Early Detection of Microvascular Dysfunction in Asymptomatic Individuals with Different Cardiovascular Diseases Risk Factors

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Thesis Summary

In health, the endothelium maintains the balance between opposing dilator and constrictor influences, while in disease, it is the common ground on which cardiovascular risk factors act and a site at which cardiovascular disease begins. Consequently, as such, the endothelial acts as a barometer of an individual's likely future cardiovascular health; however, the currently used cardiovascular diseases risk assessments scores tend to overlook the importance of endothelial dysfunction in the progress of cardiovascular diseases. Moreover, the presence and severity of endothelial dysfunction in apparently healthy individuals suggest considerable variability in pre-clinical risk that could be identified well before the onset of the disease. The earlier detection of cardiovascular diseases can improve the effectiveness of treatments and avoid long-term complications and may lead to longer survival.

This possibility has led to public health programs to recommend periodic screening examinations for detecting specific chronic diseases, for example, cancer, diabetes, and cardiovascular disease.

Therefore, this thesis aimed to investigate microvascular dysfunction as an early culprit in the pathophysiology of cardiovascular diseases in clinically healthy individuals with different cardiovascular risk factors.

As cardiovascular diseases are clearly multifactorial with risk factors that tend to cluster and interact, in an individual to determine the level of future disease risk and to increase the clinical accuracy of the conducted investigations, influences of systemic circulatory oxidative stress biomarkers on retinal microvascular function were also evaluated.

The principal sections and findings of this work are:

1. Microvascular Function and Oxidative Stress in Adult individuals with Early Onset of Cardiovascular Disease

- Prehypertension patients showed abnormal retinal vascular response to flicker light stimuli throughout the entire functional response curve for arteries. Systemic blood pressure and impairments in microvascular function at the retinal level correlated with established plasma markers for oxidative stress.

2. European Society of Cardiology/European Society of Hypertension versus the American College of Cardiology/American Heart Association guidelines on the cut-off values for early hypertension: A Microvascular Perspective

- Microvascular dysfunction was present in ESC/ESH grade 1 hypertension patients and not in stage 1 hypertension in the form of altered peripheral and retinal responses to systemic stimuli (blood occlusion, flickering light).

3. Dry Eye Disease is Associated with Retinal Microvascular Dysfunction and Possible Risk for Cardiovascular Disease

- Individuals with a positive diagnosis of Dry Eye Disease exhibit a higher risk for cardiovascular disease than age, sex-matched normal individuals. These alterations were correlated to circulatory blood cholesterols.

4. Novel Composite Early Risk Markers for Vascular Ageing and Risk for Cardiovascular Disease

- Study findings confirmed the systemic association between plasma oxysterol levels and gradual microvascular endothelial dysfunction at the retinal arterial level after the age of 30. This association was also correlated to decreased systemic antioxidant capacity without any significant change in NO concentrations.

5. Prediction of Cardiovascular Risk in Asymptomatic Individuals: A Symbolic Regression-Based Analysis of Single and Composite Vascular-Omics

- Study findings confirmed the predictive power of the telomere and retinal arteries function as a composite biomarker for predicting cardiovascular risk factors such as blood pressure, while the assessment of retinal vascular function is better predictor of chronological ageing than relative telomere length.

Key words: Endothelial dysfunction, dynamic retinal vessels analysis, oxidative stress, cardiovascular diseases, oxysterols, telomeres, Aging.

Dedication

This thesis is dedicated to my family and friends
For their endless love, support, and encouragement

To my dad Abdelmohaimen Shokr, the man who have taught me everything I stand for and managed to change me, without ever trying to do so. You have raised me to be strong and independent, however, I will always be your little girl, and I will always need you.
Thank you dad for teaching me to smile in the face of adversity and to believe in myself no matter what anyone says. I can only hope to one day become half the man you are. You are the person I will always aspire to be, love you dad. Thank you for everything.

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To Lamia, thanks for walking with me when I needed support, thanks for walking ahead of me when I needed guidance and thanks for walking behind me when I needed someone to watch my back. Thanks for understanding me better than me.

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Abbreviations

ABPM	Ambulatory blood pressure monitoring
AHA	American Heart Association
ACC	American College of Cardiology
ACCF	American College of Cardiology Foundation
ACH	Acetylcholine
ADMA	Asymmetric dimethylarginine
aTR	Adjusted temperature rebound
AU	Arbitrary Units
AUC_{TR}	Area under the curve temperature rebound
AVR	Arteriovenous ratio
BCFR	Baseline Corrected Flicker Response
BDF	Baseline diameter fluctuation
BF	Blood Flow
BFR	Baseline corrected flicker response
BH4	Tetrahydrobiopterin
BMI	Body mass index
BP	Blood pressure
bpm	Beat per minute
CAD	Coronary Artery Disease
cGMP	Cyclic guanosine monophosphate
cNOS	Constitutive nitric oxide synthase
CRAE	Central arteriolar equivalent
CRVE	Central venular equivalent
COP	Cardiac Output
CVDs	Cardiovascular Diseases
DA	Dilation amplitude
DBP	Diastolic blood pressure
DED	Dry Eye Disease
DEQ-5	Dry Eye Questionnaire
DTM	Digital thermal monitoring
DVA	Dynamic retinal vessel analysis
EEG	Electrocardiogram
EDCF	Endothelial derived constricting factor
EDRF	Endothelial derived relaxing factor
EDVF	Endothelial derived vasoactive factors

EndoPAT	Endothelial Peripheral Arterial Tonometry
eNOS	Endothelial nitric oxide synthase
EPCS	Endothelial progenitor cells
ESC	European Society of Cardiology
ET-1	Endothelin-1
FH	Family History
FDA	Food and Drug Administration
FMD	Flow mediated dilation
GLUC	Glucose
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GSR	Glutathione Reductase
GST	Glutathione Transferase
GMP	Guanosine monophosphate
H^+	Hydrogen
HDL-C	High-density lipoprotein cholesterol
HF	High frequency
HO-1	Heme oxygenase
HR	Heart rate
HRV	Heart rate variability
IDEEL	Impact of Dry Eye on Everyday Life
IL-6	Interleukin-6
IMT	Intima-media thickness
iNOS	Inducible nitric oxide synthase
IOP	Intraocular pressure
Kg	Kilogram
L	Vessel length
LDL-C	Low-density lipoprotein cholesterol
L-FMC	Low flow-mediated constriction
LTL	Leukocyte Telomere length
m^2	Square meter
MAE	Mean Absolute Errors
MAP	Mean arterial blood pressure
MC	Maximum constriction
MC%	Maximum constriction percentage
MD	Maximum dilation

MD%	Maximum dilation Percentage
mg/dL	Milligrams per deciliter
MI	Myocardial Infarction
M/L	Media-to Lumen ratio
mmHg	Millimeters of mercury
µmol	Micromole
µM	Micromole
mmol/L	Mill moles per liter
n	Blood Viscosity
NADPH	Adenine dinucleotide phosphate
nmol	Nanomoles
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
NOS	Nitric oxide synthase
O_2	Oxygen
OBF	Ocular blood flow
OCT	Optical coherence tomography
OHT	Ocular Hypertension
ONH	Optic Nerve Head
OPP	Ocular perfusion pressure
OSDI	Ocular Surface Disease Index
Oxy-LDL	Oxidized Low-density lipoprotein cholesterol
PAT	Peripheral Arterial Tonometry
PDE	Phosphodiesterases
PET	Positron emission tomography
PP	Perfusion pressure
PVR	Peripheral vascular resistance
QCA	Quantitative coronary angiography
R	Vessel resistance
r	Internal radii
RBCs	Red blood cell
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reaction time

RTL	Relative Telomere Length
RVA	Retinal vascular analyzer
S1P	Sphingosine-1
SA node	Sinoatrial node
SBP	Systolic blood pressure
SDRA	Sequential and diameter response analysis
SD	Standard deviation
sGC	Soluble guanylyl cyclase
SEE	Salisbury Eye Evaluation Questionnaire
$Slope_{AC}$	Arterial constriction slope
$Slope_{AD}$	Arterial dilation slope
$Slope_{VC}$	Venous constriction slope
$Slope_{VD}$	Venous dilation slope
SVR	Systemic vascular resistance
T-CHOL	Total Cholesterol
TG	Triglycerides
t-GSH	Total glutathione
TL	Telomere Length
tMD	Time to maximum dilation
tMC	Time to maximum constriction
TR	Temperature rebound
UM	Units of measurement
VWF	von Willebrand factor
XO	Xanthine oxidase
7-KC	7-Ketocholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
4β-OHC	4 beta-hydroxycholesterol
7β-OHC	7 beta-hydroxycholesterol
7,27-OHC	7,27-hydroxycholesterol
7,25-OHC	7,25-hydroxycholesterol

1. Introduction

Cardiovascular diseases (CVDs) are a broad group of heart and blood vessels disorders that include coronary artery diseases, heart failure, cerebrovascular diseases, rheumatic heart diseases and many other conditions.[1] They are the main leading cause of death worldwide and responsible for over 18 million deaths each year (representing 31% of all global deaths, including approximately 35% of deaths in developed countries and 32% in developing ones).[2] CVDs are directly linked to several risk factors, including diabetes, advanced age, obesity, dyslipidaemia ethnicity, family history and genetic predisposition.[3] In the past decade, early identification of those at risk gained considerable scientific attention due to its significant role in decreasing CVDs- associated mortalities and clinical complications.[4] Given this, several risk prediction models were developed; however, the reliability of these risk scores was always a constant cause of concern.[5] The majority of their validation data was based on small studies conducted in developed countries[6] and, it was found that most of them either over or underestimate the future risk of developing CVDs.[7] Moreover, these scores were mainly tested in White populations; therefore, using them to classify CVD risks in different races was found clinically misleading, increased unnecessary medical interventions or led to missing vulnerable cases.[8,9] Accordingly, in the past few years, many techniques have been developed to facilitate the assessment of cardiovascular function; however, it was challenging to accurately predict the risk of CVDs due to the difficulties associated with the invasive procedures used in visualising the heart and the blood vessels.[10,11] Subsequently, finding less invasive and more accessible ways to examine the cardiovascular system became a must. [5] Microvascular endothelial function and structure measures were of particular interest in this regard, as they have recently emerged as a potential surrogate marker of future cardiovascular events. Additionally, small arteries remodelling was found in most of the cardiovascular aetiologies and represented a relevant factor in terms of the development of target organ damage or events.[12]

Of all the examined microvascular beds, retinal vessels were found to exhibit very close features comparable to the cardiovascular system. One of the most prominent features shared by the ocular and cardiac units is the nature of their vascular supply, with the microcirculation in particular demonstrating a large number of anatomical and physiological similarities.[13] These similarities have raised the question of whether the two systems may share similar aetiologies. Subsequently, functional and structural retinal vascular analysis techniques were used to assess the endothelial function in both systems.[14] One of the most advanced techniques used is the dynamic retinal vascular analysis (RVA), which allowed direct and precise CVDs complications, and had a vital prognostic significance in individuals with established cardiac diseases.[15] However, little was published regarding its importance in predicting disease development in low risk and clinically asymptomatic individuals. Given that,

this thesis aims to explore this possible association and additionally address the question of whether functional assessment at the ocular level could prove effective as an indirect measure of systemic cardiac and vascular functions and hence whether the retinal vessels could effectively be used as a surrogate marker for subclinical cardiovascular system function.

Of particular interest to this thesis is the involvement of cofounder factors in the aetiology of cardiovascular diseases and endothelial dysfunction. Although the participation of vascular factors in the aetiology of both cardiac and vascular diseases has been increasingly realised, the nature of this involvement is still somewhat uncertain, and many questions remain. As such, this thesis additionally aims to explore the presence and aetiological relevance of physiological disorders at both the ocular and systemic levels in apparently healthy individuals to enhance our understanding of the pathological mechanisms involved in this population. Age-related vascular physiological changes and oxidative stress are of particular interest to this thesis.

To provide a foundation for the research conducted in this thesis, the following sections will first outline the normal cardiac, vascular and ocular anatomy and physiology, which is necessary to aid in interpreting this work. It will then discuss and outline the Background and current aetiological thinking, firstly for endothelial function, and secondly for the factors leading to its dysfunction and how it is connected to the development of CVDs.

1.1. Anatomy and Physiology of the Cardiovascular System

The circulatory system is a complex system that consists of two main components: the **cardiovascular** and the **lymphatic** system.[16] The cardiovascular system consists of the heart, the blood vessels and the blood, while the lymphatic system consists of lymphatic microvessels (capillaries) and the larger lymph vessels.[17]

Blood compromises approximately 7% of the body's volume; regardless of its small volume, it plays a fundamental role in controlling all body functions while distributed through the vascular system. In the last centuries, many scientists, such as the ancient Greek physician Galen, proposed that blood moves through the body like an ocean tide, first moving out from the heart and then ebbing back in the same vessels.[18] In the 1620s, after the massive developments in vascular imaging techniques, an English physician (William Harvey) was the first to describe the vascular system as a circulatory system.[18]

Using Dr Malpighi visualising method, he described the vascular system as an interconnecting network spreading all over the body.[19] Later, scientists discovered that the vascular system consists of different vessels (arteries, veins, and capillaries), forming a closed circle that begins and ends in the heart.[20]

Another wrong description of blood vessels is a system of pipes. Unlike rigid pipes, blood vessels are dynamic; they dilate, constrict and pulsate.[21] They are also the key factor in

maintaining and balancing all living cells' vital functions in the human body, e.g. reproduction, metabolism, and growth.[22] To balance all these functions, vessels must exhibit similar cellular structures yet be different enough to fulfil different physiological roles. For example, the coronary arteries in the heart are responsible for supplying oxygenated blood to the heart, while those in the kidney are required for the selective filtration of blood waste products.[16] An overview of the cardiovascular system with relevance to this thesis is given in the following sections.

1.1.1. The Heart

The heart is a muscular organ that weighs almost 350 grams and is considered the strongest muscle in the human body. It is the main organ in the cardiovascular system that contracts rhythmically and autonomously.[23]

The heart is located between the lungs in the middle of the mediastinal cavity of the thorax, and its end extends downwards to the left between the second and the fifth intercostal space. Anatomically, its cavity divided into four chambers, two atria and two ventricles separated by the cardiac valves that regulate the blood flow direction (Figure 1.1).[24]

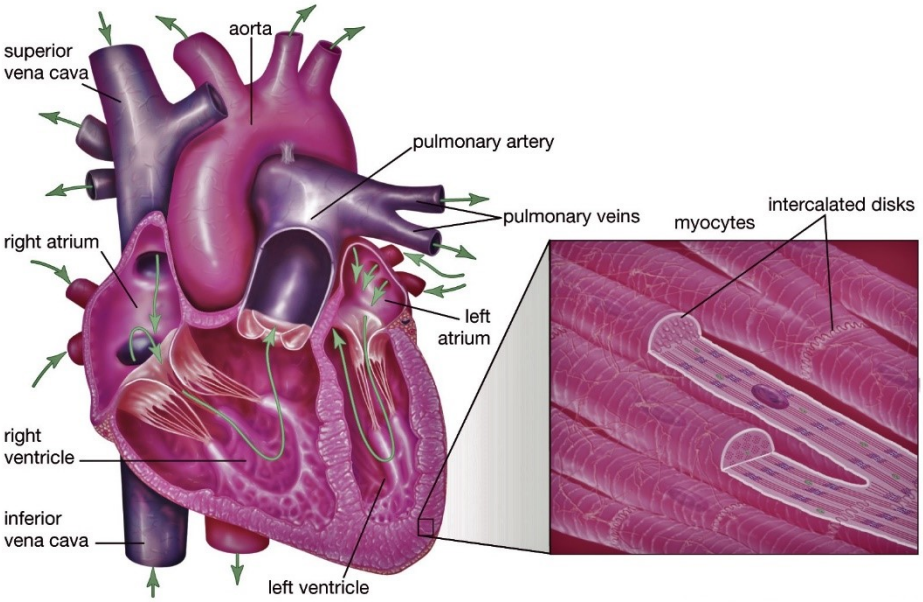

Figure 1.1: Anatomy of the Heart and the Myocardium
Adapted from Britannica website[25]

The cardiac muscles consist of three layers; the **myocardium**, which is the thickest middle layer of the heart wall. This layer lies between the single-cell endocardium layer, which lines the inner chambers. The outer epicardium, also known as the visceral pericardium, forms the

pericardium's inner layer that surrounds and protects the heart from the outside. The primary function of the pericardium is to protect the heart and prevent its over-expansion inside the thorax cavity.[22]

The inner layer of the heart wall is the **endocardium;** it lines the inner heart chambers, heart valves and is continuous with the endothelium of the large blood vessels (Figure 1.2).[24]

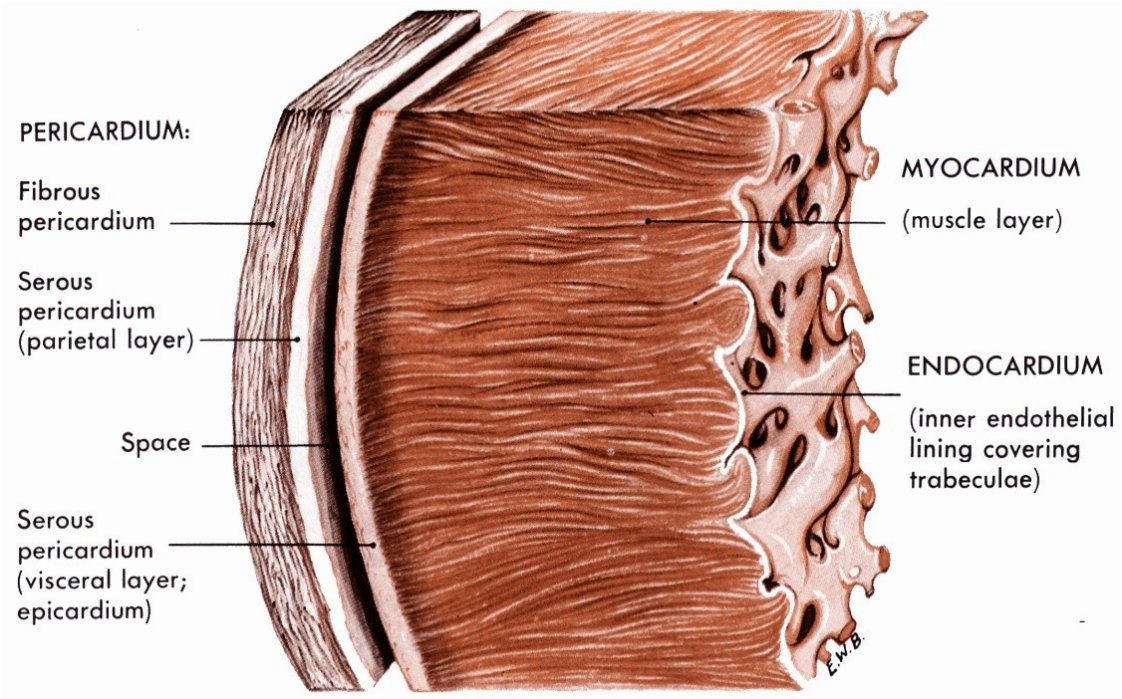

Figure 1.2: Intersection of the Heart Wall Showing the Three Cardiac Wall Layers Adapted from the Atlas of Human Anatomy [26]

1.1.2. The Conduction System and the Cardiac Cycle

One of the unique characteristics of the cardiac muscles is their conduction system which gives them the ability to depolarise and repolarise continuously. The right atrium's sinoatrial node (SAN) is the heart pacemaker; it generates electrical impulses that start a sequence of electrical excitation all over the heart and stimulate its contraction. The electrical impulses generated by the SAN stimulates the atria to contract, then it travels to the interatrial septum and stimulates the atrioventricular node (AVN).[27] The AVN is a tissue located between the right atrium and the left atrium, and it provides a pathway of electrical conduction between the atria and the ventricles.[28] The fibres in the AVN is smaller than the SAN, which cause a 0.1-second delay in conduction and give the atria the time to contract and empty its blood content before the next ventricular contraction. The impulses are then transmitted through the bundle

of His branches to the two ventricles. The Purkinje fibres then continue down to the inferior aspect of the heart before looping upwards and travelling in the lateral aspects of the right and left ventricles (Figure 1.3).[27,29,30]

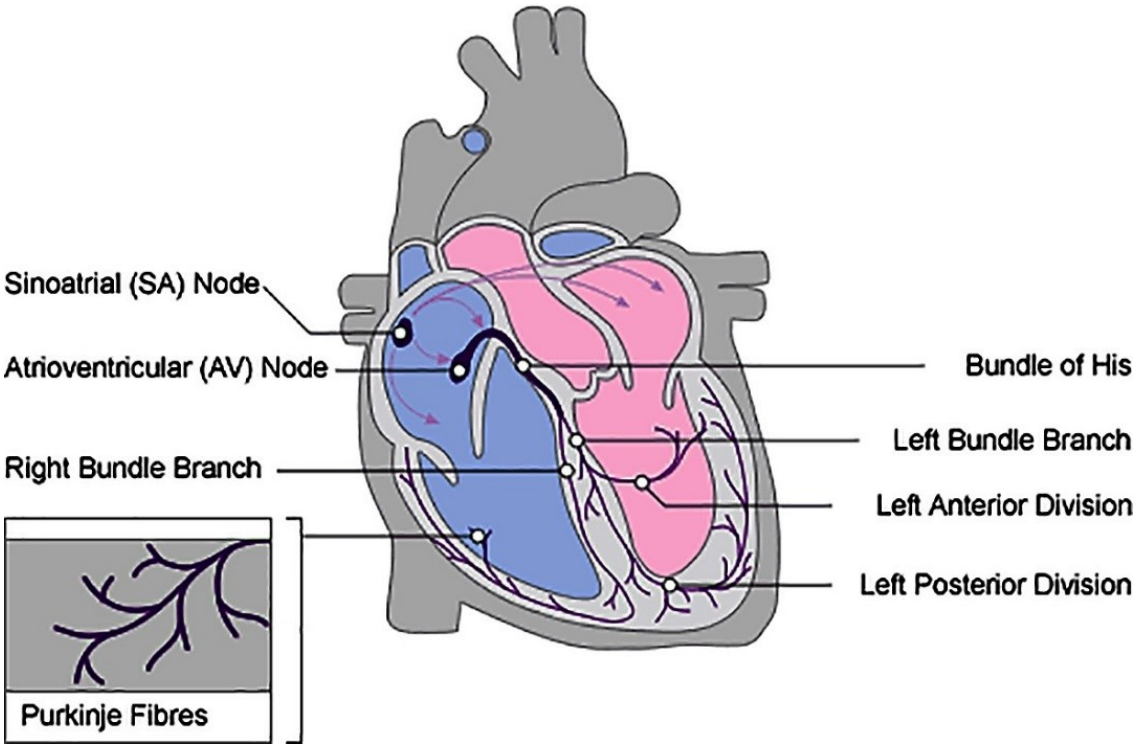

Figure 1.3: The Cardiac Conduction System

Adapted from the EMS education Website [31]

The contraction phase of the heart chambers is called systole, and the relaxation phase is referred to as diastole. One complete cycle of these events is referred to as the cardiac cycle.[32] The change of the blood pressure inside the cardiac chambers during the cardiac cycle affects the opening and closure of the interchamber valve, thereby regulating blood flow between the chambers. Although the same volume of blood is pumped per each cardiac beat, the blood pressure in the left side of the heart is almost five times the right side.[33] These changes of the intracardiac blood pressure cause blood to flow from the high-pressure to low-pressure areas in the heart (Figure 1.4).[32]

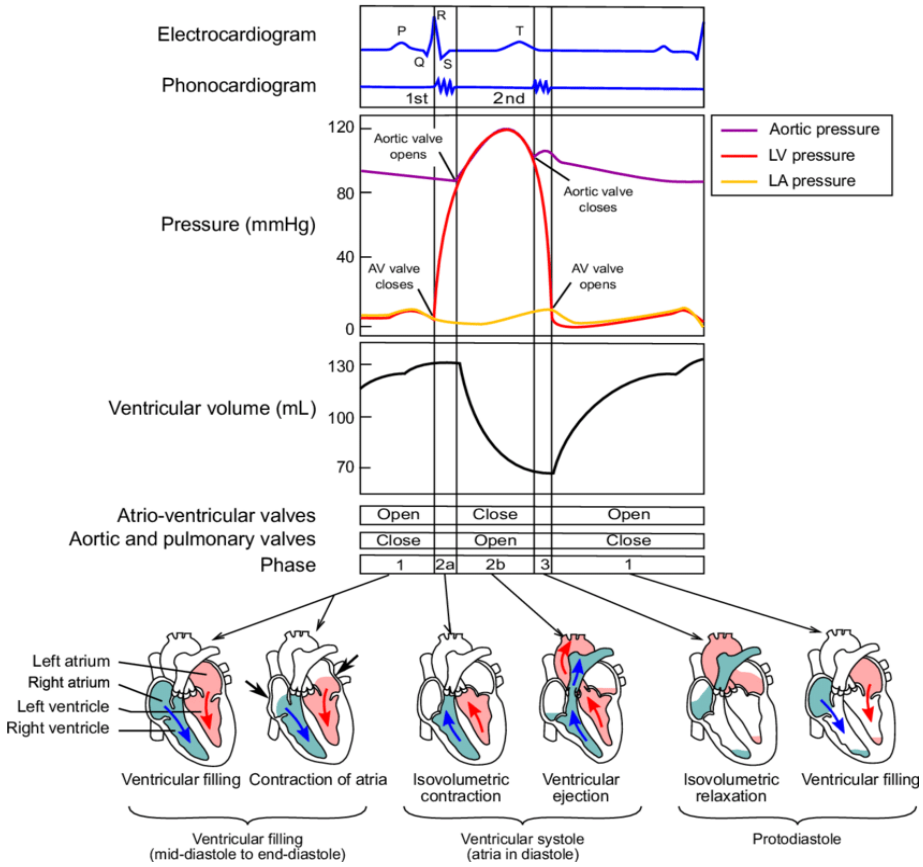

Figure 1.4: The Cardiac Cycle
Top diagram represents ECG cardiac signals, pressures and volumes.
Bottom diagram illustrates blood circulation in heart cavities at each phase.
Adapted from the Atlas of Human Anatomy [26]

1.1.3. The Vascular System

1.1.3.1. Basic Structural Features of Blood Vessels

The vascular system, with few exceptions, share common physiological and histological features. Generally, blood vessels consist of three layers; each layer is named a `tunic', a Greek word meaning a membrane covering an organ. [34,35] These layers are different in the amount of smooth muscle and elastin they contain.

Anatomically inside out these layers are (Figure 1.5):

- The tunica intima
- The tunica media
- The tunica adventitia

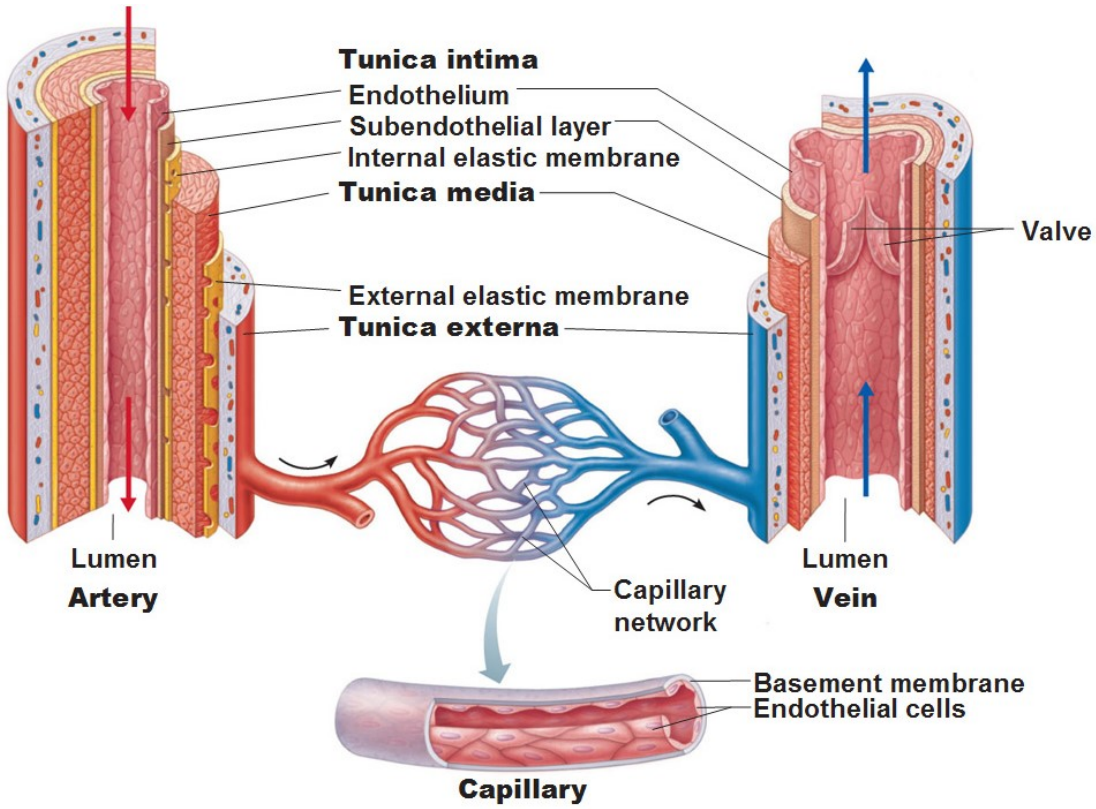

Figure 1.5: The Vascular System

Adapted from The Circulatory system dynamics [36]

The first layer (**tunica intima**) is the thinnest layer of the three, consists of a single endothelial cell layer based on a basal lamina (basement membrane).[37] Underneath is the sub-endothelial layer, a fibro-connective layer attached to an internal elastic layer that gives the endothelial cells elasticity and flexibility. The second layer in the vascular wall is the **tunica media**, consisting mainly of elastin fibres and smooth muscle cells. The tunica media is supported by the external lamina that provides its structure. The third and outermost layer of blood vessels is the **tunica adventitia**, which is entirely fibro-elastic connective tissue.[38]

In large blood vessels, simple diffusion of oxygen is minimal, and the endothelium is the only layer that can receive oxygen and nutrition from the blood carried by the vessels.[29] Arteries and veins have their own blood supply network through small thin-walled blood vessels located outside the adventitia called the vasa vasorum (vessels of the vessels) (Figure 1.6).[35,39]

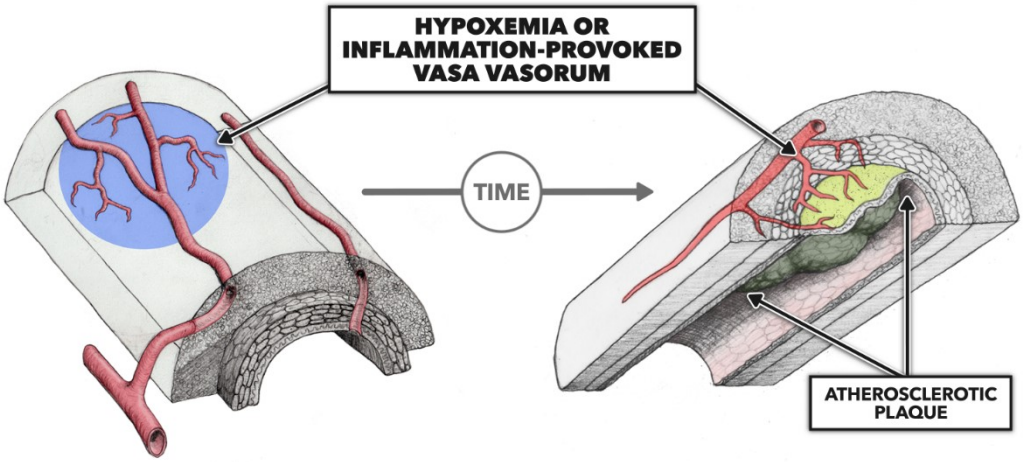

Figure 1.6: The Vasa Vasorum
Adapted from the clinical anatomy associates [40]

1.1.3.2 Arterial and Venous Components of the Circulatory System

a. Arteries and Arterioles

Arteries carry oxygenated blood ejected from the left ventricles; the only exception is the pulmonary artery that carries deoxygenated blood from the right ventricle to the lungs.[16] Based on cell histology, arteries can be classified into elastic arteries and muscular arteries.[41] Elastic arteries are also known as **conducting** arteries; they are large arteries placed near the heart and can carry large amounts of blood (e.g. the aorta). Anatomically these arteries' walls have several layers of perforated elastic membrane (Figure 7); therefore, they are more flexible to expand, and relax rapidly to accommodate changes in blood volume during the cardiac systole and diastole.[42] The large population of arteries in the body are muscular (**distributive** arteries). Their walls mainly consist of a massive layer of smooth muscles and few elastic fibres. Their primary function is to distribute an adequate amount of blood to the organs according to their needs.[42]

Arterioles are smaller in diameter blood vessels extending and branching out from the arteries leading to the capillaries. The function of these arterioles is to deliver oxygen and nutrition to the organs and reduce blood flow reaching the capillaries to prevent their damage.[43]

b. The Capillaries

The arterial vascular system transitions to the venous system through the capillary network. Capillaries are the thinnest vessels (about 0.25 mm thick) in the vascular system; they connect the arterial system to the venous tree and regulate the release of neurotransmitters at the

sympathetic synapses.[43] They also regulate the microcirculation via series of complex interactions with autacoids, hormones, and neurotransmitters released at the sympathetic synapses leading to alteration of the smooth muscle tone in the arteriolar wall.[44]

Walls of capillaries are marked histologically by a lack of smooth muscle, and instead, they have a single layer of endothelial cells surrounded by a basement membrane. [45,46]

c. Veins and Venules

After circulating in the arteries, the deoxygenated blood return to the heart via the venous network. The blood in the post-capillary venules moves to the larger veins then to the heart's right atrium.[16] Veins size can vary from 1 to 10 mm in diameter. The transition from the capillaries to the veins characterised by the reappearance of muscle cells in the tunica media and elastic fibres in the adventitia. Small and medium-sized veins contain a high percentage of muscle cells in their tunica media, while larger ones have greater connective tissue levels.[38,47] Compared to corresponding arteries, veins have thinner walls and larger lumens (Figure 1.8). With their large lumens, veins can accommodate around 60% of the body's total blood volume, and that is why they are named blood reservoirs (Figure 1.7).[38]

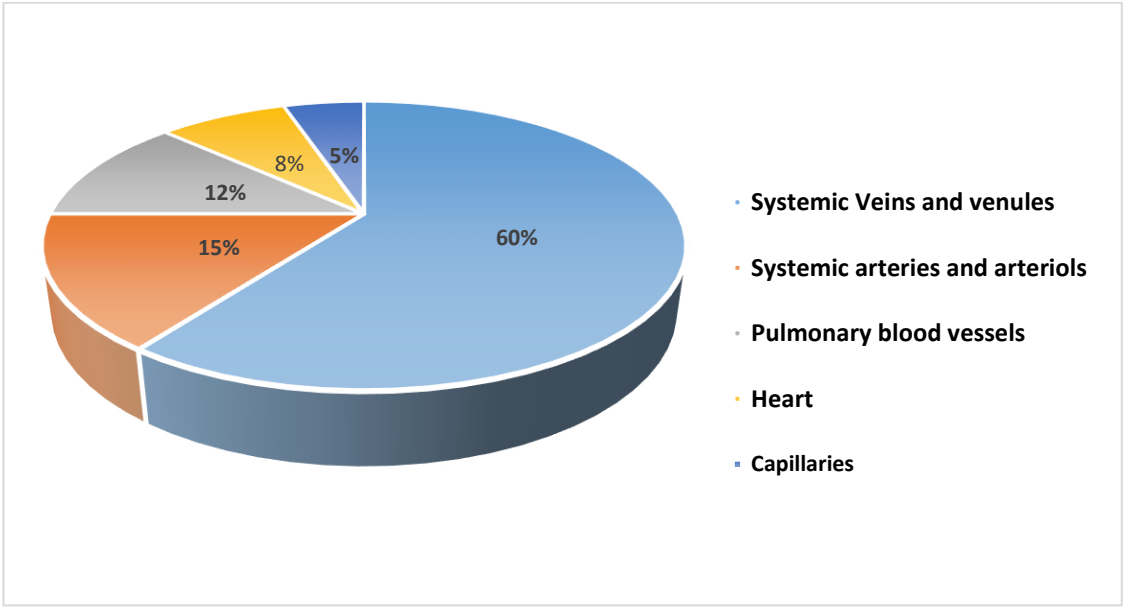

Figure 1.7: Percentage Blood Volume in the Circulatory System

The second component of the venous system is the **venules**. They are similar to veins in function; however, they are formed from united capillaries. The post-capillary venules are the most miniature and consist of a single layer endothelium. Larger venules have one or two layers of smooth muscles and a thin externa. Unlike veins, venules are highly porous, allowing

free water movement and other molecules from the blood to the tissues, including white blood cells.[38]

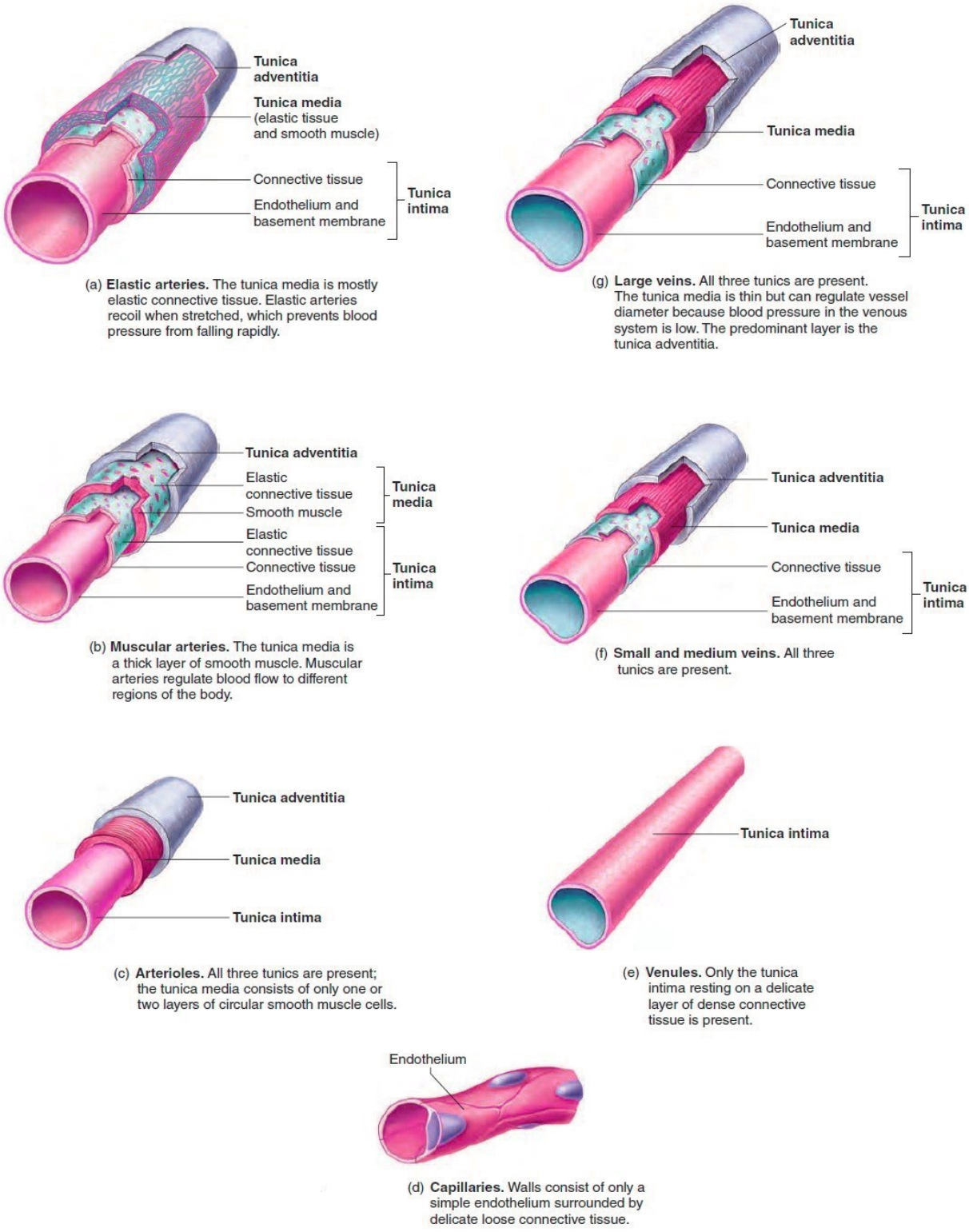

Figure 1.8: Blood Vessels Anatomy

Adapted from William D. Tucker, anatomy, blood vessels [48]

1.1.4. Physiology of Circulation

1.1.4.1. Systemic Blood Pressure

In engineering, it is well known that any fluid pumped in a closed circuit operates under pressure; the closer the liquid to the pump, the higher this pressure.[49] Blood circulation in the cardiovascular system is not an exception, as mentioned above, the heart is the pump responsible for generating the blood flow in the body; the resistance the blood face while circulating in the vessels generates the pressure and blood follow pressure gradient (flow from the higher pressure to the lower pressure areas.[50]

As explained in Figure 1.9, the highest systemic blood pressure is in the aorta, and it drops throughout its path until it reaches 0 mm Hg in the right atrium. The sharpest drop happens in the arterioles as they are the most resistant to blood flow; nevertheless, as long as there is a pressure gradient, blood continues to circulate until it completes an entire cycle back to the heart.[51,52]

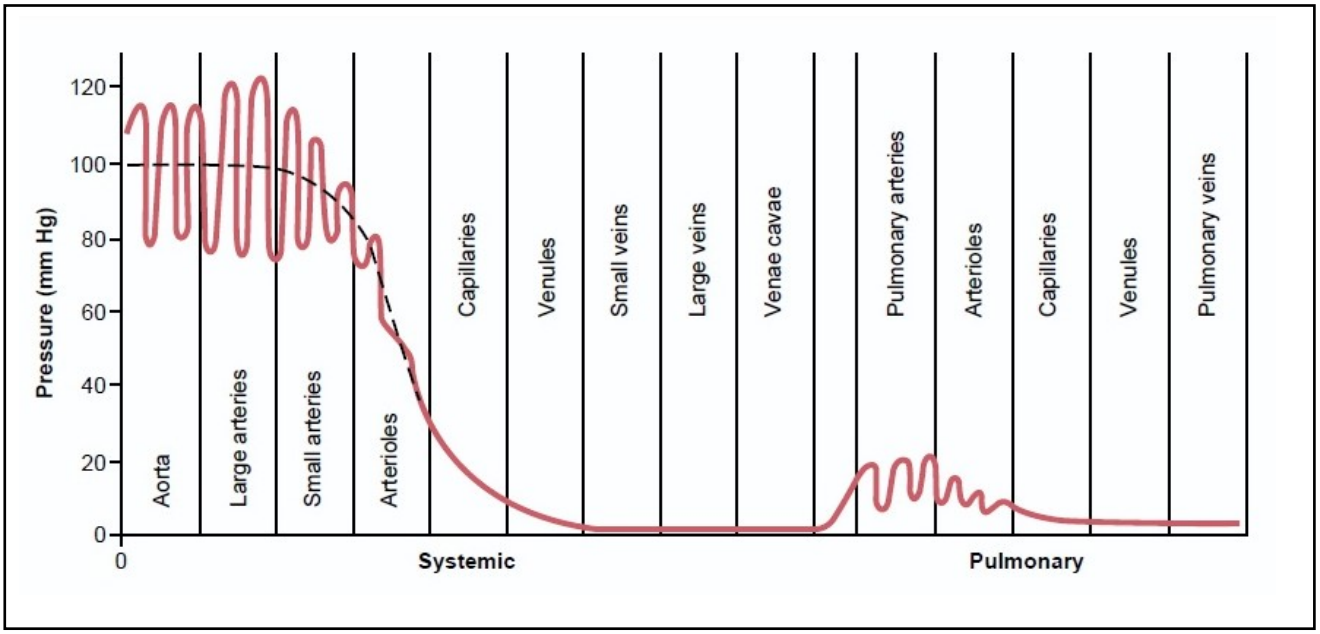

Figure 1.9: Blood Pressure Gradient in the Systemic Circulation

Adapted from Physical Characteristics of the Circulation [53]

1.1.4.2. Arterial Blood Pressure

Arterial blood pressure is a parameter that reflects the elasticity of the arterial walls and blood volume. It is a function of the cardiac output (COP) and the peripheral vascular resistance (PVR). COP depends on the myocardium contractility and end-diastolic volume, while the PVR is the arterial resistance to the blood flow, and it depends on the artery diameter, length and blood viscosity.[54] As the left ventricle contracts and pumps blood to the aorta, the aortic pressure increases until it reaches its peak. This peak is termed the "systolic blood pressure" (SBP), and its average is 120 mm Hg in healthy young adults.[55] Following pressure gradient, blood moves towards the distal arteries, and during the diastole, the walls of the elastic arteries and the aorta recoil to sustain enough pressure to keep the blood moving forward into smaller vessels. During this period, the aortic valve closes to prevent the backflow of blood into the left ventricle; sequentially, the aortic pressure drops to its lowest level (70-80 mm Hg), which is called the diastolic blood pressure (DBP).[56] The difference between the systolic and diastolic pressures is called pulse pressure (PP).

As seen in Figure 9, aortic blood pressure fluctuates with each heartbeat. A mean value of the aortic pressure is used to reflect an accurate estimation of mean arterial blood pressure (MAP), and it is used as a mirror of the tissue pressure. As diastole lasts longer than the systole; consequently, MAP is not directly the mean of the SBP and DBP. Instead, it is DBP plus one-third PP (Equation 1.1).

MAP = DBP + 1/3 (PP)

$$\boldsymbol{MAP = DBP + \frac{1}{3}\, X\, (SBP - DBP)}$$

MAP = mean arterial blood pressure

DBP = Diastolic blood pressure

SBP = Systolic blood pressure

Equation 1.1: Calculation of Mean Arterial Blood Pressure

According to the SBP and the DBP patients are classified into different hypertension classes. The most recent classification of hypertension according to the European Society of Cardiology/ European Society of Hypertension [57] and the American College of Cardiology classification of Arterial Hypertension [58] are outlined in Table 1.1.

Table 1.1: The ESC/ESH versus the ACC/AHA Hypertension Guidelines

ESC/ESH 2018			ACC/AHA 2017		
Category	**Systolic (mmHg)**	**Diastolic (mmHg)**	**Category**	**Systolic (mmHg)**	**Diastolic (mmHg)**
Optimal	<120	<80	Normal	<120	<80
Normal	120-129	80-84	Elevated BP	120-129	<80
High Normal	130-139	85-89	Stage 1	130-139	80-89
Grade 1	140-159	90-99	Stage 2	≥140	≥90
Grade 2	160-179	100-109	Hypertensive Crisis	≥180	≥120
Grade 3	≥ 180	≥110			

1.1.4.3. Arterial Blood Pressure Regulation

Arterial BP is controlled by changes in COP and systemic vascular resistance (SVR). SVR is determined by the anatomical features of the vascular system. As we explained before, the vascular structure remains relatively unchanged; however, some pathological conditions such as atherosclerosis, vascular thrombosis can affect the number of perfused blood vessels in the organs.[59] It was proven that hypertensive patients have a decrease in the anatomical number of the arterioles and the capillaries (rarefaction).

The most important factor affecting the SVR is the changes in vascular luminal diameter. According to the Poiseuille relationship, the SVR is inversely related to the vessel radius (Equation 1.2). [60]

$$R \propto \frac{nL}{r4}$$

R= Vessel resistance

L= length of the vessel

n= Viscosity of the blood

r= internal radii

Equation 1.2: The Poiseuille Relationship

Other vascular factors such as nitric oxide (NO), endothelin and prostacyclin also play an essential role in altering vessel diameter, discussed in detail in the coming sections. Similarly, some tissue factors such as adenosine and histamine can significantly affect organ blood flow when secreted from the parenchymal cells surrounding the vascular system.[61]

Finally, neurohumoral mechanisms regulated mainly by the baroreceptor and, to a lesser extent, the chemoreceptor play a significant role in regulating systemic vascular resistance and arterial pressure, particularly in certain forms of secondary hypertension.[62]

The following diagram summarises the main factors regulating blood pressure by altering systemic vascular resistance and blood volume (Figure 1.10).

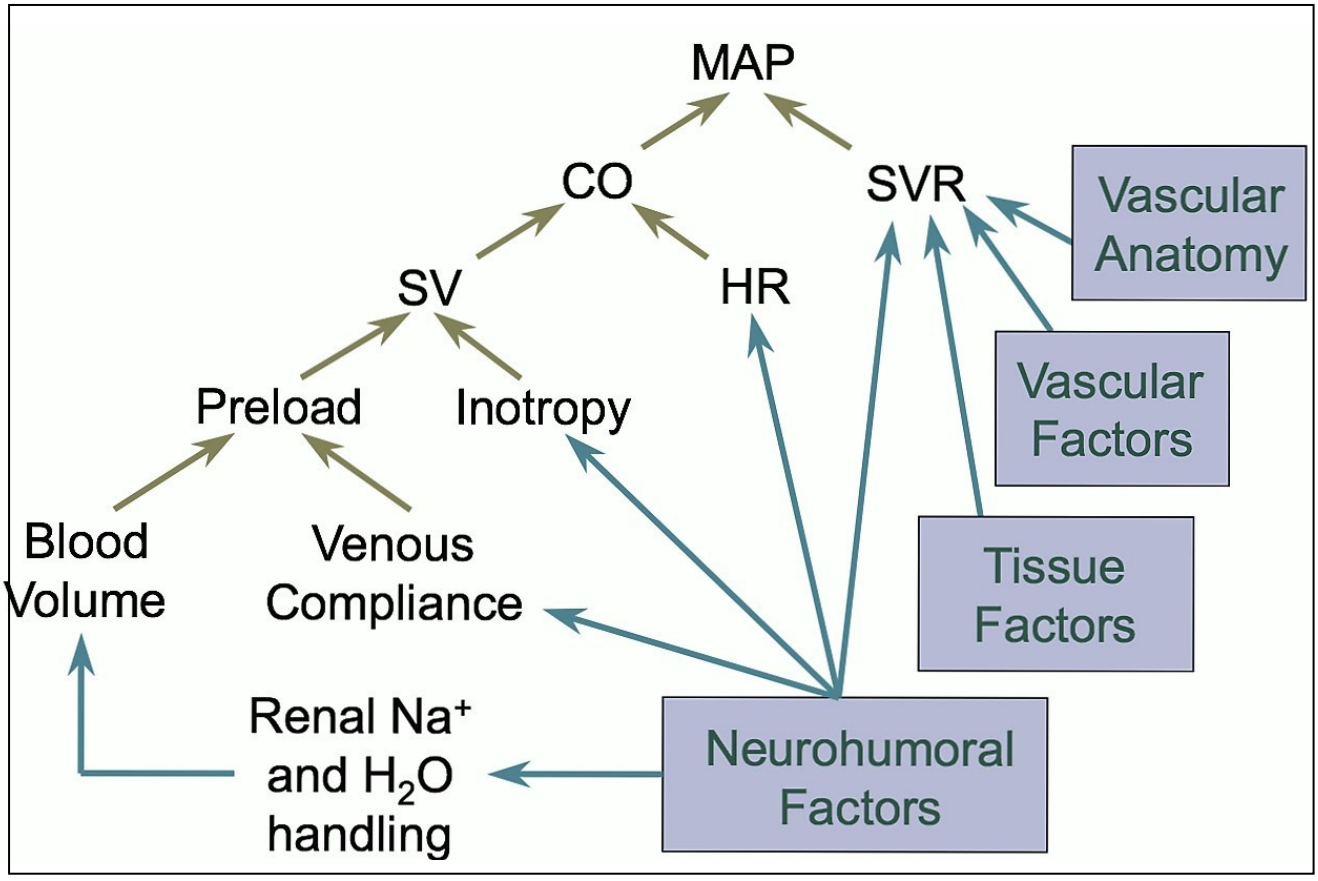

Figure 1.10: Factors Affecting Arterial Blood Pressure
Abbreviation: SV, stroke volume; CO, cardiac output; MAP, mean arterial pressure; HR, heart rate; SVR, systemic vascular resistance; Na, sodium; H2O, water.

Adapted from CV Physiology [708]

1.2. Anatomy and Physiology of the Ocular System

1.2.1. The Ocular Circulation

The eye has four different blood circuits (Figure 1.11): the ciliary body and the anterior part of the eye circulation; the retinal circulation, which lacks autonomic control similar to the brain circulation; the optic never head circulation; and the choroidal vasculature, which has the greatest autonomic innervations.[63] The retinal blood flow is completely auto-regulated and is not dependent on the blood perfusion pressure. Its blood flow is mainly dependant on the endothelium, the neural and neuroglia cells function (neurovascular coupling).[64] Unlike retinal circulation, choroidal circulation is mainly affected by psychological and physical stressors and temperature change, with cold increasing the choroid blood flow. [65] The optic nerve circulation is affected mainly by the neurovascular coupling and some other mediators diffused from the choroid. [63,66,67]

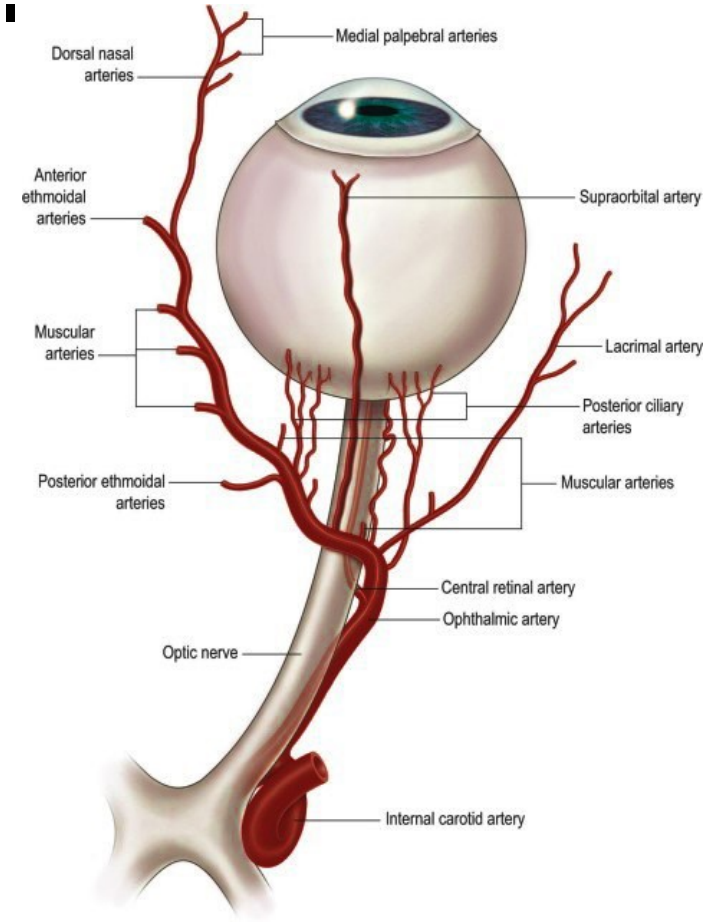

Figure 1.11: The Ocular Circulation

Adapted from Brent Siesky, Glaucoma Risk Factors: Ocular Blood Flow [68]

1.2.2. Physiology of the Ocular Circulation

Similar to the systemic circulation, blood flow in the eyes depends on the perfusion pressure (OPP) and flow resistance generated by the vessels walls (R). Following the Hagen-Poiseuille pipe flow law, the blood flow (BF) equal PP / R; however, it is difficult to apply this law to microvessels due to the changes in red blood cells (RBCs) velocity profile, local haematocrit and the difference in the shear stress between branches, junctions and others.[69]

Due to these challenges, scientists introduced another approach calculating blood flow in the ocular microcirculation based on Murray's Law.[70] According to this approach, the mean ocular PP driving blood through the eye is the mean blood pressure in the ophthalmic artery minus the pressure in the veins leaving the eye. The venous blood pressure was approximately equal to the intraocular pressure (IOP) [71], and the ocular PP is almost two-thirds of the mean arterial blood pressure. The factor 2/3 is due to the natural drop between the heart and the ophthalmic artery see Equation 1.3.[72,73]

$$OPP = \frac{2}{3}MAP - IOP$$

OPP = Ocular perfusion pressure

MAP = Mean arterial blood pressure

IOP = Intraocular pressure

Equation 1.3: Ocular Perfusion Pressure Calculation

1.2.3. The Eyes: A Window to the Heart

For years scientists dealt with the eye and the heart as two different organs with no association. However, with advances in technology and research methods, it was discovered that these two organs have more common than expected. Vessels in the eye have their own uniqueness; nevertheless, they share many features with the heart and the circulatory vessels.[347] Both vessels (retinal and cardiovascular) are exposed to the same intrinsic and extrinsic influences, and their response is the same in both organs. Hence, the vasculature of the eye is accessible and easily assessed; many scientists used it to evaluate the cardiovascular system functions.[13,74]

1.3. The Endothelium

The endothelium is the largest organ in the human body. It is a layer of simple squamous cells lining the blood/ lymphatic vessels and the heart. Its weight is around 1.5 kg in average size individuals and covers a total surface area of 4000 to 7000 square meters.[69] In the beginning, it was thought it served only as a barrier between the vascular wall and the blood. However, later it has been shown to have a pivotal role in homeostasis in micro and macrocirculation through response to humoral, neural, and mechanical stimuli. It also helps synthesise and release vasoactive substances in the body (Figure 1.12).[75,76]

As an autocrine, endocrine and paracrine organ, the endothelium interacts merely with every system in the human body. It helps supplying oxygen, nutrition, receives active metabolites and delivers them back to the circulation. Additionally, it has the ability to sense any stimuli on the vascular system (mechanical or hormonal), and in response, it regulates the vasomotor function by releasing some mediators that affect haemostasis, dilate or constrict the vessels, stimulate the inflammatory process or affect coagulation.[77]

Examples of the vasodilatory substances produced by the endothelial cells are NO, prostacyclin and endothelium-derived hyperpolarisation factors such as c-natriuretic peptides, histamine, prostaglandins, vasoactive intestinal peptide, bradykinin and nicotinic acid. [78,79] Several vasoconstrictors are also secreted from the endothelium, e.g. angiotensin II, endothelin-1, thromboxane A2 and reactive oxygen species.[80,81] Similarly, inflammatory mediators such as intercellular adhesion molecule-1 and vascular adhesion molecule-1 are produced by the endothelium tissue. Modulators of haemostasis also include plasminogen activator, tissue factor inhibitor, Von-Willebrand factor, NO, prostacyclin, thromboxane A2, plasminogen-activator inhibitor-1, and fibrinogen.[82] It also has an essential role in angiogenesis and body fluids balance.

Under normal circumstances, the total of these endothelial-dependent factors is to maintain vasoconstriction, adequate blood flow, control vascular inflammatory mediators and muscular cell proliferation.[75] Nevertheless, when vascular risk factors are present, the endothelium may adopt a phenotype that facilitates inflammation, thrombosis, vasoconstriction, and atherosclerotic lesion formation.[83]

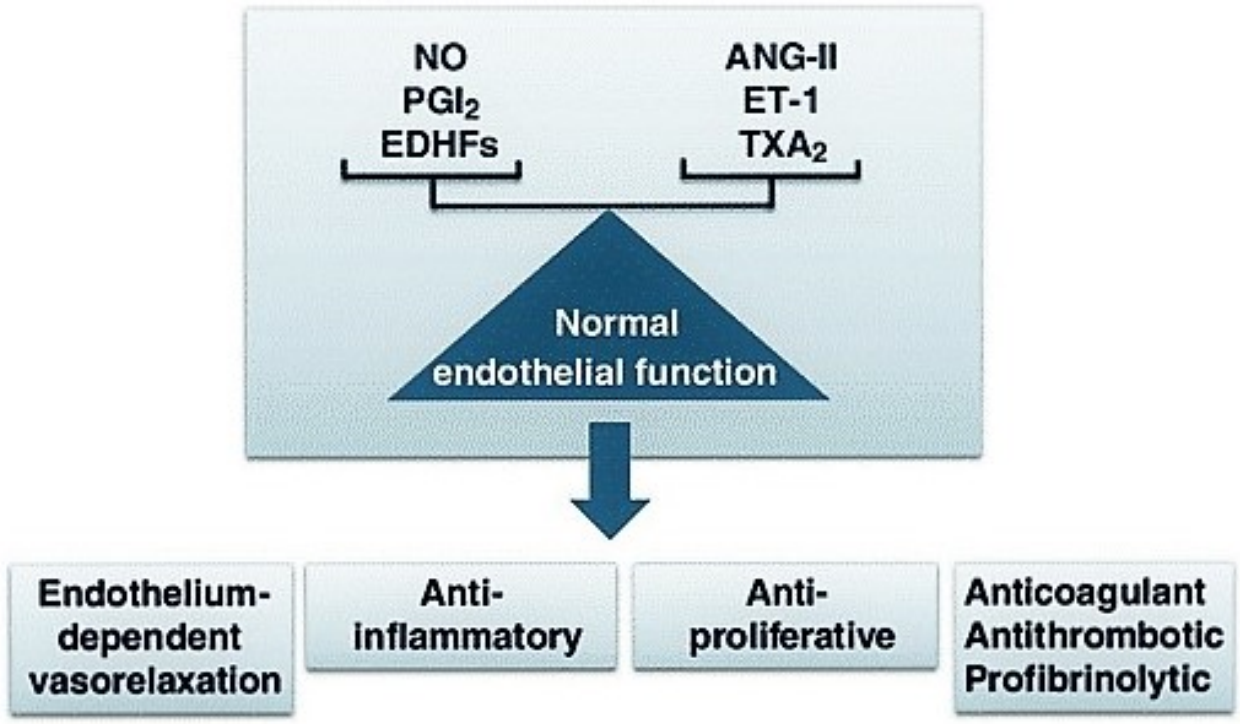

Figure 1.12: Endothelium-Derived Factors
ANG-II, angiotensin II; EDHF, endothelium-derived hyperpolarising factor; ET-1, endothelin 1; NO, nitric oxide; PGI 2, prostacyclin; TXA 2, thromboxane A 2.

1.3.1 Nitric Oxide

NO is a free radical diatomic gas synthesised and expressed in various tissues in the body, especially the cardiovascular and nervous systems.[84,85] Its properties as a free radical and gas allow it to pass easily between the cells and the tissues and react with many molecules in the body.[86–88] These biological and chemical properties enabled NO to be an ideal signalling molecule between tissues and cells and be a stimulator of many biological functions, especially in the cardiovascular system. Although the endothelium produces many other mediators, NO is considered the key molecule mediating all the endothelial functions. [82]

Its effects on the cardiovascular system, e.g. vasodilatory, anti-thrombotic and anti-inflammatory effects [89] made it gain a huge interest over the years and became the core of many pharmaceutical treatments for various vascular diseases, e.g. hypertension, preeclampsia and peripheral vascular disorders.[90,91]

It is also produced in various human body cells, including platelets, macrophages, and neuronal cells. Different concentrations of NO can activate different pathways in the human body; for example, low levels activate soluble guanylyl cyclase (sGC), and intermediate concentration (5-300 nM) activates wound healing and oncology pathways.[92] Concentrations higher than 1Um cause oxidative, nitrative and nitrosative stress.[92–94] Additionally, with

reactive oxygen species, it can cause modification of many human proteins, lipids and DNA strands.[92,95]

1.3.1.1 Generation and Regulation of NO

The endothelial production of NO is a crucial step in regulating vascular blood flow, maintaining enough vascular tone and resistance to help circulating blood around the body.[95–98] Over the years, scholars identified various mechanisms involved in the production of NO, yet until now, it has been agreed that the activity of NO synthase (NOS) is the key regulating step in NO production.[99–102] The substrate for NO synthesis is the terminal guanidine nitrogen of the amino acid L-arginine. Catalysed by the NOS enzyme family and using one molecule of oxygen(O_2), iron protoporphyrin-IX (heme complex), oxidative cofactors (reduced glutathione and tetrahydrobiopterin (BH_4)), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH as cofactors; arginine undergoes 5-electron oxidation to form L-citrulline and a free NO radical.[103]

NOS enzyme has three domains: **The oxygenase domain** binds the heme molecule, the BH4, and in which the formation of NO from arginine and O_2 takes place. **The reductase domain** uses NADPH to reduce the FAD, and FMN then transfers the free electron produced to **the oxygenase domain** through the calmodulin (CaM) binding domain (Figure 1.13).[104,105] According to the physiological and biological properties, the NOS enzyme family, can be classified into two isomers:

- **The constitutive isomers** (cNOS) present mainly in the vascular endothelial (eNOS or NOS III) and neuronal cells (nNOS or NOS I).[106,107]

- **The inducible isomers** (iNOS or Nos II) are found mainly in the macrophages [108] and the neutrophils (Figure 13). The Inducible isomers are mainly activated immunologically by exposure to bacterial endotoxin or cytokines such as interleukin-6 (IL-6) and interferons.[106,107]

Similarly, calcium ions, calmodulin and endothelial agonists such as acetylcholine and bradykinin control the stimulation of the two constructive isoforms.[109,110]

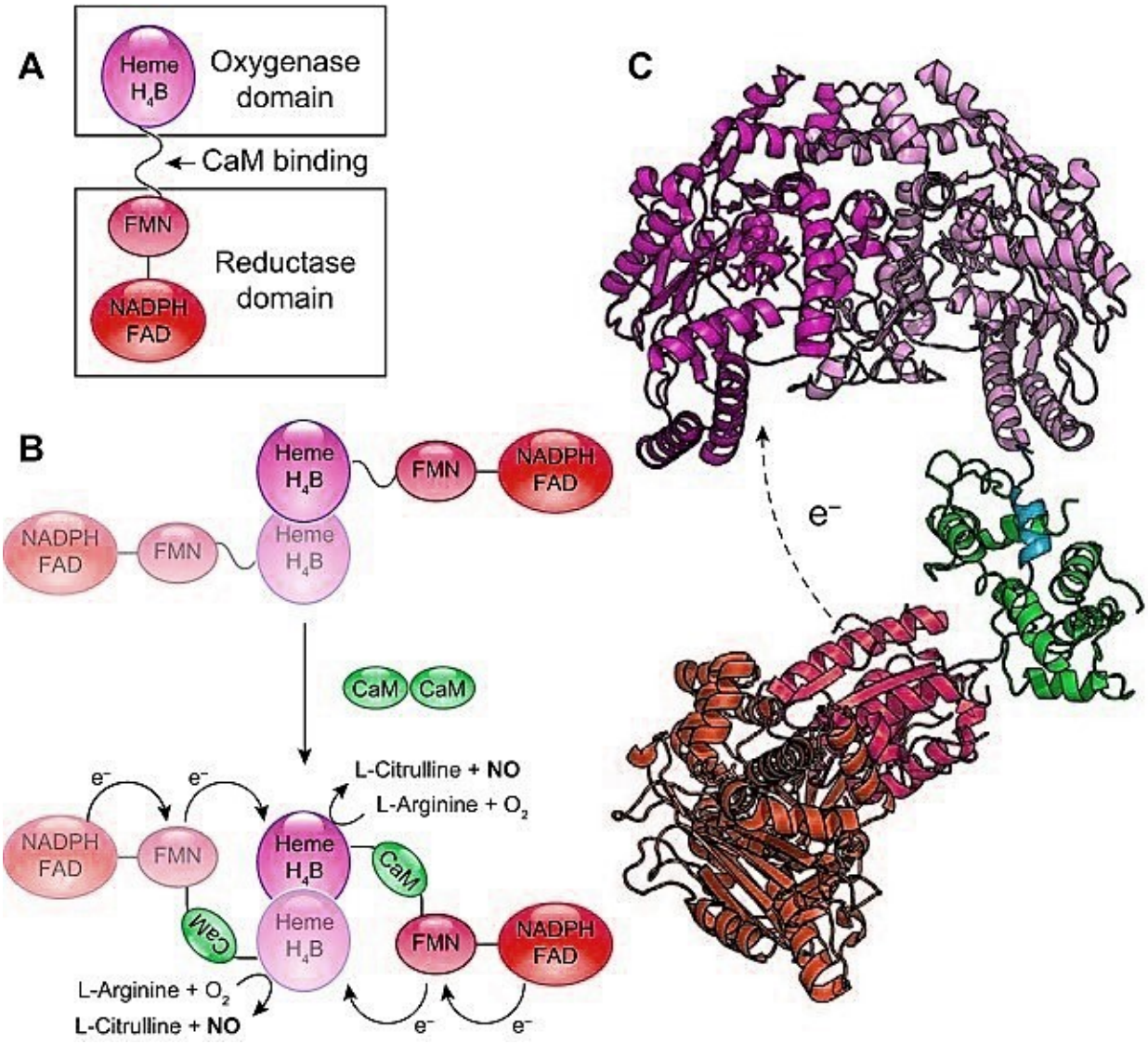

Figure 1.13: Structural Design of Nitric Oxide Synthases
A: the oxygenase domain containing the heme and BH4 and connected to the reductase domain by a calmodulin link.
The reductase domain containing FMN, FAD and NADPH as an electron source.
B: calmodulin electron transfer from the FMN domain to the heme domain.
C: three-dimensional structure model of NOS.
Adapted from Tejero J et.al. Sources of vascular nitric oxide and reactive oxygen species and their regulation.[709]

Table 1.2. Tissue Distribution of NOS Isoforms in the Body

	Tissues	Cell Types
nNOS (NOS1)	Heart, lungs, and blood vessels	ECs, VSMCs, Adventitial fibroblasts, Cardiomyocytes
iNOS (NOS2)	Heart, lungs, and blood vessels	Macrophages and other leukocytes ECs, VSMCs, Adventitial fibroblasts
eNOS (NOS3)	Heart, lungs, and blood vessels	ECs, VSMCs, Erythrocytes, Platelets, Cardiomyocytes

Table 1.2: EC, endothelial cell; eNOS, endothelial NOS; iNOS, inducible NOS; NOS, nitric oxide synthase; nNOS, neuronal NOS; VSMC, vascular smooth muscle cell.

1.3.1.2 Nitric Oxide Function

a. Modulation of the Vascular Tone

NO's effects on the vascular wall is mediated by the soluble guanylate cyclase (sGC) receptor activation. [111–113] sGC is a heterodimeric cytosolic protein composed of two subunits, α and β. Each subunit has two isoforms (α1, α2 and β1, β2) which determine the tissue distribution of the protein [114]. Each protein monomer consists of four domains the heme-binding domain, which is the N-terminal, the Per-Arnt-Sim (PAS) domain, a helical/coiled-coil motif, and a C-terminal guanylyl cyclase domain that catalyses the formation of cGMP from GTP (Figure 1.14). Once NO binds to the heme domain, it stimulates the activation of sGMP synthesis through the interaction with the C-terminal domain.[115] NO activation of sGC increases cGMP synthesis, which consequently activates phosphodiesterases (e.g. PDE5) and cGMP-dependent gated ion channels and kinases, leading to vasodilation. [116–118]

b. Regulation of Myocardial Contractility

NO is directly involved in the control of myocardial contractility. Many scientists showed that NO reduces the contractility of the cardiac muscles via cGMP dependant mechanism.[120,121] Both cNOS and iNOS are expressed in the ventricular muscles of the heart. eNOS and nNOS are constitutively expressed; they produce a minimal amount (less than 100 nM) of NO in the heart [122,123] with eNOS, particularly associated with the maintenance of basal, physiological cardiac function.[124]

Myocardium contractility depends on the amount of NO produced; at lower levels (submicromolar), it causes a positive inotropic effect. However, at higher levels (micromolar or above), it causes negative inotropic effects.[121,125] Hence, the impact of NO in the heart depends on which NOS isoform is activated and the amount of NO produced.

NO also helps maintaining adequate blood supply to the myocytes. In ischemia, the level of nNOS is upregulated, which inhibits xanthine oxidoreductase and prevents the formation of peroxide molecules.[126–128] In addition, nNOS overexpression in ischemia downregulates the L-type calcium channels, decreasing the ischemic calcium overload and protecting the heart from reperfusion injury and progression of heart failure.[129–131]

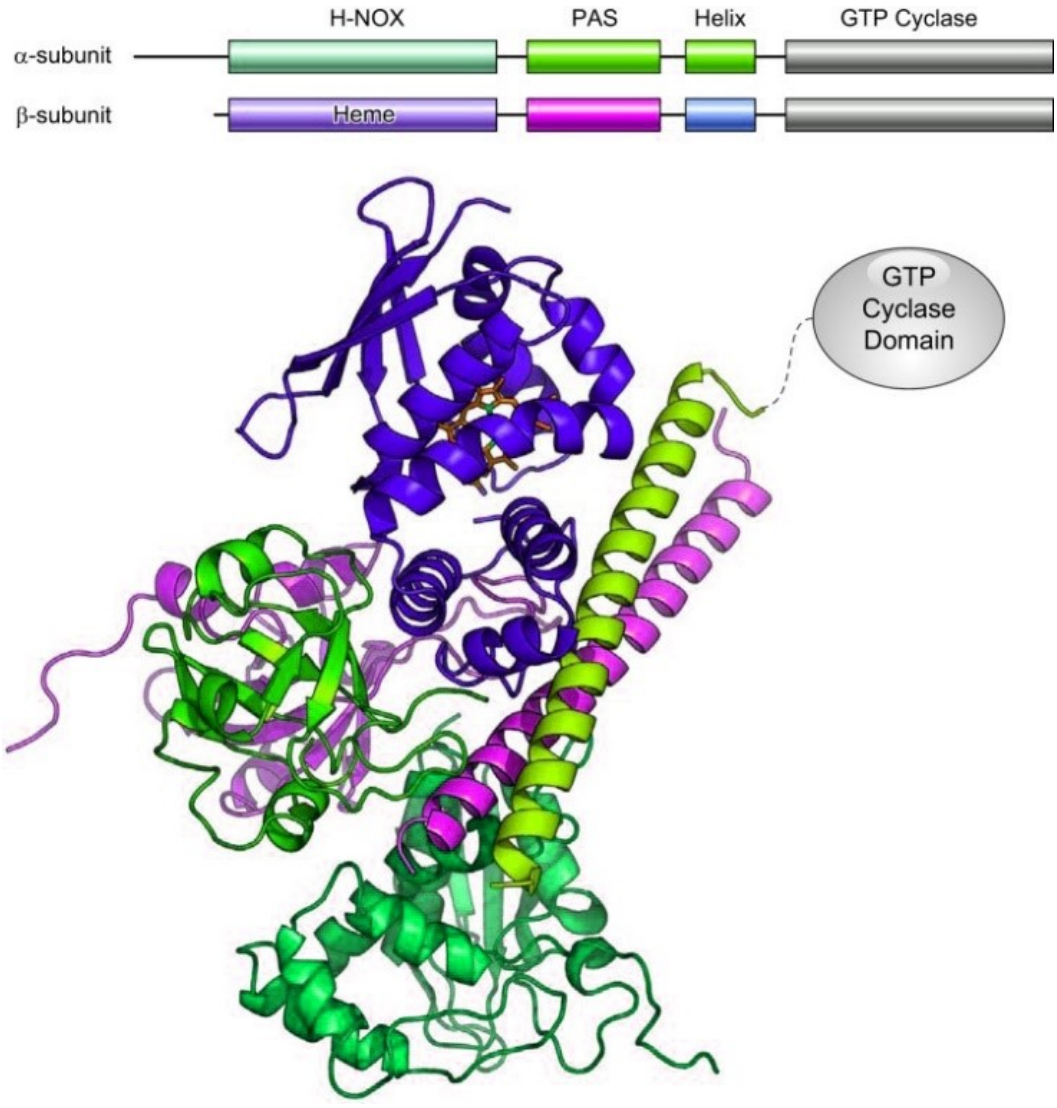

Figure 1.14: Structural Design of Soluble Guanylyl Cyclase
Top: soluble guanylyl cyclase domains in the α and β subunits.
Bottom: the interaction of the N-termini domains of the α and β subunits of soluble guanylyl cyclase.
Adapted from Childers KC. Structure/function of the soluble guanylyl cyclase catalytic domain.[119]

c. Anti-Thrombotic Effect

NO plays a vital role in endothelial protection against unusual vasoconstrictors and thrombus formation risks. The protective role of NO is also extending to the prevention of platelet aggregation and adhesion of foreign molecules to the endothelial cells. In hypercoagulopathy states, platelet aggregation stimulates the release of serotonin (5HT) and adenosine diphosphate (ADP), which augment NO production from the endothelial cells and, consequently, vasodilation.[132] The relaxation of the blood vessels increases the blood flow to the affected area and stop thrombus formation.[133] NO also potentiate the effect of prostacyclin and prevent platelet aggregation.[134,135] If the endothelial barrier is damaged, this relaxation does not occur, and platelet aggregation stimulates vasospasm by the release of thromboxane A2, serotonin and haemostasis is initiated.[133,136–139]

d. Anti-Inflammatory Effect

In recent years, increasing evidence supported the importance of NO in acute and chronic inflammation. They are also proved that treatment with NOS enzyme inhibitors reduces inflammation, especially in acute episodes where L-arginine enhances it.[140,141] It has also been shown that NOS enzyme inhibitors can attenuate immune induced vasculitis in the lung and skin vasculature.[142] Similarly, the colon expression of the NOS enzyme is augmented in inflammatory bowel diseases as ulcerative colitis and chronic ileitis.[143] In addition, scientists have reported that once the level of NO decreases, inflammatory responses leading to atherosclerosis is activated.[144,145]

Despite all these proved anti-inflammatory/inflammatory actions of NO, its exact origin in the inflammatory process is still unclear; however, scholars have suggested it might come from the endothelial cells of the blood vessels, neutrophils or macrophages.[146] NO also plays an important role in tissue damage, and it is believed to be a cytotoxic and cytoprotective agent at the same time.

It is cytotoxic by itself in normal conditions, and even in some situations, its effect is enhanced by direct interaction with oxygen free radicals to produce more cytotoxic compounds. Even though NO's cytotoxicity is well established, some scientists also proved its cytoprotective effect in some tissue injuries.[146,147] This dual functional nature of NO was explained by its vasodilator effect, which increases blood flow to the tissues and, consequently, protects it from harm.[148–151] All these properties gave NO its multifaceted role in the inflammatory cascade, from the vasodilation effects to increased permeability, oedema formation, leukocyte stimulation, to tissue cytotoxicity.[146]

iNOS was also found to be highly expressed in inflamed tissues, and its correlation with disease activity has been reported. Additionally, high doses of NO can trigger the necrotic and apoptotic pathways of cell death.[152]

e. Regulation of Endothelial-Leukocyte Interactions and Vascular Permeability

NO inhibits the neutrophil aggregation and adhesion to the endothelium. It has also been reported that it increases microvascular permeability and vascular protein leakage, which are prevalent features of acute inflammatory response.[153] The antiadhesive effects of NO on the neutrophils are related to the superoxide interaction.[154] Superoxide enhances leukocytes adhesions to the vascular endothelium, and in the absence of NO, it activates the mast cells, causing degranulation that enhance leukocyte adhesion to the endothelium. Thus, a relative imbalance in superoxide anion and/or NO levels may promote mast cell degranulation, leukocyte adherence, and leukocyte emigration, thereby inducing acute inflammation.[154]

1.3.2. Endothelial Dysfunction

Dysfunction of the endothelium has been implicated in the pathophysiology of different forms of CVDs, including hypertension, coronary artery disease, chronic heart failure, peripheral artery diseases, diabetes, and chronic renal failure.[77]

After discovering NO and its importance, endothelial dysfunction was defined as the inability of the endothelial cells to release NO and the enhancement of the endothelium-derived contracting factors (EDCF) effects.[155] Later, endothelial dysfunction was identified as the hallmark of many CVDs and the first step in a chain of events that leads to atherosclerosis and coronary artery disease.[156]

In the past decade, a plethora of studies showed that impaired vascular coagulation function, inflammatory properties, vascular growth and vascular remodelling are pathologically associated with endothelial dysfunction, with loss of NO bioavailability as the main feature in all these pathologies.[157,158] Additionally, localised endothelial dysfunction was proven to lead to induction of the tissue factor, increased release of von Willebrand factor (vWF) factor, homeostatic shift towards coagulation and inflammation.[159] All these changes were linked directly to cardiovascular diseases and complications (Figure 1.15).[156] Another feature of endothelial dysfunction that was highlighted in cardiac patients is the impairment of the endothelium barrier; this dysfunction manifested as oedema, coupled with leukocyte migration, which are the fundamental signs of inflammation.[160]

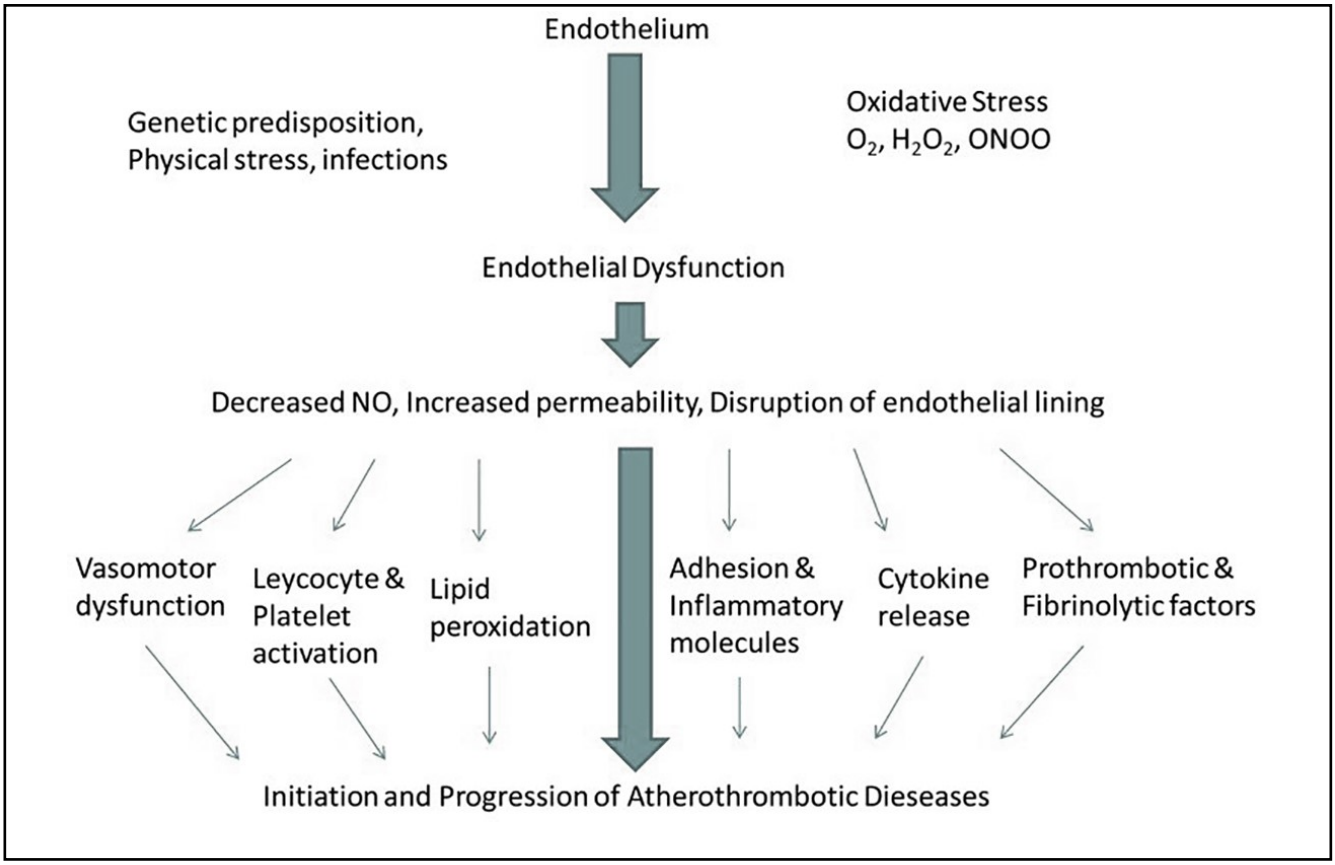

Figure 1.15: Endothelial Dysfunction and Atherothrombotic and Cardiovascular Diseases

1.4. Cardiovascular Diseases Predisposing Factors

1.4.1. Ageing

Ageing is one of the main risk factors of CVDs.[161] Vascular ageing is a new terminology used to describe vascular dysfunction, structural changes and degradation, which is the ultimate cause of end-organ damage, especially in highly perfused organs, e.g. the heart, the kidneys and the brain.[162] Although there is high inter-individual variability in disease onset and associated mortality, scholars have shown that age-dependent arterial injury typically manifests in the early fifties or sixties.[163]

Over the years, scientists proved that individuals do not age at the same pace; many factors were identified as contributors to the ageing progress, such as lifestyle and exercise [164], disease/health condition[165,166], genetic factors [167] and family history.[165] This observation led to the concept of biological ageing or physiological ageing, which describes the decline in organs function due to the advanced age effect, whereas chronological ageing is the number of years since birth (Figure 1.16). [168]

Biologically, ageing reduces the endothelium ability to dilate and contract as It decreases the endothelial ability to respond to NO and EDH. [169–171] Many researchers linked this dysfunction to the reduced production of NO, which is due to:

- Augmented arginase enzyme activity, competing with eNOS for the common substrate arginine [172–174]
- Increase the production of oxygen free radicals, which reduce the bioavailability of nitric oxide [175–177]
- Reduce the activity of eNOS [178]

Ageing was also found to affect the release of endothelium dependant prostanoids and can reduce the expression of sGC in the vascular smooth muscles.[179] Similarly, vascular contractions were reported as more prominent with any increase in age.[180,181] The upregulation of cyclooxygenase enzymes explained this by increased oxidative stress [182–184] and the augmented expression of the prostacyclin synthase gene.[184–186]

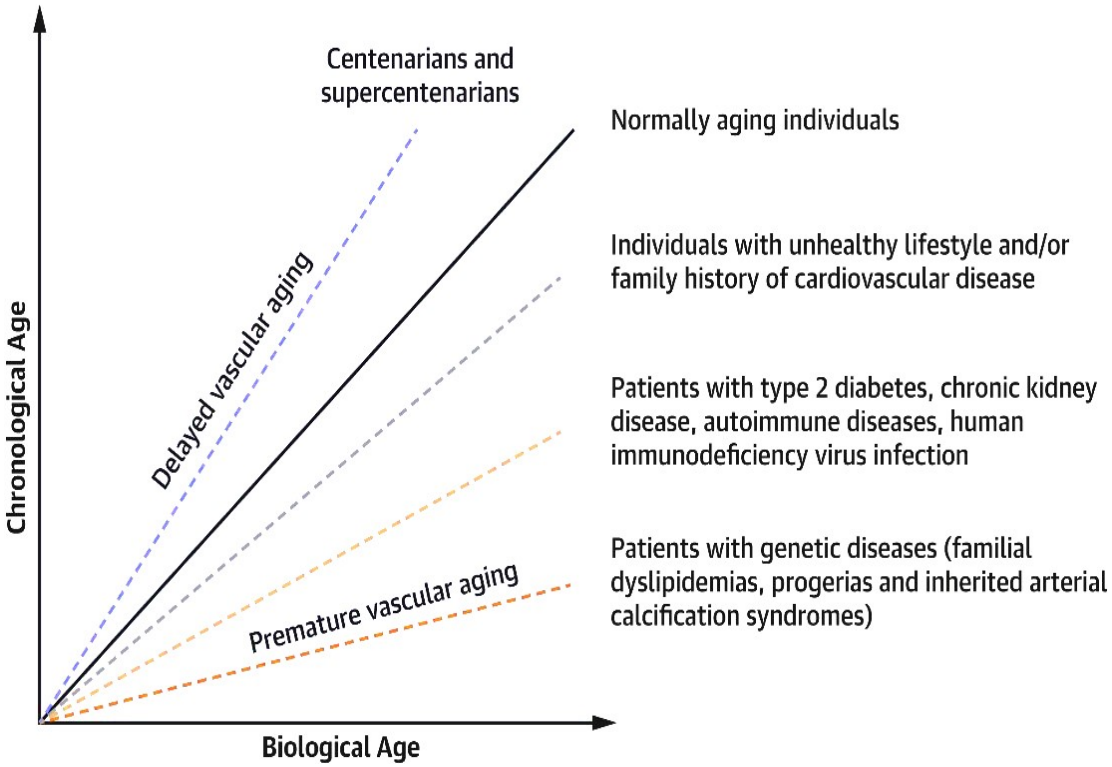

Figure 1.16: Biological versus Chronological Vascular Ageing

Adapted from Hamczyk MR et al. Biological versus chronological ageing[168]

1.4.1.1. Telomere Length (TL)

Telomeres are repetitive non-signalling DNA components at the end of the circulating peripheral blood leukocytes' chromosomes (TTAGGG hexamer in the vertebrates) (Figure 1.17).[187] Its main function is coding sequence protection from degradation. A shelterin protein complex caps the telomere to protect it from DNA damage response and several forms of double-strand break repair. This complex function depends on both the stability of the nucleoprotein structure and length. [188–190]

The telomeric sequences are lengthened by DNA exchange during the mitosis phase in cell division by a reverse transcriptase enzyme named telomerase, which elongates the telomeres to maintain their function.[191,192]

Most body cells (somatic cells) do not express telomerase; however, germ cells in the human stem cells, immune cells, and embryogenesis have active telomerase enzymes.[193] The absence or insufficient amount of telomerase results in TL attrition. Additionally, the destruction of the shelterin results in cellular senescence.[190,194]

TL shorten in proliferating cells with each physiological cell division due to the inability of DNA polymerase to complete the chromosome termini, which is reflected in age-dependent

telomere attrition.[192,195–197] Similarly, exposure to abnormal physiological conditions can also cause direct damage to the telomeric DNA, e.g. oxidative stress and inflammation, and some studies linked it to telomere attrition.[198]

1.4.1.2. Telomere Length and Oxidative Stress

Oxidative damage to the telomeric DNA is mediated by the formation of guanine, 8-oxodG, which leads to disturbances in the maintenance of telomere length. Furthermore, ROS can induce breaks in the DNA and weaken base repair.[199] As discussed before, telomeres cannot repair breaks in single strand DNA [200] making it very sensitive to the accumulation of the guanine oxide.[201–203] Additionally, non-matched bases in the telomeric sequence make it difficult for the replication mechanisms to maintain structural integrity, and oxidative stress can produce early telomere shortening regardless of age.[204]

Another critical point is the close correlation between oxidative stress and the inflammatory process in the human body.[205,206] In this context, several studies reported a negative correlation between the telomerase enzyme, TL and exposure to tumour necrosis factor-alpha.[207,208] Following the same concept, proinflammatory produced cytokines can cause telomere shortening directly.[209,210]

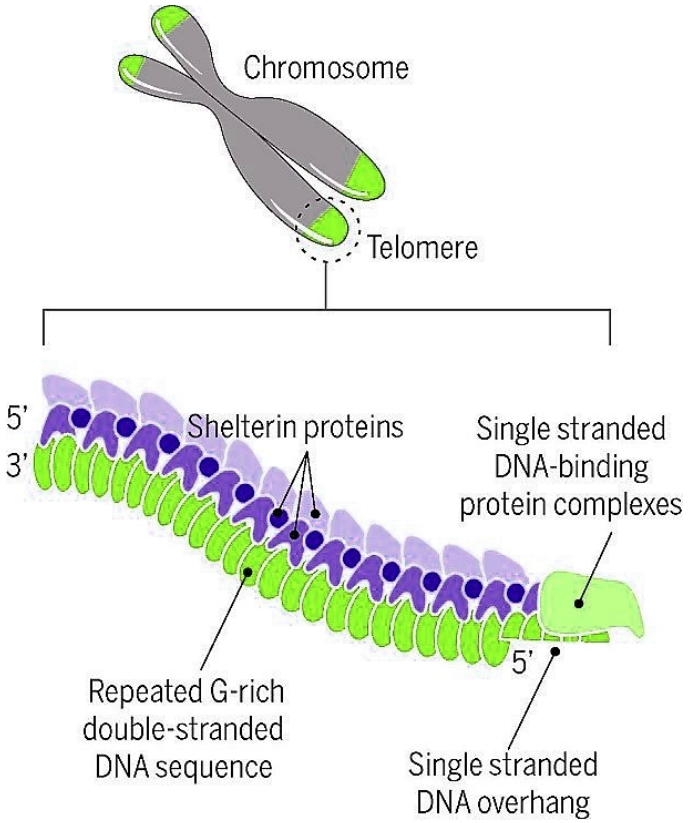

Figure 1.17: Telomere Structure

Adapted from Blackburn EH,et al. Human telomere biology[191]

1.4.1.3. Telomere Length and Cardiovascular Diseases

Independent of the age effect on the TL, studies found a possible involvement of telomeres in the initiation and progression of CVDs. It was noticed that patients with established CVDs or even early stages of myocardial infarction had shorter TL than age and sex-matched controls.[211–213] A direct correlation between TL in circulating cells such as leukocytes and TL in vascular tissues such as the aorta was also reported by some studies showing that leukocyte TL reflects telomere dynamics in tissues affected by CVDs, e.g. myocardium and coronary vessels.[214] This relation was also confirmed in coronary artery disease patients (CAD), although it was not possible to determine if it is a consequence or the cause of CAD.[213,215] However, in most cases, CAD was in the context of atherosclerosis/thrombosis and cellular senescence[216], which made scholars suggest that shorter TL can cause a certain type of senescence in both endothelial and smooth muscles cells.[215,216]

A correlation between CAD, TL and family history was also reported in a case-control study suggesting that the presence of short telomeres is a principal anomaly in atherosclerotic coronary diseases.[217] Similar correlation was found between shorter TL and increased mortality in CAD patients independent of any other risk factors.[218] Likewise, studies investigating the correlation between TL and HTN showed shorter telomere length in HTN patients compared to normotensive individuals.[198,219,220]

In addition, various CVDs risk factors were found to be strongly associated with leukocyte TL. For example, a significantly negative correlation was reported between Leukocyte TL (LTL) and the body mass index (BMI) ($r = -0.077$) and smoking history ($r = -0.087$) in a cohort of 1122 women.[221] The negative effect of smoking on LTL was confirmed later in many studies and was further correlated to early onset of chronic obstructive pulmonary disease [222–224], survival rate in smoker individuals [225] and airflow limitation in non-smokers.[226] Similarly, as a metabolic risk factor, homocysteine was found to negatively correlate to the LTL in a cohort of 1319 subject 236 and the association between BMI and decrease in LTL association was confirmed in the same study ($r = -0.106$).[227] Other risk factors such as atherosclerosis and elevated C-reactive protein were also reported to strongly correlate to shortened LTL.[218]

To conclude, currently, it is still difficult to define the telomere role in the pathophysiology of CVDs, and further research is needed to confirm the ability of its use as a prognostic factor of CVDs.

1.4.2. Blood Pressure and Shear Stress

As discussed in section 1.1.4, blood vessels are under constant mechanical loading due to the blood pressure and flow, leading to increased internal stresses, e.g., endothelial shear stress and circumferential wall stress.

These mechanical forces cause not only morphological changes of endothelium and blood vessel wall but also trigger biochemical and biological events.[228] There is considerable evidence that the increased frictional force of the blood on the vascular endothelium (wall shear stress) increases the blood flow and the pressure on the vascular endothelium, and this consequently stimulates endothelial nitric oxide synthase activity and NO production.[229–232] Additionally, it was proven that disturbed blood flow and uncontrolled blood pressure patterns cause epigenetic DNA, RNA methylation and other changes that modify gene expression, increase oxidative stress, blunt all endothelium dependant responses, and enhance atherosclerosis.[233–237]

Similarly, prolonged exposure to high blood pressure raises angiotensin I and II signalling and oxidative stress [238,239], leading to endothelial dysfunction.[240]

1.4.3. Serum Blood Lipids

Scientists have proved that high-density lipoproteins (HDL) bind to a bioactive lipid mediator named sphingosine 1-phosphate (S1P) and its endothelial receptor promoting the action of eNOS. This action induces the antioxidant enzyme heme oxygenase-1 (HO-1), leading to increased release of NO, explaining the beneficial effects of high HDL levels on the endothelial function.[241–243] Therefore, the agreement that higher HDL cholesterol levels are protective against endothelial dysfunction and CVDs. However, unlike other patients, higher HDL levels in CAD patients inhibit the action of eNOS and lose of its protective effect and stimulate platelet aggregation.[244–246]

Additionally, Hypercholesterolaemia, particularly high concentrations of low-density lipoprotein (LDL) and triglycerides (TG), were found to associate with reduced endothelial dilation.[247–250] Both the oxidised (Oxy LDL) and the carbamylated forms of LDL increase oxidative stress, which reduces the bioavailability of NO, attenuate its vasodilation effect [251–254], impair the turnover rate of eNOS [255,256] and increase asymmetric dimethylarginine (ADMA), causing vessel stiffness.[257,258]

1.4.4. Oxidative Stress

1.4.4.1. Radical Formation

Oxidative stress is the imbalance between the production of oxidant and antioxidant substances in the body. In various pathophysiological states, activation of nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), cyclooxygenase, eNOS when uncoupled with tetrahydrobiopterin (BH4) or lack of its substrate L-arginine and mitochondrial electron transport as well as inactivation of the antioxidant system, including superoxide dismutase, glutathione peroxidase, and catalase, lead to an increase in reactive oxygen species (ROS) production and decrease in ROS degradation.[259–265] The reduction of oxygen through the addition of electrons leads to the formation of several reactive species, including (Table 1.3):

- **Free radical species**, which are usually highly reactive and have one or more unpaired electrons.
- **Non-radical species** that can be derived from either oxygen or nitrogen.

In normal physiological conditions, oxygen free radicals can activate eNOS and stimulate the production of NO and vasodilation. Some of these superoxide molecules can also undergo dismutation by the superoxide dismutase enzyme to form hydrogen peroxide (H_2O_2), which can act as endothelium-derived hydrogen peroxide and stimulate vascular endothelium relaxation.[266] Additionally, the normal physiological amount of oxidative free radicals produced by the mitochondria can stimulate eNOS and help in the endothelial relaxation process.[267–270] However, under oxidative stress, the natural physiological process in the biological systems where the presence of free oxygen radicals overpowers the radical scavenging mechanisms is impaired.[271] Thus, creating an imbalance between the oxidants and the antioxidants.

Reactive ROS binds to NO molecules forming peroxynitrite, which decrease the bioavailability of NO and impair its effect on the vascular endothelial.[261,272–274] Adding to this, ROS can cause inactivation of eNOS by S-glutathionylation of the enzyme, subsequently, leads to endothelial dysfunction.[275,276] Overproduction of ROS is associated with developing various human diseases (including cancer, cardiovascular, neurodegenerative, and metabolic disorders), inflammation, and ageing (Figure 1.18).

Table 1.3: Different Types of ROS and RNS Produced in the Cell

Radicals		Non-Radicals	
Reactive Oxygen Species			
O_2^-	Superoxide	H_2O_2	Hydrogen peroxide
OH	Hydroxyl	$HOCL^-$	Hypochlorous acid
RO_2	Peroxyl	O_2	Single oxygen
RO	Alkoxyl	$ONOO^-$	Peroxynitrite
HO_2	Hydroperoxyl		
Reactive Nitrogen Species			
NO	Nitric Oxide	$ONOO^-$	Peroxynitrite
NO_2	Nitrogen dioxide	ROONO	Alkyl peroxynitrites
		N_2O_3	Dinitroge trioxide
		NO^-	Nitroxyl anion
		NO_2CL	Nitryl chloride

1.4.4.2. Radical Scavenging

The human body is equipped with a variety of antioxidants that serve to counterbalance the effect of oxidants.[277] They can be classified into two categories: enzymatic and nonenzymatic (Table 1.4).

The body's ability to respond to oxidative stress is limited. Under conditions of sustained oxidative stress or excessive ROS production, the damage induced may exceed the capacity of the antioxidants and the bodies repair mechanisms leading to more permanent cell, DNA and protein damage.[278]

Oxidative stress can be measured directly by measuring the concentration of the reactive oxygen species and plasma oxidant/antioxidant balance, or indirectly by measuring the levels of DNA/RNA damage, lipid peroxidation, and protein oxidation/nitration.[279]

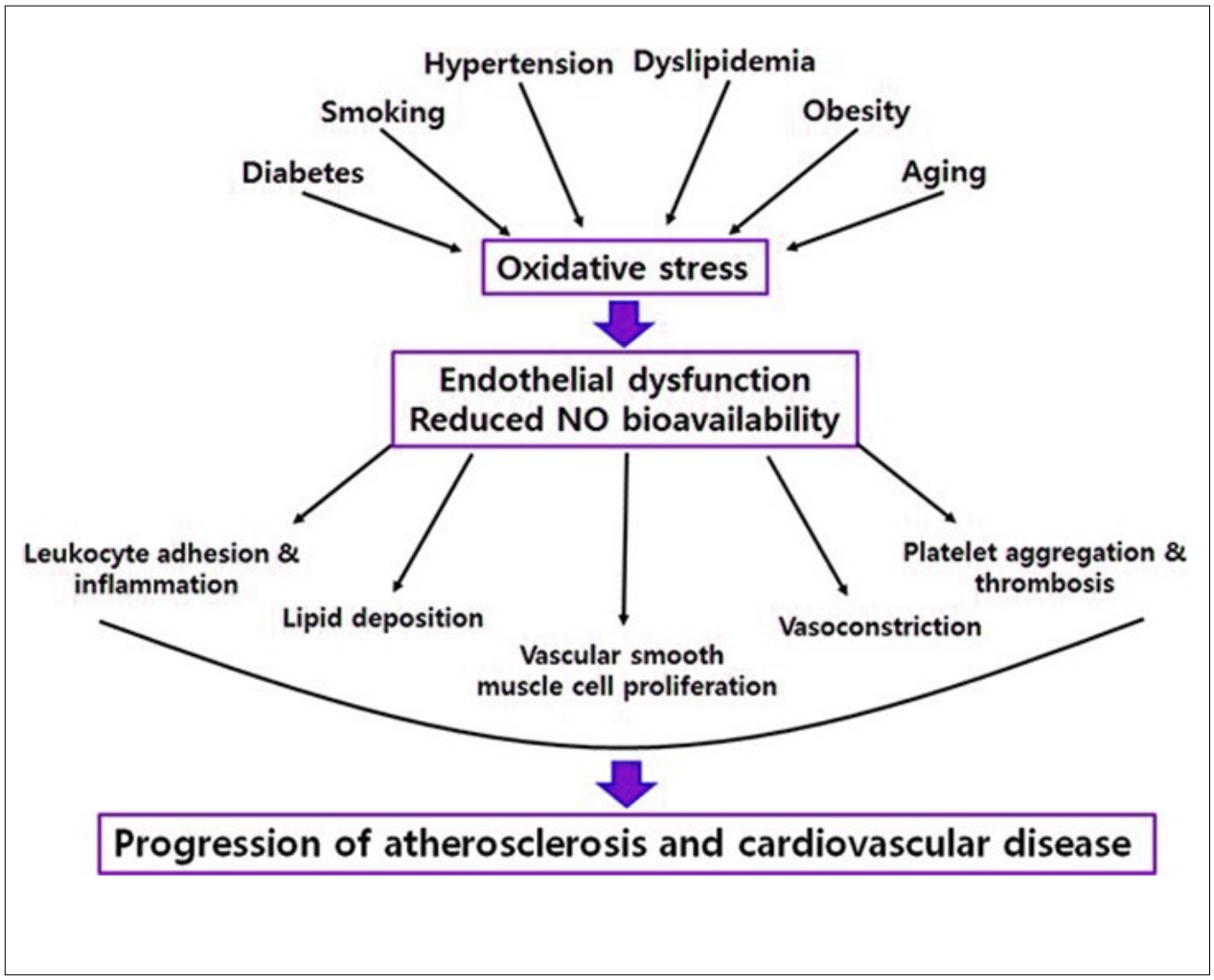

Figure 1.18: Oxidative Stress and Endothelial Dysfunction

Table 1.4: Enzymatic and Nonenzymatic Antioxidants that Protect Against ROS/ RNS Generation

Enzymatic antioxidants	Nonenzymatic antioxidants
▪ Thioredoxin ▪ Peroxiredoxins ▪ Glutaredoxin ▪ Glutathione peroxidase ▪ Reduced glutathione (GSSG) ▪ Oxidised glutathione (GSH) ▪ Glutathione reductase ▪ Catalase ▪ Peroxidase ▪ Superoxide dismutase	▪ Vitamin C, E, A ▪ Thiols ▪ B- Carotene ▪ Polyphenols ▪ NAC ▪ Zinc, selenium ▪ Glutathione ▪ Uric acid ▪ Lycopene ▪ Allyl sulfide ▪ Indoles ▪ Gallic acid ▪ Hesperidin ▪ Catechin ▪ Chrysin

1.4.4.3. Oxidative Stress Biomarkers

a. Glutathione Redox Buffer

One of the most commonly used methods to assess oxidative stress status in the body is measuring glutathione in the plasma. Glutathione has two forms; the reduced form (GSH) and the oxidized glutathione disulphide (GSSG). [280–282] The GSH/GSSG is the most efficient plasma and tissue oxidant/antioxidant system in the human body. Usually, this system is in balance; however, under oxidative stress conditions, it can be disrupted. As an antioxidant, GSH can prevent the devastating effects of ROS either directly through its oxidisation or indirectly by maintaining other cellular oxidants in a functional state.[283]

The product of GSH oxidation is glutathione disulfide. Within the cells, GSH is regenerated from GSSG by glutathione reductase using NADPH as an electron donor (Figure 1.19).[284,285]

Under normal physiological conditions, GSSG represents only around 10% of the total glutathione pool of a healthy cell.[286] However, under conditions of oxidative stress, ROS levels are raised, and hence the production of GSSG is increased significantly. As such, determining

the GSH: GSSG ratio or glutathione status of a cell is considered a good indicator of cellular redox imbalance and oxidative stress, especially as maintaining an optimal GSH: GSSG ratio is critical for cell survival. A reduced GSH: GSSG ratio would be considered indicative of oxidative stress.[287]

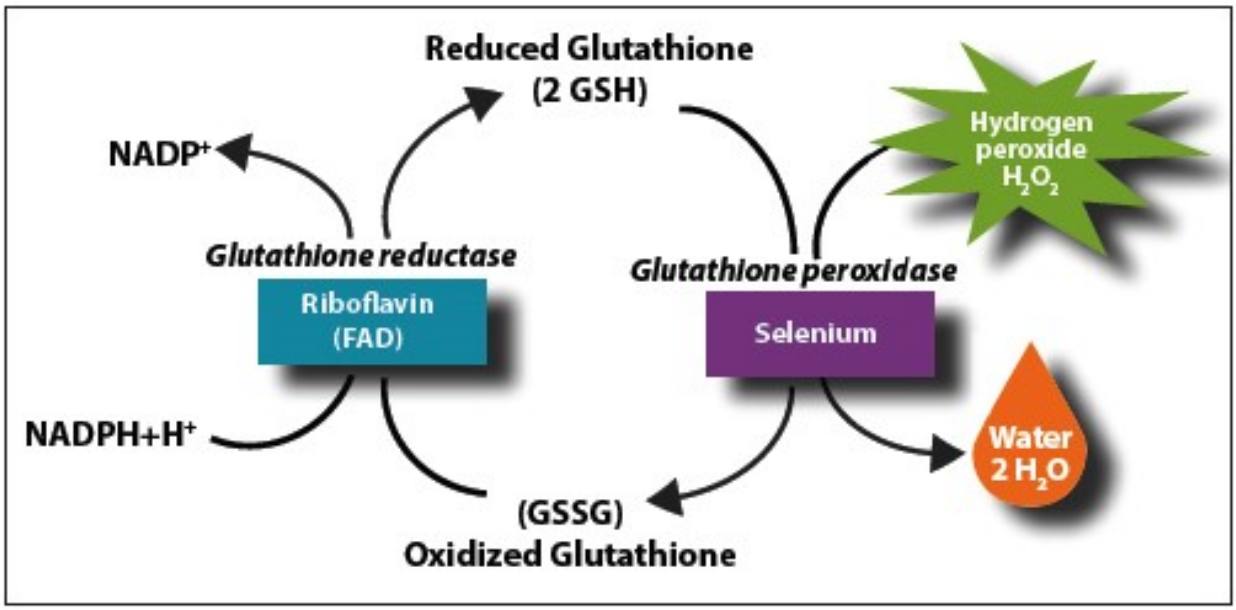

Figure 1.19: Glutathione Redox Cycle

Adapted from Faranak Ilkhani, Niacin and Oxidative Stress [288]

b. Glutathione Redox Homeostasis and its Relation to Cardiovascular Diseases

The first study to evaluate the relation between GSH and CVDs was conducted in Japan, results from this study showed that increased GSH levels decrease systolic and diastolic blood pressure and decrease the incidence of diabetes in cardiac patients. The same authors reported decreased plasma GSH levels in subjects with CVDs compared to individuals with no cardiac history.[289]

The effect of antihypertensive drugs was also evaluated in a study conducted by Chaves et al., GSH levels were decreased while GGSG increased in hypertensive patients compared to controls.[290] Additionally, GSSG levels decreased, and GSH increased after three months of the hypertension treatment, highlighting the importance of oxidative stress in the onset and progression of hypertension. [290,291] Similarly, lower plasma and right atrial appendages levels of GSH were found in CAD patients and were consistent with the severity of left ventricular dysfunction.[292] The same study also reported higher GSH levels in asymptomatic CVDs

patients compared to symptomatic patients, which was linked to more severe cardiac abnormalities in CVDs patients.[292]

Higher intraoperative GHS levels were also associated with higher prevalence of myocardial infarction, which aligns with the findings of other studies reporting the association between Glutathione S-transferase polymorphism and the incidence of myocardial infarction during cardiac surgeries.[293] The same extent, decreased glutathione peroxidase activity was inversely associated with increased risk of stroke and coronary heart diseases (Table 1.5). [294,295]

Table 1.5: Relationship between GSH levels and cardiovascular diseases

Paper	Condition	GSH association
Haruki Shimizu et al.[289]	Stroke & Myocardial Infarction	Lower plasma levels of glutathione.
Viktória Kovacs et al.[293]	Myocardial Infarction after cardiac surgery	Glutathione Transferases polymorphism.
Stefan Blankenberg et al.[295]	Myocardial Infarction/Cardiac death	Decreased level of erythrocyte Glutathione Peroxidase-1.
Thibaud Damy et al.[292]	Heart transplantation/coronary artery bypass grafting/ aortic valve replacement	Decreased level of glutathione.
Kiyotaka Kugiyama et al.[296]	Cardiac catheterization	Vasodilator effects on coronary arteries and increased blood flow.
J. Robaczewska et al.[297]	Elderly people with hypertension	Disturbed level of glutathione and enzymes involved in the synthesis of glutathione.
Josep Redón et al.[291]	Hypertension	Decreased level of glutathione; increased level of glutathione disulphide.
Felipe J. Chaves et al. [290]	Non-treated hypertension	Decreased level of OS and glutathione disulfide; increased level of glutathione
Saleem Ullah Shahid et al.[298]	Diabetes/coronary heart disease	Decreased level of glutathione; increased level of glutathione disulphide.
Tohru Hamanishi et al.[299]	Type 2 diabetes mellitus/some forms of CVD	Increased values of intima-media thickness in Pro/Leu glutathione peroxidase-1 genotype.
J. Rybka et al. [300]	Elderly on antihypertensive drugs	Increased level of glutathione and glutathione-disulfide reductase.
Josep Redón et al.[291]	Hypertension	Decreased level of glutathione; increased level of glutathione disulphide.

c. Protein Carbonyl

As discussed above, increased oxidative stress leads to proteins, lipids and DNA damage; however, protein is considered the immediate vehicle imposing oxidative stress on the cells as they are catalysts rather than stoichiometric mediators; hence, the effect of the damage to one molecule is more remarkable than stoichiometric.

During increased oxidative stress, the carbonyl groups either aldehydes or ketones, are formed on the side chains of the proteins.

The formed Carbonyl proteins are very stable, which facilitated their detection and measurements in the human plasma.[301] Although there are many potential oxidative protein modifications, only few have been studied in clinical practice. Of them, protein carbonyl was the most used, and its accumulation was linked to several diseases such as diabetes, Parkinson's disease, Alzheimer's, sclerosis and inflammatory arthritis.[302–304]

d. Protein Oxidation and Cardiovascular Diseases

Few studies evaluated the relationship between protein carbonyl and cardiovascular diseases. Descamps et al. evaluated advanced oxidation protein to predict the development of atherosclerosis in non-diabetic patients. During the follow-up period (7 years), oxidized protein products were associated with a higher incidence of ischemic heart diseases, ischemic stroke, peripheral artery diseases, and aortic aneurysms.[305] On the other hand, another two studies conducted by Mocatta et al. and Semba et al. evaluated the relation between protein carbonyl and myocardial infarction in advanced aged women. These studies supported the CVDs predictive power of protein carbonyl; however, no correlation was found regarding all causes of mortality over a five-year follow-up period. [306,307]

e. Lipid Oxidation

Lipid oxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids. Over the last four decades, an extensive body of literature regarding lipid peroxidation has shown its important role in cell biology and human health and different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide/insecticides, are sources of in vivo ROS production.[308]

The two most prevalent ROS that can affect profoundly the lipids are mainly hydroxyl radical ($HO^{\bullet}$) and hydroperoxyl ($HO^{\bullet}{}_2$).

Human sterols are prone to oxidation as they all have an unsaturated double bond between the carbon number 5 and 6 in their structure. Under the influence of reactive oxygen species

and free radicals, cholesterol molecules bind to hydroxyl, epoxide, and ketone groups to form a more polar and easily absorbed moieties.[308]

Oxidation of cholesterol is usually via two mechanisms, autoxidation or enzymatic metabolism Figure 1.20. This oxidation produces an epimeric mixture of hydroperoxides which is further disintegrated to 7α- or 7β-hydroxysterol and 7-ketocholesterol [309] while epoxidation produce 5,6α-epoxy-5α- and 5,6 β-epoxy-5β-cholestan-3β-ols and hydration of epoxysterols produces 5α-cholestane-3β,5,6β-triol.[310] Additionally, auto-oxidation of crystalline cholesterol produces the 20, 24, 25, and 26-hydroperoxides.[311]

The most common oxysterols found in human plasma are 27OH, 24OH, 3βOH and 7αOH cholesterol produced by the enzymatic reaction of the mitochondria and the endoplasmic reticulum hydroxylases.

The activity of oxysterols (pathophysiological and biomedical) is more than twice that of cholesterol, and molecules produced by the enzymatic reaction are always present in equilibrium with those formed by the non-enzymatic ones. [308] This equilibrium is disturbed in certain aetiologies and has been used as a biomarker for diagnosing and monitoring of diseases progression.

f. Oxysterols and Cardiovascular Diseases

Hypercholesterolemia and atherosclerosis are part of the primary risk factors of CVDs. Of particular note oxysterols were found to contribute to the vascular cellular dysfunction by promoting oxidative stress, inflammation and atheroma development Figure 1.21.[313]

Since the end of the 90s, efforts were exerted to evaluate oxysterols in human blood and blood vessels. In this consideration most of the investigations confirmed oxysterols presence in CVDs; specifically, 7-ketocholesterol, cholestane-3β,5α,6β-triol, 7-HC, and 5α,6α- and the 5β,6β-epoxycholesterol isomers were found in the carotid artery atherosclerotic plaques.[314]

Similarly, mass spectrometry techniques identified high plasma concentrations of cholesterol oxides (24S-Hydroxycholesterol, and 27-hydroxycholesterol) in peripheral artery diseases patients. Additionally, the tissue concentrations of 24S-Hydroxycholesterol, and 27-hydroxycholesterol were significantly correlated to the plasma levels of C-reactive protein.[315]

Elevated levels of 24S-Hydroxycholesterol were also found in the serum of individuals with genetic hypercholesterolemia and were positively associated with higher carotid intima-media thickness.[316]

Preeclampsia (PE) is another CVD associated with pregnancy; altered cholesterol metabolism and pregnancy-induced oxidative stress were confirmed as one of this disease's aetiologies.[317,318]

Higher plasma levels of cytochrome P450 and ROS derived oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol,4β-hydroxycholesterol, 20α-hydroxycholesterol

and 27-hydroxycholesterol) were confirmed in PE patients compared to normotensive pregnant women and was directly correlated to preeclampsia.[319]

Recently scientists started to study the potential application of oxysterols as indicators of the efficacy of lifestyle interventions and therapies. Preliminary animal results also showed that aerobic exercises and anti-dyslipidemic drugs (e.g. Simvastatin) decreased oxysterols plasma levels, thus suggesting antioxidant properties of the drugs and the applied interventions besides their cholesterol-lowering action.[320–323]

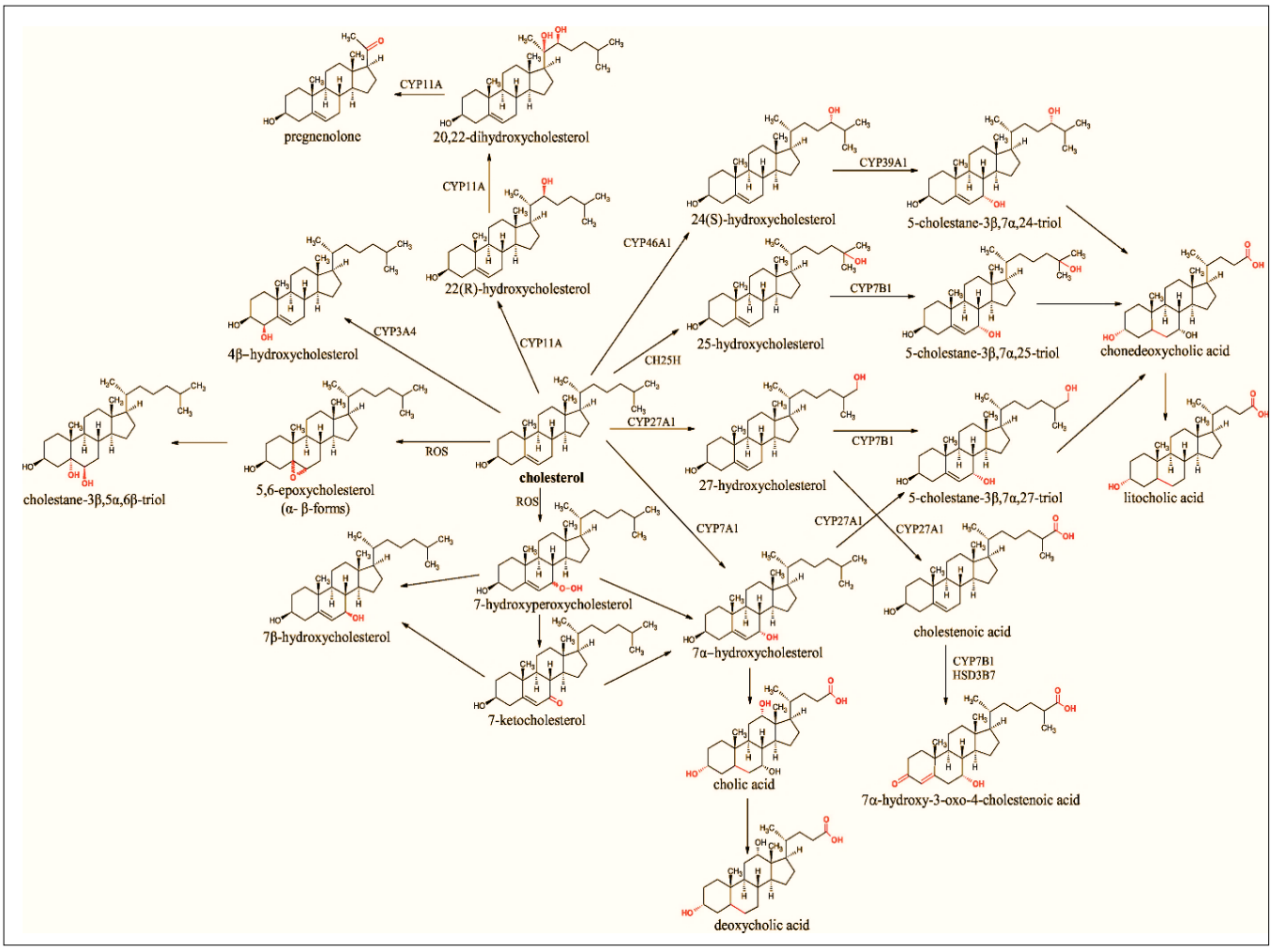

Figure 1.20: Main Products of Enzymatic or Auto-Oxidation of Cholesterol
Adapted from Leonarduzzi G et al. Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects.[312]

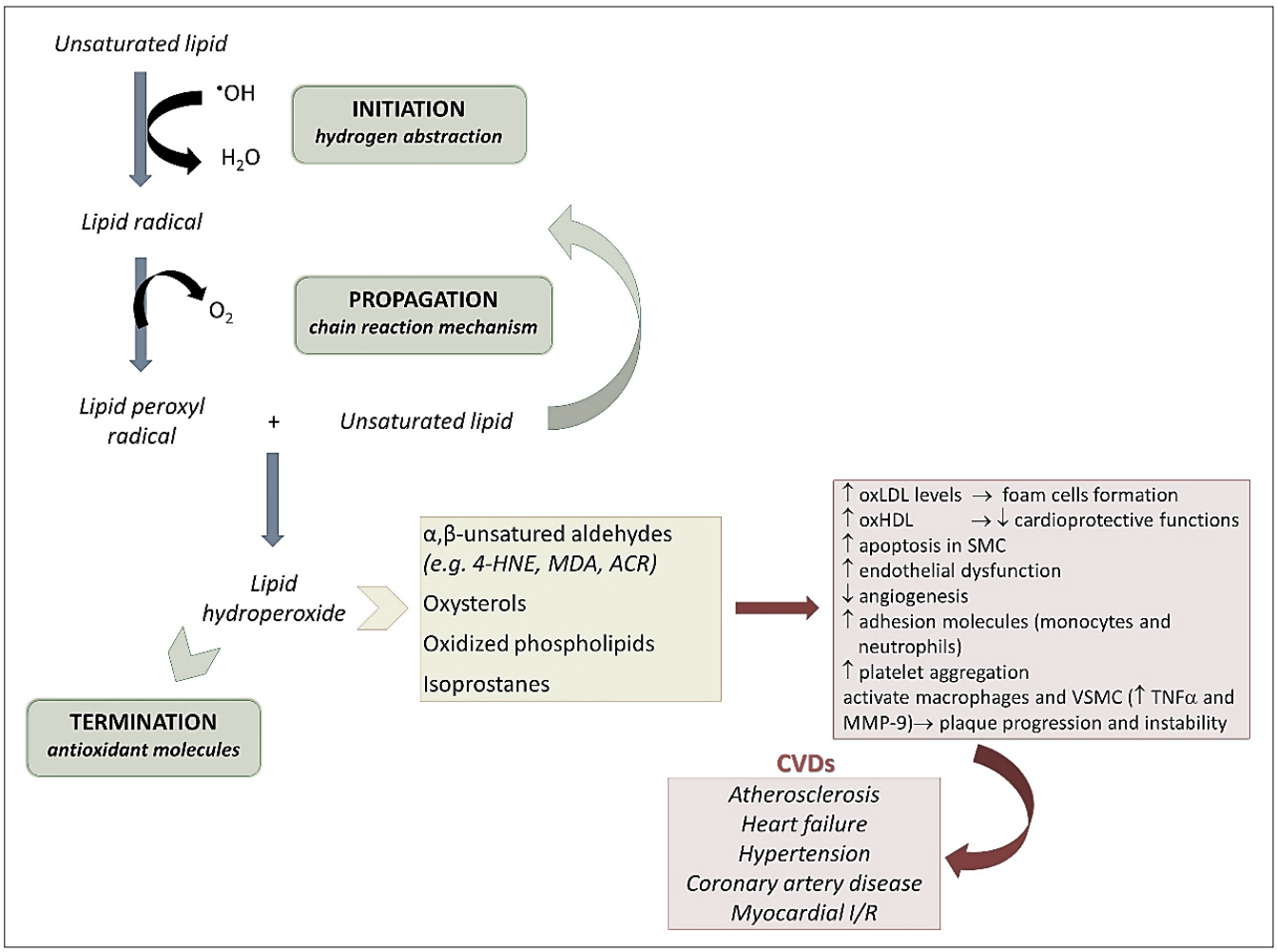

Figure 1.21: Mechanisms Involved in The Formation of Advanced Lipoxidation, Endproducts, Relevant Targets and Their Involvements in Cardiovascular Diseases. Adapted from Gianazza E. Lipoxidation in cardiovascular diseases.[324]

1.4.5. Dry Eye Syndrome

Dry eye disease (DED) is a multifactorial disease of the ocular surface (cornea, conjunctiva, accessory lacrimal glands, meibomian glands) with loss of tear film homeostasis and development of ocular symptoms. The disease is also named keratoconjunctivitis sicca, dry eye syndrome, and dysfunctional tear syndrome.[325]

The prevalence of DED ranges from five to 50% globally. This prevalence was estimated to be 2.7% in those between 18 to 34 years old and increases to 19% in those above 75 years old and was higher in women 8.8% than men 4.5%.[326]

Disease Risk factors [327–330]:

- Advanced age
- Female sex

- Decreased androgens
- Medications (antihistaminic, anticholinergics, hormonal therapy, antidepressants, antiarrhythmic drugs, ocular medications).
- Diseases (diabetes mellitus, Parkinson disease, Sjögren's syndrome)
- Contact lens
- vitamin A deficiency
- Corneal refractive surgery
- Dry environments

Severe DED can decrease visual acuity, which can limits individuals physical functioning and productivity.[331–333]

DED has a complex and multifactorial aetiology. The tear film of the eye consists of three components:

- Aqueous component
- Mucous component
- Lipid component

A healthy tear film depends on the balanced interaction between the eyelid, ocular surface and the lacrimal glands (Figure 1.22).[334]

It is also classified into two groups [325] :

- **Decreased tears production**, resulting in aqueous component deficiency DED
- **Abnormal meibomian gland physiology**, resulting in evaporative DED.

Tear film hyperosmolarity, inflammation of the ocular surface and subsequent activation of the sensory nerves of the ocular surface leads to signs and symptoms of the disease.[335,336]

As the clinical evaluation findings of DED are variable, clinicians base their diagnosis on validated questionnaires.[337] These questionnaires can also be used to monitor disease progress and treatment follow-up.[338]

The most commonly used questionnaires are:

- Ocular Surface Disease Index (OSDI): Useful in patients with more severe symptoms to monitor the response to therapy and variability in symptoms over time (Appendix 1).[339]
- Dry Eye Questionnaire (DEQ-5): a five-item questionnaire reduced from the Dry Eye Questionnaire to determine DED symptom severity (Appendix 2).[340]
- Impact of Dry Eye on Everyday Life (IDEEL): Fifty-seven questions in three modules validated in patients with DED.[341]

- Salisbury Eye Evaluation Questionnaire (SEE): a six-item questionnaire used in self-reported, population-based prevalence surveys to determine visual impairment among older adult subjects.[342]

Details of the diagnosis of DED is provided in section 3.3.9.

Recently, the relationship between DED and inflammation produced systemic illnesses such as hypertension, diabetes and dyslipidaemia was evaluated in several studies; however, further investigation is needed to assess and confirm this association and identify the possible correlation mechanisms.[344–346]

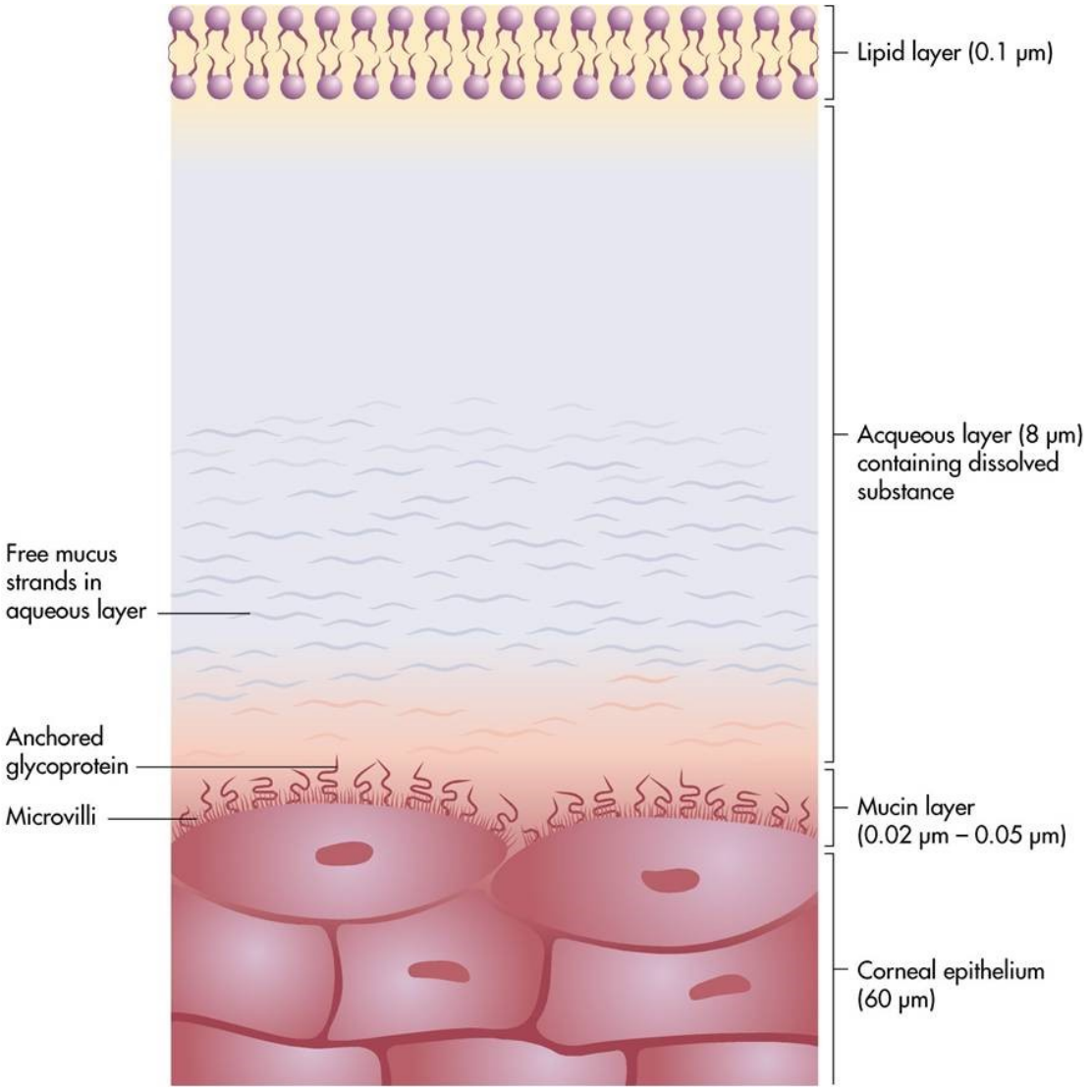

Figure 1.22: Precorneal Tear Film

Adapted from Physiology of the Eye [343]

1.5. Cardiovascular Diseases Risk Assessment

1.5.1. Assessment of Endothelial Function

Endothelial function testing can be divided into peripheral (systemic) and central (coronary) vascular assessment. Most clinically used techniques depend on the pharmacological/ physiological stimulation of NO and other vasoactive mediators release and measuring the vascular response to the endothelial dependant stimuli, e.g., increased shear stress, hyperaemia, or systemic administration of vasoactive stimulants.

The endothelial function testing technique can also be divided into invasive and non-invasive procedures.[347]

1.5.1.1. Invasive Techniques

Invasive techniques include **quantitative coronary angiography** (QCA), which measures coronary arteries luminal diameter in response to intracoronary administrated endothelial dependant vasodilator substances such as Acetylcholine (Ach). The NO-mediated vasodilation is measured by angiography or intravascular ultrasound.[348,349] Coronary microcirculation can also be assessed by **intracoronary Doppler** using a pharmacological stimulator, e.g. adenosine or nitroprusside or physiological stimuli.[350]

A similar method but dependant on thermodilation and index of microcirculatory resistance is also used to assess coronary microcirculation. This technique is identical to pharmacological and pressure-based approaches but uses **intracoronary temperature** measurements to approximate flow.[351,352]

1.5.1.2. Non-Invasive Techniques

The gold standard non-invasive technique used to assess macrovascular endothelial function is the NO-driven **flow-mediated dilation (FMD).** Using brachial artery ultrasound, it is possible to assess peripheral changes in the blood flow rate in response to increased shear stress in the arm.[353,354] Other ways of stimulating vasodilation are also used, e.g. reactive hyperaemia, exercise, psychological stress and cold presser sympathetic activation (Table 1.6).

A newer method of testing macrovascular function is the **low flow-mediated constriction (L-FMC)** which measures the endothelial response to reduced blood flow and shear stress.[355] L-FMC is a better measure of resting and basal arterial tone. The exact mechanisms of the arterial response to decreased shear stress are not fully elucidated; however, scientists believe it is partially dependant on NO.[356]

Plethysmography of the forearm circulation is one of the leading techniques used to assess peripheral endothelial dysfunction. It examines the changes in forearm blood flow in response to IV administered vascular stimuli. Because it is a semi-invasive technique (IV

administration of vasoactive substances), it is not a preferred method in clinical practice and is only used for research purposes.[357]

Microvascular endothelial function is also used to measure vascular reactivity to the same stimuli; however, typically utilising peripheral tonometry or pulse wave amplitude to approximate blood flow.

Peripheral Arterial Tonometry (PAT) is another technique that depends on measuring reactive hyperaemia response in finger pulse wave amplitude after the inflation/deflation of a blood pressure cuff to supra-systolic pressures.[358–361]

Traditional Imaging techniques are also used to assess microvascular endothelial function; for example, myocardial positron emission tomography (PET) is used in diabetic patients [362] and was verified in other patients cohorts.[363] Similarly, cardiac magnetic resonance perfusion has been used to assess endothelial function in patients without overt CAD and normal angiograms angina patients.[364]

There are other measures of endothelial function that are currently in various stages of use and design, mainly in the research realm, e.g., measures of endothelial progenitor cells (EPCs) and subtypes that assist in endothelial repair and function. These measures has been associated with the Framingham scores[365] and various measures of CVD [366], Statins [367], erythropoietin [368] and exercise. [369]

Nucleotide polymorphisms, microRNAs, urinary markers and genome sequencing were also linked to endothelial dysfunction and atherosclerosis; however, they are not clinically used.

Table 1.6: Advantages and Limitations of the Most Common Non-invasive Techniques Used to Assess Endothelial Function

Technique	Vascular bed	Advantages	Limitations	Stimulus
Flow-mediated dilation [370]	Brachial artery Conduit artery	Easy access Strong correlation with invasive epicardial vascular function Used to assess other parameters (flow, baseline arterial diameters, FMC) Inexpensive	Several measurement protocols Not standardised	Reactive Hyperaemia
Venous occlusion Plethysmography[371]	Forearm vasculature Microvasculature	Assess dose-response relation Easy access	Invasive Time-consuming	Different vasoactive substances, e.g., acetylcholine
Peripheral Arterial Tonometry [372]	Finger Microcirculation	Easy access Easy to perform Automated so decreases interpersonal variability	Influenced by non-endothelial variables	Reactive Hyperaemia

1.5.1.3. Peripheral Vascular Reactivity

Vascular reactivity is the response of blood vessels to external stimuli. It is a simple, non-imaging and non-invasive method used to assess endothelial dysfunction in primary clinical settings.

Digital thermal monitoring (DTM) is one of the most commonly used techniques for evaluating peripheral reactivity. It measures changes in finger temperature during and after arm-cuff-induced ischemia (Figure 1.23).[373,374] The Arm- cuff occlusion induces reactive hyperaemia, which correlates to brachial artery reactivity.[374]

A vital advantage of DTM is that it measures both microvascular and macrovascular reactivities.[375] Studies have shown that cutaneous micro and macrocirculation are similar to the systemic circulation a function of NO [376], and temperature rebound measured by DTM was found to correlate directly with the presence and extent of CAD.[377] It was also correlated to the Framingham risk scores and coronary artery calcification in asymptomatic populations.[378] In another study, it was directly associated with myocardial perfusion independent of cardiovascular risk factors.[379]

Additionally, it was also used to evaluate the effect of the pharmacological influences of some vasoactive drugs.[380]

Although large prospective trials are needed to establish the clinical value of fingertip DTM, the clinical implications are very promising.

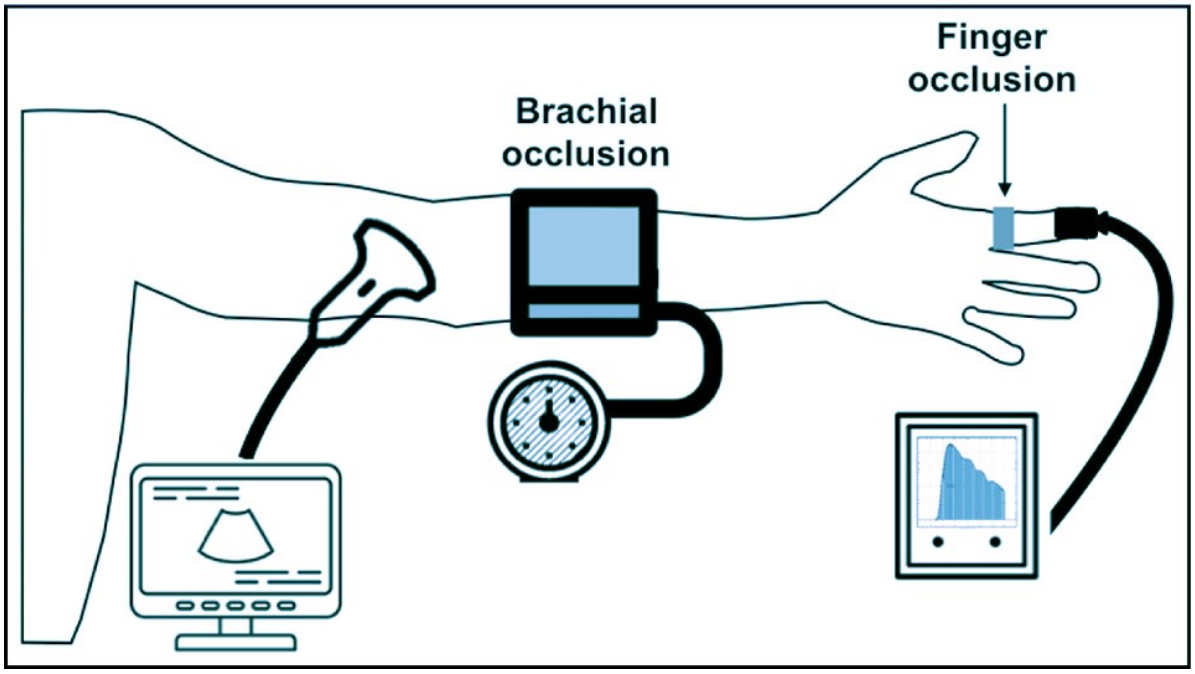

Figure 1.23: Schematic Figure Depicting Digital Thermal Monitoring

1.5.1.4. Endothelial Dysfunction as a Marker of Future Cardiovascular Risk

Does it provide prognostic information beyond commonly used risk scores?

As discussed above, many studies linked endothelial dysfunction to most CVDs' predisposing factors, e.g., systemic hypertension [381–383], dyslipidemia [384,385], diabetes [386–389], smoking [390,391], obesity [392,393], hyperhomocysteinemia [394,395] and age.[396,397] Similarly, extensive literature evidence confirmed the systemic nature of endothelial dysfunction and showed its strong correlation to the coronary arteries functions.[353,358,398] Additionally, the pathophysiology of the impaired endothelium was found to relate to vascular structural changes like the carotid intima-media thickness in individuals free from CVDs [399,400] and in less healthy individuals as hypertensive postmenopausal women.[401]

From the above, we can see that endothelial dysfunction is a confirmative link to CVDs and CVDs' risk factors. However, it is essential to ask whether endothelial dysfunction provides more clinical information beyond the routinely used assessment scores in clinical settings.

1.5.2. Cardiovascular Events Risk Scores

Over the years, many invasive methods to assess endothelial function in the coronary arteries were used as a prognostic factor for future risk of development of CVD, even in healthy individuals.[402,403] However, in primary prevention, the risk of using invasive techniques overweighted their benefit. Thus, most clinicians preferred using disease risk scores or peripheral ways to assess the future risk of developing cardiovascular diseases. The Framingham, Reynolds, PROCAM, QRISK, SCORE, JBS3, MESA, and the pooled cohort ASCVD risk calculators are examples of the most commonly used CVDs estimation risk scores in the clinical settings (Table 1.7).[404,405]

Regardless of clinicians' wide use of these risk calculators, studies showed many drawbacks associated with their use. For example, the Framingham risk score was found to overestimate CVDs risk by 37% and up to 67% in some populations.[404,405] Similarly, the Reynolds Risk Score was found to overestimate the risk by 9% to 21%, and the AHA-ACC-ASCVD risk calculator did not show improved calibration or discrimination compared with four other scores used in clinical practice.[404,405]

Results from the evaluation studies pushed clinicians to use non-invasive peripheral endothelial dysfunction evaluation methods as risk evaluators for future cardiovascular events.

Table 1.7: Comparison of Frequently Used Cardiovascular Risk Prediction Tools

	FRS	Pooled Cohort Equ.	WHO/ ISH Risk Charts	JBS3	SCORE CVD	QRISK	Reynolds	MESA
Age	✓	✓	✓	✓	✓	✓	✓	✓
Gender	✓	✓	✓	✓	✓	✓	×	✓
Ethnicity	×	✓	×	✓	×	×	×	✓
Region	×	×	×	✓	✓	✓	×	×
Diabetes	✓	✓	✓	✓	×	×	✓	✓
Smoking	✓	✓	✓	✓	✓	✓	✓	✓
FH	×	×	×	✓	×	✓	✓	✓
AF	×	×	×	✓	×	×	×	×
CKD	×	×	×	✓	×	×	×	×
RA	×	×	×	✓	×	×	×	×
BP treatment	✓	✓	×	✓	×	✓	×	✓
SBP	✓	✓	✓	✓	✓	✓	✓	✓
BMI	×	×	×	✓	×	✓	×	×
Apolipoprotein	×	×	×	×	×	×	×	×
T-Chol	✓	✓	✓	✓	✓	✓	✓	✓
HDL	✓	✓	×	✓	✓	✓	✓	✓
Lipid-lowering treatment	×	×	×	×	×	×	×	✓
CRP	×	×	×	×	×	×	✓	×
CAC score	×	×	×	×	×	×	×	✓
Psychological assessment	×	×	×	×	×	×	×	×
End Points Assessed								
CHD Death	✓	✓	×	✓	✓	✓	✓	✓
MI	✓	✓	×	✓	×	✓	✓	✓
Angina	✓	×	×	✓	×	✓	×	×
Revascularisation	×	×	×	✓	×	✓	✓	✓
Arrhythmia	×	×	×	×	✓	×	×	×
Heart failure	✓	×	×	×	✓	×	×	×
Stroke	✓	✓	×	✓	✓	✓	✓	×
TIA	✓	×	×	✓	×	✓	×	×
Intermittent Claudication	✓	×	×	✓	✓	✓	×	×
Aortic aneurysm	×	×	×	×	✓	×	×	×
Cardiac resuscitation	×	×	×	×	×	×	×	✓

Table 1.7: FH, family history; AF, atrial fibrillation; CKD, chronic kidney disease; RA, rheumatic arthritis; BP, blood pressure; SBP, systolic blood pressure; BMI, body mass index; T-Cho, total cholesterol; HDL, high-density lipoprotein; CRP, C reactive protein; MI, myocardial infarction; TIA, transit ischemic attack.

1.5.2.1. Non-Invasive Endothelial Dysfunction Assessment Techniques versus Risk Scores in the Prediction of Cardiovascular Events

FMD was one of the fundamental ways used for this purpose. It was found to predict cardiovascular events beyond traditional risk scores in some special populations, e.g., postoperative assessment of patients who underwent elective vascular surgeries [406], healthy postmenopausal women [407], patients who suffered from chest pain [408] and patients admitted for invasive evaluation of chest pain.[409] Additionally, many large-scale studies recognized the value of the use of FMD in primary prevention settings. In two studies (435 and 268 healthy subjects), brachial FMD assessment enhanced the long-term adverse cardiovascular events prediction capability of the traditional risk factor assessment.[410,411] Similarly, in the 2007 cardiovascular health study (2700 apparently healthy 72 years old participants), a five-year follow-up period showed the association of endothelial dysfunction measured by FMD and event-free survival even after adjusting for traditional risk factors.[412]

In 2009, the multi-ethnic study of atherosclerosis (3000 participants) confirmed the predictive power of FMD in White, Black, Hispanic and Chinese subjects even after adjusting for the Framingham risk factors.[413] Similarly, many other studies have confirmed the additional prognostic information the FMD provides over other traditional risk scores.[414,415] However, all these studies showed that adjustment to conventional risk factors weakened the correlation between FMD and the assessed outcomes. Additionally, many studies showed that the vascular bed in which the assessment is performed could affect the results to the extent of no correlation at all with CVD risk.[416] Similar to FMD, scholars found that adding hyperemic velocity to the Framingham Risk score led to a risk-reclassification improvement.[400,417] Acetylcholine-associated peripheral microvascular endothelium dysfunction was also associated with CV events in the elderly while FMD was not and its addition to the Framingham risk score improved risk discrimination.[418] Likewise, EndoPAT helped predict non-coronary atherosclerosis [419] and adverse cardiac events in outpatients [420] that the Framingham risk score could not predict.

From the previous discussion, we can say that macrovascular assessment is beneficial in patients with established diseases, and microvascular function is a better indicator for future risk. However, and even with all the data indicating the importance of endothelial dysfunction as a predictive value of future risk of CV events, routine use of endothelial function measurements assessment is not yet recommended by neither the European (ESC)[421,422] nor the American (AHA/ACCF) guidelines [423] and received a lower recommendation than carotid IMT measurements and coronary calcium score.

The reason against the routine use of these techniques despite their high sensitivity is that their specificity is not well established and yet remains a concern. Another limitation is the poorly standardized methodology (except EndoPAT).[424] Additionally, these techniques are a

static way to assess the endothelial, so if the endothelium function is appreciated as a dynamic process, these methods are not the best to reflect the real state of the endothelium.[424] Furthermore, the conflicting reports regarding the agreement between the FMD and the PAT results made the situation more confusing. Though some scientists found an association between the two tests, others highlighted the discordance between them.[425–427] Likewise, although IMT and calcium scores can be performed in a more standardized manner and are less impacted by transient abnormalities than endothelial function, these measures give information about the vascular structure (more established disease) rather than the function. Additionally, these measurements do not change rapidly with interventions, which is an invaluable advantage of endothelial function measurements.[428,429] Finally, cost and limited resources are other reasons limiting the wide adaptation of these modalities.

1.5.3. Assessment of the Ocular Circulation

1.5.3.1. Ocular Blood Flow Assessment

Over the years, several methods were developed to assess the ocular blood flow depending on the area of interest. Colour Doppler ultrasound imaging is one of the most used methods to assess retro-ocular vessels blood flow [430], while intra-ocular vessels can be visualised and evaluated by direct methods such as ophthalmoscope, fluorescence or indocyanine green angiography.[431] Laser Doppler velocimetry is used to quantify the blood velocity [66], and laser-flowmetry and laser-speckling are used to quantify blood flow in the capillary bed, such as the optic nerve. Similarly, thermography is used to assess the bulk flow to the eye [432], and retinal vascular analyser (RVA) is used to observe dynamic changes in blood flow over time.
A summary of the main characteristics of these methods is provided in Table 1.8.

Table 1.8: Techniques of Ocular Blood Flow Measurement and Assessment

Technique	Measured Variables	Vascular bed	Measurement Location	Advantages	Limitation
Fluorescein angiography[433–435]	Mean Circulation time, Arteriovenous passage time	Retina	Main retinal vessels	Simple, painless procedure	Exact relation to retinal blood flow unclear
Fluorescein Angiography with scanning laser [436–438]	Erythrocyte and Leucocytes velocity	Retina	Perimacular retinal capillaries	Provides detailed information on ocular perfusion	No information on vessel diameter
Laser Doppler Velocimetry combined with fundus photography [439]	Blood flow	Retina	Main retinal vessels	Capable of calculating the blood velocity, cross-sectional area, vessel diameter, and hence the total blood flow. Non-invasive	A highly skilled operator is mandatory
Laser Doppler flowmetry[440,441]	Blood flow	Optic nerve or choroid	Capillaries	One of the most widely utilised techniques. Non-invasive	Sampling depth unknown
Scanning Doppler flowmetry[442,443]	Blood flow	Retina or optic nerve	Capillaries	Non-invasive, does not require dilation, quick, direct assessment of ocular blood flow	Sampling depth unknown, exact relation to blood flow unclear
Colour Doppler imaging [444,445]	Blood velocities	Central retinal artery, posterior ciliary arteries, ophthalmic artery, Retina	Extraocular vessels	High reproducibility Fast procedures	No information on vessel diameter, can't be used for small vessels
Blue field entopic technique [446–449]	Leucocyte velocity and density	Retina	Perimacular retinal capillaries	Allows assessment of foveal perfusion.	Leucocyte movement is blood flow subjective
Pneumotonometry [450]	Changes in intra-ocular pressure during cardiac cycle	Choroid	Global	A non-invasive method of estimating the intraocular pressure	Only pulsatile flow component, standard ocular rigidity assumed
Laser interferometric measurement of fundus pulsation [451]	Changes in corneo-retinal distance during cardiac cycle	Choroid	High topical resolution	Simple, non-invasive	Only pulsatile flow component
Dynamic retinal vessel analysis[452]	Retinal vascular Diameter and reactivity	Retina	Retinal vessels	Measurement of the diameter of retinal vessels in relation to time and location. Noncontact & non-invasive method	Requires clear media, good fixation and pupil dilation. More aimed at assessing retinal vascular function than OBF
Retinal Oximetry[453–455]	Retinal vessels	Retina	Retinal metabolism	Reliable, non-invasive technology for retinal oxygen measurements	Influenced by many external factors e.g. light
Laser Speckle Flowgraphy[443]	Blood flow velocity	Retinal, choroidal vessels, circulation of the ONH	Global	A noncontact & non-invasive method	Not direct assessment of OBF

1.5.3.2. Retinal Vascular Analysis

a. Background

For years, structural changes in peripheral vessels were frequently associated with numerous CV risk factors, e.g. hypertension, diabetes[456,457], obesity [458–460], chronic kidney disease [461] and smoking. [462,463] It was also linked to the progress and development of cardiovascular events [464,465] and end-organ damage in CVD patients.[465,466]

Increased media-to lumen ratio (M/L) is the most frequently used technique to evaluate the resistance of small peripheral arteries. Wire or pressure micromygraphy was highlighted as the best approach to demonstrate an increased M/L ratio in high and low to moderate risk patients.[467] Still, unfortunately, the invasive nature of this technique prevented the wide and routine application in clinical settings. To develop alternative non-invasive approaches for evaluating the microvascular structure and function, the interest of many researchers shifted towards the retinal vascular district. The rapid development of retinal vessels investigational methodologies allowed scientists to quantify geometrical and topological changes in the arteriolar and venular trees in many cardiovascular diseases, e.g., hypertension, coronary artery disease and cardiovascular risk factors such as diabetes mellitus, ageing, smoking and obesity.

On the other hand, microcirculation in the retina can be imaged directly with plenty of non-invasive methods. Thus, it provides a window to visualize any microvascular changes that lead to the development of cardiovascular diseases.[468] Although data from retinal vascular analysis provide information about the blood vessels' structure and function; its clinical application has only recently gained attention.[469]

b. Structural Retinal Changes

Systemic CVDs and cardiovascular risk factors like arterial hypertension[470,471], coronary heart disease[472], stroke [473,474] or preeclampsia[475], as well as obesity[476] are all associated with structural vascular changes in the retina. These changes include arterioles narrowing, veins dilation and arteriovenous ratio alterations (AVR).

The development in ophthalmoscopy techniques in the 19th century opened a window to investigate the retina and its microvasculature in a simple and non-invasive way. Robert Marcus Gunn was the first to detect retinal vascular changes in systemic CVDs.[477,478] Later, Keith, Wagener and Barker associated retinal changes to the survival of hypertensive patients, and this was used later for the classification of hypertensive retinopathy (The KWB scale).[479–482] Similarly, Hubbard and his colleagues developed formulas to calculate the central retinal arteriolar/ venular equivalent (CRAE/CRVE) using retinal photography.[483] Based on these formulas, the arteriovenous ratio (AVR) was calculated in many studies as it is not dependent on the optical properties of the eye or the used camera.[484] However, the introduction of more

sophisticated fundoscopic and optical coherence tomography (OCT) techniques offered a way for early detection of retinal structural changes and future susceptibility to develop CVDs.[485–488] Later several studies reported a strong association between larger venular calibre and higher risk of future development of CVDs.[489] Additionally, the M/L ratio was found to be independently associated with CVDs in high, moderate and low-risk populations.[465,490–494]

Similarly, CRAE and AVR were found to precede hypertension's clinical appearance even in normotensive individuals and children.[495–501]

Based on the findings of these studies, the 2011 British society of hypertension guidelines recommended routine fundus examination for all hypertension/prehypertensive patients to assist in the evaluation of the future risk of CVDs.[502] However, regardless of the data supporting its importance, fundus examination value in clinical assessment was questioned. [503,504] and the following European society of cardiology guidelines recommended against its routine use in the evaluation of hypertensive patients.[505]

A list of the most commonly used measurements of retinal vascular caliber is provided in Table 1.9.

Table 1.9. Measurement of Retinal Vascular Caliber

Retinal vascular parameters	Definition	Advantages	Limitations
Arteriovenous Ratio	Ratio of the caliber of arterioles to venules.	Dimensionless, controls for magnification differences from camera lenses and refractive error.	Nonspecific. Changes in AVR can't specify if the changes in arteriolar or venular diameter or in both.
Central Retinal Artery Equivalent	An index that reflects the average width of retinal arterioles.	Reflects distinct systemic vascular disease pathways that tend to target the arterial system.	Not highly accurate. May underestimate the true internal vascular diameter.
Central Retinal Vein Equivalent	An index that reflects the average width of retinal venules.	Reflects distinct systemic vascular disease pathways that tend to target the venous system.	Not highly accurate. May underestimate the true internal vascular diameter.

c. Limitations of Structural Retinal Vascular Measurements

Regardless of scientists support for the routine use of retinal imaging in clinical settings, the technique has many limitations that limit its clinical applicability. Firstly, the used equations to calculate summarized indices from individual calibers is based on empirical models. The Knudtson-parr-Hubbard formulas were derived from analysis of population sample retinal images, and they used a root mean square deviation model that best fits the observed data.[483,506,507] Later, Knudtson et al. developed revised formulas for summarizing vascular caliber. The new formulas gained wide acceptance from scientists and demonstrated more accuracy in calculating the CRAE and CRVE.[484] Still, all these formulas were an estimated summary, not the actual value of the retinal vascular caliber. The second issue faced these formulas was that all the researchers used them to compare different groups. However, to use these measures for risk stratification in clinical practices, it should be optimised to measure individual patients.[508] That brought the magnification effect from retinal image, either by incorporating an adjusted measurement to compensate for this effect or using dimensionless measurements.[508] Ocular biometric data can be used to adjust for magnification whoever its accuracy in digital retinal images is not well established. The third limitation that was addressed by many scientists is retinal caliber measurements are usually obtained from one image, and it has been shown it vary by up to 15% depending on the point of the pulse cycle when the shoot was taken.[509] The Fourth and the most critical limitation is the lack of normal population data. Although the technique was widely used in different populations, there is still no clear identification of what should be considered normal measurement and what is abnormal. Finally, the retinal vascular caliber measurements do not reflect the 3-dimensional architecture or the functional changes of the retinal microvasculature. Therefore, the full potential of retinal image analysis in relation to the prediction of cardiovascular diseases remains undetermined.[480]

d. Functional Retinal Changes

In the last decade, dynamic retinal vascular analysis (DVA) grabbed all the attention as an innovation in endothelial function evaluation techniques. The technique and its application in clinical practice greatly impacted the assessment and risk stratification of many diseases.[452] DVA is a closed system consists of a fundus camera attached to a real-time monitor and a video camera with special software to record and analyse the vascular response to external stimulation. Scientists used many techniques to stimulate the endothelial reaction in the vascular vessels; oxygen or meduna's mixture inhalation was the first technique used to provoke retina vascular response; however, due to its side effect, scientists moved to using systemic vasoactive drugs or flickering light to activate the retinal vascular response.[510,511] Of

all the vascular stimulation methods, light stimulation was the most accepted due to its non-invasive nature and the absence of systemic side effects.[512]

Flicker-light induced vasodilation in the retinal artery was proved to be endothelium and nitric oxide dependent [513–515], and as it precedes the development of structural vascular changes, it played an important role in the diagnosis, prognosis evaluation and treatment monitoring diabetic, cardiovascular and dyslipidaemic patients.[159,516,517]

While reduced flicker light-induced vasodilation has already been demonstrated in patients with established cardiovascular diseases, [452,518,519] few studies used it to evaluate patients' future risk of developing CVDs.[513,515] Compared to static retinal imaging, DVA offers a more precise way to assess microcirculatory behaviour and identify any impairments even before developing clinical signs.

e. Retinal Vascular Functionality and Hypertension

Similar to structural retinal vascular changes, there is a huge number of evidence reporting reduced retinal arterial vasodilation in response to flicker light in people with untreated hypertension.[520] One of the first studies to evaluate retinal vessel response to flicker light stimulation was Nagel and his colleagues in 2004.[520] They reported a significant reduction in retinal vascular response to flicker stimulation in hypertensive patients compared to healthy individuals. The untreated hypertensive patients had a mean baseline-corrected flicker response of 2.2 ± 2.5% compared to 6.4 ± 2.7% with a P value of 0.001.[520] However, because of the small sample size (45 subjects) and as there was no follow-up period, researchers could not prove if these reported differences were an individual variation or due to the untreated pathology.[520] Nonetheless, another study performed by the same group showed that treatment of hypertensive is directly correlated to improvement in arterial baseline diameter, however not to flicker response after one and a half year follow-up period. These results suggested that vascular baseline diameter is a better indicator of vascular reactivity than vasodilation in hypertensive patients. [521]

Later, many studies reported impairments in other retinal vascular parameters (arterial and venous) in hypertensive patients. Machalińska and her colleagues reported a decreased arterial dilation in hypertensive patients compared to healthy controls (mean 1.31, P=0.001) and a lower vein dilation by an average of 1.32, p=0.002 after adjustment of all influential factors.[522] Additionally, the same study reported a negative correlation between arterial response to flicker and plasma C reactive protein (Rs = −0.29, p = 0.07), plasma tissue necrotising factor α (TNFα) concentrations and arterial response to flickering light stimulation (Rs = −0.39, p = 0.02).[522] There is also growing evidence suggesting a decreased maximum dilation and baseline corrected flicker response in middle age untreated hypertensive patients.[523]

f. Retinal Vascular Functionality and Dyslipidaemia

A strong association between dyslipidemia and wider venular caliber was found in many researches.[524] Scientists also reported improvement in visual acuity with LDL apheresis and control of refractory dyslipidemia.[525] Although systematic studies on flicker-induced retinal vasodilatation in hypercholesterolemia are still scarce, a reduced response to flicker has been reported in patients with hypercholesterolemia.[526] Similarly, scientists showed that LDL apheresis significantly improved flicker light-induced retinal venular dilation, indicating improved retinal venular response, endothelial function, or both.[526]

g. Retinal Vascular Functionality and Ageing

Most cardiovascular and cerebrovascular diseases are directly connected to ageing and have been linked to progressive endothelial dysfunction in the macrovessels.[527]

Microcirculation analysis using DVA showed impaired flicker light response in the elderly, suggesting a decline in the regulation of the retinal vessel diameter.[528] An abnormal constriction response was also noticed, with young individuals showing an arterial constriction response to amplitudes below baseline diameter post flicker light-induced vasodilation.[529,530] This constriction response did not appear in older individuals.[523] Similarly, in individuals with low cardiovascular risk, age-related differences in flicker-induced retinal vessel diameter change throughout the entire functional response curve for arteries and veins were reported, where gender differences mainly affected the arterial dilatory phase and are only present in young individuals.[531]

2. Research Rationale

While various biomarkers offer a disease-specific individual biological profile, none of them enables non-invasive assessments in primary care settings while being sufficiently sensitive to allow early detection and prevention.[532]

Similarly, although quantification of vascular and endothelial dysfunction is under investigation as a promising early indicator for CVD risk, assessing it implies complex techniques, high costs, and specialised settings.[533]

The Dynamic Vessel Analyser (DVA, Imedos GmbH, Jena, Germany) is one such device that enables the assessment of retinal vascular function through a non-invasive method. The use of DVA in early CVDs screening was pioneered by the supervisory team at Aston University, who was also the first to demonstrate that dynamic vascular behaviour is unique to each individual and their age group and existing pathologies.[534]

Using DVA for risk stratification of CVDs patients will also provide a method of obtaining easy-to-understand data for practitioners to use for follow-up on disease onset progression and treatment effectiveness, including personalised reports. Benefits to patients are expected over

the immediate, medium- to long-term, as the solution will provide specific and personalised warnings of CVD risk and opportunities for immediate advice regarding lifestyle changes, preventive treatments, and disease and management follow-up.
Assessing individual patients rather than the whole population will give more insight into specific risk factors and will open the door for tailored therapy plans. CVDs prevention is imperative, and the most effective path to it should include actions primarily at an individual level.

2.1. Research Aims:

Study I: To investigate the presence and impact of ocular and systemic vascular alterations in prehypertensive patients and to explore the concept of using retinal vascular analysis to identify people at risk of developing future CVDs.

Study II: To analyse retinal and peripheral microvascular function in subjects that fall into different BP groups when using either the ESC/ESH or the ACC/AHA guidelines to identify the diagnostic cut-off values for early hypertension.

Study III: To explore the presence of microvascular endothelial dysfunction (as a measure for early CVD) in individuals diagnosed with dry eye disease and its correlation with systemic lipids.

Study IV: To investigate the correlation between lipid peroxidation markers, glutathione redox ratio and retinal microvascular functional reactivity in apparently healthy individuals of various age groups.

Study V: To assess the performance of using either leucocytes telomere length or retinal microvascular dysfunction or the combination of both for predicting age and systemic blood pressure, two of the most known risk factors for CVD.

3. Subjects and Methods:

This chapter outlines the recruitment procedure, inclusion, and exclusion criteria for all the participants involved in this research. It then outlines the study protocol and investigative techniques used throughout this thesis for the assessment of ocular and systemic vascular function.

3.1. Ethical Approvals

Ethical approval was sought and received from the relevant research ethics committee(s) (National Health Service (NHS) and/or Aston University College of Health and Life 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.

3.2. Patient Recruitment

3.2.1. General Inclusion/Exclusion Criteria

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 diseases 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 cerebrovascular diseases 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 overestimation 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 retinal vessel analyser, 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.

3.3 Methods

Prior to study initiation, the author was trained to use 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 was achieved. See Table 3.1 below for a summary of the main investigative techniques used for each of the studies (1-5) in this thesis.

Table 3.1: Overview of Main Investigative Techniques/Clinical Parameters Measured

	Technique/Parameter	**Purpose**	**Study**				
			1	**2**	**3**	**4**	**5**
Ocular	-Non-contact tonometry	-IOP	✓	✓	✓	✓	✓
	-DVA	-Endothelial Function	✓	✓	✓	✓	✓
	-Non-invasive Keratograph	-Tear film and corneal assessment	-	-	✓	-	-
	- Ocular Osmolarity	- Osmolarity	-	-	✓	-	-
Clinical	-Anthropometry	-Body composition	✓	✓	✓	✓	✓
	-Sphygmomanometry	-Blood Pressure	✓	✓	✓	✓	✓
	-Ambulatory blood pressure	- 24 blood pressure	✓	✓	✓	✓	✓
Biomarkers	-Redox Index (GSH/GSSG)	- Oxidative stress	✓	✓	-	✓	✓
	-Inflammatory Markers (IL-6)	- Endothelial dysfunction	-	-	-	✓	-
	-Telomere Length	- Biological ageing	-	-	-	✓	-
	-Oxidized lipids (Oxysterol)	- Endothelial function	-	-	-	-	✓
	-Oxidized proteins	- Endothelial Function	-	-	-	-	✓
	-Lipid Panel	- Circulatory blood lipids	✓	✓	✓	✓	✓
	-Glucose	- Diabetes	✓	✓	✓	✓	✓
	-Venepuncture	- Blood withdrawal	✓	✓	✓	✓	✓
Other subjective	-General questionnaire	- Medical history/diet/lifestyle	✓	✓	✓	✓	✓
	- Framingham Risk Score	- CVDs risk	✓	✓	-	-	-
	- DEQ-5 item score	- Dry eye severity	-	-	✓	-	-
	- OSDI Scores	- Ocular surface disease	-	-	✓	-	-

3.3.1. Experimental Protocol

All measurements were performed between 8 am and 11 am following a 12 hour overnight fast, which included refining from alcohol or caffeine. All procedures were conducted in a consistent order outlined below and detailed in the following sections (Figure 3.1):

1. Suitable participant identified, approached, and provided with the study information pack
2. Procedures and risks explained, concerns addressed
3. Consent form read, understood, completed, and signed by participants
4. Preliminary assessments:
    - Demographic questionnaire
    - IOP measurement
    - Height and weight measurement
    - Baseline BP readings
    - DEQ-5 and OSDI questionnaires (DED study participants)
5. 1% Tropicamide inserted into a randomly selected eye
6. Peripheral vascular reactivity assessment
6. Fasting venous blood sample obtained by venepuncture
7. Ambulatory BP and heart rate variability (HRV) monitor fitted (Study 1&2 participants)
8. Assessment of retinal vessel reactivity (DVA)
11. Dry Eye Disease examination (DED study participants)
12. Final BP measurement

Appointment Flowchart

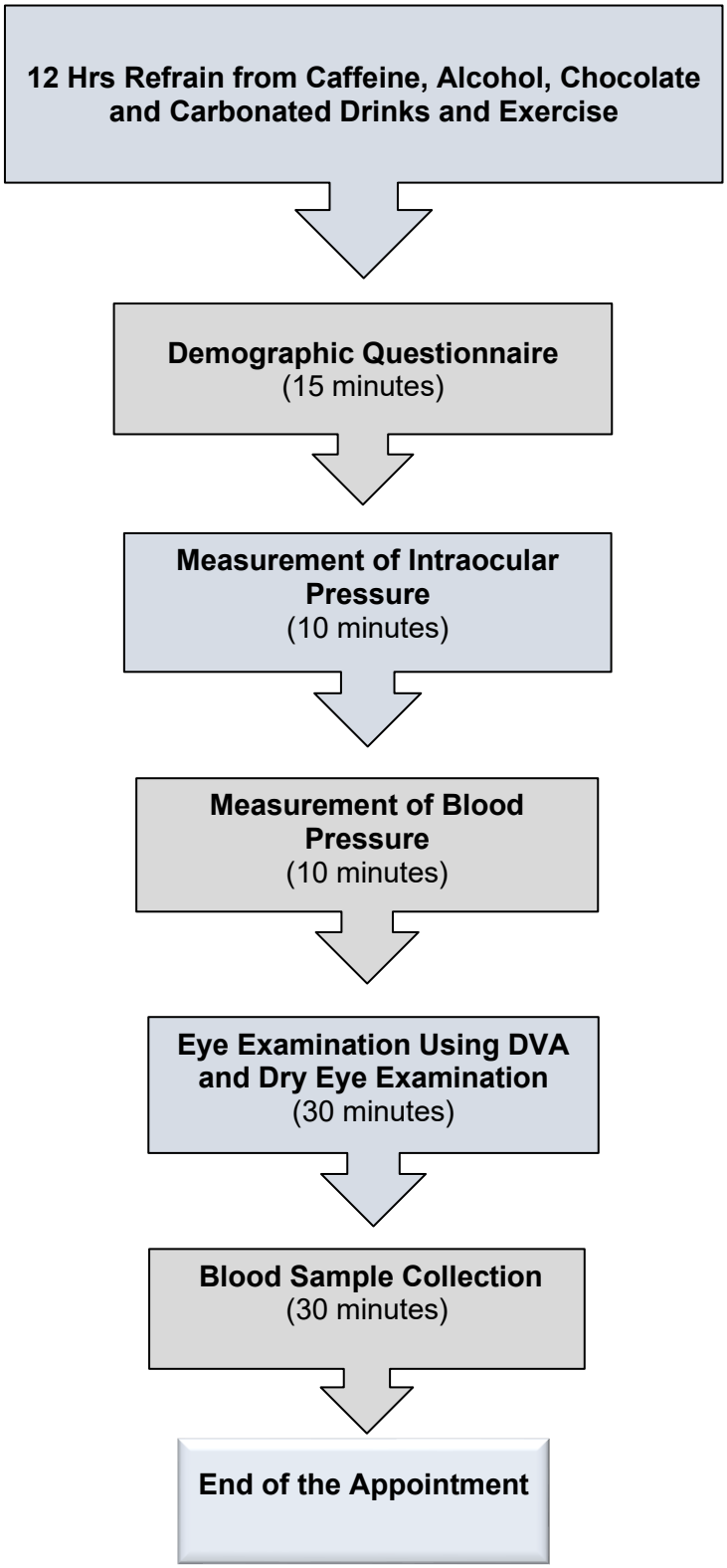

Figure 3.1: Flowchart of the Experimental Protocol

3.3.2. Preliminary Assessments

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 C. Anthropometric measures of height and weight were then recorded, and body mass index (BMI) was calculated as per (Equation 3.1).

$$\textbf{BMI= Weight (}\textit{Kg}\textbf{)/Height}^{2}\textbf{ (}\textit{m}\textbf{)}$$

Equation 3.1: Body Mass Index

3.3.3. Blood Pressure Measurement

Measurements of BP were performed on two separate occasions, one in-clinic and one out-of-clinic.[535,536]

a. Clinic Blood Pressure Monitoring

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 SBP, DBP, and heart rate (HR) were recorded.

b. Ambulatory Blood Pressure Monitoring

The BP values were confirmed by a second measurement using a computer-operated ambulatory BP and electrocardiogram (ECG) monitor (Cardiotens-01, Meditech Ltd, Budapest, Hungary). All subjects maintained their normal activity and were carefully instructed to complete a diary each time their activities changed, or when any chronic medication was taken. The 24-h BP were later downloaded and analysed using the 'Medibase' software program (Meditech). SBP as well as diastolic DBP measurements were calculated for the daytime (6 am to 10 pm) and night-time (10 pm to 6 am) intervals. At least 80% of the programmed recordings were required for a diurnal curve to be considered in the present analysis.[514,515]

Using the daytime SBP and DBP values, study participants were stratified into three subcategories: “normal”, “high normal”, and ‘’Grade I’’ as recommended by the 2018 ESC/ESH Guidelines. They were then also further classified into three other subcategories: “normal”, “elevated”, and “stage I”, as recommended by the 2017 ACC/AHA guidelines. Subjects classed as “optimal” according to the 2018 ESC/ESH guidelines were excluded, and their values were included in our pool of “normal” data.[514]

3.3.4. Intraocular Pressure Measurements

IOP readings were then obtained by way of non-contact tonometry using the Pulsair 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.

3.3.5. Ocular Vascular Assessment

Vascular dysregulation in the retina is implicated in the development of vascular diseases[537] 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.[538–542] A detailed description of this technique is provided in the following sections.

3.3.5.1. Dynamic Retinal Vessel Analysis

As described in section 1.5.3.2, the RVA system (IMEDOS GmbH, Jena, Germany) enables real-time recording of retinal vessel diameters, and can be coupled to a variety provocative stimulus 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.

3.3.5.2. Device Setup

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 is also equipped with an optoelectronic shutter placed in the 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, as illustrated in Figure 3.2.[512,529,538]

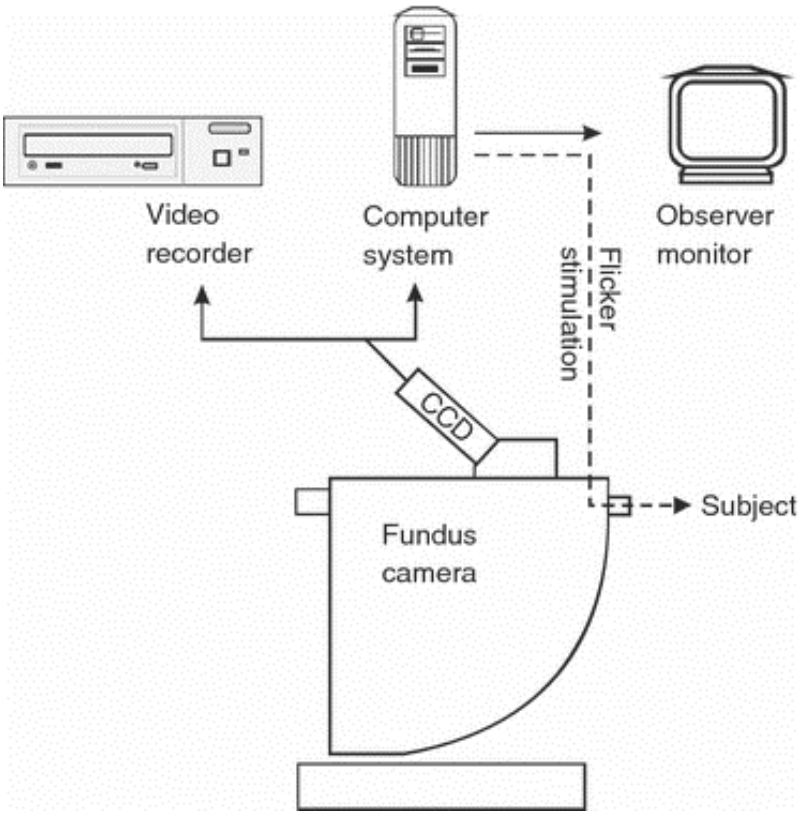

Figure 3.2: Diagrammatic Representation of the Dynamic Retinal Vessel Analyser Set Up

The DVA is capable of measuring retinal vessel diameter continuously along a selected vessel segment over a specified time. This is achieved via the analysis of the brightness profile of the retinal vessels. The brightness profile is based on the absorbing properties of the red blood cells (RBCs) within the vessel. With regard to retinal vessels, maximum absorption of light occurs at a wavelength of 400-620nm and by comparing the brightness profile of the RBC column within the vessel with that of the surrounding tissues, a continuous assessment of vessel diameter can be made. DVA, therefore, measures vessel diameter as the width of the red blood cell column within the selected vessel.

It achieves this as follows:

- The illumination light of the fundus camera enters the eye through a dilated pupil and is reflected by the different layers of the retina and retinal vessels.
- It is then delivered via the observation pathway to the CCD camera.

- The brightness profile data is then analysed by the computer system and simultaneously recorded by a high-quality video recorder so that offline analysis can be conducted at a later date if necessary.

To ensure optimal alignment and set-up, the operator can observe an image of the fundus on the computer display (Figure 3.3) and to ensure optimal contrast for vessel visualisation, a green filter is inserted into the illumination pathway of the fundus camera.
Furthermore, the device is equipped with a series of adaptive algorithms to compensate for any disruption of the vessel brightness profile by the presence of either shadowing structures from the background or reflections from the vessel surface. It also has the capability to correct for slight eye movements during assessment and can continuously monitor image quality, according to image contrast and then automatically remove any inadequate measurements from the analysis.[534,543]

3.3.5.3. Technical Specifications

The technical specifications of the RVA are summarized in Table 3.2. 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.

Table 3.2: Technical Specifications of Retinal Vessel Analyser

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

3.3.5.4. 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 3.3).

Table 3.3. 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 the absolute vessel diameter
Simultaneous assessments of multiple vessels and vessel segments	Heavily reliant on steady patient fixation over recording duration
High reproducibility [544]	Assumes no refractive error
Low variability[545]	Requires full pupil dilation

As mentioned previously, in order to assess the dynamic behaviour and autoregulatory capacity of the retinal vessels, the vessels need to be provoked. The DVA can be operated in conjunction with a variety of provocation devices, including suction cup IOP enhancement, pure oxygen breathing, inhalation of CO2, and flicker light stimulation.[543] For this thesis, flicker light stimulation has been used to assess dynamic retinal vessel reactivity, and this will be discussed in more detail in the following section.[546]

3.3.5.5. Flicker Light Stimulation

Flicker light can be considered the most natural provocation method for assessing dynamic retinal vessel behaviour and has the advantage of stimulating the retina exclusively, without the involvement of any other vascular bed. The normal vascular response to flickering light has been widely studied [523,529], and there is plentiful evidence to indicate that, under normal circumstances, flicker stimulation should lead to an increase in vessel diameter, retinal blood flow and ONH blood flow in humans.[547]

Flicker light can be defined as illumination, which alternates in brightness or colour at a frequency of approximately 1-50 Hz.[538] Electrophysiological studies have shown that the maximum sensitivity of the human visual system to flicker stimulation is obtained with a flicker frequency of between 10-20 Hz.[538] The DVA device used throughout this thesis was equipped

to generate flickering light at a sampling rate of 12.5 Hz via an optoelectronic shutter placed in the optical pathway of the camera. A sampling rate of 12.5 Hz lies within the optimum flicker frequency range and has been shown to provide appropriate retinal stimulation by numerous studies.[512,529,538]

The measurement protocol used to assess retinal microvascular reactivity to flicker light in this thesis is in accordance with that introduced by Nagel et al.[523] This protocol is widely used and recommended [534] and is outlined in the following section.

3.3.5.6. Measurement Protocol

- The fundus camera is positioned so as to obtain a uniformly illuminated fundus image through the fully dilated pupil without unwanted reflections, and the brightness is adjusted to ensure optimal contrast.
- The patient's fixation is directed, with the use of a fixation needle, so that the measurement area of interest lies in the centre of the fundus picture A region of interest is defined on the retina by the user selecting a rectangular area on the real-time monitor, which outlines the region to be studied (usually inferior to ONH).
- From within this area, a section of the inferior temporal retinal artery and a section of the inferior temporal retinal vein, located approximately 1.5-disc diameters from the ONH and approximately 0.5-1-disc diameters in length and a reasonable distance apart, are selected for analysis and monitoring (Figure 3.3).

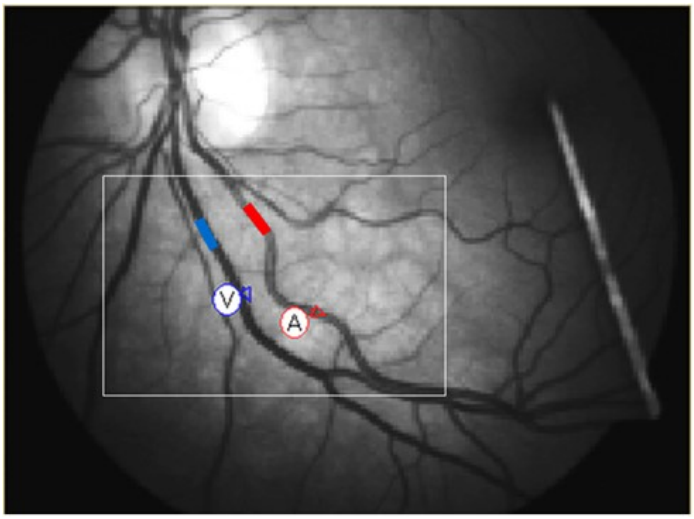

Figure 3.3: Example of Retinal Vessel Selection before Analysis

Measurement then starts automatically, and vessel diameters are continuously calculated along the length of the selected vessels over a 350 second testing period. This consists of 50

seconds of baseline measurements under still illumination (25Hz), followed by three cycles of 20-second flicker stimulation (optoelectronically generated at 12.5 Hz), each interrupted by 80 seconds of still illumination (recovery), as outlined in Figure 3.4.

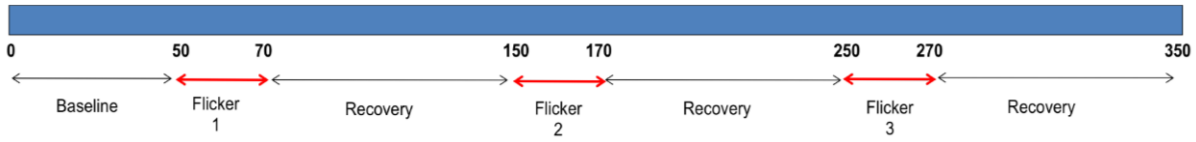

Figure 3.4: Breakdown of the 350 Second DVA Testing Period

All measurements are performed in a quiet, temperature-controlled room (22°C) following full dilation of one unselected eye (1% tropicamide, Chauvin Pharmaceuticals Ltd). The analysis is conducted in a dark room 30 mins after dilation to allow dark adaptation.

The use of a 350 second testing period with three sequential flicker cycles was initially introduced so that an averaged vessel response could be calculated and analysed to ensure stable results could be obtained over a testing period of tolerable length.[521,523] More recently, new analysis techniques have been introduced, which also consider the vessel responses to each flicker cycle individually and the overall average.[548] This is discussed in more detail in the following sections.

3.3.5.7. Normal Vascular Response

The normal retinal vessel response profile to flicker light stimulation by the DVA is illustrated in figure 3.5. Previous studies have shown that the maximum vessel response to flickering light typically occurs within 20 seconds.[512] After this time period only small increases in diameter occur. Once the flicker stimulation ends, dilation ceases immediately but rather than simply returning to baseline, the baseline is usually overshot and a vasoconstriction occurs. This overshoot has been found to start within approximately 6-10 seconds following the end of the flicker period, reaching its minimum diameter between 10-40 seconds following the end of the flicker.[512,529] The vessel diameter then returns to its baseline level (Figure 3.5). Both retinal arteries and veins respond to flicker light stimulation; however, arteries tend to show a more pronounced diameter change in comparison to veins and whilst arteries start to react immediately, veins have been shown to have approximately a 5-second delay before a response is seen.[512,538]

Deviation away from this normal vascular response profile can be indicative of vascular disease, and indeed numerous studies have already provided evidence of altered vascular response to flickering light in both ocular and systemic disease, including POAG [549], ARMD [550], diabetes [551–553] and hypertension[520]. In order to understand the implications of an impaired or altered vascular response to flicker light with regard to the development of disease, it is necessary to try and understand the mechanisms by which flicker light provokes the retinal vessels.

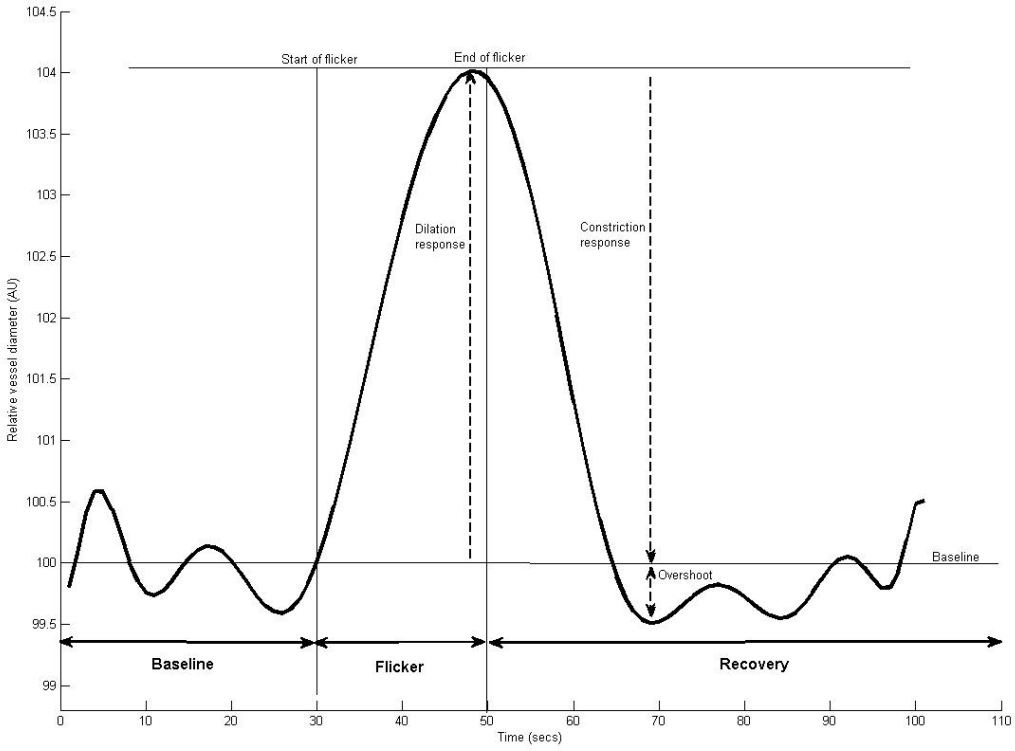

Figure 3.5: Diagrammatic Representation of a Typical Retinal Vessel Response to Flicker Light on Dynamic Retinal Vessel Analysis

3.3.5.8. Reaction Mechanism

Flicker light stimulation increases the neural demand of the retina, which, under normal circumstances, should trigger a neurovascular coupling response of the retinal microvasculature, resulting in vasodilation.[547] In general terms, an increase in the metabolic rate of the photoreceptors, following stimulation by flickering light, is thought to trigger the release of NO from the retinal vascular endothelium, bringing about an increase in vessel diameter followed by an increase in blood supply to meet the increased demand.[554] An altered vascular response to flicker light, therefore, could be indicative of impaired autoregulatory

mechanisms and/or endothelial dysfunction in the form of reduced bioavailability of NO. However, due to the complexity of the neurovascular coupling response, it is highly likely that other causative factors such as altered endothelial dependant vasoactive substances levels, altered astrocyte activity or changes in the basal tonus of the vessels could also play a role in altering the retinal vascular response to flicker. The role of these alternative factors may be particularly relevant when considering the vascular constriction response following flicker.[550]

3.3.5.9. Data Analysis

Of primary interest when analysing the retinal vascular response profile to flicker light is the percentage dilation of the vessel in response to the stimulus and the time scale across which this happens, along with the percentage constriction or overshoot of the vessel following cessation of flicker and again the time scale across which this happens.

In this thesis, such analysis of the retinal vascular response was conducted using elements of a newly defined method of DVA analysis, termed 'Sequential and Diameter Response Analysis' (SDRA) in conjunction with our novel vascular profile imaging methods. The inbuilt software of the DVA device itself does have the capability to provide an analysis of the retinal vascular response to flicker light; however, this has been identified to have a number of shortfalls and the need to move away from this traditional inbuilt software analysis and to evaluate the retinal vascular response profile in more detail has been increasingly realised by numerous authors in recent years.[534,548,550]

As described in section 3.3.5.3., 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 the 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 3.6).

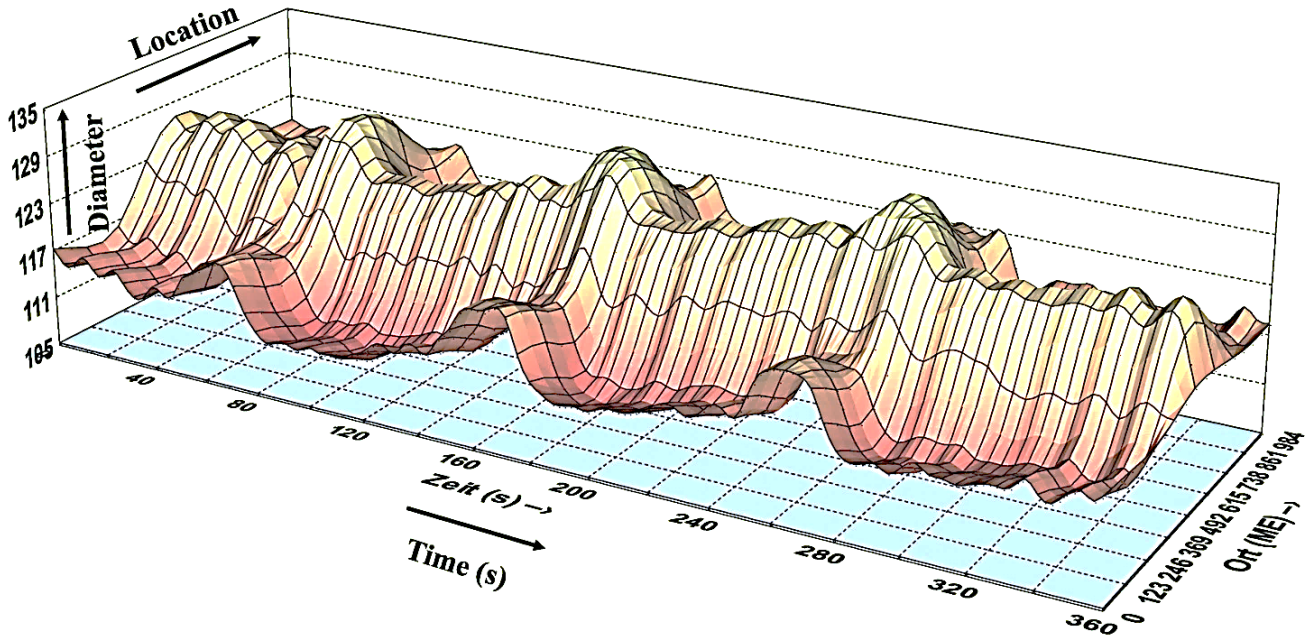

Figure 3.6: Local Temporal Course Representation of Retinal Vessel Analysis.
For each examination, a vessel segment approximately 1 mm in length and approximately 1 to 2 disc diameters away from the optic disc was selected. The vessel longitudinal section within the region of interest was scanned 25 times per second and continuous diameter data was obtained along the vessel over time, thus creating a three-dimensional matrix of values as depicted.

Indeed SDRA, first introduced by Heitmar et al. [548], was primarily designed to overcome the shortfalls of the inbuilt software analysis programme. An overview of this inbuilt DVA analysis and its limitations, along with a summary of SDRA and the novel imaging analysis methods used in this thesis, is given in the following sections.

3.3.5.10. Previous Methods of Analysis

The inbuilt DVA software calculates the vascular dilation response to flicker light by averaging all three of the stimulation cycles and then taking the average diameter from the last +/- 3 seconds of flicker stimulation as the maximum diameter response to flicker (i.e., average diameter reached between 17-23 seconds from the start of flicker taken as maximum diameter response). The shortfalls of this method largely arise due to its incorporation of both time and diameter responses and its inaccurate assumptions about the nature of the vascular response. [548]

Indeed, subjects who reach their maximum dilation outside the 17-23 second window would have their maximum dilatory response underestimated by this technique. Furthermore, averaging the results from all three flicker cycles, differences in the reaction pattern or time

course of each individual cycle cannot be determined.[548] Additionally, this method of analysis does not take in to consideration baseline fluctuation in vessel diameter (BDF), a parameter first highlighted as important in DVA analysis by Nagel et al.[520] BDF refers to the spontaneous variations in vessel diameter, which occur under normal resting conditions, as a result of vascular tone and arterial pulsation and are superimposed on the vascular response profile.[520] In order to account for the influence of BDF, Nagel et al. introduced the concept of baseline-corrected flicker response (BCFR), where BDF is accounted for by subtracting it from the dilation amplitude (DA) of the vascular response, as shown in Equations 3.2 and 3.3.[520]

$$\boldsymbol{BCFR = DA - BDF}$$

BCFR = Baseline corrected flicker response

DA = Dilation amplitude

BDF = Baseline diameter fluctuation

Equation 3.2: Baseline Corrected Flicker Response

$$\boldsymbol{DA = MD - MC}$$

DA = dilation amplitude

MD = maximum dilation

MC = maximum constriction

Equation 3.3: Dilation Amplitude

3.3.5.11. Sequential and Diameter Response Analysis (SDRA)

SDRA has the advantage of utilising the raw data set generated by the DVA device and allowing each individual flicker cycle to be considered separately. This enables a more accurate assessment of dynamic vessel response to be obtained and enables the inclusion of the parameters BDF, BCFR and DA on top of the standard percentage dilation and constriction response parameters. Additionally, it allows the time taken to reach maximum dilation and the time taken to reach maximum constriction to be determined for both the artery and vein for each individual flicker cycle.

It is important to note that analysis of individual flicker cycles is reliant on a full data set having been obtained from the participant on each subsequent cycle. If this is not achieved, for

example, due to poor patient fixation, loss of concentration or excessive blinking, calculating and analysing the average data set using SDRA is considered more reliable.

Overall the SDRA method has been validated and shown to be a sensitive measure of the vascular response to flicker light with good coefficients of variation.[548] Furthermore, it is able to overcome a number of the shortfalls of the inbuilt RVA analysis software. It has therefore been the analysis method of choice for this thesis; however, we have taken it one step further and using the principles of SDRA have developed an additional way of imaging the retinal vascular profile to allow further aspects of the vascular response to be explored.

3.3.5.12. Novel Analysis

Whilst SDRA overcomes many of the limitations of the inbuilt RVA software analysis, it still does not allow visualisation of the entire dynamic retinal vessel response profile. Furthermore, it has been suggested that, in addition to the parameters illustrated in sections 3.3.5.10 and 3.3.5.11, evaluation of the slope of both the dilation and constriction responses to flicker light could give additional important information about the state of the retinal microvasculature in health and disease. In order to address this we have developed a new method of analysing and interpreting the retinal vascular response to flickering light using Matlab (MATLAB R2010a; MathWorks Inc., Natick,MA). Our method expands on the SDRA methodology by extracting the raw response data and applying a statistical polynomial regression algorithm, implemented using the polyfit and polyval functions of the Matlab high level programming language (MATLAB R2010a; MathWorks Inc., Natick, MA).

Given the measurements y_i at times t_i , i=1 …T, we approximated y = f(t) by a polynomial of degree b 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,…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:

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

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 $p = \begin{Bmatrix} p_1 \\ . \\ . \\ . \\ p_{n+1} \end{Bmatrix}$ And $y = \begin{Bmatrix} y_1 \\ . \\ . \\ . \\ y_T \end{Bmatrix}$

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. 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[541] 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 3.4) and (Equation 3.5).

$$Slope_D = \frac{MD - Baseline}{tMD}$$

Equation 3.4: Dilation Slope

$$Slope_C = \frac{MC - MD}{tMC}$$

Equation 3.5: Constriction Slope

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 3.7. A summary of the main parameters of interest to this thesis is provided in Table 3.4.

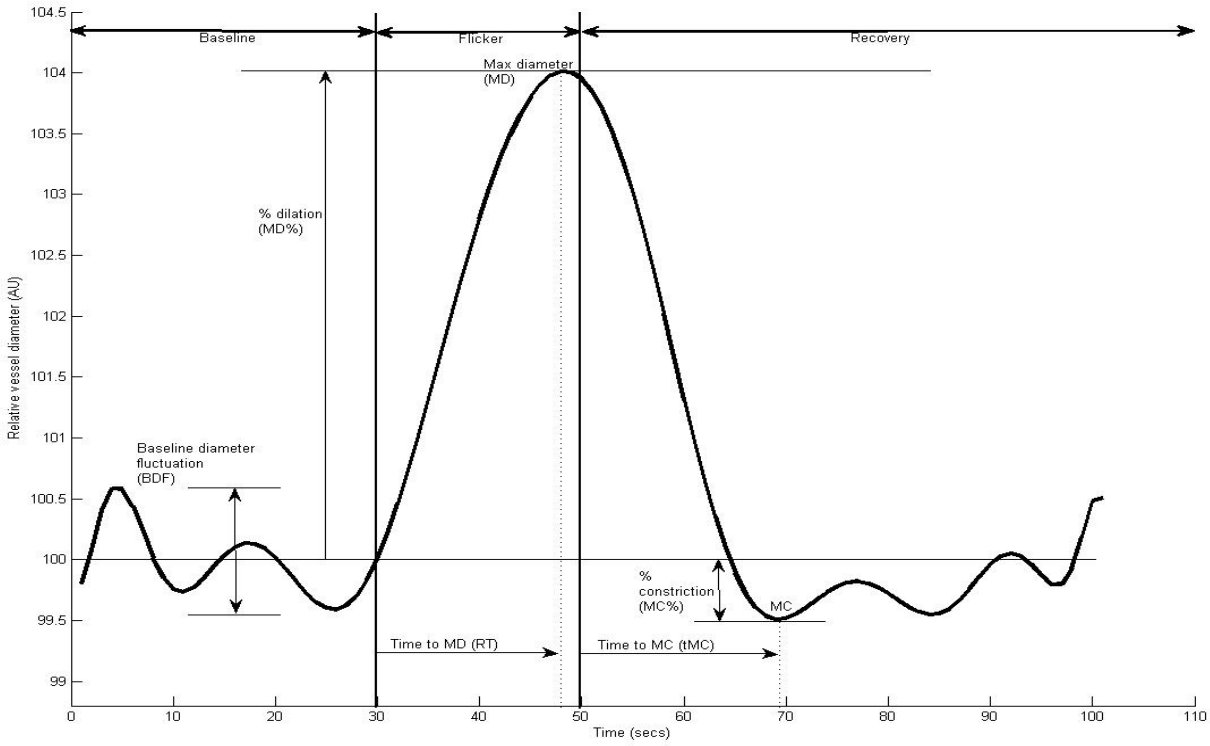

Figure 3.7: Diagrammatic Representation of the SDRA Parameters
Abbreviations: SDRA, sequential diameter response analysis; BDF, baseline diameter fluctuation; MD, maximum diameter; MD%, percent dilation relative to average baseline diameter; MC, minimum constriction diameter; MC%, percent constriction relative to average baseline diameter.

Table 3.4: 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 the 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).

3.3.6. Systemic Vascular Assessment (Digital thermal monitoring (DTM))

Peripheral microvascular reactivity at the level of the fingertips was assessed by way of DTM using VENDYS 5000BC Digital Thermal Monitoring (DTM) system (Endothelix, Inc., Houston, TX, USA). This FDA approved device consists of a computer-based thermometry system (0.006°C thermal resolution), with two special thermocouple fingertip probes designed to minimize the area of skin-probe contact and fingertip pressure. A standard sphygmomanometer cuff and a compressor unit to control cuff inflation and deflation is included to facilitate the occlusion-hyperaemia protocol

3.3.6.1. Measurement Protocol

The test is conducted with the patient at rest for 30 minutes in the supine position, in a quiet, dimmed room with an ambient temperature of 22°C to 26°C. VENDYS DTM probes are affixed to the index finger of each hand, and after a period of stabilization of basal skin temperature (defined as stabilization within a 0.05°C threshold), the temperature is measured in the index fingers of both hands (of which the right arm only is subjected to occlusion-hyperaemia) with an automated, operator-independent protocol. The right upper arm cuff is rapidly inflated to ≥50 mmHg above systolic pressure for 5 minutes and then rapidly deflated to invoke reactive hyperaemia distally.[555]

Thermal tracings are measured continuously and digitized automatically using a computer-based thermometry system with 0.006°C thermal resolution. Dual-channel temperature data is simultaneously acquired at a 1 Hz sample rate.

(Figure 3.8) shows a representative example of a temperature-time trace and the primary DTM-derived measures related to thermal debt and recovery that were recorded and calculated. Temperature rebound (TR): maximum temperature- start temperature (just before cuff inflation); adjusted temperature rebound (aTR): temperature rebound/ start temperature; area under the curve temperature rebound (AUC_{TR}): area under the curve between maximum temperature and minimum temperature. The post-occlusive adjusted temperature rebound aTR determined by the software algorithm is directly associated with the extent of the subjects vascular reactivity.[556] An aTR below 1 is considered to show poor cardiovascular reactivity, whereas an aTR of between 1 and 2 is considered intermediate, and an aTR of more than 2 is considered healthy.

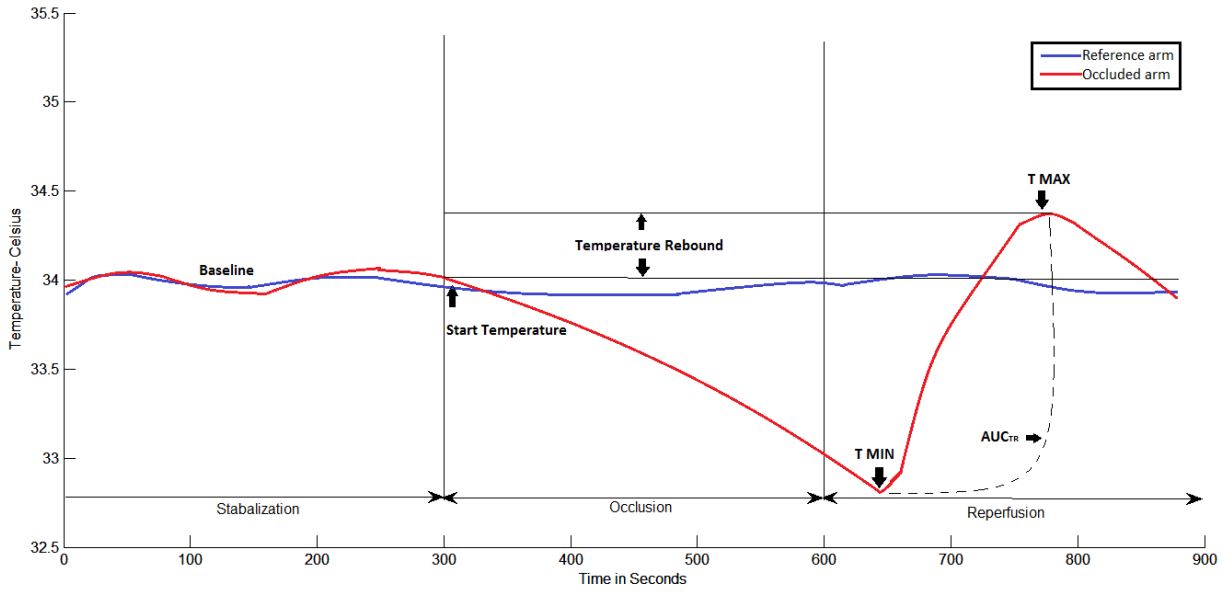

Figure 3.8: Graphical Representation of The Digital Thermal Monitor Software Analysis. T MAX, maximum temperature; TMIN, minimum temperature; AUCTR, Area under the curve temperature rebound.

3.3.7. Assessment of Anterior Ocular Structure and Function
These tests are a collaborative work with Dr Sonia Trave Huarte
3.3.7.1. Keratograph

The Keratogrpah (K5M, Optikgerate GmbH, Wetzlar, Germany), combines keratometry measuring processes with topographic mapping. It is an illumination system which has a special reflector illuminating a placido bowl which contains a series of 22 white concentric rings, and thus images obtained are reflected from this placido bowl from the patients' eye. Besides being an advanced corneal topographer it also contains additional imaging modalities to measure the anterior ocular surface. These include infra-red measurements of the Meibomian glands at 840nm, evaluation of the tear break-up time non-invasively, measuring the amount of bulbar and limbal hyperaemia, tear meniscus height, and a dynamic evaluation of the lipid layer. It also has a built-in video recorder and software to analyse the data. It can be used for future reference when evaluating the ocular surface after treatment or post-surgical interventions.

3.3.7.2. Procedure

The test is conducted in a dim room to eliminate reflections from the surface of the keratograph. The patient is in a seated position with their chin placed on the chin rest and the outer canthus is aligned with the chin-forehead reference bar. The patient focuses on the red light located directly in the centre of the concentric rings. All subsequent measurements are taken from this reference point. The following procedures described below are in the order of the protocol used in this thesis.

3.3.7.3. Tear Meniscus Height

Images obtained of the tear meniscus height (TMH) from the keratograph are taken from the lower lid. The tear meniscus can be evaluated with different options to magnify the view. The height of the tear meniscus can give an indication of the tear volume, i.e., Quantity of tear reserve. TMH should be directly measured under the pupil centre at the lower lid. The classification of normal tear reserve is as follows: good >0.2mm; normal = 0.2mm; poor < 0.2mm.[557] The software allows the user to quantify the height of the tear meniscus with an integrated ruler. An example of the assessment is presented below in Figure 3.9.

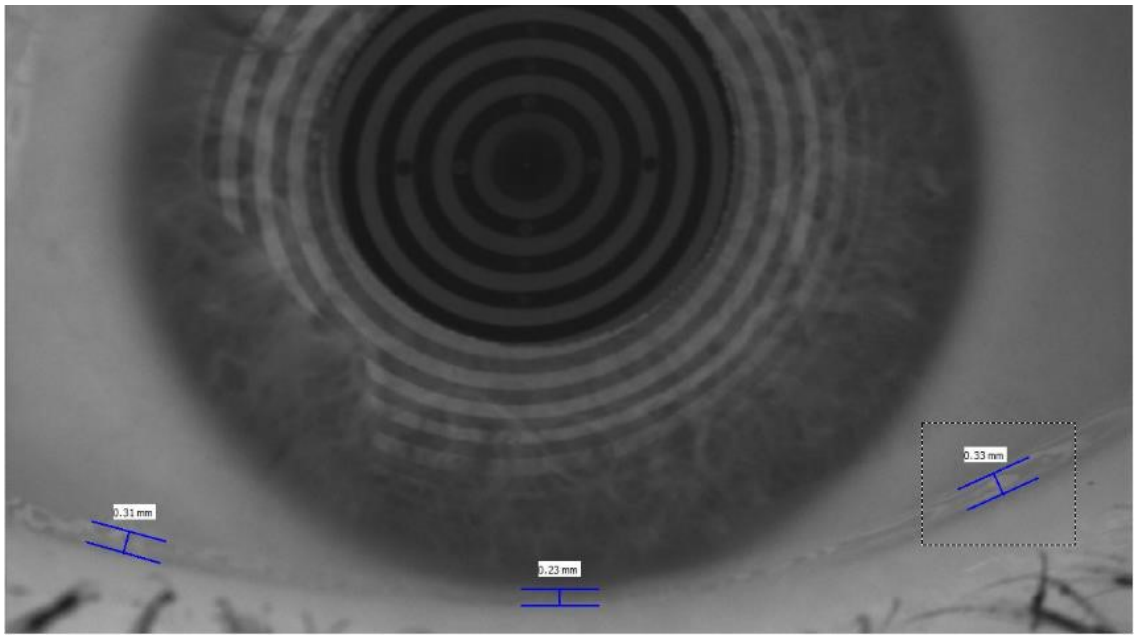

Figure 3.9. Example of TMH Measurement

3.3.7.4. Non-Invasive Keratograph Break up Time (NIKBUT)

The non-invasive break up time is evaluated and displayed in a colour coded map of the cornea Figure 3.10. Once the patient is aligned, the clinician will require the patient to blink twice consecutively. This second blink triggers the program to initiate the recording. The recording of break time up time ceases when one of two events occur; the patient blinks or there is a significant amount of distortion from the reflected image of the placido rings. The information is then encoded by the software and displayed to the clinician. The time it takes for the tears to break up can give an indication of the quality of the tear film. Normal tear break up time is > 10 seconds. Areas highlighted in red indicate an unstable tear film, and areas in green indicate a stable tear film.

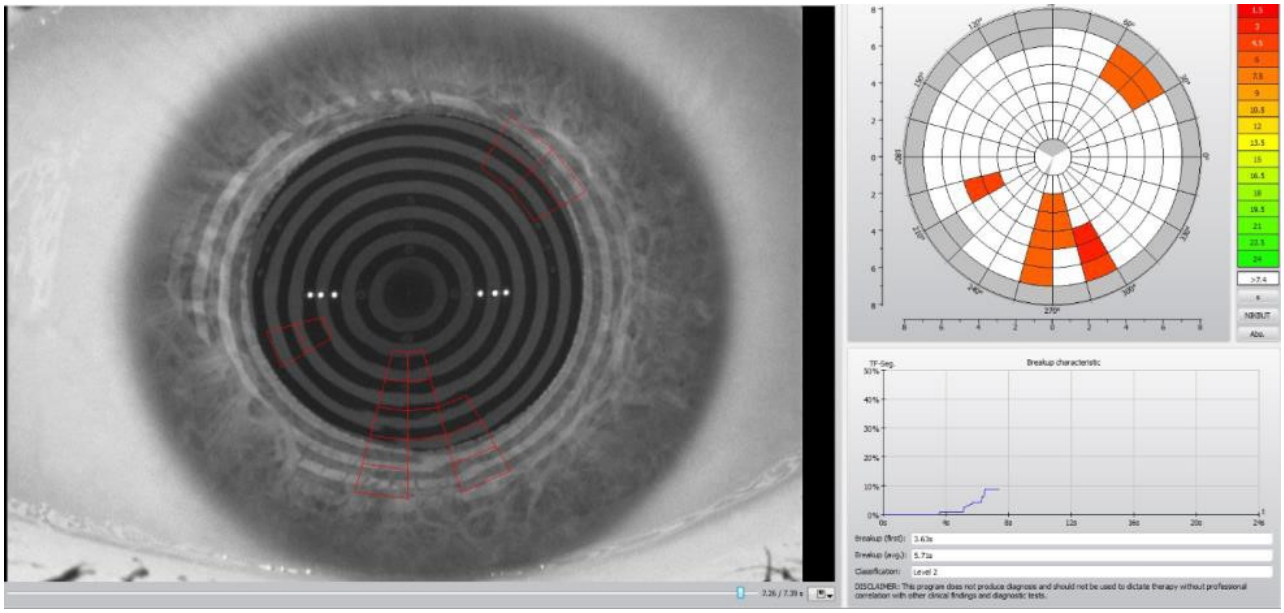

Figure 3.10. Example of NIKBUT Measurement

3.3.7.5. Dynamic Evaluation of the Lipid Layer

The evaluation of the lipid layer is performed with the patient looking directly at the centre of the placido disc Figure 3.11. Once aligned, the measurement commences, and the clinician should advise the patient to blink normally as to reveal the spread and formation of the lipid layer across the corneal surface. This measurement is usually recorded to assess the dynamic behaviour of the tear film, as well as giving an indication of the thickness of the lipid layer. If the interference pattern displays colours and structures, it is regarded as normal; however, if no colours or structures are visible it could be an indication of early tear film evaporation.

Recordings can range from 5 sec to 10 seconds or longer if the clinician deems so. There is also the ability to capture images within the video recording for analysing images later on.

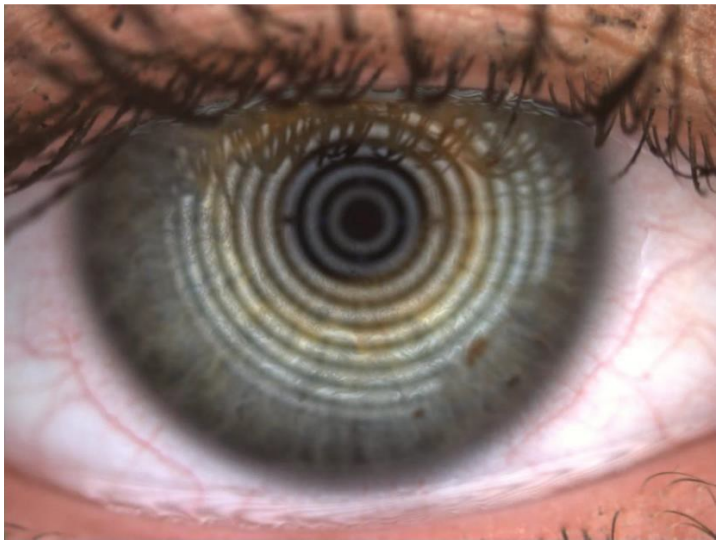

Figure 3.11. Example Measurement of Dynamic Evaluation of Lipid Layer

3.3.7.6. Redness

The Keratograph is also able to evaluate the amount of limbal and bulbar hyperaemia. This feature can record and grade redness automatically and objectively with the inbuilt software that compares the amount of redness and analyses it according to the Efron grading scale. This area analysed is based on the area percentage between the vessels and the rest of the analysed area represented in figure 3.12.

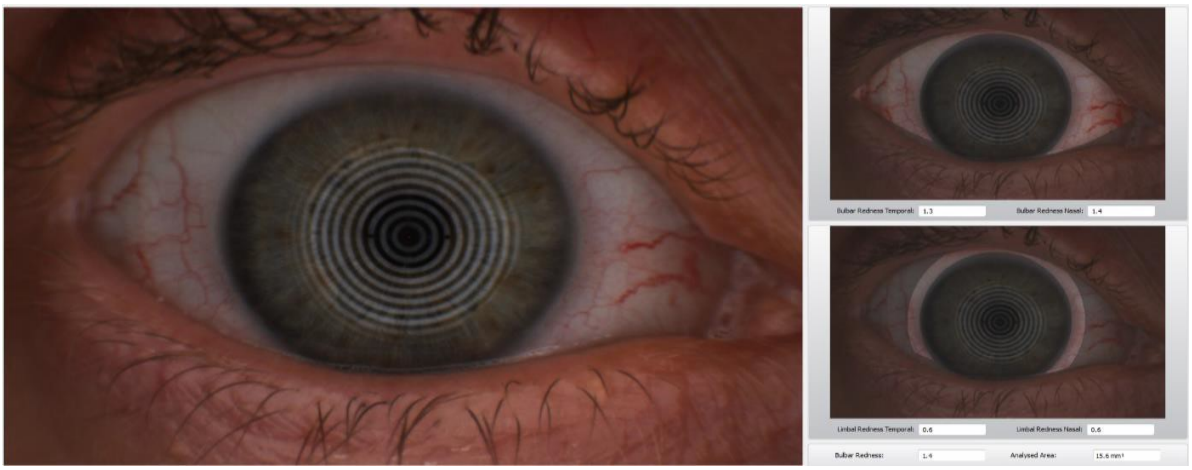

Figure 3.12. Measurement Example of Bulbar Redness

3.3.7.7. Meibography

The Meibomian glands structure and can be imaged with infra-red light. The superior lid as well as the lower lid must be everted before capturing the image. The morphological changes in the glandular tissue can be made visible using a 3D meibo scan represented in Figure 3.13. Once the image is captured the software makes a contrast enhanced image alongside a normal image which can be viewed by the clinician and is usually subjectively graded.

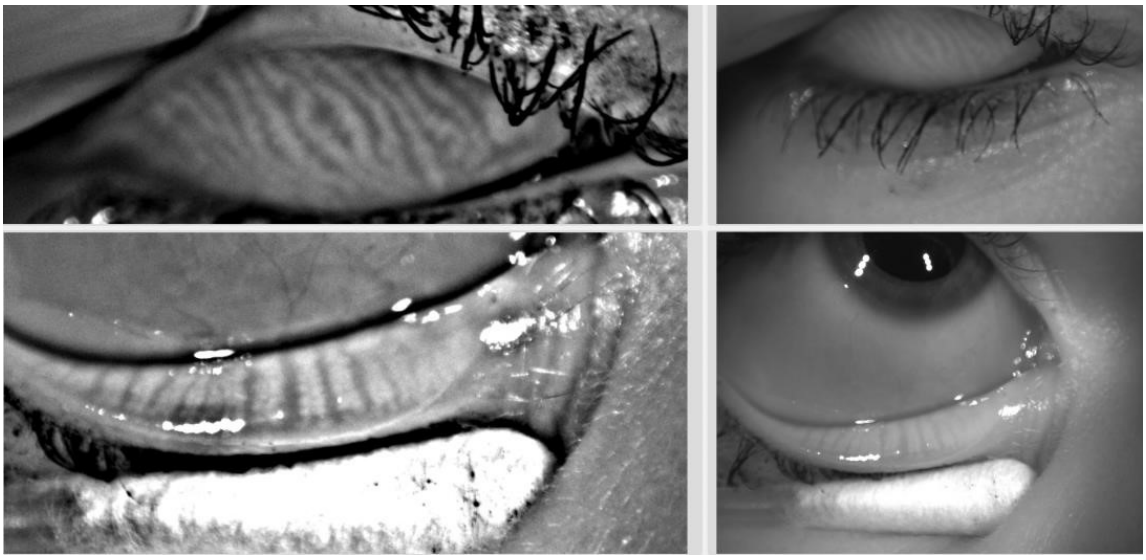

Figure 3.13. Example of Infrared Superior and Inferior Meibography

3.3.7.8. Data Analysis

The inbuilt software is capable of analysing data for a number of images taken. These are limited to the amount of Hyperaemia, which is graded according to the EFRON grading scale and the NIKBUT with the use of the placido discs. These values are generated by the software algorithm itself and have been previously used in studies that show good agreement with previous techniques and is highly repeatable and reproducible.[558,559]

The quantification of tear meniscus height is made on the actual image with the use of an inbuilt ruler which measures to 1/10th of a mm and can be graded accordingly. The classification of lipid layer thickness can be made subjectively on the interference pattern observed during the video recording. An open/closed meshwork correlates to a thickness of 13-50nm, wave or flow pattern is between 50-70nm, an amourphous pattern is between 80-90nm, Colour fringes represent a thickness between from 90-180nm, and finally a globular network represents a thickness of >200nm.

The Meibomian glands can be analysed according to a number of different protocols. Originally scales were developed on a 4 point scale to assess the level of meibomian gland drop out or a change in its morphology.[560,561] More recently, a new scale was introduced by Pult et al [562], which incorporated a 5 point scale based on the level of Meibomian gland dropout. The infrared image is captured and then analysed with the imajeJ software V1.49 (Wayne Rasband, NIH, USA). The software allows the user to trace the whole area which is going under analysis and then to trace the area where the glands drop out represented in Figure 3.14. The percentage of gland dropouts can be calculated by dividing the (Area of dropout/whole area) *100. In a further study Pult et al. [563] analysed the repeatability of objective grading and subjective grading using the Meibomian gland loss ratio and determined that intra-observer and inter-observer agreement was better in computerized grading following the subjective 5 point scale and 4 point scale.

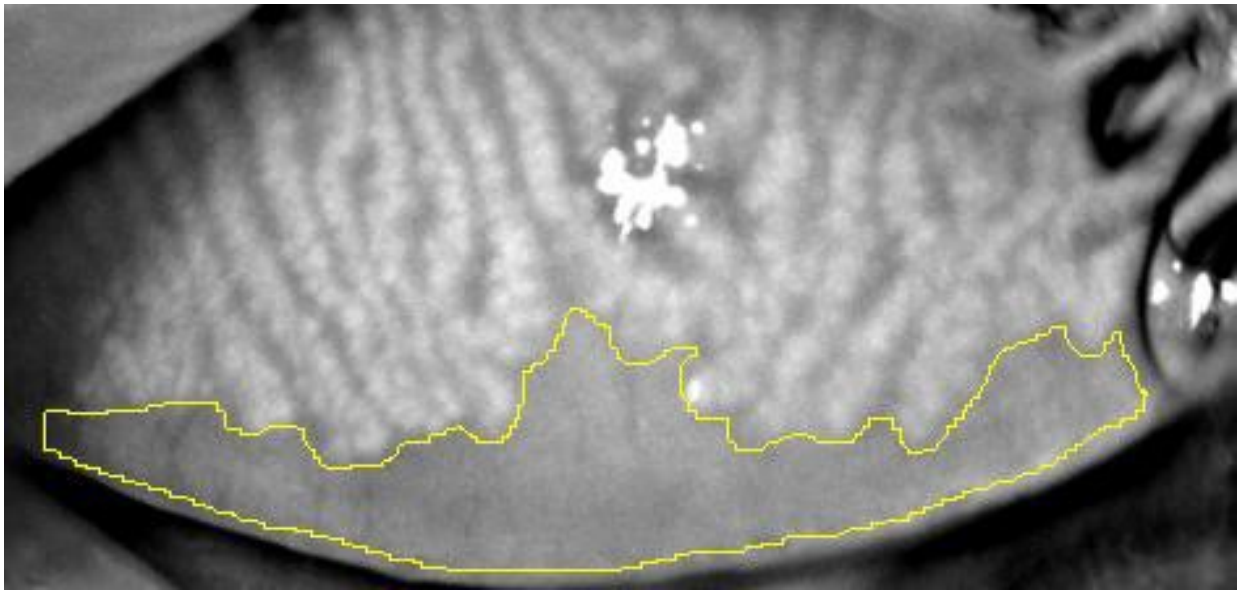

Figure 3.14. Measurement Example with Image J representing area of loss of the superior tarsus.

3.3.8. 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.

3.3.9. Blood Analysis

The fasting EDTA blood samples obtained were immediately assessed for glucose (GLUC) and TG, and plasma total cholesterol (TChol) and HDL-c using a Reflotron Desktop Analyser (Roche Diagnostics, UK). In addition, LDL-C levels were estimated as per the Friedewald equation (Equation 3.6). Aliquots of the remaining EDTA blood and plasma were then processed and stored at approximately -80ºC for further analyses.[564]

$$\boldsymbol{LDL = \frac{Total\ cholesterol - (HDL + TG)}{2.17}}$$

LDL= Low-density lipoprotein

HDL= High-density Lipoprotein

TG= Triglycerides

Equation 3.6: Friedewald Equation

3.3.10. Systemic Circulatory Markers – Biochemical Assays

3.3.10.1. Glutathione/Glutathione Disulphide Redox System

a. Glutathione

As discussed in section 1.4.4 glutathione/glutathione disulphide (GSH/GSSG) is the most abundant thiol redox system and is essential for the maintenance of redox balance in cells.[565] 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.[566] In the present thesis, glutathione levels were based on the principles of an enzymatic-recycling assay.[567,568] This technique, developed in-house and validated previously [569], 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 [570], 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.[571]

b. 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 (H2O2) to water (H2O) and the respective alcohol – a reaction that is catalysed by glutathione peroxidase (GPx) – GSH becomes oxidized glutathione (GSSG) (Equation 3.7). 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 3.8).

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSG + 2H_2O$$

Equation 3.7: Glutathione Oxidation

$$GSSG + NADPH + H^+ \xrightarrow{GSR} 2GSH + NADP^+$$

Equation 3.8: Oxidized Glutathione Reduction

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.[572] The method used is described below.

c. 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 -80oC for further analyses. Based on previous reports of sample stability, assays were conducted within 2 months of collection.[573] 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 3.15.

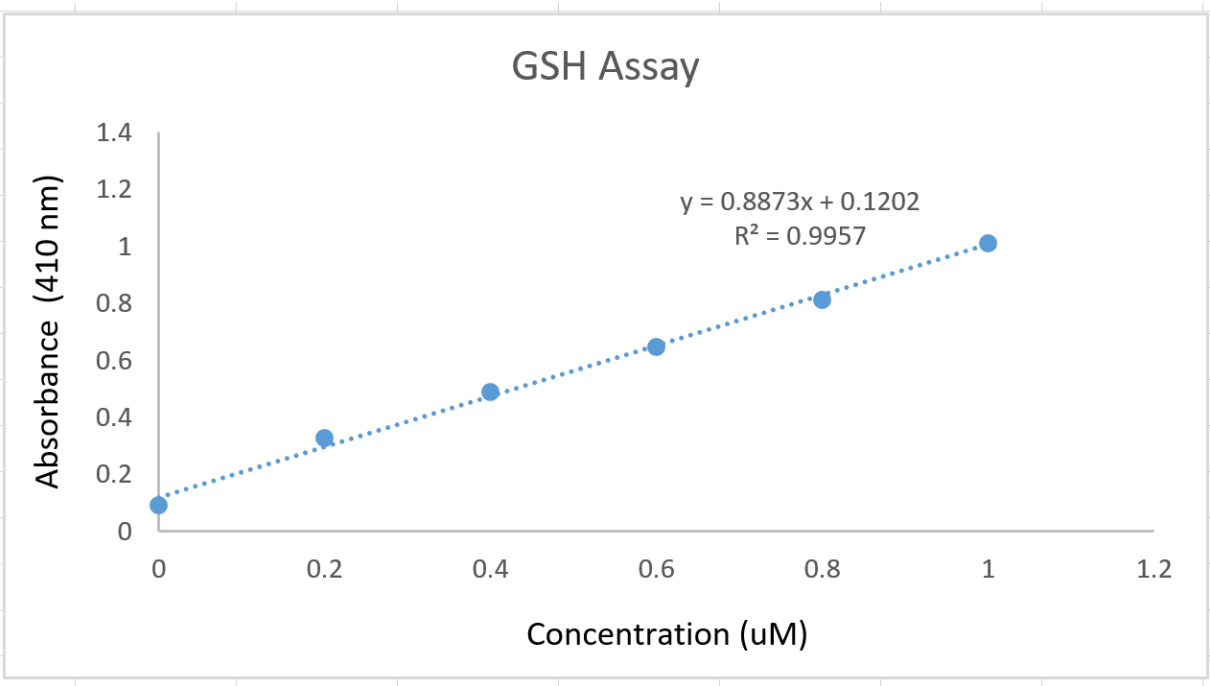

Figure 3.15: Standard Curve for the GSH Assay

d. 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. In addition, the standards and samples were pre-treated with 2-vinylpyridine (2-VP) in order to derivative GSH without interfering with GSR reaction. A summary of the GSH/GSSG recycling assay is depicted in Figure 3.16.

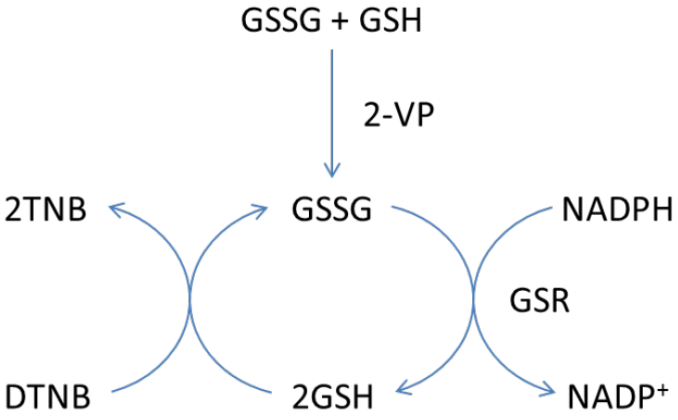

Figure 3.16. Glutathione Recycling Assay Principle

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 °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 3.17).

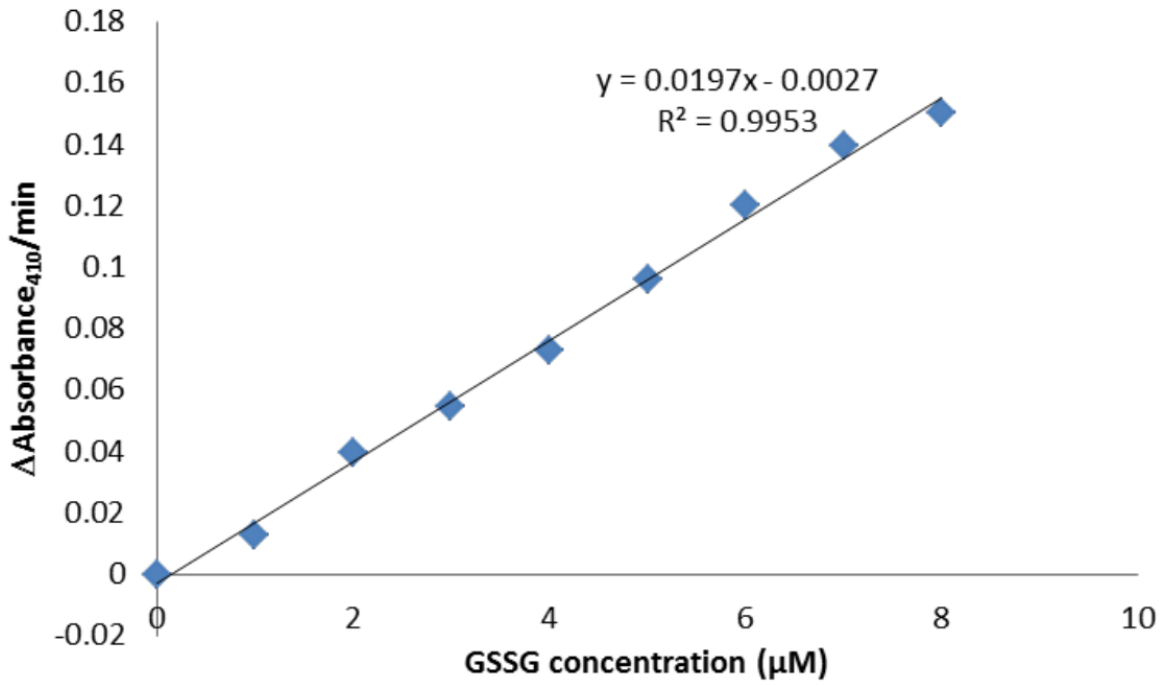

Figure 3.17: Standard Curve for the GSSG Assay

e. 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 (Equation 3.9):

$$A_{410} = Slope \times minutes \times intercept$$

Equation 3.9: Absorbance Change

The net rate is the difference between the rate at each concentration of the GSH or GSSG standard and the blank rate (Equation 3.10). The general form of the equation describing the calibration curve is:

$$Net\ rate = Slope \times GSH\ or\ GSSG + intercept$$

Equation 3.10: Net Rate Calculation

Therefore, to calculate the GSH or GSSG concentration (Equation 3.11):

$$\text{Concentration} = \frac{Net\ rate - intercept}{Slope} \times dilution\ factor$$

Equation 3.11: GSH and GSSG Concentration

Finally, the total GSH and redox index were calculated as per (Equation 3.12) and (Equation 3.13).

$$tGSH = GSH + (2 \times GSSG)$$

Equation 3.12: Total GSH Calculation

$$Redox\ index = GSH/GSSG$$

Equation 3.13: Redox Index Calculation

3.3.10.2. 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.[574] 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)[575], 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.[576] Previous reports propose that NO_2 more specifically represents a delivery source for intravascular NO and reflects acute changes in regional eNOS activity.[577] 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.

a. Protocol

Reagent preparation: the 1% sulphanilamide solution was prepared by first adding 0.5 mL phosphoric acid (H2PO4) 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 3.18) 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.

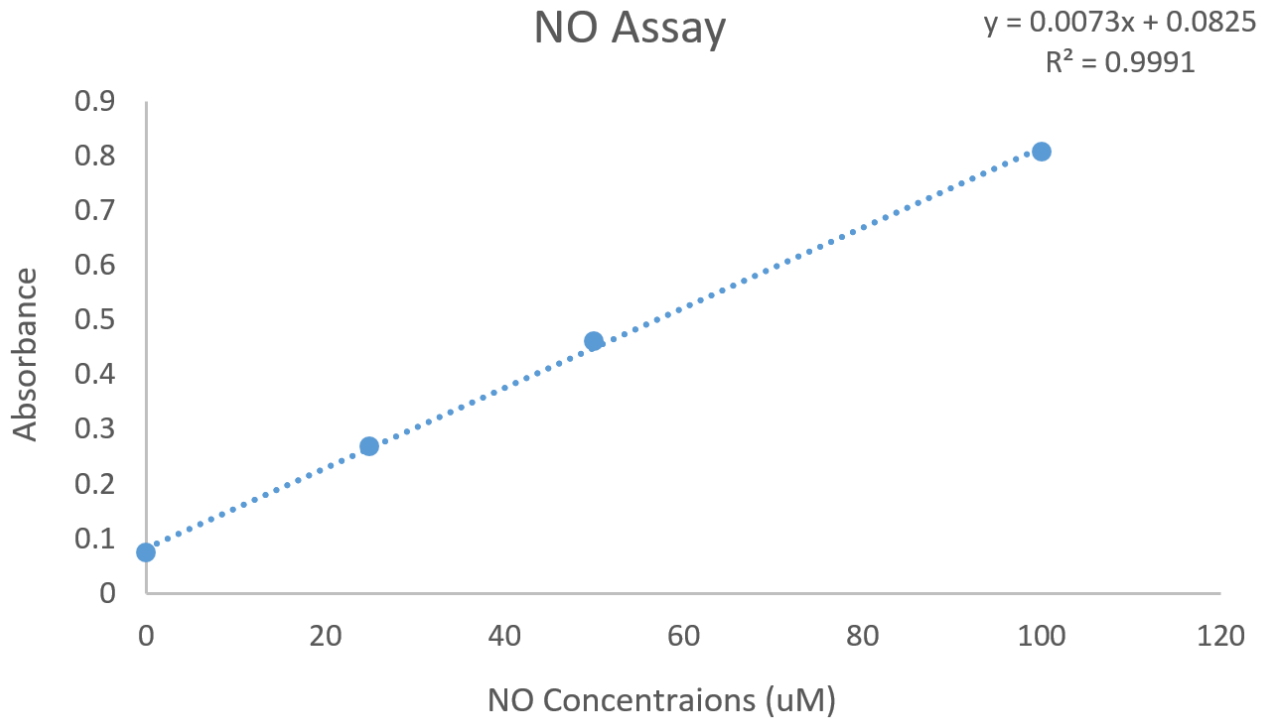

Figure 3.18. Nitrite Standard Curve Reference

3.3.10.3. Relative Telomere Length (RTL)

DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.[578]

DNA purity was detected by NanoDrop ™ 1000/c (Spectrophotometers, Thermo Fisher Scientific, USA) and samples were stored at -80°C until further analysis.

Relative telomere length was measured using real-time polymerase chain reaction (RT-PCR) according to a previously published method [579] using LightCycler® 480 Instrument (Roche Diagnostics GmbH, Germany). Briefly, primers for telomere repeats and a normalising genomic sequence (Table 3.5) was prepared in a 25 µl PCR reaction, consisting of Precision 2× qPCR Mastermix (0.025 U/µl Taq polymerase, 5 mM $MgCl_2$, dNTP mix 200 µM each dNTP) and 15 ng of template DNA. Samples for both the telomere and single-copy gene amplifications were performed in triplicate with non-template control. The ratio of telomere to the normalising genomic control sequence (T/S ratio) was calculated as previously described [579,580] to provide an indication of relative telomere length (RTL).

Table 3.5: Telomere Primers Genomic Sequence

CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT	hTelo Forward
GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT	hTelo Reverse
TCCAGGCTTTGGGCATCA	36B4 Forward
CTTTATCAGCTGCACATCACTCAGA	36B4 Reverse

3.3.10.4. Extraction and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) of Plasma Oxysterols.

This analysis is a collaborative work with Dr Irundika Dias

Oxysterols were extracted from 70 µl of human plasma, spiked with 1 ng of internal standards following the protocol described by Dias et al.[323] Plasma were mixed with 430 µl methanol, vortexed and incubated on ice for 10 min in the presence of 4 mg/ml BHT before centrifugation at 14,000 × g for 10 min. The methanolic supernatant was diluted with acidified water up to 10 % of methanol for loading onto an SPE cartridge. Oxysterols were eluted with 1.8 ml of butyl acetate and dried under a vacuum.

The lipid extracts were dissolved in 40 µL of 40:60 Methanol:H2O and analysed with a liquid chromatography UltiMate 3000 HPLC system (Dionex, Thermo Scientific Ltd.) coupled on-line with an electrospray tandem triple quadrupole-linear ion trap mass spectrometer (QTrap 5500, ABSciex) as described previously (Dias et al., 2018) with some modifications.[323] Briefly, the lipid extracts were separated using a reverse phase C18 column (100 × 3.2 mm, 5.0 µm particle size; Macherey-Nagel) with mobile phases A (70:30 Methanol:H2O with 0.1% formic acid) and B (90:10 Isopropanol: Methanol with 0.1% formic acid). The flow rate was 200 µL/ min, and the column was maintained at 45 °C.

3.3.11. Symbolic Regression

This analysis is a collaborative work with Dr Aniko Ekart and Dr Victoria Lush

To model and subsequently predict the cardiovascular risk factors of age and BP a symbolic regression-based analysis was used.[581,582] For this, we applied the GPLEARN genetic programming with symbolic regression library in python.[583]

As all these measurements are numerical values, and the relationships between these are unknown and, possibly, of nonlinear nature, symbolic regression represents an excellent framework for modelling CVD risk factors. While classical regression methods rely on a priori definition of the model structure and only provide parameters for the pre-selected models, symbolic regression derives both the model structure and its parameters automatically. The mechanism powering symbolic regression is genetic programming, a form of evolutionary computation for automatically generating computer programs or functions that best describe the data provided to it.

The practitioner only has to provide the set of functions and variables to base the model on and genetic programming will learn both the model structure and parameters automatically from the data, only including those functions and variables that are necessary. If not controlled, the models evolved by genetic programming quickly become very large and hence both time consuming to evaluate and difficult to interpret, without necessarily improving in quality. For this reason, we are applying so-called parsimony pressure to control model size. Symbolic regression has already been applied successfully in a variety of domains, recently including medical, for example an automatic machine learning approach for brain age prediction.[584] Differently from other so-called black-box artificial intelligence (AI) approaches, symbolic regression solutions will be interpretable and will enable healthcare professionals to draw conclusions about the model and the relationships it uncovers.

Models were generated for each age group separately. With this division we ensure equitable consideration of all ages, without the need to apply data augmentation methods to generate synthetic data. As symbolic regression automatically determines the most relevant features as well as the structure of the regression model, there is no need to separately apply any dimensionality reduction method.

For each age group, we compared predicted age based on the models generated on the combined telomere relative expression and artery measurements, artery measurements only and telomere relative expression only. We used k-fold cross-validation and mean absolute error (MAE) for assessing model quality.

An example age prediction tree generated by Symbolic Regression and its corresponding expression formula are shown in Figure 3.19. The tree represents best model produced for

age group 1 arterial measurements and telomere experiment (fold 5, MAE 1.157639). Table 3.6 presents Symbolic Regression feature name encodings used in prediction trees and expression formulas and their corresponding feature names.

Table 3.6: Artery measurements and telomere encodings for the Symbolic Regression formulas.

Feature Representation	Corresponding Measurements
X0	Telomere Relative expression
X1	Artery baseline
X2	Artery Baseline Diameter Fluctuation
X3	Artery Maximum Dilation
X4	Artery Time to Maximum Dilation
X5	Artery Maximum Dilation Percentage
X6	Artery Maximum Constriction
X7	Artery Time to Maximum Constriction
X8	Artery Maximum Constriction Percentage
X9	Artery Dilation Amplitude
X10	Artery Baseline Corrected Flicker Response
X11	Artery Dilation Slope
X12	Artery Constriction Slope

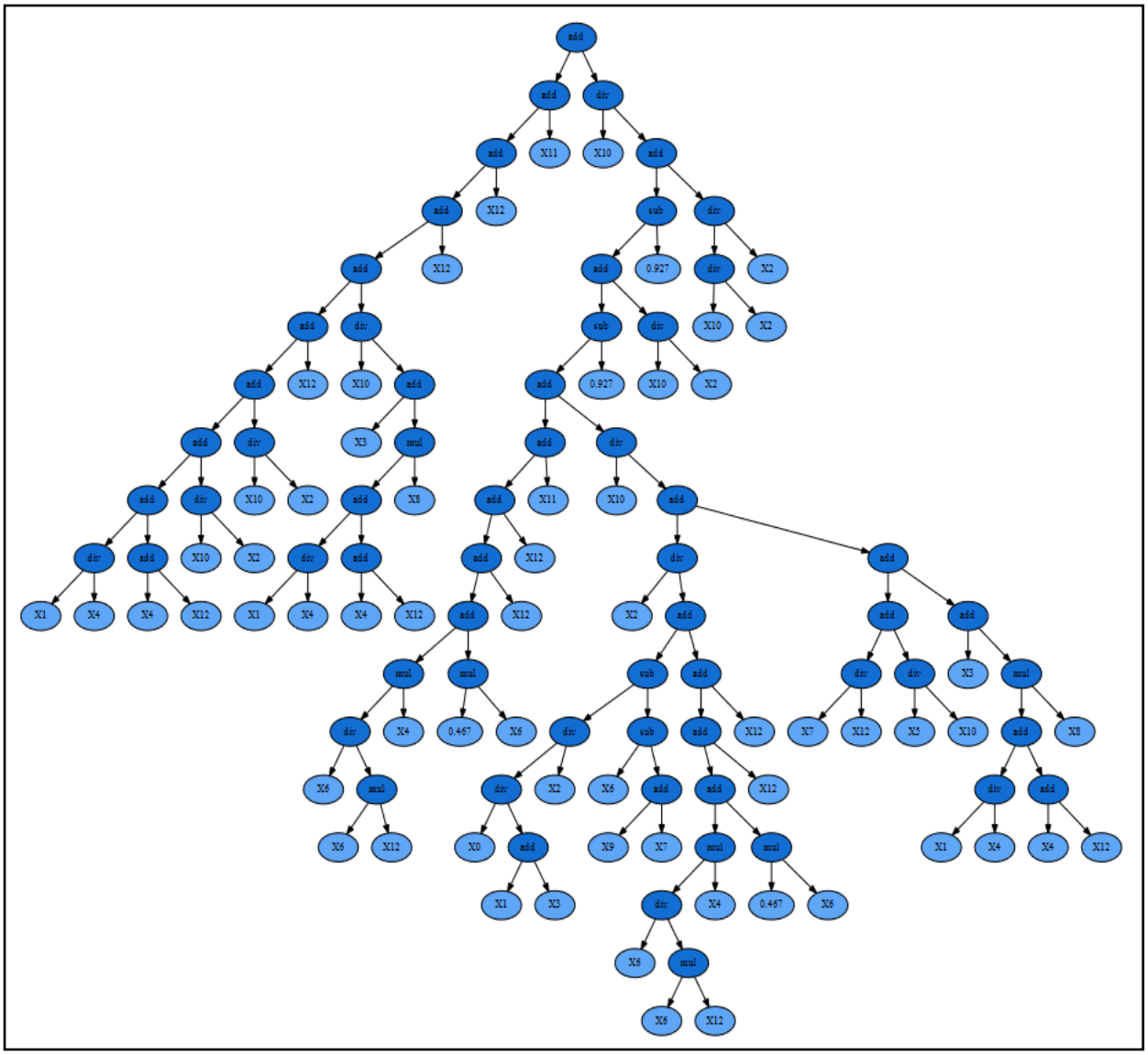

Figure 3.19: Symbolic Regression tree for age group 1, telomere + arteries experiment (MAE 1.157639). Corresponding expression formula: add(add(add(add(add(add(add(add(add(div(X1, X4), add(X4, X12)), div(X10, X2)), div(X10, X2)), X12), div(X10, add(X3, mul(add(div(X1, X4), add(X4, X12)), X8)))), X12), X12), X11), div(X10, add(sub(add(sub(add(add(add(add(add(mul(div(X6, mul(X6, X12)), X4), mul(0.467, X6)), X12), X12), X11), div(X10, add(div(X2, add(sub(div(div(X0, add(X1, X3)), X2), sub(X6, add(X9, X7))), add(add(add(mul(div(X6, mul(X6, X12)), X4), mul(0.467, X6)), X12), X12))), add(add(div(X7, X12), div(X5, X10)), add(X3, mul(add(div(X1, X4), add(X4, X12)), X8)))))), 0.927), div(X10, X2)), 0.927), div(div(X10, X2), X2)))).

3.3.12. Statistical Analysis and Sample Size Calculation

Sample size was calculated using G-power software. The first step in power calculation is seeking out published papers in the area of empirical interest that answer theoretically, conceptually, or physiologically similar research questions and use the reported values associated with the statistical results to define the population of interest. Next the researcher defined the inclusion and exclusion criteria for each study in order to define the effect size that will be taken. Probability sampling is used in experimental designs and a P-values of <0.05 were considered significant with a β error of 80%.

All data are reported as mean (SD) unless otherwise indicated. The Shapiro–Wilk test was used to determine the distribution of the data. Univariate associations were determined using Pearson's (normally distributed data) or Spearman's method (non-normally distributed data), and forward stepwise regression analyses were performed to test the influences of systemic 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. Differences between groups were subsequently assessed using one-way analysis of variance (anova) or analysis of covariance (ancova). All analyses were performed using Statistica® (version 13.3, StatSoft Inc., Tusla, OK, USA) software.

4. Study 1: Microvascular Function and Oxidative Stress in Adult Individuals with Early Onset of Cardiovascular Disease

This study has been published in Scientific Reports, Nature (Appendix 13.4.1) [514]

4.1. Abstract

Aims: The current study aims to investigate retinal vascular function and its relationship with systemic anti-oxidative defence capacity in normal individuals versus those with early hypertensive changes according to the current ESC/ESH guidelines.

Methods: Retinal microvascular function was assessed in 201 participants by means of dynamic retinal vessel analysis. Blood pressure, lipid panel, oxidized (GSH) & reduced glutathione (GSSG) were also evaluated for each participant.

Results: Individuals classed as grade 1 hypertension demonstrated higher retinal arterial baseline diameter fluctuation ($p = 0.0012$), maximum dilation percentage ($p = 0.0007$), time to maximum constriction ($p = 0.0003$) and lower arterial constriction slope ($p = 0.0131$). Individuals classed as high normal, and grade 1 hypertension also demonstrated higher time to maximum dilation than individuals classed as optimal or normal. GSH levels correlated negatively with SBP, DBP and MBP values in all participants ($p = 0.0010$; $p = 0.0350$ and $p = 0.0050$) as well as with MBP values in high normal and grade 1 hypertension ($p = 0.0290$). The levels of GSSG correlated positively with SBP, DBP and MBP values in all participants ($p = 0.0410$; $p = 0.0330$ and, $p = 0.0220$).

Conclusion: Our results point to the fact that microvascular alterations can be identifiable at BP values still considered within normal values and go in parallel with the changes observed in the level of oxidative stress.

4.2. Introduction

The assessment of the microvascular function represents an important part in establishing the pathophysiology but also the risk stratification of cardiovascular disease (CVD).[585] Indeed, endothelial dysfunction, one of the main culprits for the development of atherosclerosis, occurs much earlier at the microvascular than at the macrovascular level.[586,587] Dynamic retinal vessel analysis (DVA) was identified as a useful measure of early changes that signal endothelial dysfunction at the microvascular level. This method can also be used to identify risk for future cardiovascular pathologies in individuals at risk for [521,539,540,548,588,589] or already suffering from CVD.[550,590] This is generally possible due to the fact that the retinal microvascular response to flicker provocation is, in part, dependent on nitric oxide (NO) release[591], and compromised NO homoeostasis is known to be a key factor in endothelial dysfunction at all vascular levels. The assessment of retinal microvessels is used in research but also in clinical practice for diagnosis and follow-up of various CVD, including hypertension.[478,592] Nevertheless, the overwhelming majority of protocols that look at retinal microvessels in the course of various CVD, use static imaging to detect abnormalities associated with various degrees of pathology. This is, however, useful only when CVD already established itself and not at earlier, pre-clinical stages. In order to determine the risk at earlier stages to allow preventive measures to be adopted, using the assessment of the retinal microvascular function represents a better alternative to structural imaging because it provides integrated and dynamic data to help establishing possible CVD risk.

In certain conditions that accelerate degradation of NO, such as high oxidative stress, microvascular dilation can be severely impaired.[593] In order to counteract such effects, the human body uses various anti-oxidative mechanisms including glutathione. Therefore, any condition associated with low levels of circulating glutathione result in a higher rate of oxidative reactions that contribute towards low NO bioavailability and, consequently, to an impaired microvascular function.[539,540,594,595] We have already shown that retinal microvascular dilation and constriction responses to stress levels are influenced by systemic antioxidant capacity, and circulating markers for CVD risk in healthy individuals with low to moderate cardiovascular risk.[589] Moreover, we have suggested that, by providing an integrated and dynamic analysis of vascular function that is, indeed, specific for each individual, retinal vessel reactivity could also be used for profiling a so-called individualized vascular risk for CVD.[540,589]

Nevertheless, we have not tested this hypothesis in individuals with early stages of CVD. Therefore, the present study aims to study the retinal vascular function and its relationship with systemic anti-oxidative markers in individuals with various levels of early BP abnormalities as defined according to the 2018 European Society of Cardiology/European Society of Hypertension Guidelines.[596]

4.3. Methods

4.3.1. Study Participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). The inclusion / exclusion criteria as defined in section 3.2.1. The main study-specific inclusion criteria for this study were defined as those individuals aged above 30 years, with no current or prior history of cardiovascular, cerebrovascular diseases, peripheral vascular disease, severe dyslipidaemia, diabetes and metabolic disorders. Individuals treated for systemic hypertension as well as those using other vasoactive medications such as dietary supplements containing vitamins or antioxidants and bronchodilators were also excluded from the study.

4.3.2. General Assessment

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI = weight/height). General clinical assessments for all participants as detailed in sections 3.3.2, 3.3.4 including general health history questionnaires and IOP profiles.

4.3.3. Blood Pressure Assessment and Patients Grouping

Measurements of BP and heart activity were first performed in-clinic as described in section 3.3.3.a. They were then confirmed using a computer-operated ambulatory BP and electrocardiogram (ECG) monitor (Cardiotens-01, Meditech Ltd, Budapest, Hungary) according to the described protocol in section 3.3.3.b. Using the 24-h SBP and DBP values, study participants were then stratified into four subcategories, ‘’optimal ‘’ “normal”, “high normal” and ‘’Grade I” as recommended by the 2018 European Society of Cardiology/European Society of Hypertension arterial hypertension Guidelines.[57]

4.3.4. Dynamic Retinal Vessel Analysis

Vascular assessments of interest to this study, detailed in the methods section 3.3.5. For each participant, one unselected eye was evaluated by DVA machine. For all of the selected participants, the raw data was re-analysed using the mathematical approach for the analysis of retinal response parameters described in this thesis (section 3.3.5.12). 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 3.3.5.11).

4.3.5. Biomarkers Assays

Bloods samples drawn in the morning of the appointment. Fasting TG, T-CHOL and HDL-C were analysed according to the detailed protocol in section 3.3.9. LDL-C values were calculated as per the Friedewald equation.

4.3.6. Measurement of GSH and oxidized glutathione (GSSG)

Initial processing of blood GSH and GSSG levels were assessed by the GSH recycling assay as detailed previously in section 3.3.10.1.

4.3.7. Sample Size and Statistical Analysis

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessels reactivity was shown to be significant.[540,597] As the study design was multifactorial in nature, it was calculated that a sample size of n = 201 in each group was sufficient to provide 95% power at an alpha level of 0.05.

All statistical analyses were performed using the methods explained 3.3.12.

4.4. Results

A total number of 218 participants were initially screened for the study inclusion, of which 17 individuals were excluded based on the quality of retinal vascular image analysis. The remaining 201 participants (93 men and 108 women) were included in the final analysis and classified in one of 4 study groups using the current ESC/ESH Guidelines: group 1 (optimal BP: 56 individuals 22 men and 34women), group 2 (normal BP: 54 individuals, 35 men and 19 women), group 3 (high normal BP: 44 individuals, 28 men and 16 women) and group 4 (grade 1 hypertension: 47 individuals, 28 men and 19 women).

Table 4.1 shows the clinical characteristics of the study population. Although the first group were significantly younger than group 4, there were no statistically significant differences between the study groups with regards to gender, BMI and circulating levels of glucose, t-Chol and HDL-Chol between the study groups (all $p > 0.05$).

Nevertheless, as expected, there were statistically significant differences between the 4 study groups with regards to SBP, DBP and MBP (all $p < 0.0001$). In addition, individuals classed as high normal and grade 1 hypertension demonstrated higher levels of circulating TG ($p = 0.0021$, ANCOVA) and GSSG ($p = 0.0111$, ANCOVA) and lower levels of GSH ($p = 0.0060$, ANCOVA). Moreover, individuals classed as grade 1 hypertension demonstrated higher levels of LDL-C ($p = 00265$, ANCOVA) than the rest of the study groups.

With regards to arterial and venous retinal vascular reactivity parameters as characterized in Tables 4.2 and 4.3, all values reported are based on averaged data across three flicker cycles with the artery and vein regarded separately.

The measured retinal arteriolar parameters, after controlling for all influential covariates identified by multivariate regression analysis, and independent of age, individuals classed as grade 1 hypertension demonstrated significantly higher BDF ($p = 0.0012$, Figure 4. 1), MD% ($p = 0.0007$, Figure 4.1), tMC ($p = 0.0003$, Figure 4.1) and lower $Slope_{AC}$ ($p = 0.0131$, Figure 4.1) than the rest of the study groups. In addition, individuals classed as high normal and grade 1 hypertension also demonstrated higher tMD than individuals classed as optimal or normal ($p < 0.0001$, Figure 4.1).

There were no significant differences between participants with regard to measured venous parameters (all $p > 0.05$, Table 4.3).

4.4.1. Correlations between Vascular and Systemic Circulatory Parameters

Univariate analysis revealed that the whole blood GSH levels correlated significantly and negatively with SBP, DBP and MBP values in all participants ($r = -0.25$, $p = 0.0010$; $r = -0.17$, $p = 0.0350$ and $r = -0.22$, $p = 0.0050$, as well as with MBP values in those classed as high normal and with grade 1 hypertension ($r = -0.38$, $p = 0.0290$) but not in those classed as having optimal or normal BP ($p > 0.05$). In addition, the levels of whole blood GSSG correlated significantly and positively with SBP, DBP and MBP values in all participants ($r = 0.16$, $p = 0.0410$; $r = 0.17$, $p = 0.0330$ and $r = 0.18$, $p = 0.0220$, but not in any groups separately (all $p > 0.05$). There were no correlations between the levels of GSH and retinal vascular parameters in any of the study groups (all $p > 0.05$). However, GSSG levels correlated significantly and positively with artery BDF ($r = 030$, $p = 0.0370$) and negatively with $Slope_{AC}$ only in individuals classed as having an optimal BP level ($r = -0.37$, $p = 0.0200$).

Table 4.1. Summary of the Systemic Characteristics of the Study Participants

Variable	**Optimal (1)** (Mean +SD)	**Normal (2)** (Mean +SD)	**High-Normal (3)** (Mean +SD)	**Grade 1 (4)** (Mean +SD)	***p*-value**	**Post – Hoc**
Number	56	54	44	47	-	-
Age (years)	40.25(1.80)	42.67(1.87)	46.50(2.06)	47.60(2.03)	0.0260*	-
SBP (mmHg)	109.11(0.79)	124.59(0.82)	131.95(0.90)	145.31(0.90)	0.0000*	1<2<3<4
DBP (mmHg)	67.38(1.07)	74.71(1.11)	81.17(1.22)	87.59(1.22)	0.0000*	1<2<3<4
HR (bpm)	66.69(1.27)	67.61(1.32)	68.48(1.46)	70.24(1.46)	0.3149	-
IOP (mmHg)	14.31(0.82)	15.15(0.85)	16.76(0.93)	16.30(0.93)	0.1870	-
MAP (mmHg)	81.29(1.30)	91.33(1.35)	98.09(1.48)	104.35(1.47)	0.0000*	1<2<3<4
BMI (kg/m^2)	25.32(0.90)	26.88(1.027)	27.89(0.99)	28.21(0.88)	0.0808	-
Glucose	4.77(0.12)	4.80(0.12)	4.90(0.14)	5.01(0.13)	0.4875	-
TG (mmol/L)	1.01(0.06)	1.15(0.06)	1.29(0.07)	1.43(0.07)	0.0021*	1=2<3,4 3=4
CHOL	4.53(0.13)	4.38(0.13)	4.62(0.15)	4.80(0.14)	0.1875	-
HDL-C (mmol/L)	1.21(0.07)	1.13(0.07)	1.19(0.08)	1.16(0.07)	0.6838	-
LDL-C (mmol/L)	2.92(0.15)	3.06(0.16)	3.27(0.18)	3.59(0.17)	0.0265*	1=2=3<4
GSH (umol)	712.37(68.79)	601.47(74.18)	412.52(77.17)	428.63(83.43)	0.0060*	1=2>3,4 3=4
GSSG (umol)	36.15(7.92)	55.53(8.54)	62.49(9.75)	76.67(9.47)	0.0111*	1=2<3,4 3=4

Table 4.1: Abbreviations: SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; IOP: intraocular pressure; BMI: body mass index; TG: triglycerides; CHOL: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein; GSSG: oxidized glutathione; GSH: reduced glutathione. *Significant p-values are indicated where $p < 0.05$ was considered significant.

Table 4.2. Summary of Retinal Arterial Vascular Function Parameters

Variable	Optimal BP (1) (**Mean** +SD)	Normal BP (2) (Mean +SD)	High-Normal BP (3) (Mean +SD)	Grade 1 BP (4) (Mean +SD)	p-value Anova/ Ancova	Post-Hoc
Baseline	109.00(2.23)	110.42(2.48)	113.81(2.97)	111.77(4.45)	0.08319	-
BDF	5.36(0.40)	5.75 (0.42)	6.62(0.46)	8.12(0.46)	0.0012*	1=2=3<4
BCFR	4.62(0.40)	3.85(0.48)	4.08(0.53)	5.74(0.81)	0.2646	-
MD	114.22(2.44)	115.54(2.72)	119.86(3.25)	118.81(4.87)	0.1194	-
tMD	12.76(0.698)	14.77(0.735)	18.04(0.842)	23.10(0.79)	0.0000*	1=2<3<4
MD %	5.46(0.53)	4.31(0.58)	5.17(0.57)	7.31(0.513)	0.0007*	1=2=3<4
MC	106.14(2.39)	107.39(2.39)	110.35(3.18)	108.60(4.76)	0.2053	
tMC	23.85(1.07)	25.40(1.109)	26.77(1.22)	30.84(1.22)	0.0003*	1=2=3<4
MC%	-4.31(0.282)	-3.63(0.293)	-4.042(0.32)	-3.23(0.32)	0.1696	-
DA	9.11(0.66)	9.04(0.735)	9.46(0.88)	11.32(1.35)	0.4951	-
$Slope_{AD}$	0.69(0.066)	0.57(0.069)	0.37(0.08)	0.51(0.075)	0.1539	-
$Slope_{AC}$	-0.45(0.07)	-0.44(0.07)	-0.48(0.08)	-0.74(0.08)	0.0131*	1=2=3>4

Table 4.2: Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach MD; MD (%), percent dilation; tMC, time to reach MC; MC (%), percent constriction; DA, dilation amplitude (difference between MD and MC during flicker) $Slope_{AD}$, slope of arterial dilation; $Slope_{AC}$, slope of arterial constriction. * Significant *p*-values are indicated where $p < 0.05$ was considered significant.

Table 4.3. Summary of Retinal Venous Vascular Function Parameters

Variable	Optimal BP (1) (Mean +SD)	Normal BP (2) (Mean +SD)	High-Normal BP (3) (Mean +SD)	Grade 1 BP (4) (Mean +SD)	p-value Anova/Ancova
Baseline	146.41(3.93)	144.11(4.08)	151.22(4.50)	143.30(4.44)	0.5542
BDF	6.08(0.50)	4.769(0.60)	5.21(0.75)	6.51(0.93)	0.1913
BCFR	4.42(0.41)	4.21(0.43)	4.86(0.47)	3.88(0.47)	0.4718
MD	153.77(4.07)	152.77(4.18)	161.22(4.63)	152.10(4.59)	0.4623
tMD	19.97(0.79)	20.56(0.94)	21.18(1.198)	22.05(1.46)	0.0525
MD%	5.28(0.35)	5.44(0.37)	5.42(0.40)	6.10(0.41)	0.2231
MC	143.04(3.89)	141.74(4.038)	148.13(4.45)	140.56(4.40)	0.6051
tMC	34.30(1.29)	31.10(1.43)	32.90(1.70)	32.50(2.53)	0.4114
MC%	-1.76(0.20)	-1.57(0.22)	-1.48(0.31)	-1.90(0.38)	0.7218
DA	9.79(0.72)	8.61(0.73)	11.22(0.84)	11.16(0.83)	0.1069
$Slope_{VD}$	0.39(0.03)	0.38(0.04)	0.36(0.05)	0.41(0.056)	0.8740
$Slope_{vC}$	-0.48(0.10)	-0.39(0.11)	-0.498(0.12)	-0.83(0.12)	0.0663

Table 4.3: Abbreviations ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach MD; MD (%), percent dilation; tMC, time to reach MC; MC (%), percent constriction; DA, dilation amplitude (difference between MD and MC during flicker) $Slope_{VD}$, slope of venous dilation; $Slope_{VC}$, slope of venous constriction. * Significant *p*-values are indicated where $p < 0.05$ was considered significant.

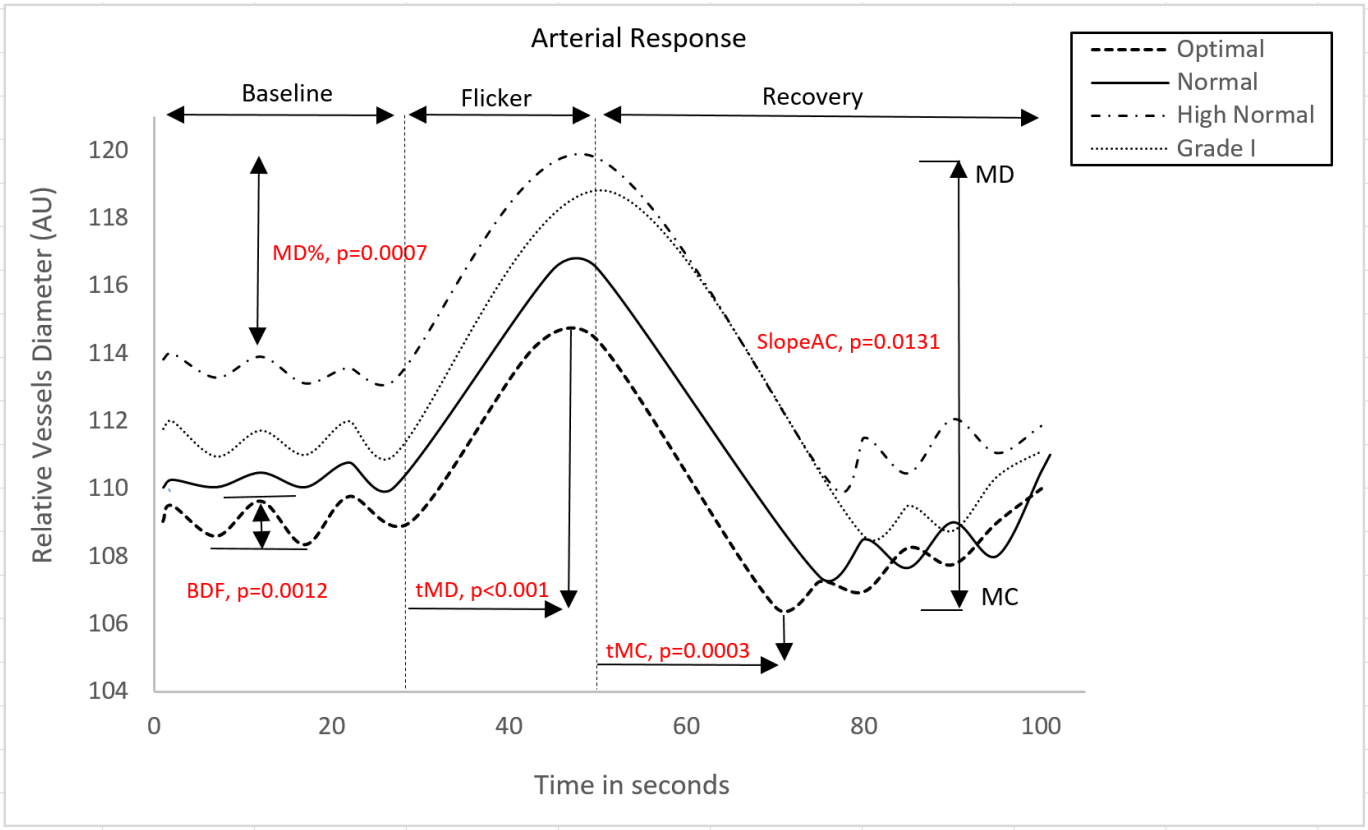

Figure 4.1: Comparison of Retinal Arterial Response Profile across Groups.
AU, arbitrary units; BDF, baseline diameter fluctuation calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings; MD%, calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker; tMD, time to reach maximum diameter during flicker; tMC, time to reach maximum constriction post flicker; $Slope_{AC,}$,calculated as (MC-MD)/(tMC).

4.5. Discussion

In the present study, we have examined the differences in retinal vascular function between individuals with normal and early stages of abnormal BP according to the current ESC/ESH guidelines. We have also looked at the relationship between systemic BP, retinal vessel reactivity and systemic antioxidant defence capacity in each of the study groups. For the first time, our results showed that individuals graded as having high normal BP values or grade 1 hypertension displayed abnormal dilatory and constrictor responses to flickering light stimulation in retinal arteries but not in the retinal veins when compared to those classed as having optimal or normal BP values.

Hypertension is a polygenic disease; however, endothelial dysfunction and enhanced oxidative stress are among the established modifiable risk factors for this disease.[598] Alteration of vascular reactivity characterized by augmented contractility and impaired relaxation is a prominent feature in hypertension. Moreover, in essential hypertension, NO availability is reduced earlier, resulting in early vascular endothelial dysfunction.[599] Indeed, our relatively young patients with high normal BP and grade 1 hypertension, according to the current ESC/EHS guidelines, demonstrated abnormal retinal microvascular dilation and constriction after stimulation with flickering light.

It has already been demonstrated that pre-hypertension is associated with retinal microvascular alterations early in life.[600] Nevertheless, it seems that these alterations can also be identifiable at BP values previously considered within normal values. Indeed, our study participants classed as having high normal BP values and without additional risks for CVD, have also exhibited signs of microvascular dysfunction at the retinal level. At these BP values, treatments for hypertension are not yet indicated; however, in the light of the above observations, additional measures to prevent further damage may be considered. Moreover, ESC/ESH also recommends treating at lower BP levels in very high-risk patients with a high–normal BP and established CVD.[57] Nevertheless, the potential impact of pushing for an earlier diagnosis is multiple, including pressures on the health system and economy.[601] Therefore, careful consideration should be given to the actual benefits of treatment in each individual separately, rather than in a given risk category. At present, all the new biomarkers for CVD risk drive the shift towards personalized medicine. Therefore, due to its personalised approach, retinal vessel reactivity can be used for profiling individualized vascular risk by providing an integrated and dynamic analysis of vascular function specific for each individual, therefore, aiding a personalised management of abnormal BP values.

As already mentioned above, increased oxidative stress is an important risk factor for the development of hypertension. Among other defence mechanisms, glutathione represents a major redox buffer.[297] It has been already shown that GSH significantly lower, and the GSSG is higher in the red blood cells in patients with hypertension but not in normal individuals.[602]

Similarly, we have also shown that whole-blood GSH levels were lower and GSSG levels were higher in individuals classed as high normal and grade 1 hypertension. These results show for the first time that such changes may actually occur at a much earlier stage than previously noticed and go in parallel in the changes observed in the retinal microvasculature, confirming that, in the presence of abnormal BP levels, the pathological inhibition of vascular relaxation involves not only NO production by endothelial cells but also a glutathione-dependent bioavailability of NO.[300] In addition, we also demonstrated that GSSG correlated with retinal arterial baseline and constriction characterisation parameters only in individuals with optimal BP levels and without additional CVD risks. It is very interesting that, although the levels of antioxidative products were abnormal in individuals with higher BP levels, the link between redox status and retinal vascular reactivity was lost in these groups. This observation links, however, to our previous results in in individuals with low to moderate CVD risk, indicating that in addition to other processes, antioxidant mechanisms support normal NO levels during flicker provocation at the retinal microvascular level.[589] However, in the present study we could not identify such relationship in individuals with higher levels of BP. As the effects of radical oxygen species (ROS) on vascular tone is dependent upon the concentration and type of species, as well as on the type of vascular beds and various experimental conditions[603], it is possible that these effects were not visible in our specific setup. Indeed, it has been proposed that there is a lack of sensitive methods to accurately evaluate the oxidative stress in the human cardiovascular system, especially when it comes to establishing the role redox stress in the small artery vasculopathy of human hypertension.[604]

4.6. Conclusion

Abnormalities of microvascular dysfunction may be an important risk factor for systemic hypertension. In addition, excessive oxidative stress is associated results in cardiovascular diseases by impairing endothelial function and, consequently, microvascular dysfunction. This complex interlink cannot be ignored, and measures to address both dysfunctions should be applied. This is especially important since, despite significant advancement in understanding the pathophysiology of human hypertension and the large number of antihypertensive drugs, strict BP control is still proving insufficient for the prevention of future vascular complications, the so-called “Hypertension Paradox”.[605] Therefore, more research is necessary to develop specific mechanism-based personalised therapies that will address the vascular redox pathobiology.[604] These therapies will possibly need to be applied at a much earlier stage when abnormalities are only functional and can still be reversed.

5. European Society of Cardiology/European Society of Hypertension versus the American College of Cardiology/American Heart Association guidelines on the cut-off values for early hypertension: a microvascular perspective

This study has been published in Scientific Reports, Nature (Appendix 13.4.2).[606]

5.1 Abstract

Aims: To investigate retinal and peripheral microvascular function in asymptomatic individuals who fall into different BP groups using either the ESC/ESH or the ACC/AHA guidelines.

Methods: Retinal and peripheral microvascular function was assessed in 358 participants by means of dynamic retinal vessel analysis and digital thermal monitoring, respectively. Blood pressure and lipid panel were also evaluated.
Retinal vascular function measured in all groups belonging to the ACC/ASH classifications were within the normal values for age-matched normal population.

Results: Individuals classed as grade 1 hypertension according to the ESC/ESH guidelines, however, exhibited a significantly decreased artery baseline (p=0.0004) and MC (p=0.040), higher $slope_{AD}$ (p=0.0018) and decreased vein MC (p=0.0446) compared to age-matched normal individuals. In addition, they also had significant lower artery baseline, artery BDF, MD and MC than individuals classed as stage 1 hypertension based on the ACC/ASH guidelines (p=0.00022, p=0.0179, p=0.0409 and p=0.0329 respectively).
Peripheral vascular reactivity (aTR) was lower in ESC /ESH grade I compared to those graded ACC/ASH stage I hypertension (p=0.0122).

Conclusion: Microvascular dysfunctions are present at multiple levels only in individuals with ESC/ESH grade 1 hypertension.
This observation could be important when deciding personalised care in individuals with early hypertensive changes.

5.2. Introduction:

One of the main differences between the latest guidelines published in 2018 by the European Society Of Cardiology/European Society of Hypertension (ESC/ESH) and those published in 2017 by The American College of Cardiology/American Heart Association (ACC/AHA) is the cut-off for what is considered elevated blood pressure (BP) and the first stage of hypertension (HTN) diagnosis.[601] As it can be expected, such difference is bound to have a high impact on clinical diagnosis and management of HTN. By using the ACC/AHA guidelines, the number of patients diagnosed with having this disease increased significantly and so did the pressure on the health system and economy. In addition, such change in practice has also a significant impact on the patients' physical and psychological wellbeing. [601] Nevertheless, it would seem sensible that, in individuals with higher risk for cardiovascular disease (CVD), early diagnosis and interventions are applied at lower BP values. Indeed, both ECC/ESH guidelines and ACC/AHA have recognised this fact and recommended considering treating patients with high CVD risk at a BP threshold lower than their current cut-off for grade/stage 1 HTN.[57] In this way, positive progress is made towards considering individual over population factors when considering someone of being at risk for CVD.

But what is the real measure of a high CVD risk? Today, the gold standard for absolute CVD risk is based on the Framingham 10-year CVD risk score (FRS).[607] Other risk scores, such as the Prospective Cardiovascular Mϋnster (PROCAM) and the European Society of Cardiology Systematic Coronary Risk Evaluation (SCORE)[608,609] are also being used for the same purpose. These calculations are based, however, only on few non-modifiable as well as some modifiable risk factors, thus limiting their ability to accurately predict the risk for CVD in all individuals. Indeed, it has been shown that they could either over- or underestimate actual risk for CVD in a large number cases.[608,610,611] Consequently, it has been suggested that other factors, such as obesity, psychosocial stress and lifestyle, to name just a few, should also be included for more precise estimations.[612] In addition, vascular endothelial function, a parameter often overlooked, is essential not only for investigations into the pathophysiology of CVD but also for better CVD risk stratification. [400,613,614] Assessing vascular endothelial function is usually performed using techniques such as ultrasound flow-mediated dilation (FMD), pulse wave analysis (PWA), plethysmography and iontophoresis.[615] These tests can, however, be complex and time-consuming, and are performed only in highly specialized services, this contributing to the lack of inclusion of this parameter in the more largely circulated risk scores. Nevertheless, parameters such as retinal microvascular function has been found to show a good association not only with various circulatory markers for CVD [514,539] , but also with other modifiable and non-modifiable risk factors for this disease such as obesity

[616], family history [513] and age.[588,589] Retinal microvascular function assessments provide instant integrated and dynamic data analysis that is specific to each individual. In addition, it also correlates with peripheral markers for endothelial dysfunction. Indeed, we have recently shown that signs of abnormal vascular function are similarly present and detectable in various microvascular beds, despite existing differences in their anatomical and physiological properties.[515] This observation is important and helps clinician to detect signs of microvascular dysfunction regardless of the method they can access to. By having a choice, practitioners can make better decisions whether to treat or not selected patients with lower BP values but with additional CVD risk, thus making decisions based on individual, rather that population risk factors, an important step towards personalised management of HTN. Nevertheless, in individuals without higher risk, the decision to treat might not be always justified. In order to shed a possible light on the validity of lowering the cut-off for the diagnosis of early hypertension in individuals without additional CVD risk, this study aimed to analyse the retinal and peripheral microvascular function in subjects that fall into different BP groups when using either the ESC/ESH or the ACC/AHA guidelines. [57,536]

5.3. Methods

5.3.1. Study Participants

Study participants were recruited through advertisements at the Vascular Research Laboratory and Health Clinics at Aston University (Birmingham, UK). The inclusion / exclusion criteria as defined in section 3.2.1. The main study-specific inclusion criteria for this study were defined as those individuals aged above 30 years, with no current or prior history of cardiovascular, cerebrovascular diseases, peripheral vascular disease, severe dyslipidaemia, diabetes and metabolic disorders. Individuals treated for systemic hypertension and those using other vasoactive medications such as dietary supplements containing vitamins or antioxidants and bronchodilators were also excluded from the study.

5.3.2. General Assessment

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI = weight/height). General clinical assessments were conducted for all the participants as detailed in sections 3.3.2, 3.3.4 including general health history questionnaires and IOP profiles.

5.3.3. Biomarkers Assays:

Bloods samples were drawn on the morning of the appointment. Fasting TG, T-CHOL and HDL-C were analysed according to the detailed protocol in section 3.3.9. LDL-C values were calculated as per the Friedewald equation.

5.3.4. Blood Pressure Assessment and Patients Grouping

Measurements of BP were performed on two separate occasions, one in-clinic and one out-of-clinic.[535,536] During the in-clinic visit, BP was measured in a quiet environment, with the patient seated for 5 min, 3 times at 1-2 min intervals using the same protocol described in section 3.3.3.a. The BP values were confirmed by a second measurement using a computer-operated ambulatory BP and electrocardiogram (ECG) monitor (Cardiotens-01, Meditech Ltd, Budapest, Hungary) as explained in section 3.3.3.b. Using the daytime SBP and DBP values, study participants were stratified into three subcategories: “normal”, “high normal”, and ‘’Grade I’’ as recommended by the 2018 ESC/ESH Guidelines. They were then also further classified into three other subcategories: “normal”, “elevated”, and “stage I”, as recommended by the 2017 ACC/AHA guidelines. Subjects classed as “optimal” according to the 2018 ESC/ESH guidelines were excluded, and their values were included in our pool of “normal” data.[514]

5.3.5. Dynamic Retinal Vessel Analysis

Vascular assessments of interest to this study, detailed in the methods section 3.3.5. For each participant, one unselected eye was evaluated by DVA. All raw data were re-analysed using the mathematical approach for the analysis of retinal response parameters described in section 3.3.5.12. 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 3.3.5.11).

5.3.6. Digital Thermal Monitoring (DTM)

The peripheral microvascular reactivity at the level of the fingertips was assessed using VENDYS 5000 BCE DTM system (Endothelix, Inc, Houston, TX, USA) according to an established protocol as mentioned in section 3.3.6.[617] The post-occlusive adjusted temperature rebound aTR determined by the software algorithm is directly associated with the extent of the subjects vascular reactivity.[618]

5.3.7. Statistical analysis

Data were analysed according to methods explained in 3.3.12.

5.4. Results

A total number of 372 participants were initially screened for study inclusion of which 14 individuals were excluded based on the quality of retinal vascular image analysis or to the fact that they did not tolerate the ambulatory BP measurements. The remaining 358 participants were included in the final analysis and classified into 3 study groups using the current

ESC/ESH Guidelines (normal BP:66 individuals; high normal BP: 40 individuals; and grade 1 hypertension: 53 individuals). A further three groups were decided based on the ACC/AHA guidelines: (normal BP: 83 individuals; Elevated BP: 46 individuals; and stage 1 hypertension: 70 individuals).

General characteristics of the study population are presented in Table 5.1. There were no significant differences in age, gender, BMI, HR, IOP, glucose, TG, cholesterol, LDL-c, HDL-c and body composition between all the study groups (all $p > 0.05$). There was a significant difference between groups in SBP and DBP ($p < 0.001$). In addition, there were also significant differences between the peripheral vascular reactivity (aTR) between ESC /ESH grade I compared to ACC/ASH stage I hypertension, with individuals graded as ESC /ESH grade I having lower aTR than those graded ACC/ASH stage I hypertension ($p=0.0122$).

Comparisons of group differences in measured retinal arterial and venous DVA parameters after flicker stimulation are presented in Table 5.2& 5.3. All values reported are based on data averaged across the three flicker cycles with arteries and veins considered separately. Values were further compared to the average normal values for the groups' corresponding age range.

5.4.1. Retinal Vascular Response:

After controlling all the influential covariates identified in multivariate analysis, there were no statistically significant differences in baseline diameter, BDF, BCFR, tMD and tMC, $slope_{AD}$ and $slope_{AC}$ between the ACC/AHA and ESC/ESH normal BP groups ($p > 0.05$, Table 5.2). Similarly, there were no significant group differences found between ACC/AHA elevated and ESC/ESH high normal BP groups ($p > 0.05$, Table 5.2). However, individuals classed as grade 1 hypertension according to the ESC/ESH guidelines had significant lower artery baseline, artery BDF, MD and MC than individuals classed as stage 1 hypertension based on the ACC/ASH guidelines ($p=0.0002$, $p=0.0179$, $p=0.0409$ and $p=0.0329$, respectively). In addition, while the retinal vascular reactivity parameters measured in individuals classed as stage 1 hypertension according to ACC/ASH guidelines were still within the normal limits for their age, the ESC/ESH grade I hypertensive group exhibited a significantly decreased artery baseline ($p=0.0004$) and MC ($p=0.040$) as well as higher $slope_{AD}$ ($p=0.0018$) compared to age matched population average (Table 5.2, Figure 5.1). Similarly, vein MC was significantly decreased in ESC /ESH grade I compared to ACC/ASH stage I hypertension ($p=0.0383$). In addition, vein MC was significantly decreased only in the ESC/ESH grade I hypertension compared to age matched population average ($p=0.0446$) (Table 5.3, Figure 5.1).

No significant group differences in any of the other measured arterial DVA parameters were identified (all $p > 0.05$).

Table 5.1: General Characteristics of the Study Population

	ACC/AHA Normal <120&<80	ESC/ESH Normal SBP:120-129 DBP:80-84	*p*-value	ACC/AHA Elevated SBP: 120-129 DBP: <80	ESC/ESH High Normal SBP:130-139 DBP:85-89	p-value	ACC/AHA Stage I SBP:130-139 DBP:80-89	ESC/ESH Grade I SBP:140-159 DBP:90-99	*p*-value
Number	83	66	-	46	40	-	70	53	-
Gender	30M:53F	34M:32F	-	29M:17F	22:18F	-	44M:37F	32M:21F	-
Age (years)	40.1.8 (1.35)	43.91 (1.70)	0.0859	42.83 (2.31)	45.80 (2.53)	0.3897	44.88(3.4)	48.77(2.7)	0.1063
SBP (mmHg)	109.43 7.08)	122.52(4.98)	0.0000*	124.17(6.88)	131.03(6.74)	0.0002*	134.61(8.72)	142.00(7.52)	0.0000*
DBP (mmHg)	68.33 (6.15)	76.15 (5.92)	0.0000*	72.76 (4.98)	83.93 (5.37)	0.0000*	83.95(4.71)	89.47(6.54)	0.0000*
HR (bpm)	68.055(9.28)	65.06(10.15)	0.0659	63.31 (9.38)	67.37(10.85)	0.1085	68.37(10.87)	69.59(10.93)	0.5467
IOP	13.30 (2.34)	13.41 (2.64)	0.8139	13.25 (2.59)	13.43 (2.25)	0.7123	14.33(2.63)	15.29(3.01)	0.0970
BMI (kg/m2)	24.69 (0.36)	25.66 (0.45)	0.0934	26.73 (0.71)	25.70 (0.78)	0.3175	26.49(4.20)	28.09(4.40)	0.0680
Body fat %	28.00(1.68)	23.76(1.76)	0.0864	23.02(1.90)	28.31(2.06)	0.0670	27.65(2.43)	31.93(3.97)	0.3651
Body water %	52.51(1.65)	51.56(1.73)	0.6907	54.25(2.30)	48.84(2.48)	0.1180	46.80(2.96)	49.44(4.84)	0.6445
Muscle mass %	47.45(2.59)	50.89(2.72)	0.1673	60.94(3.13)	55.68(3.38)	0.2599	52.75(4.29)	43.30(6.10)	0.2583
Glucose(mmol/L)	4.87 (0.55)	4.93 (0.86)	0.6178	5.07 (0.82)	5.03 (0.60)	0.8433	4.93(0.92)	5.15(1.31)	0.3356
TG (mmol/L)	1.139(0.56)	1.19 (0.61)	0.6345	1.15 (0.48)	1.25 (0.46)	0.4397	1.263(0.62)	1.44(0.80)	0.2210
T.Chol (mmol/L)	4.40 (0.78)	4.70 (1.02)	0.5642	4.88 (1.07)	4.55 (0.91)	0.2040	4.50(0.89)	4.72(0.86)	0.2516
HDL-C (mmol/L)	1.33 (0.41)	1.37 (0.39)	0.5212	1.35 (0.38)	1.23 (0.58)	0.3288	1.33(0.56)	1.21(0.44)	0.2848
LDL-C (mmol/L)	2.57 (0.91)	2.78 (0.12)	0.1639	2.10 (1.09)	2.82 (1.05)	0.5199	2.59(0.94)	2.90(0.93)	0.1131
TG/HDL	1.00 (0.81)	1.01 (0.81)	0.9561	0.98 (0.63)	1.35 (0.91)	0.0683	1.21(0.92)	1.36(0.94)	0.4460
aTR	1.60(0.13)	1.93(0.11)	0.0365*	1.70(0.11)	1.63(0.11)	0.7790	1.80(0.12)	1.20(0.20)	0.0122*

Table 5.1: Abbreviations: SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; IOP: intraocular pressure; BMI: body mass index; TG: triglycerides; T. Chol: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein; aTR: adjusted temperature rebound *Significant p-values are indicated where P<0.05 was

Table 5.2. Summary of Retinal Arterial Vascular Function Parameters

Normal Average	ACC/AHA Normal <120&<80	ESC/ESH Normal SBP:120-129 DBP:80-84	ANOVA/ANCOA *p*-value	ACC/AHA Elevated SBP:120-129 DBP: <80	ESC/ESH High Normal SBP:130-139 DBP:85-89	ANOVA/ANOVA *p*-value	ACC/AHA Stage I SBP:130-139 DBP:80-89	ESC/ESH Grade I SBP:140-159 DBP:90-99	ANOVA/ANCOVA *p*-value
Artery baseline 99.99(0.002)	99.98 (0.12)	99.88 (0.77)	0.1979	99.83(0.95)	99.97(0.92)	0.4346	99.99(0.62)	97.93(0.27)	0.0002*
Artery-BDF 5.66(2.63)	5.64 (2.650)	6.14 (3.46)	0.3461	6.28(2.87)	5.83(2.74)	0.5250	5.98(3.42)	5.02(2.41)	0.0179*
Artery-MD 103.67(2.20)	103.57(2.20)	104.07(2.24)	0.2000	104.44(2.2)	104.19(2.74)	0.5780	104.14(2.43)	102.75(4.26)	0.0409*
Artery-tMD 22.14(10.09)	21.88 (9.63)	20.87 (6.27)	0.5047	20.84(5.83)	20.27(6.44)	0.7120	19.86(6.74)	20.10(7.11)	0.9493
Artery-MD% 3.67(2.21)	3.59 (2.20)	4.20 (2.53)	0.1400	4.64(2.63)	4.48(2.75)	0.7901	4.15(2.44)	4.01(2.55)	0.1020
Artery-MC 97.51(1.89)	97.73 (0.19)	97.64 (0.25)	0.7691	97.83(1.82)	97.53(2.15)	0.5410	97.58(1.80)	96.08(4.97)	0.0329*
Artery –tMC 27.05(8.31)	27.47 (7.36)	27.88 (6.98)	0.7455	28.09(6.60)	28.05(6.44)	0.9810	27.49(6.70)	28.85(7.96)	0.3641
Artery-MC% -2.49(1.89)	-2.22 (1.84)	-2.056 (1.87)	0.6124	-1.99(1.95)	-2.14(2.14)	0.3810	-2.41(1.80)	-1.94(1.72)	0.2002
Artery-DA 6.16(2.95)	5.77 (2.87)	6.17 (2.96)	0.5191	6.61(2.95)	7.30(3.63)	0.4310	6.57(3.02)	6.71(2.91)	0.8096
Artery-BCFR 0.56(2.73)	0.30 (2.57)	0.26 (2.97)	0.9244	0.43(2.74)	0.44(3.02)	0.1681	0.67(3.07)	0.89(3.12)	0.7326
Artery-Slope$_{AD}$ 0.21(0.13)	0.21 (0.16)	0.25 (0.15)	0.1347	0.27(0.16)	0.26(0.32)	0.1902	0.28(0.20)	0.35(0.30)	0.2335
Artery-Slope$_{AC}$ -0.36(0.30)	-0.36 (0.45)	-0.30 (0.22)	0.3600	-0.31(0.22)	-0.32(0.18)	0.9603	-0.29(0.20)	-0.27(0.51)	0.2826

Table 5.2: Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach MD; MD(%), percent dilation; tMC, time to reach MC; MC (%), percent constriction; DA, dilation amplitude (difference between MD and MC during flicker) Slope$_{AD}$, slope of arterial dilation; Slope$_{AC}$, slope of arterial constriction. * Significant p-values are indicated where $p < 0.05$ was considered significant.

Table 5.3. Summary of Retinal Venous Vascular Function Parameters

Normal Average	**ACC Normal** <120&<80	**ESC Normal** SBP:120-129 DBP:80-84	**ANOVA/ANCOVA *p*-value**	**ACC Elevated** SBP:120-129 DBP: <80	**ESC High Normal** SBP:130-139 DBP:85-89	**ANOVA/ANCOVA *p*-value**	**ACC Stage I** SBP:130-139 DBP:80-89	**ESC Grade I** SBP:140-159 DBP:90-99	**ANOVA/ANCOVA *p*-value**
Vein-Baseline 99.98(0.15)	99.96(0.20)	99.90 (0.78)	0.3996	99.83(0.97)	99.99(0.002)	0.3733	99.99(0.0025)	99.91(0.0022)	0.4251
Vein-BDF 4.39(2.04)	4.427(2.22)	3.94(2.02)	0.2059	4.09(2.22)	5.149(3.48)	0.1583	4.70(2.74)	4.92(2.48)	0.7667
Vein-MD 104.34(2.26)	104.19(2.16)	104.06(2.24)	0.7239	104.01(2.19)	105.31(2.83)	0.5790	105.10(2.73)	104.61(2.245)	0.6224
Vein-tMD 22.55(6.93)	22.37(6.90)	21.53 (7.40)	0.5058	22.68(8.62)	21.25(6.46)	0.5343	19.82(5.45)	20.43(5.81)	0.4213
Vein-MD% 4.36(2.26)	4.24(2.15)	4.19 (2.61)	0.9114	4.21(2.75)	5.310(2.93)	0.1326	5.10(2.73)	4.612(2.24)	0.5127
Vein MC 98.71(1.27)	98.87(1.14)	99.20 (1.12)	0.0957	98.36(1.02)	98.70(1.51)	0.2408	98.46(1.123)	97.13(12.5)	0.0383*
Vein- tMC 31.68(5.51)	31.23(5.62)	30.15 (6.38)	0.2990	31.26(6.61)	30.54(6.34)	0.6757	30.37(5.76)	31.0384(5.20)	0.6642
Vein-MC% -1.27(1.28)	-1.09(1.18)	-0.70 (1.55)	0.0744	-0.85(0.70)	-1.29(0.81)	0.1973	-1.17(1.25)	-1.06925902	0.5289
Vein-DA 5.63(2.88)	5.325(2.69)	4.85 (2.81)	0.3302	4.86(1.69)	6.60(3.20)	0.0869	6.27(3.16)	5.6811(2.60)	0.4637
Vein-BCFR 1.23(2.19)	0.94(2.37)	0.98 (2.86)	0.9383	0.66(2.79)	1.56(2.84)	0.2202	1.52(2.49)	0.76220(1.97)	0.2591
Vein -Slope$_{AD}$ 0.23(0.13)	0.23(0.14)	0.24 (0.16)	0.5595	0.24(0.175)	0.50(1.109)	0.1881	0.39(0.74)	0.2697(0.13)	0.4007
Vein -Slope$_{VC}$ -0.24(0.15)	-0.23(0.14)	-0.20 (0.13)	0.2551	-0.20(0.13)	-0.27(0.16)	0.1062	-0.25(0.17)	-0.2318(0.16)	0.7306

Table 5.3 Abbreviations ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach MD; MD%, percent dilation; tMC, time to reach MC; MC% , percent constriction; DA, dilation amplitude (difference between MD and MC during flicker) Slope$_{AD}$, slope of venous dilation; Slope$_{VC}$, slope of venous constriction. * Significant p-values are indicated where $p < 0.05$ was considered significant.

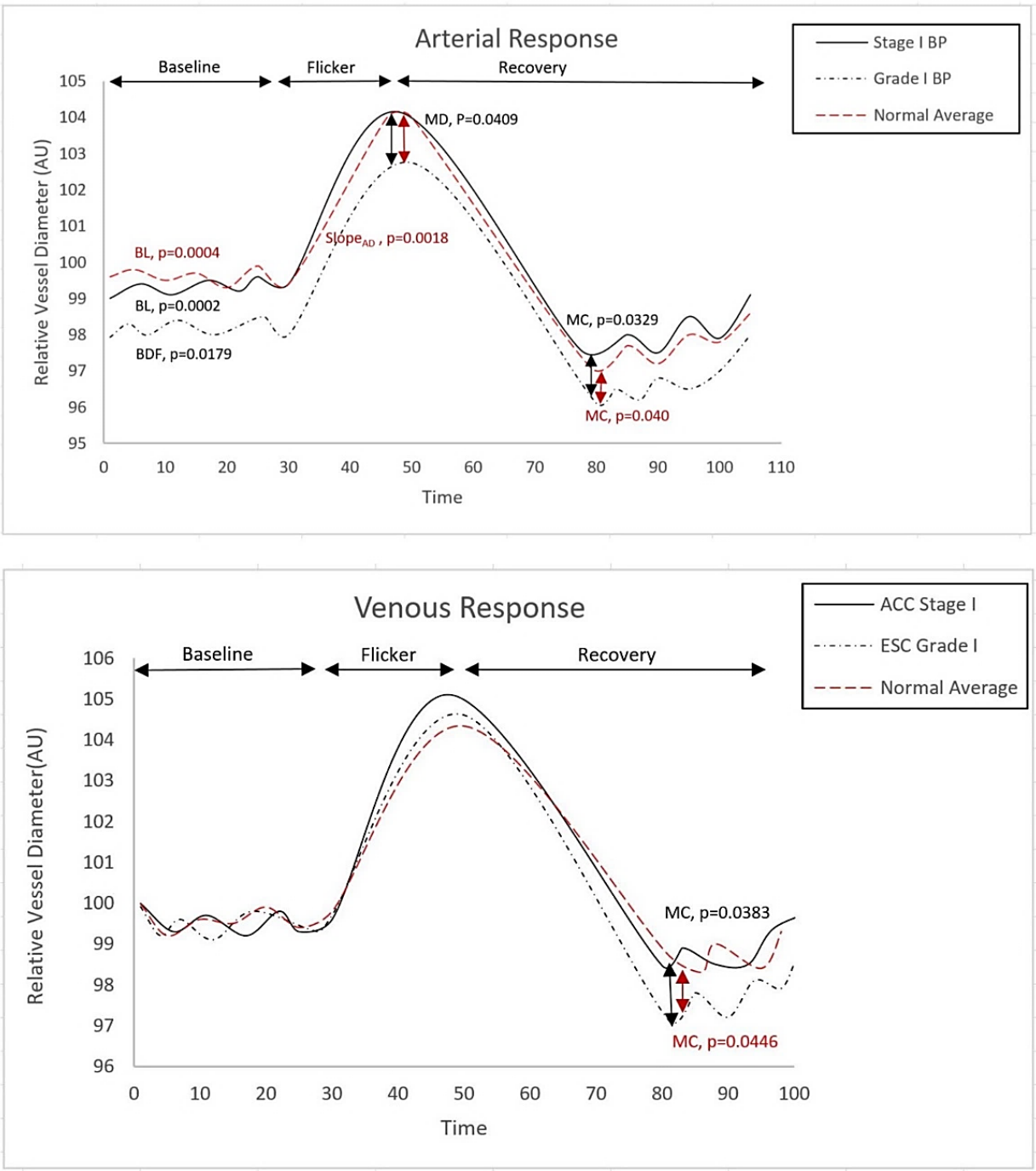

Figure 5.1: Comparison of Retinal Arterial and Venous Response Profile across Groups.

AU, arbitrary units; BDF, baseline diameter fluctuation calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings; MD%, calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker; tMD, time to reach maximum diameter during flicker; tMC, time to reach maximum constriction post flicker; $slope_{AC}$ & $Slope_{VC}$, calculated as (MC-MD)/(tMC).

5.5. Discussion:

The present study looked at the peripheral and retinal microvascular function in various BP groups divided according to either the ESC/ESH or the ACC/AHA guidelines. Our report shows, for the first time, that independent of all systemic influences, retinal microvascular dysfunction firstly manifest itself only in individuals classed as ESC/ESH grade 1 hypertension. In contrast, subjects classed as ACC/AHA stage 1 hypertension exhibited normal retinal microvascular behaviour after provocation using flickering light. In addition, ESC/ESH grade 1 hypertension individuals also exhibited lower peripheral vascular reactivity than those graded as ACC/AHA stage 1 hypertension.

It is well known that microvascular dysfunction is a parameter highly predictive of cardiovascular events. [471] Due to this quality, its early identification is extremely important, especially in individuals at risk for CVD, including those with borderline BP values or classed as grade/stage 1 hypertension. Indeed, similar to what is known for so long in the literature about the effects of systemic hypertension on microvasculature [619] in the present study we have shown that individuals classed as ESC/ESH grade 1 hypertension demonstrated abnormal peripheral and retinal microvascular function. Indeed, at the retinal vascular level, both vasodilation and vasoconstriction of the retinal arteries and veins were affected. Nevertheless, as all patients with essential hypertension are known to demonstrate microvascular endothelial dysfunction from early stages [620] , it is puzzling how individuals classed as ACC/AHA stage 1 hypertension failed to show such abnormalities. This is an important observation that requires further careful consideration since changes in diagnostic cut-off for systemic hypertension towards lower BP values in individuals without additional risk factors has a very high impact not only on the clinical practice and economy but also on each individual well-being. [601]

Beside its direct effect on the vascular functionality, hypertension is also known to cause an acceleration of the ageing process of the vascular function.[599] We have previously demonstrated that, in ageing, normal individuals there is a decline in retinal vasoregulative capacity in both dilatory and constrictory phases that was independent of any systemic influences [588] and we have linked this abnormal response to the possibility of an age-related increase in oxidative stress.[515] Nevertheless, the subjects included in the current paper were much younger that those included in our ageing studies. It is possible that, the abnormal microvascular function observed in individuals classed as ESC/ESH grade 1 hypertension was indeed due to a reduced nitric oxide (NO) availability through excess inhibition by reactive oxygen species (ROS) [621]; however, in this case, the main culprit for ROS excess was not ageing but, possibly, an increased BP. Indeed, it has been shown that, in hypertensive

patients, NO availability is reduced early in the course of the disease .[599] Although we did not measure either the level of NO or antioxidative markers in our current cohort of patients, in the light of our previous discovery showing that retinal microvascular dysfunction occurs in parallel with changes observed in the level of oxidative stress both in normal [515] and individuals with abnormal BP levels [616], it is logic to presume that similar abnormalities occurred in our current cohort of individuals with ESC/ESH grade 1 hypertension, thus contributing to the abnormal retinal microvascular function measured in these individuals.

Our ESC/ESH grade 1 hypertension also exhibited an abnormal retinal venous response to flicker. In previous studies, we have already documented that individuals with other categories of cardiovascular risk also exhibit abnormal retinal venous responses to flicker-induced provocation.[513,514,622,623] Structural retinal venular dilation has previously been implicated as a strong prediction of adverse CVD events.[624,625] Nevertheless, the abnormal functional retinal venous responses, as measured by DVA, are still to be confirmed as signs of endothelial dysfunction in their own right or as epiphenomena of retinal arterial dysfunction.[514,588]
In this particular context, our observed retinal venular dysfunctionality could reflect a compensatory adaptation following sustained arterial dilation during flicker. Further investigation is required to understand the relevance of this kind of response; however, it could be hypothesized that a change in venous calibre associated with either structural or endothelial irregularities could also be used as a marker for cardiovascular risk in individuals with early hypertension. [513,514,588]

In addition to a general retinal microvascular dysfunction, our cohort of individuals with ESC/ESH grade 1 hypertension also exhibited abnormal peripheral microvascular reactivity as assessed by DTM, showing that abnormal microvascular function is a general process in these individuals and can be detected at multiple levels despite differences in vascular physiology and methodologies.

5.6. Conclusion

This study found microvascular dysfunctions to be present at multiple levels only in individuals with ESC/ESH grade 1 hypertension and not in those classed as stage 1 hypertension according to the latest ACC/AHA guidelines. The implications of these observations could be important, especially when clinicians decide their intervention based only on controversial, borderline BP values measured in individuals without other CVD risk.

6. Dry Eye Disease is Associated with Retinal Microvascular Dysfunction and Possible Risk for Cardiovascular Disease

This study was published in Acta Ophthalmologica, Wiley (Appendix 13.4.3).[626]

6.1. Abstract

Purpose: To explore the presence of microvascular endothelial dysfunction as a measure for early cardiovascular disease in individuals diagnosed with Dry Eye Disease (DED) as compared to age-matched normal controls.

Methods: Blood pressure profiles, Body Mass Index, intraocular pressure, blood analyses for glucose and lipid metabolism markers (TG, CHOL, HDL-c, LDL-c), as well as retinal and peripheral microvascular function were assessed in twenty-five 35-50 years old with DED as diagnosed by the TFOS DEWS II criteria and twenty-five age and sex-matched controls.

Results: After controlling all the influential covariates, individuals diagnosed with DED exhibited significant lower retinal artery baseline (p=0.027), artery maximum diameter (p=0.027), minimum constriction (p=0.039) and dilation amplitude (p=0.029) than individuals in the non-dry eye group. In addition, the time to reach the vein maximum diameter was significantly longer in the DED patients than in normal controls (p=0.0052). Only in individuals diagnosed with DED, artery maximum constriction correlated statistically significantly and positively with HDL-C blood levels (p= 0.006). Similarly, artery $slope_{AD}$ correlated positively with T-CHOL and LDL-C (p= 0.006 & 0.011 respectively). Additionally, artery baseline diameter and maximum constriction were significantly and negatively correlated to T-CHOL/HDL-C ratio (p=0.032 and p=0.013 respectively) in DED individuals only.

Conclusions: Individuals with a positive diagnosis of Dry Eye Disease exhibit a higher risk for cardiovascular disease than age- and sex-matched normal individuals. Closer monitoring and prompt personalised intervention will possibly contribute to reduced cardiovascular disease morbidity in these individuals.

6.2. Introduction

Dry eye disease (DED) represents a multifactorial, chronic and debilitating pathology of the ocular surface characterized by loss of homeostasis of the tear film and accompanied by ocular symptoms. Tear film instability and hyperosmolarity, ocular surface inflammation and damage, as well as neurosensory abnormalities, play etiological roles in this disease.[325]

In addition to other risk factors, DED has previously been associated with dyslipidaemia, a group of metabolic abnormalities characterized by any or a combination of the following: raised low-density lipoprotein cholesterol (LDL-C), raised total cholesterol (TC), raised triglycerides (TG) and low high-density lipoprotein cholesterol (HDL-C).[627] Indeed, as lipid homeostasis is important for the stability of the tear film, the association between dyslipidaemia and DED is entirely justified. Moreover, disruptions of cholesterol biosynthesis are also associated with sebaceous/ Meibomian gland (MG) dysfunctions [628], another cause of tear film instability and dry eye. Dyslipidaemia also represents a significant risk factor for cardiovascular disease (CVD), especially due to its contribution to the pathogenesis of atherosclerosis in medium-sized and large arteries but also at the microvascular level.[629,630] This affects not only the anatomy of these vessels but, most importantly, their function. Indeed, at the functional level, it impairs endothelium-dependent vasodilation because of defects in nitric oxide (NO) bioavailability.[630] This has catastrophic effects on the balance between the physiological vascular dilatory and constrictory states, which, in turn, will also affect other important circulatory functions and, most importantly, vascular protection against oxidation, inflammation and thrombosis.[631] This imbalance characterises the so-called endothelial dysfunction (ED), an initial reversible step in the development of atherogenesis; nevertheless, it is also one of the most important stages in the development of CVD. Therefore, its identification, as early as possible, represents a key factor in CVD prevention.[631]

Besides having common risk factors, other relationships between DED and CVD are not clearly understood. However, as both CVD and DED disease are common and important health problems encountered frequently in the general population, a closer look at their other possible links is warranted. The present study explores the presence of microvascular ED (as a measure for early CVD) in individuals diagnosed with DED as compared to age-matched normal controls.

6.3. Subjects and Methods

6.3.1. Study Participants

Healthy individuals aged between 35 and 50-year-old were recruited for this case-control study through advertisements at the Vascular Research Laboratory and the Dry Eye Clinic, Aston University (Birmingham, UK). Ethical approval was sought from the relevant local ethics committees, and written informed consent was received from all participants prior to study

enrolment. The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki, and all study-related procedures adhered to institutional guidelines. The inclusion/exclusion criteria as defined in section 3.2.1.

6.3.2. General Assessment

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI = weight/height). General clinical assessments were conducted for all the participants as detailed in sections 3.3.2, 3.3.3.a 3.3.4 included general health history questionnaires, BP and IOP profiles.

6.3.3. Biomarkers Assays

Bloods samples were drawn on the morning of the appointment. Fasting TG, T-CHOL and HDL-C were analysed according to the detailed protocol in section 3.3.9. LDL-C values were calculated as per the Friedewald equation.

6.3.4. DED Diagnosis

Following the protocols stated in section 3.3.7. All subjects underwent dry eye assessment using a digital slit lamp and the Keratograph K5m (Oculus, Wetzlar, Germany), including objective non-invasive breakup time, taking the average of three readings and ocular surface staining with fluorescein (i-DEW Flo, Mainline, Derby, UK) and lissamine green (GreenGlo, HUB Pharmaceuticals, Plymouth, MI, USA) using the TFOS DEWS II recommended methodology. [338] Osmolarity was assessed in each eye using the Tearlab (Dallas-Fort Worth, TX, USA).

Diagnosis of dry eye was based on the latest TFOS DEWS II criteria [338], which involves positive symptoms screening with the Ocular Surface Disease Index (cut-off >+13) and one or more of non-invasive tear breakup time (<10s), hyperosmolarity (>+308mOsm/L in the higher eye or an intereye difference .8mIsm/L) and ocular surface staining (>5 corneal, >9 conjunctival punctate spots or lid margin (>=2mm length and >+25% width).

6.3.5. Dynamic Retinal Vessel Analysis

Vascular assessments of interest to this study, detailed in the methods section 3.3.5. For each participant, one unselected eye was evaluated by DVA. All raw data were re-analysed using the mathematical approach for the analysis of retinal response parameters described in section 3.3.5.12. 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 3.3.5.11).

6.3.6. Digital Thermal Monitoring (DTM)

The peripheral microvascular reactivity at the level of the fingertips was assessed using VENDYS 5000 BCE DTM system (Endothelix, Inc, Houston, TX, USA) according to an established protocol as mentioned in section 3.3.6.[617]

6.3.7. Sample size

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessel reactivity was shown to be significant. As the study design was multi-factorial in nature it was calculated that n=25 in each group was sufficient to provide 90% power with an alpha of 0.05.

6.3.8. Statical Analysis

All data were analysed according to methods explained in 3.3.12.

6.4. Results

A total number of 66 participants were initially screened for study inclusion, of which 16 individuals were excluded based on the quality of retinal vascular image analysis. The remaining 50 participants were included in the final analysis and classified into 2 study groups based on a positive diagnosis for DED: 25 individuals (12 men and 13 women) and negative diagnosis: 25 individuals (15 men and 10 women).

General characteristics of the study population are presented in Table 6.1. There were no significant differences in age, sex, BMI, HR, IOP, glucose, TG, and LDL-C, between the study groups (all $p > 0.05$). Although still within the normal range, the individuals with DED had a statistically significant higher T-CHOL (5.0 vs 4.44 mmol/L) and lower HDL-C (1.21 vs 1.58 mmol/L) values than those without DED ($p = 0.0356$ and 0.0137 respectively), albeit close to the upper limit for these parameters (Table 6.1).

There were no significant differences between the study groups with regard to the peripheral microvascular function parameters as measured using the DTM method (all $p>0.05$, Table 6.1). In addition, after controlling all the influential covariates identified using multivariate analysis, there were no significant differences between the study groups with regards to the retinal microvascular parameters BDF, BCFR, MD%, MC%, tMD and tMC, $Slope_{AD}$ and $Slope_{AC}$ (all $p > 0.05$, Table 6.2). However, individuals diagnosed with DED exhibited significant lower artery baseline diameter, artery MD, MC and DA than individuals in the non-dry eye group ($p=0.0269$, $p=0.0273$, $p=0.0386$ and $p=0.0291$, respectively, Figure 6.1). In addition, vein tMD was significantly longer in the DED patients than in normal controls ($p=0.0052$) (Table 6.3).

Only in individuals diagnosed with DED, artery MC correlated statistically significantly and positively with HDL-C blood levels ($p= 0.006$). Similarly, artery $slope_{AD}$ correlated positively

with T-CHOL and LDL-C (p= 0.006 & 0.011 respectively). Additionally, artery baseline diameter and MC were significantly and negatively correlated to T-CHOL/HDL-C ratio (p=0.032 and p=0.013 respectively) in DED individuals only (Figure 6.2).

Table 6.1: General Characteristics of the Study Population

	Dry Eye diagnosis	Non-Dry Eye	P-value
Number	25	25	-
Sex	12 Male:13 Female	15 Male:10 Female	-
Age (years)	44.1(2.5)	37.6(2.5)	0.073
BMI (Kg/m^2) [b]	26.03(1.03)	26.71(1.03)	0.645
SBP(mmHg)	116.90(4.96)	120.5(4.96)	0.611
DBP (mmHg)	68.94(3.43)	71.3(3.43)	0.629
HR (bpm)	62.14(2.10)	66.00(2.98)	0.366
IOP	13.48(0.42)	13.31(0.44)	0.776
GLUC (mmol/L)	4.81(0.15)	4.54(0.16)	0.228
TG (mmol/L)	1.01(0.07)	0.94(0.07)	0.502
T-CHOL (mmol/L)	5.0(0.172)	4.44(0.19)	0.036*
HDL-C (mmol/L)	1.21(0.109)	1.58(0.09)	0.014*
LDL-C (mmol/L)	3.15(0.23)	2.81(0.23)	0.301
T-CHOL/HDL-C	4.36(0.377)	3.086(2.34)	0.020*
aTR	1.52(0.14)	1.76(0.14)	0.237

Table 6.1: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate (in beats per minute); IOP, intraocular pressure; GLUC, glucose; TG, triglycerides; T-CHOL, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; aTR, adjusted temperature rebound.
*Significant p-values are indicated where $p < 0.05$ was considered significant.
[a] Data are presented as mean (SD) unless otherwise indicated.
[b] Calculated as weight in kilograms divided by height in metres squared.

Table 6.2: Summary of Retinal Arterial Vascular Functional Parameters

	Mean (SD)		
Parameter	**Dry Eye**	**None-Dry Eye**	**P-value**
Artery baseline	110.59(2.52)	112.72(2.51)	0.027*
Artery-BDF	5.47(0.47)	5.434(0.47)	0.954
Artery-DA[a]	8.29(0.77)	10.73(0.77)	0.029*
Artery-BCFR[b]	3.84(0.51)	4.33(0.51)	0.502
Artery-MD	118.75(2.64)	123.28(2.85)	0.027*
Artery-tMD	16.73(0.92)	15.67(0.92)	0.414
Artery-MD%	4.95(0.54)	5.56(0.53)	0.434
Artery-MC	109.50(2.38)	111.67(2.38)	0.039*
Artery-tMC	23.95(1.59)	25.71(1.58)	0.436
Artery-MC%	-3.05(0.38)	-3.63(0.38)	0.287
Artery-$Slope_{AD}$ [c]	0.41(0.04)	0.39(0.043)	0.764
Artery-$Slope_{AC}$[d]	-0.52(0.08)	-0.41(0.085)	0.361

Table 6.2. Baseline, baseline diameter; BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline-corrected flicker response; MD, artery maximum dilation; tMD, reaction time to maximum dilation diameter; MD%, percentage change in diameter from baseline to maximum dilation; MC, Maximum constriction; tMC, reaction time to maximum constriction diameter from maximum dilation diameter; MC%, percentage constriction below baseline; $Slope_{AD}$, slope of arterial dilation; $Slope_{AC}$, slope of arterial constriction.
Unless otherwise indicated, all values are expressed in arbitrary units, which approximately correspond to micrometres (μ m) in a normal Gullstrand eye.
*Significant p-values are indicated where $p < 0.05$ was considered significant.
[a] Calculated as MD – MC.
[b] Calculated as DA – BDF [523]
[c] Calculated as (MD – baseline)/tMD [541]
[d] Calculated as (MC – MD)/tMC [541]

Table 6.3. Summary of Retinal Venous Vascular Function Parameters

	Mean (SD)		
Parameter	**Dry Eye**	**None- Dry Eye**	**P-Value**
Vein-Baseline	133.62(3.92)	139.94(3.92)	0.260
Vein-BDF	5.37(0.43)	5.23(0.43)	0.822
Vein-DA[a]	9.11(0.61)	8.96(0.62)	0.867
Vein-BCFR[b]	3.73(0.43)	3.72(0.43)	0.988
Vein-MD	140.04(4.58)	149.92(4.58)	0.134
Vein-tMD	19.46(0.70)	16.40(0.70)	0.005*
Vein-MD%	4.94(0.38)	4.50(0.38)	0.415
Vein MC	130.94(4.35)	140.96(4.35)	0.110
Vein- tMC	33.49(1.61)	33.29(1.61)	0.930
Vein-MC%	-2.0(0.19)	-1.76(0.19)	0.371
Vein -$Slope_{AD}$ [c]	0.49(0.07)	0.48(0.07)	0.908
Vein -$Slope_{VC}$ [d]	-0.52(0.08)	-0.41(0.086)	0.361

Table 6.3. Baseline, baseline diameter; BDF,baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline-corrected flicker response; MD, vein maximum dilation; tMD, reaction time to maximum dilation diameter; MD%,percentage change in diameter from baseline to maximum dilation; MC, Maximum constriction; tMC, reaction time to maximum constriction diameter from maximum dilation diameter; MC%, percentage constriction below baseline; $Slope_{VD}$, slope of venous dilation; $Slope_{VC}$, slope of venous constriction.
Unless otherwise indicated, all values are expressed in arbitrary units, which approximately correspond to micrometres (μ m) in a normal Gullstrand eye.
*Significant p-values are indicated where $p < 0.05$ was considered significant.
[a] Calculated as MD – MC.
[b]Calculated as DA – BDF [523]
[c]Calculated as (MD – baseline)/tMD [541]
[d]Calculated as (MC – MD)/tMC [541]

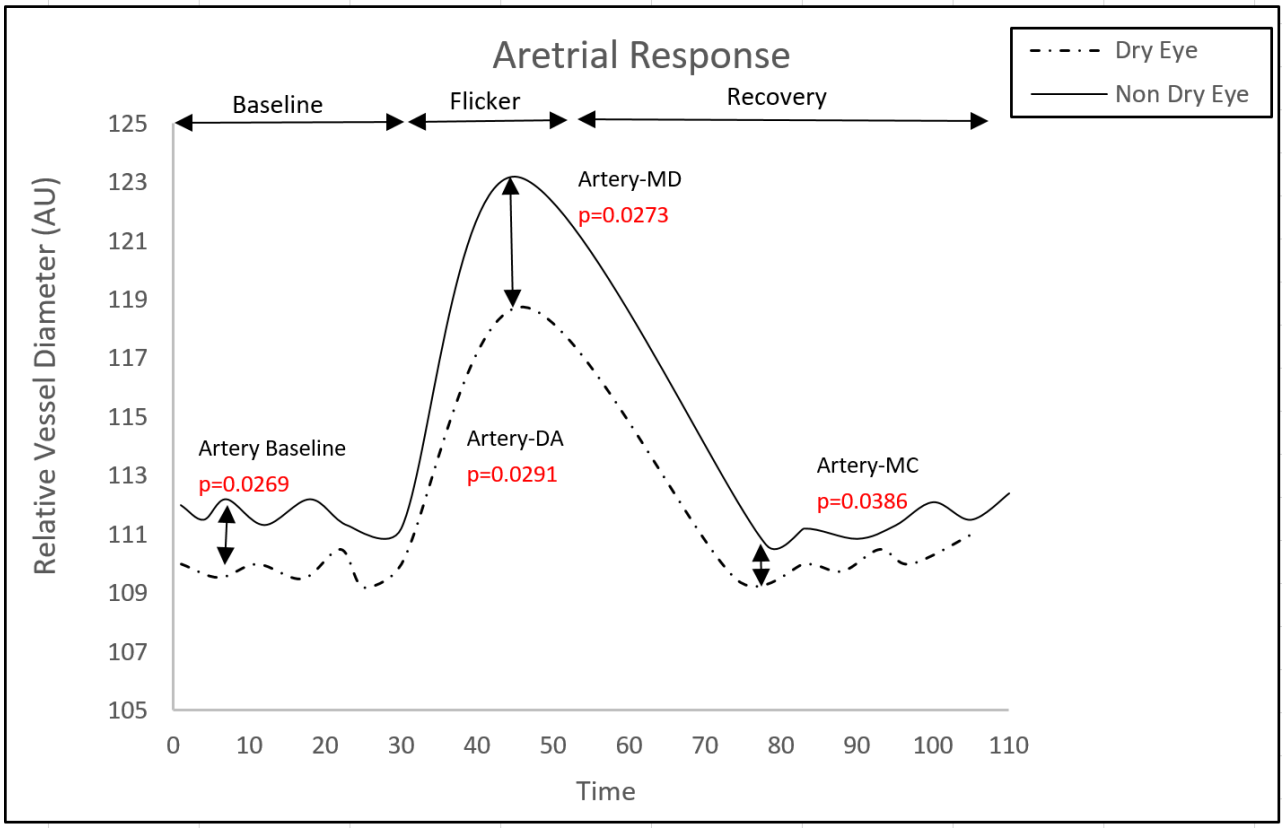

Figure 6.1: Comparison of Retinal Arterial Response Profile across Groups.
Abbreviations: AU, arbitrary units; BDF, baseline diameter fluctuation calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings; MD%, calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker; tMD, time to reach maximum diameter during flicker; tMC, time to reach maximum constriction post flicker; $slope_{AC}$, calculated as (MC-MD)/ (tMC).

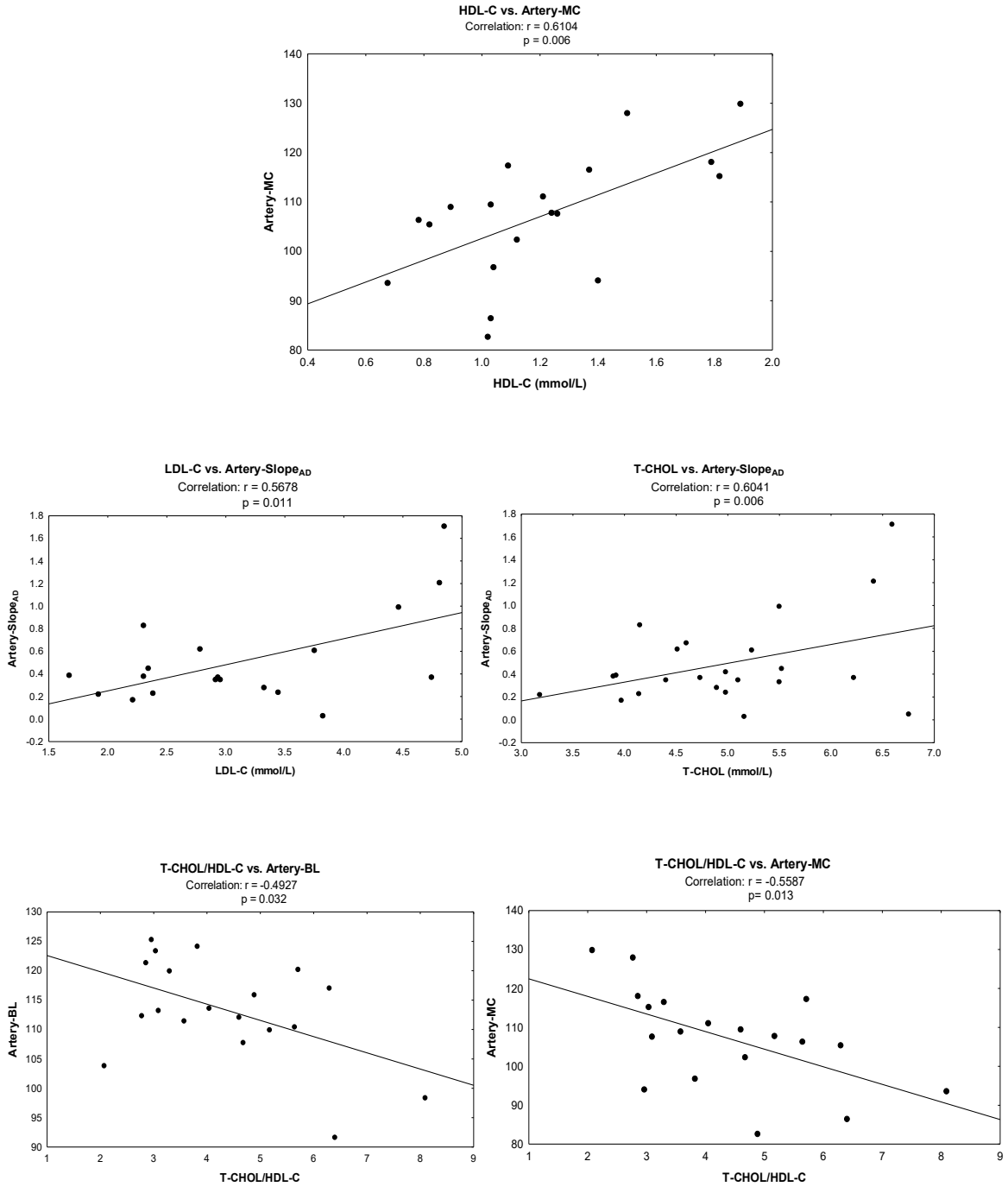

Figure 6.2: Correlation between Retinal Arterial Responses and Systemic Blood Lipids
Abbreviations: Artery- MC, Arterial maximum constriction; Artery- Slope$_{AD}$, slope of arterial dilation; Artery-BL, artery baseline diameter; Artery- MC; Arterial maximum constriction; HDL-C, high density lipoprotein cholesterol; T-CHOL, total cholesterol; LDL-C, low density lipoprotein cholesterol; T-CHOL/HDL, total cholesterol/ high density lipoprotein ratio.
Note: normalized unit values used on y-axes.

6.5. Discussion

This study examined the link between DED and CVD risk, as assessed using known circulatory markers as well as measurements of microvascular function at the retinal and peripheral level. Through this approach, it identified that individuals diagnosed with DED exhibit abnormal retinal, but not peripheral microvascular function and these abnormalities correlate with plasma levels of circulating cholesterols. To our knowledge, this is the first study to reveal that middle-aged individuals with DED have increased retinal microvascular dysfunction compared to normal, sex and age-matched controls.

Similar to other research [632–634], this study also demonstrated that individuals with DED exhibit higher, albeit at the upper normal levels of circulating T-CHOL when compared to those without DED. It has been previously proposed that the relationship between hypercholesterolemia and DED can be explained as increased levels of cholesterol in the meibomian lipid would increase its melting point, thus leading to increased viscosity and plugging of the meibomian orifice.[635] It is also important to note that individuals with DED included in our study also exhibited statistically significant lower, albeit still normal levels of HDL-C and higher T-CHOL/HDL-C ratio than the normal controls group. It is well known that HDL-C transports cholesterol from the tissues to the liver to be disposed, making it beneficial in the prevention of CVD.

Moreover, decreases in HDL-C have also been linked with endothelial dysfunction and a reduction in the bioavailability of NO.[636] In addition, T-CHOL/HDL-C ratio has previously been specified as a better indicator of premature CVD risk in than T-CHOL levels [637], making these observations very important.

The present study revealed a novel finding that, independent of other characteristic, individuals with DED exhibit abnormal retinal arterial and venous microvascular dysfunction. To date, little research looked at microvessels in individuals with DED and only at the level of conjunctival vessels. It is interesting to mention that patients with DED have been previously shown to exhibit abnormal microvascular response and reduced blood flow at the conjunctival vessels level after trigeminal stimulation, suggesting that these patients suffer of an imbalance in the autonomic nervous system (ANS).[638] Indeed, conjunctival vessels have a dual autonomic innervation.[639]

Moreover, DED has been proposed to be associated with various ANS dysfunctions as autonomic nerves are abundant in meibomian gland tissue and play an important role in regulating the secretory activities of meibomian gland in animals.[640,641] Endothelial dysfunction and ANS imbalance often co-exist in the development of various cardiovascular disease processes.[642] At the retinal microvascular level, in the absence of autonomic innervation,

metabolic and myogenic stimuli are more involved in retinal autoregulation of the microvascular calibre.[64] Although the function of retinal microvessels are not under the influence of ANS; this study suggests that both the ANS and endothelial dysregulation co-exist in individuals with DED, and the results of this imbalance are evident and can be measurable at different vascular levels. In addition, abnormalities in the retinal venous functionality have also been found in the DED group. Since retinal veins typically incite a more passive regulatory contribution to increases in blood flow whether this observation may reflect some kind of reconciliation of alterations in arterial outflow to the venous side via downstream autoregulatory mechanisms [538] is unclear at present.

The positive correlation between the retinal microvascular function parameters and the levels of circulating HDL-C in DED individuals reinforces the fact that the HDL-C has a vital role in the prevention of CVD. Indeed, retinal vascular calibres have been shown to be independently associated with risk factor variables such as age, blood pressure, HDL-C, and LDL-C. Our DED group, however, also exhibited a negative relationship between T- CHOL/HDL-C ratio and retinal artery MC. As this circulatory parameter is a strong indicator of risk for CVD [643,644], this observation is very important and, in addition to the above mentioned microcirculatory abnormalities, points to the fact that DED individuals could exhibit higher risk for CVD than age- and sex-matched normal individuals. Further follow-up studies to confirm the actual development of CVD in these individuals are warranted.

6.6. Conclusion

Significant attenuations in retinal vascular function exist and can be detected in persons diagnosed with DED. Moreover, these abnormalities correlate with known circulatory markers for CVD. Functional retinal assessments could therefore be useful for early vascular screening, possibly contributing to a reduced risk for CVD morbidity in these individuals.

7. Novel Composite Early Risk Markers for Vascular Ageing and Risk for Cardiovascular Disease

Abstract of this study has been presented in the European Network for Oxysterol Research Conference and the study was published in Antioxidants (Appendix 13.4.4).[645]

7.1. Abstract:

Abnormal redox balance is one of the main determinants of ageing, abnormal vascular function and risk for cardiovascular diseases. The aim of the present paper is to assess the relationship between various oxidative stress markers and microvascular function in individuals of various age groups, free of overt disease.

Forty-two apparently healthy individuals were included in the study (group 1: 19–30 years, group 2: 31–50 years, and group 3: 51–70 years). Parameters of microvascular function (dilation and constriction) have been assessed at the retinal microvascular level using the dynamic retinal vessel analyzer (DVA). Fasting plasma has been obtained from all subjects, and quantification of monohydroxy and dihydroxy oxysterols was performed by LC-MS/MS following reverse phase chromatography. Griess assay was used to evaluate the Nitric Oxide concentration. Glutathione redox ratio was also analyzed by means of a whole blood glutathione recycling assay. In all participants, the levels of 7-Ketocholesterol, 25-hydroxycholesterol and 7β-hydroxycholesterol correlated significantly and positively with the time to maximum arteriolar dilation. In addition, 25- hydroxycholesterol and 7β-hydroxycholesterol negatively correlated to the percentage of maximum arteriolar dilation. A negative correlation was observed for 27- hydroxycholesterol and 7β- hydroxycholesterol with microvascular arteriolar constriction. In addition, the glutathione redox ratio decreased significantly with age and correlated significantly and negatively with 7β- hydroxycholesterol and 25- hydroxycholesterol. These results suggest that oxysterol profile correlates with changes in microvascular beds function with age, and it's possible to be used as a biomarker of vascular ageing and CVD risk.

7.2. Introduction:

While we all age at the same pace chronologically, it was observed that, physiologically, individuals do not age at a similar rate.[168] This observation has led to the concept of biological ageing, which is a measure of premature progressive decline in organs' function.[646] At the vascular level, as blood vessels constantly withstand against mechanical forces, any changes to the blood components, including cells, macromolecules, ions or external pathogenic insults, could affect vessels' integrity and their performance.[647] As a result, modifiable cardiovascular risk factors such as obesity, smoking, lifestyle, as well as non-modifiable risk factors such as genetics, ethnicity and age, could affect vascular function.[648]

In the past few years, functional evaluation of the microvessels gained a lot of clinical interest since endothelial dysfunction, the main culprit for cardiovascular diseases (CVDs), was found to occur earlier at the microvessel level than the macrovessel level.[586] Accordingly, many methods were developed to evaluate functional microvascular responses to biological stimuli in different vascular beds.[649] One of these methods, the dynamic retinal vessel analysis (DVA), was highlighted as one of the most sensitive and reliable methods to measure early vascular changes that point out early endothelial dysfunction and future risk of cardiovascular pathologies in individuals with and without overt clinical symptoms.[515,548,606] It has also been shown that retinal microvascular function is modified by age.[531] However, although this is a consistently demonstrable effect, little is known about what factors in particular directly determine this outcome? We have previously demonstrated that high levels of circulating reactive oxygen species (ROS) have a direct effect on the retinal microvascular function.[514,589] Indeed, evidences suggest that oxidative stress induces cellular senescence is a key influencer of age-induced mechanical and structural vascular dysfunction [650–653] as well as of the development and progression of cardiovascular diseases (CVD). [621,654–657] Additionally, high levels of ROS also result in low levels of Nitric Oxide (NO), a well-known known critical molecule in the regulation of the vascular function and tone, therefore further contributing to the above-mentioned abnormalities.[657]

High levels of cholesterol are also an important factor affecting microvascular function at all levels, including the retinal microvascular function.[539,589,626] Cholesterol can be oxidized by non-enzymatic and enzymatic pathways.[658,659] The non-enzymatic pathway can be free-radical mediated and results in the formation of 7-hydroxycholesterol, which is further oxidized to oxysterols (7-Ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol).[309] The enzymatic oxidation by cholesterol-hydroxylase enzymes produces 4β-hydroxycholesterol, 7α-hydroxycholesterol, 24-hydroxycholesterol and 27 hydroxycholesterol) and high levels of oxysterols in plasma were highlighted as significant contributors to macrovessels atherogenesis and endothelial dysfunction.[660–663] Moreover, oxysterols have also been implicated in the pathogenesis of vascular ageing because of their marked pro-oxidant,

proinflammatory and proapoptotic properties.[664] There is an active cholesterol metabolism at the retinal level, where LDL uptake is the main source of lipids in the retina [665,666], and preclinical and clinical studies have suggested a connection between oxysterols and retinal degeneration.[667] Similarly, it can be proposed that their various effects can also be observed at the retinal microvascular level. Nevertheless, this effect has never been previously determined. Therefore, this study aims to investigate the correlation between oxysterols, glutathione redox ratio and retinal microvascular functional reactivity in apparently healthy individuals of various ages.

7.3. Materials and Methods

Healthy individuals aged between 35 and 50-year-old were recruited for this case-control study through advertisements at the Vascular Research Laboratory, Aston University (Birmingham, UK). Ethical approval was sought from the relevant local ethics committees, and written informed consent was received from all participants prior to study enrolment. The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki, and all study-related procedures adhered to institutional guidelines.

The inclusion/exclusion criteria as defined in section 3.2.1.

7.3.1. Sample Size

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessels reactivity was shown to be significant.[540,597] As the study design was multifactorial in nature, it was calculated that a total sample size of n = 42 was sufficient to provide 95% power at an alpha level of 0.05.

7.3.2. General Investigations

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI = weight/height). General clinical assessments were conducted for all participants as detailed in sections 3.3.2, 3.3.3.a 3.3.4 included general health history questionnaires, BP and IOP profiles.

7.3.3. Dynamic Retinal Vessel Analysis

Vascular assessments of interest to this study, detailed in the methods section 3.3.5. For each participant, one unselected eye was evaluated by DVA. All raw data were re-analysed using the mathematical approach for the analysis of retinal response parameters described in section 3.3.5.12. 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 3.3.5.11).

7.3.4. Biomarkers Assays:

Bloods samples were drawn on the morning of the appointment. Fasting TG, T-CHOL and HDL-C were analysed according to the detailed protocol in section 3.3.9. LDL-C values were calculated as per the Friedewald equation.

7.3.5. Measurement of GSH and Oxidized Glutathione (GSSG)

Initial processing of blood GSH and GSSG levels were assessed by the GSH recycling assay as detailed previously in section 3.3.10.1.

7.3.6. Measurement of Nitric Oxide (NO)

Nitric oxide levels were measured according to the protocol explained in section 3.3.10.2.

7.3.7. Extraction and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) of Plasma Oxysterols

Plasma oxidized lipids were extracted and measured according to the protocol previously detailed in section in section 3.3.10.4

7.3.8. Statistical Analysis

All data were analysed according to methods explained in 3.3.12.

7.4. Results:

7.4.1. Clinical characteristics

A total number of 56 participants were initially screened for study inclusion, of which 14 individuals were excluded based on the quality of retinal vascular image analysis. The remaining 42 participants were included in the final analysis and classified into 3 age groups. Group 1: 19–30 years (10 male: 6 female); Group 2: 31–50 years (8 male: 8 female); and Group 3: 51–70 years (4 male: 6 female). The general characteristics of the study population are presented in Table 7.1. There were no significant differences in sex, BMI, HR, IOP, glucose, TG, and HDL-C, between the study groups (all $p > 0.05$). Although still within the normal range, older participants had higher T-CHOL and LDL-C values than younger ones, albeit close to the upper limit for these parameters ($p = 0.0071$ and 0.0112 respectively) (Table 7.1).

7.4.2. Retinal microvascular function

After controlling all the influential covariates identified using multivariate analysis, there were no significant differences between the study groups with regard to arterial baseline, BCFR, MD, MC, tMC, $Slope_{AD}$ and all venous microvascular parameters (all $p > 0.05$, Table 7.2&7.3).

However, the post hoc analysis revealed a significantly decreased BDF, MD%, MC%, DA and $Slope_{AC}$ with age (p=0.0140, p=0.0360, p=0.0040, p=0.0230 and p=0.0055 respectively, Table 7.2, Figure 7.2). Moreover, artery tMD increased significantly with age (p=0.0254, Table 7.2). No statistically significant differences were found between retinal venous vascular function parameters among the study groups (Table 7.3).

Table 7.1: Summary of the Systemic Characteristics of the Study Participants

Variable	19-30	31-50	51-70	p-value	Post –hoc
Number	16 10M:6F	16 8M:8F	10 4M:6F	-	-
Age (years)	24.54 (1.74)	37.81 (1.57)	60.75 (2.22)	0.0001*	1<2<3
SBP (mmHg)	115.27(3.80)	114.7 (3.95)	118.5 (5.10)	0.82946	-
DBP (mmHg)	64.64 (2.54)	70.9 (2.67)	72.5 (3.44)	0.13002	-
HR (bpm)	63.17 (3.29)	68.1 (2.55)	66.18(2.43)	0.50522	-
IOP (mmHg)	12.93 (0.57)	13.23 (0.60)	14.83 (0.80)	0.14723	-
BMI (kg/m^2)	23.34 (1.13)	26.02 (1.19)	26.59 (1.53)	0.16039	-
Glucose (mmol/L)	4.41(0.21)	4.99 (0.28)	4.68 (0.28)	0.26740	-
TG (mmol/L)	0.83 (0.065)	1.0 (0.081)	0.96 (0.088)	0.22376	-
CHOL (mmol/L)	3.95 (0.17)	4.14 (0.21)	4.93 (0.24)	0.0071*	1<2<3
HDL-C (mmol/L)	1.33 (0.08)	1.44 (0.10)	1.25 (0.12)	0.50590	-
LDL-C (mmol/L)	2.28 (0.17)	2.60(0.21)	3.01 (0.24)	0.01116*	1<2<3

Table 7.1: Abbreviations: SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; IOP: intraocular pressure; BMI: body mass index; TG: triglycerides; CHOL: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein.
*Significant p-values are indicated where $p < 0.05$ was considered significant.

Table 7.2. Summary of Retinal Arterial Vascular Function Parameters

Variable	19-30	31-50	51-70	P-value Anova/Ancova	Post-Hoc
Baseline	117.44 (4.24)	116.60 (4.45)	113.41(5.74)	0.61902	-
BDF	7.06 (0.65)	5.92 (0.70)	3.49 (0.88)	0.0140*	1>2>3
BCFR	4.90 (0.72)	4.81 (0.80)	2.54 (0.90)	0.1058	-
MD	124.94 (4.13)	121.76 (4.60)	117.02 (5.20)	0.4986	-
tMD	14.67 (0.77)	16.15 (0.73)	19.29 (0.83)	0.0254*	1<2<3
MD %	5.64 (0.63)	4.50 (0.70)	3.24 (0.79)	0.0360*	1>2>3
MC	112.99 (4.32)	113.24 (4.78)	112.80 (5.42)	0.5985	-
tMC	25.39 (2.34)	28.93 (2.60)	27.523 (2.93)	0.5974	-
MC%	-3.85 (0.36)	-2.43 (0.4)	-1.70 (0.46)	0.0040*	1>2>3
DA	11.95 (1.27)	9.74 (1.40)	6.04 (1.60)	0.0230*	1>2>3
$Slope_{AD}$	0.52 (0.11)	0.51 (0.12)	0.26 (0.14)	0.3030	-
$Slope_{AC}$	-0.62 (0.062)	-0.38 (0.07)	-0.30 (0.08)	0.0055*	1>2, 2=3, 1>3

Table 7.2: Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach M.D.; M.D. (%), percent dilation; tMC, time to reach MC; MC (%), percent constriction; DA, dilation amplitude (difference between M.D. and MC during flicker) $Slope_{AD}$, slope of arterial dilation; $Slope_{AC}$, slope of arterial constriction. * Significant *p*-values are indicated where $p < 0.05$ was considered significant.

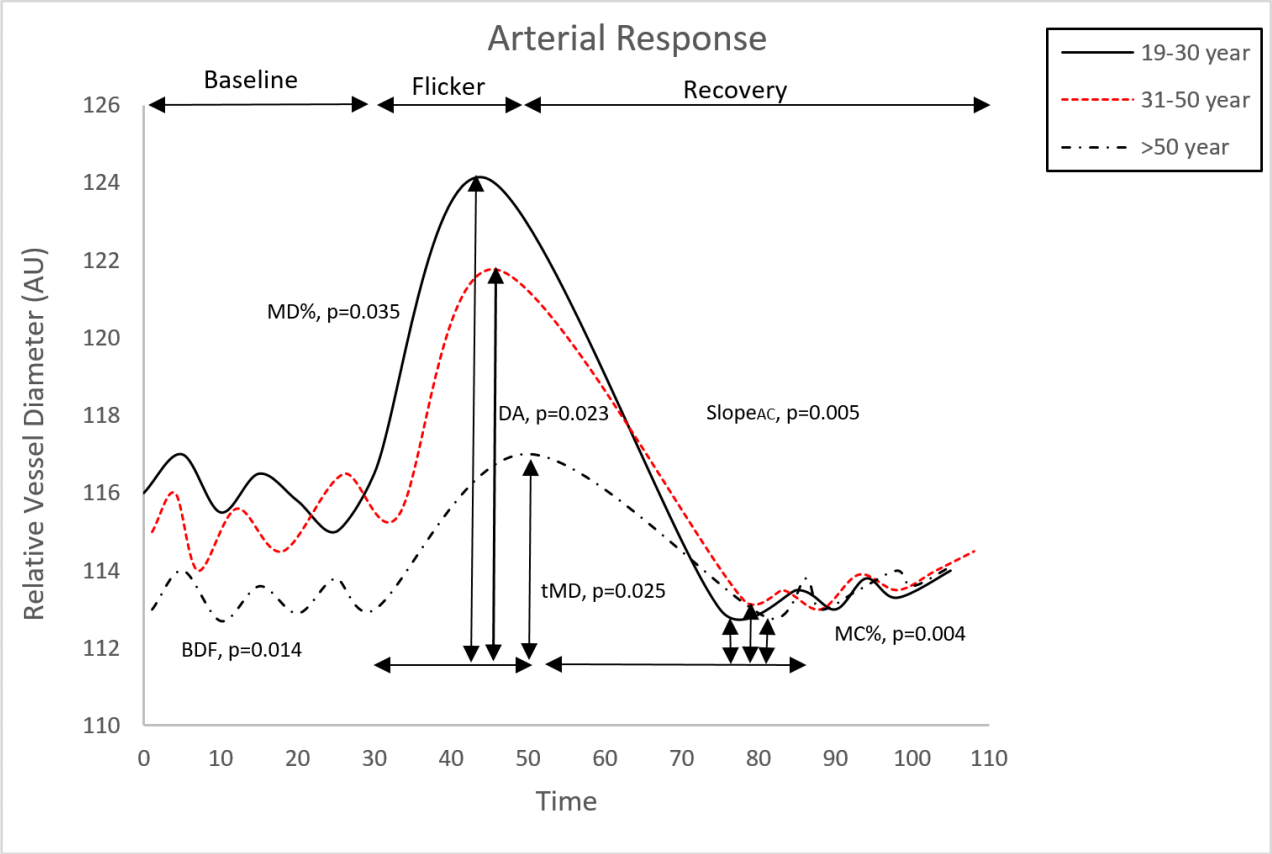

Figure 7.1: Comparison of Retinal Arterial Response Profile across Groups.
Abbreviations: AU, arbitrary units; BDF, baseline diameter fluctuation calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings; MD%, calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker; tMD, time to reach maximum diameter during flicker; tMC, time to reach maximum constriction post flicker; $slope_{AC}$, calculated as (MC-MD)/ (tMC).

Table 7.3. Summary of Retinal Venous Vascular Function Parameters

Variable	19-30	31-50	51-70	P-value Anova/Ancova	Post-Hoc
Baseline	142.47(4.25)	143.55 (4.70)	131.39 (5.33)	0.19389	-
BDF	5.45 (0.75)	5.02 (0.84)	5.61 (0.95)	0.88379	-
BCFR	3.00 (0.85)	4.87 (0.95)	3.14 (1.07)	0.30859	-
MD	149.09 (4.44)	150.26 (4.91)	138.19 (5.60)	0.22687	-
tMD	23.76 (1.47)	19.37 (1.63)	21.95 (1.85)	0.15744	-
MD %	4.66 (0.66)	4.75 (0.74)	5.15 (0.83)	0.89666	-
MC	140.64 (4.24)	140.37 (4.69)	129.44 (5.32)	0.22113	-
tMC	32.12 (2.16)	34.41 (2.40)	33.71 (2.71)	0.76678	-
MC%	-1.30 (0.45)	-2.20 (0.49)	-1.50 (0.56)	0.38622	-
DA	8.45 (1.36)	9.90 (1.50)	8.75 (1.70)	0.76728	-
$Slope_{AD}$	0.33 (0.046)	0.36 (0.051)	0.35 (0.06)	0.86531	-
$Slope_{AC}$	-0.60 (0.12)	-0.31 (0.13)	-0.39 (0.14)	0.22394	-

Table 7.3: Abbreviations ANOVA, analysis of variance; ANCOVA, analysis of covariance; Baseline, baseline diameter; BDF, baseline diameter fluctuation; BCFR, Baseline corrected flicker response; tMD, time to reach MD; MD (%), percent dilation; tMC, time to reach MC; MC (%), percent constriction; DA, dilation amplitude (difference between MD and MC during flicker) $Slope_{VD}$, slope of venous dilation; $Slope_{VC}$, slope of venous constriction. * Significant *p*-values are indicated where $p < 0.05$ was considered significant.

7.4.3. Oxidative Stress markers

After controlling all the influential covariates identified using multivariate analysis, post hoc analysis showed a significant increase in 7-Ketocholesterol (7-KC), 25 Hydroxycholesterol (25-OHC), 27 Hydroxycholesterol (27-OHC), 7β Hydroxycholesterol (7β-OHC) and 7,25 Hydroxycholesterol (7,27-OHC) with age (p=0.0051, p=0.0067, p=0.0192, p=0.0168, p= 0.0113 respectively).

The GSH/GSSG ratio decreased significantly with age among the study's populations (p=0.0279). No other significant differences were recorded (all p>0.05, table 4).

No statistically significant differences were found in the NO concentrations between the study groups (all p >0.05)

4. Correlations between vascular and systemic circulatory parameters:

Univariate analysis revealed that blood 7-KC, 25-OHC and 7β-OHC levels correlated significantly and positively with tMD in all the study participants (r=0.4318, p=0.025; r=0.0301, p=.742; r=0.4029, p=0.037 respectively).

Additionally, 25-OHC and 7β-OHC are negatively correlated to arterial MD% (r= -0.3879, p=0.046; r=-0.5324, p=0.004 respectively). Similarly, 27-OHC and 7β-OHC significantly correlated negatively with artery $Slope_{AC}$ (r= -0.2291, p=0.0250; r= -0.4576, p=0.016 respectively).

There were no correlations between the GSH redox ratio and retinal vascular parameters in any of the study groups (all p>0.05). However, GSH/GSSG levels correlated significantly and negatively with 7β-OHC and 25-OHC (r=-0.5129, p= 0.021; r= -0.4799, p= 0.032 respectively).

Table 7.4. Summary of Normalized Oxidative Stress and Inflammatory Systemic Circulatory markers

Variable	19-30	31-50	51-70	P-value Anova/Ancova	Post-Hoc
7-KC (nmol/mmol)	10.49 (1.71)	13.28 (1.17)	17.26 (1.17)	0.0051*	1<2<3
25-OHC (nmol/mmol)	16.42 (3.86)	20.86 (2.65)	30.56 (2.65)	0.0067*	1<2<3
27-OHC (nmol/mmol)	16.61 (3.05)	20.55 (2.09)	26.79 (2.09)	0.0192*	1<2<3
4β-OHC (nmol/mmol)	1.02 (0.35)	0.81 (0.24)	1.14 (0.24)	0.6054	-
7β-OHC (nmol/mmol)	6.65 (1.70)	9.86 (1.17)	12.74 (1.17)	0.0168*	1<2<3
7,27-OHC (nmol/mmol)	8.45 (2.30)	8.64 (1.57)	10.65 (1.57)	0.5986	-
7,25-OHC (nmol/mmol)	5.60 (2.60)	8.89 (1.78)	11.41 (1.80)	0.0113*	1<2<3
NO (uM)	46.45 (4.37)	43.02 (3.39)	39.73 (3.10)	0.4495	-
GSH/GSSG	27.25(7.95)	20.84 (7.95)	17.05 (8.60)	0.0279*	1<2<3

Table 7.4: Abbreviations: ANOVA, analysis of variance; ANCOVA, analysis of covariance; 7-KC, 7-Ketocholesterol; 25-OHC, 25-Hydroxycholesterol; 27-OHC, 27-Hydroxycholesterol; 4β-OHC, 4β-Hydroxycholesterol; 7β-OHC, 7β-Hydroxycholesterol; 7,27-OHC, 7,27-Hydroxycholesterol; 7,25-OHC, 7,25-Hydroxycholesterol; IL-6, interleukin-6; NO, nitric oxide; GSH, glutathione; GSSG, Glutathione disulphide; GSH/GSSG, redox ratio.

7.5. Discussion:

In the present study, we have examined the relationship between circulatory markers of oxidative stress and endothelial function with dynamic retinal microvascular changes in various age groups. Our results show, for the first time that, with age oxysterol levels change in parallel with microvascular function in individuals without overt CVD.

In accordance with the existing literature, our results demonstrated a systematic increase in plasma circulatory lipids [668] and oxidative stress markers [669,670] with age. In addition and similar to previous research, circulating oxysterols concentrations were also increased with age.[671–673] Another consistency with previously published work was the fact that our healthy and yet older individuals (above 50 years old) displayed abnormal dilatory and constrictor responses to flickering light retinal arteries stimulation in comparison to younger and middle-aged participants.[674,675]

Indeed, it is well known that healthy and young endothelium usually retains antithrombotic, vasodilatory, anti-inflammatory and antioxidant properties, which regulate the vessels response to vasodilatory stimulants.[676] One hallmark of endothelium dysfunction is the progressively impaired vasodilatory response to blood flow and vasodilating compounds.[76] It was believed that a reduced vasodilation, even at the retinal level is mainly due to a diminished bioavailability of NO. Nevertheless, in our study, the NO levels were not different between the three groups, and the impaired microvascular responses were only correlated to abnormal plasma oxysterols levels.

Although a surprise, it is possible that these circulatory biomarkers are, indeed, the initial elusive culprits for the consistently observed age-related retinal vascular dysfunction and not early abnormal NO levels.[531] This observation and hypothesis does not eliminate, however, the well-demonstrated involvement of NO early microvascular dysfunction. Indeed, oxysterols are involved in the impairment of endothelial-eNOS-dependent NO generation. Moreover, as well-known reactive oxygen species (ROS) inducers, oxysterols also contribute to a decline in NO availability [677]. Therefore, the fact that NO levels were not different between the 3 groups of individuals tested could simply mean that the oxysterol actions were the first to appear and be measured. More research is necessary to confirm our hypothesis.

Oxidative stress is known to be a major driving force in ageing and in the development of CVD. In addition, oxysterols may contribute to endothelial senescence and CVD by making the endothelial cells more susceptible to various risk factors for CVD.[664] Therefore, our observations are highly significant and emphasise the fact that the assessment of plasma oxysterols as well as the retinal microvascular dysfunction are among the earliest markers for CVD risk. As for the fact that to date the clinical tools used to assess risk for CVDs rely mainly structural vascular changes and, therefore, as a direct result, either overestimate or underestimate individualized risk.[608]

7.6. Conclusion

Our observations suggest that using DVA and plasma oxysterols levels instead of other time consuming and less sensitive methods could increase the sensitivity of CVDs risk prediction, especially in individuals free of overt CVD but with various risk factors. Additionally, as molecular and imaging biomarkers drive the shift towards personalized medicine, DVA and oxysterols levels can be used as a quick, minimally invasive method of profiling individualized vascular risk, therefore, to be used in prediction, prevention, and personalized intervention.

8. Prediction of Cardiovascular Risk in Asymptomatic Individuals: A Symbolic Regression-Based Analysis of Single and Composite Vascular-Omics

8.1. Abstract:

Aims: To assess the performance of using either leucocyte telomere length or retinal microvascular dysfunction or the combination of both for predicting age and systemic blood pressure, two of the most known risk factors for cardiovascular diseases.

Methods: The final analysis included one hundred twenty-three healthy participants with low global cardiovascular risk and similar dietary habits. Study participants were divided into three age groups (30 and below, between 31 and 50 and over 50). Parameters of microvascular function (dilation and constriction) have been assessed at the retinal microvascular level using the dynamic retinal vessel analyzer. Relative telomere length was measured using real-time polymerase chain reaction, and a symbolic regression-based analysis was used to predict the cardiovascular risk factors of age and blood pressure.

Results: The three groups were also similar regarding heart rate, body mass index, triglycerides, high-density lipoprotein, glutathione redox system (all p-value >0.05). Total cholesterol and low-density lipoprotein were significantly higher in middle age and elderly groups compared to the younger group (p= 0.0004, 0.006 and p= 0.0001, 0.001). Relative telomere length decreased significantly with age (p=0.010). There were significant group differences in arterial time to maximum dilation (p=0.005), maximum constriction (p=0.007) and maximum constriction percentage (p=0.010). Time to maximum dilation and constriction significantly decreased in the youngest and middle age groups compared with the oldest group (p = 0.012, 0.013 and p= 0.0024, 0.0004 respectively). Artery maximum constriction percentage increased in the oldest age group compared to the youngest and middle age group (p = 0.0085 and p =0.0148, respectively). In the two younger groups, the error between predicted versus actual values for age are smallest in the case of using artery measurements only, while the largest error occurred when using telomere only. In the older group, using telomere measurements only led to the lowest error. Systolic blood pressure was better predicted by telomere measurements only; however, the smallest error in diastolic blood pressure prediction occurred when both telomere and arterial measurements were used.

Conclusion: The assessment of retinal vascular function is a better predictor of chronological ageing than Relative telomere length. Nevertheless, composite biomarkers are better when predicting modifiable risk factors for cardiovascular diseases, such as systemic blood pressure.

8.2. Introduction:

At present, the identification of at-risk individuals for cardiovascular disease (CVD) is performed using risk calculators that consider population-based markers such as lipid profiles, smoking and hypertension.[678,679] Nevertheless, it has been shown the CVD predictive accuracy of such scores either over- or underestimates actual risk at the individual level.[608–610] Indeed, for complex, multifactorial disorders such as CVD, there is high individual variability in the resistance and susceptibility to pathological onset and progression.[163] Therefore, the hunt for reliable and easy to measure individual risk predictors for CVD is still on. Nevertheless, this is not an easy task. Most biomarkers, especially those used for early screening, should be easily available and fit a tight budget while being performant and reliable. Ageing is a significant risk factor for many pathologies, including CVDs.[680–682] It has been, however, admitted that individuals do not age at a similar pace.[168] This observation has led to the concept of biological ageing, which is a measure of bodily functional decline [168] and represents a parameter that can be used for the selection of the individuals in need for early CVD prevention.[683] Indeed, the assessment of biological ageing can be, possibly, the earliest available markers for CVD risk.

Both reduced telomeres' length and vascular dysfunction have been linked to ageing as well as early CVD risk detection. Indeed, reduced leukocyte telomere length (LTL), beside its age-predictive power, has also been associated with known CVD risk factors, such as positive family history, lower high-density lipoprotein cholesterol (HDL-C) levels [684], smoking and alcohol consumption.[685,686] but also with high oxidative stress levels [197] and increased endothelial cell turnover.[187] Moreover, recent studies also link telomeres length to the development of hypertension.[687] Nevertheless, the assessment of telomeres is a complicated process and is not readily available.

The assessment of vascular endothelial function is essential not only for investigations into the pathophysiology of CVD but also for better CVD risk stratification.[76,163,683] Parameters such as retinal microvascular function are easily measured, potentially in primary care settings and have been found to show a good association not only with various circulatory markers for

CVD[514,688], but also with other modifiable and non-modifiable risk factors for this disease such as obesity [616], family history [513] and age. [531,589] A highly desirable quality of this assessment is the fact that it provides instant integrated and dynamic data analysis that is specific to each individual rather to a population.[513–515,531,606,626] Therefore, its quantification in apparently healthy individuals could offer quick and reliable data on their systemic health and possible risk for CVD.

So far, both above-discussed parameters were demonstrated to be used, with certain degrees of success, as predictors for CVD risk.[5,8,689] Nevertheless, the question remains if the proportion of actual cases correctly predicted can be increased by using a combination of these two biomarkers? Indeed, combining multiple biomarkers to develop a composite biomarker with enhanced performance is an appealing perspective. Although composite biomarkers have been successfully validated in other multifactorial disorders, such as cancer [690,691], their utility in predicting CVD risk in asymptomatic individuals without obviously elevated conventional risk factors is unclear. Indeed, as previously discussed, already existing composite biomarkers such as the Framingham Risk Score (FRS) are based on population risk parameters and are not very successful in predicting early CVD.

This is largely because this score is an incremental to known risk factors.[692] The aim of the present study is to assess the performance of using either LTL or retinal microvascular dysfunction or the combination of both for predicting age and systemic BP, two of the most known risk factors for CVD.

8.3. Methods

8.3.1. Study Participants

Healthy individuals aged 18-year-old and above were recruited for this study through advertisements at the Vascular Research Laboratory, Aston University (Birmingham, UK). The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki, and all study-related procedures adhered to institutional guidelines.

The inclusion/exclusion criteria as defined in section 3.2.1.

8.3.2. General Investigations

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI = weight/height). General clinical assessments were conducted for all participants as detailed in sections 3.3.2, 3.3.3.a, 3.3.4 included general health history questionnaires, BP and IOP profiles.

8.3.3. Blood Analyses

Bloods samples were drawn on the morning of the appointment. Fasting TG, T-CHOL and HDL-C were analysed according to the detailed protocol in section 3.3.9. Low-density lipoprotein cholesterol (LDL-C) values were calculated as per the Friedewald equation.

8.3.4. Measurement of GSH and Oxidized Glutathione (GSSG)

Initial processing of blood GSH and GSSG levels were assessed by the GSH recycling assay as detailed previously in section 3.3.10.1.

8.3.5. Relative Telomere Length

RTL was measured using real-time polymerase chain reaction (RT-PCR) according to a previously detailed method in section 3.3.10.3.

8.3.6. Dynamic Retinal Microvascular Function Vessel Analysis

Vascular assessments of interest to this study, detailed in the methods section 3.3.5. For each participant, one unselected eye was evaluated by DVA. All raw data were re-analysed using the mathematical approach for the analysis of retinal response parameters described in section 3.3.5.12. 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 3.3.5.11).

8.3.7. Sample Size

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessels reactivity was shown to be significant.[540,597] As the study design was multifactorial in nature, it was

calculated that a total sample size of *n* = 120 was sufficient to provide 95% power at an alpha level of 0.05.

One hundred twenty-three healthy participants with low global cardiovascular risk (< 10% at 10 years as assessed by the FRS and similar dietary habits were included in the final analysis. We divided our study participants into three age groups (30 and below, between 31 and 50 and over 50).

8.3.8. Statistical Analysis

Data were analysed according to the methods explained in 3.3.12.

8.3.9. Symbolic Regression

A symbolic regression-based analysis was used to model and subsequently predict the cardiovascular risk factors of age and BP, as described in section 3.3.11.

8.4. Results

Table 8.1 shows the general and circulatory markers characteristics of the study population, stratified by age groups.

There were no statistically significant differences between the number of participants in each group ($p>0.05$). In addition, the number of men and women in each group was similar ($p>0.05$). The three groups were also similar with regards to HR, BMI, TG, HDL, GSH and GSSG (all p-value >0.05). However, although within the normal range, T-CHOL and LDL plasma concentrations were significantly different between the 3 study groups ($p<0.001$ and $p=0.010$), with middle age and elderly groups showing higher concentrations compared to the younger group ($p= 0.0004$, 0.006 and $p= 0.0001$, 0.001 respectively). On the other hand, RTL decreased significantly with age ($p=0.010$) Table 8.1.

8.4.1. Differences in Retinal Vascular Function

Group differences in flicker-induced retinal arterial diameter changes (DVA) are summarized in Table 8.2. All reported values are based on data averaged across the three flicker cycles, with the artery and vein regarded separately.

After controlling for influential covariates identified in multivariate analysis, there were no significant group differences in baseline diameter, BDF, DA, BCFR, MD, MD%, MC, $Slope_{AD}$ and $Slope_{Ac}$ (all $p > 0.01$, Table 8.2). There were, however, significant group differences in arterial tMD ($p=0.005$), MC ($p=0.007$) and MC% ($p=0.010$) (Table 8.2). *Post hoc* comparisons showed tMD, and tMC significantly decreased in the youngest and middle age groups compared with the oldest group ($p = 0.012$, 0.013 and $p= 0.0024$, 0.0004 respectively). Additionally, artery MC% was increased in the oldest age group compared to the youngest and middle age groups group $p = 0.0085$ and $p =0.0148$, respectively) (Figure 8.1). There were no statistically significant differences between all measured venous retinal parameters in all study groups (all $p>0.01$).

Table 8.1: General Characteristics of the Study Population

Variables	<30	31-50	>50	p-value	Post-hoc analysis
Number	40	47	36	>0.05	-
Gender	20M:20F	23M:24F	19M:17F	>0.05	-
Age (years)	24.95 (0.72)	38.28 (0.67)	58.53 (0.76)	0.0000*	1<2<3
SBP	114.9(1.84)	114.96(1.70)	121.86(1.94)	0.0129*	1=2<3
DBP	66 (1.35)	70.64(1.25)	72.83 (1.43)	0.0022*	1=2<3
HR (bpm)	66.05 (1.32)	64.94(1.22)	62.11 (1.39)	0.1133	-
BMI (kg/m^2)	24.92 (0.71)	26.07 (0.65)	26.53 (0.76)	0.2727	-
Glucose	4.43 (0.11)	4.68 (0.12)	5.00 (0.12)	0.0034*	1=2<3
TG (mmol/L)	0.83 (0.050)	0.92 (0.047)	0.98 (0.05)	0.1269	-
T-CHOL	3.94 (0.12)	4.63 (0.12)	4.51 (0.14)	<0.001*	2=3>1
HDL-C (mmol/L)	1.33 (0.06)	1.26 (0.06)	1.16 (0.06)	0.051	-
LDL-C (mmol/L)	2.21 (0.13)	3.13 (0.12)	2.92 (0.14)	0.001*	2=3>1
GSSG	31.19 (3.43)	36.80 (2.93)	28.70 (3.33)	0.169	-
GSH	348.79 (47.91)	412.13 (41.03)	410.09 (46.52)	0.549	-
RTL	0.64 (0.22)	0.09 (0.21)	-0.36 (0.24)	0.010*	1>2>3

Table 8.1: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate (in beats per minute); GLUC, glucose; TG, triglycerides; T-CHOL, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GSH, Glutathione; GSSG, Glutathione disulphide; RTL, leucocyte telomere relative lenght.
*Significant p-values are indicated where $p < 0.05$ was considered significant.
[a] Data are presented as mean (SD) unless otherwise indicated.
[b] Calculated as weight in kilograms divided by height in meters squared.

Table 8.2. Summary of Retinal Arterial Vascular Function Parameters

	Mean (SD)				
Parameter	**19-30**	**31-50**	**>50**	**P-Value**	**Post-hoc analysis**
Artery baseline	116(5.02)	114.86 (2.22)	108.46 (6.07)	0.107	
Artery-BDF	6.34 (0.41)	5.22 (0.38)	4.80 (0.44)	0.029*	
Artery-DA[a]	10.80 (0.70)	9.94 (0.66)	8.18 (0.76)	0.041*	
Artery-BCFR[b]	4.48 (0.40)	4.69 (0.37)	3.26 (0.43)	0.034*	
Artery-MD	124.11 (2.04)	118.36 (1.90)	116.11 (2.15)	0.021*	
Artery-tMD	17.44 (0.58)	17.55 (0.53)	19.89 (0.61)	0.005*	1=2<3
Artery-MD%	5.31 (0.32)	4.66 (0.29)	4.30 (0.34)	0.104	-
Artery-MC	113.17 (2.30)	109.92 (2.12)	112.80 (2.43)	0.521	-
Artery-tMC	24.65 (2.49)	23.94 (1.13)	30.75 (3.11)	0.007*	1=2<3
Artery-MC%	-3.40 (0.30)	-3.55 (0.27)	-2.70 (0.30)	0.010*	1=2>3
Artery-Slope$_{AD}$ [c]	0.45 (0.04)	0.39 (0.04)	0.38 (0.05)	0.567	-
Artery-Slope$_{AC}$[d]	-0.56 (0.04)	-0.44 (0.04)	-0.36 (0.05)	0.126	-

Table 8.2. Baseline, baseline diameter; BDF, baseline diameter fluctuation; DA, dilation amplitude; BCFR, baseline-corrected flicker response; MD, artery maximum dilation; tMD, reaction time to maximum dilation diameter; MD%, percentage change in diameter from baseline to maximum dilation; MC, Maximum constriction; tMC, reaction time to maximum constriction diameter from maximum dilation diameter; MC%, percentage constriction below baseline; Slope$_{AD}$, slope of arterial dilation; Slope$_{AC}$, slope of arterial constriction. Unless otherwise indicated, all values are expressed in arbitrary units, which approximately correspond to micrometres (μ m) in a normal Gullstrand eye.
*Significant p-values are indicated where $p < 0.05$ was considered significant.
a Calculated as MD – MC, b Calculated as DA – BDF
c Calculated as (MD – baseline)/tMD
d Calculated as (MC-MD)/ (tMC)

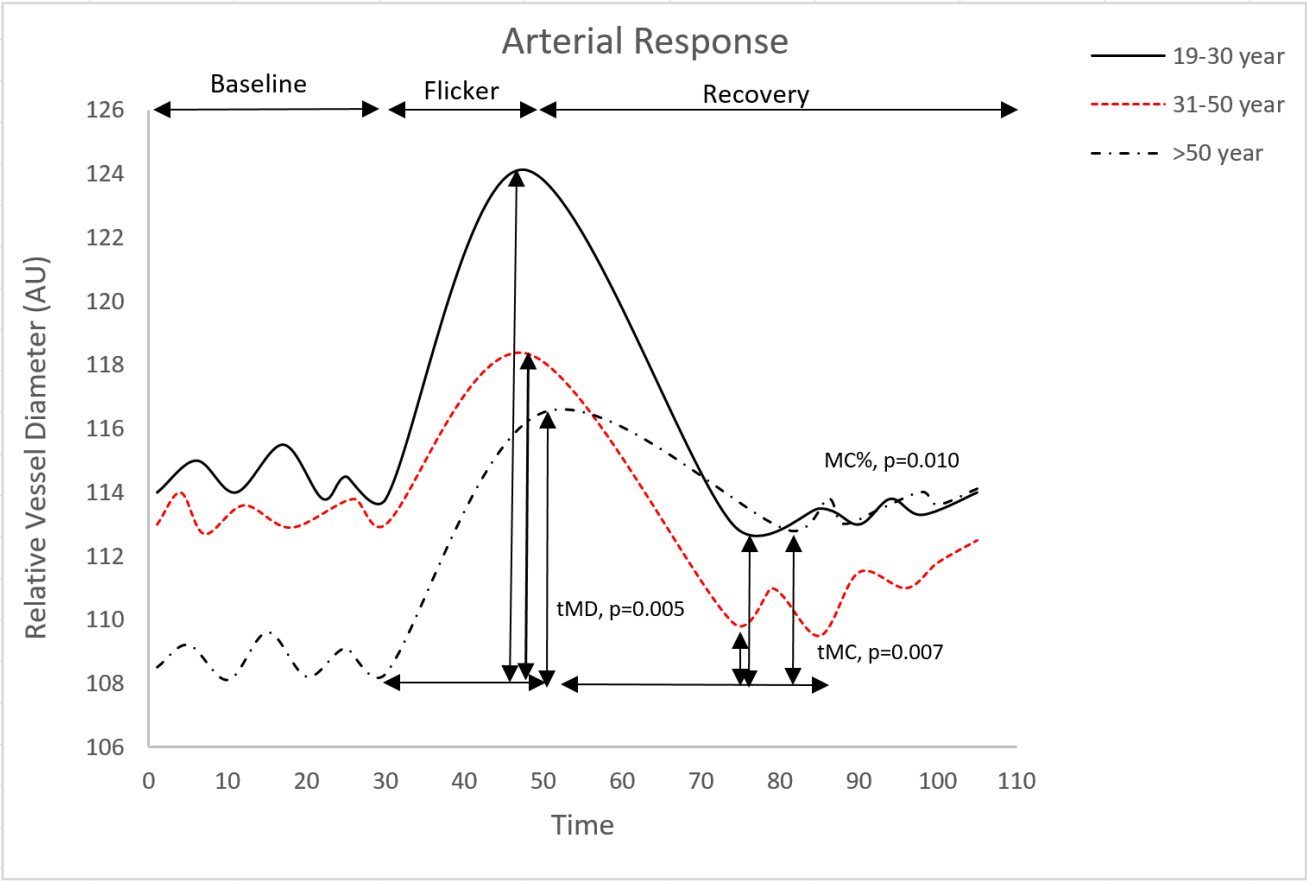

Figure 8.1: Comparison of Retinal Arterial Response Profile Across Groups.
Abbreviations: AU, arbitrary units; BDF, baseline diameter fluctuation calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings; MD%, calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker; tMD, time to reach maximum diameter during flicker; tMC, time to reach maximum constriction post flicker; $slope_{AC}$, calculated as (MC-MD)/ (tMC).

8.4.2. Symbolic Regression-based Analysis

As differences between groups were mainly perceived at the retinal arteriolar level, only these measurements have been used for our analysis. We have performed three sets of Symbolic Regression experiments for each age group, based on the combined biomarkers of arterial measurements and telomere, arterial measurements only, and telomeres only. 5-fold cross validation with 30 Symbolic Regression runs per fold was used for each set of experiments.

8.4.2.1. Age prediction

The mean absolute error (MAE) values for each age group are presented in tables 8.3a, 3b, 3c and figure 8.2. In the two younger groups, the error between predicted versus actual values for age are smallest in the case of using artery measurements only, while the largest error occurred when using telomere only. The situation was completely the opposite in the older group, where using telomere measurements only led to the lowest error.

The Friedman test for age prediction data indicated that only for the youngest age group are the differences significant (p-value 0.0295), and in this case, the Nemenyi post-hoc test indicated that at $\alpha=0.05$, only the results for arteries only and telomere only are significantly different, with p-value 0.043.

The Kruskal-Wallis test indicated that the differences are significant for Telomere + Arteries and Arteries groups in our case, and in these cases, we performed the Dunn post-hoc test to determine which groups have different means. With p-values of 0.012 and 0.000022, we established that the differences are significant for telomere and arteries, between the youngest group and the middle group 2 and between the youngest and oldest groups. In the case of arteries, all pairwise differences were indicated by the Dunn test as significant.

Table 8.3a: Mean Absolute Errors for age predictions (Age group 1).

Arteries + Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
1	1.488355		1	1.457137		1	2.150139
2	2.03976		2	1.628655		2	2.466321
3	1.634165		3	1.580925		3	3.263452
4	2.157625		4	2.038026		4	3.874383
5	1.157639		5	1.40857		5	1.776776
Average	1.695509		Average	1.622663		Average	2.706214

Table 8.3b: Mean Absolute Errors for age predictions (Age group 2).

Arteries + Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
1	2.812663		1	2.527317		1	3.204149
2	2.107845		2	2.245985		2	1.518323
3	2.790105		3	2.620622		3	3.189129
4	3.892265		4	3.516084		4	4.30067
5	2.632783		5	2.965419		5	3.701804
Average	2.847132		Average	2.775085		Average	3.182815

Table 8.3c: Mean Absolute Errors for age predictions (Age group 3).

Arteries + Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
1	4.902158		1	6.013113		1	2.392696
2	2.922922		2	5.230758		2	4.52436
3	4.003612		3	3.691483		3	3.489574
4	4.052891		4	5.85124		4	4.656914
5	6.184841		5	4.847482		5	3.400724
Average	4.413285		Average	5.126815		Average	3.692854

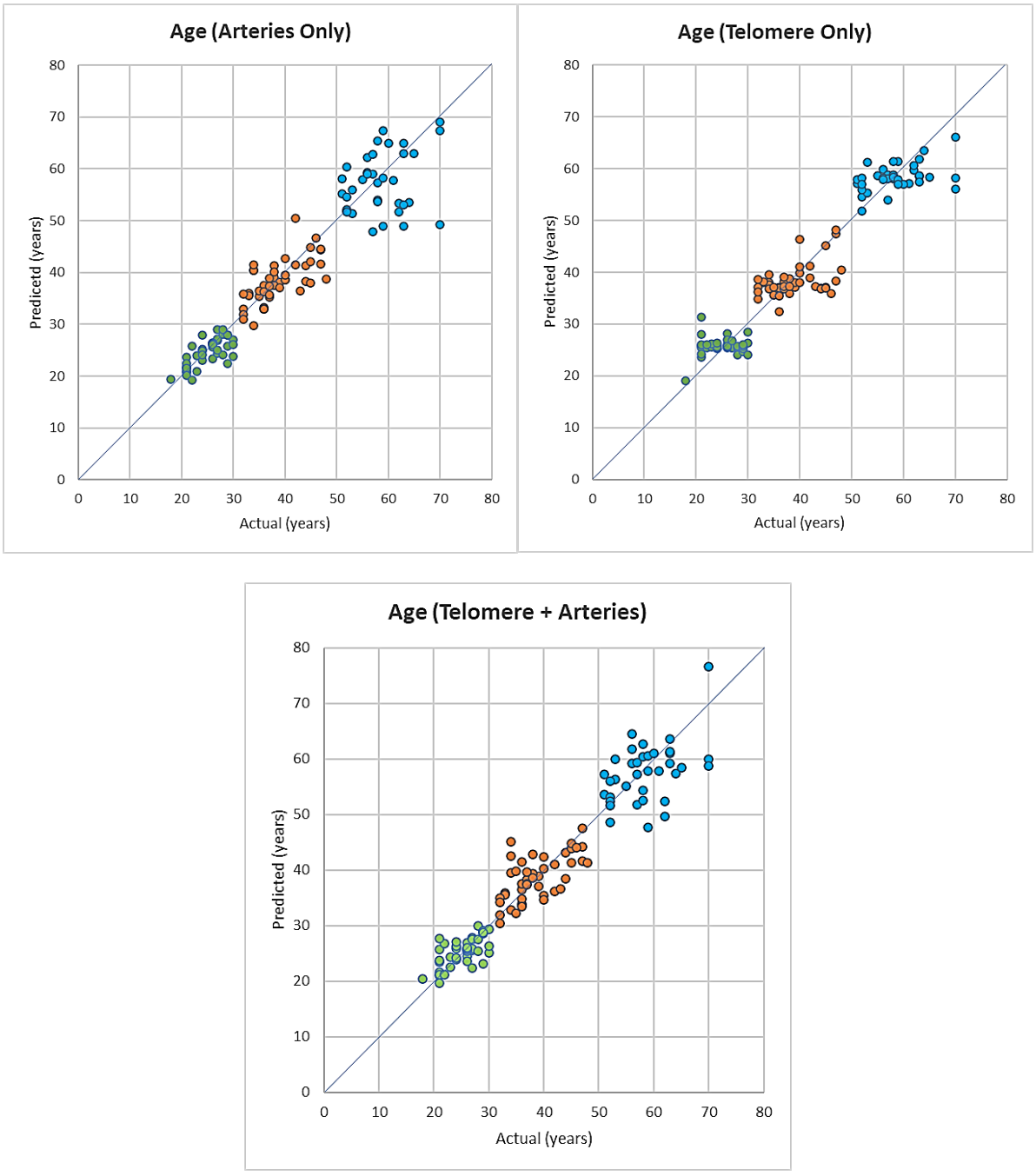

Figure 8.2. Predicted age vs actual age using models based on artery data, telomere relative expression and artery and telomere combined data.
Colour coded for the different age groups (green: 18 – 30, orange: 31 – 50, blue: 50+).

8.4.2.2. Blood Pressure Prediction

As shown in Tables 8.4a,4b,4c and 8.5a,5b,5c and figures 8.3 and 8.4, composite biomarkers are better when predicting systemic diastolic BP and using the telomeres alone gave a better prediction of the systolic blood pressure.

Table 8.4a: Mean Absolute Errors for Systolic BP predictions (Age group 1).

Arteries and Telomere			**Arteries Only**			**Telomere Only**	
Fold	MAE		Fold	MAE		Fold	MAE
1	7.60824		1	9.781193		1	9.214715
2	14.67967		2	15.0163		2	8.569096
3	8.132338		3	6.466947		3	9.16523
4	8.570601		4	14.36258		4	6.129705
5	13.69032		5	7.829224		5	9.043941
Average	10.53623		Average	10.69125		Average	8.424537

Table 8.4b: Mean Absolute Errors for Systolic BP predictions (Age group 2).

Arteries and Telomere			**Arteries Only**			**Telomere Only**	
Fold	MAE		Fold	MAE		Fold	MAE
1	8.581326		1	8.418168		1	7.495257
2	7.755753		2	7.490878		2	7.130104
3	10.37444		3	7.814303		3	5.463688
4	10.96121		4	8.422463		4	10.27759
5	8.381083		5	6.844803		5	8.355181
Average	9.210761		Average	7.798123		Average	7.744364

Table 8.4c: Mean Absolute Errors for Systolic BP predictions (Age group 3).

Arteries and Telomere			**Arteries Only**			**Telomere Only**	
Fold	MAE		Fold	MAE		Fold	MAE
1	17.1292		1	16.13368		1	12.3724
2	6.913672		2	5.783934		2	5.320442
3	8.221233		3	8.26078		3	8.073649
4	8.246475		4	6.949804		4	5.237078
5	7.398514		5	5.531346		5	6.151203
Average	9.581819		Average	8.531909		Average	7.430954

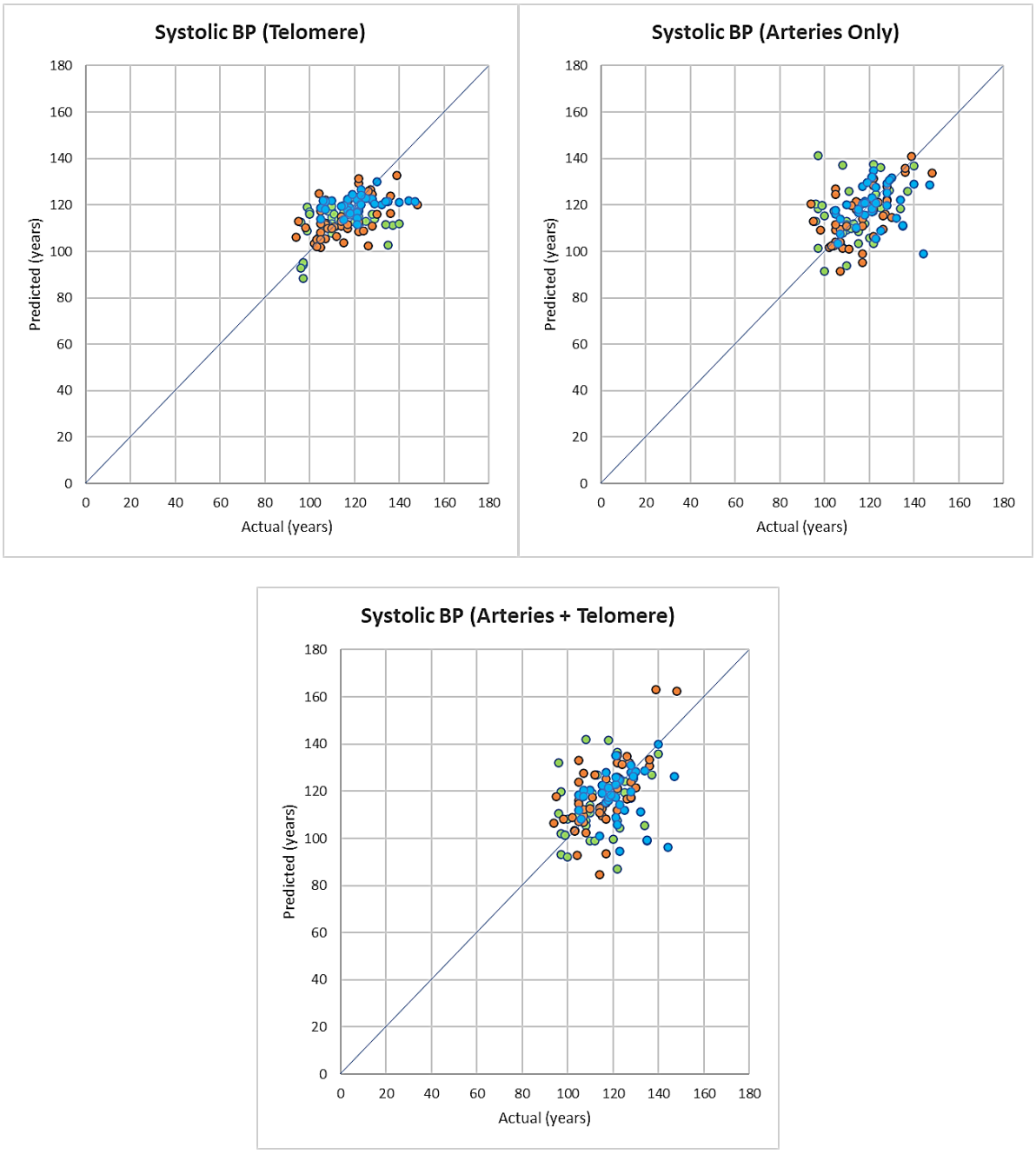

Figure 8.3: Predicted systolic blood pressure vs actual systolic blood pressure using models based on artery, telomere, artery + telomeres combined data.
Colour coded for the different age groups (green: 18 – 30, orange: 31 – 50, blue: 50+).

Table 8.5a: Mean Absolute Errors for Diastolic BP predictions (Age group 1).

Arteries and Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
27	5.110043		23	6.43493		1	6.644792
26	6.032743		3	7.535935		2	5.476441
4	4.814686		14	4.070671		3	5.058709
4	4.77134		11	4.221734		4	5.449827
22	4.172079		23	3.580786		5	4.426252
Average	4.980178		Average	5.168811		Average	5.411204

Table 8.5b: Mean Absolute Errors for Diastolic BP predictions (Age group 2).

Arteries and Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
1	4.733007		1	5.99453		1	4.215411
2	4.618492		2	6.014625		2	5.779917
3	7.849373		3	8.224371		3	5.561008
4	6.65106		4	8.225318		4	5.896184
5	2.782652		5	3.812176		5	8.340123
Average	5.326917		Average	6.454204		Average	5.958529

Table 8.5c: Mean Absolute Errors for Diastolic BP predictions (age group 3).

Arteries and Telomere			Arteries Only			Telomere Only	
Fold	MAE		Fold	MAE		Fold	MAE
1	8.175021		1	7.292121		1	7.451285
2	6.597758		2	5.685044		2	6.589668
3	4.907752		3	5.379021		3	7.05904
4	3.739657		4	5.28883		4	4.040507
5	5.058974		5	6.01973		5	3.199624
Average	5.695832		Average	5.932949		Average	5.668025

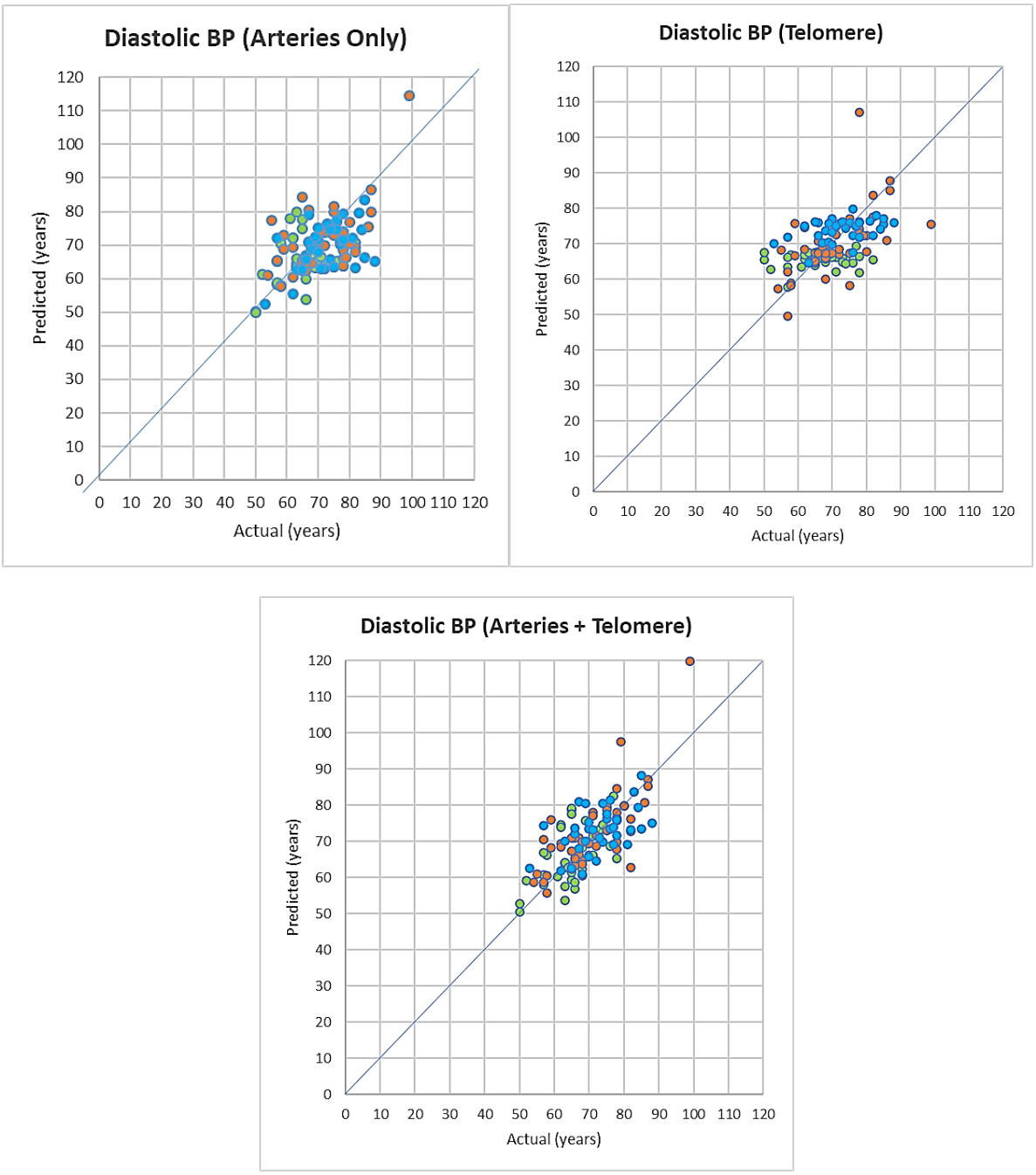

Figure 8.4: Predicted diastolic blood pressure vs actual diastolic blood pressure using models based on artery, telomeres and artery + telomeres combined data.
Colour coded for the different age groups (green: 18 – 30, orange: 31 – 50, blue: 50+).

8.5. Discussion

Our results show, for the first time, that the assessment of retinal vascular function is better predictor of chronological ageing that RTL. Nevertheless, using composite biomarkers are better when predicting modifiable risk factors for CVD, such as systemic BP.

In line with our previous reports [531], we have again demonstrated that in healthy individuals, retinal vascular function parameters are affected by the chronological age, therefore being a good predictor for vascular dysfunction associated with this physiological process. In addition, and as already published [693–695], RTL values has also declined with age in our groups. Nevertheless, as many studies imply, individuals do not age at a similar pace, a concept included into the definition of the so-called "biological age", which is considered a measure of each individual's health status and, possibly, their health span, things that cannot always be predicted by looking at the chronological age only.[696] Indeed, assessing biological age can help identify those individuals at risk from preventable diseases, such as CVD, through early applications of healthy-ageing intervention programs. They can also be used for selecting suitable candidates for clinical trials designed to delay the onset and the progression of ageing-associated pathological conditions.[697] Therefore; it is understandable why the need of measuring biological ageing is so important.

Many studies use various circulatory markers in association with chronological age to try to predict everyone's biological age; however, this approach is not ideal, as using chronological age into this type of calculation is already skewing the results in the wrong direction. The use of RTL only as a marker of biological ageing is also criticised as recent research show that RTL per se represent only a rough estimate of the ageing rate and can hardly be regarded as a clinically important risk marker for age-related pathologies and mortality.[697] Indeed, the process of RTL shortening with age is very complex, and the exact mechanisms underlying this process are not yet established.[697–699] Indeed, our study revealed that for younger individuals (groups 1 and 2), even when it came to predicting the actual chronological age, the largest error was given when using the RTL measurements only. A much better prediction was obtained when the RTL values were combined with the retinal vascular function. However, what was particularly noticeable was that when using retinal arterial function measures alone, the prediction error was the lowest. However, the situation was completely the opposite when we considered individuals over 50 years old when assessing the RTL had the best prediction success when it came to chronological ageing. There is no clear explanation for this finding, and more research needs to be done to understand the exact mechanism behind it. Nevertheless, we can hypothesise that, in older individual, vascular function is a less predictable measurement due to the heterogeneity of their vascular health status. Indeed, in this age group, the influence of genetic, environmental and socioeconomic factors that had

influenced the function of their bodies can be, probably, perceived more and, therefore, will result in a large variability when it comes to assessing their vascular health and using is as a predictor of their chronological ageing.

The level of blood pressure is another risk factor for CVD, and many attempts were made to possibly predict these, even using retinal vascular imaging.[700] Nevertheless, these methods have limitations as they use static imaging, and the computer will perceive structural changes that are present when the disease is already present, even if at the subclinical level. By assessing retinal vascular function rather than structure, we believe that our method will be far superior in this respect. Nevertheless, for the SBP and for all age groups, the assessment of RTL was far superior to that of retinal vascular function or of the combination of RTL and retinal vascular function. A similar situation was also found for DBP values, apart from younger individuals were using the retinal arterial function in combination with RTL assessment and was a much better predictor of the actual values.

This is a very interesting discovery. Telomere length has been previously associated with the development of hypertension. However, this parameter has never been used to predict BP values in asymptomatic individuals. More research is necessary to determine the validity of our findings.

9. General Summary and Discussion

Assessment of systemic vasculature is a crucial step in the early diagnosis and management of CVDs. The currently used vascular investigating techniques provided an invaluable understanding of many cardiovascular pathologies; however, they are either expensive or technically complicated to be used in the widespread screening of individuals at risk. Likewise, the commonly used risk scores though beneficial from a public health perspective; however, they have more inferior predictive value for the individual patient. As a result, a simpler and more accessible technique is required.

With the close correlation between the macrovascular blood supply in the cardiovascular system and the microvasculature of the retina and as both networks share close regulatory processes, it was thought that retinal vascular analyser could be used to assess the systemic vascular function. Owing to the homology between the two networks, any changes in the retinal vessels may reflect similar changes in the cardiac vasculature.

DVA also has a unique advantage owing to its ability to simultaneously and directly visualize and assess both the microvascular function and structure. It can also provide detailed vascular profiling at the individual rather than population level, which could guide the shift towards personalised medicine, especially in cases at high risk to develop CVD despite having low predicted cardiovascular risk score.

This thesis focused on analysing the microvascular function as reflected by the retinal vessels to assess whether microcirculatory dysfunctions can be present and detected at an early stage in otherwise healthy individuals with different cardiovascular risk factors. As the evaluation of one vascular bed can be limiting, in this thesis, we sought to provide a broader picture of systemic vascular health using additional validated surrogate markers for vascular endothelial structure, function and plasma oxidative stress status. It was thus hypothesised that outcomes substantiating the relevance of retinal vascular function against these measures could foster new strategies in cardiovascular diseases risk screening, prevention, and management in asymptomatic individuals.

In summary, the findings of this work were:

9.1. Microvascular Function and Oxidative Stress in Adult individuals With Early Onset of Cardiovascular Disease

Vascular remodelling represented by alterations in the endothelial structure and function of resistance vessels is well identified and assessed in cardiovascular patients. However, questions remain around individuals who are classified as having early-onset CVDs, such as high normal blood pressure and grade 1 hypertension. These classes of hypertension are not subjected to medical treatment, and routine follow-up techniques are not suitable for assessing

their preclinical stages of the diseases. Additionally, to date, no studies evaluated the microvascular function in this population, and our understanding of when the first recognizable signs of vascular dysfunction occur in patients with overt CVDs, or those at risk is limited.

Therefore, this study (chapter 4) aimed to evaluate for the first time the microvascular function in different hypertensive patients classified according to the European Society of Cardiology guidelines. Additionally, as oxidative stress and its role in the progression to more severe stages of hypertension are highlighted as a well-established causative factor of macrovascular dysfunction, we assessed if the degree of endothelial dysfunction discovered could relate to GSH redox system imbalance in these patients.

Following DVA assessment, alteration in the retinal vessels' response was detected in the high-normal and grade 1 hypertension groups in the form of higher baseline diameter fluctuation, impaired dilation responses, and post-flicker under-constrictions compared to individuals in the optimal BP group. In parallel with these changes, alterations in the glutathione redox buffering system were found. Furthermore, GSH and GSSG levels were found to correlate with SBP, DBP and MBP values in all the study participants.

Although we can only hypothesize the exact cause of the early signs of microvascular dysfunction in these groups, our findings support the early onset of CVDs in the microvascular level before the macrovascular level. Furthermore, this study confirmed that functional changes in the microvasculature are an early sign of losing macrovascular function and structure later in life.

While structural and functional changes in the macrovascular level are easy to assess and identify clinically, it is crucial to identify these vascular anomalies when the disease is in the subclinical phase to allow superior disease management. Thus, this study suggests that retinal vascular assessment could be a valuable tool in identifying early cardiovascular risks in individuals with early stages of CVDs. Additionally, since dysfunctional retinal responses correlated with an established cardiovascular risk indicator (oxidative stress), it could act as a helpful screening marker to identify asymptomatic individuals who could benefit from early targeted and individualized therapy.

9.2. European Society of Cardiology/European Society of Hypertension versus the American College of Cardiology/American Heart Association guidelines on the cut-off values for early hypertension: A Microvascular Perspective

The most recent and widely used guidelines for the diagnosis and management of high blood pressure are the 2017 American College of Cardiology (ACC)/American Heart Association (AHA) guidelines and the 2018 European Society of Cardiology (ESC)/European Society of Hypertension (ESH) guidelines. Although both guidelines adopted the same technique in

measuring BP, both have different cut-off values to diagnose hypertension (130/80 vs 140/90) and different levels to which BP should be reduced in hypertensive patients. These differences in the level of HTN that require treatment added a lot of pressure on the healthcare systems worldwide, increased the health insurance costs and increased unreimbursed physician time for visits. The ACC/AHA argues that patients' early treatment can help slow the progress of the disease and the development of diseases end-organ complications. On the other hand, the ESC claim that the risk of extrapolation to lower levels recommended in the ACC guidelines could result in overtreatment.

To assess the clinical validity of lower BP cut-off, we conducted the study presented in chapter 5 to compare the microvascular function in two beds (the retinal vessels and the peripheral microvascular beds) in healthy individuals free from CVDs risk factors who fall into different BP groups classified using both the ESC/ESH or the ACC/AHA guidelines. The analysis of the two vascular beds confirmed the initiation of microvascular dysfunction with blood pressure 140/90 and above in the form of an altered dynamic response profile and a steeper arterial dilation slope and venous constriction slope following cessation of flicker while significant peripheral vascular changes were only noticeable in the grade 1 BP group.

The Correlation between these two different vascular beds in individuals classified as ESC/ESH grade 1 hypertension only supports the introduction of higher BP targets when managing hypertension. It also supports the solo introduction of non-pharmacological treatments in individuals with BP below 140/90 and the routine use of retinal vascular analysis as a variable specific for each individual to help monitoring therapeutic responses in hypertension patients.

9.3. Dry Eye Disease is Associated with Retinal Microvascular Dysfunction and Possible Risk for Cardiovascular Diseases

Dry eye disease is an ocular surface disease that involves multiple interacting predisposing mechanisms. Several systemic clinical conditions were reported to negatively impact the ocular surface and lead to DED. Of these medical conditions, metabolic conditions such as diabetes mellitus and dyslipidaemia and CVDs such as peripheral vascular disorders, stroke and ischemic cardiac diseases were found to be positively associated with DED.[701] However, the type and strength of this association were neither consistent nor clear in the published literature.[633,702,703] Moreover, most of these researchers based their diagnosis on signs and symptoms with no clinically objective ocular examinations. For this reason, our study in chapter 6 aimed to assess functional retinal and peripheral microvascular responses to flickering light in healthy and DED patients. Due to the numerous systemic associations of

DED [704], this study also included assessments of systemic circulatory biomarkers of vascular function.

The study's main finding was that individuals with dry eye disease exhibited impaired microvascular responses on the retinal beds, which was represented by the inability to reach proper arterial baseline diameter before and after the flicker stimulation and signs of arterial stiffness demonstrated as impaired constriction and dilation responses after stimulation. All these observed vascular impairments were significantly and positively correlated to systemic plasma lipids concentrations.

Although the impairments in retinal vascular function were not associated with peripheral arterial stiffness, this lack of significance can be justified by the pilot nature of the study as well as autoregulatory mechanisms of the retinal vessels and the absence of systemic compensatory mechanisms in this level which allow the appearance of signs of microvascular dysfunction in the retinal bed earlier than the peripheral microvascular beds.[705]

In this regard, further validation research in larger cohorts is recommended, and studies to evaluate the impact of the treatment of DED risk factors on the retinal and peripheral vasculature are still needed.

9.4. Novel composite early risk markers for vascular ageing and risk for cardiovascular diseases

The close association between ageing and vascular morphological and molecular alterations such as increased wall thickness, loss of elasticity and reduction of NO haemostasis has been verified extensively.[706] Consequently, the risk of developing cardiovascular diseases increases in the elderly.[707] Currently published literature highlighted imbalances in the redox status and consequent impairment of NO signalling as main determents of age-dependent vascular endothelial dysfunction. However, to date, no study examined the relationship with regard to microcirculation. Using oxysterols as a marker of oxidative stress, the study in chapter 7 examined the potential influences of established increased oxidation markers, systemic antioxidation defence capacity on retinal microvascular function in healthy individuals classified into different age groups. Study findings confirmed the systemic association between plasma oxysterol levels and gradual microvascular endothelial dysfunction at the retinal arterial level after the age of 30. This association was also correlated to decreased systemic antioxidant capacity (redox index) without any significant change in NO concentrations. This direct relationship between oxysterols and functional retinal response to stress and based of the risk category of individuals included in this study suggest the high sensitivity of the combination between oxysterols plasma concentration and DVA as a composite biomarker of early vascular ageing. Nevertheless, beside the action of local factors,

a better understanding of the possible systemic influences on microvascular retinal function in individuals without any CVD risks is still needed.

9.5. Prediction of Cardiovascular Risk in Asymptomatic Individuals: A Symbolic Regression-Based Analysis of Single and Composite Vascular-Omics

As discussed before, current clinically used risk prediction models are typically based on limited predictors with sub-optimal performance across all patient groups. Having established the presence of retinal vascular dysfunction in patients without overt clinical CVDs, an investigation into whether it can predict CVDs future risk was the next logical step. Hence, the study in section 8 aimed to examine the significance of the assessment of retinal vascular function alone and in combination with relative telomere length in predicting CVDs major risk factors (age, BP). On investigation, older age groups exhibited increased systemic arterial stiffness and alterations in retinal arterial reactivity to flicker light stimulation in the form of an altered dynamic response profile. Additionally, our results also confirmed the benefits that can be reached by using a composite measurement of retinal vascular responses and relative telomere length as a predictive biomarker of CVDs major risk factors such as age and blood pressure. These observations are of great scientific importance as combining these markers to currently used risk scores could be a cornerstone of preventative cardiology, which is the new era of interest in the international cardiovascular guidelines.

10. General Conclusion & Limitations

Studies in this thesis confirmed the usefulness of functional retinal vascular analysis using DVA machine as a surrogate endpoint of endothelial dysfunction and a marker of future risk of development of CVDs in apparently healthy individuals. However, it is appreciated that the included research in this thesis is still subject to some potential limitations. For instance, all the included studies were cross-sectional in nature, so follow up periods couldn't be established. Further longitudinal studies are recommended to investigate the progressive association between microvascular dysfunction and the development of clinical vascular and cardiac diseases. Further evaluation of the predictive ability of the suggested composite CVDs risk stratification markers is also recommended to strengthen the clinical value of routine functional retinal vascular assessment and oxidative stress biomarkers analysis in primary clinical settings as an easy non-invasive CVDs risk stratification tools.

The validity and reliability of the research findings should be tested in special populations such as African Americans, Asian, children and cancer survivors, as it is known these individuals have higher susceptibility to CVDs and abnormal responses to commonly used medications and interventions. Larger sample size is needed to confirm the external validity of the findings.

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, dynamic retinal functional assessments provide an opportunity to explore the relationship between cardiovascular risk factors, microvascular endothelial dysfunction, and associative cardiovascular risk. The presented results open up several avenues that can be explored in future work.

11. Future Directions

11.1. Individuals-focussed Studies

Despite statistically significant results being demonstrated in this thesis, it would be interesting to determine individual's risk of developing CVDs using the retinal vascular function parameters. The generated 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 genetic risk factors or ethnic susceptibility. Identifying these individuals will enable early management of the modifiable risk factors and accordingly, prediction, diagnosis, treatment and, eventually, prevention of CVDs can be matched to individuals based on genetics, environmental risk factors and lifestyle.

11.2. Improving the Predictive Power of Currently Used Risk Scores

Owing to the poor predictive power of the currently used risk scores, large cohorts should be established to assess whether functional retinal vascular parameters could improve the predicted results of the clinically used risk models. Early identification of at-risk individuals will facilitate early clinical intervention and slow down the progression of the disease.

11.3. Expansion of Preliminary Data

Results of the included studies confirmed the clinical significance of using retinal arteries functional parameters in combination with oxysterols as an early vascular ageing biomarker and relative telomeres length as a predictor of cardiovascular risk factors; however, conducting a larger prospective cohort study could enhance the validity of our findings with regard to exposures, confounders, and endpoints and could also minimize the recall error. Furthermore, expansion of CVDs risk predictive biomarkers could offer additional information of both aetiological and clinical relevance with regard to CDVs.

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13. Appendices

13.1. Appendix a. Ocular Surface Disease Index (OSDI)

Have you experienced any of the following during the last week:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light?	4	3	2	1	0
2. Eyes that feel gritty	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	NA				
5. Poor vision?	NA				

Have problems with your eyes limited you in performing any of the following during the last week:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	
6. Reading?	4	3	2	1	0	NA
7. Driving at night?	4	3	2	1	0	NA
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	NA
9. Watching TV?	4	3	2	1	0	NA

Have your eyes felt uncomfortable in any of the following situations during the last week:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	
10. Windy conditions?	4	3	2	1	0	NA
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	NA
12. Areas that are air conditioned?	4	3	2	1	0	NA

13.2. Appendix b. Dry Eye Questionnaire (DEQ-5)

1. Questions about **EYE DISCOMFORT**:
   a. During a typical day in the past month, **how often** did your eyes feel discomfort?
      0 Never
      1 Rarely
      2 Sometimes
      3 Frequently
      4 Constantly
   b. When your eyes felt discomfort, **how intense was this feeling of discomfort** at the end of the day, within two hours of going to bed?

Never have it	Not at all Intense				Very Intense
0	**1**	**2**	**3**	**4**	**5**

2. Questions about **EYE DRYNESS**:
   a. During a typical day in the past month, **how often** did your eyes feel dry?
      0 Never
      1 Rarely
      2 Sometimes
      3 Frequently
      4 Constantly
   b. When your eyes felt dry, **how intense was this feeling of dryness** at the end of the day, within two hours of going to bed?

Never have it	Not at all Intense				Very Intense
0	**1**	**2**	**3**	**4**	**5**

3. Question about **WATERY EYES**:
   During a typical day in the past month, **how often** did your eyes look or feel excessively watery?
      0 Never
      1 Rarely
      2 Sometimes
      3 Frequently
      4 Constantly

Score: 1a + 1b + 2a + 2b + 3 = Total

___+___+___+___+___= ____

13.3. Appendix c. Patient Examination Sheet

Patient ID: [FS | dd | mm | yyyy] Examination date: [dd | mm | yyyy]

First name: [ ] Date of birth: [dd | mm | yyyy]
Surname: [ ] Sex: [f|m]

Ethnicity: ( )Asian ( )African ( )Caucasian ( )Chinese
( )Latino ( )Oriental ( )South-Asian ( )Other
Phone: [ ] Email: [ ]

Optometry

Intraocular pressure left: 1. [ ] 2. [ ] 3. [ ]

Intraocular pressure right: 1. [ ] 2. [ ] 3. [ ]

Blood pressure

Systolic blood pressure: [ ] Diastolic blood pressure: [ ]

Pulse per minute: [ ]

Lifestyle

Smoking: ( )never smoked ( )smoked for [ ] years

( )been smoking for [ ] years, [ ] cigarettes/day

Physical activity: [ ] times a week for [ ] hours with [low|medium|high] intensity
Alcohol: [uk units/week] Healthy diet: [yes|more-or-less|no]
Sleeping: [ ] hours per night

Medical conditions

[ ]Allergies [ ]Eczema [ ]Arthritis [ ]Meningitis [ ]Constipation [ ]Cardiovascular disease
[ ]Uveitis [ ]Anaemia [ ]Epilepsy [ ]Faintness [ ]Hypertension [ ]High blood pressure
[ ]Headache [ ]Stroke [ ]Glaucoma [ ]Depression [ ]Heart attack [ ]Shortness of breath
[ ]Diabetes [ ]Asthma [ ]Joint pain [ ]Migraines [ ]High cholesterol [ ]Low blood pressure
Other: 1. [ ] 2.[ ]
3.[ ]

Medications

1.[ ] 2.[ ] 3.[ ]

Family history of medical conditions

[F|M]Allergies [F|M]Anaemia [F|M]Arthritis [F|M]Asthma [F|M]Cardiovascular disease
[F|M]Constipation [F|M]Depression [F|M]Diabetes [F|M]Eczema [F|M]Low blood pressure
[F|M]Faintness [F|M]Headache [F|M]Heart attack [F|M]Glaucoma [F|M]High cholesterol
[F|M]Joint pain [F|M]Meningitis [F|M]Migraines [F|M]Uveitis
[F|M]Shortness of breath

[F|M]Stroke
[F|M]Epilepsy
[F|M]Hypertension
[F|M]High blood pressure

Other: 1. [F|M] [_____] 3. [F|M] [_____]
2. [F|M] [_____] 4. [F|M] [_____]

Anthropometric measurements
Height: [cm] Body fat percentage: [0-100] Muscle mass: [kg] Bone mass: [kg]
Weight: [kg] Body water percentage: [0-100]

Endothelix
aTR: [_____] TR: [_____] TMP-AUC: [_____] aTMP-AUC: [_____]

Bloods
Total cholesterol: [mmol/L] Glucose: [mmol/L]
HDL cholesterol: [mmol/L] Triglycerides: [mmol/L]

Checklist
DVA: [ ] Body fat%, muscle mass%, BMI: [ ]
Visualis: [ ] Information consent signed?: [ ]
Bloods: [ ] Overnight fasting? [ ]
Endothelix: [ ]

13.4. Publication

13.4.1. Study 1

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Microvascular function and oxidative stress in adult individuals with early onset of cardiovascular disease

Hala Shokr[1], Irundika H. K Dias[2] & Doina Gherghel[1*]

The current study aims to investigate retinal vascular function and its relationship with systemic anti-oxidative defence capacity in normal individuals versus those with early hypertensive changes according to the current ESC/ESH guidelines. Retinal microvascular function was assessed in 201 participants by means of dynamic retinal vessel analysis. Blood pressure, lipid panel, oxidized (GSH) & reduced glutathione (GSSG) were also evaluated for each participant. Individuals classed as grade 1 hypertension demonstrated higher retinal arterial baseline diameter fluctuation ($p = 0.0012$), maximum dilation percentage ($p = 0.0007$), time to maximum constriction ($p = 0.0003$) and lower arterial constriction slope ($p = 0.0131$). Individuals classed as high normal and grade 1 hypertension also demonstrated higher time to maximum dilation than individuals classed as optimal or normal. GSH levels correlated negatively with SBP, DBP and MBP values in all participants ($p = 0.0010$; $p = 0.0350$ and $p = 0.0050$) as well as with MBP values in high normal and grade 1 hypertension ($p = 0.0290$). The levels of GSSG correlated positively with SBP, DBP and MBP values in all participants ($p = 0.0410$; $p = 0.0330$ and, $p = 0.0220$). Our results point to the fact that microvascular alterations can be identifiable at BP values still considered within normal values and go in parallel with the changes observed in the level of oxidative stress.

The assessment of the microvascular function represents an important part in establishing the pathophysiology but also the risk stratification of cardiovascular disease (CVD)[1]. Indeed, endothelial dysfunction, one of the main culprits for the development of atherosclerosis, occurs much earlier at the microvascular than at the macrovascular level[2,3]. Dynamic retinal vessel analysis (DVA) was identified as a useful measure of early changes that signal endothelial dysfunction at the microvascular level. This method can also be used to identify risk for future cardiovascular pathologies in individuals at risk for[4–9] or already suffering from CVD[10,11]. This is generally possible due to the fact that the retinal microvascular response to flicker provocation is, in part, dependent on nitric oxide (NO) release[12], and compromised NO homoeostasis is known to be a key factor in endothelial dysfunction at all vascular levels.

The assessment of retinal microvessels is used in research but also in clinical practice for diagnosis and follow-up of various CVD, including hypertension[13,14]. Nevertheless, the overwhelming majority of protocols that look at retinal microvessels in the course of various CVD, use static imaging to detect abnormalities associated with various degrees of pathology. This is, however, useful only when CVD already established itself and not at earlier, pre-clinical stages. In order to determine the risk at earlier stages to allow preventive measures to be adopted, using the assessment of the retinal microvascular function represents a better alternative to structural imaging because it provides integrated and dynamic data to help establishing possible CVD risk.

In certain conditions that accelerate degradation of NO, such as high oxidative stress, microvascular dilation can be severely impaired[15]. In order to counteract such effects, the human body uses various anti-oxidative mechanisms including glutathione. Therefore, any condition associated with low levels of circulating glutathione result in a higher rate of oxidative reactions that contribute towards low NO bioavailability and, consequently, to an impaired microvascular function[5,8,16,17]. We have already shown that retinal microvascular dilation and

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13.4.2. Study 2

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European Society of Cardiology/ European Society of Hypertension versus the American College of Cardiology/American Heart Association guidelines on the cut-off values for early hypertension: a microvascular perspective

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The aim of this study was to investigate retinal and peripheral microvascular function in asymptomatic individuals that fall into different BP groups when using either the ESC/ESH or the ACC/AHA guidelines. Retinal and peripheral microvascular function was assessed in 358 participants by means of dynamic retinal vessel analysis and digital thermal monitoring, respectively. Blood pressure and lipid panel were also evaluated. Retinal vascular function measured in all groups belonging to the ACC/ASH classifications were within the normal values for age-matched normal population. Individuals classed as grade 1 hypertension according to the ESC/ESH guidelines, however, exhibited a significantly decreased artery baseline ($p = 0.0004$) and MC ($p = 0.040$), higher $slope_{AD}$ ($p = 0.0018$) and decreased vein MC ($p = 0.0446$) compared to age matched normal individuals. In addition, they also had significant lower artery baseline, artery BDF, MD and MC than individuals classed as stage 1 hypertension based on the ACC/ASH guidelines ($p = 0.00022$, $p = 0.0179$, $p = 0.0409$ and $p = 0.0329$ respectively). Peripheral vascular reactivity (aTR) was lower in ESC /ESH grade I compared to those graded ACC/ASH stage I hypertension ($p = 0.0122$). The conclusion of this study is that microvascular dysfunctions is present at multiple levels only in individuals with ESC/ESH grade 1 hypertension. This observation could be important when deciding personalised care in individuals with early hypertensive changes.

One of the main differences between the latest guidelines published in 2018 by the European Society of Cardiology/European Society of Hypertension (ESC/ESH) and those published in 2017 by The American College of Cardiology/American Heart Association (ACC/AHA) is the cut-off for what is considered elevated blood pressure (BP) and the first stage of hypertension (HTN) diagnosis[1]. As it can be expected, such difference is bound to have a high impact on clinical diagnosis and management of HTN. By using the ACC/AHA guidelines, the number of patients diagnosed with having this disease increased significantly and so did the pressure on the health system and economy. In addition, such change in practice has also a significant impact on the patients' physical and psychological wellbeing[1]. Nevertheless, it would seem sensible that, in individuals with higher risk for cardiovascular disease (CVD), early diagnosis and interventions are applied at lower BP values. Indeed, both ECC/ESH guidelines and ACC/AHA have recognised this fact and recommended considering treating patients with high CVD risk at a BP threshold lower than their current cut-off for grade/stage 1 HTN[2]. In this way,

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13.4.3. Study 3

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Dry eye disease is associated with retinal microvascular dysfunction and possible risk for cardiovascular disease

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ABSTRACT.

Purpose: **To explore the presence of microvascular endothelial dysfunction as a measure for early cardiovascular disease in individuals diagnosed with dry eye disease (DED) as compared to age-matched normal controls.**

Methods: **Systemic blood pressure, Body Mass Index, intraocular pressure, blood levels of glucose (GLUC), triglycerides, cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)] as well as retinal and peripheral microvascular function were assessed in twenty-five 35–50 year olds with diagnosed with DEDa (using the TFOS DEWS II criteria) and 25 age and sex-matched controls.**

Results: **After controlling all the influential covariates, individuals diagnosed with DED exhibited significant lower retinal artery baseline ($p = 0.027$), artery maximum diameter ($p = 0.027$), minimum constriction ($p = 0.039$) and dilation amplitude ($p = 0.029$) than controls. In addition, the time to reach the vein maximum diameter was significantly longer in the DED patients than in normal controls ($p = 0.0052$). Only in individuals diagnosed with DED, artery maximum constriction correlated statistically significantly and positively with HDL-C blood levels ($p = 0.006$). Similarly, artery $slope_{AD}$ correlated positively with T-CHOL and LDL-C ($p = 0.006$ & 0.011 respectively). Additionally, artery baseline diameter and maximum constriction were significantly and negatively correlated to T-CHOL/HDL-C ratio ($p = 0.032$ and $p = 0.013$ respectively) in DED individuals only.**

Conclusions: **Individuals with positive diagnosis of DED exhibit abnormal retinal microvascular function and possible higher risk for CVD.**

Key words: cardiovascular disease – dry eye disease – microvascular function – retinal vessels

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Introduction

Dry eye disease (DED) represents a, multifactorial, chronic and debilitating pathology of the ocular surface characterized by loss of homeostasis of the tear film and accompanied by ocular symptoms. Tear film instability and hyperosmolarity, ocular surface inflammation and damage as well as neurosensory abnormalities play etiological roles in this disease (Craig et al. 2017).

In addition to other risk factors, DED has previously been associated with dyslipidaemia, a group of metabolic abnormalities characterized by any or a combination of the following: raised low-density lipoprotein cholesterol (LDL-C), raised total cholesterol (TC), raised triglycerides (TG) and low high-density lipoprotein cholesterol (HDL-C) (Musunuru 2010). Indeed, as lipid homeostasis is important for the stability of the tear film, the association between dyslipidaemia and DED is entirely justified. Moreover, disruptions of cholesterol biosynthesis are also associated with sebaceous/Meibomian gland (MG) dysfunctions (Bu et al. 2019), another cause of tear film instability and dry eye. Dyslipidaemia also represents a significant risk factor for cardiovascular disease (CVD) especially due to its contribution in the pathogenesis of atherosclerosis in medium-sized and large arteries but also at the microvascular level (Pereira 2012; Padró, Vilahur & Badimon 2018). This affects not only the anatomy of these vessels but, most importantly, their function. Indeed, at the functional level, it impairs endothelium-dependent vasodilation because of defects on nitric oxide (NO) bioavailability (Padró, Vilahur & Badimon 2018). This has catastrophic effects on the balance between the physiological vascular dilatory and constrictory states, which, in turn, will also affect other important circulatory

13.4.4. Study 4

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Article

Oxysterols and Retinal Microvascular Dysfunction as Early Risk Markers for Cardiovascular Disease in Normal, Ageing Individuals

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Abstract: The aim of the present paper is to assess the relationship between oxysterol levels and retinal microvascular function in individuals of various age groups, free of clinically evident diseases. Forty-two apparently healthy individuals were included in the present study (group 1: 19–30 years, group 2: 31–50 years, and group 3: 51–70 years). Retinal microvascular function was assessed using the dynamic retinal vessel analyzer (DVA, IMEDOS GmbH, Jena, Germany). Fasting plasma was obtained from all subjects and quantification of monohydroxy and dihydroxy oxysterols assessment was performed using LC-MS/MS following reverse phase chromatography. A Griess assay was used to evaluate the Nitric Oxide (NO) concentration in all individuals. The glutathione redox ratio was also analyzed by means of whole blood glutathione recycling assay. In all participants, the levels of 7-Ketocholesterol, 25-hydroxycholesterol and 7β-hydroxycholesterol correlated significantly and positively with the time to maximum arteriolar dilation. In addition, 25-hydroxycholesterol and 7β-hydroxycholesterol negatively correlated to the percentage of maximum arteriolar dilation. A negative correlation was observed for 27-hydroxycholesterol and 7β-hydroxycholesterol with microvascular arteriolar constriction. These results suggest that, with age, abnormal oxysterol levels correlate with early changes in microvascular bed function. This relationship could signal early risk for cardiovascular diseases (CVDs) in an ageing population.

Keywords: oxidative stress; oxysterols; cardiovascular disease risk

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1. Introduction

The assessment of microvascular function gained a lot of clinical interest since endothelial dysfunction, the main culprit for CVDs, was found to occur earlier at the microvessel level than the macrovessel level [1]. Accordingly, many methods were developed to evaluate functional microvascular responses to biological stimuli in different vascular beds [2]. One of these methods, the dynamic retinal vessel analysis (DVA), was highlighted as one of the most sensitive and reliable methods to measure early vascular changes that point out early endothelial dysfunction and future risk of cardiovascular pathologies in individuals with and without overt clinical symptoms [3–5]. Moreover, it has also been shown that retinal microvascular function is modified by age [6]. Nevertheless, although this is a consistently demonstrable effect, little is known about what factors in particular directly determine this outcome. We have previously demonstrated that high levels of circulating reactive oxygen species (ROS) have a direct effect on retinal microvascular function [7,8]. Indeed, the evidence suggests that oxidative stress-induced cellular senescence is a key influencer of age-induced mechanical and structural vascular dysfunction [9–12] as well as of the development and progression of CVDs [13–17]. Additionally, high levels of