of endothelial therapies.

4.1.4.2.4. Obesity

Microvascular changes are implicated in the pathogenesis of weight gain [343] and abnormal flicker-evoked responses have been identified in obese adults [262] and children [344]. Endothelial dysfunction is hypothesized as one of the underlying mechanisms in the association between obesity and retinal microvascular alterations [345], and it is likely that interactions between oxidative stress, increased leptin levels, and NO dysregulation are also involved. The observed abnormalities in flicker-evoked responses further highlight the potential of DVA as an indirect measure or predictor of systemic endothelial dysfunction. However, whether DVA has the potential for improving the detection of subclinical CVD in obese individuals remains to be investigated. It is likely that each quantitative DVA parameter reflects varied pathogenic processes, and further understanding of the associations between vessel response parameters and clinical parameters in obesity (body mass index, waist-to-hip ratio, abdominal circumference) is still needed before DVA can be considered as an advantageous diagnostic tool in this context.

4.1.4.2.5. Ocular / systemic vascular dysregulation

Vascular dysregulation is defined as an inappropriate constriction or inadequate dilation of the microcirculation when stimulated. Global vascular dysregulation can simultaneously affect various organs including the brain, heart, fingers, and eyes, whereas primary vascular dysregulation (PVD) refers to the occurrence of vascular dysregulation in the absence of underlying disease [346]. Studies show a greater number of spatial irregularities in the retinal vessels of PVD subjects [347] and increased pulse-wave propagation indicating a higher degree of vessel stiffness [348], without recognisable morphological changes. Indeed, there is a growing body of evidence that links PVD with the development and progression of ocular vascular complications such as glaucoma [349], with disturbed autoregulation being the common underlying mechanism. Interestingly, DVA studies also show attenuated responses to flicker in healthy PVD subjects [323], comparable to that observed in glaucoma patients [263].

4.1.4.2.6. Ageing

Cardiovascular and cardiovascular-related pathologies, such as diabetes, hypertension, and hypercholesterolemia tend to be more common in older persons, and it has been shown that ageing is associated with progressive endothelial dysfunction in the macrocirculation [350].

However, the effect of ageing on flicker-evoked retinal responses is yet to be adequately defined. The few existing reports do indicate that there is an age-related decline in the regulation of retinal vessel diameter [308]. A difference in the constriction response has also been noted, where younger, healthy individuals exhibit arterial constriction responses to below baseline diameter post-flicker induced vasodilation [257, 312]. This over-constriction appears to be absent in older individuals [258], but requires further validation studies to be confirmed. As such, the inconsistencies in the few existing reports raise the question as to whether or not age has an effect on flicker-evoked retinal responses [258, 260]. This is, in part, addressed by the study in Chapter 7.

4.1.4.2.7. Ethnicity

There is substantial evidence demonstrating racial/ethnic differences in cardiovascular risk [351]. In a recent published study, delays in flicker-induced arterial vasodilation responses were associated with elevated TG levels in a sample of otherwise healthy SAs, indicating endothelial dysfunction or an increased susceptibility to CVD in these groups of individuals. Nevertheless, scarcity in the availability of normative data and prospective retinal studies specific to SAs presently precludes the interpretation of ranges and thresholds for retinal vascular function parameters that can be used as surrogate risk indicators in this ethnic group. As such, the lack of other ethnicity-based DVA studies warrants further investigations in groups of healthy individuals to assess whether DVA is an accurate predictor of CVD risk. This will, in part, be addressed by the study described in Chapter 9.

A summary of relevant DVA studies using a similar flicker assessment protocol to this thesis in individuals with risk factors for CVD is presented in Table 4.5.

Table 4.5. Relevant DVA studies in individuals with risk factors for CVD				
Risk factor	**Author**	**Year**	**Subjects**	**Main Outcomes**
Ageing	Kneser [308]	2009	52 healthy volunteers	Reduced retinal arterial (not venous) vasoregulative amplitude in older participants is attributed to diminished vasoconstriction rather than vasodilation capacity.
	Heitmar [260]	2010	78 healthy volunteers	Negligible influences of age on retinal vascular function parameters (evaluated as individual flicker cycles)
Atherosclerosis	Al-fiadh [271]	2014	259 patients with atherosclerotic risk factors and CAD	Retinal microvascular function is correlated with retinal microvascular structure and signs, independent of atherosclerotic risk factors (age, gender and other risk factors)
Hypertension	Nagel [261]	2006	9 hypertensives patients 11 controls	Baseline retinal arterial diameter negatively correlated with change in mean arterial blood pressure during follow-up visits
Hypercholesterolemia	Reimann [325]	2009	21 hypercholesterolemic patients	Reduced retinal arterial and venous reactivity is significantly improved in retinal veins after LDL-c apheresis (attributed to wider basal retinal venular caliber than serum lipids)
Diabetes (type II)	Mandecka [281]	2007	172 diabetic patients 53 controls	Retinal arterial dilation reduced in response to flicker provocation in diabetics
	Nguyen [318]	2009	139 diabetic patients 103 controls	Retinal arterial and venous dilation reduced in response to flicker provocation in diabetics
	Lott [329]	2013	22 pre-diabetic patients 25 diabetic patients 19 healthy volunteers	Reductions in retinal arterial and venous dilation in response to flicker provocation parallel reductions in insulin sensitivity
	Lim [330]	2014	279 diabetic patients	Reduced retinal arterial and venous dilation response correspond with severity of retinopathy
Smoking	Heitmar [260]	2010	21 otherwise healthy smokers	Arterial baseline diameter fluctuations (before flicker provocation) are increased in smokers compared to non-smokers
Ethnicity	Patel [309]	2011	45 White Europeans 45 South Asians (UK population)	Lower HDL-c levels and higher fasting TG correlate with delayed retinal arterial dilation responses in SAs compared to WEs.
Obesity	Kotliar [262]	2011	46 obese individuals 46 controls	Retinal arterial dilation is delayed and reduced in obese patients

Abbreviations: CAD, coronary artery disease; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; SA, South Asian; TG, triglycerides; WE, White European.

4.1.4.2.8. Sleep-disordered breathing

Several ophthalmic conditions are associated with sleep disturbances and sleep-disordered breathing (sleep apnoea) [352]. Benign signs, such as droopy eyelids can be common early indicators of sleep deprivation, whereas other sequelae may a signal a more serious underlying condition or increased risk for CVD. Obstructive sleep apnoea (OSA) is a relatively common sleep disorder that is characterized by repetitive upper airway collapses during sleep, and is increasingly becoming recognized as a prominent risk factor for CVD [353-355]. The cardiovascular consequences of obstructive sleep apnoea (OSA) may involve both the macro- and microvessels, however, little is known about the mechanism by which OSA influences the microvasculature.

Static retinal studies indicate that the severity of OSA is correlated with retinal venular widening rather than arteriolar narrowing and that the cardiovascular complications of OSA may be mediated by microvascular disease related to endothelial dysfunction [356]. In contrast, an earlier study reports no retinal vessel abnormalities associated with sleep-disordered breathing [357]. The use of the arteriolar-to-venular ratio as the endpoint in the aforementioned study, however, overlooks the possibility that retinal arteriolar narrowing and venular widening are separate phenomena that can reflect varied physiological processes [358]. As such, existing studies currently offer incomplete evidence to infer any causal associations.

On the other hand, there is strong evidence for endothelial dysfunction in OSA. Endothelium-dependent vasodilation is reduced in patients with sleep-disordered breathing [301] and a reduction in NO levels is associated with hypoxia [359] caused by repetitive upper airway collapses. Currently, however, there are no published DVA studies that have investigated flicker-evoked responses in OSA patients, and prospective studies to further our understanding of the associations between retinal venular widening and the progression of cardiovascular complications in OSA are still needed. Moreover, whether CVD risk associated with retinal vascular factors in OSA is dependent on age, gender, ethnicity, or genetic factors, remains to be demonstrated. Chapter 11 of this thesis investigates flicker-evoked responses in patients with OSA, as assessed by DVA.

4.1.5. Further research

The close associations between flicker-evoked retinal responses and cardiovascular risk factors have been highlighted by a number of studies. Moreover, reduced retinal vasodilation is exhibited in a number of clinical groups with known endothelial dysfunction, suggesting that DVA assessment may help unravel more of the pathophysiology of systemic vascular conditions yet to be understood. Functional abnormalities in DVA responses reported early in the course of diabetes before clinically apparent retinopathy also illustrates the potential in DVA for CVD risk prediction. Nevertheless, besides the need for reproducibility data, as discussed previously, several other issues currently limit the use of DVA solely for research purposes.

First, from previous studies, it is evident that the flicker response is closely related to endothelial function, and variations in metabolic demand and neurovascular coupling play important roles in optimising retinal blood flow. While the involvement of NO in the regulation of retinal vascular tone has been established (see section 4.1.2.6), whether the sources of basal NO secretion and ad-hoc secretion on demand of neurovascular coupling are the same, remains to be investigated as N-monomethyl-L-arginine is a non-specific inhibitor of endothelial and neuronal NOS [254]. Therefore, although the DVA response has been characterised in healthy and diseased states, whether the measured outcomes reflect neural or vascular components in the examined condition is still unclear. The implications of this mainly pertain to retinal diseases with neural and vascular complications. For instance, although diabetes research has mainly focused on pathophysiological vascular changes, an increasingly popular view is that diabetes is essentially a neurodegenerative disease leading to vasculopathy [318]. Given the correlations between vascular abnormalities and neuronal dysfunction in diabetes [294, 360], the effect of hyperglycaemia on the neurovascular coupling response during flicker should be investigated to fully exploit the potential of DVA in unravelling the pathophysiology of diabetes and diabetic retinopathy. To date, no DVA studies have directly examined the relationships between retinal vascular caliber, blood flow, and neural activity as one assembly. Moreover, besides alterations in larger retinal vessels, functional changes in retinal microvessels may also play an equally important role in vascular disease, however; DVA is recommended for the assessment of retinal vessels of at least 90 μm, thus, the contribution of microvascular changes in comparison to larger vessels is unclear.

Second, despite the associations demonstrated, there is no standardised classification of normal versus attenuated flicker-light induced changes and generally no reference data with regards to age, sex, BMI, BP, and lipid markers. Therefore, further studies are still needed to characterise retinal vessel behaviour during flicker-stimulation and to derive a set of DVA response parameters in individuals free of overt systemic disease that can be consistently referenced across studies. Additional DVA parameters, beyond the pre-defined vasodilation and vasoconstriction parameters should also be explored, which may require experimentation with varying recording lengths and repetitions of each flicker cycle. Further analyses of functional vessel properties and morphological changes may help clarify the regulation of retinal blood flow in vascular disease [264]. The experimental studies described in Chapters 7 through 10 of this thesis characterise the effects of age, sex, FH, ethnicity (SA), and systemic circulatory influences (lipids and oxidative stress markers) on flicker-evoked retinal responses in individuals free of overt systemic diseases.

Third, the vascular associations between DVA parameters and systemic factors seen in conditions such as diabetes and hypertension have not been validated in other cardiovascular conditions of known endothelial dysfunction, such as OSA. Moreover, in conditions already examined, the value of DVA over static retinal assessments is yet to be investigated. Experimental studies could also be useful for evaluating the contribution of other key factors involved in the flicker-evoked vessel response and that are altered in OSA, such as induced retinal hypoxia [361] or O_2 provocation [362]. Chapter 11 of this thesis investigates flicker-evoked changes in retinal vessel diameters in patients with untreated moderate to severe OSA.

Finally, the cross-sectional nature of existing DVA studies pose severe limitations when considering the temporality of the associations reported in the literature. This could be addressed in further longitudinal studies to validate the predictive capacity of DVA. Since there are yet no simple, non-invasive methods to assess the coronary microcirculation in CVD screening, research in this field remains attractive and warranted. However, it is still unclear whether DVA, as a singular approach, is sufficient for evaluating systemic vascular function. The value of multiple vascular assessments, and whether DVA is superior as a predictor of vascular dysfunction, in comparison to other established endothelial assessments, should also be ascertained. Indeed, further validating work remains to be undertaken before DVA may be considered clinically beneficial over

traditional markers and structural retinal parameters for cardiovascular risk prediction and stratification.

5. Rationale

Chapter 1 of this thesis highlights the main drawbacks associated with conventional cardiovascular risk scoring systems i.e., the majority of patients visiting primary care facilities with no signs or symptoms typical of CVD, and with one or none of the traditional cardiovascular risk factors, would be placed in the low predicted risk score category but at least 50% of these patients are likely to suffer from cardiovascular-related morbidity and mortality [363]. Despite poor predictive value for the individual patient, cardiovascular risk scoring systems are still important in the clinical decision-making process, and the management of individuals in the intermediate or low-risk categories proves to be particularly challenging. However, with the continued expansion of biomarker discovery platforms, markers that correlate with disease risk and severity have become indispensable for individualized patient care and management strategies.

Chapter 2 of this thesis discussed the importance of vascular markers and available assessments of endothelial function that can improve upon current cardiovascular risk detection strategies. Endothelial assessments widely used as surrogate markers of vascular risk include assessments of vessel stiffness (PWA), structure (c-IMT), and function (FMD). Imaging techniques based on ultrasound are useful for large vessel evaluations, although smaller vessel or microvessels are thought to be affected much earlier in the course of disease progression, and consequently imaging tools to evaluate small vessel structure and function are of incremental clinical value. This chapter, therefore, also explored the expanded spectrum of vascular assessments to include functional retinal vessel assessments. Retinal photographs have long since been used in clinical practice but emerging advances in technologies (RVA) have provided an opportunity to examine functional changes in retinal vessels with the added advantage of obtaining information on developing rather than established vascular pathology. Continuous retinal diameter measurements using the RVA system have since been applied in several studies to evaluate microvascular function, and many of these studies link a diminished or delayed retinal vasodilatory capacity (in response to a physiological stressor, DVA) with endothelial dysfunction. Nevertheless, in considering the incremental value of a vascular marker as it applies to a specific purpose or in combination with other vascular phenotypic measures, a vital part in refining our judgement includes an understanding of the underlying

physiology (Chapter 3) and the actual technical measurement (Chapter 4). An overview of existing functional retinal studies in Chapter 4 also highlights important considerations when applying this technique into clinical context. Research in this area is rapidly growing, and while clinical testing seems to be a promising endeavour an evolving gap in the literature presently precludes the adequate translation of this tool for routine clinical use.

From a risk management perspective, the concept of early risk detection is crucial, and one of the central aims of the studies in this thesis is to evaluate whether functional retinal assessments could be useful for profiling individualized vascular risk. The main advantage of the functional retinal assessment chosen is that it provides integrated and dynamic analysis of vascular function as a variable specific for each individual. As this concept may be particularly important for screening and intervention in individuals with underappreciated cardiovascular risk, the studies in this thesis include subsets of otherwise healthy individuals but with low to moderate cardiovascular risk, and also with select subtle but appreciable risk factors not commonly incorporated into widely used cardiovascular risk scoring systems. The main risk factors of interest to this thesis are age, FH, SA ethnicity, oxidative stress, and sleep-disordered breathing (OSA), as they all share an intimate association with endothelial dysfunction and with the development and progression of CVD. Moreover, the influence of these risk factors on retinal vascular function has not been adequately defined in existing functional retinal studies, as discussed in Chapter 4.

Finally, the assessment of one vascular bed, such as with DVA, could provide limited information, and pre-clinical abnormalities at various vascular levels could collectively confer greater vascular risk. Therefore, the studies in this thesis, in addition to carefully devised retinal measurement and assessment protocols, also include assessments of select clinical parameters and large artery structure and function in addition to the retinal assessment, to provide a more comprehensive picture of vascular health in the selected groups of participants. It was, thus, postulated that outcomes substantiating the relevance of retinal vascular function against these additional measures could foster new strategies in individualized vascular risk profiling and in a wider context cardiovascular screening, prevention, and management.

The principle sections and aims of this work are described below and the investigative techniques used in each experimental study are detailed in the methods section (Chapter 6).

5.1. Main study aims

Study 1: **Ageing effect on retinal vascular function**. The main aim of this study is to compare and contrast the retinal microvascular response to flicker, as assessed by DVA, in young (19-30yrs), middle-age (31-50yrs), and older (51-70yrs) individuals with low cardiovascular risk (FRS < 10%).

Study 2: Retinal vascular function in healthy individuals with a family history (FH) of cardiovascular disease. The main purpose of this study is to compare and contrast the retinal microvascular response to flicker in healthy individuals with and without a FH of CVD in a first-degree relative.

Study 3: Ethnic differences in retinal vascular function. The purpose of this study is to investigate ethnic differences in retinal vascular function and their relationship to traditional risk indicators for CVD. The study includes a middle-aged cohort (35 – 55 years) of otherwise healthy migrant UK South Asians (SAs) and age- and gender-matched White-Europeans (WEs).

Study 4: Systemic circulatory influences on retinal microvascular function. The purpose of this study is to investigate the relationship between retinal microvascular function (as assessed by DVA) and circulatory markers for CVD risk and systemic anti-oxidative defence capacity in healthy, middle-age individuals with low to moderate (FRS < 20%) cardiovascular risk.

Study 5: Retinal vascular function in individuals with obstructive sleep apnoea: a preliminary study. The purpose of this study is to investigate the relationship between known markers of cardiovascular risk and vascular function parameters measured at the retinal and systemic levels in patients with untreated moderate to severe OSA.

6. Methods

The five experimental studies described in this thesis used observational, cross-sectional designs. This chapter outlines the study approval procedures, recruitment, and inclusion/exclusion criteria. This chapter also describes the general patient visit protocol, and technical details of the assessments.

6.1. Ethical approvals

Ethical approval was sought and received from the relevant research ethics committee(s) (National Health Service (NHS) and/or Aston University Life and Health Sciences). Written informed consent was received from all subjects prior to study enrolment and all procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki.

6.2. Patient recruitment

6.2.1. General inclusion / exclusion criteria

Healthy volunteers were recruited through advertisements at the Health and Vision Sciences Clinic and Vascular Research Laboratory at Aston University (Birmingham, UK). General study inclusion criteria were defined as age ≥ 18 years; no known history of cardio-, cerebro- or related vascular disease; not to be taking any vasoactive medications; absence of symptoms or history of any other disease that had not been resolved before study inclusion; and willingness and capacity to freely provide informed consent. Study exclusion criteria were positive diagnosis of cardio- or cerebro-vascular disease such as CAD, heart failure, arrhythmia, stroke, or transient ischaemic attacks; peripheral vascular disease; severe dyslipidaemia (plasma triglycerides > 6.00 mmol/L or cholesterol levels > 7.00 mmol/L); diabetes; smoking; inflammatory conditions such as rheumatoid arthritis; as well as other metabolic disorders or chronic diseases that required treatment, and frequent use of vasoactive medications such as dietary supplements containing vitamins or antioxidants and bronchodilators. Potential participants were also screened for ocular diseases and excluded from the study if they had a refractive error of more than ± 3DS and more than ± 1DC equivalent (to address minification or magnification which can cause under- or over-estimation of the retinal vessel diameter), elevated intraocular pressures (> 21 mmHg), retinal disease, a history of intraocular surgery, cataract or any other media opacities preventing adequate examination with the RVA, as well as, any retinal or neuro-

ophthalmic disease affecting the ocular vascular system. Further study-specific recruitment and inclusion / criteria are defined in each chapter as appropriate.

6.3. Investigative techniques

Prior to study initiation, the author was trained on the use of each investigative technique in this thesis. For techniques that required more than one training session, a series of supervised preliminary examinations on 10 to 15 volunteers was undertaken to ensure that an adequate level of competency had been achieved. See Table 6.1 below for a summary of the main investigative techniques used for each of the studies (1-5) in this thesis.

Table 6.1. Overview of main investigative techniques / clinical parameters measured

LEVEL		TECHNIQUE/PARAMETER	PURPOSE	STUDY				
				1	2	3	4	5
Ocular		Non-contact tonometry	IOP reading	+	+	+	+	+
		DVA	Endothelial function	+	+	+	+	+
Systemic	Clinical / Vascular	Anthropometry	BMI	+	+	+	+	+
		Sphygmomanometry	BP	+	+	+	+	+
		c-IMT	Atherosclerotic risk	+	+	-	+	+
		PWA (AIx)	Arterial stiffness	+	-	-	-	+
		FMD	Endothelial function	-	+	-	-	+
		24h BP / HRV	HT / ANS function	-	-	-	-	+
	Circulatory markers	Venepuncture	Blood withdrawal	+	+	+	+	+
		Glucose profile	IGT / DM	+	+	+	-	+
		Triglyceride profile	Lipid profile	+	+	+	+	+
		Cholesterol (total), HDL-c, LDL-c	HC	+	+	+	+	+
		Redox index: GSH/GSSG	Endothelial function	-	-	+	+	+
		NO	Endothelial function	-	-	-	-	+
		ET-1	Endothelial function	-	-	-	-	+
Other		FRS	Cardiovascular risk	+	+	+	+	+
		General questionnaire	Health history / diet / lifestyle	+	+	+	+	+
		Questionnaires (SF-36®, FOSQ, ESS)	QoL /sleep quality	-	-	-	-	+

Abbreviations: DVA, dynamic retinal vessel analysis, IOP, intraocular pressure; BMI, body mass index; BP, blood pressure; c-IMT, carotid intima-media thickness; PWA, pulse-wave analysis; AIx, augmentation index; FMD, flow-mediated dilation; HRV, heart rate variability; HT, hypertension; ANS, autonomic nervous system; IGT, impaired glucose tolerance; DM, diabetes mellitus; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HC, hypercholesterolemia; GSH, reduced glutathione; GSSG, oxidized glutathione; NO, nitric oxide; ET-1, endothelin-1; FRS, Framingham risk score; SF-36, short-form 36; FOSQ, Functional Outcomes of Sleep Questionnaire; ESS, Epworth Sleepiness Scale; QoL, quality of life.

6.3.1. Study protocol

All study-related assessments were performed between 8 and 11 am following an overnight fast for at least 10 to 12 hours, which included refraining from alcohol and caffeine. Study procedures were performed as outlined in the flowchart (Figure 6.1) with details on techniques, procedures, and data analysis provided in the following sections.

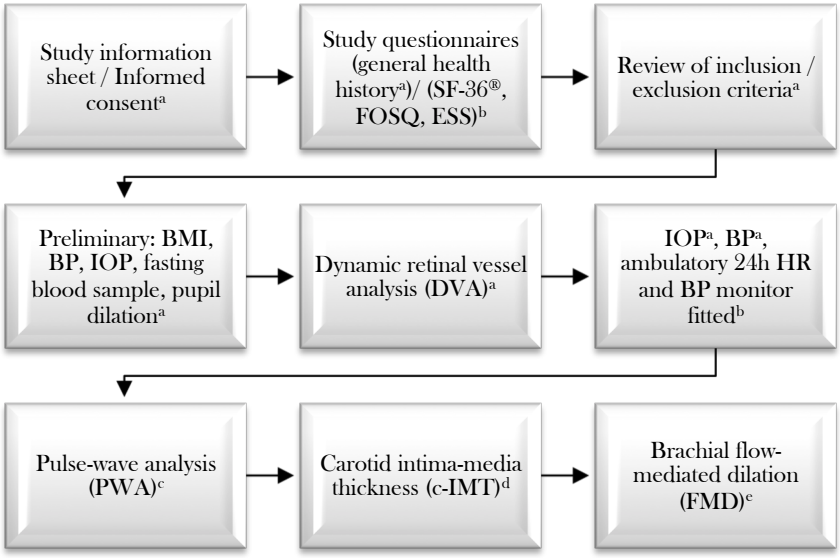


Figure 6.1. Overview of patient visit protocol
Typical order of assessments during patient visits and assessments specific to each study.
[a] study 1-5
[b] study 5
[c] study 1 & 4
[d] study 1, 2, 4, & 5
[e] study 2 & 5

6.3.2. Preliminary assessments

6.3.2.1. General health history

Participants who met the inclusion criteria and had provided informed consent were requested to complete a demographic and general health history questionnaire detailing their age, gender, ethnicity, personal and family history of illness, medication, daily diet, tobacco and alcohol consumption, and physical activity routine, as well as, menstrual cycle details for women. A template of the general health questionnaire is provided in Appendix A. Anthropometric measures of height and weight were then recorded and body mass index (BMI) was calculated as per (Equation 6.1).

$$BMI = \frac{Weight\ (kg)}{Height^2\ (m)}$$ **(Equation 6.1)**

6.3.2.2. Blood pressure profiles

Following a short acclimatization period where participants were requested to sit quietly and comfortably for 5 minutes, a baseline BP reading was obtained using an automated BP monitoring device (UA-767, A & D Instruments Ltd., Oxford UK). The procedure involved a BP cuff fastened snuggly around the upper arm above the elbow with the forearm elevated to approximately heart level and supported. The automated device was then initiated and three subsequent readings of the systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were recorded.

6.3.2.3. Intraocular pressure measurements

IOP readings were then obtained by way of non-contact tonometry using the Puls*air* system (Keeler Ltd., UK) and in accordance with the manufacturer's guidelines. Briefly, the patient was positioned seated comfortably. The patient's eye was located through the device's eyepiece at a distance of approximately 25 cm. The handheld device was then moved closer to the patient with the other operator hand rested on the patient's forehead (to prevent the device from inadvertently touching the eye). Upon proper alignment, the device automatically fires a puff of air and takes a reading. Following an average of at least three satisfactory readings, the procedure was repeated on the alternate eye.

6.3.2.4. Blood sampling

After ensuring that the patient had fasted for at least 10-12 hours, blood samples were drawn from the antecubital fossa vein for subsequent analyses. The author was trained and certified on all aspects of the blood draw procedure prior to obtaining blood samples. Briefly, the procedure involved placing a tourniquet around the patient's forearm approximately 2 cm above the proposed collection site and the desired vein was inspected and located via palpation. The skin over the selected area was then sterilized and the vein was anchored below the collection site. The needle of the syringe assembly, held parallel to and alongside the vein, was quickly inserted into the vein and blood was aspirated into EDTA Vacutainer® tubes. The tourniquet was released just as the last collection tube was filled, the needle was deftly withdrawn and pressure was simultaneously applied to the site.

The patient was instructed to continue applying light pressure on the site until bleeding, if any, had ceased.

6.3.2.5. Blood analysis

The fasting EDTA blood samples obtained were immediately assessed for glucose (GLUC) and TG, and plasma total cholesterol (CHOL) and HDL-c using a Reflotron Desktop Analyser (Roche Diagnostics, UK). In addition, LDL-c levels were estimated as per the Friedewald equation [364] (Equation 6.2).

$$LDL = \frac{CHOL_{total} - HDL - TG}{2.17} \qquad \textbf{(Equation 6.2)}$$

Aliquots of the remaining EDTA blood and plasma were then processed and stored at approximately -80^{o}C for further analyses of blood glutathione, and plasma NO and ET-1 levels as detailed in section 6.3.6.

6.3.3. Framingham risk scores (FRS)

Cardiovascular risk assessments are often based on subjective lifestyle and general health history profiles, which are used to determine the presence of related risk factors such as obesity, hypercholesterolemia, diabetes and or smoking. The FRS is a gender-specific algorithm originally developed to estimate an individual's 10-year risk of developing CVD or CHD [19] and has also been validated for use across ethnic groups [365]. In the present thesis FRS for CVD was calculated using the National Heart, Lung and Blood Institute (NHLBI) worksheet, which is based on age, gender, CHOL, HDL-c, SBP, treatment for hypertension, smoking, and diabetes [366]. The equations and coefficients used in the worksheet are provided in (Table 6.2). The risk score can be derived as a percentage (1 point, 6%; 2 points, 8%; 3 points, 10%; 4 points, 12%; 5 points, 16%; 6 points, 20%; 7 points, 25%; 10 points or more, > 30%); typically stratified as low risk (< 10%), intermediate risk (10-20%), or high risk (> 20%) [367].

Table 6.2. Framingham risk score equations and calculations

Variable (X)	Regression Coefficient (β)		Equations / Calculations
	M	F	
Log of age	3.06117	2.32888	$CVD\ risk = 1 - \delta^{e^{(\sum \beta_i X_i - \alpha)}}$
Log of CHOL	1.12370	1.20904	
Log of HDL-C	-0.93263	-070833	
Log of SBP if not treated	1.93303	2.76157	**10-year risk for women:**
Log of SBP if treated	1.99881	2.82263	$1 - 0.95012^{\exp(\sum \beta_i X_i - 26.1931)}$
Smoking	0.65451	0.52873	
Diabetes	0.57367	0.69154	
Risk period factor (δ)	0.88936	0.95012	**10-year risk for men:**
Average risk (α)	23.9802	26.1931	$1 - 0.88936^{\exp(\sum \beta_i X_i - 23.9802)}$

Abbreviations: CHOL: total cholesterol (mg/dl); HDL-C: high-density lipoprotein cholesterol (mg/dl); SBP: systolic blood pressure; β: regression coefficients [24]; δ: risk period factor [24]; α: average risk [24]; X: variables.

6.3.4. Ocular vascular assessment

Vascular dysregulation in the retina is implicated in the development of vascular diseases [346] and typically refers to an inadequate vascular adaptation to the particular needs of the organ; usually characterized as insufficient dilation/constriction or excessive dilation/constriction in response to a physiological stressor. DVA using flicker-light stimulation is one of the most widely used methods for assessing retinal microvascular reactivity [256, 309 310, 320, 368] and a detailed description of this technique is provided in the following sections.

6.3.4.1. Dynamic retinal vessel analysis

As described in section 4.1.1, the RVA system (IMEDOS GmbH, Jena, Germany) enables real-time recording of retinal vessel diameters, and can be coupled to a variety provocative stimuli to evaluate endothelial functioning of the vasculature. Flicker-light stimulation was selected as the provocation method of choice in this thesis. Typically, studies using the RVA system with an integrated flicker simulator are referred to as dynamic retinal vessel analysis (DVA) studies.

6.3.4.2. Device set-up

The device setup comprised of a fundus camera (FF450, Carl Zeiss, Germany), a charged coupling device (CCD) camera, a high-resolution video recorder, a real time monitor, and a dedicated personal computer with analysis software. The device was also equipped with an optoelectronic shutter placed in optical pathway of the camera to generate flickering light at a sampling rate of 12.5 Hz, which lies within the optimum flicker frequency range and has previously been shown to induce appropriate retinal stimulation [256, 257, 312].

6.3.4.3. *Technical specifications*

The technical specifications of the RVA are summarized in Table 6.3. The resolution of the device limits the accurate measurement of vessel diameters to vessels with diameters greater than 90 μm and the temporal resolution of the device is 40 ms, such that 25 video frames are captured per second (i.e. sampling rate = 25 Hz) for the duration of a recording. For the studies in the present thesis, the image field or camera angle was set at a 30^{o} angle and all size related measurements are expressed in 'units of measurement' (UM), whereby 1 UM is equivalent to 1 μm in a normal emmetropic eye [141].

Table 6.3. Technical specifications of retinal vessel analyser

PARMETER	VALUE
Measurement range	90 μm
Measurement resolution	< 1 μm
Temporal resolution	≥ 40 ms
Image field angle	30^{o}
Recording time	350 seconds (can be up to 10 minutes)
Maximum length of vessel segment	3 mm
Spatial resolution (along vessel segment)	180 μm

6.3.4.4. *Advantages and limitations*

With a growing number of studies citing the use of the RVA system, some of the inherent advantages and limitations of its use are important and accordingly summarized below (Table 6.4).

Table 6.4. Advantages and limitations of retinal vessel analyser

ADVANTAGES	LIMITATIONS
Non-invasive	Media opacities compromise image quality
Optimal spatial and temporal resolution to measure provocation responses	Uses standardized measurement or relative units rather than absolute vessel diameter
Simultaneous assessments of multiple vessels and vessel segments	Heavily reliant on steady patient fixation over recording duration
High reproducibility [266]	Assumes no refractive error
Low variability [268]	Requires full pupil dilation

6.3.4.5. *Procedure*

Based on technical considerations mentioned above and potential influential factors on flicker-evoked responses (section 4.1.2.5) all participants were required to refrain from alcohol and caffeine. All recordings were performed in a quiet temperature controlled room with consistent ambient lighting. Since a physiological correlation is known to exist between the two eyes of the same individual [265, 369], a randomly selected eye of each participant was chosen for further evaluation. Pupil dilation required for the assessment was achieved with 1% Tropicamide (Chauvin Pharmaceuticals Ltd., UK). To ensure optimal alignment and patient set-up, an image of the fundus was displayed on the computer screen and a green filter inserted into the illumination pathway of the fundus camera was adjusted for optimal contrast sensitivity. Eye movements were controlled for with the use of a visual fixation target and positioned so that the region of interest was in the centre of the fundus image. Once the uniformly illuminated fundus image was obtained, a rectangular region of interest (usually inferiorly to the ONH) was selected on the real-time monitor. Within this region a segment along the inferior temporal retinal artery and inferior temporal retinal vein, approximately 0.5-1 mm in length and approximately 1 to 2 disc diameters from the ONH were selected for recording and analysis as depicted in Figure 4.1.

6.3.4.6. *Flicker-stimulation protocol*

Flicker protocol 1 (section 4.1.2.1) was used to assess retinal microvascular reactivity in this thesis, which in accordance with that introduced by Nagel et al. [258, 265] and widely recommended by other experts in the field [214]. This automated 350-second protocol was initiated following optimal illumination and vessel selection, and consisted of a 50-second baseline recording (under still illumination 25 Hz), followed by 3 successive cycles of flicker stimulation (opto-electronically generated at 12.5 Hz) distinguished as 20 seconds of stimulus interrupted by an 80-second recovery period (Figure 6.2).

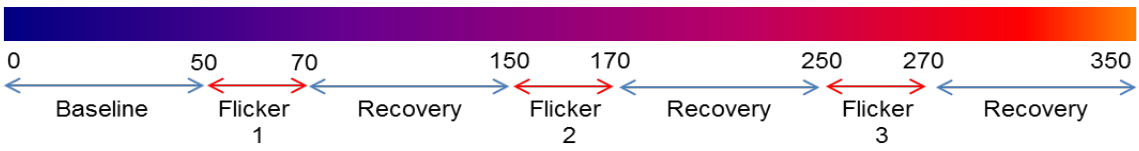


Figure 6.2. Schematic representation of flicker stimulation protocol duration

The three sequential cycles were initially introduced to ascertain an averaged vessel response over a recording period of tolerable length. Some studies, however, also consider vessel responses to each flicker cycle individually. Parameters to describe vessel behaviour, that can be derived and calculated for each flicker cycle or averaged across cycles, have also been developed (section 4.1.2.3). Those of particular interest to this thesis are discussed below in more detail (section 6.3.4.7).

6.3.4.7. Data analysis

As described in section 6.3.4.3, the spatially and temporally defined segments of the selected vessels translate into 25 captured video frames per second and for the duration of the recording (350 seconds). These data include 25 diameter recordings per second (25 x 350) as well as a spatial recording along length of segment drawn (usually 1 mm), which is then compressed by the device software to provide an averaged spatial-temporal reading or local temporal course for each vessel (Figure 6.3).

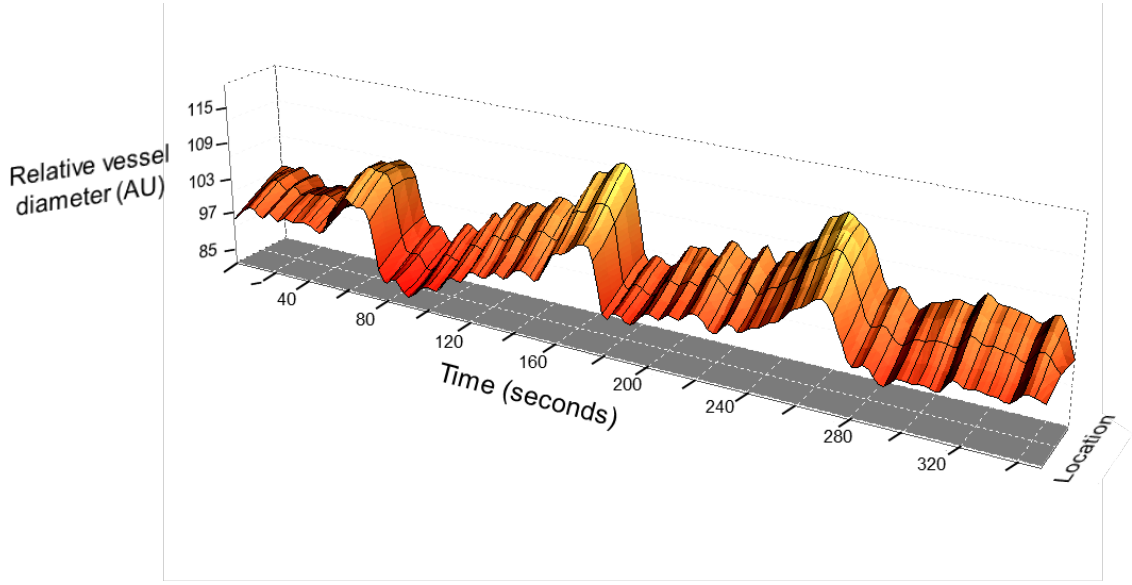


Figure 6.3. Local temporal course representation of retinal vessel analysis
For each examination, a vessel segment approximately 1 mm in length and approximately 1 to 2 disc diameters away from the optic disc was selected. The vessel longitudinal section within the region of interest was scanned 25 times per second and continuous diameter data was obtained along the vessel over time, thus creating a three-dimensional matrix of values as depicted.

6.3.4.8. Data visualization and response parameters

In this thesis, further expansions of the SDRA method (section 4.1.2.3.2) were adopted to evaluate the entire dynamic vessel response profile. Recently, our lab published a description of the use of a statistical polynomial regression algorithm that can be applied to the raw response data from the RVA device software, and implemented using the "polyfit"

and "polyval" functions in Matlab (Mathworks, Inc., USA) to create a visualization plot [320]. The statistical algorithm used for data visualization in this thesis was constructed and implemented in consultation with an experienced statistician (Dr Aniko Ekart).

Given the measurements y_i at times $t_i, i = 1, \dots T,$ we approximated $y = f(t)$ by a polynomial of degree n as:

$$p(t) = p_1 {t_1}^n + p_2 t^{n-1} + \cdots + p_n t + p_{n+1}$$

The polyfit function locates the coefficients $P_1, P_2, \dots P_n, P_{n+1}$, such that the error $\sum_{i=1}^{T} (y_i - p(t_i))^2$ is minimized.

This involves solving the system of equations:

$$\begin{cases} p_1 {t_1}^n + \cdots + p_n t_1 + p_{n+1} = y_1 \\ \qquad\qquad\qquad\qquad \vdots \\ p_1 {t_T}^n + \cdots + p_n t_T + p_{n+1} = y_T \end{cases}$$

If we denote ${t_i}^{n-j+1} = v_{ij}$ then $V = (v_{ij})$ is the Vandermonde matrix and the least squares problem to be solved can be written as $V_p = y$

with the vectors $p = \begin{cases} p_1 \\ \vdots \\ p_{n+1} \end{cases}$ and $y = \begin{cases} y_1 \\ \vdots \\ y_T \end{cases}$

The polyval function was then used to calculate the fitted polynomials that ultimately provided us with curves representative of the dynamic vascular response profile, which could then be used for analysis (Figure 6.4).

The degree of the polynomial, *n*, is an adjustable parameter. In this case, *n* = 20 was applied for consistency as this provided the closest fit polynomials on the data points.

Also based on the principles of SDRA in a more recent study our lab introduced slope [320] as an additional parameter to more accurately describe vessel behaviour as slope characterizes the interaction between the change in vessel diameter and the rate at which this change occurs. Slope can be determined independently for both the dilation (Slope $_D$)

and constriction (Slope $_C$) components and in both arteries and veins as per (Equation 6.3) and (Equation 6.4.)

$$Slope_D = \frac{MD - baseline}{tMD} \quad \textbf{(Equation 6.3)}$$

$$Slope_C = \frac{MC - MD}{tMC} \quad \textbf{(Equation 6.4)}$$

Visualization plots can be created using the algorithm for each individual flicker cycle as well as an averaged or composite response plot for all flicker cycles, with the artery and vein regarded separately. An example of a visualization plot is illustrated in Figure 6.4.

A summary of the main parameters of interest to this thesis is provided in Table 6.5.

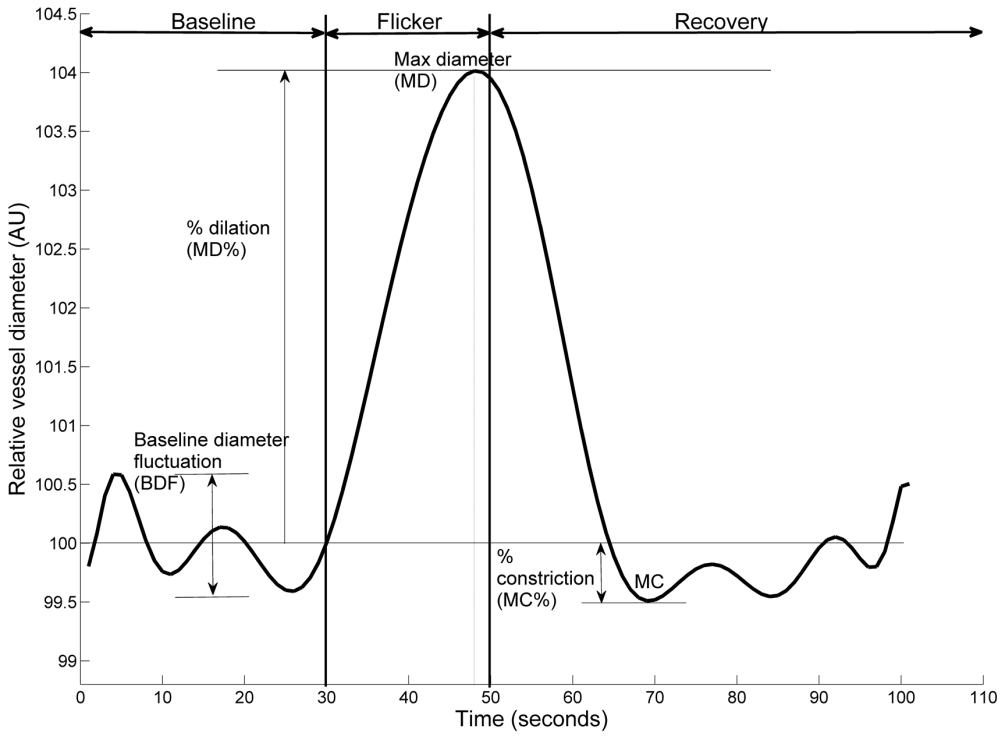


Figure 6.4. Diagrammatic representation of the SDRA parameters
Abbreviations: SDRA, sequential diameter response analysis; BDF, baseline diameter fluctuation; MD, maximum diameter; MD%, percent dilation relative to average baseline diameter; MC, minimum constriction diameter; MC%, percent constriction relative to average baseline diameter.

Table 6.5. Summary of DVA parameters calculated and used for analysis

ACRONYM	PARAMETER	DESCRIPTION / CALCULATION
Baseline	Baseline diameter	Average diameter during baseline recording
BDF	Baseline diameter fluctuation	Maximal range of diameter measurements during baseline
MD	Maximum diameter	Point of maximum dilation following onset of flicker
MD%	Percentage dilation	Percentage change in vessel diameter relative to baseline ((MD – Baseline) / Baseline)*100
MC	Minimum diameter	Point of maximum constriction after MD (post-flicker)
MC%	Percentage constriction	Percentage change in vessel diameter relative to baseline ((MC – Baseline)) / Baseline)*100
tMD*	Reaction Time	Time (seconds) taken to reach MD following onset of flicker
tMC*	Constriction Time	Time (seconds) taken to reach MC from MD post-flicker
DA	Dilation amplitude	MD – MC
BCFR	Baseline corrected flicker response	Change in vessel diameter taking into consideration baseline diameter fluctuation (DA – BDF)
Slope $_D$	Dilation slope	(MD – baseline) / tMD
Slope $_C$	Constriction slope	(MC – MD) / tMC

*Since the tMD and tMC values derived using the matlab algorithm reflect the absolute time at MD or MC, these values were expressed so as to only account for the exact number of seconds taken to reach MD since the onset of flicker (i.e. time at MD - 30 seconds) and the time in seconds taken to reach MC from MD (i.e. time at MC - time at MD).

6.3.5. Systemic vascular assessments

Although circulatory abnormalities have been known to occur in microvessels before they occur in larger vessels, there is a possibility that vascular changes in the retinal microcirculation may represent an ocular manifestation of a generalized systemic disorder. A number of systemic endothelial assessments were therefore selected in this thesis to provide a more comprehensive picture of an individual's vascular status. Namely, assessments of, systemic arterial stiffness, atherosclerotic vessel changes, and systemic endothelial function were achieved by way of PWA, c-IMT, and brachial FMD, respectively.

6.3.5.1. Pulse-wave analysis

Interpretation of the arterial pulse has become an important part of the clinical evaluation process with the introduction of non-invasive high-fidelity tonometers that measure intravascular pulse. PWA represents a blend of nineteenth century sphymography with cuff sphygmomanometry and is among the most widely used non-invasive techniques as one that enables a more accurate assessment of the arterial pulse contour and the relative degree of vessel stiffness. With the combination of high-fidelity tonometers and

mathematical algorithms it is now possible to characterize arterial hydraulic properties by generating pulse pressure waveforms, identifying systolic and diastolic periods, and generating indices of ventricular-vascular interactions [370].

6.3.5.1.1. Procedure

In the present thesis, PWA was conducted in accordance with an established protocol [371] using the validated SphygmoCor device (AtCor Medical /PWV Medical Pty Ltd, Australia). The patient's radial pulse was first located just below the wrist creases at the base of the thumb and the SphygmoCor transducer or high-fidelity pressure sensor was flattened over this site with slight pressure to generate a signal representative of the intravascular pulse in the radial artery. Reasonable confidence in readings was gained when pressure waves were consistent from beat to beat and with characteristics to be expected in the artery (sharp upstroke to the first systolic peak, sharp cleft and near-exponential pressure decay in late diastole). Figure 6.5 shows typical radial waveforms obtained with this device in individuals of varying age.

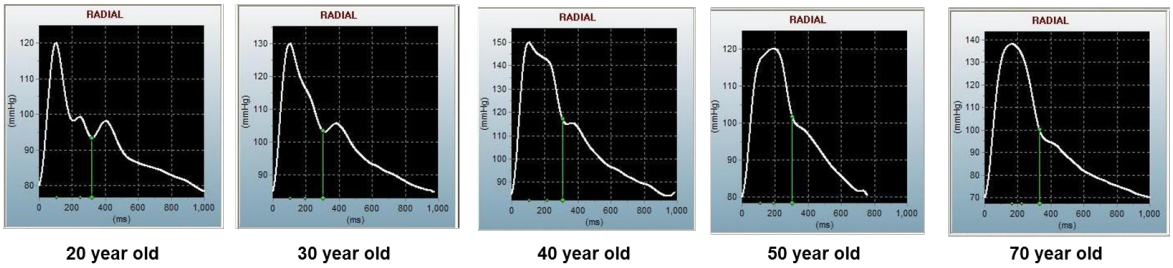


Figure 6.5. Radial pulse waveforms in individuals of varying age

The pulsatile radial waveform was then calibrated against SBP and DBP readings by the in-built software, and mathematically transformed using a transfer function to reconstruct the aortic waveform from which a range of central cardiovascular parameters can be derived (Figure 6.6).

6.3.5.1.2. Technique principles

The principle of the PWA technique is based on the generation and analysis of the central aortic pressure waveform, which can provide information about the stiffness of the systemic vasculature. The reconstructed aortic waveform is derived from two components, namely a forward pressure wave generated during ventricular contractions and incident pressure waves that are reflected back from peripheral vascular beds and bifurcations in the

vasculature (Figure 6.7a). In elastic vessels the reflected waves return to the aortic root in diastole supplementing coronary perfusion, and the sum of the outgoing and reflected wave create the waveform profile illustrated in Figure 6.7b. The speed at which the outgoing and reflected waves travel is dependent on the stiffness of the arteries along which they travel such that in stiffer arteries the waves will be reflected back at a much quicker rate (Figure 6.7c). As the outgoing and reflected waves are added (Figure 6.7d), there are three important implications: (i) increased central systolic and pulse pressure (Figure 6.7e) which can occur without any changes in cuff BP values; (i) increased left ventricular (LV) load (Figure 6.7f); (iii) decreased coronary artery perfusion in diastole (Figure 6.7g). Increased arterial stiffness therefore independently increases the risk of all three major cardiovascular outcomes.

The augmentation index (AIx) value generated by the device software is a widely used research parameter and considered to be a sensitive indicator of arterial stiffness [372]. In the present thesis AIx was therefore the main parameter of interest. Influences of age (Figure 6.5) and gender have been known to exist, with AIx values being greater in women and older individuals [373].

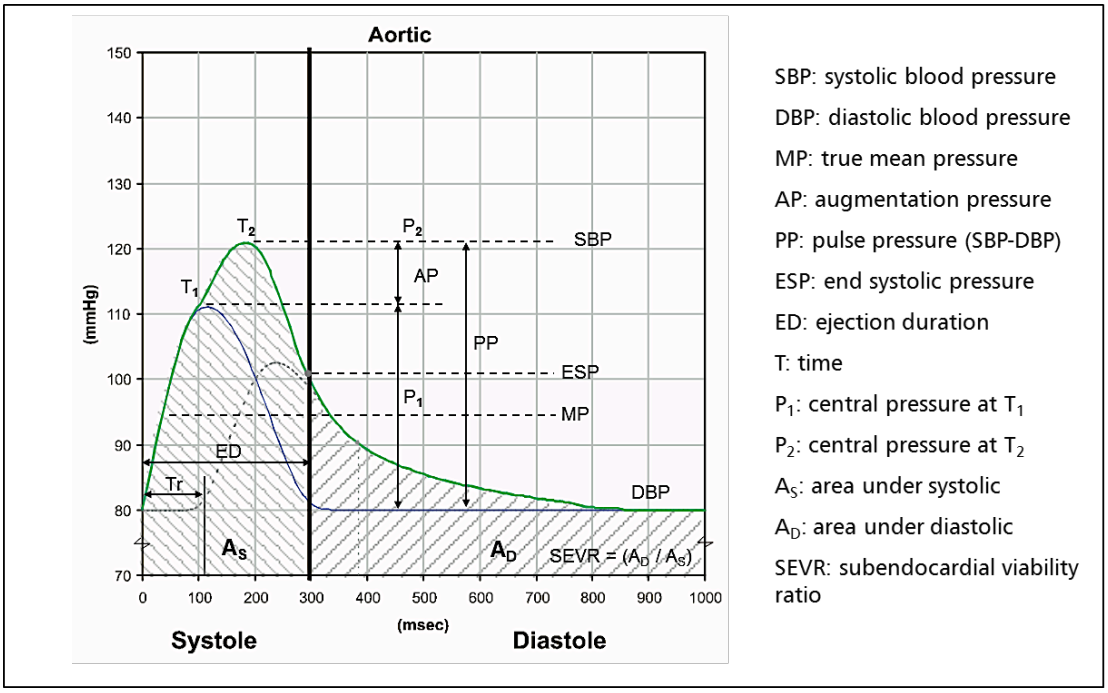


Figure 6.6. Central cardiovascular parameters calculated by the SphygmoCor software

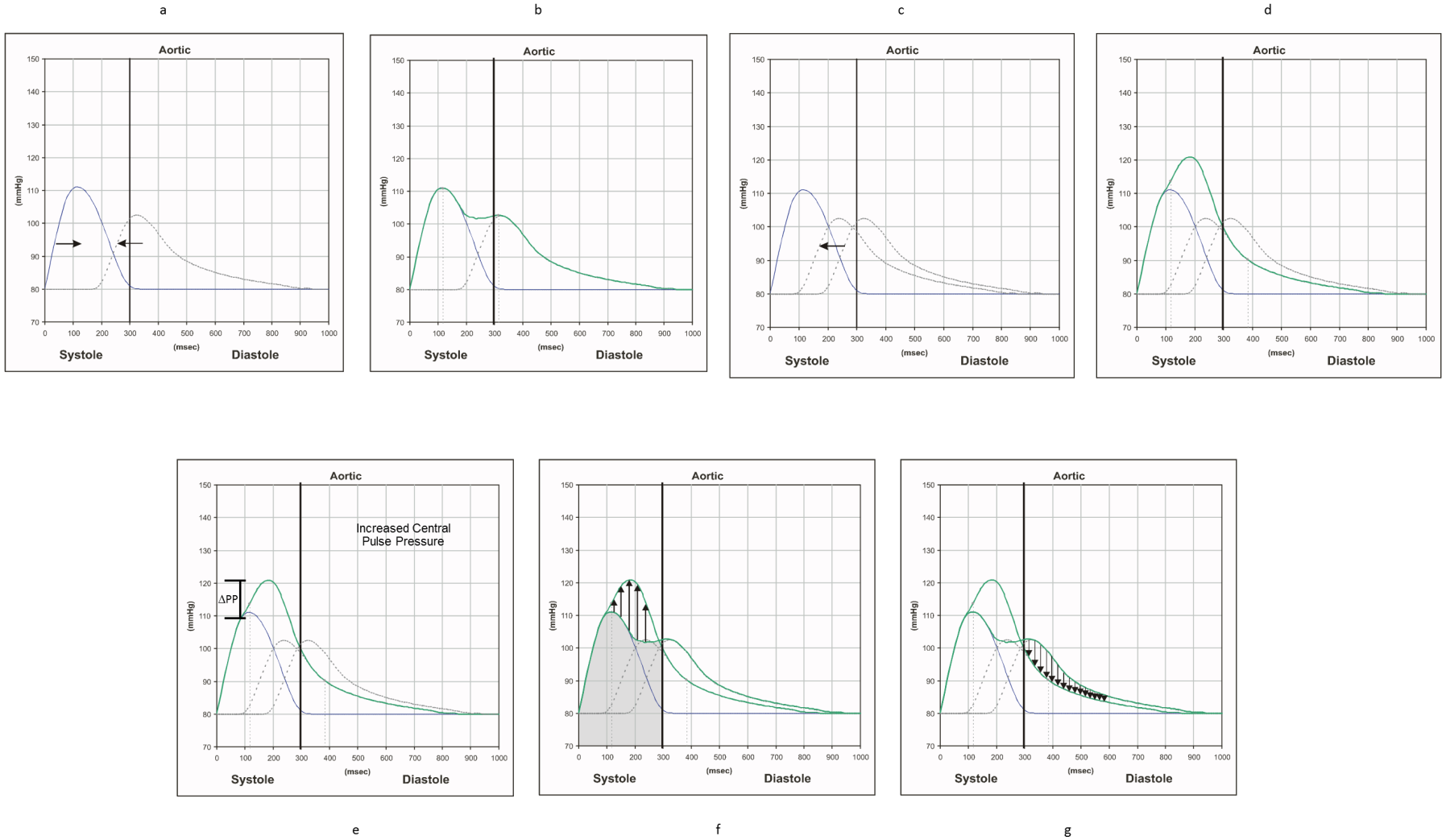


Figure 6.7. Summary of pulse-wave analysis technique principle

6.3.5.1.3. Advantages and limitations

PWA is minimally invasive, and poses relatively low risk to the patient. Preliminary findings have been promising demonstrating the technique to be both accurate and reproducible with good repeatability and low inter-observer variation [372, 374, 375]. While applanation of the artery is not difficult to master, the technique does entail training and frequency of use, as it is possible to obtain unusual or even inverted waveforms when the sensor is positioned incorrectly. One of the main weaknesses of the techniques is with regards to validating the waveforms with cuff BP readings versus invasive intra-arterial pressure recordings, and as such any attempts to determine the ascending aortic pressure waveform will be limited by inaccuracies in cuff BP measurements [371]. Since the software generates quality control indices, only those readings obtained with an operator index of greater than 80 were accepted to ensure reliable measurement.

6.3.5.2. Carotid intima-media thickness

Thickening of the inner vascular lumen in the common carotid artery is a commonly observed phenomenon in patients with early atherosclerotic disease [376]. As such, c-IMT measurements are widely used in clinical research as a direct measure of carotid arteriosclerosis and an indirect measure of the presence of generalized atherosclerosis [377]. By definition c-IMT refers to the distance between the luminal-intimal surface and the medial-adventitial interface of the common carotid artery. The temporal sequence of changes in c-IMT measures are thought to result from decreases in the bioavailability of NO, and increases in ET-1 levels, which in turn can result in vSMC proliferation [378, 379] and subsequent luminal changes in the vascular wall. Normal c-IMT values can vary from 0.05 cm in young adults to 0.08 cm in the elderly based on data from large cross-sectional studies [380]. According to the Atherosclerosis Risk in Communities Trial, c-IMT values above the threshold of 0.1 cm confer a significant risk for CHD [381]. Furthermore, c-IMT correlates with the FRS [382], has been demonstrated to offer predictive value with regard to future cardiovascular complications [383, 384], and shares a close relationship with a number of risk factors for CVD including dyslipidaemia [385], ageing [386], and hypertension [387].

6.3.5.2.1. Procedure

In the present thesis, c-IMT measurements were conducted in accordance with a well-established protocol [388] using high-resolution B-mode ultrasonography (Siemens, Acuson Sequoia®, UK). The patients were in a resting position with their head turned towards one side and neck slightly extended. Typically, high-resolution ultrasound imaging reveals a

double-line pattern in the inner vessel lumen (Figure 6.8). c-IMT measurements were then taken from central region of the inferior wall of the artery using the in-built software calliper system at a site proximal to the bifurcation.

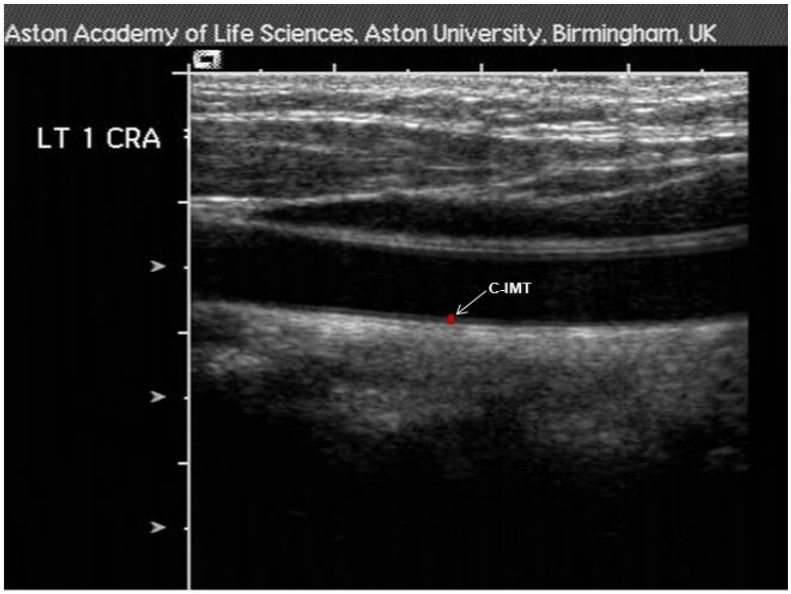


Figure 6.8. Ultrasound image of carotid intima-media measurement site

6.3.5.2.2. Advantages and limitations

The c-IMT measurement technique is generally appreciated as a non-invasive assessment that offers highly reproducible results. Nevertheless, the subjective nature of the assessment leading to an increased risk of inter-observer variability, and the potential benefits over automated alternatives have been questioned [389]. Intra-observer variability, however, has been shown to be small and therefore manual c-IMT measurements are still considered to be valid [388]. Previous studies have suggested that age, systolic BP and weight can influence c-IMT measurements, and therefore these parameters were controlled for when considering the results in the present thesis.

6.3.5.3. Flow-mediated dilation

The presence of endothelial dysfunction at the systemic level could provide important information about the involvement of the macrovasculature in the development and progression of CVD in asymptomatic individuals, and brachial FMD is considered the gold standard technique for assessing systemic endothelial function [390]. FMD refers to the dilation of a vessel in response to increased blood flow, which exerts a shear stress stimulus on the vessel wall. As such, shear stress triggers NO release in conduit arteries in

response to reactive hyperaemia. Endothelial function in the brachial artery closely correlates with that of the coronary arteries in the same patient [391].

For the purpose of this thesis FMD was detected by means of a high-resolution ultrasound imaging system (Siemens®, Acuson Sequoia, UK). This technique enables real-time image acquisition of body structures, based on sound pulses transmitted into body and the frequency of the reflected waves that are dependent on the speed and movement of the reflective surface. In blood vessels this principle is based on the Doppler shift induced by moving RBCs. The Doppler equation (Equation 6.5) 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 pulse 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:

$$Shift = \frac{2(f.V_{blood}.\cos\theta)}{V_{sound}} \qquad \textbf{(Equation 6.5)}$$

6.3.5.3.1. Technical considerations

To ensure reliability and reproducibility the FMD technique is subject to the optimization of several vessel site selection, technical, and patient-related factors [392]. Optimal patient preparation requires a fasting state which includes no alcohol or tobacco consumption for at least 6 hours prior to the study; refraining from exercise; refraining from any food or beverage containing caffeine, polyphenols, vitamins, for at least 12 hours, as well as withholding vasoactive medications if possible on the morning of the study. Technical factors include test performance in a quiet, temperature controlled room and at the same time of day (for multiple tests); a 10-minute acclimation period with the patient in supine position, the arm resting comfortably with cradle support, and the imaged artery at heart level; cuff placement approximately 1-2 cm distal to the elbow; a 5-minute occlusion duration time; and cuff inflation to at least 50 mmHg above systolic pressure. With regards to vessel segment selection the brachial arterial is the recommended vessel of choice as smaller arteries (< 2 mm) are difficult to image and absolute diameter changes correspond to large relative changes; and sites must be replicated for repeated measurements and anatomical landmarks should be used. For optimal image acquisition, a longitudinal section with a clear interface between the near and far arterial wall should be achieved; a stereotactic adjustable prop would ensure image quality; baseline diameter

recordings should be conducted for at least 1 minute; and automated edge-detection software should be used.

6.3.5.3.2. Procedure

The FMD technique was carried out in accordance with previously published guidelines for assessment [91]. The patient was positioned supine and following a brief acclimatization period, the arm was extended in a comfortable position and the brachial artery was imaged above the antecubital fossa in the longitudinal plane using high-resolution ultrasonography with a 7 mm, 8 MHz linear-array transducer (Siemens; Acuson Sequoia®, UK). A clear segment of the vessel with visible anterior and posterior intimal interfaces between the lumen and vessel wall was then selected for continuous imaging. Vessel diameters were continually recorded from the selected region of interest using a specialised wall-detection and artificial neural networking software (VIA® Software, UK). Based on published recommendations, a baseline image was acquired for 2 minutes, following which a BP cuff positioned at the forearm was inflated to a supra-systolic pressure (50 mmHg above systolic) for 5 minutes; effectively occluding blood flow through the brachial artery, inducing hypoxia, and causing dilatation of downstream resistance vessels. Thereafter, image acquisition was carried out through the cuff inflation phase and continued for an additional 2 minutes post-cuff deflation (hyperaemia). A summary of the FMD protocol is illustrated in Figure 6.9.

6.3.5.3.3. Data analysis

The VIA® wall detection software automatically detects and tracks the anterior and posterior artery walls within the user defined region of interest and processes the B-mode images acquired at a rate of 25 frames per second. The diameter recordings for the first 2 minutes of baseline acquisition (3000 frames) and the 2 minutes post-cuff deflation (3000 frames) were selected from the raw data, and compressed using Matlab (Mathworks, Inc., USA) to determine the average diameter during baseline and the peak diameter during reactive hyperaemia. FMD was then expressed as a percentage change in vessel diameter from average baseline diameter ($AD_{baseline}$) to the point of maximal artery dilation during hyperaemia ($MD_{hyperaemia}$) as per (Equation 6.6).

$$FMD = \left(\frac{MD_{hyperaemia} - AD_{baseline}}{AD_{baseline}}\right) 100$$ **(Equation 6.6)**

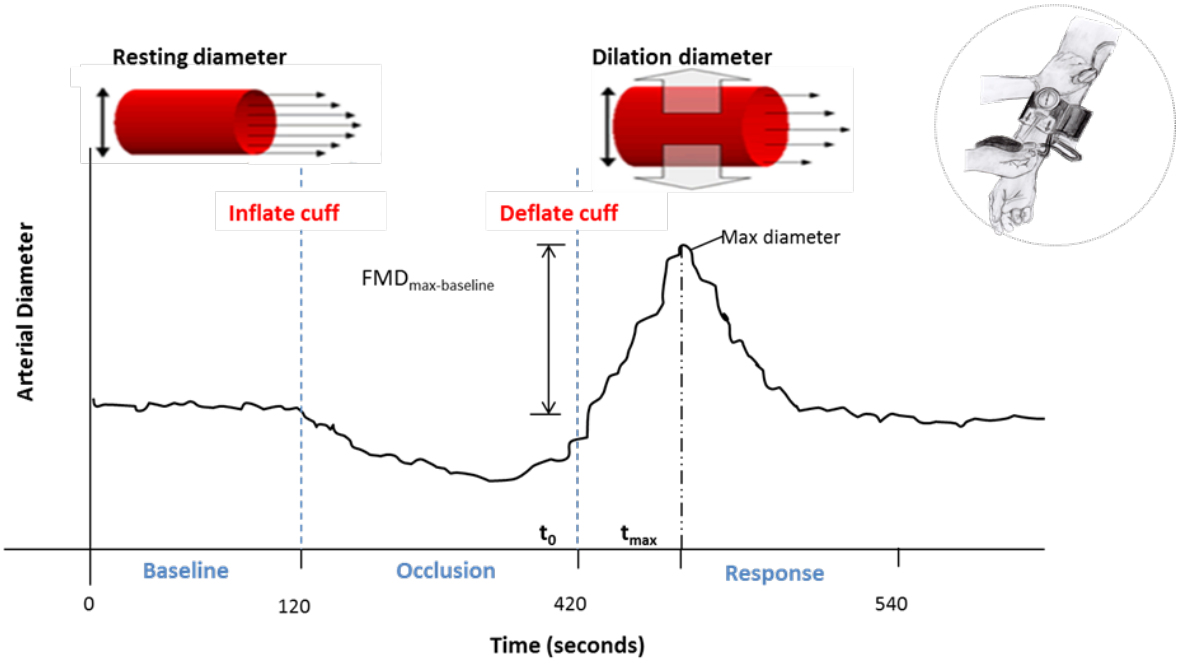


Figure 6.9. Diagrammatic representation of FMD technique
Abbreviations: FMD, flow-mediated dilation; t_0, time at cuff deflation; t_{max}, time at maximal dilation diameter.

6.3.5.3.4. Advantages and limitations

The application of this technique can be challenging and requires extensive training and standardization. As detailed previously [393] the optimization of several technique- and patient-related factors is also essential. The main advantages and limitations of the technique are summarized in Table 6.6.

Table 6.6. Main advantages and limitations of FMD

ADVANTAGES	LIMITATIONS
Non-invasive	Challenging to perform well
Correlates well with coronary artery endothelial function [394, 395]	Protocols and standardization across studies is varied
Cost-effective	Several patient-based factors to consider
Assessment of additional parameters such as flow, baseline arterial diameters, and flow-mediated constriction	Potential for inter-observer variability

6.3.6. Systemic circulatory markers – Biochemical assays

6.3.6.1. Glutathione

Glutathione/glutathione disulphide (GSH/GSSG) is the most abundant thiol redox system and is essential for the maintenance of redox balance in cells [396]. Cellular glutathione mainly exists in reduced form (GSH) which affords an elegant mechanism for redox control of metabolic processes, the failure of which has important implications for optimal endothelial function [397]. In the present thesis, glutathione levels were based on the principles of an enzymatic-recycling assay [398, 399]. This technique, developed in-house and validated previously [400], relies on the reaction of free thiol groups with a sulfhydryl reagent DTNB (5,5'-dithiobis-2-nitrobenzoic acid) to form the yellow derivative (TNB, 5'-thio-2-nitrobenzoic acid), which can then be measured via absorbance spectrophotometry at 410 nm. The blood GSH and GSSG concentrations measured in this thesis were in good agreement with literature data in control patients in the ranges of 150 – 1500 μM and 1 to 500 μM, respectively [401], and suggests that the experimental conditions reported in this thesis are suitable for the analysis of total glutathione and glutathione disulphide concentrations in whole blood. The validity and reliability of the spectrophotometric method of detection has also previously been established [402].

6.3.6.2. Glutathione recycling assay principle

An evaluation of systemic oxidative stress status was performed in this thesis through the determination of circulating reduced glutathione (GSH) in fasting venous blood samples. GSH, a tripeptide (γ-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues. During the reduction of hydrogen peroxide (H_2O_2) to water (H_2O) and the respective alcohol – a reaction that is catalysed by glutathione peroxidase (GPx) – GSH becomes oxidized glutathione (GSSG) (Equation 6.7). GSSG can, in turn, be recycled to back to GSH in the presence of glutathione reductase (GSR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) (Equation 6.8).

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} GSSG + 2H_2O \qquad \textbf{(Equation 6.7)}$$

$$GSSG + NADPH + H^+ \xrightarrow{GSR} 2GSH + NADP^+ \qquad \textbf{(Equation 6.8)}$$

During increased oxidative stress in cells, the ratio of GSH/GSSG decreases as a consequence of GSSG accumulation. The measurement of total GSH, GSSG and the GSH/GSSG ratio is therefore considered a useful indicator of oxidative stress status. The assays and analysis described below were all conducted by the author and optimized in-house according to previously reported and validated methods [403]. The method used is described below.

6.3.6.2.1. GSH assay

Protocol: To minimize auto-oxidation of thiols or the enzymatic reduction of disulphides, sample processing was carried out immediately whereby a 30 µL aliquot of the blood sample was pre-treated with 33.3 µL of 100 mg/mL SSA (5-sulfosalicylic acid) and 936.7 µL of sodium phosphate buffer (pH 7.5) to release GSH via cellular disruption and protein precipitation. The sample was centrifuged at 13,000 rpm for 5 minutes, and aliquots of the supernatant were stored at -80^{o}C for further analyses. Based on previous reports of sample stability, assays were conducted within 2 months of collection [404]. GSH standards were prepared from 0 to 80 µM in increments of 20 µM with the same final concentrations of SSA (1%) as in the samples. To each well of a 96-well plate 150 µL of daily buffer (125 mM sodium phosphate, 6.3 mM disodium EDTA and 0.3 mg/mL NADPH), 50 µL of 6mM DTNB solution, and 25 µL of standards and samples were added in triplicate. The plate was incubated for 3 minutes at 37 ^{o}C following which 25 µL of GSR was added to each well. Any GSSG formed was thereby recycled to GSH by GSR in the presence of NADPH and the plate was read at 410 nm at 0, 1, 2, 3, and 5 minutes. Standard curves of the GSH concentration were generated using a linear regression program (Microsoft Excel, Microsoft Corporation, USA). An example of a standard curve is provided in Figure 6.10.

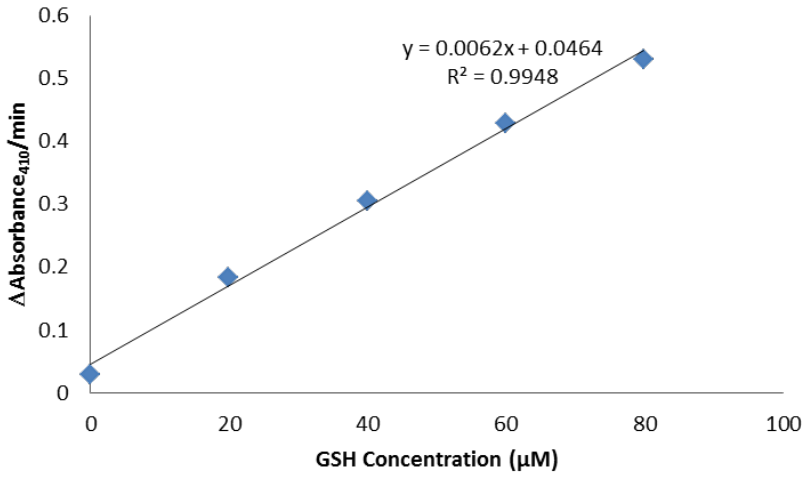


Figure 6.10. Standard curve for the GSH assay

6.3.6.2.2. GSSG assay

The accurate measurement of GSSG has in the past been more challenging due to lower concentrations in tissues and the lack of effective methods to prevent the oxidation of GSH during sample preparation. For the measurement of GSSG levels, therefore, the reagents used were the same as those described above for the GSH assay and in addition the standards and samples were pre-treated with 2-vinylpyridine (2-VP) in order to derivatize GSH without interfering with GSR reaction. A summary of the GSH/GSSG recycling assay is depicted in Figure 6.11.

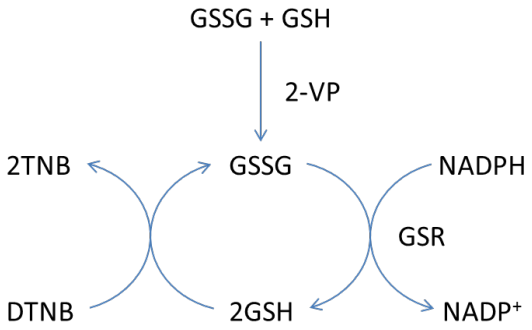


Figure 6.11. Glutathione recycling assay principle

Protocol: GSSG standards were prepared from 0 to 10 µM in 1µM increments. In addition, the 100 µL aliquots of the standards and samples pre-treated with 2-VP were adjusted to a pH of 7.5 with triethanolamine (TEA). The assay was then carried out as described above for GSH where 25 µL of standards and samples were added in triplicate to a 96-well plate containing 150 µL of daily buffer and 50 µL of DTNB in each well, incubated at 37 ^{o}C for 3 minutes, treated with 25 µL GSR and read at 0, 1, 2, 3, and 5 minutes. Standard curves of the GSSG concentrations were similarly generated using the linear regression program described above (Figure 6.12).

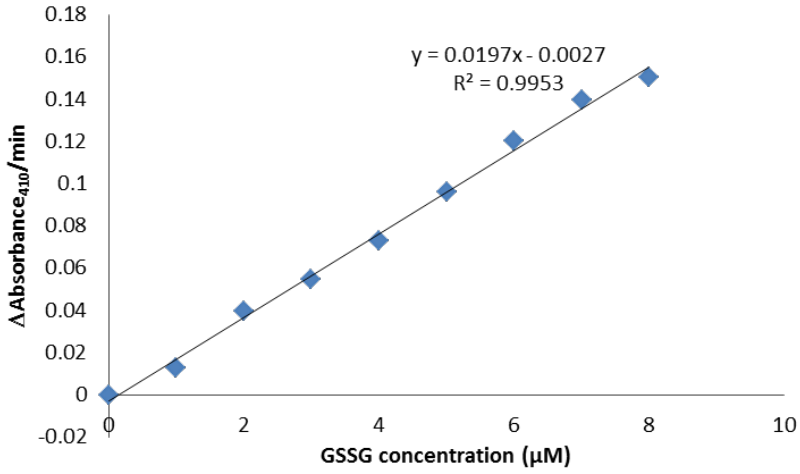


Figure 6.12. Standard curve for the GSSG assay

6.3.6.2.3. Analyte concentration calculations

The GSH and GSSG concentrations were based on the net reaction rate, construction of the standard curves, and calculation of the analyte concentrations in the samples from which total GSH levels and the redox index were then calculated as follows.

The change in absorbance (A) at 410 nm is a linear function of the analyte concentration in the reaction mixture:

$$A_{410} = slope\ x\ minutes\ x\ intercept \quad \textbf{(Equation 6.9)}$$

The net rate is the difference between the rate at each concentration of the GSH or GSSG standard and the blank rate. The general form of the equation describing the calibration curve is:

$$Net\ rate = slope\ x\ GSH\ or\ GSSG + intercept \quad \textbf{(Equation 6.10)}$$

Therefore, to calculate the GSH or GSSG concentration:

$$= \frac{Net\ rate - intercept}{slope}\ x\ dilution\ factor \quad \textbf{(Equation 6.11)}$$

Finally the total GSH and redox index were calculated as per (Equation 6.12) and (Equation 6.13).

$$tGSH = GSH + (2xGSSG) \quad \textbf{(Equation 6.12)}$$

$$redox\ index = GSH/GSSG) \quad \textbf{(Equation 6.13)}$$

6.3.6.3. ELISA assay for endothelin-1

ET-1 is a molecule best known for its actions as a potent vasoconstrictor [405]. Plasma ET-1 levels in this thesis were determined using the commercially available QuantiGlo® human ET-1 enzyme-linked immunosorbent assay (ELISA) (R&D Systems Europe Ltd, Abingdon UK).

The principle of the assay is based on an immunoassay technique whereby an antibody specific for ET-1 is coated onto a microplate which binds any ET-1 present in the standards and samples pipetted into the plate. An enzyme-linked antibody specific for ET-1 is then added to the wells followed by a luminol/peroxidase substrate solution. The luminescence emitted during this step is proportional to the amount of the ET-1 bound in the initial step and can be quantitatively measured in relative light unit (RLU) using a luminometer. The materials and reagents provided in the kit are listed in Table 6.7.

Table 6.7. List of reagents used for ET-1 assay

MATERIALS / REAGENTS	DESCRIPTION
ET-1 microplate	96-well plate coated with rat monoclonal antibody against ET-1
ET-1 conjugate	21 mL mouse monoclonal antibody against ET-1 conjugated to horseradish peroxidase
ET-1 standard	2.5 ng synthetic human ET-1
Assay diluent	11 mL buffered protein base with preservatives
Calibrator diluent	21 mL buffered protein base with preservatives
Wash buffer concentrate	100 mL of a 10-fold concentrated solution of buffered surfactant
Glo reagent A	4 mL luminol
Glo reagent B	8 mL hydrogen peroxide

A summary of the procedure (detailed in the manufacturer's guide) is provided below.

6.3.6.3.1. Protocol

Reagent preparation: 100 mL of Wash Buffer Concentrate was diluted in deionized or distilled water to prepare 1000 mL of Wash Buffer. 1 part Glo Reagent A (4 mL) was added to 2 parts Glo Reagent B (8 mL) at least 1 hour prior to assay.

Standard preparation: The standard was reconstituted with 1.0 mL of deionized or distilled water to produce a stock solution of 2500 pg/mL. For the standards 900 µL of Calibrator Diluent was first pipetted into a tube and 600 µL of Calibrator Diluent was pipetted into the remaining tubes. The stock solution was then used to produce the dilution series and final standard concentrations (Figure 6.13) with the 250 pg/mL standard as the highest standard concentration and Calibrator Diluent as the zero standard (0 pg/mL).

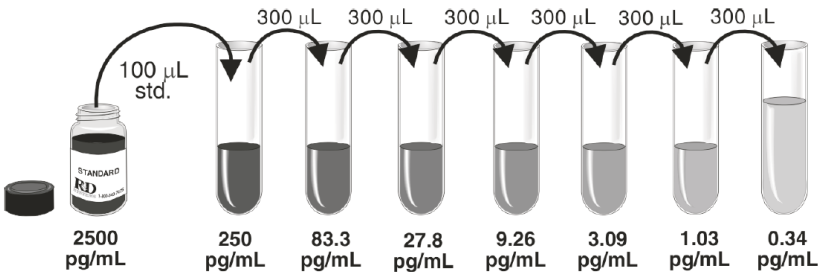


Figure 6.13. Standard preparation for ET-1 assay

Procedure: 100 µL of Assay Diluent was added to each well, followed by 100 µL of standard, control, or sample to designated wells. The microplate was covered and incubated for 1.5 hours at room temperature on a microplate shaker. Each well was then aspirated and washed with Wash Buffer, repeating the process three times for a total of four washes. After the last wash, any remaining buffer was aspirated or decanted. Then, 200 µL of ET-1 conjugate was added to each well, and the plate was incubated for a further 3 hours at room temperature on the shaker. The aspiration/wash step was then repeated and 100 µL of Working Glo Reagent was added to each well. The plate was incubated for 5 - 20 minutes at room temperature and protected from light before determining the relative light units (RLU) of each well with luminometer parameters set at 1.0 min lag time; 0.5 sec/well-read time; summation mode; auto gain on.

Results calculation: The RLU for the standards was plotted against the concentration of the standards to determine the ET-1 concentration in each sample. If the samples were diluted, the concentration read from the standard curve was multiplied by the dilution factor accordingly.

6.3.6.4. Griess assay for nitrite

Disturbances in NO bioavailability or production are thought to be responsible for functional vascular alterations associated with endothelial dysfunction and atherosclerosis [406]. Nevertheless, the transient and volatile nature of NO makes it unsuitable for most analytical assessment in complex matrices such as blood and plasma. Thus endothelial NOS activity is typically assessed as the plasma concentration of nitrite (NO_2) and nitrate (NO_3) [407], based on observations that NO is converted to NO_2^- and NO_3^- when inhaled or added to blood since NO_2^- is oxidized to NO_3^- by haemoglobin [408]. Previous reports propose that NO_2^- more specifically represents a delivery source for intravascular NO and reflects acute changes in regional eNOS activity [409]. The Griess assay is based on a diazotization reaction and detects the presence of nitrite. In order to measure both NO_2^- and NO_3^- the NO_3^- must be enzymatically converted to NO_2^-. The Griess reagent system includes a 1% sulphanilamide in 5% phosphoric acid solution, 0.1% N-(1-napthyl)ethylenediamine dihydrochloride (NED) in distilled water and a nitrite standard stock solution of 0.1 mmol/L sodium nitrite in distilled water.

6.3.6.4.1. Protocol

Reagent preparation: the 1% sulphanilamide solution was prepared by first adding 0.5 mL phosphoric acid (H_2PO_4) to 10 mL of distilled water and dissolving 0.1 g sulphanilamide. The 0.1% NED solution was prepared by dissolving 0.01 g in 10 mL. For 1 mL of 100 µM (0.1 M) nitrite standard solution, 6.9 mg of sodium nitrite was dissolved in 1 mL of distilled water based on formula weight (FW) calculations i.e. mg = (mM x FW x mL) / 1000).

Standard preparation: twenty-four wells of the 96-well plate were designated for the nitrite standard reference curve (Figure 6.14) 100 µl of the 100 µM nitrite solution was added to three wells in row A, and 50 µl of the appropriate buffer (phosphate buffered saline, PBS) was pipetted into 3 wells in rows B-H. A serial dilution was then carried out (50 µl/well) in triplicate down the plate to generate the nitrite standard reference curve (100, 50, 25, 12.5, 6.25, 3.13, 1.56 µM), discarding the 50 µl from the 1.56 µM set of wells. The final volume in each well was 50 µl, and the nitrite concentration range was 0-100 µM.

Procedure: 50 µl of each experimental sample was then added to the remaining wells in triplicate, followed by 50 µl of sulphanilamide solution to all wells (standards and samples). The plate was incubated for 5-10 minutes at room temperature protected from

light before adding 50 µl of the NED solution to all wells. The plate was incubated for a further 5-10 minutes at room temperature protected from light and the absorbance was read using plate reader (absorbance spectrophotometry) with a filter of 550 nm. A standard curve was generated to determine the NO_2^- concentration in each sample.

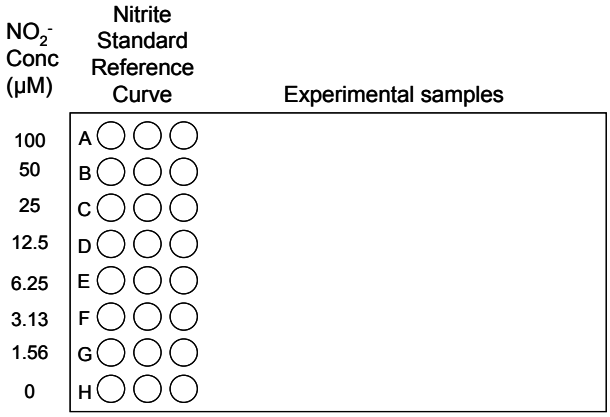


Figure 6.14. Nitrite standard curve reference

A publication of this work in Acta Ophthalmologica is provided in Appendix B.

7. Study 1: Ageing effect on retinal vascular function

7.1. Abstract

Purpose: To compare and contrast the retinal microvascular response in young (19-30yrs), middle-age (31-50yrs), and older (51-70yrs) individuals with low cardiovascular risk.

Methods: Retinal vascular function was assessed by way of DVA in 57 young, 75 middle-age, and 62 older subjects. In addition, BP profiles, blood analyses for glucose and lipid metabolism markers (TG, CHOL, HDL-c, LDL-c), arterial stiffness (AIx), c-IMT, and FRS was assessed in all participants.

Results: The overall retinal arterial dilation amplitude (DA) and post-flicker percent constriction (MC%) were significantly decreased in the oldest group compared to the middle-aged ($p = 0.028$; $p = 0.021$) and youngest group ($p = 0.003$; $p = 0.026$). The arterial constriction response slope ($Slope_{AC}$) was also decreased in the oldest group compared to the youngest group ($p = 0.027$). On the venous side, MC% was decreased in the middle-aged and oldest groups in comparison to the youngest group ($p = 0.015$; $p = 0.010$, respectively). Additionally, arterial DA ($p = 0.007$) and MD% ($p < 0.001$) were higher in men in comparison to women, but only in the youngest group. Although AIx and c-IMT scores increased with age, the observed differences in retinal vascular function parameters were independent of these systemic parameters.

Conclusion: In otherwise healthy individuals, there are age differences in retinal vascular function throughout the entire functional response curve for arteries and veins. Gender differences mainly affect the dilatory phase and are only present in young individuals.

7.2. Introduction

It is well known that the incidence and prevalence of CVD increases exponentially with age [48-50]. At present, the current identification of at-risk individuals for primary prevention efforts relies on classical risk factors for CVD such as lipid profiles, smoking, and hypertension [410-412]. Although some of these variables increase with age, the predictive accuracy of traditional cardiovascular risk estimates that include the aforementioned variables, such as the Framingham model, the Prospective Cardiovascular Mὺnster (PROCAM) score and the European Society of Cardiology Systematic Coronary Risk Evaluation (SCORE) can over- or under-estimate actual risk in a large number of individuals [413-415]. Therefore, other measures such as genetic, inflammatory and coagulation markers, as well as, various tests for subclinical disease have been sought [416-418]. The primary aim of these new markers is to offer individualized biological profiles rather than profiles for population groups; a concept that is crucial for prediction, prevention, and personalised interventions that address individualized risk [419]. Nevertheless, most of these markers are not yet available in daily clinical practice. Moreover, since most of these new markers need complex assessments and assays, there is still a need for simple yet reliable non-invasive tests that correlate with the severity of CVD, and that can be applied in primary screening settings. The quantification of vascular and endothelial dysfunction [390] is a recently emerging early marker for cardiovascular risk and is usually achieved by employing techniques such as ultrasound FMD, PWA, plethysmography and iontophoresis [420]. These tests can however be complex and time consuming and are still only performed in highly specialized services. Among the various methods developed to measure microvascular function, DVA features as a non-invasive method that enables continuous recordings of retinal arterial and venous diameter changes in response to flicker-light stimulation. The main advantage of DVA assessment is that it provides an integrated and dynamic data analysis that is specific to each individual. In addition, its output has proven to be modified not only by overt disease but also in the presence of more subtle risk factors for CVD [262, 281, 300, 318-320, 325, 368, 421, 422]. Therefore, it is possible to use the assessment of retinal microvascular function as an early marker for vascular and endothelial dysfunction.

Besides pathologies, however, normal ageing can also influence retinal microvascular dynamics as assessed by DVA. Indeed, recent data allude to an age-related decrease in retinal arterial response profiles [259], and a general decline in overall dilation amplitudes during flicker-light stimulation [258, 308, 322]. Nevertheless, in order to provide a better understanding of the individual vascular dynamics and how this can be modified by

various pathologies, a more complex analysis is needed in healthy individuals of various age groups to allow the inclusion of not only the vasodilatory indices but also the dynamics of the constriction response, as well as the capacity of re-establishing a pre-flicker diameter after cessation of stress. Therefore, the present study seeks to characterize the entire retinal microvascular response to flicker provocation in apparently healthy individuals of various age groups using a more detailed approach for the analysis retinal vascular function parameters as detailed in section 6.3.4.

7.3. Methods

7.3.1. Study participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). In addition, participants were selected from a laboratory database of individuals considered for previous case-control studies if they fit the inclusion / exclusion criteria as defined in section 6.2.1. The main study-specific inclusion criteria for this study were defined as those individuals aged above 18 years, with no current or prior history of cardiovascular disease. Based on preliminary assessments and FRS calculations, any individuals identified as having moderate or high FRS were excluded from the final analysis.

7.3.2. General assessments

General clinical assessments for all participants as detailed in section 6.3.2 included general health history questionnaires, BMI, BP and IOP profiles, and circulatory markers including GLUC, TG, CHOL, HDL-c, LDL-c (section 6.3.2.5). Additionally, FRS was calculated based on age, gender, SBP, CHOL, HDL-c, diabetes and smoking status [26, 365, 423] (section 6.3.3) and only participants classified as having low risk (< 10%) for developing CVD [367] were included in the final analysis.

7.3.3. Vascular assessments

Vascular assessments of interest to this study, detailed in the methods section, include DVA (section 6.3.4.1), c-IMT (section 6.3.5.2) and PWA (section 6.3.5.1). For each participant, one unselected eye was evaluated by way of DVA. For any participants selected from the laboratory database, all raw data was re-analysed using the mathematical approach for the analysis of retinal response parameters described in this thesis. The following parameters, averaged across three flicker cycles and with the arteries and veins regarded separately, were evaluated in this study: baseline diameter, BDF, DA, BCFR,

MD%, MC%, tMD, tMC as well as dilation (Slope $_D$) and constriction slopes (Slope $_C$) (section 6.3.4.8). With regards to systemic vascular parameters, c-IMT scores (right vs. left) corresponding to eye selected (right vs. left) for DVA assessment were evaluated and the AIx, as computed by the PWA software, was used as the primary measure of systemic arterial stiffness.

7.3.4. Sample size calculations

Based on previous studies [258, 308] normal expected retinal dilation and constriction responses to flicker-light were estimated to be approximately 3.64 ± 1.84 % and 2.87 ± 1.39 %, respectively; and a change in 30 to 50% has been reported to be clinically significant, therefore a similar difference between groups was expected in this study. Previous studies also report an exponential relationship between AIx and age [424] and a linear relationship between c-IMT scores and age [425, 426]. Since it was determined that analysis of variance (ANOVA) or covariance (ANCOVA) would be required in this study, and given the uniqueness of the comparisons being made with regards to retinal and systemic vascular parameters, sample size calculations were based on a number of assumptions. Based on Cohen's [427] standardized classification of effect sizes: small effect = 0.10; medium effect = 0.25; large effect = 0.40 it was expected that a medium effect size of at least 0.25 would be observed, and in order to provide 80% power with the number of study groups specified as 3, and an alpha-level set at 0.05, a sample size of n = 159 was recommended. Sample size calculations were performed using the G*Power software [428] (University of Kiel, Version 3.1.6, Germany).

7.3.5. Statistical Analysis

All statistical analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). The Shapiro-Wilk test was used to determine the distribution of the data. Multivariate analysis was performed to determine the influence of age, BMI, BP, and circulating and systemic markers on the measured variables. Differences between groups were subsequently assessed using one-way ANOVA or ANCOVA followed by Tukey's post-hoc analysis. A p-value of less than 0.05 was considered significant except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons.

7.4. Results

7.4.1. Study participants

In total, 236 healthy volunteers were initially screened for study inclusion of which 42 individuals were excluded based on moderate or high FRS (>10%), incomplete data sets, or if the quality of the DVA recording was poor. The remaining 194 healthy participants were included in the final analysis and classified into one of three age groups (Group 1: 19 to 30yrs; Group 2: 31 to 50yrs; Group 3: 51 to 70yrs). The number of participants in each group was similar (Group 1: 57; Group 2; 75; Group 3; 62, Chi-square test: $p = 0.295$), as was the distribution of male (M) and female (F) participants within each group (Group 1: M = 27, F = 30; Group 2: M = 42, F = 33; Group 3: M = 33, F = 29, Chi-square test $p = 0.612$).

7.4.2. Clinical characteristics

Table 7.1 summarizes the clinical characteristics of the study population. There was a significant overall difference in age ($p < 0.001$), BMI ($p = 0.002$), SBP ($p = 0.002$), DBP ($p = 0.001$), HR ($p < 0.001$), MAP ($p = 0.001$), IOP ($p < 0.001$), CHOL ($p = 0.002$), HDL-c ($p = 0.007$), LDL-c ($p < 0.001$), FRS ($p < 0.001$), AIx ($p < 0.001$), and c-IMT scores ($p < 0.001$) between the age groups. There were no statistically significant differences in OPP ($p = 0.089$), GLUC ($p = 0.102$), or TG levels ($p = 0.161$). The post-hoc comparisons revealed that age, FRS and c-IMT scores significantly increased across each group (all $p < 0.001$). In addition, in comparison to the youngest group BMI, DBP, IOP, and LDL-c were higher in the middle-age ($p = 0.023$, $p = 0.013$, $p = 0.019$, and $p = 0.006$, respectively) and older ($p = 0.002$, $p = 0.001$, $p < 0.001$, and $p = 0.001$, respectively) groups. Additionally, SBP and AIx were higher in the oldest group compared to the youngest ($p = 0.009$; and $p < 0.001$, respectively) and middle-aged groups ($p = 0.003$; and $p = 0.010$, respectively). Finally, with regards to HR, MAP, CHOL and HDL-c the middle-aged group did not significantly differ from the youngest or oldest group (all $p > 0.05$), however, HR ($p < 0.001$) and HDL-c ($p = 0.009$) were lower, and MAP ($p < 0.001$) and CHOL ($p = 0.001$) were higher in the oldest age group in comparison to the youngest group.

Table 7.1. Summary of clinical data

Variable	Mean (SD) Group (1) (19-30yrs)	Group (2) (31-50yrs)	Group (3) (51-70yrs)	*p*-value	Significance
N	57	75	62	0.295	-
Gender	27M : 30F	42M : 33F	33M : 29F	0.612	-
Age (years)	26 (3)	40 (6)	56 (5)	<0.001*	1 < 2 < 3
BMI (kg/m²)	24.11 (3.84)	26.00 (3.74)	26.69 (4.69)	0.002*	1 < 2, 3
SBP (mmHg)	116 (13)	117 (12)	123 (13)	0.002*	1, 2 < 3
DBP (mmHg)	71 (9)	76 (11)	77 (10)	0.001*	1 < 2, 3
HR (bpm)	71 (11)	67 (8)	64 (8)	<0.001*	1 > 3
MAP	85.94 (9.33)	89.63 (10.67)	92.92 (10.26)	0.001*	1 < 3
IOP (mmHg)	13 (2)	14 (3)	15 (2)	<0.001*	1 < 2, 3
OPP	44.69 (6.06)	45.97 (7.08)	47.44 (7.09)	0.089	-
GLUC (mmol/L)	4.80 (0.74)	4.92 (0.68)	5.09 (0.78)	0.102	-
TG (mmol/L)	1.04 (0.47)	1.22 (0.65)	1.18 (0.50)	0.161	-
CHOL (mmol/L)	4.18 (0.77)	4.49 (0.89)	4.75 (0.97)	0.002*	1 < 3
HDL-C (mmol/L)	1.44 (0.50)	1.38 (0.41)	1.22 (0.38)	0.007*	1 > 3
LDL-C (mmol/L)	2.25 (0.75)	2.71 (0.86)	2.82 (0.91)	<0.001*	1 < 2, 3
FRS %	0.74 (0.48)	3.41 (2.41)	8.25 (2.71)	<0.001*	1 < 2 < 3
AIx	10 (9)	15 (12)	22 (12)	<0.001*	1, 2 < 3
c-IMT (mm)	0.46 (0.01)	0.56 (0.01)	0.63 (0.02)	<0.001*	1 < 2 < 3

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; IOP, intraocular pressure; OPP, ocular perfusion pressure; GLUC, glucose; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; FRS%, Framingham risk score; AIx, augmentation index; c-IMT, carotid intima media thickness. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

7.4.3. Retinal vascular function

7.4.3.1. Arterial response

After controlling for all influential covariates identified in multivariate analysis, there were no significant differences across age groups with regards to baseline diameter, BDF, BCFR, MD%, tMD, tMC, and $Slope_{AD}$, (all ANCOVA $p > 0.01$, Table 7.2). Nevertheless, there was a significant difference between age groups in arterial DA ($p = 0.003$), MC% ($p < 0.001$) and $Slope_{AC}$ ($p < 0.001$) (Figure 7.1A). Post-hoc comparisons revealed DA and MC% to be significantly decreased in the oldest age group compared to the middle ($p = 0.028$; $p = 0.021$, respectively) and youngest group ($p = 0.003$; $p = 0.026$, respectively). Additionally, $Slope_{AC}$ was decreased in the oldest age group compared to the youngest group ($p = 0.027$), with the middle-age group not differing significantly from the youngest ($p = 0.525$) or oldest group ($p = 0.216$).

7.4.3.2. Venous response

There was an overall significant difference in venous MC% across groups (ANOVA $p = 0.002$, Figure 7.1B) with post-hoc comparisons showing MC% to be decreased in the middle ($p = 0.015$) and older ($p = 0.010$) age groups compared to the youngest group. After controlling for all influential covariates identified in multivariate analysis no significant differences in any of the other measured venous DVA parameters were identified across groups (ANCOVA, $p > 0.05$, Table 7.2).

7.4.3.3. Gender comparisons

Within-group gender comparisons in the measured retinal arterial and venous DVA parameters are displayed in Table 7.3. The results revealed overall arterial MD% to be significantly higher in men compared to women for the total study population (M: 4.42 ± 2.51 vs. F: 3.84 ± 2.27, $p = 0.011$, Figure 7.2A). Within-group comparisons showed arterial DA ($p = 0.007$) and MD% ($p < 0.001$), to be significantly higher in men compared to women belonging to the youngest age group (Figure 7.2B) but not between men and women in the middle-aged and oldest groups (all $p > 0.01$, Figure 7.2C & Figure 7.2D). There were, however, no significant gender differences in the measured retinal venous DVA parameters for the study population (all $p > 0.01$, Table 7.3, Figure 7.3A-D).

Table 7.2. Summary of retinal vascular function parameters

DVA parameter	Mean (SD) Group (1) (19-30yrs)	Group (2) (31-50yrs)	Group (3) (51-70yrs)	*p*-value	Significance
Arteries:					
Baseline	99.89 (0.76)	99.97 (0.20)	99.98 (0.14)	0.488	-
BDF	6.06 (3.29)	5.93 (2.59)	5.54 (2.78)	0.093	-
DA	7.04 (3.61)	6.59 (2.72)	5.36 (2.36)	**0.003***	1, 2 > 3
BCFR	1.05 (3.03)	0.90 (2.65)	0.03 (2.36)	0.083	-
MD%	4.38 (3.00)	4.09 (2.22)	3.82 (2.04)	0.036	-
MC%	-2.67 (2.32)	-2.41 (1.67)	-1.37 (1.77)	**<0.001***	1, 2 > 3
tMD (seconds)	22 (9)	20 (8)	21 (7)	0.105	-
tMC (seconds)	24 (9)	28 (9)	29 (8)	0.041	-
$Slope_{AD}$	0.23 (0.15)	0.27 (0.16)	0.28 (0.41)	0.063	-
$Slope_{AC}$	-0.42 (0.35)	-0.27 (0.57)	-0.23 (0.20)	**<0.001***	1 > 3
Veins:					
Baseline	99.89 (0.76)	99.98 (0.13)	99.96 (0.20)	0.490	-
BDF	4.83 (2.78)	3.99 (1.63)	4.64 (2.82)	0.114	-
DA	5.80 (3.33)	5.25 (2.53)	5.51 (2.78)	0.557	-
BCFR	1.05 (2.67)	1.30 (2.29)	0.92 (2.55)	0.097	-
MD%	4.31 (2.19)	4.59 (2.43)	4.46 (2.74)	0.794	-
MC%	-1.61 (1.70)	-0.81 (1.10)	-0.75 (1.16)	**0.002***	1 > 2, 3
tMD (seconds)	23 (8)	21 (6)	22 (7)	0.129	-
tMC (seconds)	28 (9)	30 (7)	29 (7)	0.390	-
$Slope_{VD}$	0.23 (0.15)	0.25 (0.14)	0.26 (0.17)	0.391	-
$Slope_{VC}$	-0.25 (0.17)	-0.19 (0.15)	-0.22 (0.16)	0.087	-

Abbreviations: Baseline, average baseline diameter: BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percent dilation (during flicker); MC%, percent constriction (post-flicker); tMD, time taken to reach maximal dilation (MD) diameter; tMC, time taken to reach maximal constriction (MC) diameter; $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction. *Significant *p*-values are indicated in bold where $p < 0.01$ was considered significant.

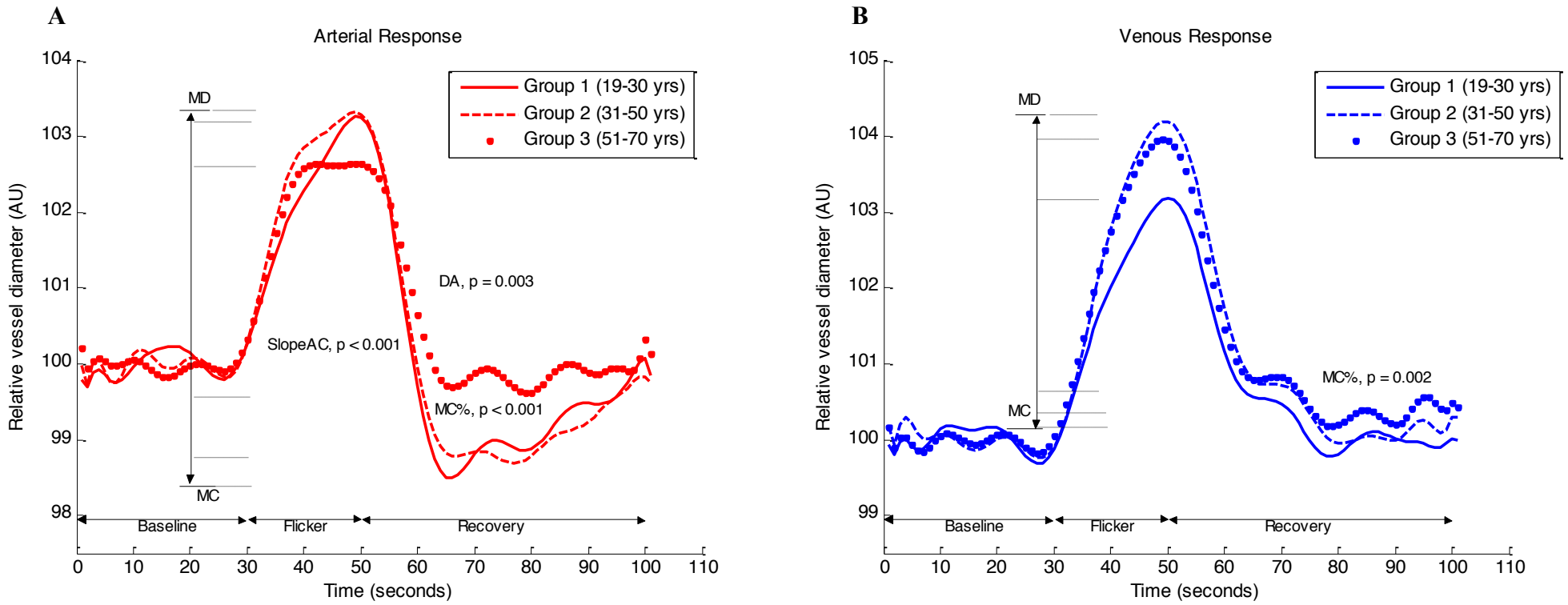


Figure 7.1. Comparisons of retinal vascular response profiles.
(A) arterial response, (B) venous response.
Abbreviations: AU, arbitrary units; DA, dilation amplitude; MD, maximal dilation diameter; MC, maximal constriction diameter; MC% percent constriction; $Slope_{AC}$, constriction slope.

Table 7.3. Summary of gender differences in retinal vascular function parameters

	Mean (SD)											
	Group 1 (19-30yrs)				**Group 2** (31-50yrs)				**Group 3** (51-70yrs)			
	M	**F**	***p*-value**	**sig.**	**M**	**F**	***p*-value**	**sig.**	**M**	**F**	***p*-value**	**sig.**
Arteries:												
Baseline	99.79 (1.07)	100.00 (0.01)	0.288	-	99.99 (0.07)	99.95 (0.27)	0.414	-	99.97 (0.19)	100.00 (0.01)	0.306	-
BDF	6.95 (3.50)	5.36 (2.77)	0.059	-	5.99 (2.40)	5.92 (2.48)	0.895	-	5.05 (1.57)	6.02 (3.09)	0.137	-
DA	8.21 (4.01)	5.72 (2.75)	**0.007***	M > F	6.38 (2.69)	7.01 (2.82)	0.298	-	5.53 (2.27)	5.43 (2.64)	0.872	-
BCFR	1.26 (3.44)	0.48 (2.64)	0.336	-	0.46 (2.94)	1.09 (2.16)	0.277	-	0.50 (2.15)	-0.37 (2.55)	0.120	-
MD%	5.85 (3.29)	3.06 (1.80)	**<0.001***	M > F	3.93 (2.08)	4.61 (2.53	0.175	-	3.97 (1.96)	3.71 (2.14)	0.592	-
MC%	-2.37 (2.82)	-2.66 (1.88)	0.654	-	-2.45 (1.55)	-2.41 (1.68)	0.895	-	-1.56 (1.72)	-1.72 (1.73)	0.687	-
tMD(sec)	23 (10)	21 (7)	0.222	-	19 (7)	22 (8)	0.080	-	20 (7)	21 (8)	0.316	-
tMC (sec)	23 (10)	26 (8)	0.148	-	29 (7)	25 (9)	0.027	-	29 (8)	27 (8)	0.232	-
$Slope_{AD}$	0.29 (0.18)	0.20 (0.17)	0.071	-	0.27 (0.16)	0.27 (0.17)	0.991	-	0.28 (0.31)	0.31 (0.49)	0.757	-
$Slope_{AC}$	-0.50 (0.28)	-0.35 (0.37)	0.081	-	-0.26 (0.13)	-0.50 (0.72)	0.022	-	-0.24 (0.14)	-0.29 (0.19)	0.289	-
Veins:												
Baseline	99.79 (1.07)	100.00 (0.01)	0.292	-	99.97 (0.16)	99.97 (0.18)	0.889	-	99.97 (0.19)	99.97 (0.18)	0.926	-
BDF	5.28 (3.37)	4.52 (2.05)	0.299	-	4.18 (1.53)	3.87 (1.85)	0.410	-	4.41 (2.39)	4.85 (3.01)	0.501	-
DA	5.47 (3.57)	6.03 (3.04)	0.519	-	5.67 (2.60)	5.09 (2.40)	0.292	-	4.82 (2.37)	6.01 (2.87)	0.059	-
BCFR	0.23 (2.93)	1.61 (2.20)	0.046	-	1.52 (2.40)	1.27 (2.05)	0.608	-	0.41 (2.35)	1.26 (2.77)	0.162	-
MD%	4.60 (3.04)	4.42 (2.43)	0.804	-	4.55 (2.28)	4.41 (2.17)	0.770	-	3.99 (2.35)	5.07 (2.23)	0.048	-
MC%	-1.39 (2.09)	-1.61 (1.26)	0.631	-	-0.69 (0.76)	-1.12 (1.08)	0.039	-	-0.83 (0.86)	-0.94 (1.13)	0.649	-
tMD (sec)	26 (10)	21 (7)	0.021	-	20 (6)	22 (5)	0.154	-	21 (6)	24 (9)	0.109	-
tMC (sec)	25 (10)	31 (7)	0.012	-	31 (7)	29 (7)	0.277	-	30 (7)	27 (8)	0.124	-
$Slope_{VD}$	0.23 (0.17)	0.24 (0.14)	0.814	-	0.27 (0.13)	0.24 (0.13)	0.335	-	0.23 (0.15)	0.40 (0.94)	0.026	-
$Slope_{VC}$	-0.27 (0.17)	-0.25 (0.16)	0.605	-	-0.22 (0.14)	-0.19 (0.10)	0.419	-	-0.20 (0.12)	-0.27 (0.13)	0.286	-

Abbreviations: Baseline, average baseline diameter: BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percent dilation (during flicker); MC%, percent constriction (post-flicker); tMD, time taken to reach maximal dilation (MD) diameter; tMC, time taken to reach maximal constriction (MC) diameter; $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction. *Significant *p*-values are indicated in bold where $p < 0.01$ was considered significant.

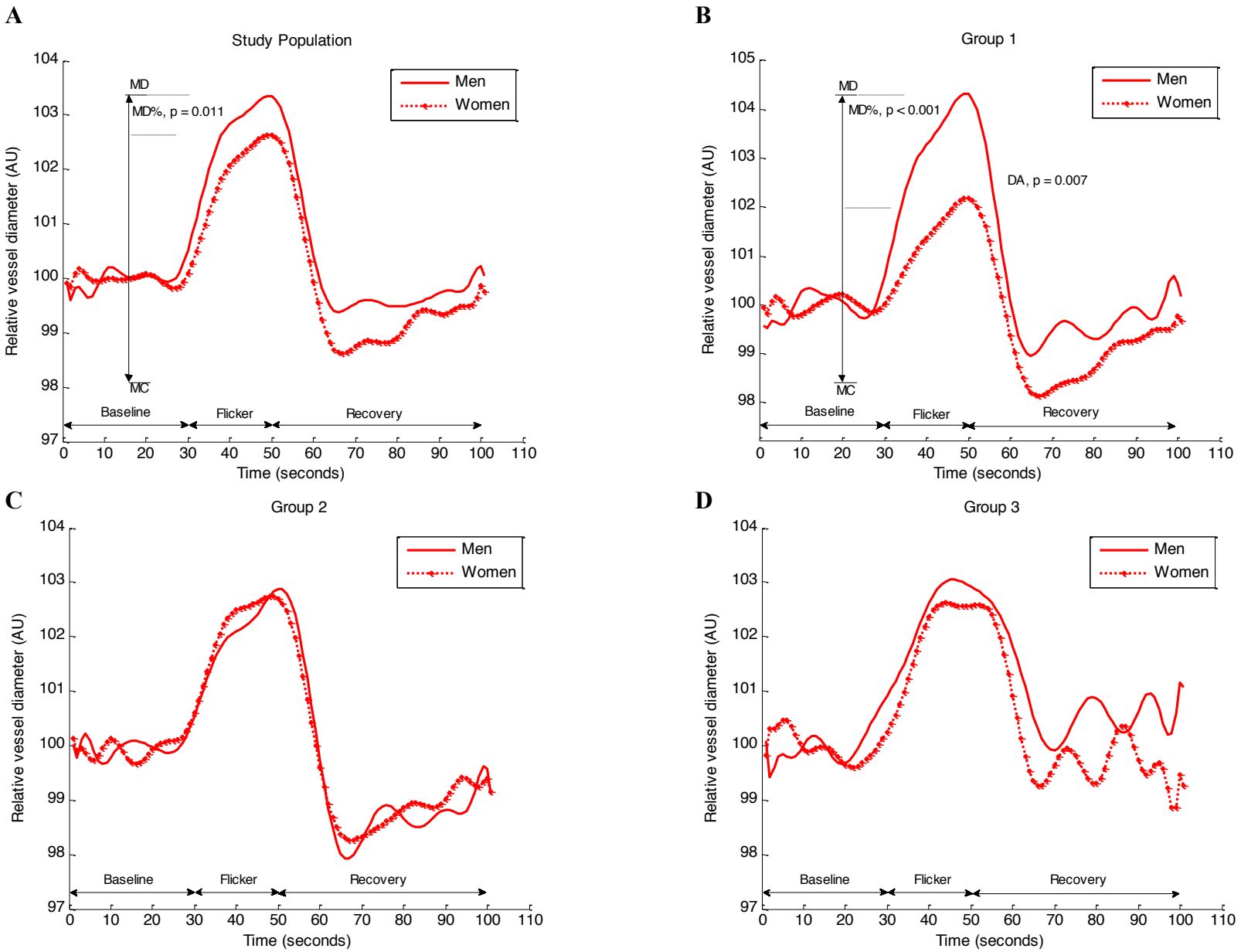


Figure 7.2. Retinal arterial response profiles stratified by gender.
(A) Study population; (B) Group 1; (C) Group 2; (D) Group 3.
DA, dilation amplitude; MD, maximal dilation diameter; MD%, percent dilation; MC, maximal constriction diameter.

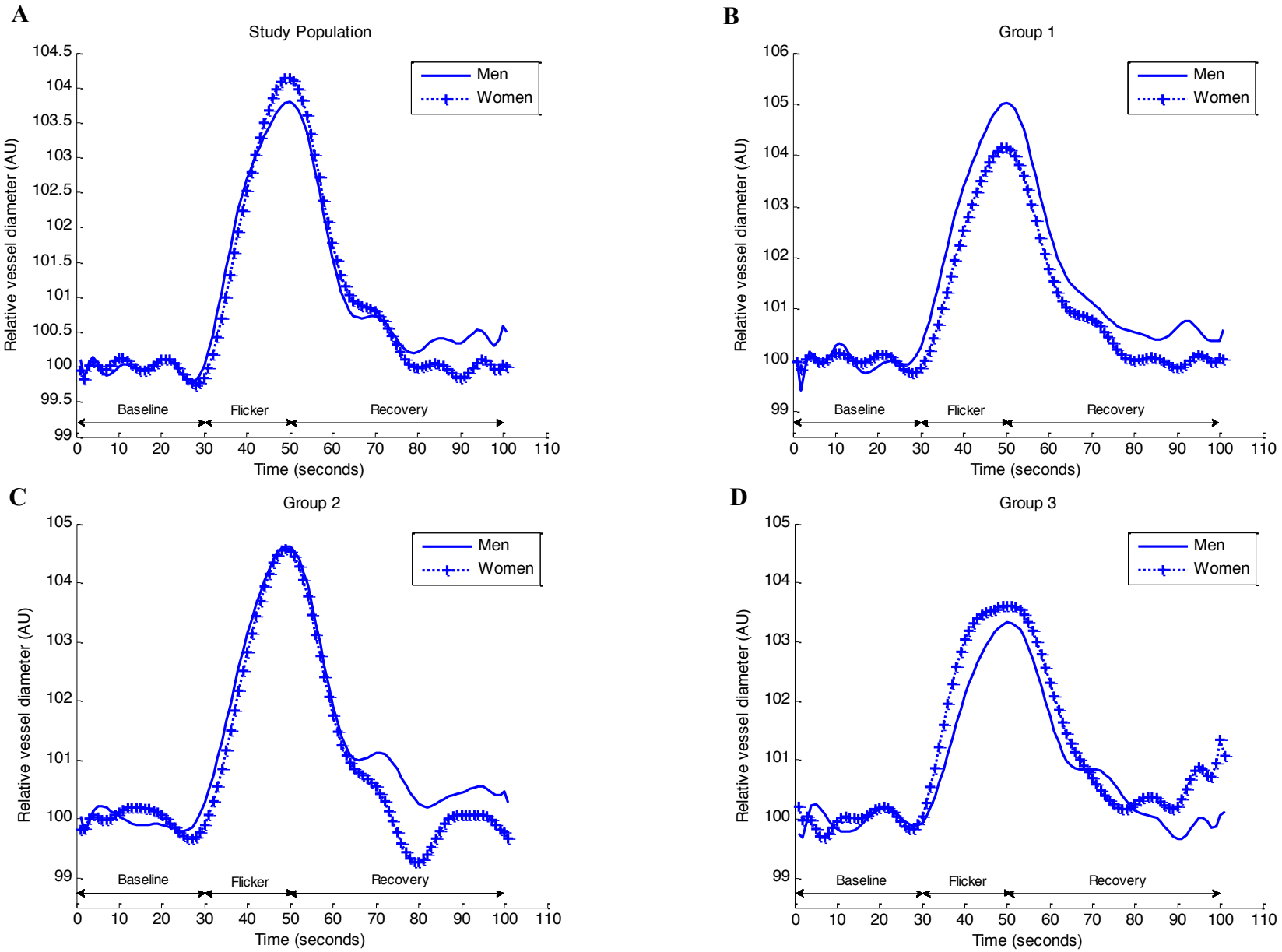


Figure 7.3. Retinal venous response profiles stratified by gender.
(A) Study population; (B) Group 1; (C) Group 2; (D) Group 3

7.5. Discussion

In the present study, a specific computational model was used to evaluate the entire dynamic response of retinal microvessels after flicker stimulation in a sample of individuals with low CVD risk (< 10%) belonging to various age groups. The results show that independent of systemic vascular influences, older healthy individuals displayed abnormal dilatory and constrictory responses to flickering stimulation in retinal arteries and veins. Additionally, in younger individuals gender had an influence on retinal arterial and venous dilation, but this effect was lost in the older groups.

It is known that decreased vessel distensibility and focal narrowing occur in ageing vessels independently of other arteriosclerotic risk factors [96, 429, 430]. Despite various adaptations to vascular structural remodelling and changes in viscoelastic properties that occur with ageing, there could still be individual limitations in functional vascular reserves that may only be evident during responses to provocative stressors. In line with previous research [258, 308], this study shows an age-related decline in retinal vasoregulative capacity which mostly corresponded with an attenuated response in the constrictory phase, that was independent of other systemic vascular influences. It was previously hypothesized that in ageing vessels the retinal vascular adaptive response may be attributed to a re-setting of vessels' average working points within which the points of maximum dilation and constriction tend to occur [308]. The cause of this shift in vessel behaviour remains unclear; however, a possible contender is a high level of oxidative stress that occurs with ageing and is a known cause of senescent endothelial dysfunction [431]. Indeed, in otherwise healthy individuals with low to moderate cardiovascular risk, retinal microvascular dilation and constriction responses to stress levels are influenced by systemic antioxidant capacity [432] (Chapter 8). Although the levels of antioxidant molecules were not determined in this study, it can be hypothesized that similar interactions take place in all individuals with similar CVD risk.

Other factors such as age-related vascular stiffness can also be involved. An understanding of microcirculatory responses with regards to systemic haemodynamic parameters is important as the combination of arterial stiffening and ensuing hypertension [433] can offset the stiffness gradient between ventricular and vascular interactions and augment pressure pulsatility penetrating into the microvasculature. In the present study groups the AIx, a measure of peripheral arterial stiffness, was higher in older than in middle-aged or younger individuals, although the age-related decline in the retinal vessels' dilation and constriction phases demonstrated in this study occurred independently of this systemic measure. It

cannot, however, be excluded that local microvascular stiffness played some role in the present findings and this remains to be established.

In previous studies in our lab, increases in pre-flicker baseline diameter fluctuations [422], decreases in the arterial corrected flicker response [310] and dilation capacity [311], as well as, venous dilation capacity [320], and enhanced post-flicker vasoconstrictions [320, 422] in groups with various levels of cardiovascular risk, have already been documented. In the present study, in individuals with low risk, decreases in the post-flicker constrictory phase of retinal veins were apparent in the middle-aged and older individuals. The role of the venular circulation in CVD has previously received limited attention until unexpected associations implicated retinal venular dilation rather than arteriolar narrowing as a stronger predictor of adverse vascular phenomena [128, 301, 434]. These studies have since stimulated an increased interest in retinal venular physiology although it remains unclear whether changes detected by DVA represent separate causal pathways of endothelial dysfunction or are epiphenomena of the autoregulatory response. In such context, it could be possible that the observed decrease in post-flicker venous diameter returns to baseline reflects a compensatory adaptation following sustained arterial dilation during flicker. Further investigation is still required to understand the relevance of the observed abatements in the re-establishment of post-flicker venous diameters, it however could be hypothesized that changes in venous caliber associated with structural or endothelial irregularities could also be used as a marker for ageing and associative cardiovascular risk.

The dynamic behaviour of retinal microvessels also appears to be affected by more than just the ageing functional state of the endothelium. In the present study, gender differences in the retinal vasoregulative response were lost with ageing. Sex hormones influence both the vascular tone and blood flow in various organs and tissues, including the retinal vessels [435]. Taking into consideration the above, gender differences in vascular tonus and blood flow are to be expected due to changes in hormonal status across the life span of individuals. Indeed, in the present study an overall gender difference in arterial MD% was observed; however with younger men exhibiting higher MD% values than age-matched women. To our knowledge, this is the first study to observe gender differences in DVA measurements in healthy individuals, and as oestrogens upregulate NO production and supress the effect of vasoconstrictors such as ET-1 [436], the results are somewhat unexpected. As the retinal vascular response to flickering light is also a neurovascular coupling driven response [255] and sex hormones can exert effects on other cells in the neurovascular unit such as neurons and astrocytes [437], it is possible that the gender

differences in the retinal vascular function do not truly match those assessed by other methods that measure resting blood flow and vascular tone. Nevertheless, the expected ageing-related blunt in the gender differences in vascular reactivity was still apparent in the present results. Further study is, however, still necessary in order to clarify the mechanism of gender differences in retinal vascular function. The possible influences of diet and lifestyle factors such as exercise should also be considered.

7.6. Conclusion

Although age is a non-modifiable cardiovascular risk factor, it can be useful for identifying individuals at increased risk. Functional retinal changes with age are already apparent in otherwise clinically healthy individuals, and this study demonstrates that age and gender are variables to be considered when assessing the retinal vascular response to flicker. The entire functional retinal response curve (to include vessel behaviour after stress) should also be evaluated when assessing risk. Moreover, pathological changes must be carefully differentiated from age-related functional hyperaemic responses in retinal vessels. Nevertheless, the present study also has implications for identifying risk in individuals that exhibit functional irregularities, which could be equated to signs of premature vascular ageing. The possible causes of altered vascular functionality, such as structural vascular changes, endothelial dysfunction, reduced synthesis and release of vasoactive substances, decreased sensitivity to reactive metabolites, and or diminished neurovascular coupling activity must also be considered.

8. Study 2: Retinal vascular function in healthy individuals with a family history of cardiovascular disease

8.1. Abstract

Purpose: To compare and contrast retinal microvascular function in response to flicker provocation in healthy individuals with and without a FH of CVD.

Methods: Retinal vascular function was assessed by way of DVA in 75 healthy individuals aged between 30 and 66 years, who were classified into two groups based on the presence (FH positive, n = 38) or absence (control, n = 37) of familial CVD. General assessments in all participants included BP profiles, blood analyses for glucose (GLUC) and lipid metabolism markers (TG, CHOL, HDL-c, LDL-c), and FRS. Other vascular assessments also included c-IMT scores and brachial FMD as a measure of systemic endothelial function.

Results: In comparison to controls, FH positive subjects showed decreased retinal arterial baseline diameter fluctuation (BDF), percent dilation (MD%), and overall constriction response slope ($Slope_{AC}$) (p = 0.001; p = 0.001; and p < 0.001, respectively), and increased arterial percent constriction (MC%, p = 0.008). On the venous side, baseline corrected flicker response (BCFR) and dilation response slope ($Slope_{VD}$) were decreased in the FH positive group (p = 0.009 and p = 0.010, respectively). There were no significant differences between groups in c-IMT scores or FMD parameters (all p > 0.05); however, lower HDL-c levels in the FH positive group (p = 0.030) correlated with the observed increases in retinal arterial MC% (r = -0.52, p = 0.002).

Conclusion: In low-risk individuals with FH of CVD macrovascular function is preserved, however, impairments in microvascular function identified at the retinal level correlate with established plasma markers for cardiovascular risk.

8.2. Introduction

The vascular endothelium plays an important role in regulating vessel tone [438]. In addition, it also acts in preventing inflammation, platelet aggregation, and leukocyte adhesion. Ageing, smoking, dyslipidaemia, hypertension, obesity, type II diabetes, and chronic inflammatory diseases can all result in a dysfunctional endothelium, a condition in which NO levels are reduced and the levels of ROS are increased [390]. Moreover, in addition to local vasoconstrictions and tissue ischaemia, endothelial dysfunction is a well-known major culprit in the onset of atherosclerosis and CVD [439, 440]. Data from clinical and observational studies, however, indicate that endothelial dysfunction can precede the development of atherosclerosis and be detected even in individuals without overt clinical disease [441-444].

It has already been documented that a FH of CVD increases the risk for circulatory pathologies [445-448], moreover, signs of endothelial dysfunction are present in those with familial risk factors [449, 450]. Despite this, endothelial dysfunction and FH are not among variables commonly incorporated into cardiovascular risk assessment models such as the FRS. Such inclusions may be particularly important for identifying at-risk individuals with an inherited vascular vulnerability, but for whom the overall predicted risk score is a misattribution. Emerging vascular markers that improve traditional risk assessments show promise in identifying vulnerable vascular phenotypes, and in addition to providing more detailed biological profiles, could be pivotal in the shift towards personalized interventional approaches for individualized disease risk. Imaging vascular markers are of particular clinical interest, owing to the need for less invasive tests, and endothelial properties are commonly evaluated using techniques such as ultrasound FMD and carotid scans. However, the quantification of endothelial function or dysfunction can be complex and; there is still a need for reliable vascular indices that correlate with the extent and severity of risk and that can be applied in a clinical setting.

A functional microvascular assessment such as DVA can be performed quickly, and offers reliable information on vascular function in patients suffering from various systemic conditions. The main advantage of this technique is the opportunity to derive integrated and dynamic vessel response parameters that are specific to each individual. In addition, vascular dysfunctions in smaller more susceptible vessels may be present before they can be detected in larger vessels; therefore, microvascular assessments are generally considered to be of added clinical value. Indeed, retinal vascular function studies in

patients with diabetes [281, 318], hypertension [258, 300], and hypercholesterolemia [262, 309, 325] have shown a reduced retinal vessel response to flicker-light stimulation (an endothelium-mediated response), suggesting that endothelial dysfunction measured at the retinal level could serve as an indirect marker of systemic vascular status [254]. Little is, however, known about when the first signs of vascular dysfunction occur or can be detected in patients with, and or at risk for disease and the extent to which such dysfunctions are present in otherwise healthy, but at-risk individuals, could influence the potential to decrease future vascular risk as well as identify non-responders to endothelial therapies. Using FH as an early indicator of vascular risk, this study sought to investigate the relative influence of FH on retinal microvascular function and systemic vascular parameters. Studies addressing this question in low-risk patients but with a positive FH of CVD, to the best of our knowledge, have not yet been published.

8.3. Methods

8.3.1. Study participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). Study specific inclusion criteria were as those defined in section 6.2.1. FH positive individuals were identified through self-report questionnaire if they indicated the presence of CVD in a first-degree relative. In addition, a subset of age- and gender-matched subjects who reported no FH of CVD on the self-report questionnaires were selected as study controls.

8.3.2. General assessments

General clinical assessments for all participants as detailed in section 6.3.2 included general health history questionnaires, BMI, BP and IOP profiles, and circulatory markers including GLUC, TG, CHOL, HDL-c, and LDL-c (section 6.3.2.5). Additionally, FRS was calculated based on age, gender, SBP, CHOL, HDL-c, diabetes and smoking status [26, 365, 423] (section 6.3.3) and only participants classified as having low risk (< 10%) for developing CVD [367] were included in the final analysis.

8.3.3. Vascular assessments

Vascular assessments of interest in this study (detailed in the methods section) included DVA (0), c-IMT (section 6.3.5.2) and FMD (section 6.3.5.3). For each participant, one unselected eye was evaluated by way of DVA and the following parameters were averaged

across three flicker cycles with the arteries and veins regarded separately: baseline diameter, BDF, DA, BCFR, MD%, MC%, tMD, tMC as well as dilation (Slope $_D$) and constriction slopes (Slope $_C$) (section 6.3.4.8). With regards to systemic vascular parameters, only c-IMT scores corresponding to eye selected (right vs. left) for DVA assessment were evaluated; and for FMD assessment, the diameter recordings for the first 2 minutes of baseline acquisition and 2 minutes post-cuff deflation were selected from the raw data and compressed using Matlab (Mathworks, Inc., USA) to determine the average brachial diameter during baseline ($AD_{baseline}$), the peak diameter during reactive hyperaemia ($MD_{hyperaemia}$), and endothelium-dependent FMD (FMD_{ED}) expressed as the percentage change in vessel diameter relative to baseline values (Equation 6.6).

8.3.4. Sample size calculations

Sample size calculations were performed using the G*Power software [428] (University of Kiel, Version 3.1.6, Germany). The sensitivity and reproducibility of DVA assessments in healthy subjects has been reported previously [141, 266, 269] and the normal expected retinal responses to flicker-light stimulation have been reported to be around 6.9 ± 2.8 % in arteries and 6.5 ± 2.8 % in veins [256, 268]. In previous studies of otherwise healthy patients with risk factors for CVD, including hypertension and pre-diabetes a 30-40% alteration in this response has been reported to be significant [12, 14] and a similar difference between controls and those with positive familial CVD was anticipated in this study. Additionally, FMD studies have shown a 33 ± 5.2 % reduction in FMD response in those with familial risk factors [451]. Since it was determined that *t*-tests or ANCOVA would be required and given the multifactorial nature of the study design, a sample size of $n = 34$ per group was recommended as sufficient to provide 95% power with an alpha-level of 0.05 and a large effect size of at least 0.80. The effect size conventions were selected based on Cohen's standardized effect sizes [427]: small effect = 0.20; medium effect = 0.50; large effect = 0.80.

8.3.5. Statistical analysis

All statistical analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). The Shapiro-Wilk test was used to determine the distribution of the data. Multivariate analysis was used to test the influence of age, BMI, BP, and circulating and systemic markers on the measured variables. Differences between groups were subsequently assessed using independent samples *t*-tests or ANCOVA as appropriate. Within-group correlations between retinal vascular function parameters and systemic

parameters were explored using Pearson's or Spearman's rank method as appropriate. A *p*-value of less than 0.05 was considered significant except in certain cases where a stricter *p*-value of less than 0.01 was adopted in order to correct for multiple comparisons.

8.4. Results

8.4.1. Study participants

In total, 102 participants were screened for eligibility of which 27 participants were excluded if they had moderate or high risk for CVD based on FRS scores (>10%), if the quality of DVA recordings was poor, and or if the presence or absence of FH could not be identified in the self-report questionnaires. The remaining 75 healthy participants with low FRS scores (0-10%) and who fit the inclusion criteria were classified into one of two groups based on the presence or absence of familial CVD (FH positive: $n = 38$, controls: $n = 37$, respectively). The number of participants in each group was similar (Chi-square $p = 0.909$) as was the within-group distributions of male (M) and female (F) participants (FH positive: M = 20; F = 18, Control: M = 22, F = 15, Chi-square $p = 0.551$).

8.4.2. Clinical characteristics

A summary of group differences in clinical characteristics and systemic vascular assessments is presented in Table 8.1. There were no significant group differences in age, BMI, SBP, DBP, HR, MAP, IOP, OPP, GLUC, TG, CHOL, LDL-c, TG: HDL-c and FRS between groups (all $p > 0.05$). Those with a positive FH of CVD, however, exhibited lower levels of HDL-c ($p = 0.030$) and higher CHOL: HDL-C ratios ($p = 0.021$).

8.4.3. Systemic vascular function

In multivariate regression analysis age significantly and positively influenced c-IMT scores ($\beta = 0.29$, $p = 0.047$). After correcting for other known influential covariates including BP and BMI, there were no significant group differences in c-IMT scores ($p > 0.05$). Similarly following correction for influential covariates, including age, BP and CHOL: HDL-c, there was no significant difference in $AD_{baseline}$, $MD_{hyperaemia}$, or FMD_{ED} in those with and without a positive FH of CVD (all $p > 0.05$, Table 8.1).

8.4.4. Retinal vascular function

8.4.4.1. Arterial response

After controlling for influential covariates in ANCOVA models, statistically significant differences in arterial BDF ($p = 0.001$), MD% ($p = 0.001$), MC% ($p = 0.008$), and $Slope_{AC}$ ($p < 0.001$) were identified between the two study groups (Table 8.2). FH positive individuals exhibited significantly lower BDF, MD%, and $Slope_{AC}$ and increased MC% compared to their counterpart healthy controls (Figure 8.1A). No significant group differences in any of the other measured arterial DVA parameters were identified (all $p > 0.05$).

8.4.4.2. Venous response

After controlling for influential covariates in ANCOVA models as appropriate, group comparisons showed statistically lower venous BCFR ($p = 0.009$) and dilation response $Slope_{VD}$ ($p = 0.010$) in the FH positive group in comparison to controls (Table 8.2; Figure 8.1B). No significant group differences in any of the other measured venous DVA parameters were identified (all $p > 0.05$).

Table 8.1. Summary of group differences in clinical and systemic vascular parameters

Variable	Mean (SD)		*p*-value	Significance
	FH positive (1)	Control (2)		
N	38	37	0.909	-
Gender	20M : 18F	22M : 15F	0.551	-
Age (years)	47 (11)	45 (11)	0.468	-
BMI (kg/m²)	27.13 (4.45)	26.75 (4.72)	0.727	-
SBP (mmHg)	121 (11)	121 (12)	0.752	-
DBP (mmHg)	74 (9)	76 (9)	0.227	-
HR (bpm)	65 (10)	68 (8)	0.096	-
MAP	89.36 (8.90)	91.29 (9.05)	0.356	-
IOP (mmHg)	14 (2)	14 (3)	0.439	-
OPP	45.15 (6.36)	46.91 (5.54)	0.206	-
GLUC (mmol/L)	4.95 (1.07)	5.08 (0.58)	0.539	-
TG (mmol/L)	1.14 (0.44)	1.15 (0.62)	0.958	-
CHOL (mmol/L)	4.68 (1.02)	4.55 (0.98)	0.578	-
HDL-C (mmol/L)	1.16 (0.41)*	1.39 (0.45)	**0.030***	1 < 2
LDL-C (mmol/L)	3.00 (0.97)	2.67 (0.92)	0.156	-
TG/HDL-c	1.11 (0.56)	1.00 (0.84)	0.515	-
CHOL/HDL-c	4.39 (1.36)*	3.61 (1.36)	**0.021***	1 > 2
FRS %	5.91 (4.16)	4.85 (3.78)	0.105	-
c-IMT	0.55 (0.11)	0.58 (0.11)	0.318	-
$AD_{baseline}$ (mm)	4.16 (0.93)	4.44 (0.88)	0.174	-
$MD_{hyperaemia}$ (mm)	4.61 (1.12)	5.14 (1.26)	0.060	-
FMD_{ED}	10.88 (9.42)	12.13 (8.63)	0.520	-

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; IOP, intraocular pressure; OPP, ocular perfusion pressure; GLUC, glucose; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; FRS%, Framingham risk score; c-IMT, carotid intima media thickness; $AD_{baseline}$, brachial baseline diameter; $MD_{hyperaemia}$, maximum brachial diameter during hyperaemia; FMD_{ED}, endothelium-dependent flow-mediated dilation. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 8.2. Summary of group differences in retinal vascular function parameters

DVA parameter	Mean (SD)		*p*-value (*t*-test/ANCOVA)	Significance
	FH positive (1)	Control (2)		
Arteries:				
Baseline	100.02 (0.05)	100.01 (0.06)	0.552	-
BDF	5.13 (2.10)	6.50 (2.88)	**0.001***	1 < 2
BCFR	1.04 (2.00)	1.12 (3.07)	0.882	-
DA	6.16 (2.67)	7.51 (2.76)	0.015	-
MD%	3.74 (2.25)	5.22 (2.50)	**0.001***	1 < 2
MC%	-2.42 (1.87)	-2.29 (2.31)	**0.008***	1 > 2
tMD (seconds)	19 (6)	22 (8)	0.048	-
tMC(seconds)	29 (6)	26 (8)	0.064	-
$Slope_{AD}$	0.24 (0.12)	0.30 (0.15)	0.070	-
$Slope_{AC}$	-0.25 (0.13)	-0.38 (0.25)	**<0.001***	1 < 2
Veins:				
Baseline	100.00 (0.04)	100.00 (0.03)	0.775	-
BDF	3.94 (1.78)	3.79 (1.68)	0.011	-
BCFR	1.40 (2.42)	2.35 (1.83)	**0.009***	1 < 2
DA	5.33 (2.91)	6.09 (2.60)	0.242	-
MD%	4.44 (2.74)	5.43 (2.38)	0.045	-
MC%	-0.90 (1.59)	-0.66 (1.04)	0.461	-
tMD (seconds)	21 (6)	20 (4)	0.200	-
tMC (seconds)	28 (7)	31 (8)	0.058	-
$Slope_{VD}$	0.23 (0.13)	0.29 (0.12)	**0.010***	1 < 2
$Slope_{VC}$	-0.23 (0.16)	-0.23 (0.14)	0.960	-

Abbreviations: Baseline, average baseline diameter: BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percent dilation (during flicker); MC%, percent constriction (post-flicker); tMD, time taken to reach maximal dilation (MD) diameter; tMC, time taken to reach maximal constriction (MC) diameter; $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction. *Significant *p*-values are indicated in bold where $p < 0.01$ was considered significant.

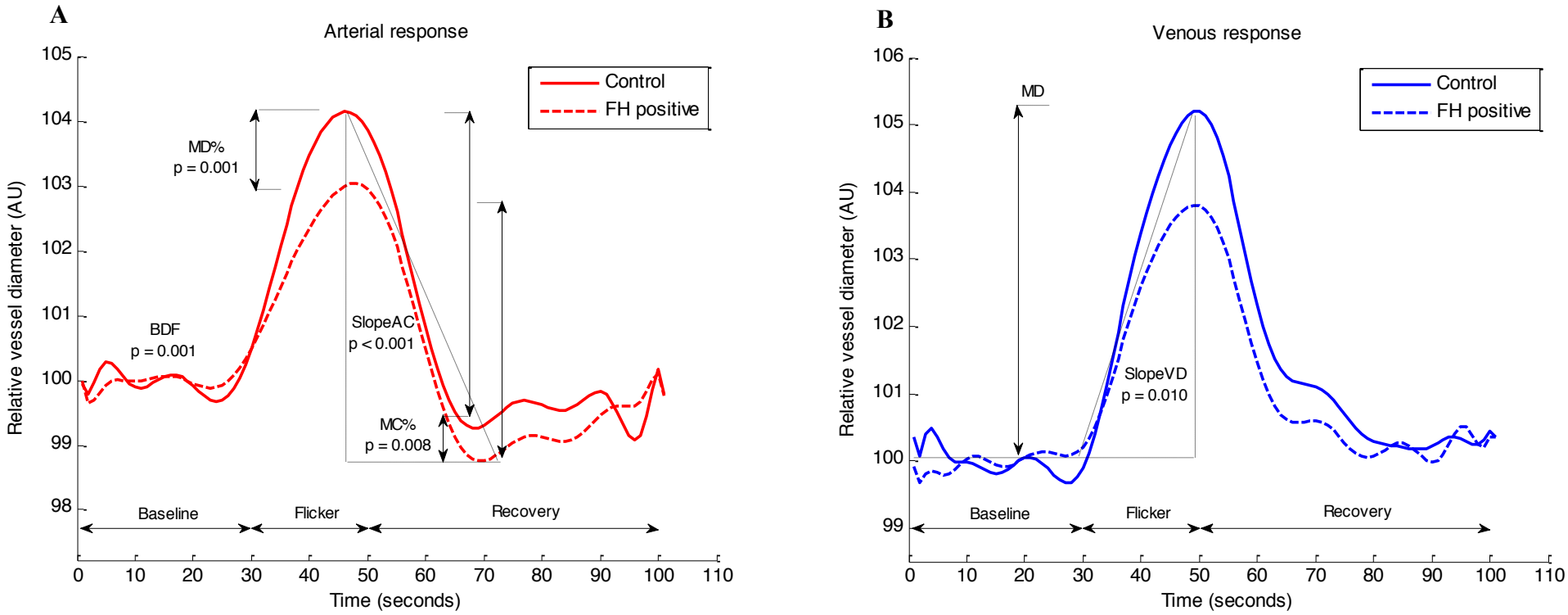


Figure 8.1. Comparisons of retinal vascular response profiles.
(A) arterial response; (B) venous response.
Abbreviations: AU, arbitrary units; BDF, baseline diameter fluctuation; DA, dilation amplitude; FH, family history; MD, maximal dilation diameter; MD%, percent dilation; MC%, percent constriction; $Slope_{AC}$, arterial constriction slope; $Slope_{VD}$, venous dilation slope.

8.4.4.3. *Correlations between retinal and systemic parameters*

In the FH positive group increased arterial MC% was associated with decreased HDL-c (r = -0.52, p = 0.002, Figure 8.2), however, a similar trend was not observed in the control group ($p > 0.05$). No other significant within-group correlations were identified ($p > 0.05$).

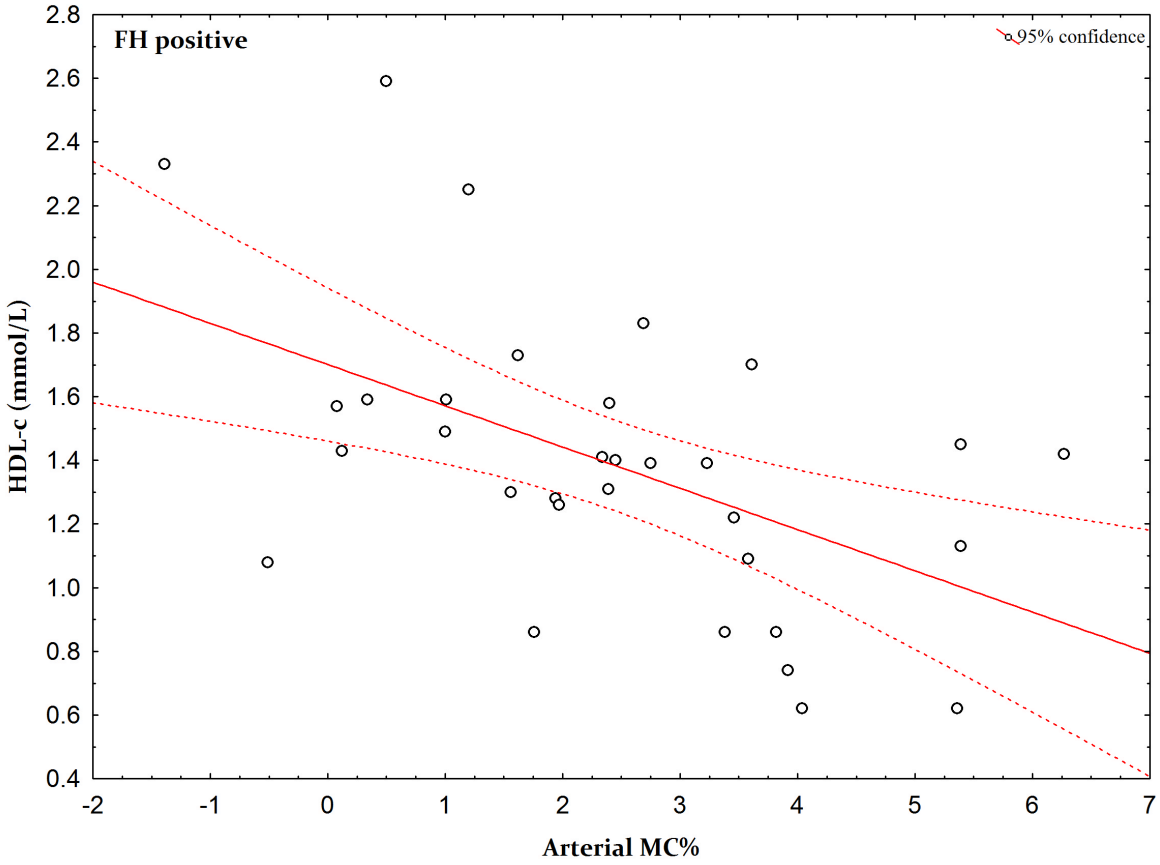


Figure 8.2. Correlation between HDL-c and arterial MC% in the FH positive group.
Abbreviations: FH, Family history; HDL-c, high-density lipoprotein cholesterol; MC%, percent constriction.

8.5. Discussion

In the present study, it has been shown that otherwise healthy individuals with a FH of CVD could still present with early detectable signs of vascular dysfunction at the retinal microvascular level. This was evident as normal c-IMT scores and FMD responses but attenuated retinal vascular reactivity in response to flicker-stimulation in the FH positive group.

The importance of detecting when the first signs of vascular dysfunction occur in at-risk individuals is well established. Other studies have reported peripheral microvascular changes, such as reduced coronary flow [452] and skin reactive hyperaemia in subjects with FH of hypertension [453] and diabetes [454], showing that microvascular abnormalities are present and detectable in individuals who are 'at-risk' for CVD. Furthermore, known vascular risk factors such as dyslipidaemia [455], and elevated blood pressure [456] tend to aggregate in families but whether this is due to a genetic determinant or the presence of endothelial dysfunction is not fully understood. Indeed, endothelial dysfunction is not only a consequence of vascular disease, but also a significant contributor to the development of vascular disorders. Likewise, based on the findings of the present study FH in itself appears to be an independent vascular risk factor and poor microvessel function appears to be a requisite inherited phenotype for cardiovascular risk in patients with familial risk factors [74-79].

In the present study, functional irregularities in the retinal response to flicker were identified as decreased arterial and venous dilation responses and post-flicker arterial over-constrictions in FH positive subjects. No structural or functional changes were however identified in the systemic vessels. It is known that the microcirculation represents the first vascular area to be affected by pre-clinical signs of endothelial dysfunction [457-460] and early vascular disturbances could be expected to occur in the microvessels before they can be detected in larger vessels. The presence of a functional correlation between brachial (FMD) and retinal arterial function (arterial dilation) has been described previously [300], though this was not corroborated in the present study. It is however known that micro- and macrovascular functions are governed by different physiological mechanisms and early vascular dysfunctions occurring at multiple vascular levels need not occur simultaneously. The absence of confounding vascular risk factors such as hypertension, dyslipidaemia, and diabetes in the present study sample may also offer some explanation as to lack of detectable differences in macrovascular parameters. Nevertheless, functional endothelial assessment at the microcirculatory and macrocirculatory levels is important to evaluate as

dysfunctions can be diffuse, and the temporal relationship between risk factors and the initiation and progression of vascular dysfunctions can differ in FH positive individuals with varying risk factor profiles. Further work to assess the relationships between distinct vascular beds would also help provide a better clinical picture as to whether generalized 'vascular breakdown' corresponds with a higher risk for cardiovascular complications. Nevertheless, the subtle but appreciable functional retinal irregularities identified in this study indicate that retinal vessel assessment could become an important investigative tool in everyday clinical practice for early vascular risk detection. Moreover, in comparison to FMD, retinal vascular function can be examined quickly and non-invasively.

A further understanding of the complex relationships between vascular dysfunctions at multiple levels, as well as, their interaction with metabolic, lifestyle, and genetic factors in subjects at increased risk could also support early interventions for the prevention of CVD. Likewise, in the present study reduced retinal vascular function in FH positive subjects implicates local endothelial dysfunctions, and the correlation identified between HDL-c levels and attenuated retinal vascular function is consistent with what has previously been reported at the macrovascular level in those with familial risk factors [461]. Moreover, improvements in functional retinal responses to flicker with HDL-c restoration have previously been explained by interactions between lipid levels and the bioavailability of NO [462], and studies in our lab have also shown a measurable reduction in retinal vascular function that correlates with oxidative stress status [310, 463] (Chapter 10) and plasma markers for cardiovascular risk (LDL-c) [10]. NO contributes to the regulation retinal vessel tone [254] and endothelial dysfunction due an abnormal secretion or action of NO is an early feature of vascular disease. Nevertheless, in order to adequately clarify the mechanisms underlying functional retinal vascular impairments attributed to familial risk further considerations of associated co-morbidities are still needed.

Age is an important vascular risk factor [48-50], and FH positive individuals are at an increased risk for disease onset at an earlier age [464]. Based on the ageing effects on retinal vascular function reported in Chapter 7, retinal arterial diameters could be expected to reach a similar dilation diameter during flicker and a baseline value that corresponds with a larger post-stimulation diameter as reported in middle-age individuals, whereas middle-age FH positive subjects of the present study exhibit decreased arterial dilation responses during flicker, and arterial over-constrictions post-flicker compared to age-matched controls. Similar deviations in retinal arterial behaviour that correspond with reduced vasodilation and increased vasoconstrictions are, however, reported in other high-risk

groups such as patients with diabetes [281] and obesity [262], and a reduced capacity to respond to changes in perfusion pressure has been interpreted as an increase in vascular rigidity of retinal arteries. Likewise, in FH positive subjects spontaneous variations in baseline diameters before the onset of flicker (BDF) were reduced when compared to controls. The exact nature of arterial over-constrictions in FH positive individuals during the re-establishment of baseline diameter needs further clarification. However, the presence of an adaptive mechanism that enables the vessel to adjust baseline diameters, in keeping with metabolic demand and forecasting a need for dilation, has been proposed to exist in healthy retinal vessels [256] [140] that may subsequently be a feature that is absent or offset in dysfunctional vessels [262]. Also of note in FH positive subjects of the present study, was a decreased retinal venous dilation response slope – a composite parameter that could reflect a decrease in retinal venous dilation diameter and or a delay in reaching maximal dilation diameter. Retinal veins are however thought to be involved in more passive autoregulatory functions [256], such as the refinement of active reactions evoked on the arterial side. There also remain particular inconsistencies concerning venous responses with some studies showing less pronounced venous dilations as part of the normal vascular response profile [257], and or no significant differences between normal arterial and venous responses [294]. However, structural changes in arterial and venous caliber have been proposed to independently reflect varied pathophysiological processes [358], for instance, the significant associations between wider retinal veins and increased risk for stroke [465-467]. In patients with established disease, reduced arterial compliance is linked with wider retinal venous caliber [468], which is in turn associated with impaired adaptive retinal regulation [318]. In light of these studies, it could be speculated that specific attenuations in retinal vascular function parameters could signal key vascular alterations associated with the presence of non-modifiable risk factors such as ageing and FH, and further investigation is still required to understand the relative importance of attenuated venous responses in FH positive individuals. A potential study limitation, however, is that the age at disease onset of the first-degree relative of the participants included in this study was not recorded. Nevertheless, the results of the present study indicate that familial risk is associated with alterations in retinal vascular function.

8.6. Conclusion

With traditional cardiovascular risk scoring systems such as the FRS known to severely underestimate relative risk [413, 414], the findings of the present study suggest that functional retinal assessments provide an improved diagnostic capability for the detection of

subclinical microvascular dysfunction. Although larger prospective studies are needed to validate the data analysis approach used in this study, the quantitative parameters and time course of the temporal vessel response characterize vessel behaviour during and after the cessation of stress. This approach could be promising in the diagnosis of vascular dysfunction in established disease as well as in furthering the concept of early vascular screening and prevention strategies in at-risk groups - an important step towards the development of individualized and targeted endothelial therapies.

9. Study 3: Ethnic differences in retinal vascular function

9.1. Abstract

Purpose: To investigate ethnic differences in retinal vascular function and their relationship to traditional risk indicators for CVD. The present study was undertaken to include a middle-aged cohort (35 – 55 years) of otherwise healthy migrant UK South Asians (SAs) and age- and gender-matched White-Europeans (WEs).

Methods: Retinal vascular function was assessed by way of DVA in 126 healthy SAs (n = 61) and WEs (n = 65). Other clinical assessments in all subjects included, BMI, BP and IOP profiles, fasting GLUC, TG, total, HDL, and LDL cholesterol, as well as, circulating oxidative stress markers (GSH, GSSG, and redox index). In addition, an ethnicity-based modification of the FRS, specific to British ethnic and minority groups (ETHRISK), was used to re-calculate a relative cardiovascular risk score.

Results: In comparison to WEs, SAs exhibited higher TG ($p < 0.001$) and lower HDL-c ($p < 0.001$) levels resulting in higher TG/HDL-c ($p < 0.001$) and CHOL/HDL-c ratios ($p < 0.001$). The overall cardiovascular risk was estimated to be low (>10%) in both groups, however, ETHRISK was significantly higher in SAs ($p = 0.012$). In addition, SAs had higher circulating GSSG levels ($p = 0.002$), and lower redox index ($p = 0.025$). With regards to retinal vascular function parameters, SAs exhibited an overall reduction in the arterial baseline corrected flicker response (BCFR, $p = 0.002$) in comparison to WEs. Additionally, a significant decrease in retinal arterial dilation (MD%) in SAs ($p = 0.007$) was associated with increased CHOL/HDL-c ($r = -0.28$, $p = 0.026$) and the subsequent delay in the re-establishment of post-flicker baseline diameter (tMC, $p = 0.003$) was associated with redox index ($r = -0.37$, $p = 0.003$). Moreover, SAs also exhibited significant post-flicker over-constrictions (MC%) in retinal arteries and veins ($p < 0.001$; $p = 0.002$, respectively).

Conclusion: In otherwise healthy middle-aged SAs, with low estimated cardiovascular risk, there are signs of impaired retinal vascular function that correlate with systemic antioxidant stress status and established plasma markers for cardiovascular risk.

9.2. Introduction

People of South Asian (SA) descent (tracing ancestry to India, Sri Lanka, Pakistan, and Bangladesh) constitute one of the largest minority populations in the UK. Studies have shown that migrant SAs in the UK are not only prone to vascular pathologies such as metabolic syndrome [469, 470], obesity and dyslipidaemia [471], insulin resistance [472, 473], and type II diabetes [474], but that they also tend to have a 50% higher risk of developing CVD than their White European (WE) counterparts [475]. A more recent meta-analysis study indicates that increased cardiovascular-related mortality rates in SAs are due to a higher incidence rather than worse prognosis [476]. Consequently, strategies for early screening and primary prevention in this community are therefore essential.

Although conventional cardiovascular variables confer risk in SAs as in other populations [477], they still do not adequately explain excess risk in this group [80, 478-480]. On the other hand, endothelial dysfunction is regarded as key biological mechanism by which cardiovascular risk factors exert their propensity for vascular pathology, and when considering risk stratification measures, pre-clinical modifications in vascular function that signal risk before the clinical manifestation of CVD, could facilitate more effective preventative approaches in at-risk or high-risk groups. Indeed, known markers of atherosclerosis and vascular disease (inflammation and endothelial dysfunction) can be present in SAs as early as childhood [481], and in comparison to more conventional screening methods, show promise in identifying vascular irregularities in the presence of more subtle cardiovascular risk factors.

Even apparently healthy SAs exhibit features of attenuated endothelial function in the macrocirculation [83]. However, as microvascular dysfunctions are thought to occur much earlier in the course of disease progression [460], their assessment may be equally important for disease screening and prevention. In addition, physiological stressors that incite alterations in blood flow may trigger vascular responses that are comparable across vascular categories and therefore microcirculatory assessments could provide an indirect measure of the macrocirculation [482]. Fortunately, normal endothelial function can be restored, and as more aggressive preventive measures and treatments have already been proposed in SAs [483], functional microvascular assessments, with the possibility of detecting early endothelial dysfunction, could facilitate the implementation of primary preventive measures that might reduce the occurrence and/or slow progression of vascular disease in SAs, as well as, identify non-responders to targeted therapies.

The retina, being vulnerable to alterations in blood flow regulation, is also particularly well-suited for non-invasive study of the microcirculation, and functional retinal assessments have already been endorsed as a useful tool for identifying pre-clinical vascular dysfunctions [271] [140, 213]. Moreover, previous studies have further characterized functional retinal behaviour in obesity [262], pre-diabetes [310] and type II diabetes [329], all of which are conditions that SAs are particularly prone to develop.

More recently, our laboratory identified ethnic differences in retinal microvascular function [309] but in a younger sample of SAs. The aim of the present study was therefore to confirm these findings in a middle-aged sample of SAs and WEs and include the evaluation of additional retinal vascular function parameters. In addition to the regular description of the dilatory retinal response to flicker [281, 344], the ensuing re-establishment of baseline diameter and time course of vascular reactivity [256, 257] suggest that the form of the dynamic response curve and vessel behaviour can vary depending on vessel type and disease [258, 319]. A more detailed evaluation of retinal vascular function parameters, using the specific data analysis and visualization model described in this thesis (section 6.3.4.8), is an approach that has successfully been applied by our laboratory to study retinal vascular function parameters in other pathologies [320, 368], as well as, in the studies described in the preceding chapters of this thesis. This present study therefore sought also to provide comparably complex considerations in the descriptions and interpretation of retinal vascular function parameters in SAs and WEs. In parallel, it is known that endothelial dysfunction is commonly associated with oxidative stress, which has in turn been linked to high-fat diets in SAs [484]. On this basis, the investigation of the relationship between retinal vascular function and established risk indicators for CVD in SAs and WEs also included measures of systemic oxidative stress status.

9.3. Methods

9.3.1. Study participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK) and selected from a laboratory database of individuals considered for previous case-control studies. The study specific inclusion criteria were as those defined in section 6.2.1 and only individuals over the age of 35 years and of WE or SA descent that fit the defined inclusion criteria were selected for the final analysis.

9.3.2. General assessments

Ethnicity was confirmed through self-report questionnaire, and other general clinical assessments for all participants, as detailed in section 6.3.2, included a general health history review, BMI, BP and IOP profiles, and circulatory markers including GLUC, TG, CHOL, HDL-c, LDL-c (section 6.3.2.5), as well as, GSH and GSSG by way of glutathione recycling assay (section 6.3.6.2). In addition to FRS calculations, an ethnicity-based modification of the FRS (ETHRISK®) [27] specific to British ethnic and minority groups was used to re-estimate risk scores in this study.

9.3.3. Vascular assessment

Retinal vessel reactivity was measured with the RVA system as described in section 6.3.4. All measurements were performed in a quiet, temperature-controlled room (22^{o}C) following full dilation of one unselected eye with 1% Tropicamide (Chauvin Pharmaceuticals Ltd). The statistical polynomial regression algorithm (section 6.3.4.8) was applied to the raw response data to explore the entire functional dynamic response curve and the following parameters were then determined for each flicker cycle and averaged across flicker cycles, with the artery and vein regarded separately: baseline diameter, BDF, DA, BCFR, MD%, MC%, tMD, tMC, as well as, dilation and constriction slopes for both the arteries ($Slope_{AD}$ and $Slope_{AC}$) and veins ($Slope_{VD}$ and $Slope_{VC}$).

9.3.4. Sample size calculations

Based on previous studies, normal expected retinal arterial responses to flicker-light stimulation have been around 6.9 ± 2.8 % [256, 268] and a change of 30% with a SD of 2.5 % in retinal vessel reactivity has been shown to be significant [316, 485]. Additionally, in our previous lab study a difference of approximately 20% in retinal vessel reaction times (tMD) was found between WEs and SAs. Since it was determined that *t*-tests or ANCOVA would be required in this study in order to provide 95% power with an alpha-level of 0.05 and with a SD of 2.5% a total sample size of n = 84 was recommended. Sample size calculations were performed using the G*Power software [428] (University of Kiel, Version 3.1.6, Germany).

9.3.5. Statistical analysis

All statistical analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). Distributions of continuous variables were determined by the Shapiro-Wilks test. Multivariate analysis was performed to test the influence of age, BMI, BP, and circulating

markers on the measured variables. Group differences in mean values for each of the measured variables were compared using independent samples *t*-tests or ANCOVA as appropriate. Within-group univariate associations were evaluated using the Pearson correlation method. A *p*-value of less than 0.05 was considered significant except in certain cases where a stricter *p*-value of less than 0.01 was adopted to control for multiple comparisons.

9.4. Results

9.4.1. Clinical characteristics

Of the 173 individuals screened for inclusion, 47 individuals were excluded from the final analysis if they were below the age of 35 (since the ETHRISK is only validated in those above 35 years of age), or if the quality of the DVA recording was poor. The general demographics of the remaining 126 individuals included in the study (SAs: $n = 61$; WEs: $n = 65$) are summarized in Table 9.1. The distribution of male and female subjects was similar (53% male, Chi-square $p = 0.391$), as well as, within groups (SA: 67% male, WE: 57% male, Chi-square $p = 0.238$). There were no significant differences in age, BMI, SBP, DBP, HR, MAP, IOP, OPP, GLUC, CHOL, LDL-c, GSH, tGSH, and FRS, between groups (all $p > 0.05$). The SA group however, exhibited higher TG ($p < 0.001$), TG/HDL-c ($p < 0.001$), CHOL/HDL-c ($p < 0.001$), GSSG ($p = 0.002$), and ETHRISK ($p = 0.012$), and lower HDL-c ($p < 0.001$) and redox index ($p = 0.025$) compared to WEs.

9.4.2. Retinal vascular function

All values reported are based on averaged DVA data across three flicker cycles with the artery and vein regarded separately. After controlling for influential covariates in ANCOVA models as appropriate, statistically significant differences in BCFR ($p = 0.002$), MD% ($p = 0.007$), MC% ($p < 0.001$) and tMC ($p = 0.003$) were identified between the two study groups. SAs exhibited significantly lower BCFR and MD%, and increased MC% and tMC compared to counterpart healthy WEs (Table 9.2, Figure 9.1A). No significant group differences in any of the other measured arterial DVA parameters were identified (all $p > 0.01$). On the venous side, group comparisons also showed significantly increased MC% ($p = 0.002$) in SAs compared to WEs (Table 9.2, Figure 9.1B). No significant group differences in any of the other measured venous DVA parameters were identified (all $p > 0.01$).

Table 9.1. Summary of clinical data

Variable	Mean (SD)		*p*-value	Significance
	SAs (1) *n* = 61	**WEs (2)** *n* = 65		
Age (years)	45 (7)	47 (8)	0.351	-
BMI (kg/m²)	26.05 (3.92)	24.90 (3.90)	0.100	-
SBP (mmHg)	119 (13)	119 (11)	0.913	-
DBP (mmHg)	75 (11)	74 (10)	0.624	-
HR (bpm)	68 (10)	68 (8)	0.823	-
MAP	89.19 (11.24)	88.67 (9.27)	0.775	-
IOP (mmHg)	13 (3)	13 (3)	0.733	-
OPP	46.02 (6.59)	45.83 (6.54)	0.875	-
GLUC (mmol/L)	4.89 (0.77)	4.93 (0.69)	0.725	-
TG (mmol/L)	1.31 (0.64)	1.00 (0.34)	**< 0.001**	1 > 2
CHOL (mmol/L)	4.51 (0.85)	4.40 (0.89)	0.481	-
HDL-c (mmol/L)	1.19 (0.44)	1.47 (0.44)	**< 0.001**	1 < 2
LDL-c (mmol/L)	2.71 (0.81)	2.47 (0.86)	0.110	-
TG/ HDL-c	1.30 (0.86)	0.77 (0.46)	**<0.001**	1 > 2
CHOL/HDL-c	4.22 (1.46)	3.22 (1.04)	**<0.001**	1 > 2
tGSH (µmol/L)	1030 (642)	973 (676)	0.635	-
GSH (µmol/L)	814 (584)	850 (621)	0.743	-
GSSG (µmol/L)	108 (103)	62 (51)	**0.002**	1 > 2
Redox index	14 (12)	20 (17)	**0.025**	1 < 2
FRS (%)	3.18 (3.40)	2.87 (2.55)	0.571	-
ETHRISK (%)	6.79 (6.10)	3.97 (2.50)	**0.012**	1 > 2

Data are presented as mean (SD). Abbreviations: SA, South Asians; WE, white Europeans; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; IOP, intraocular pressure; OPP, ocular perfusion pressure; GLUC, fasting glucose; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; tGSH, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; redox index, ratio of GSH:GSSG, FRS, Framingham risk score; ETHRISK, ethnicity-based modification of FRS. $p < 0.05$ was considered significant.

Table 9.2. Summary of group differences in retinal vascular function parameters

DVA parameter	Mean (SD)		*p*-value	Significance
	SAs (1)	WEs (2)		
Arteries:				
Baseline	99.89 (0.06)	99.92 (0.56)	0.025	-
BDF	5.73 (2.94)	5.69 (2.61)	0.028	-
BCFR	0.75 (2.92)	1.29 (2.67)	**0.002***	1 < 2
DA	6.48 (2.85)	6.84 (3.37)	0.518	-
MD%	4.01 (2.49)	4.36 (2.68)	**0.007***	1 < 2
MC%	-2.47 (1.55)	-2.10 (1.87)	**<0.001***	1 > 2
tMD (seconds)	22 (9)	20 (8)	0.139	-
tMC(seconds)	28 (8)	25 (9)	**0.003***	1 > 2
$Slope_{AD}$	0.24 (0.15)	0.26 (0.20)	0.037	-
$Slope_{AC}$	-0.37 (0.30)	-0.36 (0.48)	0.091	-
Veins:				
Baseline	100.00 (0.04)	99.90 (0.71)	0.013	-
BDF	4.48 (2.48)	4.08 (1.75)	0.299	-
BCFR	1.50 (2.41)	1.26 (2.16)	0.560	-
DA	5.92 (3.14)	5.32 (2.57)	0.244	-
MD%	4.71 (2.58)	4.43 (2.45)	0.529	-
MC%	-1.21 (1.34)	-0.93 (1.36)	**0.002***	1 > 2
tMD (seconds)	21 (7)	22 (7)	0.767	-
tMC (seconds)	29 (8)	29 (8)	0.922	-
$Slope_{VD}$	0.27 (0.15)	0.28 (0.25)	0.025	-
$Slope_{VC}$	-0.24 (0.17)	-0.22 (0.13)	0.055	-

Abbreviations: Baseline, average baseline diameter: BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline corrected flicker response; MD%, percent dilation (during flicker); MC%, percent constriction (post-flicker); tMD, time taken to reach maximal dilation (MD) diameter; tMC, time taken to reach maximal constriction (MC) diameter; $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction. *Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

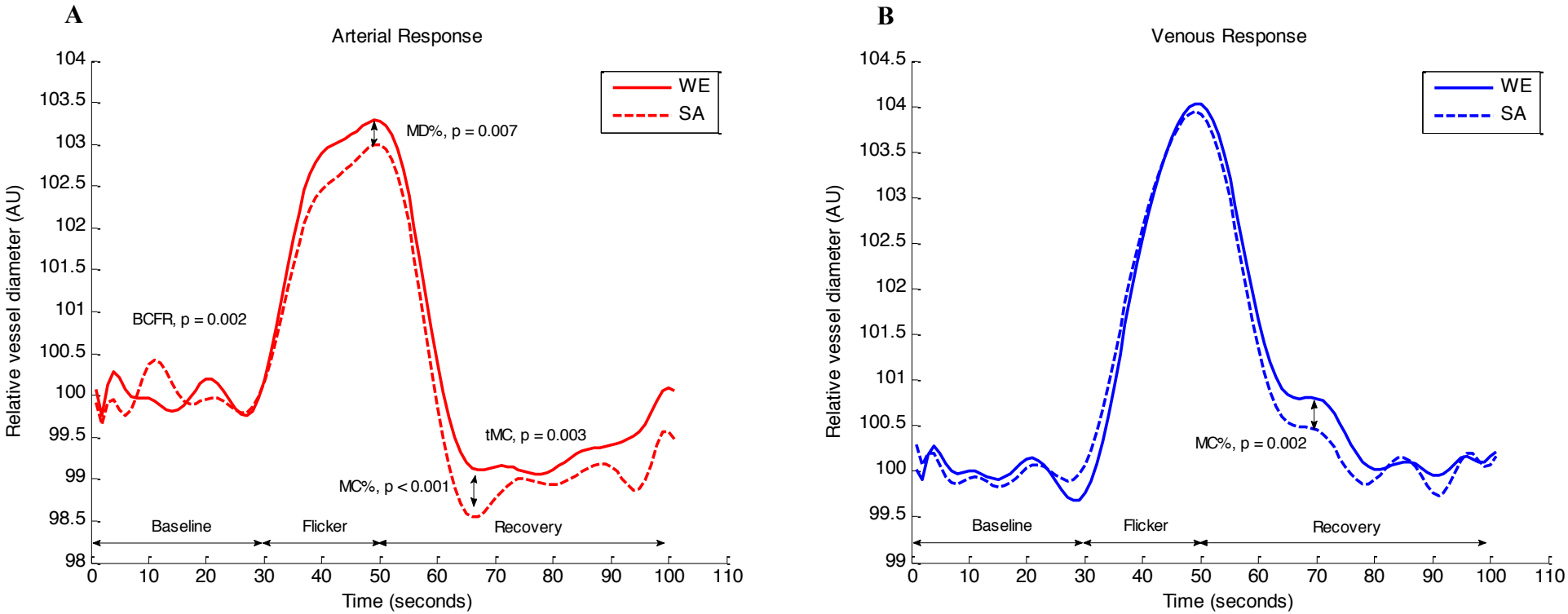


Figure 9.1. Retinal arterial and venous response profiles across groups
(A) arterial response; (B) venous response
Abbreviations: WE, White European; SA, South Asian; BCFR, baseline corrected flicker response; MD%, percent dilation; MC%, percent constriction; tMC, time taken to reach maximu constriction diameter.

9.4.3. Within-group correlations

In within-group correlations, decreased arterial MD% was associated with increased CHOL/HDL-c (r = -0.28, p = 0.026) in SAs (Figure 9.2A). Additionally, longer arterial tMC was associated with decreased redox index (r =-0.37, p = 0.003, Figure 9.3A). Similar trends were, however, not observed in WEs (p > 0.05).

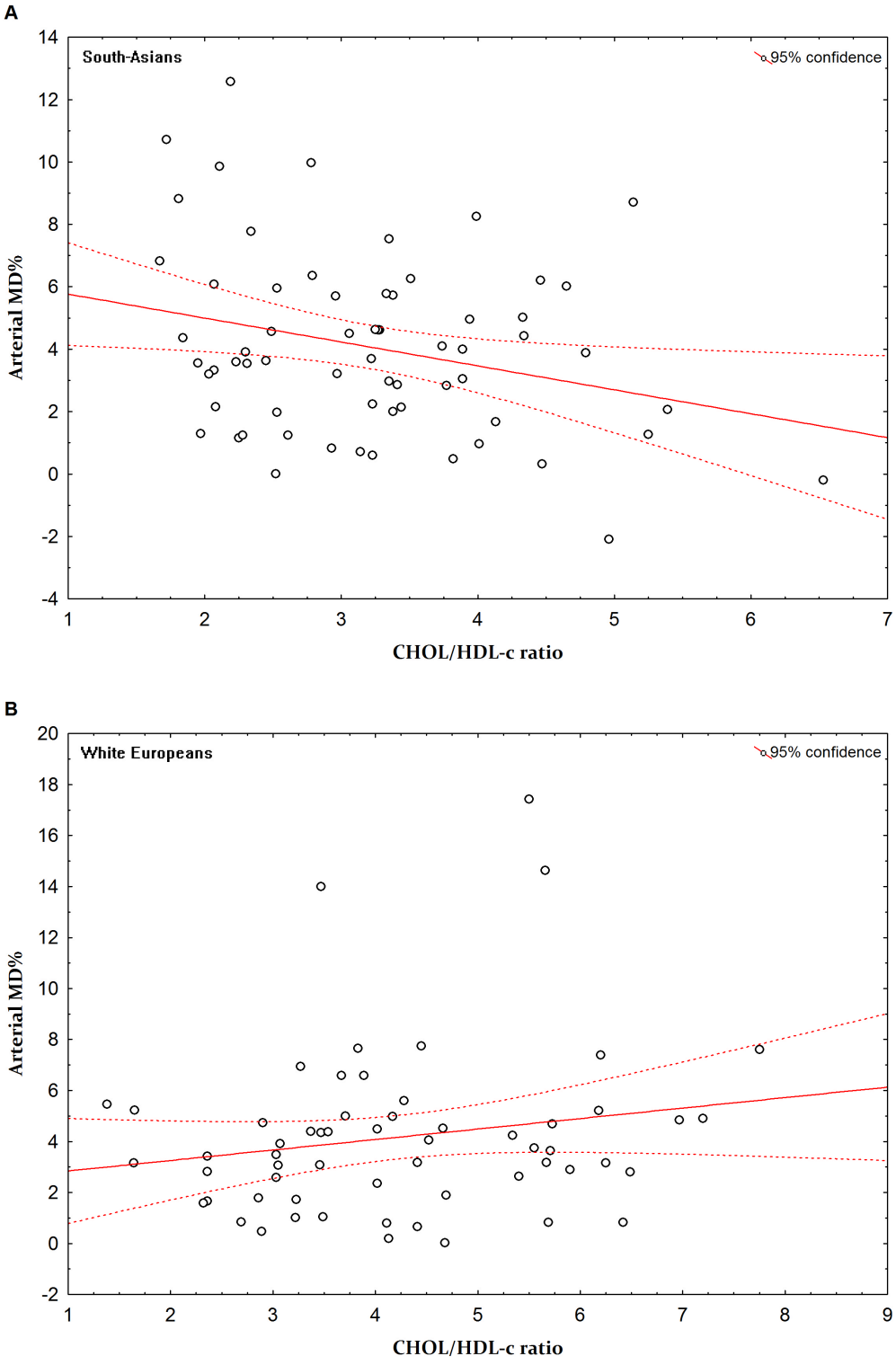


Figure 9.2. Correlations between retinal arterial dilation and lipids
Abbreviations: MD%, percent dilation; CHOL, total cholesterol, HDL-c, high-density lipoprotein cholesterol.

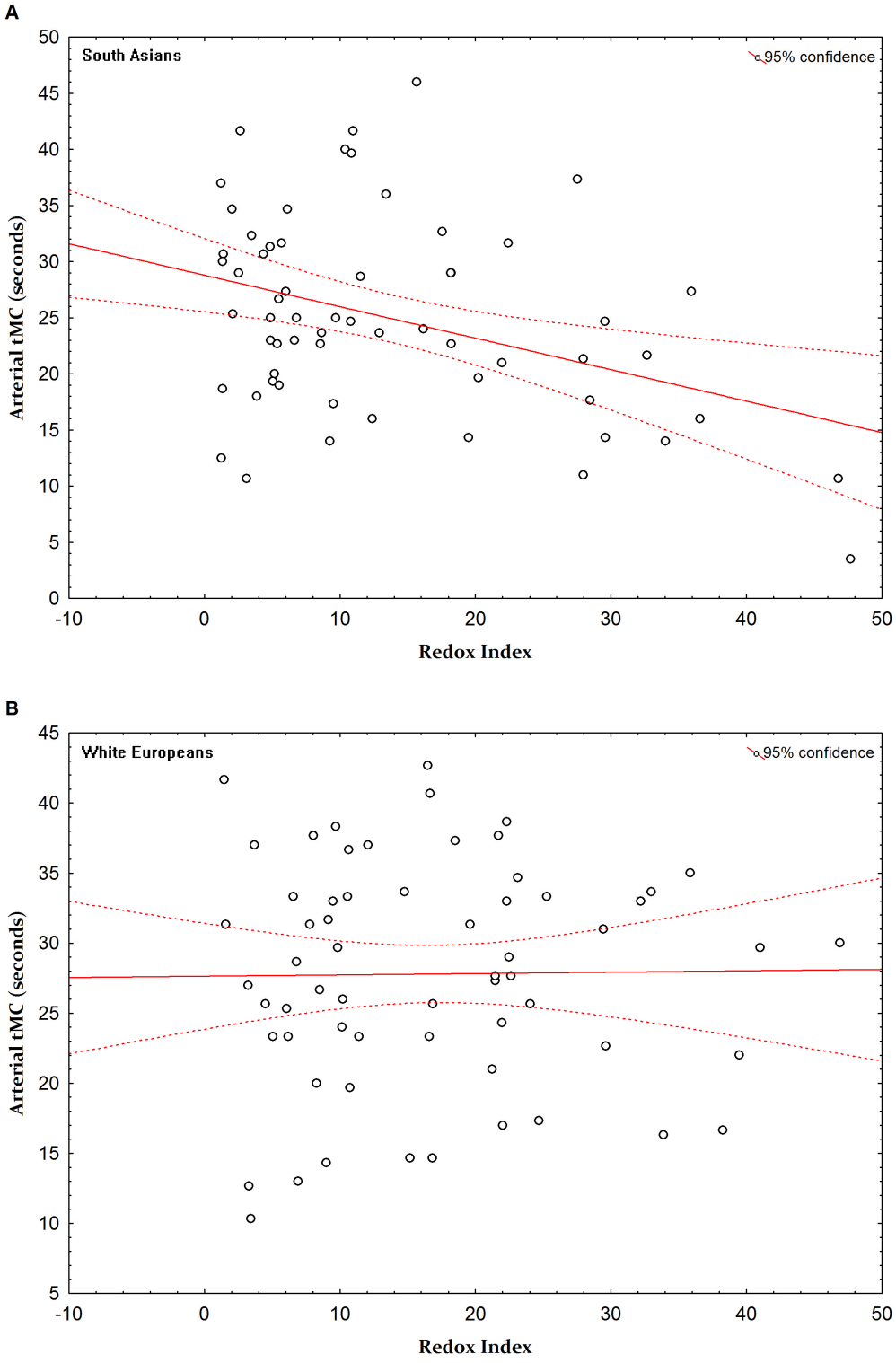


Figure 9.3. Correlations between retinal arterial constriction time and redox index
Abbreviations: tMC, time take to reach maximum constriction diameter, redox index; ratio of reduced (GSH) to oxidized (GSSG) glutathione.

9.5. Discussion

The findings in this study show that in comparison to age- and gender-matched WEs, healthy SAs present with an early risk for vascular disease as evidenced by increased ethnicity-adjusted cardiovascular risk scores, and selective impairments in retinal vascular function that were consistent with a clinical pattern of early dyslipidaemia and oxidative stress. Using a specific computational model to evaluate the entire dynamic retinal vessel profile, this study was also able to corroborate previous findings in SAs, and identify additional irregularities in specific retinal vascular function parameters including; reductions in arterial dilation responses to stress, delays in the re-establishment of baseline diameter, and post-flicker arterial and venous over-constrictions during the cessation of stress.

Previous studies have demonstrated that SAs exhibit impaired vascular function in the peripheral circulations [83, 484, 486], however, microvascular dysfunctions can precede those occurring in larger vessels, and consequently vascular reactivity tests that enable the assessment of microvascular endothelial function are of increased clinical value [487]. Functional retinal assessments such as DVA allow dynamic evaluations of vessel behaviour in response to provocative stressors – analogous to how functional vessel behaviour is assessed in large arteries. Indeed, studies evaluating flicker-evoked retinal responses have reported vascular impairments in patients with known cardiovascular risk factors including diabetes [296, 316, 318, 344], smoking [260, 273], obesity [262] and hypertension [258, 264]; thereby demonstrating the utility of DVA as an adequate clinical tool for evaluating altered vessel functionality in vascular diseases. More recently, a previous study using the same method reported features of altered retinal microvascular function in younger healthy SAs that also signal an early risk for vascular pathology [309]. The findings of the present study, comprising an older sample of SAs, substantiate these earlier reported differences, and identify additional irregularities in the dilation and constriction components of retinal vessel behaviour that could be attributed to a more detailed analysis of vascular function parameters, as well as, the age range of the participants selected. While the exact aetiology of retinal vascular disturbances in SAs remains to be clarified, possible contenders are structural vascular changes, abnormal lipid profiles, and oxidative stress, all of which are associated with endothelial dysfunction or alterations in vasoactive mediators such reductions in the bioavailability of NO or an exhaustion of NO reserves.

Consistent with earlier reports, the current sample of SAs exhibited decreased HDL-c and elevated TG levels. A growing number of studies show anomalies in classic lipid profiles

such as concentrations of smaller and less protective HDL-c particle sizes [488] even in SAs with normal HDL-c levels [489], and decreases in HDL-c have in turn been linked with endothelial dysfunction [490], and a reduction in the bioavailability of NO [491]. Moreover, SAs are susceptible to hypertriglyceridemia-induced endothelial injury at an earlier age [492]. In the present study it was considered that abnormal lipid profiles were likely to have an influence on retinal vascular function, possibly implicating a common progression that involves both the macro- and microcirculations. Indeed, reduced arterial dilations corresponded with increased CHOL/HDL-c ratios, which has previously been specified as a better indicator of premature CVD risk in SAs than total cholesterol levels [493]. The precise mechanisms involving such changes, however, warrant further investigations. Nevertheless, the current observations support what is already known and has been proposed with regards to lowered thresholds when evaluating CVD risk in SAs. Whether this, in turn, could influence thresholds in vascular function studies is an interesting avenue for further exploration and reinforces the need for normative vascular function parameter values specific to ethnic groups. Ethnic variations in macrovessel function are indeed recognized, and it is highly interesting that similar changes are detectable at a much earlier stage at the level of the microcirculation that correlate with establish plasma markers for CVD risk in SAs.

Multiple lines of evidence also show that SAs are more vulnerable to the unfavourable effects of hyperglycaemia- and dyslipidaemia-induced oxidative stress, at lesser thresholds of glucose [494, 495] and lipids [82]. The combined effect of abnormal glutathione and circulating lipids could therefore amplify endothelial dysfunction at all vascular levels, including the retinal vessels [309, 311]. This possibility is important for screening and intervention, and may further underscore the functional retinal irregularities observed in SAs in this study. In the present study signs of retinal microcirculatory dysfunctions correlated with the redox index in SAs. The ratio of GSH to GSSG or 'redox index' is an indicator of circulatory oxidative stress status [496-498] as GSSG accumulation can result in endothelial cell apoptosis [499, 500], as well as, accelerate the vascular ageing process and risk for vascular pathologies [501]. It is possible that high levels of ROS diffusing into the microcirculation, that also seek to quench NO, result in a local overproduction of GSSG that consequently alters the balance of redox status and incites abnormal vascular reactivity [502-506]. Furthermore, the increased retinal arterial and venous over-constrictions in SAs follow what has previously been observed in habitual smokers [239], a condition associated also with oxidative stress [507]. Smokers have been shown to suffer a 'chronic vasodilation' due to an alteration in vasoactive substances [260], and oxidative stress and ROS diminish

with smoking cessation [507]. The redox-associated delays in the re-establishment of baseline diameters in SAs in this study are therefore not surprising results when considering other vascular haemodynamic findings that indicate a shift in vasomotive responses during the cessation of stress and as a result of interacting mechanisms. The contribution of some degree of local retinal microvascular stiffness [492] that is already present in our middle-aged cohort of SAs is also possible. These tentative associations implicate not just an autoregulative but biochemical involvement in the precipitation of selective retinal function impairments observed in the SA group. Nevertheless, all of the above stated hypotheses would need to be substantiated and further validation studies are still necessary.

9.6. Conclusions

The results of the present study show that in comparison to age- and gender-matched WEs, SAs may be at increased risk for vascular disease as evidenced by increased ethnicity-adjusted cardiovascular risk scores; moreover, selective impairments identified at the retinal level in this group were consistent with a clinical pattern of early dyslipidaemia and oxidative stress. Thus, the present study findings support the use of novel vascular markers that can be used as proxies, in lieu of conventional risk assessments, to detect the presence of vascular risk in at-risk populations or individuals in whom conventional definitions of risk can be a misattribution. The suggested method for functional retinal assessment has already been validated in both healthy and at-risk individuals with minor vascular pathologies [260] and represents a suitable practice for the screening and monitoring of established vascular disease. With the literature emphasising a need for cardiovascular screening strategies that can overcome the difficulties associated with screening at a population level, techniques that are simple, non-invasive, and easy to apply in clinical or primary care settings are gaining interest. In this regard, functional retinal assessment is a particularly attractive tool [216].

The interplay between retinal vascular function parameters and biochemical markers for endothelial function and oxidative stress, however, illustrates the complex nature of vascular risk in SAs in this study; and at present it is not clear whether genetic, environmental, dietary or lifestyle factors contribute to the expression of the observed irregularities. Nevertheless, the data presented in this study provide an opportunity for further work to validate whether the use of the functional retinal assessments can be beneficial for ethnic vascular screening, as well as, for monitoring therapeutic approaches and established vascular disease in multi-ethnic societies.

A publication of this work in Acta Ophthalmologica is provided in Appendix C.

10. Study 4: Systemic circulatory influences on retinal vascular function

10.1. Abstract

Purpose: To investigate the relationship between retinal microvascular function and circulatory markers for CVD risk and systemic anti-oxidative defence capacity in healthy middle-age individuals with low to moderate cardiovascular risk**.**

Methods: Retinal vascular function was assessed in 102 healthy, middle-age participants by way of DVA. General assessments in this study included BMI, BP, and IOP profiles, lipid levels (TG, CHOL, HDL-c, LDL-c), and blood glutathione levels in reduced (GSH) and oxidized (GSSG) form. Additionally, FRS and c-IMT scores were assessed in all participants.

Results: Retinal arterial baseline diameter fluctuation (BDF) was independently, significantly and negatively influenced by LDL-c levels (β = -0.53, p = 0.027). Moreover, the arterial dilation slope ($Slope_{AD}$) was independently, significantly and positively associated with redox index (GSH: GSSG ratio, β = 0.28, p = 0.016), while the arterial constriction slope ($Slope_{AC}$) was significantly and negatively influenced by blood GSH levels (β = -0.20, p = 0.042), and positively associated with FRS (β = 0.25, p = 0.009). On the venous side, BDF and dilation amplitude (DA) were also significantly and negatively influenced by plasma LDL-c levels (β = -0.83, p = 0.013; and β = -0.22, p = 0.028, respectively).

Conclusion: In otherwise healthy individuals with low to moderate cardiovascular risk; systemic antioxidant capacity, and circulating plasma markers for cardiovascular risk influence retinal microvascular dilation and constriction responses to stress.

10.2. Introduction

Functional assessments of microvessels are of particular clinical interest since endothelial dysfunction, one of the main culprits for the development of atherosclerosis, is thought to occur much earlier at the microvascular than at the macrovascular level [459, 460]. Consequently, several methods have been developed to assess functional responses in various microvascular beds. Among those, DVA was recently identified as a useful measure of early changes that signal endothelial dysfunction and risk for future cardiovascular pathologies in individuals with and without overt disease [260, 261, 281, 309-312, 317, 320]. This is generally possible due to the fact that the retinal microvascular response to flicker provocation is, in part, dependent on NO release, [254] and compromised NO homeostasis is known to be a key factor in endothelial dysfunction at all vascular levels. However, other mechanisms such as altered metabolic demand and neurovascular coupling are also largely involved in the retinal vascular response to flicker [143]. Indeed, previous studies in our lab have already demonstrated that retinal microvascular function as measured by DVA is affected in patients with Alzheimer's disease, a pathological process largely associated with disturbed neurovascular coupling. [368]. Nevertheless, it has been hypothesized that abnormal neurovascular coupling in such context could also represent a direct consequence of endothelial dysfunction [143].

At the systemic level it is known that degradation of NO by free radicals generated during oxidative stress may impair vasodilation [438], and therefore result in vascular dysfunction. The human body uses a complex anti-oxidative defence mechanism involving glutathione among other factors to combat stressors such as high oxygen flux. Consequently, any condition associated with low levels of circulating glutathione results in a higher rate of oxidative reactions that contribute towards low NO bioavailability, with important consequences on the normal regulation of systemic haemodynamics [508]. In parallel, it is also known that abnormal lipid metabolism and insulin resistance are linked to microvascular endothelial dysfunction and increased CVD risk. The combined effect of low glutathione and abnormal circulating lipids could therefore amplify endothelial dysfunction at all vascular levels, including the retinal vessels [309, 311]. This possibility may be important for screening and early intervention, particularly in individuals free of overt vascular disease, but who belong to a group when age-induced cardiovascular complications start to occur. Indeed, previous reports indicate that nearly 50% of individuals who suffer a fatal cardiovascular event remain asymptomatic, or do not display any symptoms, prior to death [363]. It is also known that traditional risk scoring systems such as the FRS can over- or under-estimate actual risk in a large number of individuals [414], and

despite this risk scores such as the FRS are still largely used for disease prognosis. As other more sensitive biomarkers emerge [413, 416-418], it seems that using these in addition to risk scoring systems could increase the likelihood of early CVD detection [509-512]. This represents an important need in the current concept of prediction, prevention, and personalised interventions that target individualized risk for certain diseases [419]. Following the trend of modern research, cardiovascular medicine has also embraced the study of "omics" (genomics, metabolomics, proteomics) [418], where detailed biological profiling of individuals rather than population groups has been crucial in initiating the move to personalised medicine. As biomarkers that form an important component of these strategies comprise anything from genetic markers to imaging tests, functional microvascular assessment could also be included as it provides integrated and dynamic data to aid in establishing possible CVD risk in an individual. Indeed, previous published studies [260, 309, 310, 463], as well as, those described in this thesis have already demonstrated that assessing retinal microvascular function is an easy, non-invasive method that facilitates early vascular risk detection in asymptomatic individuals. Nevertheless, beside the action of local factors, a better understanding of the possible systemic influences on microvascular retinal function in individuals with low to moderate CVD risk is still needed. The aim of this study is to investigate the influences that circulatory markers for CVD risk and systemic anti-oxidative defence capacity could have on retinal microvascular reactivity, in healthy middle-aged individuals with low to moderate cardiovascular risk.

10.3. Methods

10.3.1. Study participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). Only those individuals who fulfilled the inclusion criteria as defined in section 6.2.1, and were over the age of 40 were included in the final analysis.

10.3.2. General assessments

All study participants underwent preliminary assessments (section 6.3.2) that included a general health history review, BMI, BP and IOP profiling, and assessment of circulatory markers GLUC, TG, CHOL, HDL-c, and LDL-c, as well as, GSH and GSSG by way of the glutathione recycling assay (section 6.3.6.2).

10.3.3. Vascular assessments

Vascular assessments in this study included c-IMT scores (section 6.3.5.2) and DVA assessment in one unselected eye (section 6.3.4). The following retinal vascular function parameters, averaged over three flicker cycles and with the artery and vein regarded separately, are reported in this study: BDF, DA, MD%, tMD, tMC as well as dilation and constriction slopes for both the arteries ($Slope_{AD}$ and $Slope_{AC}$) and veins ($Slope_{VD}$ and $Slope_{VC}$).

10.3.4. Sample size calculations

Since DVA parameters estimated using the polynomial regression fitting methods have not previously been correlated with oxidative stress markers sample size calculations were based on a previous study correlating glutathione levels and indices of microvascular function in the coronary microcirculation [513]. In order to detect a correlation of at least 0.3 with 95% power and alpha-level of 0.05, a sample size of $n = 95$ was recommended. All sample size calculations were performed using the G*Power software [428] (University of Kiel, Version 3.1.6, Germany).

10.3.5. Statistical analysis

All statistical analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). Distributions of continuous variables were determined by the Shapiro-Wilks test. In cases where normality of the data could not be confirmed appropriate data transformations were made or non-parametric statistical alternatives were used. Group differences were assessed using the Student's *t*-test for independent variables (normal distributions). Univariate associations were determined using Pearson's (normally distributed) or Spearman's method (non-normally distributed), and forward stepwise regression analyses were performed to test the influence of age, BMI, SBP, DBP, HR, IOP, c-IMT, and circulating markers on the measured variables. In multivariate regression models the β coefficient value was considered to answer the question of which of the independent variables has a greater effect on the dependent variable as β refers to the SD change in the dependent variable per SD increase in the predictor variable, and is particularly useful when variables are measured in different units. A *p*-value of less than 0.05 was considered as statistically significant.

10.4. Results

10.4.1. Clinical characteristics

One hundred and two healthy participants over the age of 40 years with low to moderate cardiovascular risk (5-20 % assessed by the FRS) were included in the final analysis. Table 10.1 shows the demographic characteristics of the study population, stratified by gender. Men exhibited higher DBP ($p = 0.002$), and FRS ($p < 0.001$), and lower total CHOL ($p = 0.001$), and HDL-c ($p < 0.001$). There were no significant differences in age, BMI, SBP, HR, IOP, TG, LDL-c, and c-IMT. Although in the present study sample, women had lower levels of GSSG ($p = 0.03$), there were no significant differences in tGSH, GSH, or redox index between men and women (all $p > 0.05$).

10.4.2. Retinal vascular function

With regard to arterial and venous retinal vascular function parameters as characterized in Table 10.2, all values reported are based on averaged data across three flicker cycles with the artery and vein regarded separately. There were no significant differences between male and female participants in this study (all $p > 0.05$). Univariate analyses revealed that age correlated significantly and negatively with retinal arterial DA ($r = -0.24$, $p = 0.015$), and positively with $Slope_{AC}$ ($r = 0.23$, $p = 0.021$). BMI also correlated negatively and significantly with the retinal arterial BDF ($r = -0.20$, $p = 0.040$). Plasma LDL-C levels correlated negatively and significantly with arterial BDF and DA ($r = -0.29$, $p = 0.004$; $r = -0.23$, $p = 0.020$, respectively) and venous BDF and DA ($r = -0.24$, $p = 0.018$; $r = -0.22$, $p = 0.028$, respectively). Blood GSH levels correlated significantly and negatively with $Slope_{AC}$ ($r = -0.20$, $p = 0.041$) and positively with overall retinal arterial DA ($r = 0.25$, $p = 0.010$), while the redox index similarly positively influenced $Slope_{AD}$ ($r = 0.27$, $p = 0.008$). The FRS correlated negatively with arterial BDF ($r = -0.21$, $p = 0.36$), and positively with $Slope_{AC}$ ($r = 0.26$, $p = 0.010$) and $Slope_{VC}$ ($r = 0.22$, $p = 0.026$).

Forward stepwise multiple regression analysis revealed arterial BDF to be independently, significantly and negatively influenced by LDL-c ($\beta = -0.53$, $p = 0.027$, Figure 10.1A) and on the venous side BDF ($\beta = -0.83$, $p = 0.013$, Figure 10.1B) and DA ($\beta = -0.22$, $p = 0.028$, Figure 10.1C) were also independently, significantly and negatively influenced by circulating LDL-c levels. Also on the arterial side, $Slope_{AD}$ was independently, significantly and positively associated with redox index ($\beta = 0.28$, $p = 0.016$, Figure 10.2), while $Slope_{AC}$ was significantly and negatively influenced by blood GSH levels ($\beta = -0.20$, $p = 0.042$, Figure 10.3A) and positively associated with FRS ($\beta = 0.25$, $p = 0.009$, Figure 10.3B).

Table 10.1. Summary of clinical data

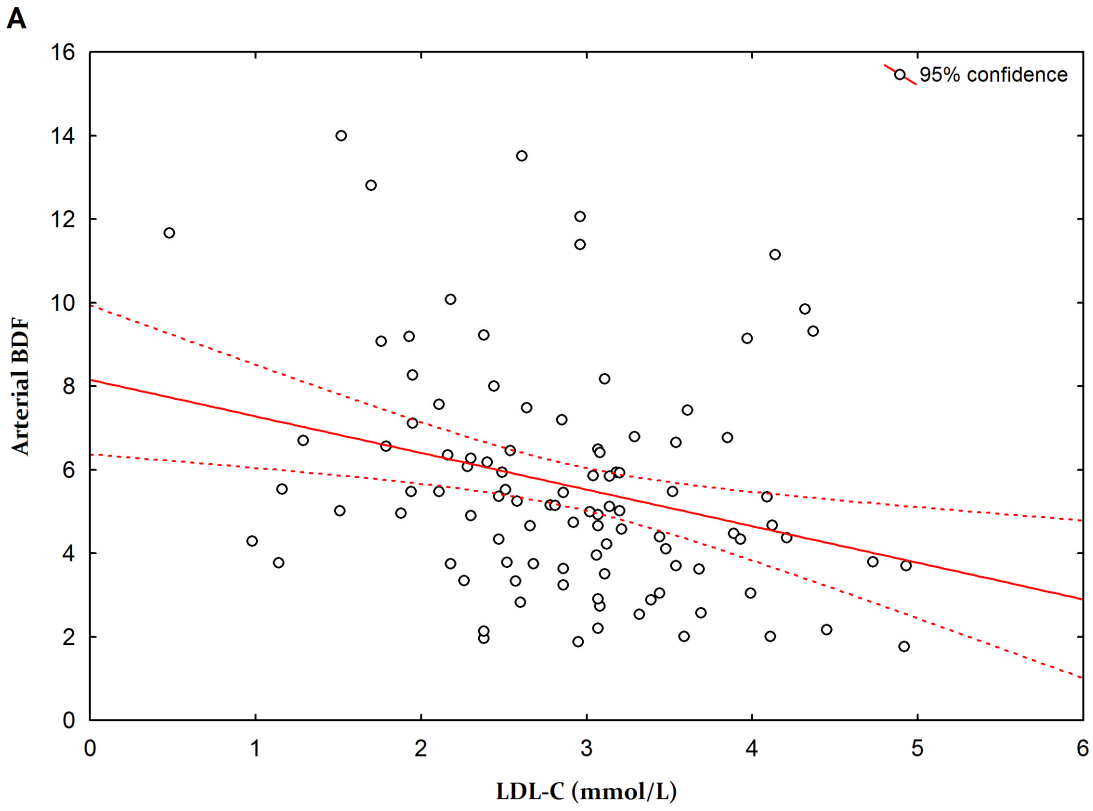
Variable	Mean (SD)		*p*-value	Significance
	Men (1) (n = 54)	**Women (2)** (n = 48)		
Age (years)	52 (8)	53 (7)	0.271	-
BMI (kg/m²)	27.05 (4.17)	25.56 (3.89)	0.070	-
SBP (mmHg)	124 (14)	120 (15)	0.149	-
DBP (mmHg)	80 (10)	73 (11)	**0.002***	1 > 2
HR (bpm)	65 (9)	64 (7)	0.495	-
IOP	14 (3)	14 (2)	0.904	-
TG (mmol/L)	1.22 (0.55)	1.06 (0.34)	0.088	-
CHOL (mmol/L)	4.47 (0.80)	5.05 (0.96)	**0.001***	1 < 2
HDL-c (mmol/L)	1.18 (0.36)	1.49 (0.37)	**<0.001***	1 < 2
LDL-c (mmol/L)	2.74 (0.79)	3.07 (0.97)	0.056	-
tGSH (µmol/L)	1117 (772)	935 (643)	0.200	-
GSH (µmol/L)	935 (713)	813 (586)	0.353	-
GSSG (µmol/L)	85 (73)	61 (48)	**0.030***	1 > 2
Redox index	15 (11)	20 (22)	0.140	-
c-IMT (cm)	0.07 (0.01)	0.06 (0.01)	0.064	-
FRS (%)	9.46 (5.94)	5.59 (4.35)	**<0.001***	1 > 2

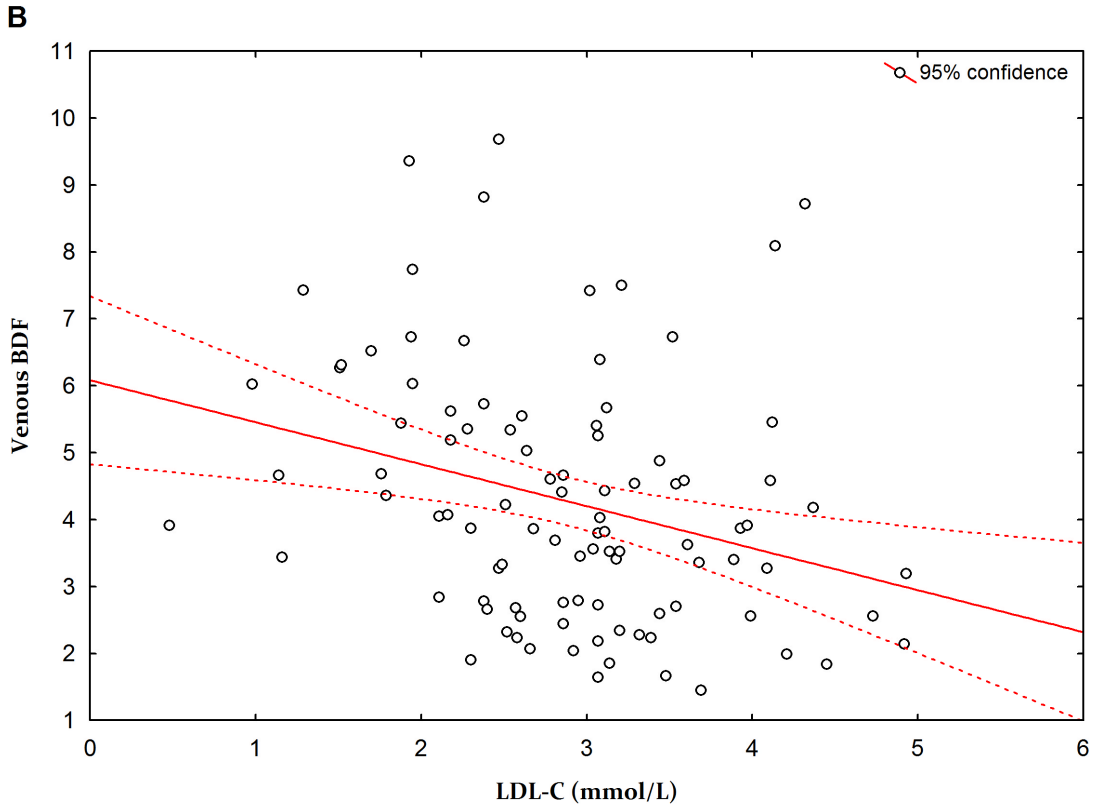
Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; IOP, intraocular pressure; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; tGSH, total GSH; GSH, reduced glutathione; GSSG, oxidized glutathione; Redox index, GSH: GSSG ratio; c-IMT, carotid intima media thickness; FRS%, Framingham risk score. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 10.2. Summary of retinal vascular function parameters

DVA parameter	Mean (SD)		*p*-value	Significance
	Men (n = 54)	Women (n = 48)		
Arteries				
BDF	5.45 (2.14)	6.08 (3.63)	0.282	-
DA	8.05 (3.65)	7.77 (3.90)	0.704	-
MD%	5.15 (2.42)	4.71 (2.69)	0.385	-
tMD (seconds)	19 (6)	20 (8)	0.216	-
tMC (seconds)	28 (8)	27 (10)	0.767	-
$Slope_{AD}$	0.37 (0.24)	0.34 (0.25)	0.567	-
$Slope_{AC}$	-0.25 (0.11)	-0.31 (0.20)	0.060	-
$Slope_{AC}$, NU	1.15 (0.54)	1.02 (0.52)	0.195	
Veins				
BDF	4.38 (1.77)	4.30 (2.26)	0.866	-
DA	6.92 (2.93)	6.89 (2.94)	0.971	-
MD%	7.54 (5.07)	5.85 (3.18)	0.060	-
tMD (seconds)	20 (5)	22 (6)	0.099	-
tMC (seconds)	31 (9)	30 (9)	0.587	-
$Slope_{VD}$	0.32 (0.16)	0.49 (0.14)	0.408	-
$Slope_{VC}$	-0.23 (0.14)	-0.24 (0.13)	0.135	-
$Slope_{VC}$, NU	1.49 (0.74)	1.36 (0.61)	0.365	

Abbreviations: BDF, baseline diameter fluctuation; DA, dilation amplitude; MD%, percent dilation relative to baseline diameter; tMD, time taken to reach MD; tMC, time taken to reach maximum constriction (MC) diameter; $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction; NU, normalized units. $p < 0.05$ was considered significant.


A
95% confidence
Arterial BDF
LDL-C (mmol/L)
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6


B
95% confidence
Venous BDF
LDL-C (mmol/L)
1
2
3
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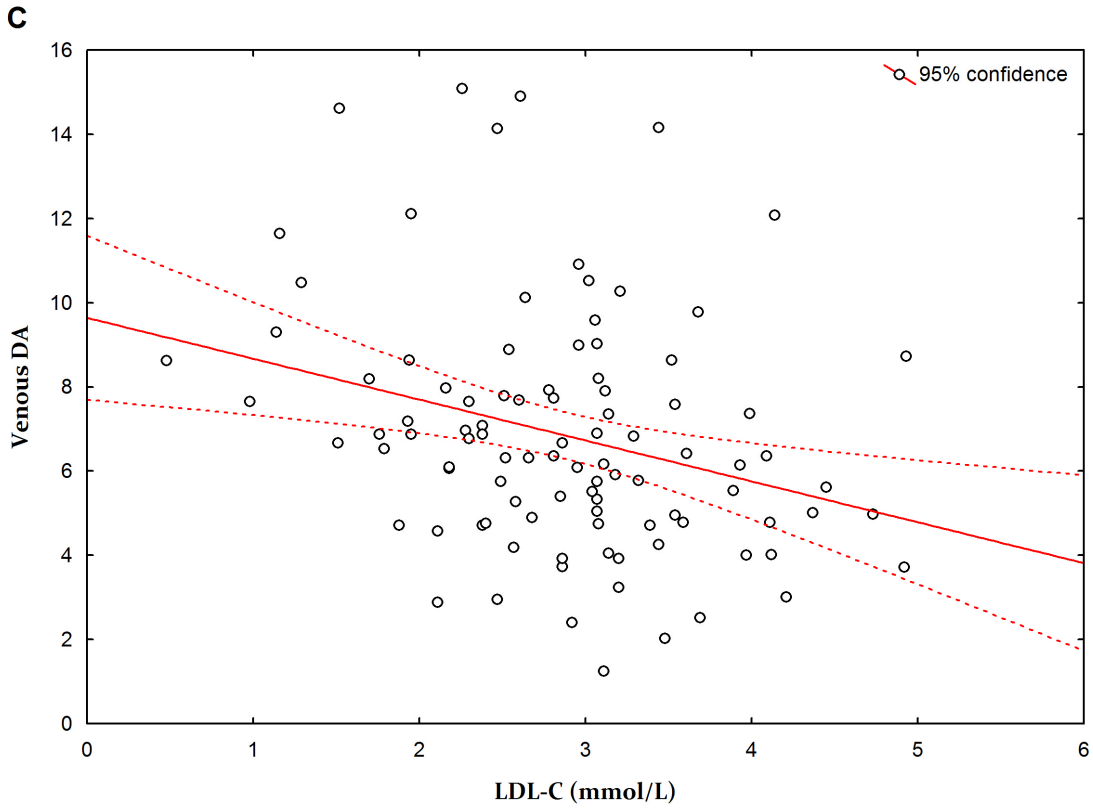

Figure 10.1. Relationship between retinal vascular function parameters and lipid levels.
(A) arterial BDF vs. LDL-c; (B) venous BDF vs. LDL-c; (C) venous DA vs. LDL-c. Abbreviations: BDF, baseline diameter fluctuation; DA, dilation amplitude; LDL-c, low-density lipoprotein cholesterol.

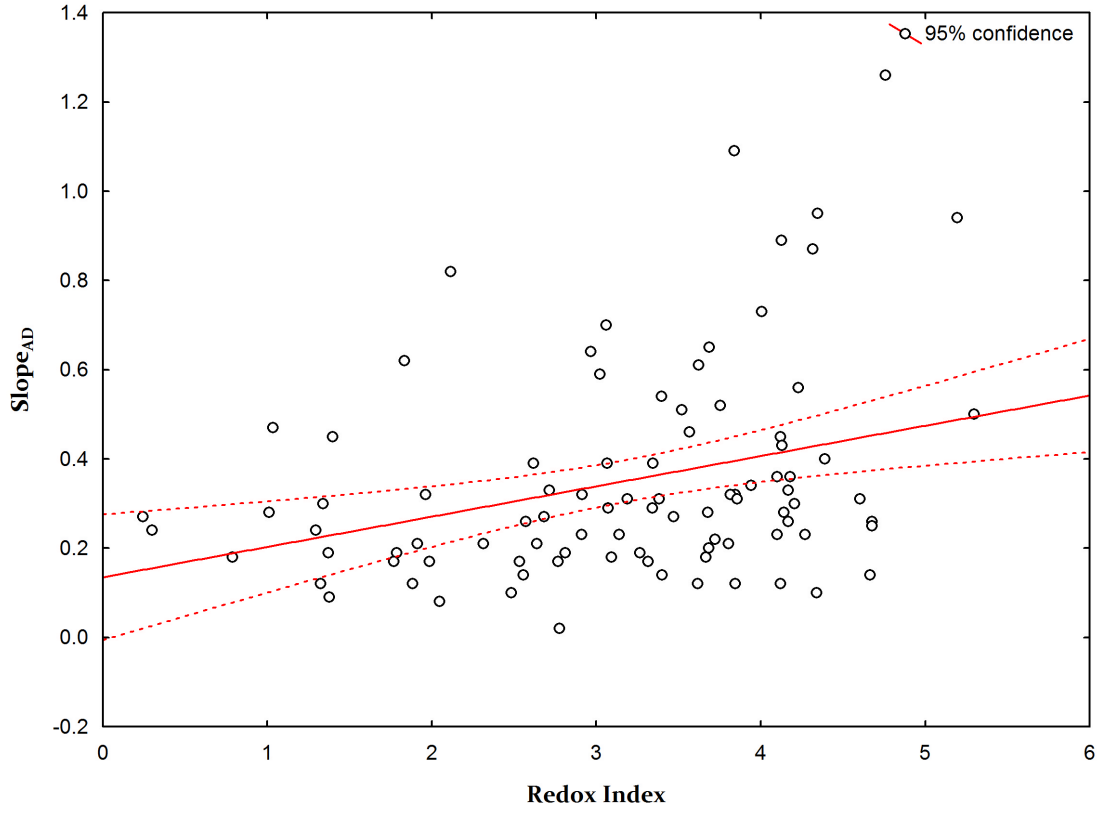

Figure 10.2. Relationship between retinal arterial dilation slope and redox index
Abbreviations: $Slope_{AD}$, retinal arterial dilation response slope; redox index, ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) levels.

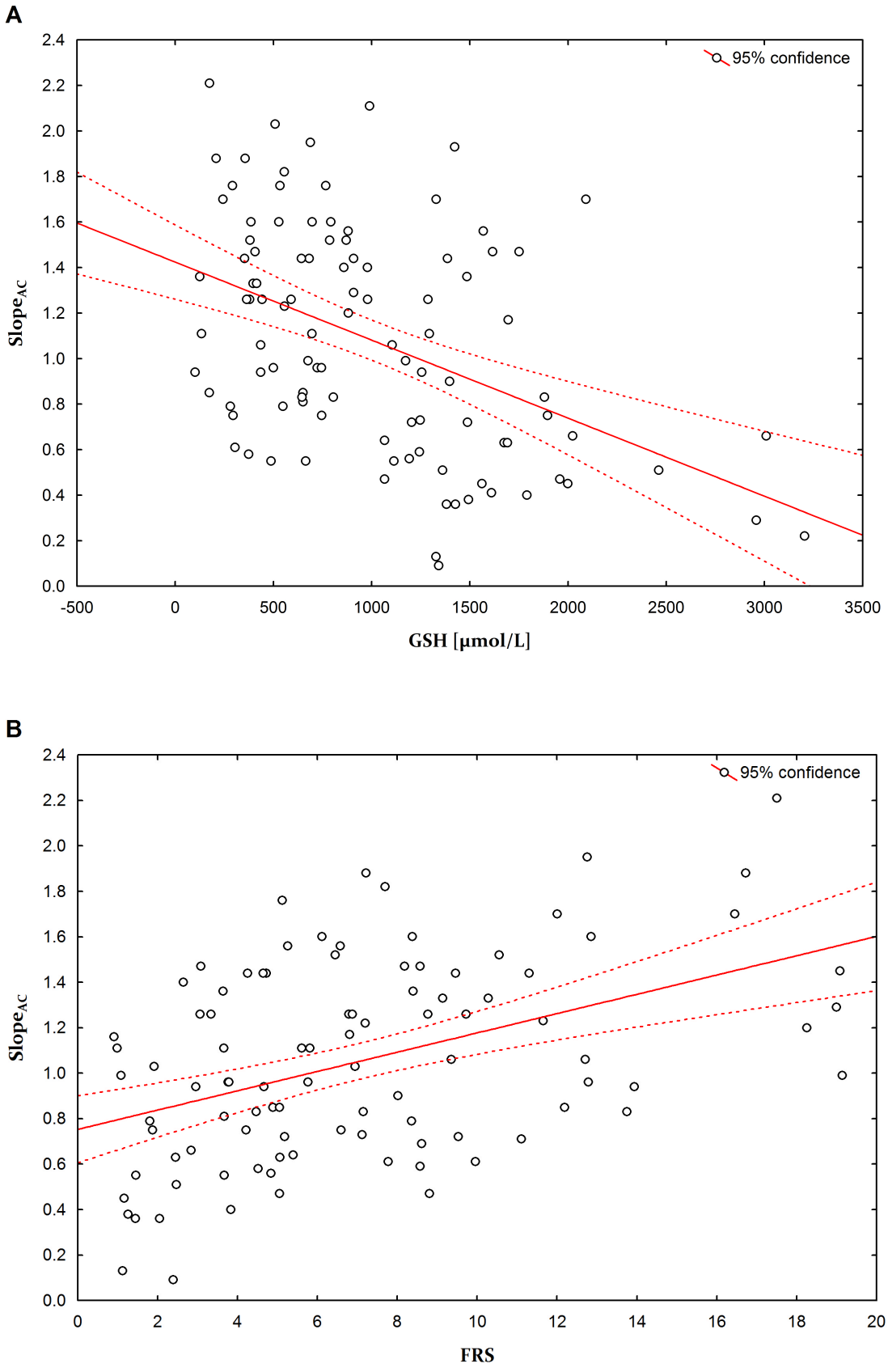

Figure 10.3. Relationship between retinal arterial Slope$_{AC}$, GSH, and FRS
(A) Slope$_{AC}$ vs. GSH; (B) Slope$_{AC}$ vs. FRS. Abbreviations: Slope$_{AC}$, retinal arterial constriction response slope; GSH, reduced glutathione; FRS, Framingham risk score. Note: normalized unit values used on y-axes.

10.5. Discussion

The present study examined the relationship between retinal vessel reactivity, systemic antioxidant defence capacity and circulating markers of cardiovascular risk in individuals free of systemic disease, but with low to moderate FRS for CVD. Systemic 'redox' status (GSH: GSSG) positively influenced retinal arterial dilation capacity ($Slope_{AD}$) during flicker while systemic GSH levels negatively influenced retinal arterial constriction capacity ($Slope_{AC}$). In addition, decreases in arterial and venous BDFs before flicker provocation were identified in individuals with increased LDL-c levels. High LDL-c levels also negatively influenced the overall retinal venous DA during flicker.

In this study, and in line with previous research [514, 515], men exhibited higher BP and FRS, as well as, lower total CHOL and HDL-c compared to age-matched women. Nevertheless, despite the observed differences there were no significant independent influences of these parameters on retinal vascular function parameters in either men or women ($p > 0.05$).

Lower levels of blood GSSG were also observed in women when compared to age-matched men. While a number of studies have revealed a relationship between redox balance and sex hormone patterns [516-522], clinical reports of gender or sex differences in glutathione levels are inconsistent with some studies reporting higher levels of GSH in men [523] and others reporting no sex differences in plasma or blood glutathione levels [524]. These discrepancies may possibly arise due to variations in assay techniques. Previous reports also reveal some degree of variability [311, 400, 403], however, with the method used in our own research being identical, these inconsistencies could also be attributed to other factors such as age. Indeed, the present study included a slightly younger group than our lab's previous published reports [311, 403]. Nevertheless, taken together, these observations suggest that gender might have a less consistent effect on blood glutathione levels than other confounding variables [400]. Moreover, there were no differences with regards to retinal microvascular function parameters between men and women included in this study.

The present study reports for the first time a link between retinal arterial dilation during stress ($Slope_{AD}$) and redox index. From a microvascular standpoint endothelial health and redox balance are associative attributes [525, 526]. Indeed, it is well known that excessive levels of ROS are linked to an impaired endothelium-dependent dilation [527-529]. Typically, a decrease in the ratio of circulating reduced glutathione (GSH) relative to its oxidized form (GSSG) or 'redox index' has been proposed as an indicator of oxidative imbalance [530] and oxidative stress status [496-498]. Therefore, a positive relationship between $Slope_{AD}$ and redox

index, could indicate that in addition to other processes, antioxidant mechanisms support normal NO secretion during flicker provocation at the retinal microvascular level. This possibility is further emphasized by the other novel discovery of a negative relationship between blood GSH levels and $Slope_{AC}$. Indeed, thiol oxidations during interactions with ROS are associated with endothelial dysfunction and increased vasoconstriction [531]. This effect has been previously reported at the coronary [531], renal [532], and cerebral levels [533]. Similarly, retinal microcirculatory reactivity also seems to be influenced by circulating levels of thiols. Interestingly, $Slope_{AC}$ also correlated with a more traditional risk assessment, namely FRS. Previous studies have observed similar relationships between established cardiovascular risk scores such as FRS and other measures of vascular function including peripheral arterial tonometry [534], digital thermal monitoring [535], and whole-body MR angiography [536]. Additionally, retinal vascular calibres have been shown to be independently associated with risk factor variables constituted in the FRS such as age, BP, HDL-c, and LDL-c [120]. The present study, however, demonstrates not only a quantitative relationship between vascular function parameters and systemic risk scores, but more specifically, also a functional one based on the speed and amount of vascular reactivity defined by slope in the vascular response curves. This relationship characterizes the dynamic responsiveness of any vascular bed to increased demand, a parameter that determines each person's vascular reserve. This observation is particularly important since most of the efforts to prevent CVD are currently focused on modifying populational risk factors that often either over- or underestimate individualized risk [414]. The assessment of the dynamic retinal vascular response to flicker represents a non-invasive way to determine the individual's functional vascular capacity and, therefore, the individual's likelihood to develop disease or to respond to vasoactive therapies currently used in treating CVD. The present study observations therefore, suggest that individual vascular response profiles when used alongside traditional risk factors might improve the accuracy of CVD prediction [537, 538].

Additional results of the present study also disclosed a negative relationship between LDL-c and arterial and venous BDF. LDLs are particularly susceptible to oxidative modifications and oxidized low-density lipoproteins (ox-LDLs) can inhibit the synthesis and release of endothelial NOS resulting in the decreased bioavailability of NO [63], as well as the increased expression of ET-1 and adhesion molecules [539, 540]; all of which further contribute to the down-regulation of local retinal vascular tone and vessel stiffness [541]. On this basis, it could be hypothesized that the oxidative modification of LDL-c maybe

associated with changes in the elastic properties of the vessel wall, reflected at the retinal vascular level as decreases in spontaneous variations of vessel diameter during normal resting conditions [312]. Indeed, ox-LDLs have been shown to impair vasomotor function of the coronary microcirculation [542]. Moreover, LDL-c apheresis has been shown to improve endothelium-dependent vasodilation in hypercholesterolemia patients [543], and to positively influence myocardial [544], cerebral [545], and retinal [546] blood flow. A negative influence of LDL-c on the overall venous DA was also observed. Since retinal veins typically incite a more passive regulatory contribution to increases in blood flow [256], whether the overall decreases in DA may reflect the reconciliation of alterations in arterial outflow to the venous side via downstream autoregulatory mechanisms is unclear at present.

In the present study, no correlations between measures of systemic macrovascular integrity (c-IMT) and retinal microvascular function abnormalities were found. Interestingly, this finding is in concordance with that of a number of recent studies which have similarly demonstrated no direct correlation between anomalies identified at the macro- and micro-vascular levels in various disease states [313, 547, 548], as well as, with what was observed in the studies described previously in this thesis. In addition, while c-IMT is a marker for structural abnormalities our measured retinal vascular parameters assess function, which is affected first in the course of CVD. It is possible that in individuals with higher CVD risk than those included in the present study a positive correlation between anomalies identified at the macro- and micro-vascular levels would become apparent. Nevertheless, early identification of individuals at risk for CVD should be done when the disease is subclinical and well before structural changes begin to appear. Indeed, vascular changes in various beds appear to have common determinants since retinal vascular calibres have been shown to be associated with renal function in apparently healthy subjects [549]. With this in mind, the direct relationship observed between systemic anti-oxidative defence capacity, plasma markers for CVD risk, and retinal dynamic vascular responses in apparently healthy individuals points toward more complex mechanisms that regulate the vascular response to stress at this level. In addition, it shows that assessing the retinal microvascular response in individuals free of overt vascular disease, but with a certain degree of CVD risk that is either age-related or due to other variables included in the FRS, could be a very sensitive indicator of each individual's specific risk. As molecular and imaging biomarkers drive the shift towards personalized medicine, retinal vessel reactivity can be used for profiling individualized vascular risk by providing an integrated and dynamic analysis of vascular

function as a variable specific for each individual and, therefore, to be used in prediction, prevention, and personalised intervention.

10.6. Conclusion

In conclusion this study demonstrates that in healthy individuals, but with low to moderate cardiovascular risk, systemic antioxidant capacity and plasma markers for relative cardiovascular risk influence retinal microvascular dilation and constriction responses to stress at both arterial and venous levels. As assays for measuring circulatory oxidative stress markers can be complex and need specialized laboratories, it is tempting to propose that a simple, non-invasive retinal vascular function assessment could be used as a surrogate indicator for an individual's systemic capacity of dealing with the damaging effects of excess LDL-c, as well as, free radicals and, consequently, for their risk of endothelial dysfunction and future CVD.

11. Study 5: Retinal vascular function in individuals with obstructive sleep apnoea: A preliminary study

11.1. Abstract

Purpose: To investigate the relationship between known markers of cardiovascular risk and vascular function parameters measured at the retinal and systemic levels in patients with untreated moderate to severe obstructive sleep apnoea (OSA).

Methods: Eighteen age- and gender-matched subjects who underwent polysomnography assessments (PSG) were included in this study. Patients with moderate to severe OSA were defined as those with an apnoea/hypopnea index (AHI) > 15 (n = 9) and subjects with an AHI < 5 were included as study controls (n = 9). General assessments included BMI, BP and IOP profiles, circulating markers (GLUC, TG, CHOL, HDL-c, LDL-c) and oxidative stress and endothelial markers (GSH, GSSG, NO, ET-1). Additional assessments also included full blood a count, urinalysis, 24-hour BP and HR monitoring, and FRS. Systemic vascular assessments included AIx by way of PWA, c-IMT and FMD; and retinal vascular function was assessed by way of DVA. All participants also completed a series of study questionnaires including the Short Form Health Survey (SF-36), Functional Outcomes of Sleep Questionnaire (FOSQ), and Epworth Sleepiness Scale (ESS).

Results: In comparison to controls, BMI (p = 0.002), 24-hour HR (p = 0.027), AIx (p = 0.024), and white blood cell (WBC) count (p = 0.048) was significantly higher in the OSA group. There was however no significant difference between groups in other systemic parameters (all $p > 0.05$). With regards to retinal vascular function, OSA subjects exhibited delayed reaction times in response to flicker (tMD) (p = 0.049) and decreased dilation amplitude (DA) (p = 0.003), dilation slope ($Slope_{AD}$) (p = 0.003), and post-flicker constriction MC% (p = 0.014). Additionally, $Slope_{AD}$ correlated negatively with BMI (r = -0.46, p = 0.045) in the OSA group. Further evaluation of the arterial response parameters for individual flicker cycles in the OSA group showed that baseline diameter fluctuation (BDF) and tMD were both significantly increased during the third flicker cycle compared to the first flicker cycle (p = 0.046 and p = 0.035, respectively), and MC% was significantly decreased in the third flicker cycle compared to the first (p = 0.022) and second (p = 0.028) flicker cycles.

Conclusion: Individuals with untreated moderate to severe OSA exhibit signs of increased arterial stiffness and sympathetic drive. Retinal microvascular responses to flicker were

also attenuated in the OSA group, which correlated with increases in BMI and were consistent with decreases in retinal vessel capacity to respond to stress.

11.2. Introduction

Sleep disturbances and sleep-disordered breathing are increasingly becoming recognised as important risk factors for the development and progression of CVD, given the known associations between blood flow regulation and sleep-wake cycle. In parallel, a number of ocular conditions can also be linked with sleep disturbances and / or sleep-disordered breathing [352]. Benign signs, such as droopy eyelids are common early indicators of sleep deprivation, whereas other sequelae may signal a more serious underlying condition. Obstructive sleep apnoea (OSA) is a relatively common form of sleep-disordered breathing. OSA is characterised as recurrent upper airway collapses during sleep leading to cardiopulmonary and neuro-physiological changes such as intermittent hypoxia, pulse rate variation, and sleep disruption [550]. OSA has also been linked with the occurrence of cardiovascular complications [551-557]. Cardiovascular consequences of OSA may involve both the macro- and microvessels, however, little is known about the mechanism by which OSA influences the microvasculature. Consequently, the early detection of vascular risk could denote an essential step in preventing the development of vascular disease in this group of patients.

While a cardiovascular risk assessment is not routinely conducted in patients with sleep disorders, research indicates that OSA patients exhibit selective impairments in macrovascular [558], as well as, microvascular endothelial function [559-561]. It is known that smaller blood vessels are more susceptible to the effects of unfavourable exposures [382], and consequently functional vascular assessments that can identify early changes in the microcirculation could be used as a measure of local vascular dysregulations, as well as, an indirect measure of systemic vascular complications [562]. At the retinal level, static studies show the severity of OSA to be correlated with retinal venular widening supporting the hypothesis that cardiovascular complications of OSA may be mediated by microvascular disease related to endothelial dysfunction [356]. In contrast, an earlier study found no retinal vessel abnormalities associated with sleep-disordered breathing [357]. The use of the arteriolar-to-venular ratio as the endpoint in the aforementioned study, however, overlooks the possibility that retinal arteriolar narrowing and venular widening are separate phenomena that can reflect varied physiological processes [358]. As such, existing retinal studies currently offer incomplete evidence to infer any causal associations.

On the other hand, there is strong evidence for endothelial dysfunction in OSA. Endothelium-dependent vasodilation is reduced in patients with sleep-disordered breathing [301] and a reduction in NO levels is associated with hypoxia [359] caused by repetitive upper airway collapses. As discussed previously (Chapter 4), functional retinal assessments can detect the presence of early vascular dysfunctions, even in the absence of overt vascular disease [260, 309, 310, 463] (corroborated by other studies described in this thesis). There are, however, limited data on functional retinal assessments in OSA patients that presently preclude the adequate definition of retinal vascular function parameters specific to this group. Although, both microvascular [559] and macrovascular [558, 563-566] changes can be expected there is also still limited knowledge regarding the simultaneous occurrence of these dysfunctions at multiple vascular levels, and their relative importance in individuals with more subtle or underappreciated cardiovascular risk factors such as OSA. Naturally, if a patient is highlighted as having an adverse vascular profile, that patient may be at a higher risk of future adverse cardiovascular outcomes. Nevertheless, the temporal relationship between OSA and the development and progression of manifest CVD also suggests that the threshold of risk may vary amongst OSA patients. Consequently, few studies address this issue.

The aim of this study was to investigate the presence of known markers for CVD risk in subjects with untreated moderate to severe OSA and assess their relationship to structural and functional vascular parameters assessed at the retinal and systemic levels.

11.3. Methods

11.3.1. Study participants

Prior to study initiation, ethical approval was sought and received from the Heart of England NHS Foundation Trust (HEFT) ethics committee, as well as, the Aston University research ethics committee. Written informed consent was received from all participants prior to study enrolment and all study procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki.

All patients referred to the Birmingham Heartlands Respiratory Physiology and Biomedical Research Centre Sleep Unit (Birmingham, UK) for the possibility of OSA, and who underwent overnight Polysomnography (PSG) assessment were considered for this study. A team of sleep physiologists examined all recruited study patients and only those classified as having OSA where continuous positive airway pressure (CPAP) therapy was

indicated as the first-line treatment were considered for the study. Patients who had undergone the overnight PSG but who were not considered to be suffering from OSA were included as study controls.

Since one of the original aims of this study was to assess vascular function in OSA patients before and after 6 months of CPAP therapy, the diagnosis of OSA and treatment assignment was initially masked from the author, and both OSA and suitable controls were selected by the attending sleep physiologist (Dr Dev Banerjee). All participants were provided with detailed information about the study, and allowed at least 24 hours to consider their enrolment. Participants who provided consent were then requested to attend a study assessment at the Vascular Research Laboratory (Aston University), prior to the initiation of OSA treatment. As a 6-month follow-up study assessment was originally intended, and owing to the masked nature of the study design, participants were instructed not to divulge whether or not they would undergo CPAP therapy. The present study, however, only reports data that was analysed from the patients' baseline visit due to technical difficulties with study assessments at 6 months, as well as, loss to follow-up in some cases.

11.3.2. Inclusion / exclusion criteria

All PSG assessments undertaken at the sleep clinic included 2-channel Electroencephalography (EEG), 2-channel Electrooculography (EOG), 1-channel submental electromyography (EMG), respiratory and abdominal movements via chest and abdominal belts, nasal pressure via pressure sensor, and oximetry using a finger oximetry probe. Sleep stages and respiratory parameters were scored by the attending sleep physiologist according to standard American Academy of Sleep Medicine guidelines [550]. The AHI was referred to as the average number of apnoeas (complete cessation of airflow for at least 10 seconds) and hypopneas (reduction in airflow of at least 30% accompanied by at least a 4% blood oxygen desaturation in the preceding 30 seconds, and a reduction in chest wall movement and/or arousal) per hour of sleep. Patients with an AHI > 15 or greater per hour were defined as having moderate to severe OSA and were included in the study and those with an AHI < 5 per hour were defined as not having OSA and were included into the study as controls. Patients with an AHI of at least 5 but less than 15 per hour were, however, excluded from the study. Other study exclusion criteria were similar to those defined in section 6.2.1 and included history of smoking; unstable CVD such as CAD, valvular heart disease and heart failure; chronic lung disease such as chronic

pulmonary obstructive disease (COPD) and bronchiectasis; renal failure, previous history of OSA treatment such as CPAP therapy; other sleep disorders such as insomnia, narcolepsy, and shift-work related sleepiness; and history of vasoactive medications known to affect vascular and or endothelial function.

11.3.3. General assessments

General preliminary assessments were as those detailed in section 6.3.2 and included BMI, SBP, DBP, HR, MAP, IOP and OPP. In addition, FRS was determined for all study participants.

11.3.3.1. Study questionnaires

All study participants completed the general health history questionnaire, as well as, subjective quality of life (QoL) and sleep questionnaires (Appendix A) as detailed below.

11.3.3.1.1. Short Form-36 (SF-36®)

The SF-36® [567] is a validated, 36-item questionnaire used as measure of subjective health status and quality of life (QoL). The instrument provides an 8-scale summary measure of health and well being, as well as, physical and mental health. Figure 11.1 illustrates the taxonomy in the construction of the SF-36® scales, which is comprised of three levels: (1) items; (2) eight scales that combine between 2 and 10 items each; and, (3) two summary measures that aggregate the scales. All but one of the 36 items (self-reported health transition) is used to score the eight SF-36® scales (Table 11.1).

Table 11.1. Scoring the SF-36

SF-36 SCALES	QUESTIONS	CALCULATION
Physical Functioning (PF)	3, 4, 5, 6, 7, 8, 9, 10, 11, 12	
Role-Playing (RP)	13, 14, 15, 16	$\text{Mean} = \frac{\sum_{i=}^{n} a_i}{n}$
Bodily Pain (BP)	21, 22	
General Health (GH)	1, 33, 34, 35, 36	$\text{Mean}_w = \frac{\sum_{i=}^{n}[\{t1_i + t2_i\}a_i]}{\sum_{i=}^{n}[t1_i + t2_i]}$
Vitality (VT)	23, 27, 29, 31	
Social Functioning (SF)	20, 32	a_i = value of each question n = number of questions w = weighted (mean)
Role-emotional (RE)	17, 18, 19	
Mental Health (MH	24, 25, 26, 28, 30	

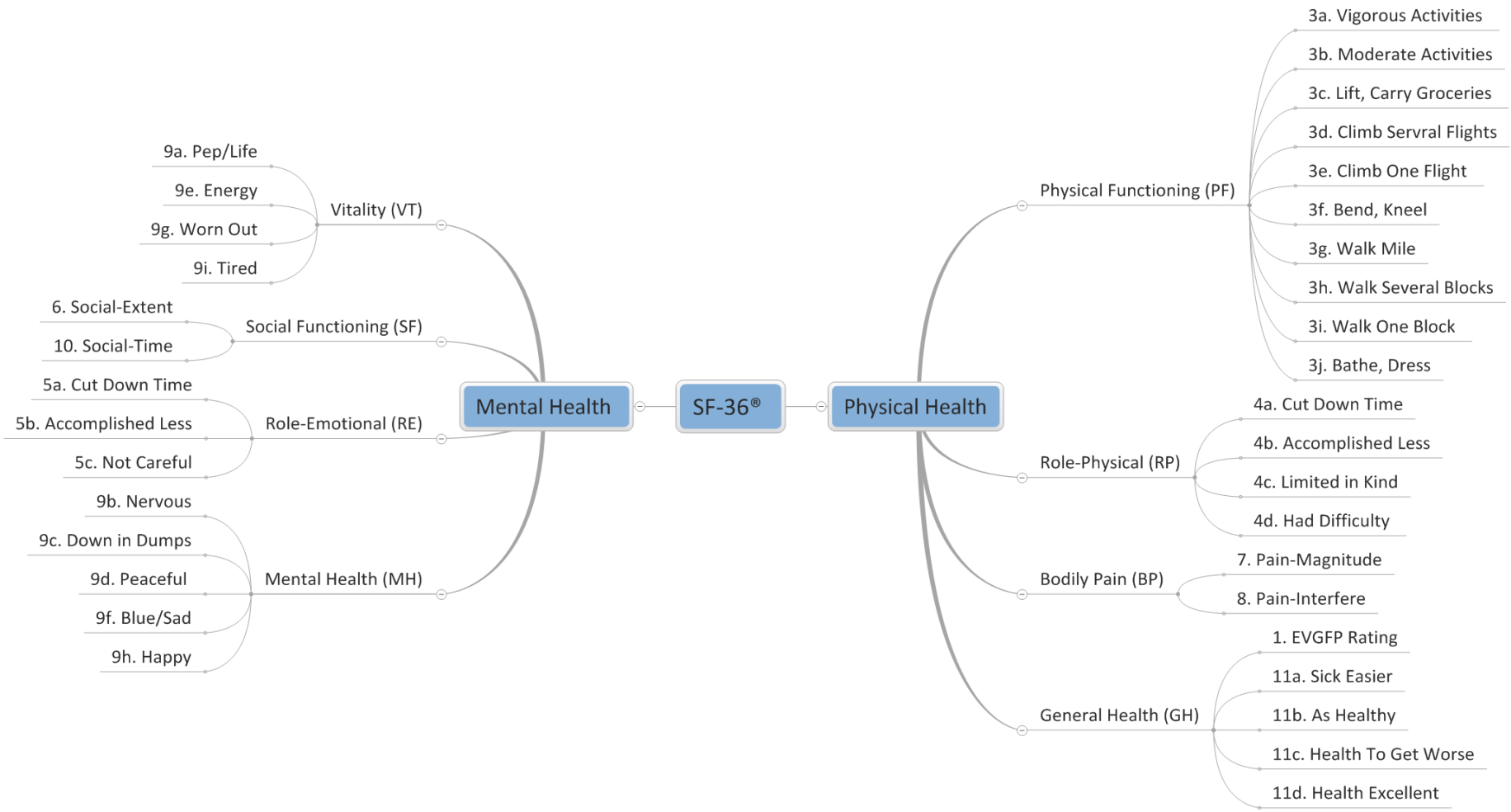

Figure 11.1. SF-36® measurement model

The score range for health transition is between 1 and 5, for the eight sub-scores between 0 and 100, and for the physical and mental component scores standardization provides population means and an SD of 50 ± 10. The raw scores for each subscale were transformed to scores that ranged from 0 to100%, according to the formula: transformed score = [(raw scale score − lowest possible score)/possible score range] ×100%, as recommended [568]. Higher scores in each subscale are consistent with a better QoL.

11.3.3.1.2. Functional outcomes of sleep questionnaire (FOSQ)

The FOSQ is a 30-item self-report questionnaire designed to measure the impact of excessive sleepiness on multiple activities of daily living, conceptually defined as functional status, and a number of studies support the validity and reliability of FOSQ as an outcome measure in clinical trials [569-579]. The FOSQ is comprised of five dimensions: activity level, vigilance, intimacy and sexual relationships, general productivity, and social outcome. Each of these dimensions is rated on a 4-pointscale. The questions pertaining to sexual intimacy and relationships were excluded from the questionnaire in the present thesis owing to the personal nature of the questions, and possible pitfalls associated with non-response to these items [580]. A weighted-item mean score (Table 11.2) was computed for each of the remaining four domains along with a total FOSQ score out of 16. Lower scores were associated with a greater impact of sleepiness on daily activities [580-582].

Table 11.2. Scoring the FOSQ

FOSQ Scale	QUESTIONS	CALCULATON
Productivity	1,2,3,4,8,9,10,11	$\text{Mean} = \frac{\sum_{i=}^{n} a_i}{n}$
Social Outcome	12,13	$\text{Mean}_w = \frac{\sum_{i=}^{n}[\{t1_i + t2_i\}a_i]}{\sum_{i=}^{n}[t1_i + t2_i]}$
Activity	5,14,15,16,22,23,24,25,26	a_i = value of each question n = number of questions w = weighted (mean)
Vigilance	6,7,17,18,19,20,21	

11.3.3.1.3. Epworth sleepiness scale (ESS)

The ESS is an 8-item questionnaire designed to measure daytime sleepiness or 'somnoficity' [583]. With significant correlations between ESS scores and sleep latency measures [584], the ESS has been used as a validated measure of sleep propensity in adults. The questionnaire comprises a 4-point Likert scale format where respondents are asked how likely they would be to doze during eight varying scenarios of daily living from

"sitting and reading" to "watching TV", with possible scores ranging from 0 (would never doze) to 3 (high chance of dozing), and with the possibility of yielding a total score between 0 and 24. A higher level of daytime sleepiness was indicated in scores greater than 16.

11.3.3.2. Blood chemistry and urinalysis

Blood samples drawn from the antecubital fossa vein were collected into appropriate Vacutainer® tubes and sent to the Birmingham Heartlands Hospital blood pathology unit (Birmingham, UK) for further analyses including full blood count (FBC), serum glucose, triglycerides, cholesterols (total, HDL-c, LDL-c,) and glycated haemoglobin (HbA1c). An additional set of urine samples were also collected and sent to the pathology unit for assessment of albumin/creatinine ratio (ACR). A sample of EDTA blood was retained and analysed by the author for circulating levels of reduced (GSH) and oxidized (GSSG) glutathione, ET-1, and NO as described in section 6.3.6.

11.3.4. Blood pressure monitoring

Ambulatory blood pressure monitoring was conducted using the computer-operated Meditech CardioTens device (Cardiotens-01, Meditech Ltd, Budapest, Hungary), which has been validated in previous studies [313, 585, 586]. The device, which is worn around the waist, is connected to a BP cuff placed around the forearm. This device measures BP automatically using an oscillometric method and can store 1000 BP measurements and a total of 4 to 5 hours of electrocardiography (ECG) recordings. In the event of a faulty reading, the device is programmed to re-inflate a second time, which helps to avoid missing data points. Automated BP readings were conducted at intervals of 15 minutes during the day and every 30 minutes at night during the 24-hour recording period (Figure 11.2). Day and night periods were also customized to each individual with regards to variations in sleep wake schedules. The 24-h BP data were later downloaded and analysed using the 'Cardiovisions' software program version 1.18.0 (Meditech), and the day, night and 24-hour SBP, DBP, and HR were recorded.

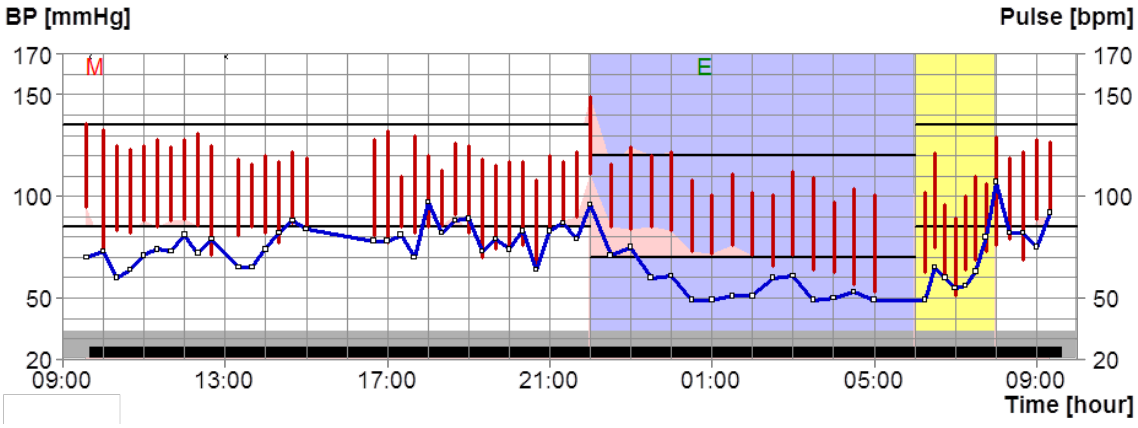

Figure 11.2. Blood pressure profiling over 24 hours
This figure represents an example of a 24-h blood pressure (BP) profile generate by the Cardiovisions software. The horizontal axis indicates the time. The red lines indicate the recorded BP data, with the pulse rate indicated by empty squares connected by a blue line. The background colours indicate the corresponding morning (yellow), day (white), and night (blue) periods.

11.3.4.1. Heart rate variability analysis

The Cardiotens-01 device is also able to simultaneously monitor and analyse ECG signals and stores heart rate variability (HRV) data [587]. For HRV analysis the device requires at least 5 minutes of continuous ECG data recording with proper electrode placement on the chest. A frequency-domain analysis of HRV was performed using a series of validated algorithms in the device software (Cardiovisions version 1.18.0, Meditech, Hungary). An example of normal range power spectral density (PSD) and compressed spectral array charts generated by the software using frequency domain analysis is provided in Figure 11.3. In normal individuals cyclic changes in HR are associated with a higher frequency (HF) (0.2–0.4 Hz) and mediated by the PNS. Conversely, variations due to changes in BP that result from changes in baroreceptor activity are typically low in frequency (LF) (0.0–0.04 Hz), and mediated via the SNS. The LF/HF ratio is thus said to represent sympathovagal balance of ANS function [588, 589].

In the present study, low-frequency, HF and LF/HF parameters were calculated for both the active and passive periods of the recording since transient variations in HRV have been validated as measures of short-term changes in autonomic tone [590]. An additional software parameter: the HRV triangulation index (HRVti), which is determined using a mathematical approach to plot each beat-to-beat interval as a function of the previous interval and involves pattern-based categorization of the shape of the plot for the derivation of HRV [591, 592] (Figure 11.4), was also recorded in this study.

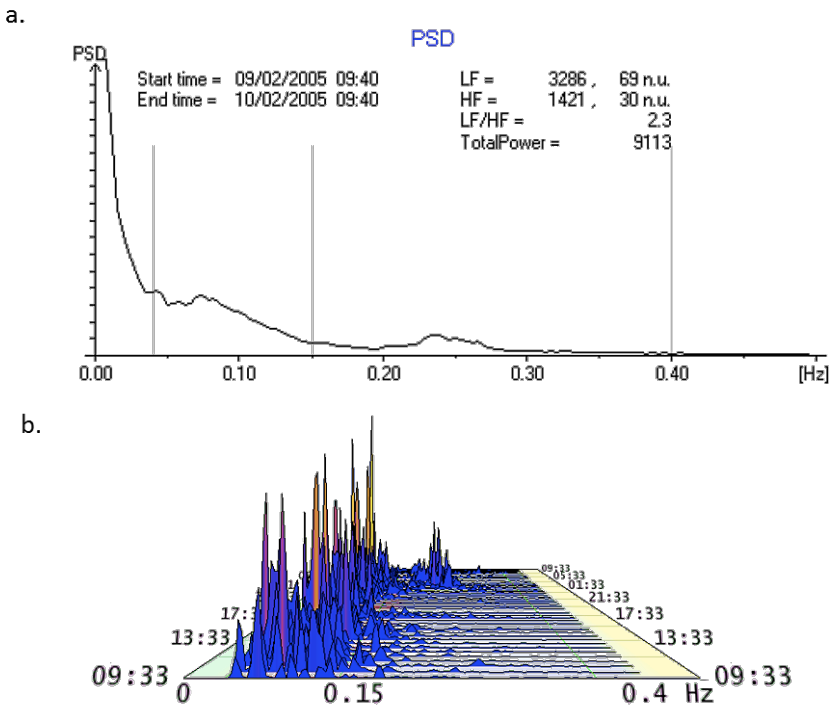

Figure 11.3. Normal HRV power spectral density and compressed spectral array charts
(a) The horizontal axis represents the frequency (Hz); the vertical axis shows the power spectral density (PSD). The input range, total power, low frequency (LF) and high frequency (HF) power components and the LF/HF ratio are also displayed on the chart. The grey vertical lines on the chart mark the very low frequency (VLF), LF, and HF components (0-0.14 Hz, 0.14-0.15 Hz, 0.15-0.4 Hz, respectively). **(b)** The compressed spectral array chart is a summation of PSD graphs. The horizontal axis represents the frequency (Hz). Depending on the resolution the graph is based on 30, 20 or 15-minute time ranges for the 24-hour period which translates into 2, 3 or 4 PSD per hour.

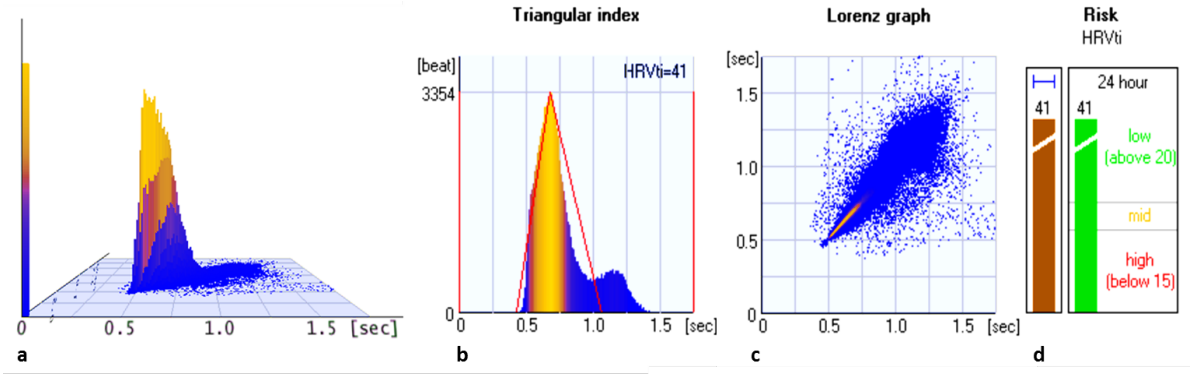

Figure 11.4. The HRV 3-D Lorenz graph and histogram frame
(a) The 3-D Lorenz graph analyses pulse rate variability using a geometric method using a time interval input, which can be selected in the tachogram frame. The horizontal (X) and vertical (Y) axes represents the R-R distance in milliseconds (msec) and z-axis shows the number of heart beats in the X, Y category; **(b)** the height of the triangular interpolation (HRVti) plot corresponds to the most frequent category; **(c)** the interpolations area selected is displayed on the Lorenz graph; **(d)** the risk diagram shows the number of HRVti, the left column represents the values of the time interval selected and the column on the right shows the 24-hour value. HRVti risk is categorized as low (above 20), mid (15-20) or high (0-15).

11.3.5. Vascular assessments

Vascular assessments in this study included DVA (section 6.3.4.1), PWA (section 6.3.5.1), c-IMT (section 6.3.5.2) and FMD (section 6.3.5.3).

11.3.6. Sample size calculation

All sample size calculations were performed using the G*Power software [428] (University of Kiel, Version 3.1.6, Germany). With regards to retinal vascular function, the measurement parameters and polynomial regression fitting methods used in this thesis have not previously been reported in OSA patients. Sample size calculations were therefore based on previous studies, which share similar protocols with that of the present study. On the basis of functional retinal studies using DVA, a change of 30% with a SD of 2.5% has been shown to be clinically significant [258]. With regards to FMD, a brachial reactive hyperaemic response of 10.93% with a standard deviation of 2.59 % is considered normal on the basis of previous research and an approximately 30% alteration in this response has been shown to be clinical significant in OSA patients [593, 594]. It was anticipated that *t*-test and repeated measures ANOVA would be required in this study, therefore in order to provide a statistical power of at least 80% with an alpha-level set at 0.05, it was estimated that a sample size of 10-14 participants per group would be required (14 DVA within/between ANOVA, 10 FMD *t*-test).

11.3.7. Statistical analysis

All statistical analyses were performed using Statistica® software (StatSoft Inc., Version 9, USA). Differences in mean values between groups were compared by independent samples *t*-test for continuous variables. Multivariate analysis was performed to investigate possible influences of age, gender, BMI, BP, and circulating markers on the measured variables. Differences between groups in retinal and systemic vascular function parameters were computed by *t*-test or analysis of covariance (ANCOVA) where applicable. Comparisons of retinal function parameters for individual flicker periods were carried out by two-factor repeated-measures analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

11.4. Results

Of the 21 participants originally recruited to the study 3 participants were excluded based on poor quality of DVA recordings. The remaining 18 adults (11 men and 7 women), aged between 43 and 67 years (mean age 55) were selected for the final analysis, and included 9 OSA and 9 control participants. Although the number of participants was below the intended target in each group, statistical significance was still observed.

11.4.1. General characteristics and systemic data

Table 11.3 provides a summary of general characteristics, clinical data and systemic parameters. BMI levels were significantly higher in the OSA group compared to controls ($p = 0.002$). Comparisons of clinical measurements also showed significantly higher WBC count in the OSA group ($p = 0.048$) compared to controls (Table 11.4). There were, however, no other significant group differences in blood or urine chemistry measures (all $p > 0.05$). A summary of comparative differences in quantitative (PSG) and qualitative sleep assessments (FOSQ and ESS), and QoL (SF-36) questionnaires is provided in Table 11.5. With regards to PSG, AHI values were significantly higher in the OSA group ($p < 0.001$). There were, however, no significant group differences in SF-36, FOSQ, or ESS scores (all $p > 0.05$).

After correcting for influential variables, there were also no significant differences in c-IMT scores, FMD, or 24-hr SBP, DBP and LF/HF ratios between OSA and control subjects. However, 24 HR ($p = 0.027$) and AIx values ($p = 0.024$) were both higher in OSA subjects in comparison to controls (Table 11.6).

11.4.2. Retinal vascular function

11.4.2.1. Averaged response

Retinal vascular function parameters were averaged across three flicker cycles with the artery and vein regarded separately (Table 11.7). After correcting for influential covariates identified in multiple regression models arterial tMD ($p = 0.049$) was longer, while DA ($p = 0.003$), $Slope_{AD}$ ($p = 0.003$), and MC% ($p = 0.014$) were decreased in the OSA group compared to controls (Figure 11.5). No other group differences were identified in any of the other measured averaged retinal arterial and venous parameters (all $p > 0.05$).

11.4.2.2. Individual flicker cycles

Each flicker cycle was then considered individually with the artery and vein regarded separately and significant between- and within-group differences were identified with repeated measures ANOVA. In between-group comparisons, arterial tMD was significantly longer ($p = 0.048$) and arterial MC% was significantly decreased ($p = 0.013$) in the OSA group when compared to controls, but only during the third flicker cycle (Figure 11.6A). In within-group comparisons specific to the OSA group, arterial BDF ($p = 0.046$) and tMD ($p = 0.035$) were both significantly increased during the third flicker cycle compared to the first flicker cycle, and arterial MC% was significantly decreased in the third flicker cycle compared to the first ($p = 0.022$) and second ($p = 0.028$) flicker cycles (Figure 11.6B). No other significant between- or within-group differences were identified for any of the other measured retinal arterial and venous parameters (all $p > 0.05$).

11.4.2.3. Significant correlations

In within-group correlations, BMI significantly and negatively correlated with averaged $Slope_{AD}$ in OSA subjects ($r = -0.46$, $p = 0.045$). A similar trend was, however, not observed in the control group and no other significant correlations were identified (all $p > 0.05$).

Table 11.3. Summary of clinical data

Variable	Mean (SD) Group (1) OSA	Mean (SD) Group (2) Control	*p*-value (*t*-test)	Significance
N	9	9	-	-
Gender (% male)	77	68	-	-
Age (years)	53 (7)	53 (8)	0.767	-
BMI (kg/m²)	34.07 (6.44)	26.07 (3.25)	**0.002***	1 > 2
SBP (mmHg)	130 (13)	123 (14)	0.293	-
DBP (mmHg)	80 (7)	81 (11)	0.668	-
HR (bpm)	74 (7)	67 (11)	0.085	-
MAP	96.27 (7.89)	95.37 (11.43)	0.833	-
IOP (mmHg)	15 (2)	14 (2)	0.239	-
OPP	49.55 (5.79)	50.08 (8.07)	0.863	-
GLUC (mmol/L)	4.64 (0.58)	4.61 (0.61)	0.930	-
TG (mmol/L)	2.13 (1.55)	1.34 (0.50)	0.194	-
CHOL (mmol/L)	5.23 (1.32)	5.19 (0.86)	0.947	-
HDL-C (mmol/L)	1.23 (0.53)	1.21 (0.35)	0.952	-
LDL-C (mmol/L)	3.02 (1.05)	3.36 (0.78)	0.478	-
TG/HDL-C	2.15 (1.97)	1.25 (0.73)	0.246	-
CHOL/HDL-C	4.65 (1.61)	4.54 (1.31)	0.880	-
tGSH (µmol/L)	975 (348)	1334 (522)	0.105	-
GSH (µmol/L)	828 (317)	1163 (472)	0.095	-
GSSG (µmol/L)	74 (22)	86 (43)	0.463	-
Redox index	11 (3)	17 (11)	0.169	-
ET-1 (pg/mL)	1.26 (0.15)	1.20 (0.09)	0.496	-
NO (µmol/L)	6.46 (2.89)	5.21 (2.19)	0.394	-
FRS (%)	10.04 (4.23)	8.87 (5.99)	0.651	-

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; IOP, intraocular pressure; OPP, ocular perfusion pressure; GLUC, glucose; TG, triglycerides; CHOL, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; GSH, reduced glutathione; GSSG, oxidized glutathione; tGSH, total GSH; Redox index, GSH:GSSG; ET-1, endothelin-1; NO; nitric oxide; FRS, Framingham risk score.
* Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 11.4. Summary of clinical data contd.

Variable	Mean (SD) Group (1) OSA	Mean (SD) Group (2) Control	*p*-value (*t*-test)	Significance
Basophil count (10^9/L)	0.05 (0.02)	0.03 (0.01)	0.114	-
Eosinophil count (10^9/L)	0.27 (0.24)	0.14 (0.06)	0.249	-
Lymphocyte (10^9/L)	2.00 (0.44)	1.58 (0.21)	0.052	-
Monocyte (10^9/L)	0.55 (0.18)	0.43 (0.11)	0.201	-
Neutrophil (10^9/L)	3.78 (1.47)	2.42 (0.69)	0.056	-
Total WBC (10^9/L)	6.65 (2.02)	4.68 (1.06)	**0.048***	1 > 2
RBC count (10^2/L)	5.90 (2.67)	4.67 (1.52)	0.289	-
Haemoglobin (g/L)	122.37 (51.07)	114.30 (57.48)	0.803	-
Haematocrit	0.44 (0.04)	0.44 (0.07)	0.785	-
Platelet count (10^9/L)	244.22 (70.29)	233.0 (71.38)	0.768	-
MCV (fL)	90.20 (8.00)	93.52 (8.57)	0.458	-
MCH (pg/cell)	29.28 (1.71)	29.60 (0.85)	0.679	-
HbA1c (%)	5.70 (0.45)	5.78 (0.34)	0.717	-
Na^+ (mmol/L)	140.88 (2.59)	139.86 (2.85)	0.481	-
K^+ (mmol/L)	4.34 (0.44)	4.30 (0.49)	0.900	-
Urea (mmol/L)	4.56 (1.13)	4.94 (0.97)	0.505	-
Creatinine (μmol/L)	80.38 (17.85)	79.43 (15.18)	0.914	-
Albumin (g/L)	41.00 (1.41)	41.14 (3.63)	0.924	-
Globulins (g/L)	31.29 (3.15)	31.14 (3.02)	0.932	-
Total Bilirubin (μmol/L)	14.57 (8.85)	11.43 (6.02)	0.452	
Total Protein (g/L)	72.29 (4.23)	72.43 (4.54)	0.952	-
ALP (U/L)	74.29 (13.62)	65.29 (6.32)	0.139	-
Urine creatinine (mmol/L)	17.25 (5.96)	10.30 (9.25)	0.276	-
Urine ACR (mg/mmol)	4.33 (5.58)	1.03 (0.85)	0.368	-

Abbreviations: WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; HbA1c, glycated haemoglobin; Na^+, sodium; K^+, potassium; ALP, alkaline phosphatase; ACR, albumin/creatinine ratio. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 11.5. Summary of group differences in sleep and QoL assessments

Variable	Mean (SD) Group (1) OSA	Mean (SD) Group (2) Control	*p*-value (*t*-test)	Significance
PSG				
AHI (*n*/h)	42 (24)	2 (2)	**<0.001***	1 > 2
SF-36				
Physical functioning	67.50 (20.00)	78.50 (16.51)	0.219	-
Role, physical	62.50 (35.36)	72.50 (36.23)	0.565	-
Bodily pain	63.13 (26.88)	66.80 (23.70)	0.762	-
General health	61.25 (13.56)	55.40 (20.29)	0.495	-
Vitality	51.88 (23.44)	54.00 (25.65)	0.862	-
Social functioning	64.06 (36.25)	78.75 (28.29)	0.348	-
Role, emotional	70.84 (37.54)	86.67 (32.20)	0.350	-
Mental health	78.00 (19.94)	66.80 (20.05)	0.255	-
FOSQ				
General productivity	2.99 (0.74)	3.37 (0.63)	0.244	-
Social outcome	2.78 (1.10)	2.94 (0.86)	0.718	-
Activity level	3.24 (0.76)	3.71 (0.34)	0.091	-
Vigilance	3.39 (0.86)	3.65 (0.58)	0.443	-
Total FOSQ	15.49 (3.85)	17.09 (2.59)	0.298	-
ESS	7.00 (5.52)	5.43 (4.12)	0.540	-

Abbreviations: PSG, polysomnography; AHI, apnoea/hypopnea index; *n*/h, number of episodes per hour; SF-36, short-form 36, FOSQ, functional outcomes of sleep questionnaire; ESS, Epworth sleepiness scale. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 11.6. Summary of systemic vascular function parameters

Parameter	Mean (SD)		*p*-value (*t*-test/ANCOVA)	Significance
	Group (1)	Group (2)		
	OSA	Control		
24h BP and HRV				
SBP	121 (9)	119 (14)	0.761	-
DBP	72	74	0.653	-
HR	75 (6)	67 (7)	**0.027***	1 > 2
LF/HF ratio	2.48 (0.47)	3.76 (2.85)	0.351	-
logLF/HF ratio	0.39 (0.08)	0.46 (0.36)	0.675	-
HRVTi	38.38 (15.13)	42.83 (17.17)	0.616	-
c-IMT				
R-IMT (cm)	0.76 (0.14)	0.68 (0.12)	0.119	-
L-IMT (cm)				
PWA				
AIx	25 (10)	18 (12)	**0.024***	1 > 2
FMD				
$AD_{baseline}$ (mm)	4.59 (0.72)	4.67 (1.26)	0.860	-
$MD_{hyperaemia}$ (mm)	5.08 (0.74)	5.28 (1.24)	0.672	-
FMD_{ED} (%)	11.33 (10.43)	14.17 (10.24)	0.562	-

Abbreviations: BP, blood pressure; HRV, heart rate variability; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LF, low-frequency bands; HF, high-frequency bands; LF/HF ratio, used as a marker of sympathovagal balance; HRVTi, triangulation index; c-IMT, carotid intima-media thickness; R, right; L, left; PWA, pulse-wave analysis; AIx, augmentation index; FMD, flow-mediated dilation; AD, average baseline brachial diameter; $MD_{hyperaemia}$, maximum brachial diameter during hyperaemia; FMD_{ED}, endothelium-dependent flow-mediated dilation. *Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

Table 11.7. Summary of averaged retinal vascular function parameters

DVA parameter	Mean (SD)		*p*-value	Significance
	Group (1)	Group (2)		
	OSA	Control		
Arteries:				
Baseline	99.87 (0.56)	99.81 (0.37)	0.784	-
BDF	5.87 (2.61)	5.80 (2.34)	0.951	-
BCFR	-1.10 (3.64)	-0.20 (2.46)	0.546	-
DA	4.80 (2.09)	6.20 (2.22)	**0.003***	1 < 2
MD%	3.64 (2.08)	3.99 (1.48)	0.690	-
MC%	-0.91 (3.14)	-2.24 (3.24)	**0.014***	1 < 2
tMD (seconds)	23 (11)	18 (9)	**0.049***	1 > 2
tMC (seconds)	28 (9)	29 (15)	0.764	-
$Slope_{AD}$	0.22 (0.19)	0.43 (0.46)	**0.003***	1 < 2
$Slope_{AC}$	-0.20 (0.09)	-0.28 (0.18)	0.270	-
Veins:				
Baseline	99.75 (0.74)	99.88 (0.37)	0.661	-
BDF	5.24 (2.82)	5.30 (4.54)	0.972	-
BCFR	-0.20 (3.10)	-0.21 (2.75)	0.997	-
DA	4.98 (2.80)	5.03 (1.93)	0.964	-
MD%	3.74 (2.09)	3.77 (1.96)	0.980	-
MC%	-1.22 (1.06)	-1.31 (1.50)	0.990	-
tMD (seconds)	24 (6)	23 (10)	0.880	-
tMC (seconds)	31 (6)	30 (9)	0.747	-
$Slope_{VD}$	0.21 (0.13)	0.23 (0.18)	0.808	-
$Slope_{VC}$	-0.23 (0.15)	-0.20 (0.13)	0.756	-

Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, baseline corrected flicker response; DA, dilation amplitude; MD%, percent dilation; MC%, percent constriction; tMD, reaction time to MD; tMC, reaction time to MC (time between MD and MC); $Slope_{AD/VD}$, slope of arterial/venous dilation; $Slope_{AC/VC}$, slope of arterial/venous constriction. * Significant *p*-values are indicated in bold where $p < 0.05$ was considered significant.

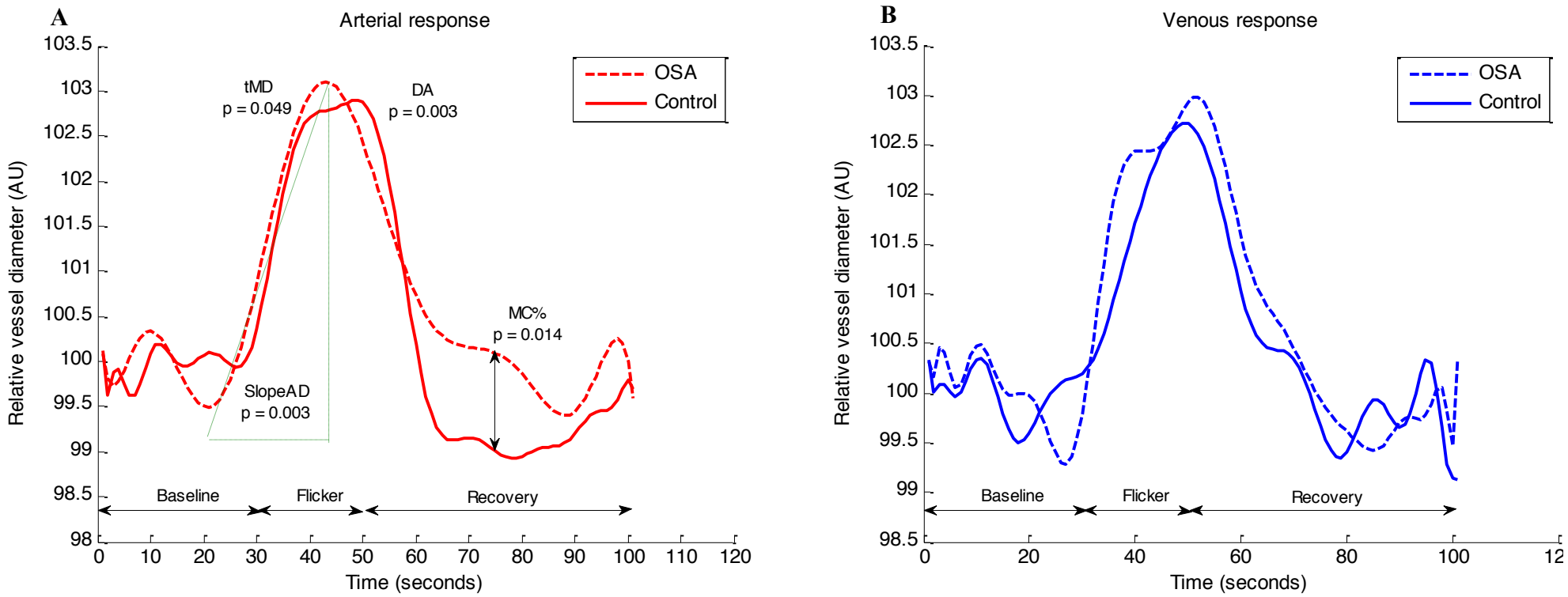

Figure 11.5. Group comparisons of averaged retinal vascular response profiles
(A) arterial response; (B) venous response
Abbreviations: AU, arbitrary units; DA, dilation amplitude; tMD, time to maximal dilation diameter; MC% percent constriction; $Slope_{AD}$, arterial dilation slope

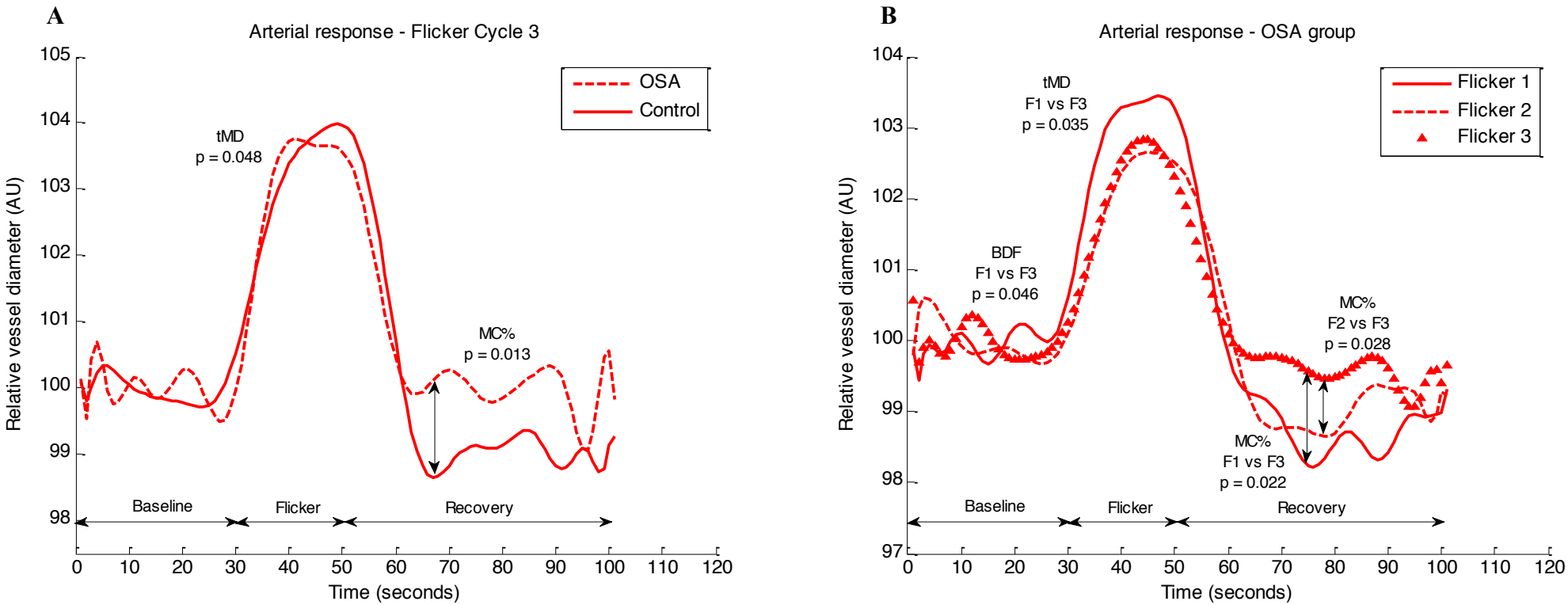

Figure 11.6. Significant within- and between-group differences
(A) between-group comparison of arterial response for the third flicker cycle; (B) within-group comparison of arterial response during each flicker cycle in the OSA group
Abbreviations: AU, arbitrary units; BDF, baseline diameter fluctuation; tMD, time to maximal dilation diameter; MC% percent constriction

11.5. Discussion

The results of this preliminary study indicate that OSA subjects with untreated moderate to severe sleep apnoea, and low to moderate cardiovascular risk, exhibit signs of systemic arterial stiffness, increased sympathetic drive, and retinal vascular dysfunction.

An important finding of this study is the presence of selective arterial impairments in response to stress at the retinal microvascular level in OSA subjects (greater fluctuations in arterial baseline diameter, delayed reaction times, and abatements in the re-establishment of baseline diameter). Previous studies reporting retinal abnormalities in OSA patients are those detected with static fundus assessments [595], and in patients with confounding risk variables such as obesity [596] and diabetes [597]. The inherent static nature of the assessment also conveys more information on established rather than developing disease. On the other hand, functional abnormalities may only be revealed when the vascular system is stressed, and the present study reports, for the first time, such changes at the retinal microvascular level in OSA patients with no apparent signs of structural retinal disease. While the precise mechanisms underlying these observations in our pilot sample need further elucidation in larger cohorts, some hypotheses can still be formulated.

It is well known that vascular disturbances occurring in a particular vascular bed are not secluded and both the origination and risk for further wider vascular dysfunctions can be triggered by common underlying mechanisms, which can act in a concerted manner in the macro- and microcirculations. In the present study, OSA patients exhibited increased arterial stiffness, which has previously been linked to vascular remodelling in retinal vessels [598], further underscoring the importance of the pulsatile component of organ perfusion and the interrelationships between the macro- and microcirculations. In the present study, however, attenuations in retinal vascular function appeared to be independent of increased arterial stiffness observed in the OSA group, though this could be attributed to the pilot nature of the study design and would need to be confirmed in a larger cohort. It cannot, however, be excluded that local microvascular stiffness or generalized increases in vascular tone played some role in these findings as there were also indications of increases in 24-hour heart rate in OSA patients, corroborating previous reports of augmented cardiac sympathetic drive in these groups of patients [599]. Notably, the episodic respiratory abnormalities in OSA can trigger hypoxemic or hypercapnic episodes, and the subsequent re-oxygenation phenomena have been known to have a profound effect on vascular function [600], both of local and widespread systemic consequence, and including autonomic and haemodynamic aspects [601-604]. At the retinal level, where autoregulatory

mechanisms take over, however, it is more probable that vascular disturbances are linked to local variations in vasoactive factors.

A key role is reserved for NO in the regulation of retinal vessel tone [254] and intermittent hypoxia in OSA can induce free radical production [605, 606] which seek to quench NO, and can then, in turn, impair vascular endothelial function [560, 593, 607-614]. A reduced retinal arterial dilation response slope was observed in our OSA subjects, which was associated with higher BMI levels in this group. This finding is consistent with reports of a higher incidence of OSA in individuals with increased BMI [615], as well as, with studies showing that a reduction in the bioavailability of NO maybe responsible for obesity-induced vascular complications [616]. A closer evaluation of retinal vascular function parameters during individual flicker cycles, however, revealed attenuated vascular function in retinal arteries to be more distinct during the third flicker cycle in OSA subjects. It is possible that delayed vessel reactions during the third flicker cycle represent an early sign of vascular dysfunction due to decreases in the bioavailability of NO or an exhaustion of NO reserves following the first two flicker cycles [617]. Alterations in vessel reactivity and elasticity could also reflect disequilibrium in local vasodilatory and vasoconstrictory influences, signalled by the fluctuations in arterial baseline diameters (before the onset of flicker) in OSA patients.

Systemic circulatory assessments of NO levels, however, did not show any significant differences between groups in this study, but whether the sources of NO secretion in the regulation of vascular tone and *ad hoc* NO secretion during flicker are the same still remains to be established. An additional finding of this study was the attenuation of retinal arterial vasoconstrictive responses following flicker in OSA subjects, which is comparable to what has previously been reported in conditions associated with hypoxia [339]. However, it is unclear whether this observation could be a consequence of vessel behaviour during stress and possibly reflects the vessel's inclination to remain in a distended state in response to provocation. Moreover, the diminished capacity to re-establish baseline diameter, to some extent, follows what was reported in Chapter 5 with regards to the ageing effect in middle-aged individuals. Nevertheless, the observed attenuations in dilation capacity indeed signal the presence of vascular dysfunction that is distinct from normal vascular responses in ageing vessels.

An interesting observation in this study was the increases in WBC counts in the OSA group. Increases in circulating WBCs represent a nonspecific marker of inflammation, and

structural retinal studies show that inflammation and endothelial dysfunctions are common events in the development of retinal vascular changes [101]. While the independent or concerted effects of these processes remain to be confirmed, the observed alterations in retinal vascular function in OSA patients appear to be underscored by subtle but appreciable alterations in vascular tone or compliance. Blood flow studies [241] also show that increases in circulating WBCs can influence flow regulation in retinal vessels [618], and it could therefore be suggested that WBC aggregation or adherence may have influenced retinal vessel reactivity in OSA subjects. Nonetheless, all of the above stated hypotheses require further clarification and research, and the preliminary design of this study, with small numbers of patients in the control and OSA groups, can therefore represent a drawback. The findings of this study, however, open up several research avenues that remain to be explored, particularly with regards to the influence of dietary and lifestyle factors based on the noted association between OSA and BMI levels. Further studies to evaluate the impact of OSA treatment on retinal and systemic vascular function could also inform early preventative care and management strategies aimed at reducing the risk of cardiovascular complications in this patient group.

11.6. Conclusion

Significant attenuations in retinal vascular function exist and can be detected in patients with untreated moderate to severe OSA, and before the appearance of functional vascular changes in larger vessels. Functional retinal assessments could therefore be useful for early vascular screening in these and other groups of at-risk or high-risk patients, and for monitoring vascular function in response to targeted therapies.

12. General Summary & Discussion

The importance of vascular risk factors in the development and progression of cardiovascular diseases has been studied extensively; nevertheless assessments of vascular function are still underappreciated in the current scheme of cardiovascular risk appraisals, and even fewer translate into clinical management strategies. Moreover, risk assessment engines, though useful from a public health perspective, have poorer predictive value for the individual patient. The continued search for additional risk markers, that improve conventional risk assessments, further illustrates a growing need in current platform of individualized screening and prevention strategies. In this regard, detailed vascular profiling, at the individual rather than population level, could guide the shift towards personalised medicine, especially in cases that go on to develop CVD despite having low predicted cardiovascular risk.

Vascular imaging studies are of particular clinical interest, and on the basis that microvessels are an early target in the onset and progression of vascular diseases, this thesis has been concerned with assessing retinal microvascular function to investigate whether microcirculatory dysfunctions can be present and detected at an early stage in otherwise healthy individuals with risk factor profiles consistent with low to moderate cardiovascular risk. As the evaluation of one vascular bed can be limiting, the studies in this thesis also sought to provide a more comprehensive picture of vascular health with the use of additional validated surrogate markers for vascular structure and function, and cardiovascular risk (c-IMT, PWA, FMD, ANS function, FRS). It was thus postulated that outcomes substantiating the relevance of retinal vascular function against these measures could foster new strategies in cardiovascular screening, prevention, and management. The main findings of the studies in this thesis are summarized below.

12.1. Ageing effect on retinal vascular function

A publication of this work is provided in Appendix B.

Age is one of the most important non-modifiable risk factors for CVD, and a key adaptation associated with altered structure and functionality of ageing vessels, is in response to endothelial dysfunction [53, 54, 619]. The study described in Chapter 5 of this thesis investigated the ageing effect on retinal microvascular function in response to provocation on the basis that even in otherwise healthy individuals, problematic vascular coping mechanisms may only be apparent when the vascular system is stressed. Since pathological

changes must be carefully differentiated from normal vascular ageing, this study provided a more in-depth analysis of the retinal vascular response parameters in low-risk individuals belonging to various age groups. Relatively few studies have adequately defined the effects of ageing on the flicker-evoked retinal responses, and there is generally a lack of reference data with regards to retinal vascular function parameters in different age groups. There are also inconsistencies in the few existing reports as to whether or not age has indeed an effect on flicker-evoked retinal responses [258, 260, 308]. Most retinal function studies also tend to examine the dilation component of the reactive vessel profile while responses following the cessation of stress are less well examined. This study therefore also examined the dynamics of the constriction response and retinal vessel capacity in re-establishing baseline diameters after the cessation of stress.

Using this more detailed approach for the analysis, a significant effect of age was identified in the arterial constriction component. In younger individuals, as part of the normal vascular response profile, flicker-evoked vasodilations were followed by constriction responses to amplitudes below baseline levels before reaching equilibrium, and this was consistent with what has been reported in other studies including younger subsets of participants [257, 312]. A novel finding of this study, however, was a possible gender or sex influence in younger persons that was not identified in the other older age groups. The absence of a sex influence in older subjects was somewhat unexpected given the changes in hormonal status that occurs across the lifespan. However, rather than these effects being lost with ageing, it could alternatively be suggested that younger women exhibit less pronounced arterial dilation, and that the variation between men and women in the retinal vasoregulative response to stress with age may correspond with the known variation in vascular risk between men and women. For instance, cardiovascular diseases tend predominantly to be male health concern [620], however, more recent studies suggest that CVD risk in women is severely underestimated by traditional risk scoring systems like the FRS [621]. Moreover, microcirculatory dysfunctions in the coronary vessels are identified as contributing factors to poor prognosis in women with no signs of obstructive disease [60]. Concurrently, the findings in the present study could offer support for the hypothesis that sex hormones may contribute to or amplify microcirculatory sex-specific differences occurring with age, and as such functional retinal studies could be a more sensitive indicator of endothelial function and vascular risk specific to men or women. Even more important is the extent to which these microcirculatory anomalies signal the clinical debut of vascular disease in both men and women, and in either case the early identification of

risk is paramount. In addition to longitudinal follow-up studies, future studies that independently examine the ageing effect between younger and older women, and younger and older men could therefore be useful in furthering our understanding of the independent influences of age and sex on retinal vascular function.

An additional observation in this study was the absence of post-flicker arterial and venous over-constrictions in older subjects. Based on previous conflicting reports [269, 300], it was unclear whether over-constrictions during the re-establishment of baseline diameter, may or may not be absent in older subjects, however, older participants indeed exhibited decreased capacity for the re-establishment of baseline diameter. It is known that decreased vessel distensibility and focal narrowing occur in ageing vessels independently of other arteriosclerotic risk factors [96, 100, 429], and even though vascular adaptations to structural remodelling and changes in viscoelastic properties may be adequate, any limitations in functional vascular reserves may only be evident during responses to provocative stressors. Likewise in the present study, though AIx values were increased in older participants signalling increased arterial stiffness, retinal vasodilatory capacity in response to flicker stimulation was preserved. It is plausible that the functional mechanisms involved in *ad hoc* NO secretion during flicker stimulation are still preserved, but as basal NO secretion decreases with age [622-624] vessel behaviour favours sustained distension when the vascular system is stressed and the absences in post-flicker over-constrictions reflect the aftereffects of this behaviour. Nevertheless, the exact mechanisms responsible for these age-associated alterations remain to be investigated and since ageing and endothelial dysfunction progress in parallel, functional changes as reflected at the level of the microcirculation may provide further insight into the pathogenic processes in CVD. For instance, other studies in this thesis comprising an older subsets of participants demonstrate a shift in vessel behaviour towards a more exaggerated constrictive state post-flicker, and while the causes or consequences of this shift in vessel behaviour remain to be investigated, a possible contender is oxidative stress, which on the one hand is a known cause of endothelial cell senescence and dysfunction and on the other hand is also recognized as a consequence of accelerated vascular ageing.

12.2. Retinal vascular function in healthy individuals with a family history of cardiovascular disease

Family histories of disease reflect not only the presence of risk but also bridge the gap between genetics and genomics and complex gene and environment interactions that influence vascular risk [445, 446, 625]. Patients with a history of familial CVD are at increased

risk for atherosclerosis and exhibit attenuated endothelial function [445, 626, 627]. For example, studies in conduit vessels such as the brachial artery and epicardial vessels have reported impairments in endothelium-dependent vasomotor responses in those with familial risk factors [449, 450]. With the tendency of other vascular risk factors (hypertension, hyperglycaemia, or hyperlipidaemia) to aggregate in families inherited vascular risk can be mediated by a number of ways, but the relative contribution of FH as an independent risk factor is still not adequately understood. It is recognized that presence of confounding factors in families with and without CVD may be unequally distributed, and generally the risk associated with FH is mainly considered in patients with established disease, overlooking the possibility that FH may be more important in identifying subsets of at-risk groups who are otherwise considered low risk. Indeed, a previous study examining the independent effect of FH found a greater aggregate incidence and earlier age of disease onset, in relatives of individuals with low cardiovascular risk compared to higher risk patients. These data suggest that FH is still important in low risk cases, where other risk factors may be absent or negligible, and moreover that the threshold for clinically overt disease is likely to be lower in this group [446].

To date there are no studies that have charted the association between FH and retinal vascular function, however, functional retinal abnormalities are apparent in patients with cardiovascular risk factors that can aggregate in families [258, 281, 300, 318,262, 309, 325]. Collectively these studies show that retinal function can be useful for studying systemically induced vascular complications, however, the question still remains whether functional retinal assessment is convenient for evaluating early CVD risk in groups with more subtle cardiovascular risk factors and no apparent signs of systemic vascular complications. Moreover, our understanding about when the first recognizable signs of vascular dysfunction occur in patients with disease, and or in those at risk for disease, is limited. The study described in Chapter 6 therefore investigated retinal microvascular function in response to flicker provocation, and its relationship to macrovascular structural and functional tests, in otherwise healthy individuals with low cardiovascular risk profiles but with familial CVD. In these individuals, signs of retinal vascular dysfunction were characterized as decreases in arterial baseline diameter fluctuations, regulative amplitudes, and dilation responses, as well as, post-flicker over-constrictions in both arteries and veins. Moreover, while systemic vascular function (c-IMT and FMD) was preserved, increases in arterial constrictions correlated with decreases in HDL cholesterol levels in FH positive individuals. It cannot, however, be excluded that in individuals with higher CVD risk than

those included in the present study a positive correlation between anomalies identified at the macro- and micro-vascular levels would become apparent. Nevertheless, the early identification of vascular risk should occur when the disease is subclinical and well before structural changes begin to appear. The findings of the present study therefore suggest that functional retinal assessment could be useful for the early identification of vascular risk, particularly since dysfunctional retinal responses correlated with an established cardiovascular risk indicator (HDL-c) that was decreased but still above the clinical threshold considered for cardiovascular risk. A further understanding of the relationships between endothelial function and familial risk may also be an important step towards the early identification of asymptomatic at-risk individuals who could benefit from early targeted and individualized therapies, and identify non-responders to therapy.

12.3. Ethnic differences in retinal vascular function

The unique phenotypic profile of SAs appears to differentiate them from other ethnic groups with regards to cardiovascular risk [628, 629] and SAs suffer higher cardiovascular-related mortality [475, 630, 631]. Although it is apparent that other conventional cardiovascular factors such as lipid levels confer risk in SAs, traditional risk assessments still do not adequately explain excess risk in this ethnic group. With the continued search for additional risk markers, a greater emphasis is being placed on techniques that are simple, non-invasive, and easy to apply in clinical or primary care settings. Consequently, non-invasive vascular markers that can be used as proxies to detect the presence of risk in at-risk populations or individuals in whom conventional definitions of risk can be a misattribution are of added clinical value. In this regard, functional retinal assessment is a particularly attractive tool and has already been validated in both healthy and at-risk individuals with minor vascular pathologies. The study described in Chapter 7 therefore sought to evaluate the relationship between retinal microvascular function and cardiovascular risk in SAs.

The main study findings showed that low-risk middle-aged SAs exhibit attenuated retinal arterial dilation responses to flicker that were associated with higher total to HDL cholesterol ratios (a well-accepted marker of vascular risk in SAs), as well as, post-flicker over-constrictions in both arteries and veins. A delay in the re-establishment of arterial baseline diameter also correlated with systemic redox status. SAs are indeed considered to be more susceptible to the unfavourable effects of dyslipidaemia-induced oxidative stress and, moreover, at lower thresholds of lipids. The combined effect of abnormal glutathione and circulating lipids could therefore amplify endothelial dysfunction at all vascular levels,

including the retinal vessels. This possibility is important for screening and intervention, and may further underscore the functional retinal irregularities observed in SAs in this study. These tentative associations also implicate not just an autoregulative but biochemical involvement in the precipitation of selective functional retinal impairments observed in the SA group in this study. The interplay between retinal vascular function parameters and biochemical markers for endothelial function and oxidative stress, however, illustrates the complex nature of vascular risk in SAs; and at present it is not clear to what extent genetic, environmental, dietary or lifestyle factors could have contributed to the expression of the observed irregularities. A scarcity in the availability of normative and prospective retinal studies specific to SAs also precludes the interpretation of ranges and thresholds for retinal vascular function parameters that can be used as surrogate risk indicators in this group. Nevertheless, the data presented in this study provide an opportunity for further work to validate whether the use of functional retinal assessments can be beneficial for ethnic vascular screening, as well as, for monitoring therapeutic approaches and established vascular disease in multi-ethnic societies.

12.4. Systemic circulatory influences on retinal vascular function

A publication of this work is provided in Appendix C.

The intimate association between oxidative stress, atherosclerosis, and cardiovascular risk has been substantiated extensively however; few studies have examined this relationship with regards to the microcirculation. Microvessels are often affected much earlier in the course of disease progression and there is evidence that shows systemic contributions to pathological oxidative stress in the retina and oxidative stress-related contributions to retinal vascular alterations [632]. The POLA study [633] was recently among the first to show an independent association between biomarkers of oxidative stress and retinal vessel calibres. Functional retinal assessments can, however, convey more information on developing rather than established disease. The study described in Chapter 8 therefore investigated the potential influences of established plasma markers for vascular risk and systemic anti-oxidative defence capacity on retinal microvascular function, in otherwise healthy individuals but with low to moderate cardiovascular risk.

The main study findings were that systemic antioxidant capacity (redox index) and plasma markers for cardiovascular risk (LDL-c) influenced retinal microvascular function at both arterial and venous levels. In particular, in low- to moderate-risk individuals the retinal arterial dilation slope was influenced by redox index, while the constriction response slope was influenced by GSH levels; and in retinal arteries and veins LDL-c levels negatively

influenced spontaneous variations in resting baseline diameters (BDF) before the initiation of flicker.

The direct relationship observed between systemic anti-oxidative defence capacity, plasma markers for CVD risk, and retinal dynamic vascular responses in apparently healthy individuals points toward more complex regulatory mechanisms that are involved in the retinal vascular response to stress. Nevertheless, based on the risk category of individuals included in this study, functional retinal assessment could indeed be a more sensitive indicator of individualized risk. As emerging biomarkers and vascular imaging markers drive the shift towards personalized medicine, retinal vascular function can be used for individualized risk profiling as it can provide an integrated and dynamic analysis of vascular function as a variable specific to each individual. Nevertheless, beside the action of local factors, a better understanding of the possible systemic influences on microvascular retinal function in individuals with low to moderate CVD risk is still needed.

12.5. Retinal vascular function in individuals with obstructive sleep apnoea: a preliminary study

There are several lines of evidence that suggest sleep patterns could affect vascular tone and endothelial function even in healthy subjects – based on shear stress, plasma catecholamine levels [634], and altered sympathetic activity [635]. Patients with sleep disordered breathing such as OSA are recognized as being increasingly susceptible to endothelial dysfunction [636, 637] which can contribute to the genesis of and progression of atherosclerotic cardiovascular phenomena, also frequently encountered in this group of patients [638]. One mechanism involves OSA propagating endothelial dysfunction through hypoxia, ROS generation, as well as, sympathetic activation during frequent awakenings; which are in turn associated with vasoconstriction and thrombosis [85]. There is also compelling but conflicting evidence of systemic influences on retinal vessels calibres in OSA patients. Nevertheless, the static nature of retinal assessments in these cases conveys more information on established disease rather than adaptive vascular responses to stress that can be identified before the onset of structural phenomena. There is however limited data on functional retinal responses in OSA as no studies have directly characterized retinal vascular function in OSA patients. The study in Chapter 9 therefore evaluated the functional retinal vascular response to flicker in untreated moderate to severe OSA patients. Given the numerous systemic associations of OSA, this study also included

assessments of systemic structural, functional, and circulatory markers of vascular function.

The main study findings were that subjects with moderate to severe untreated OSA exhibited delayed responses to flicker, as well as, a diminished capacity to re-establish baseline diameter post-flicker. It is well known that vascular disturbances occurring in a particular vascular bed are not secluded and both the origination and risk for further wider vascular dysfunctions can be triggered by common underlying mechanisms. OSA patients did display signs of increased arterial stiffness, which has previously been linked to vascular remodelling in retinal vessels [598]. Although attenuations in retinal vascular function appeared to be independent of arterial stiffness this lack of significance could be attributed to the pilot nature of the study design, and it cannot be excluded that local microvascular stiffness played some role in these findings. Likewise, the possibility of increased vascular tonus is further corroborated by the increased 24-hour pulse rates in OSA patients. Nevertheless, at the retinal level, where autoregulatory mechanisms take over, it is more probable that vascular disturbances are linked to local variations in vasoactive factors. In this regard, further evaluation of the vascular function parameters for individual flicker cycles in OSA patients showed attenuations in retinal responses to be more distinct during the third flicker cycle compared to the first and second flicker cycles. It is possible that delayed vessel reactions during the third flicker cycle represent an early sign of vascular dysfunction due to decreases in the bioavailability of NO or an exhaustion of NO reserves following the first two flicker cycles [617]. Alterations in vessel reactivity and elasticity could also reflect a possible disequilibrium in local vasodilatory and vasoconstrictory influences, signalled by increased fluctuations in arterial baseline diameters (before the onset of flicker) in OSA patients. Systemic assessments of NO levels, however, did not show any significant differences between groups in this study and it is unclear whether the sources of NO secretion in the regulation of vascular tone and that supplied *ad hoc* on demand of neurovascular coupling are the same. The pilot design of this study, with relatively small numbers of patients in both the control and OSA groups, however represent a drawback and further validation studies in a larger cohort are still needed. In addition, studies that evaluate the impact of OSA treatment on retinal and systemic vascular function could also inform early preventative care and management strategies aimed at reducing the risk of cardiovascular complications in this patient group.

13. Conclusions & Future Directions

The studies in this thesis show that functional retinal assessment is useful as a surrogate marker for vascular function and could represent a suitable practice for the screening and monitoring of established vascular disease. It is however appreciated that the studies devised in this thesis are still subject to a number of potential limitations. For instance, due to the cross-sectional nature of the study designs, causal relations cannot be established, and further longitudinal studies with regular follow-up intervals and outcome analyses that investigate the temporal associations between vascular function and the development and progression of disease would strengthen the value of functional retinal assessment as a cardiovascular risk stratification tool. It is also acknowledged that much of the variability in specific retinal vascular function parameters in the general population remains to be explained as the role of conventional risk factors in determining retinal vascular reactivity across the range usually found in the general population and not just at extremes of the range remains to be quantified. Nevertheless, as supported by findings in this thesis functional retinal assessments provide an opportunity to explore the relationship between vascular risk factors, endothelial dysfunction, and associative cardiovascular risk, and the presented results open up several avenues that can be explored in future work.

13.1. Population-based studies

To determine the role of retinal vascular function for the purposes of screening and intervention in sub-categories of at-risk individuals, larger cohorts of patients need to be established in future studies. The FRS was used in the present studies to categorize relative cardiovascular risk, which is a multivariable proxy often considered by clinicians in evaluation of CVD risk. However, owing to the shortcomings of predictive risk scores such as the FRS, future work should examine the direct relationship between retinal microvascular reactivity and FRS in multivariable regression models to evaluate whether the retinal vascular function parameters could improve upon predictive risk models. This data would help interpret the inter-relationships between risk factors and further evaluate risk severity in sub-categories of at-risk individuals such as those with familial risk, or SAs and other ethnic minorities, and moreover with those with overlapping categories of risk (for example, SAs with familial risk).

13.2. Data analysis

Further work is also needed to establish a normative reference database with regards to age, gender or sex, and ethnicity to evaluate the clinical significance of individual retinal response parameters. It is evident that the timing of the dilation and constriction responses and the curve of vessel behaviour during and after provocation can vary depending not only on vessel type but also in the presence of various risk factors. Most functional retinal studies using DVA include these parameters but tend to focus on reactive responses during flicker likely owing to the complexities involved in interpreting a biological control loop. Nevertheless, the consideration of the both dilatory and constrictory response components and the calculation of the dilation and constriction slopes were successfully applied in the present studies and can further extend the development of algorithms and data-mining applications in larger cohorts.

As part of preliminary work, the retinal vascular function parameter data collected in this thesis aided in the development of a 'Vascular Analysis Software' tool (developed by the laboratory of Dr. Aniko Ekart, School of Engineering and Applied Sciences, Aston University, Birmingham UK) that could be applied to characterize deviations in retinal arterial and venous behaviours. As described in this thesis, the analysis of DVA parameters required manual extraction of the raw from the device software and implementation of a Matlab algorithm to (i) calculate each of the vascular response parameters for arteries and veins separately and for each patient; (ii) generate vascular response curves for individual flicker cycles for each vessel and for each patient; (iii) generate averaged vascular response curves for each vessel for each patient; (iv) generate comparative vascular response curves (averaged across all patients) to be included in a particular study. As such, the purpose of the software tool is automated report generation for individual patients based on the same Matlab algorithm used in this thesis. The software enables the addition of new patients to the database, which can be continually updated. Moreover, each patient's data can then be compared with that of any existing patient in the database, as well as, with the averaged normal data for his / her age group with the artery and vein regarded separately. A sample report that could potentially be generated by the software is provided in Figure 13.1.

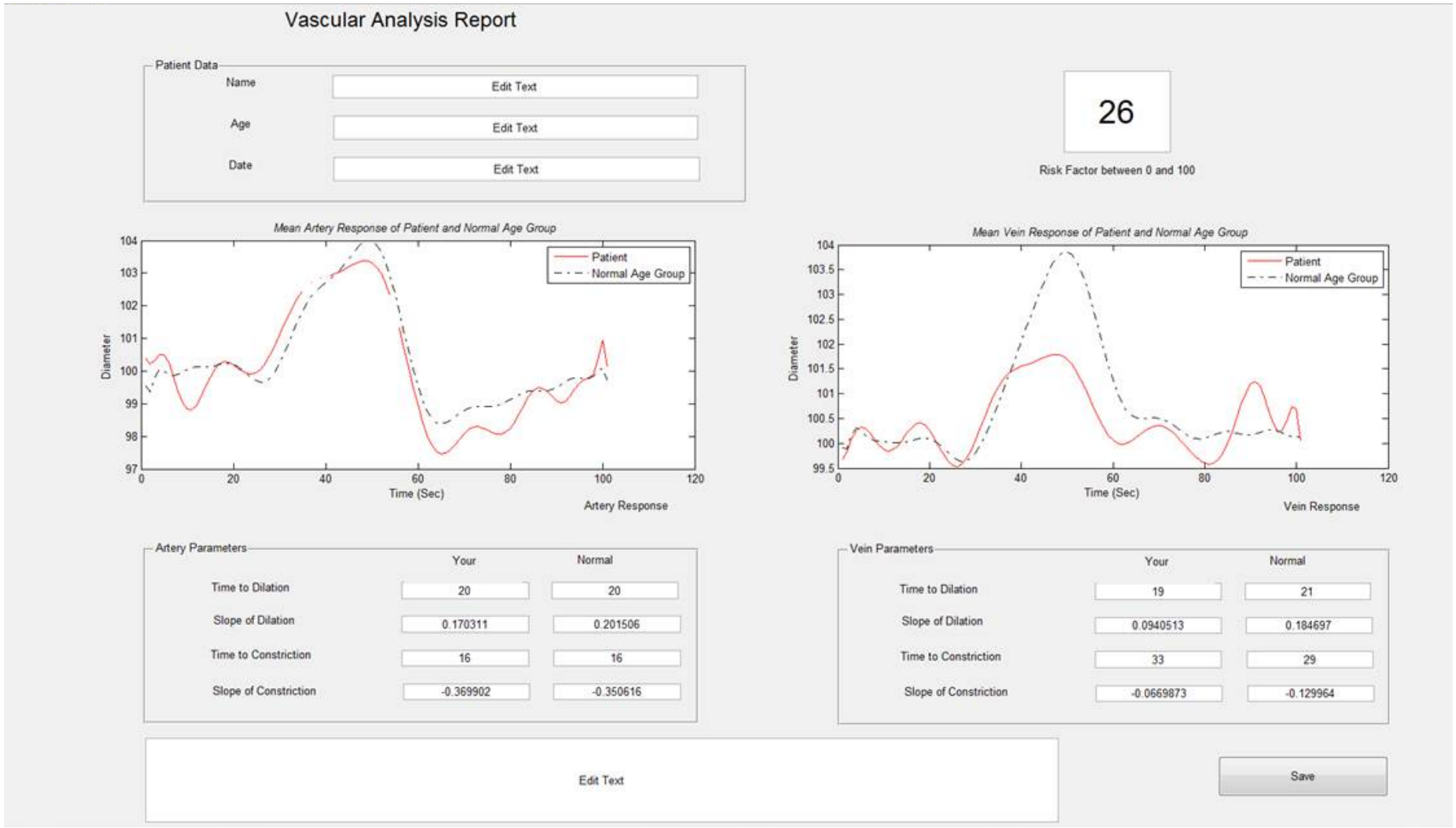

Figure 13.1. Example of a vascular analysis report

The Vascular Analysis Software, however, is still in developmental phase and remains to be validated in a larger subset of individuals (with varying risk factor profiles). Further work is therefore still needed to develop normative reference database across age, gender, and ethnic categories, as well as, a disease-specific database across a variety of disease stages using the parameters presented in this thesis. The composition of a reference database also has strong clinical implications since arterial versus venous responses can reflect independent and varied pathological phenomena.

At present comparisons made between retinal and systemic functional assessment are also limited. With regards to brachial endothelial function, the percentage change in FMD was the primary parameter of interest in this thesis and a further expansion of this analysis to include parameters such as diameter fluctuations, response times, and dilation and constriction slopes, which are more closely associated with retinal vascular function parameters, could enhance the evaluation of systemic endothelial responses as well as enhance comparative evaluations.

Nevertheless, based on the findings of this thesis it is apparent that modifiable and non-modifiable cardiovascular risk factors have independent and significant effects on retinal vessel behaviour and the preliminary results presented here offer a characterization of these effects that could be taken into account in further observational studies and when considering normal versus exaggerated vessel responses and in detecting uncharacteristic shifts in vessel behaviour. The present findings also have implications for population-based screening studies for other vascular disorders and in at-risk groups, as well as, for evaluating primary preventative strategies and therapeutic efficacy in overt diseases. While further work is still needed to validate its clinical use, if proven, functional retinal assessment may become an important clinical tool in the assessment and risk stratification of cardiovascular disease.

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Appendix A: Questionnaires

Aston University

Patient ID:

DEMOGRAPHIC / HEALTH QUESTIONNAIRE

Name *(Last, First, M.I.):*	❒ M ❒ F	**DOB:**

Ethnicity:

WHITE: ❒ English ❒ Scottish ❒ Welsh ❒ Irish ❒ British ❒ Other: _____

ASIAN: ❒ Indian ❒ Sri Lankan ❒ Pakistani ❒ Bangladeshi ❒ Other: _____

MIXED: ❒ Caribbean ❒ White & Caribbean ❒ White & Asian ❒ Other: _____

GP Name & Contact:	**Date of last physical exam:**

PERSONAL HEALTH HISTORY

History of:
(check any that apply):

❒ Diabetes ❒ Glaucoma ❒ Macular degeneration ❒ Sleep Apnoea ❒ Hypertension ❒ Cardiovascular Disease

Other conditions	❒ Coronary Artery Disease (CAD)	❒ Hypotension
	❒ Heart Failure	❒ Bradycardia
	❒ Recent Head injury	❒ Kidney or Liver Disease
	❒ Cold hands and feet	❒ Fainting / Dizziness

List any medical problems that other doctors have diagnosed

Have you ever had a blood test at the GP/Clinic/ Hospital	❒ Yes	No

List your prescribed drugs and over-the-counter drugs, such as aspirin, vitamins E, and inhalers

Name the Drug	Strength / Dose	Frequency Taken

Allergies to medications

Name the Drug	Reaction You Had

Aston University

Patient ID:

HEALTH HABITS AND PERSONAL SAFETY

ALL QUESTIONS CONTAINED IN THIS QUESTIONNAIRE ARE OPTIONAL AND WILL BE KEPT STRICTLY CONFIDENTIAL.

Exercise	❒ Sedentary (No exercise)				
	❒ Mild exercise (i.e., climb stairs, walk 3 blocks, golf)				
	❒ Occasional vigorous exercise (i.e., work or recreation, less than 4x/week for 30 min.)				
	❒ Regular vigorous exercise (i.e., work or recreation 4x/week for 30 minutes)				
Diet	Are you dieting?			❒ Yes	❒ No
	If yes, are you on a physician prescribed medical diet?			❒ Yes	❒ No
	# of meals you eat in an average day?				
	Rank salt intake	❒ High	❒ Med	❒ Low	
	Rank fat intake	❒ High	❒ Med	❒ Low	
Caffeine	❒ None	❒ Coffee	❒ Tea	❒ Cola	
	# of cups/cans per day?				
Alcohol	Do you drink alcohol?			❒ Yes	❒ No
	If yes, what kind?				
	How many drinks per week?				
	Are you concerned about the amount you drink?			❒ Yes	❒ No
	Have you considered stopping?			❒ Yes	❒ No
	Have you ever experienced blackouts?			❒ Yes	❒ No
	Are you prone to "binge" drinking?			❒ Yes	❒ No
	Do you drive after drinking?			❒ Yes	❒ No
Tobacco	Do you use tobacco?			❒ Yes	❒ No
	❒ Cigarettes – pks./day	❒ Chew - #/day	❒ Pipe - #/day	❒ Cigars - #/day	
	❒ # of years	❒ Or year quit			

OTHER PROBLEMS

Check if you have, or have had, any symptoms in the following areas to a significant degree and briefly explain.

☐ Skin	☐ Chest/Heart	☐ Recent changes in:
☐ Head/Neck	☐ Back	☐ Weight
☐ Ears	☐ Intestinal	☐ Energy level
☐ Nose	☐ Bladder	☐ Ability to sleep
☐ Throat	☐ Bowel	☐ Other pain/discomfort:
☐ Lungs	☐ Circulation	

Aston University

Patient ID:

FAMILY HEALTH HISTORY

	AGE	SIGNIFICANT HEALTH PROBLEMS		AGE	SIGNIFICANT HEALTH PROBLEMS
Father			**Children**	☐ M ☐ F	
Mother				☐ M ☐ F	
Sibling	☐ M ☐ F			☐ M ☐ F	
	☐ M ☐ F			☐ M ☐ F	
	☐ M ☐ F		**Grandmother** *Maternal*		
	☐ M ☐ F		**Grandfather** *Maternal*		
	☐ M ☐ F		**Grandmother** *Paternal*		
	☐ M ☐ F		**Grandfather** *Paternal*		

WOMEN ONLY

Age at onset of menstruation:		
Date of last menstruation:		
Period every _____ days		
Heavy periods, irregularity, spotting, pain, or discharge?	☐ Yes	☐ No
Number of pregnancies _____ Number of live births _____		
Are you pregnant or breastfeeding?	☐ Yes	☐ No
Have you had a D&C, hysterectomy, or Cesarean?	☐ Yes	☐ No
Any urinary tract, bladder, or kidney infections within the last year?	☐ Yes	☐ No
Any blood in your urine?	☐ Yes	☐ No
Any problems with control of urination?	☐ Yes	☐ No
Any hot flashes or sweating at night?	☐ Yes	☐ No
Do you have menstrual tension, pain, bloating, irritability, or other symptoms at or around time of period?	☐ Yes	☐ No

MEN ONLY

Do you usually get up to urinate during the night?	☐ Yes	☐ No
If yes, # of times _____		
Do you feel pain or burning with urination?	☐ Yes	☐ No
Any blood in your urine?	☐ Yes	☐ No
Have you had any kidney, bladder, or prostate infections within the last 12 months?	☐ Yes	☐ No

Thank you for completing this questionnaire

Aston University

Patient ID:

FUNCTIONAL OUTCOMES SLEEP QUESTIONNAIRE (FOSQ)

Some people have difficulty performing everyday activities when they feel tired or sleepy. The purpose of this questionnaire is to find out if you generally have difficulty carrying out certain activities because you are too sleepy or tired.

In this questionnaire, when the words "sleepy" or "tired" are used, it means the feeling that you can't keep your eyes open, your head is droopy, that you want to "nod off", or that you feel the urge to take a nap. These words do not refer to the tired or fatigued feeling you may have after you have exercised.

DIRECTIONS: Please put a (✓) in the box for your answer to each question. Select only **one** answer for each question. Please try to be as accurate as possible. All information will be kept confidential.

Name *(Last, First, M.I.):*	**AGE:**	**Today's Date:**

	0 **(I don't do this activity for other reasons)**	(4) **No difficulty**	(3) **Yes, a little difficulty**	(2) **Yes, moderate difficulty**	(1) **Yes, extreme difficulty**
1. Do you have difficulty concentrating on things you do because you are sleepy or tired?		❒	❒	❒	❒
2. Do you generally have difficulty remembering things because you are sleepy or tired?		❒	❒	❒	❒
3. Do you have difficulty finishing a meal because you become sleepy or tired?		❒	❒	❒	❒
4. Do you have difficulty working on a hobby (for example: sewing, collecting, gardening) because you are sleepy or tired?	❒	❒	❒	❒	❒
5. Do you have difficulty doing work around the house (for example: cleaning house, doing laundry, taking out the trash, repair work) because you are sleepy or tired?	❒	❒	❒	❒	❒
6. Do you have difficulty operating a motor vehicle for short distances (less than 100 miles) because you become sleepy or tired?	❒	❒	❒	❒	❒
7. Do you have difficulty operating a motor vehicle for long distances (greater than 100 miles) because you become sleepy or tired?	❒	❒	❒	❒	❒
8. Do you have difficulty getting things done because you are too sleepy or tired to drive or take public transportation?	❒	❒	❒	❒	❒

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Patient ID:

	0 **(I don't do this activity for other reasons)**	(4) **No difficulty**	(3) **Yes, a little difficulty**	(2) **Yes, moderate difficulty**	(1) **Yes, extreme difficulty**
9. Do you have difficulty taking care of financial affairs and doing paperwork (for example: writing checks, paying bills, keeping financial records, filling out tax forms, etc.) because you are sleepy or tired?	❒	❒	❒	❒	❒
10. Do you have difficulty performing employed or volunteer work because you are sleepy or tired?	❒	❒	❒	❒	❒
11. Do you have difficulty maintaining a telephone conversation because you become sleepy or tired?	❒	❒	❒	❒	❒
12. Do you have difficulty visiting with your family or friends in <u>your</u> home because you become sleepy or tired?	❒	❒	❒	❒	❒
13. Do you have difficulty visiting with your family or friends in <u>their</u> home because you become sleepy or tired?	❒	❒	❒	❒	❒
14. Do you have difficulty doing things for your family or friends because you are sleepy or tired?	❒	❒	❒	❒	❒

	(4) **No**	(3) **Yes, a little**	(2) **Yes, moderately**	(1) **Yes, extremely**
15. Has your relationship with family or work colleagues been affected because you are sleepy or tired?	❒	❒	❒	❒
In what way has your relationship been affected?				

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	0 **(I don't do this activity for other reasons)**	(4) **No difficulty**	(3) **Yes, a little difficulty**	(2) **Yes, moderate difficulty**	(1) **Yes, extreme difficulty**
16. Do you have difficulty exercising or participating in a sporting activity because you are too sleepy or tired?	❒	❒	❒	❒	❒
17. Do you have difficulty watching a movie or videotape because you become sleepy or tired?	❒	❒	❒	❒	❒
18. Do you have difficulty enjoying the theatre or a lecture because you become sleepy or tired?	❒	❒	❒	❒	❒
19. Do you have difficulty enjoying a concert because you become sleepy or tired?	❒	❒	❒	❒	❒
20. Do you have difficulty watching TV because you are sleepy or tired?	❒	❒	❒	❒	❒
21. Do you have difficulty participating in religious services, meetings or a group or club, because you are sleepy or tired?	❒	❒	❒	❒	❒
22. Do you have difficulty being as active as you want to be in the <u>evening</u> because you are sleepy or tired?		❒	❒	❒	❒
23. Do you have difficulty being as active as you want in the <u>morning</u> because you are sleepy or tired?		❒	❒	❒	❒
24. Do you have difficulty being as active as you want in the <u>afternoon</u> because you are sleepy or tired?		❒	❒	❒	❒
25. Do you have difficulty keeping pace with others your own age because you are sleepy or tired?		❒	❒	❒	❒
	(1) **Very Low**	(2) **Low**	(3) **Medium**	(4) **High**	
26. How would you rate your general level of activity?	❒	❒	❒	❒	

Thank you for completing this questionnaire

Aston University

Patient ID:

SF-36 HEALTH SURVEY

INSTRUCTIONS: This survey asks your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.
Please answer every question by marking the answer indicated. If you are unsure about how to answer a question, please give the best answer you can.

Name *(Last, First, M.I.):*	**AGE:**	**Today's Date:**

1. In general would you say your health is:	❒ Excellent (1) ❒ Very Good (2) ❒ Good (3) ❒ Fair (4) ❒ Poor (5)
2. Compared to <u>one year ago</u>, how would you rate your health in general <u>now</u>?	❒ Much better now than one year ago (1) ❒ Somewhat better than one year ago (2) ❒ About the same as one year ago (3) ❒ Somewhat worse than one year ago (4) ❒ Much worse now than one year ago (5)

3. The following questions are about activities you might do during a typical day. Does your health <u>now limit you in these activities</u>? If so, how much? (Mark each answer with a ✓)

	Yes, Limited A Lot (1)	Yes, Limited A Little (2)	No, Not Limited At All (3)
a. **Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports.	❒	❒	❒
b. **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling or playing golf	❒	❒	❒
c. Lifting or carrying groceries	❒	❒	❒
d. Climbing **several** flights of stairs	❒	❒	❒
e. Climbing **one** flight of stairs	❒	❒	❒
f. Bending, kneeling or stooping	❒	❒	❒
g. Walking **more than a mile**	❒	❒	❒
h. Walking half a mile	❒	❒	❒
i. Walking one hundred yards	❒	❒	❒
j. Bathing or dressing yourself	❒	❒	❒

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4. During the past 4 weeks, have had you had any of the following problems with your work or other regular daily activities as a result of your physical health? (Mark **one** answer with a ✓ for each question)

	YES (1)	NO (2)
a. Cut down on the **amount of time** you spent on work or other activities	❒	❒
b. **Accomplished** less than you would like	❒	❒
c. Were limited in the **kind** of work or other activities	❒	❒
d. Had **difficulty** performing the work or other activities (for example, it took extra effort)	❒	❒

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)? (Mark **one** answer with a ✓ for each question)

	YES (1)	NO (2)
a. Cut down on the **amount of time** you spent on work or other activities	❒	❒
b. **Accomplished less** than you would like	❒	❒
c. Didn't do work or other activities as carefully as usual	❒	❒

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

❒ Not at all (1)
❒ Slightly (2)
❒ Moderately (3)
❒ Quite a bit (4)
❒ Extremely (5)

7. How much bodily pain have you had during the past 4 weeks?

❒ None (1)
❒ Very mild (2)
❒ Mild (3)
❒ Moderate (4)
❒ Severe (5)
❒ Very severe (6)

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

❒ Not at all (1)
❒ A little bit (2)
❒ Moderately (3)
❒ Quite a bit (4)
❒ Extremely (5)

Aston University

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9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks. (Mark **one** answer with a ✓ for each question)

	All of the time (1)	Most of the time (2)	A good bit of the time (3)	Some of the time (4)	A little of the time (5)	None of the time (6)
a. Did you feel full of life?	❒	❒	❒	❒	❒	❒
b. Have you been a very nervous person	❒	❒	❒	❒	❒	❒
c. Have you felt so down in the dumps that nothing could cheer your up?	❒	❒	❒	❒	❒	❒
d. Have you felt calm and peaceful?	❒	❒	❒	❒	❒	❒
e. Did you have a lot of energy?	❒	❒	❒	❒	❒	❒
f. Have you felt downhearted and blue?	❒	❒	❒	❒	❒	❒
g. Did you feel worn out?	❒	❒	❒	❒	❒	❒
h. Have you been a happy person?	❒	❒	❒	❒	❒	❒
i. Did you feel tired?	❒	❒	❒	❒	❒	❒

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

- ❒ All of the time (1)
- ❒ Most of the time (2)
- ❒ Some of the time (3)
- ❒ A little of the time (4)
- ❒ None of the time (5)

11. How TRUE or FALSE are each of the following statements for you? (Mark **one** answer with a ✓ for each question)

	Definitely True (1)	Mostly True (2)	Don't Know (3)	Mostly False (4)	Definitely False (5)
a. I seem to get ill more easily than other people	❒	❒	❒	❒	❒
b. I am as healthy as anybody I know	❒	❒	❒	❒	❒
c. I expect my health to get worse	❒	❒	❒	❒	❒
d. My health is excellent	❒	❒	❒	❒	❒

Thank you for completing this questionnaire

Aston University

Patient ID:

EPWORTH SLEEPINESS SCALE (ESS)

Note: The next few questions are a simple measure of how sleepy you are **during the day**. They are **not** a measure of how tired or fatigued you are. For each scenario mark one answer with a ✓:

High chance of falling asleep **= 3**
Moderate chance of falling asleep **= 2**
Slight chance of falling asleep **= 1**
Never likely to fall asleep **= 0**

Name *(Last, First, M.I.):*	**AGE:**	**Today's Date:**

During an ordinary day (assume you are not at work for example); from the following situations, **how likely are you to doze off to sleep**:

	(3) Highly Likely	(2) Moderately Likely	(1) Slightly Likely	(0) Unlikely
Sitting and reading	❒	❒	❒	❒
Watching TV	❒	❒	❒	❒
Sitting inactive in a public place (for example theatre or meeting)	❒	❒	❒	❒
As a passenger in a car for an hour without break	❒	❒	❒	❒
Lying down to rest in the afternoon when circumstances permit	❒	❒	❒	❒
Sitting and talking to someone	❒	❒	❒	❒
Sitting quietly after lunch (no alcohol)	❒	❒	❒	❒
In a car, while stopped for a few minutes in the traffic	❒	❒	❒	❒

Thank you for completing this questionnaire

Appendix B: Publication

Appendix C: Publication

Appendix D: Research Presentations