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Ocular and systemic vascular function in human ageing

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OCULAR AND SYSTEMIC VASCULAR FUNCTION IN HUMAN AGEING

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Synopsis

The importance of vascular risk factors in various age-related pathologies has been extensively researched. Nevertheless, the haemodynamic disturbance occurring in various ocular and systemic vascular beds to impact upon ocular function remained largely unknown. The purpose of the following studies was to explore the presence and impact of both ocular and systemic vascular dysregulation as well as biochemical vascular risk factors in healthy elderly individuals and patients with age-related macular degeneration. Furthermore, the possible role was played by circulatory oxidative stress and its relationship with endothelial dysfunction at both ocular and systemic levels has also been investigated. There were four principal sections to the present work:

1. **To assess the relationship between ocular and systemic anti-oxidative defence in healthy individuals**

The principal findings of this work were:

- It has been shown that MPOD significantly and positively related with circulatory GSH levels.

2. **To investigate macro- and microcirculation and oxidative stress in early AMD patients without overt systemic disease**

The principal findings of this work were:

- AMD patients exhibit abnormal macrocirculation compared to the controls.
- Blood GSSG level was significantly higher in early AMD patients than controls.
- AMD patients showed abnormal microcirculation at retinal level compared.
- In early AMD patients, retinal venous RT positively correlated with blood GSSG levels.

3. **To assess the relationship between ocular vascular function and circulatory markers of endothelial dysfunction and CVD risk**

The principal findings of this work were:

- Age had a positive effect on ET-1, vWF and slope of retinal arterial constriction in otherwise healthy individuals.
- Even in otherwise healthy individuals, retinal arterial vascular function showed a significant correlation with circulatory markers of endothelial dysfunction and CVD risk.

4. **To assess age-related changes in ANS and vascular function, and their relationship to retinal vascular function parameters**

The principal findings of this work were:

- Elderly individuals demonstrated abnormal circadian changes of PSNS activity compared to middle-aged group.
- Elderly groups showed higher ET-1 and vWF level as well as C-IMT and AIx, and also impaired retinal vascular function compared to the middle-aged group.
- In the elderly group, impaired retinal vascular function significantly correlated with the dysregulation of PSNS activity.

Keywords: Retinal vascular function, oxidative stress, endothelial function, age-related macular degeneration.

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List of Abbreviations

2-VP	2-vinyl pyridine
3-NT	3-nitrotyrosine
AIx	Augmentation index
AMD	Age-related macular degeneration
ANS	Autonomic nervous system
AO	Aorta
ATP	Adenosine triphosphate
AVN	Atrioventricular node
BDF	Baseline diameter fluctuation
BFR	Baseline corrected flicker response
BMI	Body mass index
BP	Blood pressure
BRB	Blood-retinal barrier
CAD	Coronary artery disease
CBF	Cerebral blood flow
CCA	Common carotid artery
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CHOL	Total cholesterol
C-IMT	Carotid intima-media thickness
CNS	Central nervous system
CO_2	Carbon dioxide
CRA	Central retinal artery
CVD	Cardiovascular disease
DA	Dilation amplitude
DBP	Diastolic blood pressure

DNA	Deoxyribonucleic acid
DTNB	5.5 dithiobis-2-nitrobenzoic acid
DVA	Dynamic vessel analysis
ECG	Electrocardiogram
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamide tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthases
ETs	Endothelins
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_A	$Endothelin_A$ (endothelin receptor A)
ET_B	$Endothelin_B$ (endothelin receptor B)
FMD	Flow mediated dilation
GC	Guanylate cyclase
GPx	Glutathione peroxidise
GSH	Glutathione
GSR	Glutathione reductase
GSSG	Oxidized form of glutathione
HDL-C	High-density lipoprotein cholesterol
HF	High-frequency
H_2O_2	Hydrogen peroxide
HR	Heart rate
HRV	Heart rate variability
ICA	Internal carotid artery
iNOS	Inducible nitric oxide synthases
IOP	Intraocular pressure

IQR	Inter-quartile range
K^+	Potassium
LA	Left atrium
LDL-C	Low-density lipoprotein cholesterol
LF	Low-frequency
LV	Left ventricle
MBP	Mean blood pressure
MC	Maximum constriction
MD	Maximum dilation
MP	Macular pigment
MPOD	Macular pigment optical density
NFL	Nerve fibre layer
nNOS	Neuronal nitric oxide synthases
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthases
O_2	Oxygen
O_2^-	Superoxide anion
OA	Ophthalmic artery
OD	Optical density
OH^-	Hydroxyl radical
ONH	Optic nerve head
OPP	Ocular perfusion pressure
oxLDL-C	Oxidized form of low-density lipoprotein cholesterol
ΔP:	Augmentation pressure
PA	Pulmonary artery
PCAs	Posterior ciliary arteries
RPE	Retinal pigment epithelium

PSNS	Parasympathetic nervous system
PV	Pulmonary vein
PWA	Pulse wave analysis
RA	Right atrium
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reaction time to reach the maximum dilation to flicker
RV	Right ventricle
SAN	Sinoatrial node
SBP	Systolic blood pressure
SD	Standard deviation
SDRA	Sequential and diameter response analysis
$Slope_{AC}$	Slope of arterial constriction
$Slope_{AD}$	Slope of arterial dilation
$Slope_{VD}$	Slope of venous dilation
$Slope_{VC}$	Slope of venous constriction
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SSA	5-sulphosalicylic acid
SVC	Superior vena cava
TG	Triglycerides
t-GSH	Total glutathione levels
TEA	Triethanolamine
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal polypeptide
vWF	von Willebrand factor

Statement of Authenticity

This thesis represents the work of Lu Qin during a 3 year postgraduate research at School of Life and Health Sciences (Ophthalmic Research Group), Aston University, Birmingham, UK. This work has not been presented in any previous application for a degree and all work was performed by the undersigned unless otherwise stated in the text. The author has no commercial interest in any of the equipment or laboratory methods used in this work.

Chapter 1

Introduction

1.1 Background

The number of aged people has increased during the past 50 years in the developed countries (Wood et al., 2005), as the life also improved reaching today in England 82 years for women and 77 years for men (Hayes et al., 2011); compared with 49 years for women and 45 years for men in 1901 (Hicks J, 1999). However, despite an improvement in the living condition elderly individuals still face higher risk of chronic diseases that can dramatically affect their quality of life.

Ageing is a primary risk factor for dysfunctions in all systems and organs of the human body including the eye and the blood vessels, thus resulting in high rates of disability and mortality. In addition, it also results in biochemical imbalances with high levels of oxidative stress (Cai et al., 2000;Khandhadia and Lotery, 2010), chronic inflammation (Nagineni et al., 2012) and endothelial dysfunction (Lip et al., 2001) that all contribute further to various ocular and systemic morbidities. Indeed, ocular diseases such as cataract (Haveman et al., 2011), glaucoma (Leske et al., 2008) and age-related macular degeneration (AMD) (Jonasson et al., 2011) occur with an increased frequency in elderly individuals. Systemically, ageing results in dilation, stiffening and thickening of the arteries as well as a reduction in cardiac function (Lee and Oh, 2010;Sawabe, 2010). Moreover, ageing is accompanied by a reduction in parasympathetic tone even in the absence of disease, resulting in a high risk for hypertension (Charkoudian and Rabbitts, 2009). The endothelium power to counteract the sympathetic-induced vasoconstriction is also diminished in the elderly, therefore contributing to the increased cardiovascular risk and mortality in these individuals. Moreover, a high blood pressure (BP) variability associated with an impaired ability of the body to counteract it, could also contribute to the higher risk for acute circulatory events in elderly population. This haemodynamic imbalance could affect multiple organs, including the eye.

The role of systemic circulatory disturbances in the aetiology of age-related ocular diseases resulting in blindness, such as AMD and glaucoma have been extensively studied (Caprioli et al., 2010;Choudhury et al., 2011;Gherghel et al., 2010;Sato et al., 2006a); however, changes in retinal microcirculation that occur with ageing in the absence of systemic disease have been addressed by far fewer investigating (Klein et al., 2007;Zaliuniene et al., 2008). Moreover, the effect of ageing on the vascular function in retinal circulation and its relationship to systemic circulatory abnormalities and biochemical imbalances has not been properly studied.

Therefore, gaining more knowledge in this area could be important for better prevention and early diagnosis of blinding diseases and their systemic vascular associations; in addition, a comprehensive study of the link between vascular microcirculatory changes with other systemic and biochemical markers for ageing-related disturbances would be necessary for patient-tailored preventive and/or therapeutic intervention to decrease the risk for morbidity and mortality in patients at risk. The purpose of the present work was to study these complex relationships in both healthy elderly individuals and in patients suffering from AMD.

For the purpose of interpretation of this thesis, understanding the anatomy and physiology of the systemic and ocular circulation and their regulatory mechanisms are required. A summary of these is also presented in the first part of this report.

1.2 Ageing and cardiovascular system's health

1.2.1 Anatomy of cardiovascular system

The present research concentrates on both systemic and ocular circulation in healthy elderly and in patients with AMD. Cardiovascular activity and BP in general are great

important for ocular circulation. Therefore, an understanding of the cardiovascular anatomy and physiology is necessary in this study.

The cardiovascular system is a part of the circulation system, which distributes bloods to the whole body. The main components of the cardiovascular system are the heart and blood vessels (arteries, capillaries and veins). The cardiovascular system includes two circulations:

- Pulmonary circulation: the heart (right side) receives deoxygenated blood from the body at low pressure and pumps it to the lungs.
- Systemic circulation: the heart (left side) receives oxygenated blood away from the lungs and pumps it at high pressure to the body.

The human heart as a pump is responsible for maintaining the blood circulation around the vascular network of the body. It consists of four chambers: the left and right atrium, and left and right ventricle (Figure 1.1). The right atrium receives deoxygenate blood from the body through the superior vena cava, and the deoxygenate blood is then ejected to the right ventricle, and then into the pulmonary artery and into the pulmonary circulation (Gray, 1985). After the pulmonary circulation, oxygenated blood returns to the left atrium through the pulmonary vein, and then is pumped to the left ventricle, aorta, and into the systemic circulation to supply oxygen and nutrient to all tissues and organs (Gray, 1985).

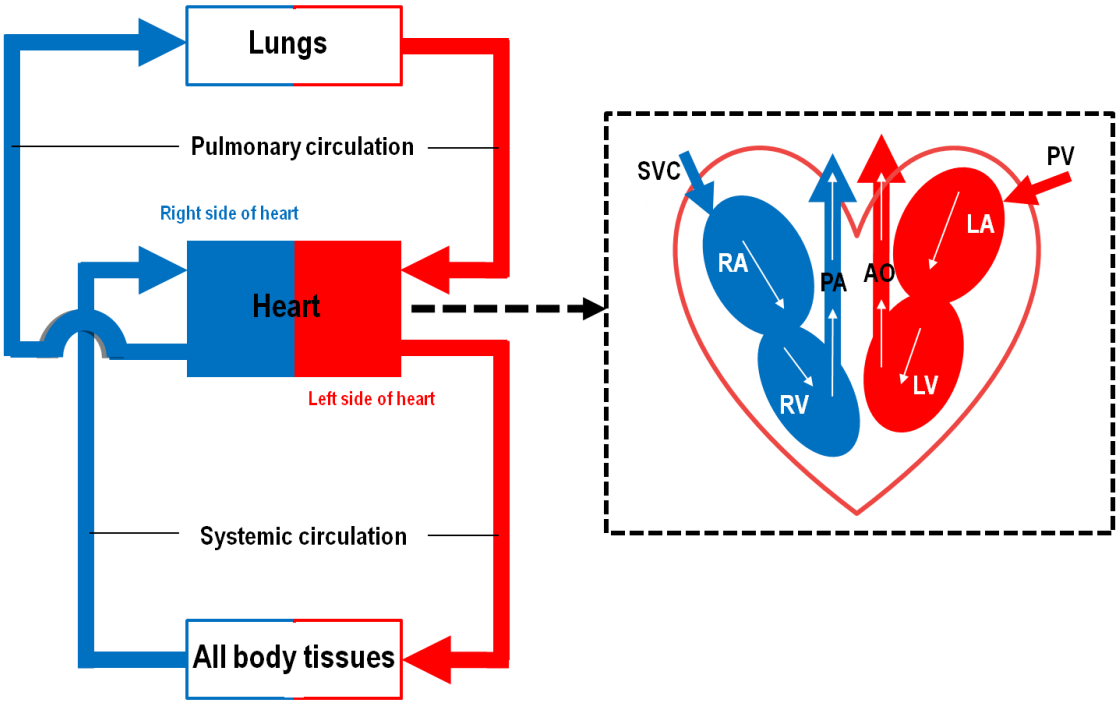


Figure 1.1: Systemic circulation shows red colour is oxygenated blood, and blue colour is deoxygenated blood. Adapted it according to Gray (Gray, 1985)**.** SVC: superior vena cava; RA: right atrium; RV: right ventricle; PA: pulmonary artery; PV: pulmonary vein; LA: left atrium; LV: left ventricle; AO: aorta.

1.2.1.1 Vascular structure

Systemic circulation is the part of the circulatory system which carries oxygenated blood away from the heart to the body, and returns deoxygenated blood back to the heart. The blood vessels acts as a key part of the vascular circulation, and play a vital role in the movement of blood throughout the body. The human vessels are divided to three varieties: arteries, capillaries and veins. In general, most human blood vessels usually consist of three histological tunics (membranes) from the outermost layer of the blood vessel inward. Anatomically, they are the tunica adventitia, the tunica media and the tunica intima (Figure1.2).

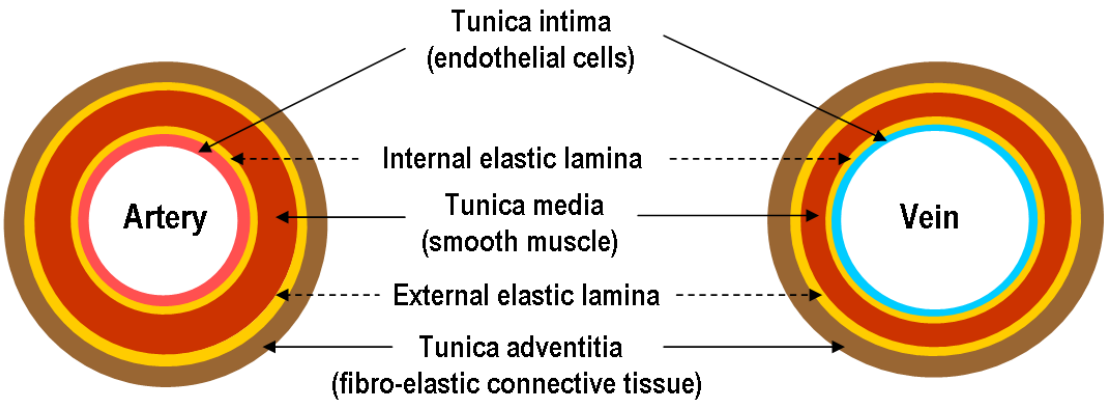


Figure 1.2: Diagram showing the layers of vessels. Adapted it according to Pugsley and Tabrizchi (Pugsley and Tabrizchi, 2000)**.**

Tunica adventitia The outermost layer of the blood vessel. It is almost entirely made of fibro-elastic connective tissue, which provides stability for blood vessels. It also contains nerves that supply the vessel (Pugsley and Tabrizchi, 2000).

Tunica media The thickest layer as mainly contains smooth muscle cells and elastin fibers. These cell layers tend to be more highly organized in larger arteries and play an important role in the movement of blood. Another layer that is common to this membrane of the blood vessel is an external elastic lamina, which provides structural support (Pugsley and Tabrizchi, 2000).

Tunica intima The innermost and thinnest constituent layer, and is made up of a single layer of endothelial cells and surrounded by an internal elastic lamina, which organized by a thin layer of sub-endothelial fibro-elastic connective tissue that provides flexibility and stability for endothelial cells (Pugsley and Tabrizchi, 2000).

The layer of endothelial cells forms the endothelium, and performs an important role in

maintaining vascular homeostasis (Kedzierski and Yanagisawa, 2001;Rubanyi and Botelho, 1991;Rubanyi, 1993;van Hinsbergh et al., 1983) through a number of important primary regulatory functions:

- Synthesis of collagen I, fibronectin, laminin and growth factors that contribute to tissue synthesis and catabolism.
- Production of intracellular adhesion molecules, vascular cell adhesion molecules, and E-selectin promoting leukocyte recruitment and activation.
- Hydrolysis of low-density lipoprotein in order to aid lipoprotein metabolism.
- Production and release of coagulant and anticoagulant factors as well as vasodilator and vasoconstrictor factors to aid haemostasis.

1.2.1.2 Coronary arteries

The coronary arteries are the first blood vessels which branch off from the aorta. The coronary arteries extend from the aorta to the heart walls supplying blood to the atriums, ventricles and septum of the heart. There are two main coronary arteries: right coronary artery and left coronary artery (Figure 1.3). The right coronary with one major branch (posterior descending artery) mainly supply oxygenated blood to right atrium and ventricle as well as the interventricular septum, in some cases they may also supply parts of the left atrium and left ventricle (Gray, 1985). The left coronary artery directs oxygenated blood to the two major branches: left anterior descending artery and left circumflex artery (Figure 1.3). The left anterior descending artery mainly nourishes the ventricles and left atrium as well as to the anterior portion of the interventricular septum; whereas the left circumflex artery mainly nourishes the ventricles and left atrium (Gray, 1985). The venous blood is drained via the coronary veins, which follow a distribution similar to the main coronary arteries.

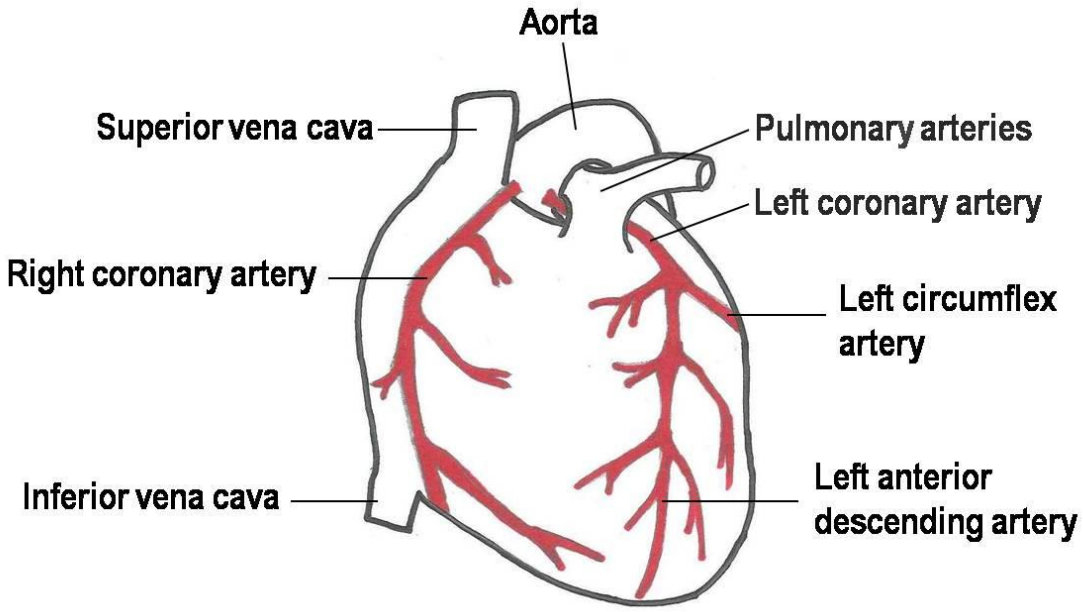


Figure 1.3: Vascular supply of heart via coronary arteries.

1.2.1.3 Common carotid arteries

Oxygenated blood originating from the pulmonary circulation, is pumped to the body via the aortic arch (a part of AO). The major arteries supplying the upper body are from the upper border of aortic arch, including the brachiocephalic artery, left common carotid artery and left subclavian artery (Gray, 1985). Indeed, the principal arteries of supply to the head and neck are the two common carotid arteries, one for each side. The left and right common carotid arteries follow the same course excepting their origin. The left common carotid artery originates from the aortic arch, and the right arises from the brachiocephalic artery (Gray, 1985). They ascend in the neck and each divides in two branches: the external carotid artery which supplies the exterior of the head, the face and the greater part of the neck; in addition, the internal carotid artery, supplying to a great extent the parts within the cranial and orbital cavities including the ocular circulation such as ophthalmic artery and its branches that supply all the structures in the orbit as well as

some structures in the nose and face (Gray, 1985). More details of ocular vessels supply will be described in Section 1.3.

The blood in ocular or cerebro-circulation through the local capillaries and microcirculation to drain into the internal jugular veins (left and right internal jugular veins) (Gray, 1985), which collect the deoxygenated blood from the head and neck as well as ocular venous drainage. Ultimately, those blood drain into the right atrium (RA) through the superior vena cava (SVC) (Gray, 1985).

1.2.2 Vascular physiology

Blood vessels play an important role to transport blood throughout the body due to their both elastic and conduit properties. The blood vessels, particularly arteries have a degree of ability to regulate their inner diameter by vasodilation and vasoconstriction of the smooth muscular layer, which is mediated by endothelial-dependent factors [55]. This changes the blood flow to downstream tissues and organs, and is controlled by the autonomic nervous system (ANS). Oxygen and other nutrients carried by the blood from the heart to arteries, and to the capillaries which enable the actual exchange of nutrients between the blood and the tissues, and then the veins carry the deoxygenated blood from the capillaries back toward the heart (Levick, 2000). This circulating blood exerts a force to the vascular walls that is also named BP, which is one of the principal vital signs.

1.2.3 Cardiac physiology

1.2.3.1 Cardiac cycle

The cardiac cycle consists in electrical and mechanical events that result in rhythmic atrial and ventricular contractions that pump blood into the systemic and pulmonary circulations (Lilly, 2003).

Electrical events The cardiac muscle, also called the myocardium, undergoes automaticity and rhythmic contractions coordinated by the sinoatrial node (SAN, also known as the cardiac pacemaker), which is located in the upper wall of the right atrium (Gray, 1985). In the normal human adult heart, the SAN generates oscillations of approximately 70 beats per minute. From SAN, the electrical excitation spreads over the atria and when it reaches the junction between the atria and the ventricles, it excites another electrical centre named the atrioventricular node (AVN). The ventricular contraction is the result of transmission of the excitatory signal along a bundle of modified cardiac fibres known as the Purkinje bundle (Levick, 2000).

Mechanical events The ventricular contraction (systole) is the beginning of the cardiac cycle. As the ventricles start contraction, the inside pressure of ventricles increased rapidly and soon exceed the inside the aorta and pulmonary artery, leading to the blood is ejected into the pulmonary and systemic circulations (Levick, 2000). After the ejection, the pressure decrease and ventricles relax and refilled with blood, the process referred to as diastole; then the cycle repeats itself.

1.2.3.2 Autonomic control of cardiac rhythm

The heart is innervated by both the sympathetic and parasympathetic divisions of the ANS; heart rate (HR) monitoring is thus a reflection of their modulating effect on the

cardiac pacemaker cells (Goldberger, 1999). During the day, there is an augmentation of sympathetic tone, while vagal (parasympathetic) tone is highest during resting conditions (Goldberger, 1999). Sympathetic activation results in an increase in HR, conduction system velocity and contractility (Freeman, 2006;Stiell et al., 1992), while parasympathetic innervation slows the HR via the direct activation of acetylcholine (Pumprla et al., 2002).

1.2.4 The electrocardiogram (ECG)

The electrocardiogram (ECG) is a non-invasive technique to measure cardiac activity. It provides the information about both on cardiac structure and function.

1.2.4.1 Sequence of normal cardiac activation

Each cardiac cycle (heart beat) is shown by an ECG, consists of three major parts (Levick, 1995) (Figure 1.4):

- **P wave** is the first positive wave of the cardiac cycle. It represents atrial contraction (depolarization).
- **QRS complex** represents the rapid contraction of the ventricles/ventricular muscle cells. A typical QRS complex includes three continues waves: *Q-wave* is the first downward wave of the QRS complex; followed by an upward *R-wave* which is a narrower and taller wave; and *S-wave* is defined as a downward wave following the R-wave (Lilly, 2003).
- **T wave** represents the recovery (repolarization) of the ventricles.

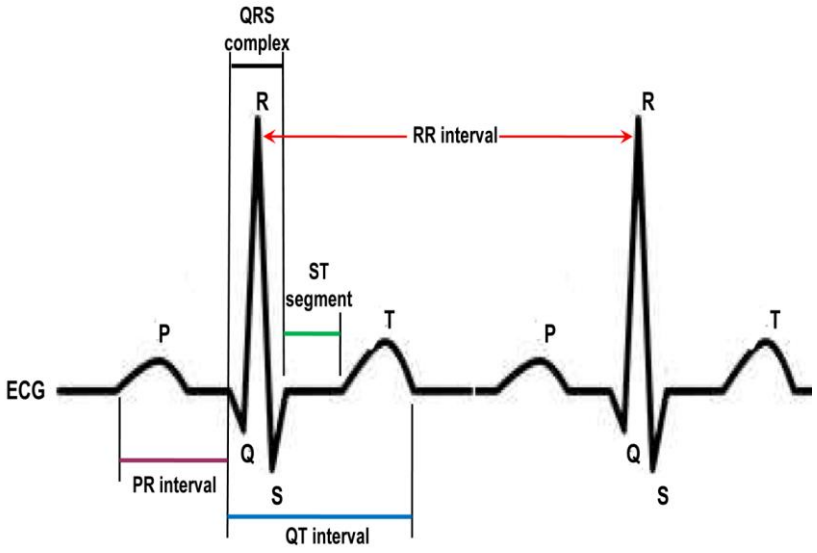


Figure 1.4: The cardiac cycle is expressed by the ECG graph. ECG: electrocardiogram**.** PR interval: the time measured from the beginning of the P wave to the beginning of the QRS complex; RR interval: the time measured between an R wave and the next R wave; QT interval: the time from the beginning of the QRS complex to the end of the T wave.

1.2.4.2 Technical considerations

ECG is recorded on either a paper or computer monitor. The ECG graph represents with time on horizontal (X) axis and voltage on vertical (Y) axis. The graph paper has a background pattern of 1mm squares. As at a paper speed of 25mm/s, therefore each 1mm horizontal line separation represents 0.04 seconds. The voltage is expressed in millivolts (mV), and each 1mm vertical line separation represents 0.1 mV (Figure 1.5).

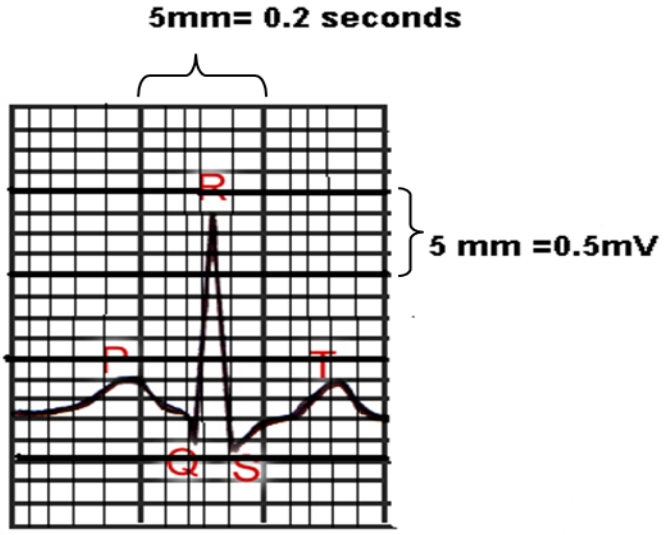


Figure 1.5: An electrocardiogram strip. Each 1 mm on the vertical axis represents 0.1 mV and each 1mm on the horizontal axis represents 0.04 seconds.

1.2.4.3 ECG analysis

ECG is altered whenever there is a cardiac abnormality or disease. Therefore, a careful analysis is crucial for detection of pre-clinical and/or clinical conditions. The below parameters can be accessed through the ECG (Lilly, 2003):

- HR is the number of heartbeat in one minute. It is calculated according to one of the formulas (Equation 1.1) through the ECG:
- Heart rhythm: the normal heart rhythm is represented at HR between 60-100 beats/minute which is referred as a normal sinus rhythm.
- Major waves and their abnormalities.
- Intervals (PR, QRS complex, QT) (Figure 1.4). Normal PR interval is between 0.12 and 0.2 sec; QRS complex is less than 0.1 sec; QT interval is between 0.3 and 0.43 sec, or < ½ RR interval (Lilly, 2003).

- ST-segment and T wave abnormalities. These occur whenever there is a difference between myocardial oxygen demand and supply (myocardial ischaemia) and are represented by:
    - ST depressions, in subendocardial ischaemia and metabolic abnormalities;
    - ST elevations, in cardiac infarct and pericarditis (inflammation of the pericardium).

$$HR = \frac{25\text{mm/sec} \times 60\text{ sec}}{\text{Number of mm between 2 consecutive beats}}$$

$$HR = \frac{1500}{\text{Number of small squares between 2 consecutive beats}}$$

HR: heart rate (beats/minute)

Equation 1.1: The calculation of heart rate through an electrocardiogram strip.

1.2.5 Systemic blood pressure

During the contraction (systole) and expulsion of the blood from the left ventricle, the aortic pressure rises from its resting value of about 80 mm Hg (DBP-diastolic BP) to about 120 mm Hg (SBP-systolic BP). It is this pressure that is measured with a sphygmomanometer. However, these values can vary due to various factors such as (Sunthareswaran, 2002):

- Ageing; this causes an increase in BP due to a concomitant decrease in arterial compliance. As a rule, SBP is equal to 100 mmHg plus age in years;
- Sleep;

- Exercise can increase BP due to either increase in cardiac output or as a result of the pressor response;
- Stress increases BP due to a higher sympathetic tone during periods of stress.

In 1978, Millar-Craig et al. first described a circadian pattern of BP variation (Millar-Craig et al., 1978). The classic description states that in healthy young subjects after waking, SBP rises rapidly by 20 to 25 mmHg and DBP by 10 to 15 mmHg, throughout the rest of the day levels are then highest in the late afternoon, beginning to decline in the evening and attaining lowest levels during sleep (Baumgart, 1991;Hermida et al., 2007;Smolensky and Haus, 2001). In addition, recent data also shows that BP exhibits two peaks in day-time: one around 9am and one around 7pm; and also men exhibit higher BP and lower HR than women (Hermida et al., 2007). This variation in systemic BP is due to mainly to external factors, such as physical activity, temperature, emotional state, diet, consumption of alcohol and caffeine as well as sleep-night routine (Baumgart, 1991;Hermida et al., 2007). Several attempts have been made to eliminate these external influences and determine the “true” circadian variation in BP. Previous studies reported that subjects during 24-h of bed rest and found that the circadian rhythm of BP had a smaller amplitude compared to those reported under normal, ambulatory conditions (Mann et al., 1979;van den Meiracker et al., 1988). And later reports found that subjects confined to 24-h bed rest without sleep and with meals distributed evenly across a 24 hour period, there was no circadian rhythm in BP (Kerkhof et al., 1998;Van Dongen et al., 2001). Nevertheless, the effect of endogenous variables such as variations in the ANS activity, and in the levels of biological active substances should not be neglected. It has been shown that plasma levels of catecholamines (Akerstedt and Froberg, 1979) and muscular sympathetic nerve activity (Somers et al., 1993) decrease at night. Moreover, there is a decrease in plasma cortisol, resulting in reduced vascular sensitivity to catecholamines (Reis, 1960). The nocturnal reduction in BP could further exacerbated by a lower venous return due to translocation of blood to the peripheral circulation (Cranston, 1964) and the effects of the

recumbent posture typically adopted during sleep which suppresses renal sympathetic activity and the renin-angiotensin system, and facilitates sodium and water excretion (Takakuwa et al., 2001).

1.2.6 Vascular function

Vascular tone/function is mediated by vascular smooth muscle cells, and mostly is influenced by a range of endothelium-derived factors through vasodilation and vasoconstriction. It has been shown that endothelium not only regulates vascular tone, but also plays a role in the coagulation processes, maintaining vascular structure, and mediating inflammatory and immunological responses (Davies and Hagen, 1993;Rubanyi and Botelho, 1991). Vasoactive, endothelium-derived factors include prostanoids, nitric oxide (NO) and NO-containing compounds, smooth muscle cell hyperpolarization factors, and endothelin. Furthermore, the local renin-angiotensin system in the vessel wall is also involved in vasomotor regulation (Blazquez-Medela et al., 2010). These endothelium released factors can be divided into endothelium-derived relaxing factor and endothelium-derived constricting factors (Dorner et al., 2003).

1.2.6.1 Endothelium-derived relaxing/dilating factor

Production of the so-called endothelium-derived relaxing factor (EDRF) was first described by Furchgott and Zawadzki in 1980 (Furchgott and Zawadzki, 1980). They observed that the vasodilation induced by acetylcholine is dependent on the presence or absence of vascular endothelium. They concluded that the endothelium produces an EDRF, and plays a key role in the effects of acetylcholine on vascular tone. Recently, it has been proved that EDRF-mediated vasodilation response is present also in the rat retinal arterioles (Mori et al., 2011).

Initially believed to be the same as EDRF, NO was also recognized for its vasodilation properties (Palmer et al., 1987) and its roles in systemic and ocular vascular physiology have been investigated in depth (Dorner et al., 2003;Haefliger et al., 1994a;Haefliger et al., 1994b;Kiss et al., 1999;Koss, 1999;Schmetterer and Polak, 2001).

The NO has a very short half-life, and is synthesized from oxygen and L-arginine by NO synthases (NOS). Three different isoforms of NOS have been identified including endothelial isoform (endothelial NO synthase, eNOS), neuronal isoform (nNOS) and inducible isoform (iNOS) (Arnal et al., 1999). NO release could be regulated by shear stress (Arnal et al., 1999) which described as a frictional force acting in the direction of blood flow on the surface of endothelium, and pressure-stretch which acts perpendicularly to the vascular wall and affects endothelial cells and smooth muscle cells (Michiels, 2003). The increase in blood flow that can induce the shear stress and leads to an increased NO release from endothelial cells (Xiao et al., 1997). Shear stress causes a rapid eNOS activation and upregulates eNOS gene expression by transcription activation of the eNOS promoter (Xiao et al., 1997).

NO also can diffuse to the vascular smooth muscle cells where it stimulates soluble guanylate cyclase (GC) resulting in an accumulation of cyclic guanosine monophosphate (cGMP) and subsequent vasodilaton (Denninger and Marletta, 1999). NO also play a key role to control the blood pressure, as verified that infusion of a NOS inhibitor such L-NMMA, can increases the blood pressure through the regulation of vasodilation and vasoconstriction (Fleming et al., 1996).

Other roles of NO include:

- Anti-oxidative and neuroprotective effects by interaction with reactive free radicals (Mastrodimou et al., 2008;Tsuda, 2009).
- Mediating toxicity after excess glutamate release (Osborne et al., 1999).

- Inhibition of the rolling and adhesion of leukocytes in micro-vessels (Hickey, 2001).
- Regulation of intestinal motility (Huang et al., 1993).
- Role in penile erection (Chuang et al., 1998).

Since NO has such complex and varied roles, any disturbances in its homeostasis could have important consequences. Indeed, it has been shown that systemic NO deficiency can result in (Avogaro et al., 2011;Bhutto et al., 2010;De Caterina et al., 1995;Kaur and Halliwell, 1994;Pigozzi et al., 2011):

- Excessive vasoconstriction
- Increased oxidative stress
- Inflammation
- Platelet aggregation and thrombosis
- Leukocyte activation and infiltration

There has been extensive research into the role of NO on ocular circulation (Dorner et al., 2003;Kiss et al., 1999;Koss, 1999;Mastrodimou et al., 2008;Schmetterer and Polak, 2001;Totan et al., 2001). In animal studies, NO has been able to modulate the contractile tone of retinal pericytes (Haefliger et al., 1994b) and Müller cells (Kawasaki et al., 1999). The human ocular angioarchitecture is, however, different from that of other species. Human experiments indicate that the arterioles and capillaries are in a constant state of vasodilatation, which is maintained by continuous release of NO (Dorner et al., 2003;Kiss et al., 1999;Schmetterer and Polak, 2001).

1.2.6.2 Endothelium-derived constricting factors

Aside their roles in vascular relaxation, endothelial cells also produce potent vasoconsticting substances. The most potent endothelial vasoconstictive factors are endothelins (ETs), a group of 21-amino acid peptides. The endothelins family consists of

three isoforms, which are ET-1, ET-2 and ET-3. Endothelins are not only produced by endothelial cells (Pernow and Wang, 1997), but can be also generated from vascular smooth muscle cells (Resink et al., 1990) and cardiomyocytes (Suzuki et al., 1993). ET-1 generated by vascular endothelial cells has vasoactive properties that affect both systemic and peripheral circulation (Angerio and Kot, 1997).

There are two major classes of endothelin receptors in the blood vessels: endothelin$_A$ (ET_A) and the endothelin$_B$ (ET_B) receptors. The ET_B has been further subdivided into ET_{B1} and ET_{B2} based on their pharmacological responses (Leite-Moreira and Bras-Silva, 2004). In vascular smooth muscle cells, the activation of ET_A receptors evokes marked and sustained vasoconstriction, while ET_B could mediate NO production. ET-1 and ET-2 have a similar affinity for the ET_A subtype, whereas ET-3 has much lower affinity for the ET_A receptor than for ET_B. Binding ET-1 causes vasoconstriction through activation of ET_A which expressed in smooth muscle cells (Kedzierski and Yanagisawa, 2001;Michiels, 2003). Binding to ET_B receptor can mediate ET-1-induced NO release from endothelial cells and an accompanying vasodilation response, which opposes the vasoconstricting action of ET. This explains the transient vasodilation that usually occurs prior to the vasoconstriction (Lanzl et al., 2011).

In the choroid, both ET_A and ET_B receptors have been found, and could be involved in the modulation of choroidal blood flow (MacCumber and D'Anna, 1994;Wollensak et al., 1998). In choroid, the interaction between ET_B receptor-mediated NO vasodilation and ET_A receptor-mediated ET vasoconstriction could occur, although choroidal blood vessels appear to be more sensitive to NO than ET in overcoming resistance (Kiel, 1999).

1.2.7 Pathophysiology of vascular disease - endothelial dysfunction

Endothelial dysfunction refers to an impairment of endothelium-dependent vasodilation caused by a loss of NO bioavailability in vessels wall (Virdis et al., 2010). This endothelium-dependent vasodilation represents a protective mechanism against time-dependent changes in the body's haemodynamics; any disturbances in this mechanism could, therefore, represent an important risk factor for circulatory ischaemic events (Giraldez et al., 1997;Shaw et al., 2001;Zeiher et al., 1995). Indeed, endothelial dysfunction indicates a generalised alteration in cells function resulting in an imbalance of vasodilation and vasoconstriction, impaired endothelial control of inflammation and fibrinolysis and abnormal expression of vascular adhesion molecules (Michiels, 2003;Virdis et al., 2010).

There are a number of factors that can contribute towards endothelial dysfunction. These include an insufficient NO release or bioavailability, overproduction of reactive oxygen species (ROS) or ROS are insufficiently degraded that leads to an accelerated inactivation of NO (Cai and Harrison, 2000). In addition, high levels of vasoconstrictors (Donato et al., 2009;Loot et al., 2009;Rubanyi and Botelho, 1991;Szabo et al., 2004) (ET-1, angiotension II) can also play a role. The resultant endothelial dysfunction has major consequences on the vascular health that in turn further down-regulates the endothelial function (Schram and Stehouwer, 2005). Ultimately, endothelial dysfunction triggers a set of chain reaction causing biochemical changes that finally lead to vascular structural changes and overt systemic vascular diseases (Bartolomucci et al., 2011;Bartolomucci et al., 2011;Pigozzi et al., 2011;Rubanyi, 1993).

1.2.8 Endothelial dysfunction and arterial stiffness

Arterial stiffness is often used to describe the loss of intrinsic elastic properties of the arteries. Endothelial dysfunction is considered an initiating event in adhesion molecule expression, leading to leukocyte recruitment into the subendothelial space. In the intimal area, macrophage lipid accumulation and secretion of pro-inflammatory mediators, and together with vascular smooth muscle cells proliferation and their intimal infiltration that accelerate the process of atherosclerosis and arterial stiffness (Nedeljkovic et al., 2003).

The term ‘arterial compliance’ refers to the ability of large arteries to distend in response to a given pressure. The aorta and its major branches act as an elastic reservoir, buffering pulsatile flow from left ventricular ejection, and ensure a smoother, less-pulsatile flow to reach the peripheral vasculature (Oliver and Webb, 2003). Most of the arterial tree has both elastic and conduit properties, although one or other tends to predominate in any given arterial segment. For example, the ascending aorta is predominantly elastic and the peripheral, more muscular, arteries are predominantly conduit vessels. Any abnormality in arterial compliance could represent a marker of arterial stiffness and, therefore, repositions the site of pulse wave reflections. As the blood from the heart travels towards to a peripheral artery of decreasing diameter, a reflected force (wave) is created and towards to the heart (Oliver and Webb, 2003). When the arteries are compliant (e.g. a normal healthy young subject), the reflected arterial waves arrive at the central aorta during late systole to diastole (Figure 1.6), thus augmenting DBP and coronary artery filling (Oliver and Webb, 2003). As the large arteries stiffen (e.g. advanced age and cardiovascular risk factors), wave reflection occurs earlier so that SBP is augmented and DBP reduced (Figure 1.6). Therefore, this reflected pulse pressure augmentation could represent an indicator of arterial stiffness, and can be measured using the SphygmoCor device and pulse wave analysis (PWA). More details about PWA are offered in Section 2.3.4.

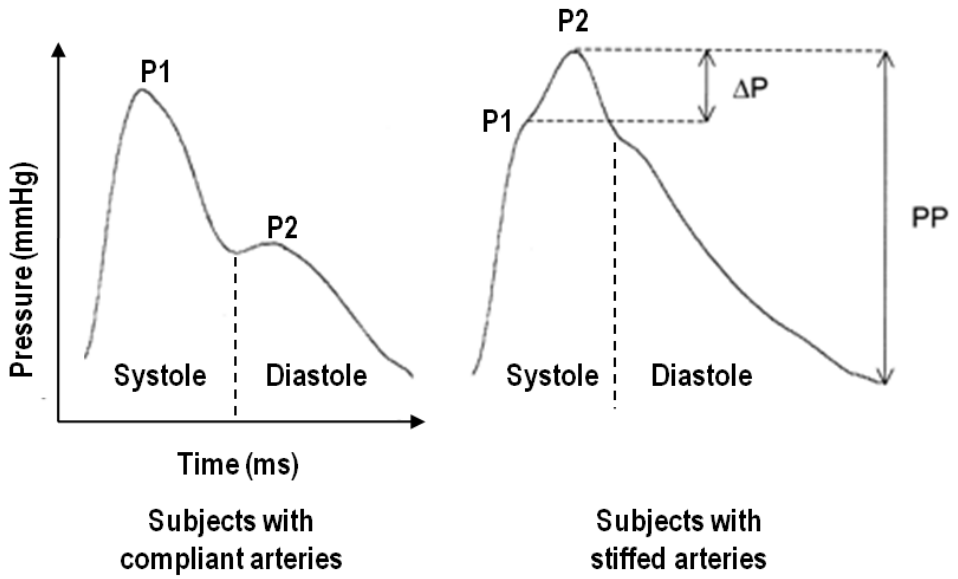


Figure 1.6: Representation of reflected pulse pressure augmentation in subjects with and without arterial stiffness. When the large arteries are compliant the initial systolic pressure wave, P1, traveling from the heart to the periphery, is responsible for peak SBP. P2, is reflected pressure waves, arrive at the central aorta in diastole, augmenting DBP and coronary artery filling. Whereas large arteries stiffen, wave reflection occurs earlier and therefore SBP is augmented and DBP falls. PP: pulse pressure; ∆P: the difference between P2 and P1, represents for the pulse pressure augmentation. In general, ∆P is negative in healthy young subjects, but is positive in subjects with advanced age or increasing cardiovascular risk and arterial stiffness.

1.2.9 Autonomic nervous system (ANS)

The ANS, along with the endocrine system with which it is closely allied, controls the body's internal environment. Its name, 'autonomic' indicates that it is almost entirely independent of conscious control. The ANS is divided into two primary systems, the sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS), which differ in anatomy, pharmacology, and behavioural outcome. The SNS, first studied by Bernard in 1849 (Alquier and Kahn, 2004) readies the organism for emergency and

conflict, whilst PSNS is primarily concerned with the conservation of energy and the maintenance of the body during periods of rest. The ANS is also under the influence of other important substances and mediators, such as peptides, cytokines and NO that together with the hormonal connections controls most of the organs of the human body, including the eye (Trachtman, 2010).

The ANS function can be measured using a number of methods (Table 1.1). For the purpose of this thesis, the heart rate variability (HRV) was employed for measurement of ANS function and is described in Section 2.3.2.

Technique	Description
heart rate variability (HRV)	• Frequency-domain analysis of HRV (HF and LF parameters) (Iellamo et al., 2006); • Time-domain analysis of HRV-analysis or RR intervals (Yildiz et al., 2011).
Provocation tests	• Valsalva maneuver (Hetzel et al., 1999); • Pressor drug infusion (Goldberger, 1999) (Goldberger, 1999)
Other techniques	• Plasma levels of norepinephrine for the overall level of sympathetic activity (Cryer, 1977); • Microneurography (Hagbarth, 1979); • Cardiac norepinephrine spillover for the sympathetic firing rate in the heart (Esler, 1993); Blood pressure variability (Lanfranchi and Somers, 2002).

Table 1.1: Autonomic assessment tests. HF: high frequency; LF: low frequency.

1.2.9.1 Interaction between ANS and endothelial function: the vascular tone

In healthy individuals, the ANS works closely with the vascular endothelium to insure a stable vascular tone. In response to local factors, the vascular endothelium continuously releases vasodilating factors (e.g., NO, endothelium-derived hyperpolarizing factor, prostacyclin, substance P, acetylcholine) which counteract the vasoconstrictor effect of the sympathetic activation (Harris and Matthews, 2004). The balance between these opposing forces acts on the vascular smooth muscle cells to maintain the appropriate vascular tone (Burnstock, 1990). Although the vascular endothelial cells do not receive direct ANS innervation, it still can be influenced through the activation of its own alpha2- and beta- adrenoreceptors that results in NO release and vasodilation (Guimaraes and Moura, 2001). In addition, the PSNS can contribute to acetylcholine release and vasodilation (van Zwieten and Doods, 1995), while the SNS can stimulate the release of endothelium-derived vasoconstrictors such as endothelin-1 (ET-1) (Bacic et al., 1992;Harris and Matthews, 2004) (Figure 1.7). More recently study has demonstrated that nNOS also contributes directly to the regulation of human vascular tone (Seddon et al., 2008). Maintaining a balance between all these factors is crucial for an appropriate perfusion with blood and, any disturbance that occurs in either ANS or endothelial function is ultimately reflected in impairments that occur in both systems (Harris and Matthews, 2004). Indeed, some diseases such as glaucoma, cardiovascular disease, hypertension and congestive heart failure, have been associated with both abnormalities of ANS regulation (Edwards et al., 2011;Gherghel et al., 2007;Lensch and Jost, 2011) and abnormalities of endothelial function (Hornig et al., 1996;Li et al., 1997;Resch et al., 2009;Su et al., 2008). It is difficult to determine however whether a dysfunction in one system may have driven a dysfunction in the other and if so which occurred first, or whether both systems have developed a dysfunction independently as part of the disease process. In young patients with essential hypertension, both ET-1 levels and sympathetic

activation were higher (Kuklinska et al., 2010). Moreover, in patients with congestive heart failure, studies have found that ET-1 levels correlate negatively with some measures of HRV (Aronson et al., 2001). Nevertheless, other external influences, such as the level of oxidative stress and the ageing effect on the vascular function, should also be considered when studying the complex interaction between ANS and endothelial function in health and disease. Indeed, an altered vascular tone may result in higher vulnerability of various organs including the eye to damage from free radicals or inflammatory stress (Hollyfield et al., 2008).

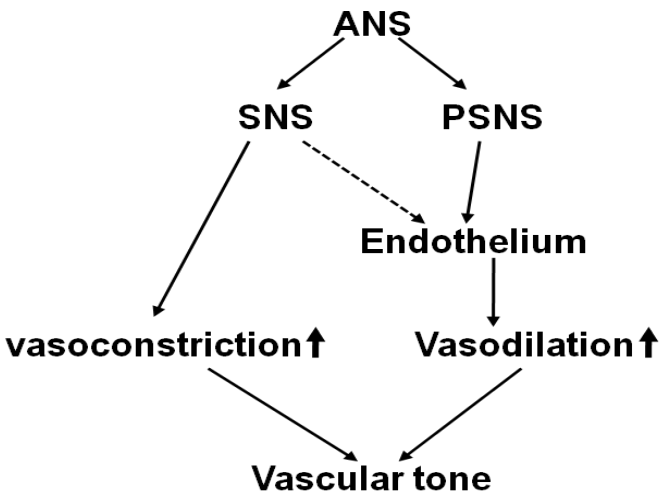


Figure 1.7: The interactions between the ANS and endothelium to regulate vascular tone. ANS: autonomic nervous system; SNS: sympathetic nervous system; PSNS: parasympathetic nervous system.

1.2.10 Ageing effect on cardiovascular system

Ageing associates with a redox imbalance manifested as high levels of oxidative stress (Jones, 2006a;Junqueira et al., 2004), inflammation and endothelial dysfunction (Ogita and Liao, 2004) and, therefore, confers a background for functional and structural disturbances in both macro- and microcirculation in subjects at risk (Nedeljkovic et al.,

2003). Indeed, dilatation, stiffening, and thickening of the arteries as well as a reduction in cardiac functions are widely reported as ageing-associated changes of the vasculature (Lee and Oh, 2010;Sawabe, 2010). Although few previous reports suggested that sympathetic vasoconstrictor outflow increases with age (Charkoudian and Rabbitts, 2009;Fagius and Wallin, 1993), more studies demonstrated a decrease in both PSNS and SNS controlled function including cardiovascular function are one of the major characteristic of senescence (Amano et al., 2001;Antelmi et al., 2004;Bigger et al., 1996;Choi et al., 2006;Corino et al., 2007;Hrushesky et al., 1984;Oida et al., 1999;Yu et al., 2010). The endothelium power to counteract the sympathetic-induced vasoconstriction is also diminished in elderly, therefore contributing to the increased cardiovascular risk and mortality in these individuals. All this changes are reflected by depressed HRV and an increased BP variability in older individuals (Monahan, 2007). HRV depression reflects into a lower capacity physical activity; however, as physical activity is important in maintaining a good HRV during ageing (De Meersman and Stein, 2007;Tsuji et al., 1994), the entire process enters a vicious circle. Moreover, a high BP variability associated with an impaired ability of the body to counteract it, could also contribute to the higher risk for acute circulatory events in elderly population. This haemodynamic imbalance could affect multiple organs, including the eye. This hypothesis will be discussed later in the present thesis.

Previous studies demonstrated that a decreased NO production due to ageing was associated with various risk for vascular pathologies (Donato et al., 2009;Jin and Loscalzo, 2010;Vallance and Chan, 2001). Indeed, in physiological quantities, NO has important protective roles; however, in large quantities it can also be toxic, being included (together with peroxynitrite -$ONOO^-$) in the so-called reactive nitrogen species (RNS), which represent very strong oxidant and can accelerate damage associated with ageing. The role of oxidative stress in vascular pathologies will be discussed in Section1.2.11.

1.2.11 Oxidative stress and vascular function

In 1956, Denham Harman suggested that free radical-induced oxidative stress is produced during aerobic respiration that causes cumulative oxidative damage to cells and tissues (Harman, 1956), resulting in ageing and mortality (Beckman and Ames, 1998). Generally, free radicals are defined as unpaired electrons of some molecules and atoms. Free radicals are unstable and highly reactive because the unpaired electrons tend to form pairs with other electrons. The free radical theory is only concerned with free radicals such as superoxide anion (O_2^-) strictly (Harman, 1956). Indeed, one and two electrons reduction of O_2 generates O_2^- and hydrogen peroxide (H_2O_2); further, O_2^- and H_2O_2 together can generate the hydroxyl radical (OH^-) (Beckman and Ames, 1998). Therefore free radical has been enlarged to include oxidative damage from H_2O_2 and OH^- refers to ROS.

As ROS are always generated during the organism respires in an aerobic environment and highly reactive, it could damage various molecules in the human body. The target elements include cellular proteins and lipids, as well as DNA (deoxyribonucleic acid) causing biological error that could induce to apoptosis and therefore, accelerate ageing process (Kelly, 2011).

In fact, an antioxidant defence could against the harmful action of ROS. Cells can synthesize some enzymes to circumvent ROS such as superoxide dismutase (SOD) and glutathione peroxidase, small molecule antioxidants such as glutathione, as well as other antioxidants are obtained from nature through nutrition such as vitamin C and E, and carotenoids (Junqueira et al., 2004). These act as antioxidants can prevent or repair oxidative damage on cells and tissues of body.

However, if ROS are produced excessively or antioxidants are defective, the balance between formation and removal is broken, resulting in oxidative stress (Junqueira et al., 2004). Consequently, excessive ROS can attack cells and tissues (Stadtman, 2006), thus causing various age-related diseases at both ocular (Beatty et al., 2000;Winkler et al., 1999) and systemic levels (Higashi et al., 2009). For example, AMD, which is the due to oxidative stress-induced angiogenesis (the growth of blood vessels from pre-existing vasculature), and leading to blood and protein leakage from the neovascular (new growth of small blood vessels) below the macular. Therefore, oxidative stress could refer as a 'harmful state of the body', which arises when oxidative reactions exceed antioxidant reactions due to the imbalance between them.

Oxidative stress is defined by the presence of pathological levels of ROS relative to the antioxidant defence. Excessive production of ROS can lead to DNA mutation, lipid peroxidation, protein damage and ultimately cell death via necrosis or apoptosis (Carmody and Cotter, 2001;Ueda et al., 2002). This Oxidative stress can induce endothelial dysfunction, leads to a series of reaction causing adhesion molecule expression and secretion of pro-inflammatory mediators, consequently influence vascular tone and function (Nedeljkovic et al., 2003).

In the late 1980s, in the course of defining the mechanisms of endothelial control of blood flow (Castro and Freeman, 2001), another free radical species, NO, was described (Mulsch et al., 1992). It has also been shown that excessive production of NO can be cytotoxic. The rapid reaction between NO and O_2^- yields peroxynitrite ($ONOO^-$) an extremely potent oxidizing species (Koppenol et al., 1992). Peroxynitrite, referred to as RNS, is assumed to react preferentially with CO_2 *in vivo* to produce nitrogen dioxide (NO_2) and trioxocarbonate radicals. It also nitrates tyrosine residues of the proteins, stimulating the production of 3-nitrotyrosine (3-NT), which disrupts the normal function of the proteins.

Nitric oxide and secondary species derived from it are known as RNS (Castro and Freeman, 2001).

1.2.11.1 Reactive species in human pathology

In certain situations free radicals can be generated in an exaggerated manner and can injure tissues and organs by interacting with their anatomy, physiology or genetic activity (Valencia et al., 2002). Oxidative stress has been implicated in a large number of human diseases including different autoimmune diseases (Ahsan et al., 2003;Buhl et al., 1989), alcoholic liver disease (Videla and Guerri, 1990), cancer (Beutler and Gelbart, 1985), as well as in ischaemia-reperfusion injury (Ferdinandy and Schulz, 2003;Nanetti et al., 2011). Oxidative stress also results in vascular endothelial dysfunction (Ashfaq et al., 2008;Nedeljkovic et al., 2003;Ogita and Liao, 2004;Zalba et al., 2007). It has been demonstrated that patients with poor endothelial function are at increased risk for developing cardiovascular and cerebral ischaemic events (Cai and Harrison, 2000;Pigozzi et al., 2011;Zalba et al., 2007).

1.2.11.2 Oxidative stress and ageing

Ageing is a process of degeneration of tissues with a decrease in cell population, which could lead to a decline in organ function over time and an increase in the risk of development of disease, even death (Harman, 2001). These senescent changes can contribute to the development of genetic defects, disease and the innate ageing process; those are the risk factors for disease and death especially in the developed countries (Harman, 2006). There are many theories have been considered for the ageing process, such as, telomere shortening (Kruk et al., 1995), senescence genes in the DNA (Harman, 2006) and degeneration of proliferation capability of cells, as well as oxidative stress causing harmful accumulation as a toxic factor to reduce the life span of the cells and

organism and further accelerate ageing process (Harman, 1993;Harman, 2006). However, not a single theory can explain all the mechanisms of ageing, some studies reported that free radial-induced oxidative stress plays a vital role for ageing process and ageing-related pathologies (Beckman and Ames, 1998;Harman, 2006;Junqueira et al., 2004).

This oxidative stress-induced endothelial dysfunction may further result in vasoconstriction (loss of vasodilation), platelet aggregation, inflammation, vascular collagen and protein synthesis due to insufficiency of NO bioavailability effecting on vascular tone and function (Nedeljkovic et al., 2003) and therefore, influences arterial stiffness (Zieman et al., 2005) (Figure1.8). Therefore, oxidative stress also plays an important role to vascular tone function.

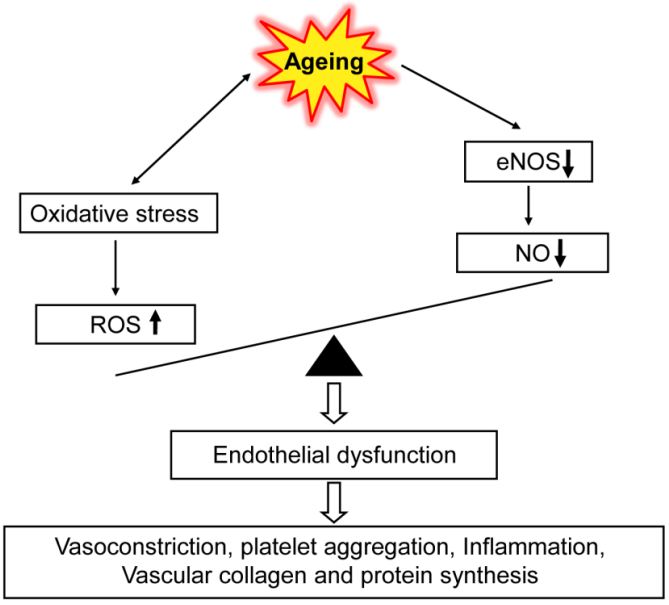


Figure 1.8: Representation of a relationship between oxidative stress and NO, and their mediated actions on vascular function. ROS: reactive oxygen species; NO: nitric oxide; eNOS: endothelial NO synthase.

1.3 Anatomy of ocular vessels

1.3.1 Retrobulbar vascular supply

The retrobulbar ocular vessels (Figure 1.9) are comprised of ophthalmic artery (OA), central retinal artery (CRA) and ciliary arteries. The OA, which represents the first branch of the internal carotid artery (ICA), provides most of the blood supply to the orbit and to some of the surrounding scalp (Wang et al., 1998). It enters the orbit through the *optic foramen*, below and lateral to the optic nerve and then passes over the nerve to reach the medial wall of the orbit. From this point the OA is situated horizontally, beneath the superior oblique muscle.

The CRA represents a direct branch of the OA. It enters the medial aspect of the optic nerve approximately 5-15 mm behind the globe; then it travels inside the optic nerve before appearing at the surface of the optic disc. The CRA provides four retinal arterioles (one for each quadrant of the retina), and several small branches within the anterior optic nerve.

The ciliary arteries are divided in three groups: short posterior, long posterior and anterior ciliary arteries. In normal population there are 1 to 5 posterior ciliary arteries (PCAs); they branch from the OA and are then grouped into medial and lateral trunks situated either side of the optic nerve. Each main PCA divides into approximately 10 to 20 short PCAs just before or after entering the posterior sclera (Harris et al., 1998). They supply the choroid, and ciliary processes. The anterior ciliary arteries leave the OA and run a short course within the *rectus* muscles bellies; then travel forward and reach the anastomotic circle in the ciliary muscle and the major arterial circle of the iris, supplying mainly the anterior uvea (Buchi, 1996).

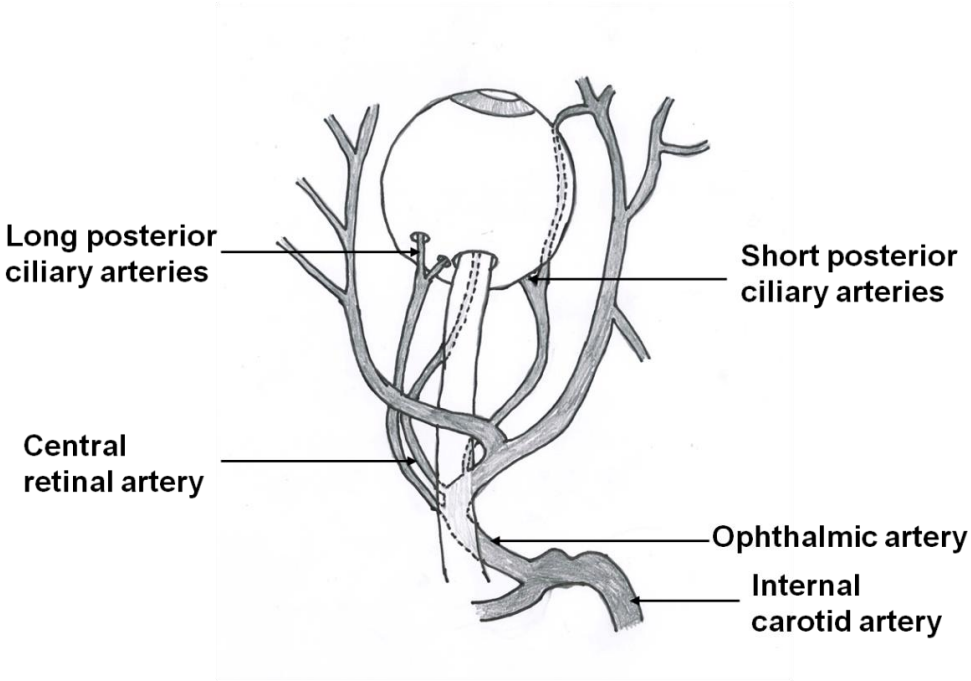


Figure 1.9: The anatomy of ocular blood arteries. Adapted it according to Snell and Lemp (Snell and Lemp, 1998).

1.3.2 Retinal circulation

Retinal vessels are supplied by the CRA, which branches to form four retinal arterioles, one for each quadrant of the retina (Gray, 1985). These arterioles are situated within the nerve fibre layer (NFL) and give rise to an extensive capillary network that supplies the inner two thirds of the retina.

There are strong resemblances between the retinal and the brain circulations; the principle difference is that the retinal circulation has no autonomic innervation (Bill and Sperber, 1990). The retina is protected from toxic molecules by a blood-retinal barrier similar to the blood-brain barrier; this is due to the presence of tight junctions between retinal capillary endothelial cells, which prevent leakage of proteins, lipid molecules and small water-soluble metabolic substrates (Sagaties et al., 1987;Simo et al., 2010;Strauss,

2005). The retinal endothelial cells are surrounded by a single layer of muscular pericytes, which help in altering local vascular resistance (Ciulla et al., 2002;Sims, 1986).

Due to their functional structure, the retinal endothelial cells are considered to be a major component of the inner blood-retinal barrier (BRB). The endothelium maintains retinal blood flow and vessel tone by releasing vasoactive factors (NO, ET-1) have been already described in Section 1.2.6.1 and 1.2.6.2.

1.4 Regulation of ocular blood flow

An adequate blood supply is a basic requirement for all tissue beds to remain healthy. In order to maintain a constant perfusion, the cardiovascular system has the ability to adjust the vascular resistance. In 1902, Bayliss suggested that despite variation in systemic BP, arteries could preserve constant blood flow through the tissues due to the existence of vascular tone provided by vascular smooth muscle or other contractile elements in the vessels' wall (Bayliss, 1902). This mechanism is called autoregulation and represents the mechanism that allows tissues and organs to get a proper blood supply despite varying haemodynamic conditions (Orgul et al., 1999;Qrgul et al., 1995).

The vascular tone is adjusted by vasoactive nerves and circulating hormones, as well as endothelial factors, myogenic and metabolic factors. These adjustments are described below.

1.4.1 Metabolic autoregulation

The metabolic hypothesis of blood flow autoregulation suggests that perfusion and tissue metabolism are tightly coupled. Therefore, any reduction in arterial blood flow results in an increase of vasodilator metabolites in the affected tissue; this could be the result of either

insufficient washout or increase in the production of metabolites, or both (Kontos et al., 1978;Orgul et al., 1999;Sullivan and Johnson, 1981). The metabolic products that may influence the vascular tone include adenosine, potassium (K^+), carbon dioxide (CO_2), pH and osmolality changes (Ishizaka and Kuo, 1997). Moreover, changes in tissue oxygen (O_2) levels can also influence vascular tone (Johnson, 1986).

1.4.1.1 The role of CO_2

In the cerebral circulation, decreased arterial blood oxygen saturation is compensated by increased cerebral blood flow (CBF) and consequently the oxygen supply to the brain normally remains unchanged (Hayakawa et al., 1996). Hypercapnia results in cerebral arterial dilation (Bari et al., 1998;Wei et al., 1980), decreased cerebral resistance, and increased CBF (Hoffman et al., 1996;Narayanan et al., 2008). Changes in blood gas levels influence the retinal and optic nerve blood flow in a manner similar to that in the cerebral circulation. In the eye, hypercapnia results in increased choroidal, retinal and retrobulbar blood flow (Deutsch et al., 1983;Roff et al., 1999), just as acute hypoxic stress might result in increased ocular haemodynamic parameters (Mullner-Eidenbock et al., 2000).

1.4.1.2 The role of O_2

The retina receives O_2 from two anatomically separated sources, the choroidal and retinal circulations. The retinal microcirculation oxygenates only the inner retina down to the inner nuclear layer, while the choroidal circulation oxygenates the rest of the retina and the pigment epithelium.

Autoregulatory mechanisms in the inner retina maintain O_2 at constant values during systemic hyperoxia or hypoxia (Wangsa-Wirawan and Linsenmeier, 2003). This process is

of extreme importance since the intraocular vessels are very sensitive to changes in O_2. Hyperoxia results in a strong vasoconstriction of the inner retinal arterioles, in a manner similar to cerebral circulation (Harris et al., 1996;Wangsa-Wirawan and Linsenmeier, 2003), while hypoxia induces vasodilation of the retinal arterioles similar to that observed in cerebral blood flow studies (Eperon et al., 1975;Kogure et al., 1970;Wangsa-Wirawan and Linsenmeier, 2003). The haemodynamic response to hyperoxia in the retinal circulation is believed to be mediated via endothelin (Takagi et al., 1996).

1.4.2 Myogenic autoregulation

By means of myogenic autoregulation, blood flow through different tissues is maintained despite changes in BP (Johnson, 1986). The myogenic mechanism is triggered by variation in the transmural pressure during moderate variations in BP, and is achieved by changes in the vascular resistance (Alm and Bill, 1973). The exact mechanism involved in myogenic regulation is still unknown. It seems, however, that extracellular calcium plays an important role; moreover, different endothelial factors could partially mediate the myogenic control of the blood flow (Davies and Hagen, 1993;Davies and Tripathi, 1993).

1.4.3 Neurogenic control of vascular tone

The ANS supplies a large network of vasomotor nerve fibres; these fibres are directed towards the uvea, the PCAs, and the extra-ocular portion of the CRA (Ehinger, 1966;Laties, 1967). Vessels in the retina and prelaminar portion of the optic nerve, however, have no neural innervation (Laties, 1967;Ye et al., 1990). Consequently, stimulation of the cervical sympathetic chain results in vasoconstriction in the uvea but has no effect on retinal or anterior optic nerve blood flow (Alm, 1977;Bill et al., 1977;Weiter et al., 1973).

Neurogenic regulated ocular circulation seems be mediated by a multitude of substances such as acetylcholine (Riva et al., 1994), noradrenaline (Riva et al., 1994), NO, substance P, calcitonin gene-related peptide, neuropeptide Y, and adenosine triphosphate (ATP), vasoactive intestinal polypeptide (VIP) (Nilsson and Bill, 1984;Wiencke et al., 1994). The role played by each of these compounds in the physiology of ocular circulation is, however, still poor understood.

Although adrenergic receptors of the α-1, α-2, β-1, and β-2- types have been found in retinal vessels (Ferrari-Dileo, 1988;Forster et al., 1987), stimulation of sympathetic nervous system does not influence retinal and optic nerve blood flow (Beausang-Linder and Hultcrantz, 1980). However, stimulation of the sympathetic nervous system plays an important role in regulation of blood flow towards the eye. It has been suggested that the probable role of the sympathetic innervation is in preventing overperfusion during an increase in systemic BP, thereby assisting myogenic autoregulatory mechanisms (Bill et al., 1977).

1.4.4 Endothelial regulation of the vascular tone

This mechanism has already been described in Section 1.2.6.1 and Section 1.2.6.2.

1.4.5 Neurovascular coupling

Neurovascular coupling refers to the relationship between local neural activity and subsequent changes in local blood flow. The amount and spatial location of blood flow changes are strongly associated with changes in neural activity through complex mechanisms of communication between neurons, astrocytes and vascular cells, especially endothelial cells (Lecrux and Hamel, 2011).

Astrocytes are characteristic star-shaped neuroglia cells in the brain and spinal cord. They are anatomically positioned close to both the vascular smooth muscle cells and microvessels putting them in the ideal location to mediate neuronal activity and many of the endothelial pathways, such as ET-1 and NO and have also been demonstrated in astrocytes (Filosa, 2010). Additionally astrocytes have also been linked to the re-establishment of vasomotor tone following neuronal stimulation (Filosa, 2010). Those studies suggest that astrocytes act as a mediator and play an important role in neurovascular coupling. Several studies have found that neurovascular coupling is also present in the retina through a complex mechanisms of communication between neurons, astrocytes and retinal vessels (Falsini et al., 2002;Lanzl et al., 2011;Riva et al., 2005). A study by Metea and Newman also suggested that flicker-evoked retinal hemodynamical changes through promoting astrocytes express eNOS leading to NO mediate endothelial-dependent vasomotor response in retina (Metea and Newman, 2006). Although NO is generally considered to be a vasodilating agent, NO levels may regulate whether neuronal activity results in vasodilation and/or vasoconstriction in the retina through a complex process (Metea and Newman, 2006). Initially, arteries are dilated in response to light (or flicker) in nominally zero NO conditions, and constricted as NO levels increased (Metea and Newman, 2006). Thereby, NO also plays an important role in the neurovascular coupling through modulating astrocytes regulation of these vasomotor responses in the retina.

1.5 Retinal pigment epithelium (RPE)

RPE represents a pigmented monolayer of hexagonally shaped cells of neural origin firmly attached to the underlying choroid and overlying the photoreceptor layer of the inner retina (Figure 1.10). Through the release of various diffusible agents, it protects the outer retina from excessive light-generated ROS and maintains retinal homeostasis, in addition to regenerate the outer segment; moreover, it regulates the transport of nutrients and waste

products to and from the retina through the expression and activity of specific protein (Alfaro et al., 2005;O'Shea, 1998;Simo et al., 2010).

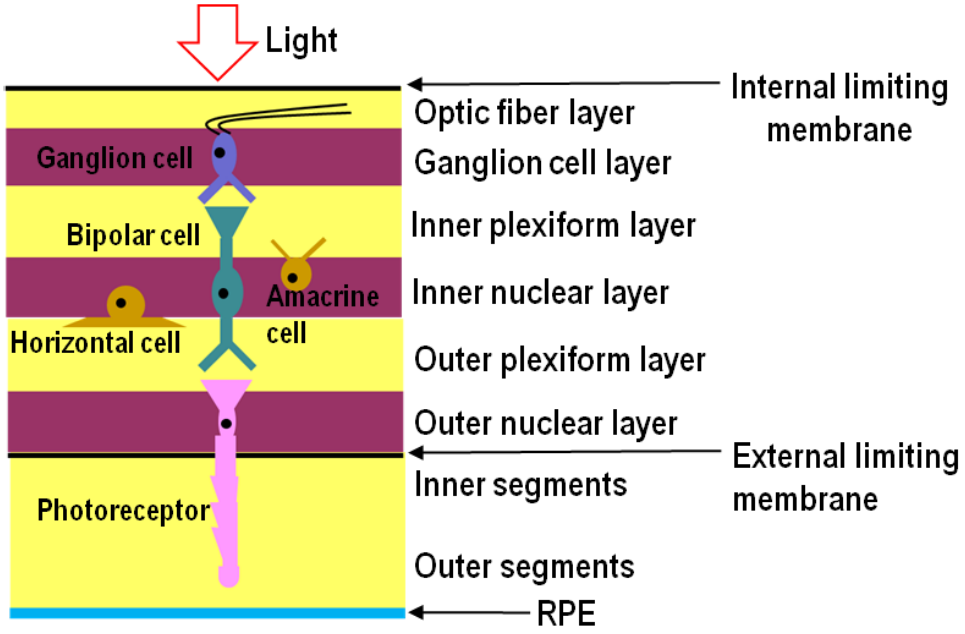


Figure 1.10: Diagram showing the layers of the retina. RPE: Retinal pigment epithelium. Adapted it according to Purves et al. (Purves et al., 2001).

Physiologically, the RPE is part of the outer of the BRB, the inner BRB being formed by the retinal vascular endothelium (Simo et al., 2010). The adjoining RPE cells and the retinal endothelial cells are joined tightly together to control fluids and to prevent toxic molecules and plasma components enter to the retina, and consequently to prevent those substances to impact the ocular metabolism (Sagaties et al., 1987;Strauss, 2005). Indeed, the RPE maintains the outer third of retinal metabolism through transporting nutrients and removing the waste products of photoreceptor metabolism to be cleared by the choroidal circulation (Mannermaa et al., 2006). Thereby, RPE as both a selective barrier and a regulator of the photoreceptor layer, plays a key role in its maintenance of a stable retinal environment (Figure 1.11).

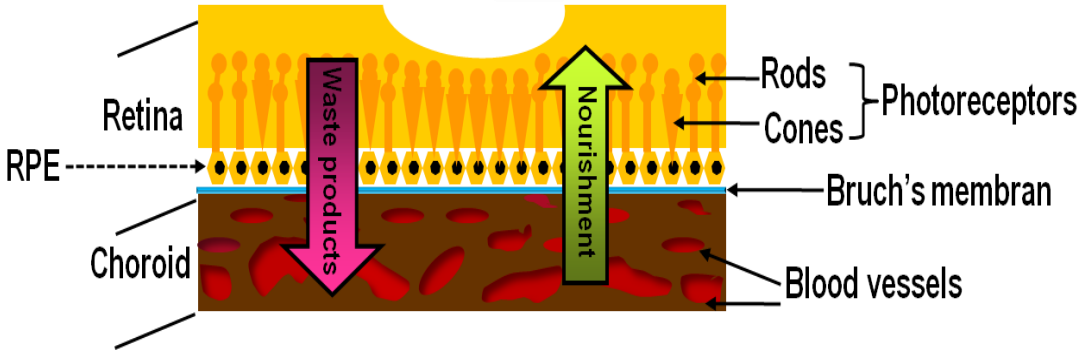


Figure 1.11: Representation of the cross section of the retina. RPE: retinal pigment epithelium.

1.6 Macula

Macula represents a 5.5 mm diameter area located roughly in the center of the retina, temporal to the optic disc (Figure 1.12). Near its center is the fovea, approximately 1.5 mm in diameter (Leung, 2008) that contains the densest concentration of cone and rod photoreceptor cells and is responsible for central and high resolution vision. In the 0.2 mm diameter of the central fovea (foveola) only contains cone cells and is responsible for sharp central vision (Gray, 1985). The rod cells which assist the dark vision are relatively sparse in foveola and then gradually increase towards to the periphery within macular region whereas the cone cells progressively decreases (Leung, 2008). In the absence of its own retinal blood vessels, the fovea has to receive oxygen from the vessels in the choroid. However, this blood supply alone is not sufficient for the high foveal metabolic demand during bright light exposure and oxidative stress; thus, the fovea exists in a state of hypoxia when under bright illumination, as a result, it may suffer the consequence of enzymatic failure over time with the accumulation of metabolic debris and lipofuscin (O'Shea, 1998), terms drusen.

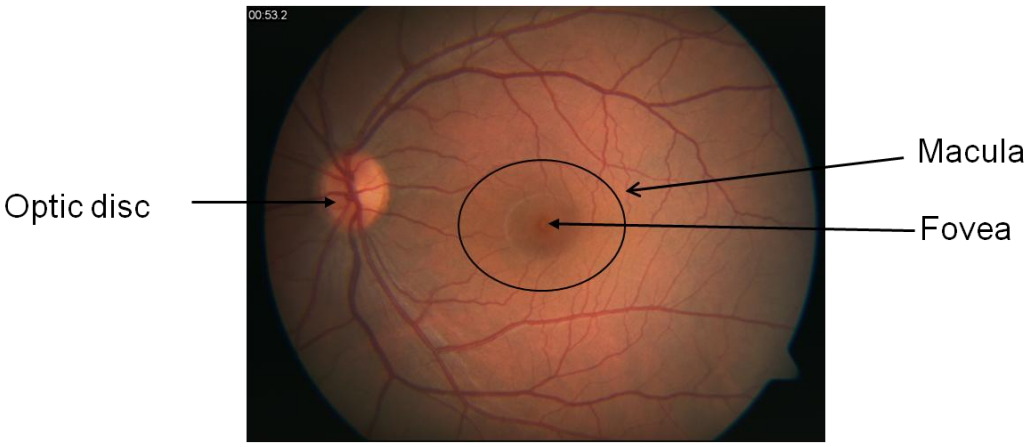


Figure 1.12: A colour photograph of a normal fundus. The area within the black circle is the macula. The center of macular is the fovea.

1.6.1 The macular pigment (MP)

In 1945, Wald was first to identify the MP as belonging to the Xanthophyll family (Wald, 1945); later, Bone separated the carotenoids from the macula and consistently showed the presence of three components: Lutein (L) [(3R,3'R,6'R)-β,ε-Carotene-3,3'-diol], zeaxanthin (Z) [(3R,3'R)-β,β-Carotene-3,3'-diol] and zeaxanthin's stereoisomer, mera-zeaxanthin (MZ) [(3R,3'R)-β,β-Carotene-3,3'-diol] (Bone et al., 1985;Bone et al., 1993). L, Z and MZ are not able to be produced within the human body, and therefore, they have to be provided by dietary intake. Indeed, some reports also confirmed an increased optical density of MP with oral anti-oxidant supplementation (Connolly et al., 2010;Johnson et al., 2008;Thurnham, 2007). This increase is steady and slow, and needs more than 140 days to reach a peak level (Landrum et al., 1997). In addition, the supplements' levels decrease at a slow rate after cessation being shown to remain present for a period of at least 6 months (Hammond et al., 1997).

Human MP peaks at the centre of the fovea, and gradually decreases with increasing eccentricity (Leung, 2008). The absorption spectrum of MP peaks at 460nm (blue light)

(Algvere et al., 2006;Wu et al., 2006), so that plays a role as a broadband filter to protect the macula against the photo-oxidative effects of damaging blue light (short wavelength light) in the 440 to 460 nm range (Pease et al., 1987;Young, 1988).

1.7 Effects of ageing on ocular circulation

Ageing clearly causes biological effects that could reduce functional capacities and enhance the risk of developing diseases. These declines necessarily include many variations within the eye; for example, decreased ocular blood flow (Ravalico et al., 1996), increased lipids and fat accumulate in the macular and Bruch's membrane (Booij et al., 2010), increased calcification of elastic fibres and collagen fibres in Bruch's membrane (Booij et al., 2010), and vulnerable vascular beds (Ehrlich et al., 2009). The results of these multiple alterations with ageing on the eye contribute to some diseases such as AMD and glaucoma.

1.7.1 Ageing effect on ocular blood flow

Within addition to systemic vascular changes, ageing is also strongly related with changes in the ocular structure and function and with development of ocular diseases such as AMD (Bhutto et al., 2010). In addition, it also results in reduction of retrobulbar (Groh et al., 1996;Harris et al., 2000;Williamson et al., 1995), choroidal (Grunwald et al., 1998;Ramrattan et al., 1994) and optic nerve (Greenfield et al., 1995;Groh et al., 1996) circulation with various consequences on the ocular health. Indeed, it has been demonstrated that ageing results in an increase in the resistance of ocular vessels which results from the increase of systolic blood pressure and systolic flow velocity in the ophthalmic arteries (Ehrlich et al., 2009), and leading to a higher velocity and decreased blood flow in the retinal microcirculation (Boehm et al., 2005). In retrobulbar and optic nerve circulation, some reports shown that reduced blood flow velocities were also

correlated with advancing age (Greenfield et al., 1995;Groh et al., 1996;Rizzo et al., 1991). Moreover, Kida et al. study found that a significant diurnal to nocturnal decrease in optic nerve head blood flow with ageing due to the elderly population having a less sensitive autoregulatory system at night (Kida et al., 2008). Additionally, some reports suggested that an increase of ocular perfusion pressure (OPP) (Ramrattan et al., 1994) and decreased fundus pulsation amplitude to be associated with advancing age in the choroidal circulation (Ramrattan et al., 1994;Ravalico et al., 1996).

1.7.2 Ageing effect on retina: age-related macular degeneration (AMD)

With ageing, the reduction in the number of photoreceptor cells causes a widespread reduction in visual function (Gao and Hollyfield, 1992;Sun et al., 2007). In addition, there is a decrease in melanin (Feeney-Burns et al., 1984;Weiter et al., 1986), and increased lipofuscin accumulation, both factors contributing to a higher risk of developing AMD (Feeney-Burns et al., 1984;Weiter et al., 1986), which is an ocular degenerative disease of the central retina (macula) and is the most common cause of vision loss in people aged over 50 in developed countries (Jager et al., 2008).

1.7.2.1 Pathophysiology of AMD

The pathology of AMD is dominated by age-associated degeneration changes involving in the RPE, Bruch's membrane and choriocapillaris (Young, 1987). The primary lesion in AMD appears to the RPE, possibly due to a high rate of molecular degradation and basal deposits in Bruch's membrane that represent the early pathological changes in this disease (Coleman et al., 2008). The combination of these deposits with further changes within the RPE, leads to the formation of drusen (Sarks et al., 2007). Hard drusen (less than 63 μm in diameter with discrete borders) deposits composed of a hyaline-like

material are located between the RPE and Bruch's membrane (Bird et al., 1995;Klein et al., 2004). In general, soft drusen (more than 63 μm in diameter with indistinct borders) are larger and related with detachment of the RPE and diffusely abnormal Bruch's membrane alterations (Bressler et al., 1994;Sarks et al., 1999). The soft drusen could contribute to further damage to the retina, RPE and choroid, and therefore leading to choroidal neovascularisation or cell death in the RPE.

1.7.2.2 Risk factors of AMD

1. Age

The incidence of AMD dramatically increases with age. Geographic atrophy and neovascular AMD are unlikely to occur under the age of 55, but more frequently occurs after 75 years old (Klein et al., 2004).

2. Smoking

Smoking was discovered to be a major risk factor for development and progression of AMD (Clemons et al., 2005;Klein et al., 2006). Specially, increased chance of development of exudative AMD was found in smokers (Complications of Age-related Macular Degeneration Prevention Trial (CAPT) Research Group., 2008;Tamakoshi et al., 1997).

3. Diet

Dietary fat intake and alcohol consumption can effect on risk of developing AMD (Knudtson et al., 2007;Seddon et al., 2003b). Greater body mass index (BMI) was established to increase the risk of progression of AMD (Seddon et al., 2003a). Antioxidant micronutrient intake has been shown to reduce the risk of AMD (van Leeuwen et al., 2005).

4. Genetics

Genetic factors have been investigated, as family history is an established risk factor for AMD (Klein et al., 1994). Ethnicity has been suggested as an effect, such that white population were significantly more likely than Afro-Caribbeans to develop large drusen and focal pigmentation and to progress from medium- to large-sized drusen or pigment abnormalities within macular area (Chang et al., 2008). And also neovascular AMD is less widespread in darkly pigmented races compared to white race (Klein et al., 2006).

5. Oxidative stress and vascular factors

High levels of oxidative stress (Cai et al., 2000;Khandhadia and Lotery, 2010), chronic inflammation (Nagineni et al., 2012), and endothelial dysfunction (Lip et al., 2001) have also been involved in the pathogenesis of this disease. The retina is an organ of high consumption of oxygen and its exposure to visible light; therefore, the retina is particularly susceptible to oxidative stress (Beatty et al., 2000;Organisciak et al., 1998). Moreover, some studies reported that a reduction in risk for AMD in people who consumed food rich in the antioxidant vitamins A, C and E (Chow, 1991;Seddon et al., 1994;Smith et al., 1999), and also the Eye Disease Case Control study found that low plasma level of vitamin C were associated with increased risk of AMD (Eye Disease Case-Control Study Group, 1993). In addition, AMD is associated with significantly reduced levels of plasma glutathione reductase which is required for regeneration of glutathione (Cohen et al., 1994).

Data from the Rotterdam Eye (Vingerling et al., 1995), Beaver Dam Eye (Klein et al., 1993) and Cardiovascular Health Studies (Klein et al., 2003) showed that AMD is associated with subclinical signs of cardiovascular disease (CVD). In addition, the Atherosclerosis Risk in Communities (ARIC) Study found that patients suffering from AMD are at higher risk than age-matched healthy controls for developing overt pathologies, such as coronary heart disease (CHD) and stroke (Wong et al., 2006;Wong et al., 2007),

all leading to a decreased survival rate (Tan et al., 2008). By screening more than 1.5 million subjects over 65 years of age, Liao et al (Liao et al., 2008) also found that independent of demographic factors and comorbidity, the risk of incident stroke was higher in AMD patients compared to controls.

Risk factors for AMD	Description	Reference
Age	Advancing age	The International ARM Epidemiological Study Group [252], The Beaver Dam Eye Study [278], and VanNewkirk et al. study [279].
Smoking	Strong association between the risk of developing AMD and the number of cigarettes smoked.	The Beaver Dam Eye Study [280], Delcourt et al. study [281], and Khan et al. study [282].
Ethnic origin	White individuals have high risk of developing AMD than African people.	The Age-Related Eye Disease Study Research Group [283], Mukesh et al. study [284], Friedman et al. study [285].
Genetic factors	Family history is associated with AMD	Haddad et al. study [286], and Scholl et al. study [287].
Nutrition	High dietary intake of fat; low dietary intake of antioxidants, such as carotenoids.	Eye Disease Case-Control Study Group [267], and Van Leeuwen et al. study [262]
Iris colour	Light iris colour has been considered as a risk factor for AMD	Taylor et al. study [288], and Delcourt et al. study [289].
Gender	Females may have a higher risk of AMD.	Smith et al. study [290], and Chakravarthy et al. study [291].
Light	Light-induced oxidative stress has been proposed is risk fator for AMD.	Taylor et al. studys [288,292].
Complement factor H,	This gene is located on chromosome lq32.	Haines et al. study [293], Edwards et al. study [294], and Klein et al. study [295].
LOC387715 (ARMS2)/HtrA1	High temperature requirement factor A-1, located on chromosome 10q26.	Yates et al. study [296], and Rivera et al. study [297].

Table 1.2: The summarized risk factors for age-related macular degeneration (AMD).

1.7.2.3 Clinical Manifestations

The clinical hallmark and usually the first clinical finding of AMD is the presence of drusen (Figure 1.13), small yellow-white deposits of acellular, polymorphous debris between the RPE and Bruch's membrane. The drusen may be found in both macula and peripheral retina (Klein et al., 2004). Drusen are categorised as small (<63 μm in diameter), medium (63 to 124 μm) and large (>125 μm) (Jager et al., 2008). Drusen are also categorised as hard or soft on the basis of the appearance of their borders. Hard drusen presents discrete borders and is no more than 63 μm in diameter; conversely, soft drusen generally have indistinct borders and a diameter larger than 63 μm (Bird et al., 1995;Klein et al., 2004).

In most cases of AMD, drusen occurs in both eyes (Klein et al., 1992). However, the presence of only few small, hard drusen is not considered to be diagnostic for AMD and they are often seen in people aged over 50 years as part of normal ageing process (Jager et al., 2008). However, the presence of drusen over 63 μm in diameter represents a high risk for AMD (Fine et al., 2000). Excess drusen can lead to damage to the RPE. Some studies found that the damage of the RPE and a chronic aberrant inflammatory response could result in large areas of RPE atrophy with clearly visible choroidal vessels (called geographic atrophy), and the neovasculogenesis (new formation of blood vessels) and angiogenesis (the growth of new blood vessels from pre-existing vasculature) due to expression of vascular endothelial growth factor (VEGF) (de Jong, 2006;Donoso et al., 2006;Hageman et al., 2001). Choroidal neovascularisation may extend through breaks in Bruch's membrane causing haemorrhage, fluid exudation, lipid deposition, detachment of the RPE from the choroid, fibrotic scars, or a combination of all these signs (Grossniklaus and Green, 2004).

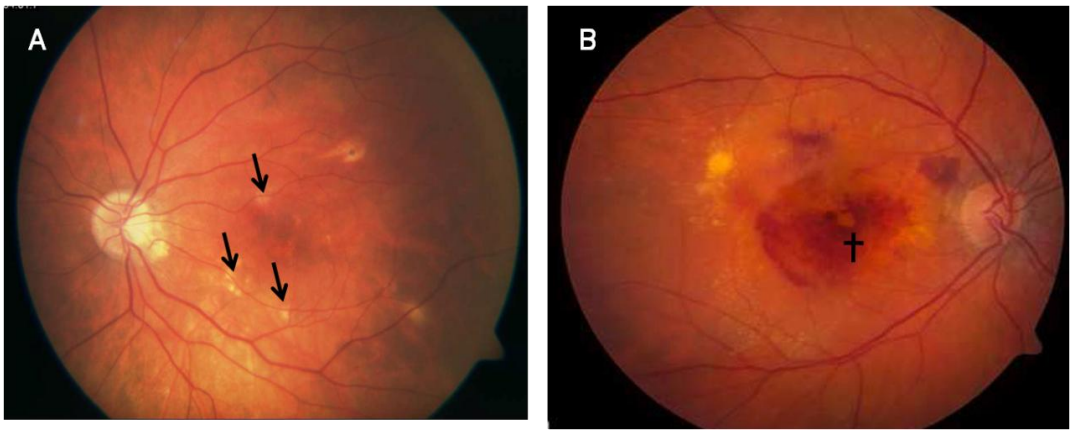


Figure 1.13: Colour fundus images of age-related macular degeneration. (A) Dry AMD. (B) wet AMD. Arrow, drusen. †, bleeding, leaking and scarring caused by neovascularization.

1.7.2.4 Classification of AMD

AMD have been categorised as two stages: early, whereby visual symptoms are inconspicuous (Hogg and Chakravarthy, 2006), and late stage, in which severe visual loss is present. Furthermore, late AMD is subdivided in two main types: dry (or geographic atrophy or non-exudative); and wet (or neovascular or exudative), characterised by choroidal neovascularisation (Kanski, 2003).

There are multiple systems that have been developed to grade AMD. The two main systems are the International Classification and Grading System (Bird et al., 1995), and the Wisconsin Age-Related Maculopathy grading system (Klein et al., 1991). The grading systems have used standard sizes of concentric circles to define the location of AMD lesion (Bird et al., 1995;Klein et al., 1991) (Figure 1.14), and standard measuring templates to assess size of drusen (Bird et al., 1995;Klein et al., 2004) (Figure 1.15).

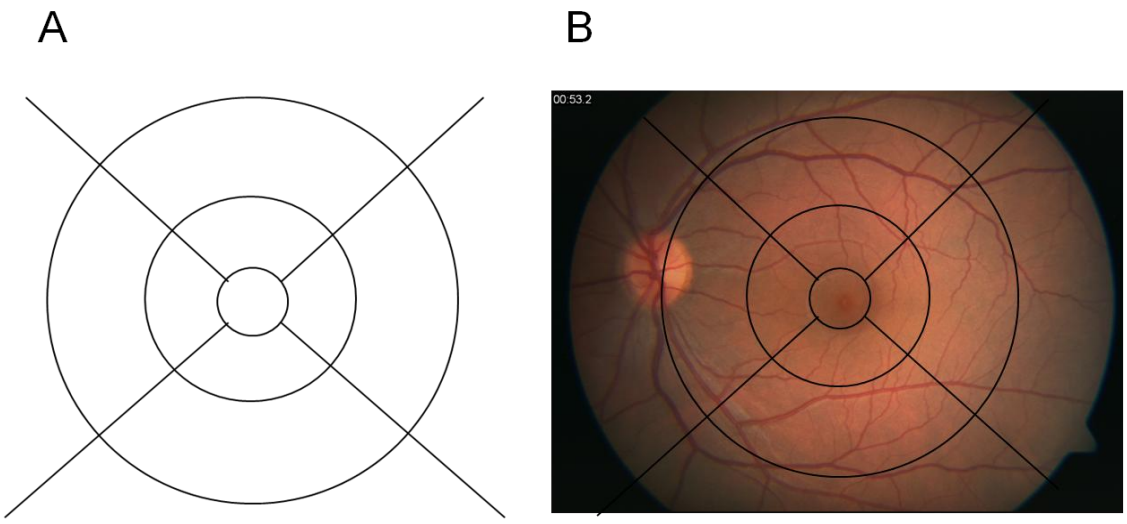


Figure 1.14: The standard grading grid is used for estimation of lesion area. (A) A standard grid used to define subfields in the macula. These circles represent respectively the central, middle and outer subfield. The circles should be adjusted by a diameter of a normal optic disk which depends on the fundus camera used, as clinical convention of considering the diameter of the average optic disk to be 1500 μm. Therefore, the diameter of the innermost circle of this grid corresponds to 1000 μm (2/3 optic disk), and the middle and outer circle is respectively 3000 μm (2 times optic disk) and 6000 μm (4 times optic disk) in diameter (Klein et al., 2004). The spokes may used to help in centring the grid on the macula and in estimating the length of a lesion. **(B)** The standard grid is placed on stereoscopic fundus photographs, centred on the fovea. The sizes of the circles should be adjusted that depends on the fundus camera used.

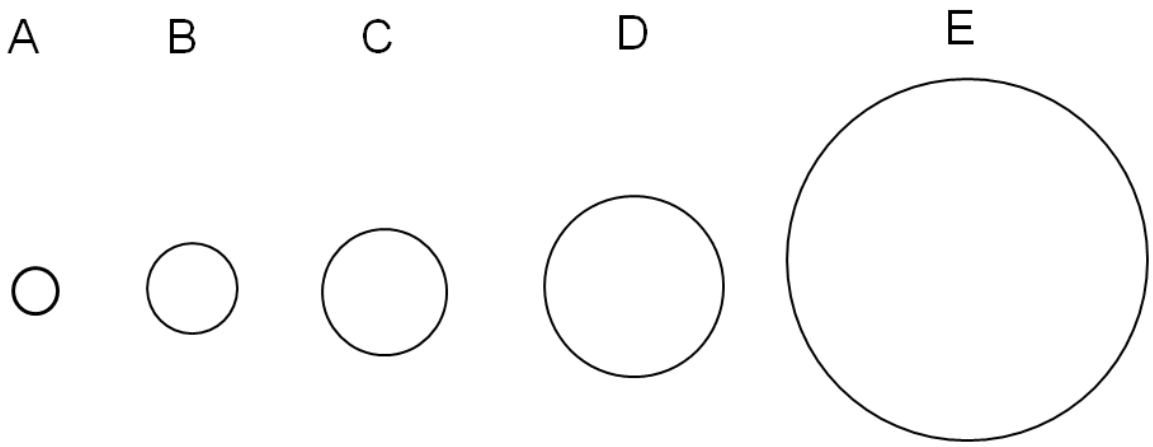


Figure 1.15: The standard measuring templates to assess size and area of drusen.

Figure 1.15 shows the standard measuring templates composed of circles of varying size diameter is used to estimate size of drusen, area involved by drusen, and area involved by other AMD lesion and other abnormalities in fundus. The circles A, B, C, D and E are used for all subfields. The circles should be adjusted on a transparent sheet depends on the fundus camera used, so that they are 1/24, 1/12, 1/8.6, 1/6 and 1/3 diameter of optic disk, corresponding to diameters in average fundus of 63 μm, 125 μm, 175 μm, 250 μm and 500 μm (Bird et al., 1995). All circles could be used for assess the size of drusen. Circle A distinguishes between small and large drusen. Circle B and C could be used for estimating area occupied by increased retinal pigment associated with AMD (the presence of granules or clumps of grey or black pigment in or beneath the retina) or RPE depigmentation. Circle D and E could be used for estimating area of geographic atrophy or neovascular AMD.

Wisconsin Age-Related Macular Degeneration grading system is widely used to grading AMD (Cheung et al., 2007a;Cheung et al., 2007b;Tan et al., 2008;Wong et al., 2006), and this category of AMD include early and late stages (Klein et al., 2006) (Table 1.3).

Stage	Definition
Early AMD	The presence of either soft drusen alone, retinal pigment epithelium depigmentation alone, or a combination of soft drusen with pigmentary abnormalities in the absence of late AMD.
Late AMD	The presence of exudative AMD or pure geographic atrophy.

Table 1.3: Overview of early and late stages of AMD. The soft drusen are defined as lesions with a diameter larger than 63μm. The pigmentary abnormalities (either increased retinal pigment or RPE depigmentation) are defined as present or absent.

1.7.2.5 Endothelial dysfunction in AMD

Endothelial dysfunction refers to an impairment of endothelium-dependent vasodilation caused by a loss of NO bioavailability in the vessel wall. It also plays an important role for the development of AMD, as endothelial abnormalities leading to defects on Bruch's membrane or the choroidal vasculature (Archer and Gardiner, 1981) and further resulting in the AMD process (Grossniklaus and Green, 2004;Zarbin, 2004). In Lip et al. study showed that in AMD subjects, there is a significantly increased level of plasma von Willebrand factor (vWF) which released when endothelial cells damaged and therefore, it is a marker of endothelial dysfunction (Lip et al., 2001). This may suggest that impaired endothelial function is related to the pathogenesis of AMD. This may hypothesis that the higher level of vWF in AMD caused by oxidative stress-induced endothelial dysfunction, and will be discussed in later present thesis.

1.8 Summary

Ageing is a primary factor to influence function and structure of both ocular and systemic vascular network thus contributing to the occurrence of age-related diseases, such as AMD and cardiovascular disease. Nevertheless, to date only few studies have investigated the influence of ageing on vascular function in both macro- and micro-circulation in health and disease. Moreover, the effect on the ocular health of systemic factors, such as oxidative stress that are enhanced by ageing has scarcely been studied. The purpose of the below work is to fill in all the gaps in knowledge and offer a better overview of the effect of ageing on the vascular ocular health.

Chapter 2

Materials and Methods

2.1 Recruitment of participants

Healthy subjects and AMD patients were recruited by advertising at Aston University, Birmingham, UK. Ethical approval was sought from the local ethics committee, and written informed consent was received from all participants prior to enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

2.1.1 Exclusion criteria

Systemic and ocular exclusion criteria are listed below:

- Smoking
- Any history of chronic systemic disease including autoimmune diseases (Ahsan et al., 2003), alcoholic liver disease (Videla and Guerri, 1990), cancer (Beutler and Gelbart, 1985), and diabetes mellitus (Beard et al., 2003).
- Subjects suffered from cardio- or cerebro-vascular disease, such as coronary artery disease, heart failure, arrhythmia, stroke and transient ischemic attacks.
- The presence of inflammatory conditions (i.e. rheumatoid arthritis and systemic lupus erythematosus)
- Subjects receiving hormone replacement therapy.
- Patients diagnosed with ocular diseases such as cataract and glaucoma.

2.2 Subjects preparation and experimental protocol

The participants were asked to fast for 12 hours prior to the date of the study, and could only drink water in the morning of the date of the test. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits and fruit juice, and any nutritional supplements (Jones et al., 1992). In addition, subjects were asked to abstain from caffeinated

beverages and chocolate and from alcohol for at least two hours before the visit. All investigations were performed in the same day and approximately 2 – 2.30 hours for each participant. All the participants underwent standardized anthropometric measurements for height and weight, and then used to calculate BMI (weight/height^2). In addition, the participants were required to complete a questionnaire on their general health, daily diet, physical activity, smoking and alcohol consumption. The experimental schedule is exhibited in Figure 2.1.

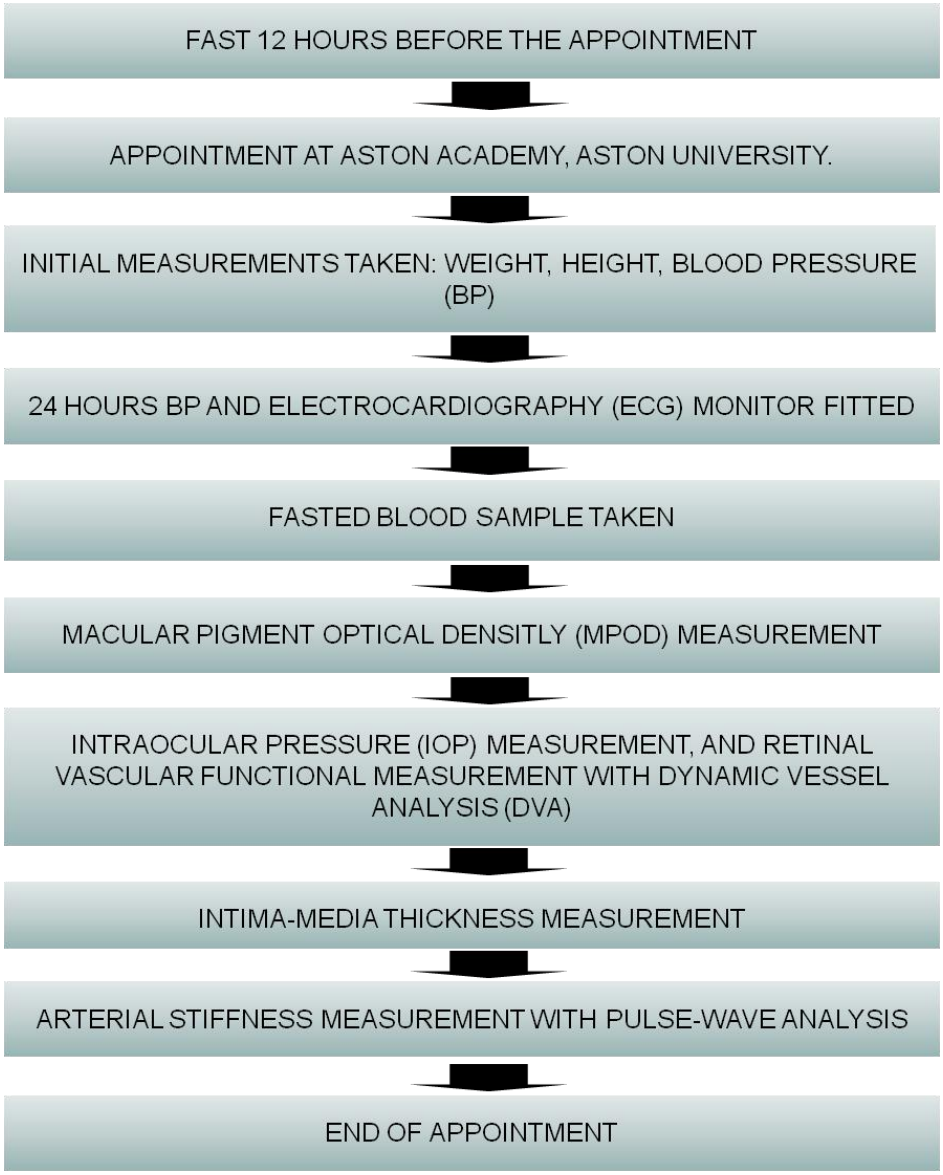


Figure 2.1: A flowchart of the measurements performed.

2.3 Assessments of systemic parameters

2.3.1 Blood pressure (BP) measurement

BP was measured in each subject in the morning of the study, with a BP monitor (UA-779, A7D Instruments Ltd., Oxford, UK). In preparation for this measurement, each subject rested in a sitting position for approximately 10 minutes in a quiet room to achieve sufficient mental and physical calm. The SBP and DBP were measured three times (1 minute apart). The average readings for SBP and DBP were then used to calculate the mean BP (MBP) (Equation 2.1).

$$MBP = 1/3\ SBP + 2/3\ DBP$$

- **MBP:** mean blood pressure
- **SBP:** systolic blood pressure
- **DBP:** diastolic blood pressure

Equation 2.1: The calculation of mean blood pressure.

2.3.2 24-hour BP and HRV assessment

Ambulatory BP monitoring provides a better prediction of major cardiovascular events than occasional measurements performed in the physician's consulting room (Ernst and Bergus, 2003;O'Brien, 2003;Verdecchia et al., 2004). This method not only provides a better estimate of the true BP profile of the participants, but also helps avoid the so-called "white-coat" effect encountered so often in the clinical practice, especially in elderly individuals (Pickering et al., 2002).

2.3.2.1 CardioTens: an ambulatory BP and ECG monitor

The device used for the purpose of this thesis was a computer-operated ambulatory BP and ECG monitor (Cardiotens-01, Meditech Ltd, Hungary; and representative by PMS Instruments, Maidenhead, UK). This device measures BP automatically using an oscillometric method. The device can be connected to the serial port of a computer with an optoelectronic interface. CardioTens can store 1000 BP measurements and a total of 4 to 5 hours of ECG recordings. In the event of a faulty reading, the device is programmed to reinflate a second time, which helps to avoid missing data points. The monitor is also able to analyze ECG signals from beat to beat in real time and stores full HR, as well as HRV data. Moreover, it is the only ambulatory BP and ECG monitor capable of triggering BP recordings during episodes of abnormal cardiac activity (Uen et al., 2003).

The final data reading consists on graphical (Figure 2.2) or worksheet-like displays, printouts and statistical analysis for evaluation.

2.3.2.2 The principle of ANS function assessment through HRV

During normal resting conditions, the sinusal rhythm is highly irregular and this behaviour is apparent when HR is examined on a beat-to-beat basis. This HRV is the result of complex changes that occur in physiological parameters such as respiration, BP, body temperature, metabolic rate, hormonal levels, and sleep cycles, reflected by ANS controlled function. As a result, the analysis of HRV has been extensively applied both in the investigation of normal physiology and in pathological conditions.

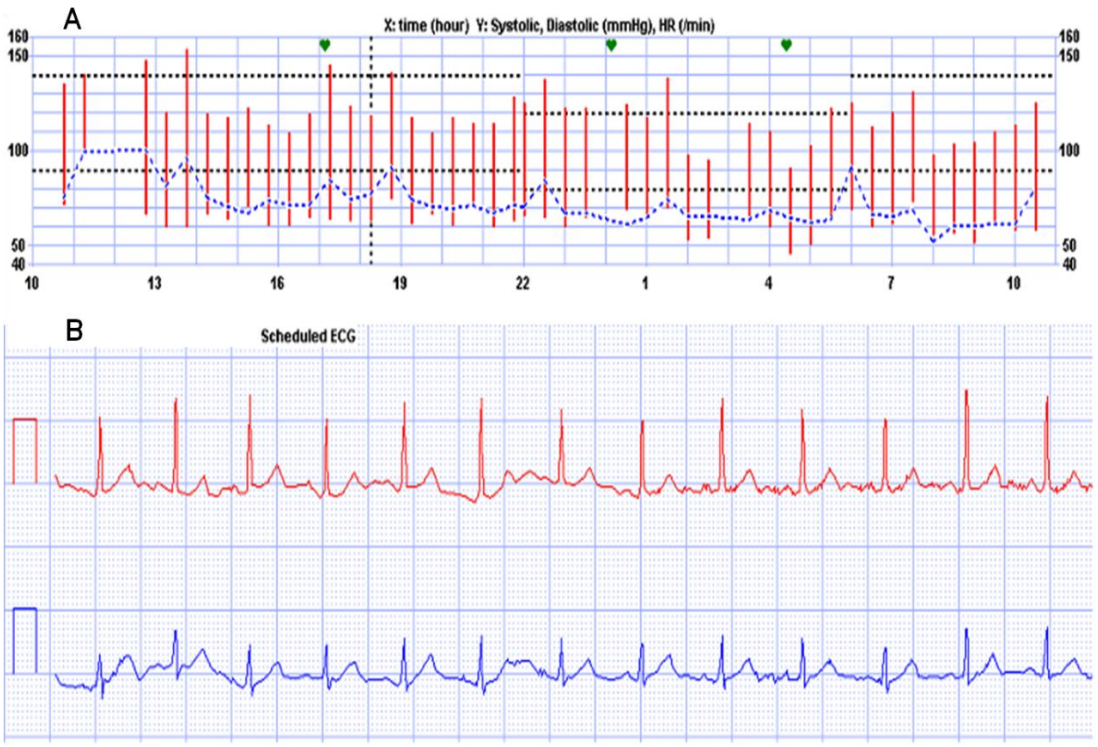


Figure 2.2: Representation of 24-hour blood pressure and electrocardiogram recordings using CardioTens-01 (graphic display). (A) A sample of 24-h blood pressure readings (vertical lines) and heart rate curve (interrupted line). (B) A sample of electrocardiogram.

The principal domains in which HRV analysis has provided useful results are:

- Clarifying the role of the ANS in regulating the cardiovascular response to changes in posture, stress and exercise (Malliani et al., 1991).
- Exploring the physiology of normal ageing and identifying those at risk of premature cardiac diseases (Whitsel et al., 2001).
- Assessing the autonomic function in a variety of non-cardiac diseases such as diabetes mellitus (Ewing et al., 1980;Ewing et al., 1991;O'Brien et al., 1991), renal diseases (Vita et al., 1988), chronic liver disease, respiratory disorders (Watson et al., 1999) and neurological diseases (Rapenne et al., 2000).

- Evaluating cardiovascular diseases such as myocardial infarction (Flapan et al., 1993), chronic cardiac failure (Nolan et al., 1992) and hypertension (Singh et al., 1998).
- In occupational health to explore the potential elevated cardiovascular risk in shift workers.

HRV can be assessed either by using a frequency-domain or a time-domain analysis of ECG recordings. For the purpose of the present work, the first method was applied that is fundamentally categorized into high-frequency (HF: 0.15-0.40 Hz) and low-frequency (LF: 0.04-0.15 Hz), which assesses ANS function by estimating the predominance of the sympathetic and parasympathetic divisions of the ANS (Kitney and Rompleman, 1980). In normal individuals cyclic changes in HR occur in association with respiration and this HF cyclical HRV is mediated by the PSNS (Pagani et al., 1986). However, cyclic variations due to changes in BP result from changes in baroreceptor activity and are typically LF in nature, being mediated via the both SNS and PSNS (Akselrod et al., 1981;Amano et al., 2001;Berger et al., 1989;Fukuba et al., 2009;Porges, 2007) (Figure 2.3). The ratio of LF to HF (LF/HF) represents the dynamic of the HRV signal and is considered to measure the sympathovagal balance of ANS function (Choi et al., 2006;Diedrich et al., 2003;Pagani and Malliani, 2000).

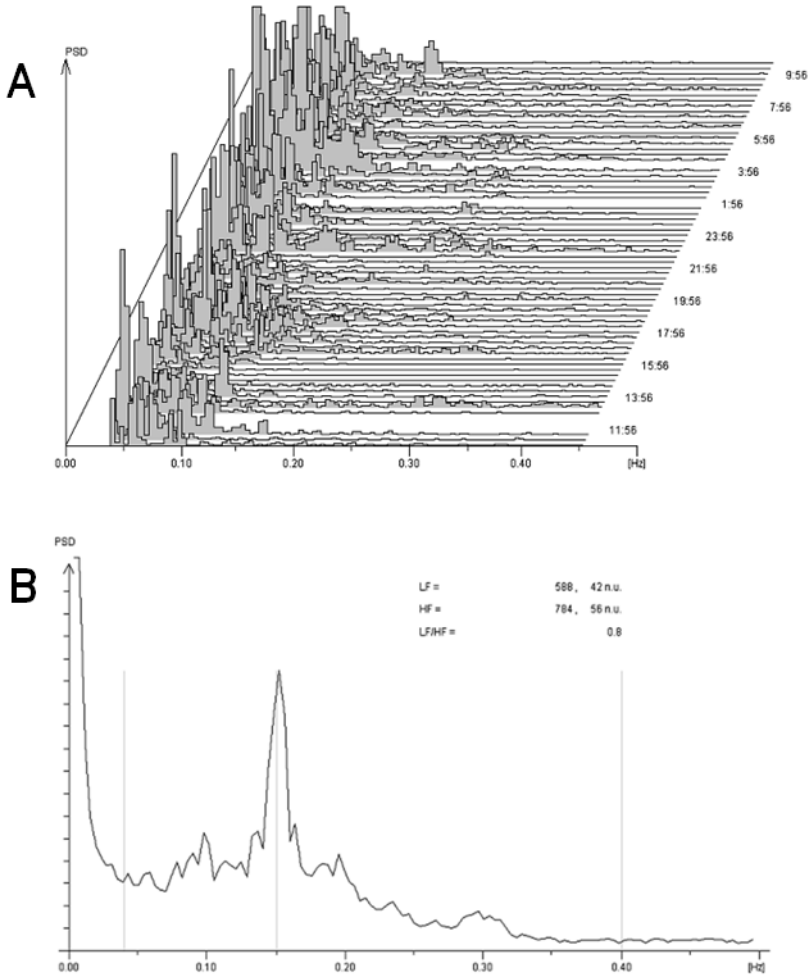


Figure 2.3: Representation of analyzing heart rate variability (HRV) in the frequency domain. (A) The compressed spectral array (CSA), which represents a set of 72 power spectral density (PSD) charts calculated with 20-minute intervals for 24 hours, based on the first 4 minutes of each 20-minute interval. The vertical axis represents PSD and the horizontal axis represents the frequency in hertz (Hz). (B) PSD is automatically calculated using fast fourier transform algorithm. The vertical axis represents PSD and the horizontal axis represents the frequency. LF: low frequency component; HF: high frequency component.

2.3.2.3 CardioTens measurement

Measurements were performed in ambulatory conditions. For each subject the BP was measured every 15 minutes over the day time (the time is defined as the true wake time) and 30 minutes over the night time (the time is defined as true sleep time). The 24 hour BP and the ECG data were downloaded and analysed using the Cardiovision 1.7.2 software (PMS Instruments Ltd., Maidenhead, UK). Average day time (active period), average night time (passive period) and overall (average 24 hours) SBP and DBP were assessed. Active and passive periods were determined for each participant based on the true wake and sleep times recorded by individual self-report.

Heart rate variability HRV values were calculated from the ECG recordings using the Cardiovision 1.7.2 software (PMS Instruments Ltd., Maidenhead, UK) after the recordings were uploaded into a computer. For the purpose of this thesis, the frequency domain parameters (LF, HF and LF/HF ratio) were collected. These parameters were automatically calculated for both the active and passive periods of the recording via the Cardiovision 1.7.2 software. Circadian changes in LF, HF and LF/HF parameters were also calculated as active period minus passive period values. A sample of such recording is presented in Appendix 1.

2.3.3 Carotid intima-media thickness (C-IMT) measurement

C-IMT is the thickness between tunica intima and tunica media of a carotid artery. C-IMT (Figure 2.4) is a well established surrogate marker of atherosclerosis and associate with cardio-/ cerebro-vascular diseases (Greenland et al., 2000;Poredos, 2004). For the purpose of the present thesis, C-IMT was applied to assess the cardio-/ cerebro-vascular risk and the extent of the atherosclerosis. The thickness greater than 1 mm was considered as increased C-IMT, and it will accelerate atherosclerosis (Gasparyan, 2009),

leading to increase the risk of developing cardio-/cerebro-vascular events (Greenland et al., 2000).

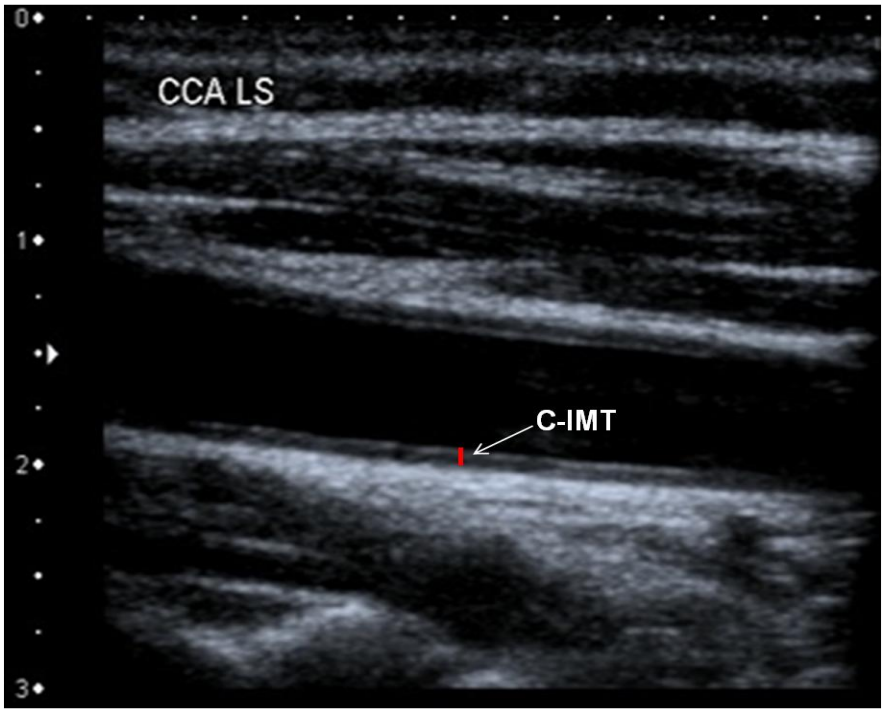


Figure 2.4: An image of left common carotid artery. The red bar is showing where the C-IMT was measured.

C-IMT was measured using the high-resolution B-model ultrasound system, with an 8MHz linear probe (Siemens; Acuson Sequoia, UK) applied on the left common carotid artery (CCA). For steps, the participant was required face-up lying on the test bed with head supported, and using the 8MHz linear probe performed on the left CCA where a region free of plaque and the double-line pattern was observed (Figure 2.4), a scanned image obtained and then was analysed subsequently to determine the C-IMT according to Mannheim Carotid Intima-Media Thickness Consensus (Touboul et al., 2007).

2.3.4 Assessment of arterial stiffness- Pulse wave analysis (PWA)

The measurement of arterial stiffness was assessed by using the SphygmoCor device and PWA. It is a non-invasive assessment of arterial stiffness lends itself to a wide range of applying in the research area and clinical setting (Matsui et al., 2004;Oliver and Webb, 2003).

Augmentation index (AIx) was used as a measure of arterial stiffness by PWA. It indicates the amount by which the aortic pressure is increased by the peripheral reflection of the blood flow. In the peripheral arteries, the outgoing systolic pulse wave is reflected back towards the heart and adds to ('augments') the central aortic pressure in late systole (O'Rourke et al., 2001;Oliver and Webb, 2003). The amount by which the aortic pressure is increased by this phenomenon is the augmentation pressure (∆P). The AIx represents the aortic ∆P as a percentage of the aortic 'pulse pressure' (PP) (Equation 2.2) (Oliver and Webb, 2003). Therefore, AIx (∆P/PP) indicates the combined influence of large artery pulse wave velocity, peripheral pulse wave reflection and endothelial function (O'Rourke et al., 2001;Oliver and Webb, 2003;Wilkinson et al., 2002). As AIx varies with heart rate it is commonly adjusted to a 'standard heart rate' of 75 beats per minute (Wilkinson et al., 2000). The AIx increased with arterial stiffness (Oliver and Webb, 2003).

$$AIx = \Delta P / PP \times 100\%$$

- AIx: augmentation index
- ∆P: augmentation pressure
- PP: pulse pressure

Equation 2.2: The calculation of the augmentation index.

2.3.4.1 The procedure of PWA measurement

The measurement used a high-fidelity tonometer to place on the radial artery. The shapes of the pulse waves were captured electronically using the SphygmoCor pulse wave analysis system and a laptop computer. Pulse waves captured over a 12-s period were used to produce an average pulse wave contour. The SphygmoCor system converts the peripheral waveform to an aortic waveform (Figure 2.5) using a proprietary algorithm which called “general transfer function” (O'Rourke et al., 2001), and AIx was generated automatically.

The quality of the pulse waves captured both visually on screen and also numerically with the device’s in-built quality index score (QI %, based on average pulse height, pulse height variation and diastolic variation). In order to ensure reliability of measurements, only those readings obtained with high quality (QI % is not less than 80 %) were considered acceptable.

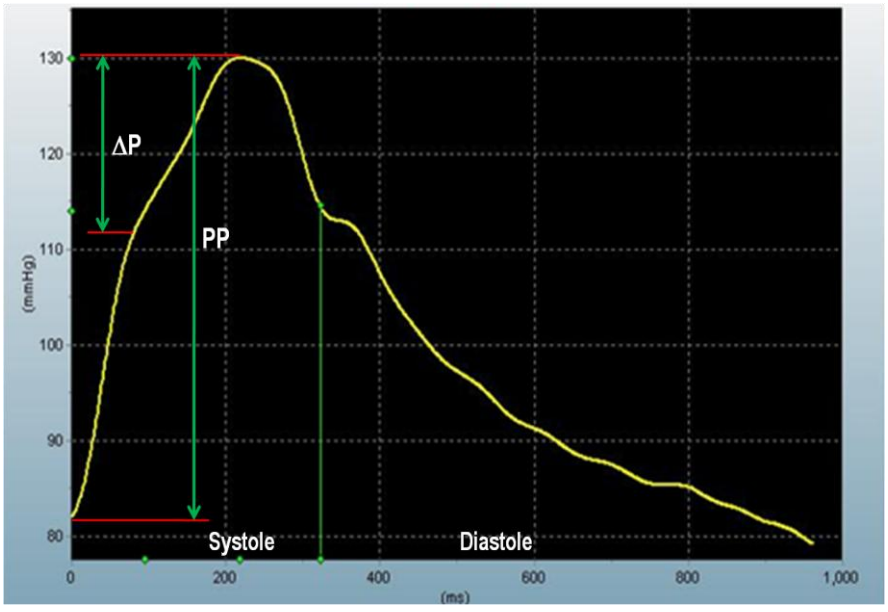


Figure 2.5: Cardiovascular indices derived from Pulse Wave Analysis. ΔP: augmentation pressure; PP: pulse pressure.

2.4 Assessment of ocular circulation - Dynamic retinal vessel analysis (DVA)

The retinal vascular functionality was reflected through the retinal vascular reactivity to flicker provocation which was measured using the dynamic vessel analysis (DVA) (IMEDOS Systems UG, Germany) (Figure 2.6). This is a computer controlled instrument consists of a high resolution fundus camera (FF450 plus, Zeiss GmbH, Jena, Germany), which can continuously measure retinal vessels diameter along a selected vascular segment over a defined time. An essential part of the DVA is the fundus camera, which includes illumination and the observation optical pathway. For the purpose of real-time analysis in retinal vascular diameter, the fundus was recorded onto the charge-coupled-device (CCD, a detected light signal is converted to digital imaging) chip of the video camera; moreover, the fundus image can be inspected on the real-time monitor. An optoelectronic shutter is inserted in the camera, interrupts the observation light (530–600 nm), and produces a bright-to-dark contrast ratio of at least 25:1; therefore the flicker frequency is generated at 25Hz. The optoelectronic shutter was controlled by a special program running on the DVA computer, and participants were visually aware of the flickering light.

Therefore, DVA used to assess the retinal vascular auto-regulation mechanisms by measuring retinal vascular dilation responded to flickering stimulus, as an indirect provocation to the retinal vessels due to ocular metabolic increase. For the purpose of this present thesis, the reactivity capability of selected vessel (arteries and veins) segments in a flickering light provocation was assessed using DVA, to reflect the retinal vascular/ endothelial function.

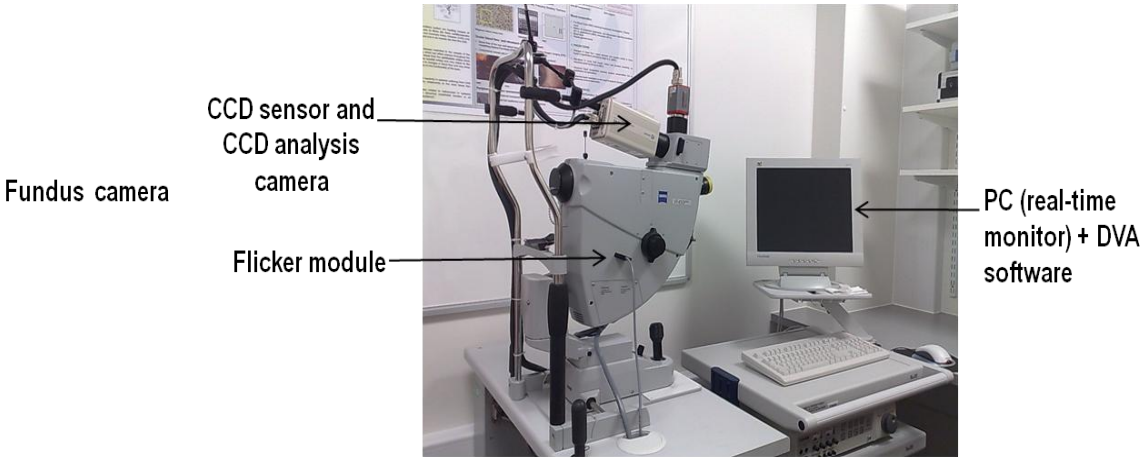


Figure 2.6: Dynamic Vessel Analysis (DVA) system and main components.

Reaction mechanism Flickering stimulus can increase the retinal blood flow and metabolic demand (Garhofer et al., 2003), and therefore indirectly affect the retinal endothelial function and vascular tone (Dorner et al., 2003). An increase of the retinal metabolic demand cause NOS to be released by endothelial cells of retinal and choroidal blood vessels (Dorner et al., 2003); as consequence there is an increase of NO in the retina circulation that cause the observed retinal vascular dilation. After stimulus removal, the retinal vascular diameter recovers to baseline values. In a normal subject, maximum dilation is reached after approximately 20 seconds of flickering stimulus. After the flicker stimulus is stopped the arteriolar diameters decrease below the baseline, reaching a minimum diameter after approximately 10-40 seconds (Polak et al., 2002). This over reactive constriction after the flicker stimulation is thought to be triggered by an overshooting regulatory mechanism of the retinal vascular endothelium. Some previous studies reported that an average of 80 seconds of recording is needed for the diameter to recover to baseline values after the flickering light stopped (Heitmar et al., 2008;Heitmar et al., 2010;Polak et al., 2002).

Therefore, DVA not only measure retinal vessels diameter and also could evaluate ocular endothelial function through measuring retinal vessel diameter responses to a flicker light because NO as a vascular dilating factor released from ocular endothelium caused by flickering stimulation. Therefore, measuring the retinal vessel responses (vasodiation and vasoconstriction) to the flicker light could indicate the ocular vascular/endothelial function indirectly. More details about the mechanisms involved in the retinal vascular reactivity response post-flickering light were offered in the Section 4.6.1.

2.4.1 The DVA measurements protocol

For the present research, measurements were performed in one randomly selected eye of each participant. Retinal vascular reactivity of both arteries and veins were assessed by the DVA system. In order to obtain a good quality measurement, pupils were dilated using tropicamide 1.0%. The camera needs to be set at 30 degrees image field angle to obtain a clear fundus image with good contrast and illumination (without reflections).

The examination was conducted in a dark and temperature controlled room. After full pupil dilation was reached, the participant was asked to focus on the tip of a fixation bar within the camera while the fundus was examined under green light. An artery and vein segment of approximately 1-1.5 mm in length were selected, where were location within a circular area of between half and two disc diameters without crossing or bifurcation for the measured segments (Kotliar et al., 2004;Nagel and Vilser, 2004;Nagel et al., 2004;Nguyen et al., 2009) (Figure 2.7). Once the selected segments were identified, the reactivity of selected segments in diameter was automatically tracked by the DVA.

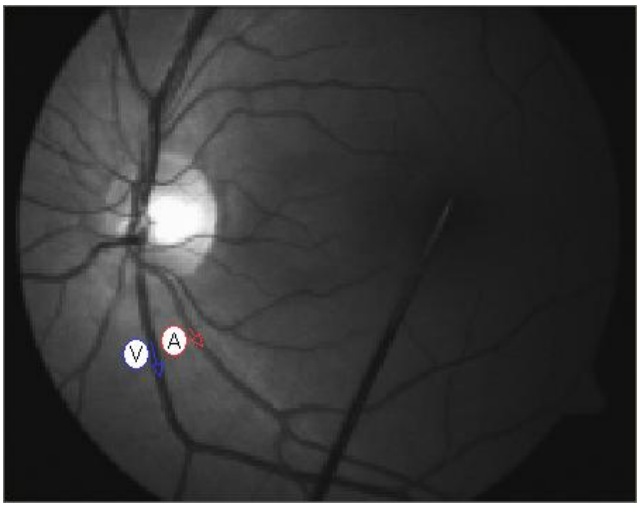


Figure 2.7: An example of the measurement locations for arteriolar (A) and venular (V) segments.

This vascular reactivity was assessed continuously over 350 seconds according to an established protocol in previous studies (Heitmar et al., 2008;Heitmar et al., 2010). In brief, the steps of the measurement were: 50 seconds still illumination (baseline recording) followed by three cycles of 20 seconds flicker provocation and 80 seconds (recovery time) still illumination (Figure 2.8). When the eye blinked, the vessel segments were not measured and measurement restarted once again, then the vessel segments were automatically re-identified.

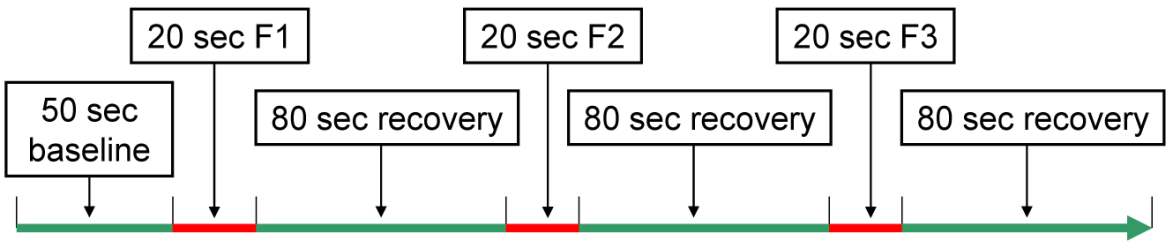


Figure 2.8: The measurement protocol of dynamic retinal vessel assessment. F1: the first flickering stimulus; F2: the second flickering stimulus; F3: the third flickering stimulus.

The diameter of the vessel segments was calculated automatically, and then created a dynamic curve (Figure 2.9). The calculated diameter was saved as a Microsoft Excel file, and these raw data sets were used for further analysis which described detailed in next.

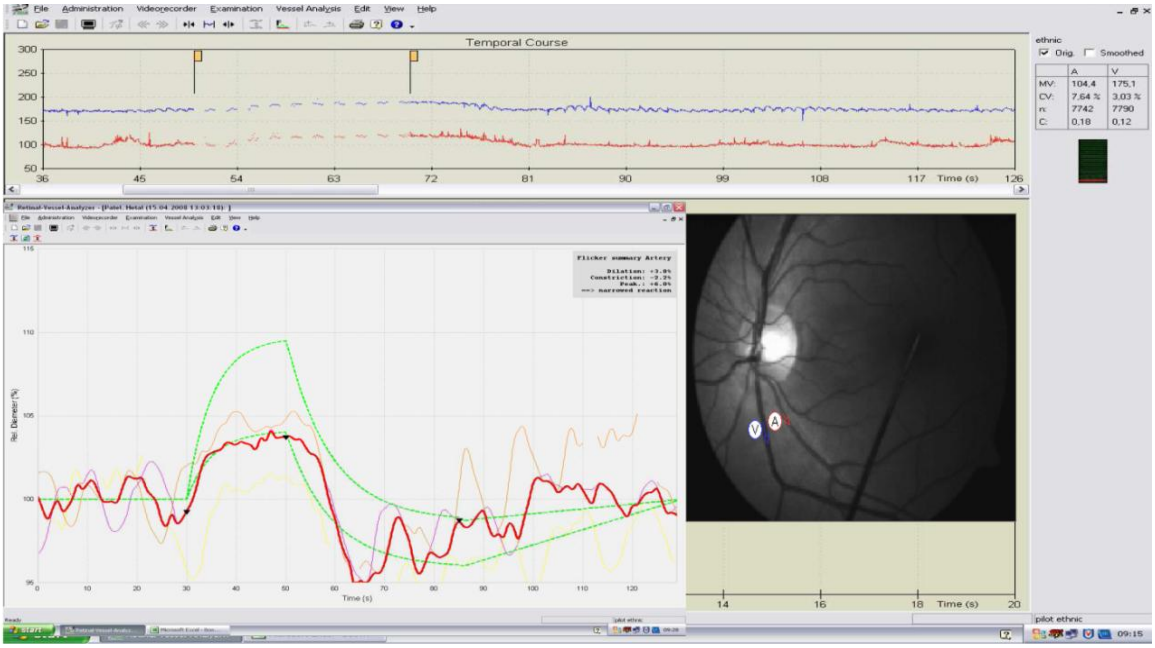


Figure 2.9: An illustration of dynamic analysis (next to the fundus image, red curve is the retinal arterial dynamic). The time course related the point of diameter on the top of the screen (arterial diameters shown in red, and venous diameter shown in blue).

2.4.2 Analysis of DVA

Retinal vessel dynamics were determined using elements of the newly defined method of 'Sequential and Diameter Response Analysis' (SDRA) (Heitmar et al., 2010). This method overcomes the limitations of the inbuilt DVA software analysis which has been demonstrated to make inaccurate assumptions about the nature of the vascular response, by using the raw data set and allowing an enhanced analysis of vascular reaction patterns.

However, the SDRA method mainly described the retinal vessel diameter change and the time course to reach the point of maximum dilation in response flicker stimulation, but not describes the response pattern (Garhofer et al., 2010). It is possible that similar to research performed on human brain arterioles (Bauser-Heaton and Bohlen, 2007;Chan et al., 2008), retinal blood vessels have a fixed maximum dilation that is similar to a rubber band, a vessel with a certain diameter has elastic properties that permit various diameter fluctuations (dilatation and constriction) up to a certain amount. As retinal vessels have similarities to those in the brain, it could be expected that a similar type of vascular reactivity as the one described above occurs in retinal arterioles and veins. However, the diameter fluctuations could be fast or slow to reached the maximum dilation and constriction that regulated by the vascular/ endothelial function, and therefore, the analysis of "slope" could directly represent how fast the retinal vessel reaching the maximum dilation and constriction, and indirectly could reflect the retinal vascular / endothelial function. For this reason, a novel approach was developed for the first time in the present thesis and is described detailed below.

2.4.2.1 Novel approach: slope analysis

In order to fully explore the nature of the entire dynamic response profile of both the dilation and constriction responses of the retinal microvasculature to flicker light stimulation, rather than just focusing on the dilation response of the vasculature, the SDRA methodology was expanded by using the raw response data and applying a statistical polynomial regression algorithm, which was implemented using the `polyfit` and `polyval` functions in 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 n as

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

in a least squares sense. The `polyfit` function locates the coefficients $p_1, p_2, ..., p_n, p_{n+1}$ such that the error $\sum_{i=1}^{T}\left(y_i - p(t_i)\right)^2$ is minimized. This involves solving the system of equations

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

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

where the vectors $p = \begin{pmatrix} p_1 \\ . \\ . \\ . \\ p_{n+1} \end{pmatrix}$ and $y = \begin{pmatrix} y_1 \\ . \\ . \\ . \\ y_T \end{pmatrix}$.

The `polyval` function was used to calculate the values of the fitted polynomials which ultimately provided us with curves representative of the dynamic vascular response profile which could then be used for analysis. The polynomial regression algorithms were performed on each of the 3 flicker cycles individually as well as on the averaged data for each individual participant, as illustrated in Figure 2.10. To ensure reliability, averaged data were used in all analysis in this study as complete recording of each individual flicker cycle was not always obtained in all cases. The degree of the polynomial, *n*, is an adjustable parameter. In the present work, $n = 20$ was applied, as this value provided the closest fit polynomials on the data points.

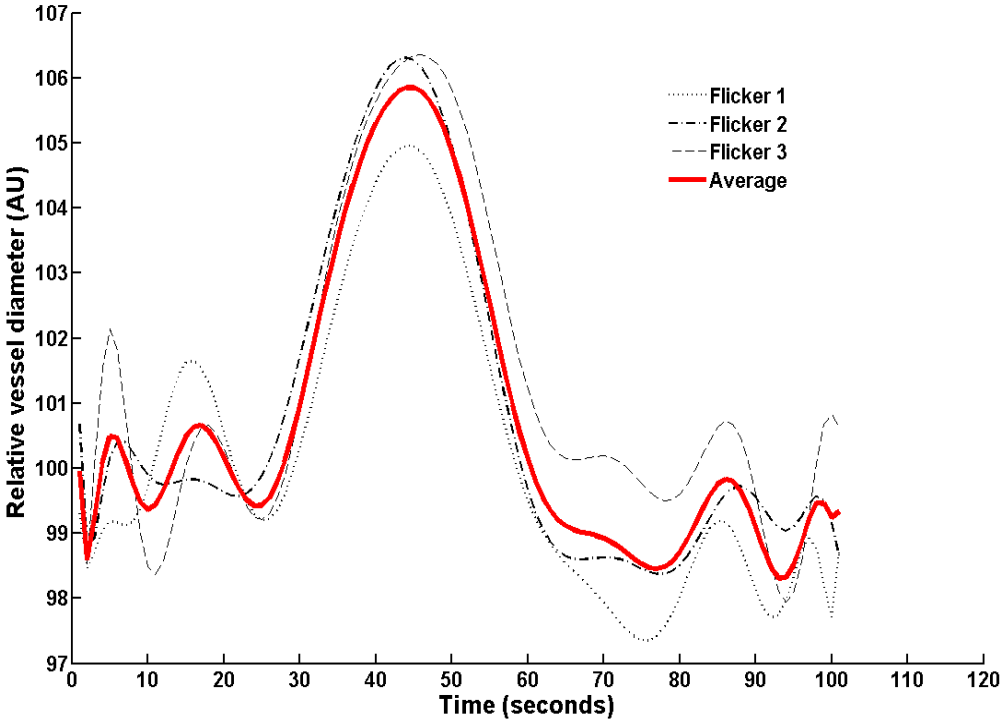


Figure 2.10: Representation of the polynomial fitted curves generated for each individual participant through Matlab from the raw data of each of the three flicker cycles and their averages.

The parameters were calculated from the generated averaged profiles depicted in Figure 2.11, and used for further analysis. In addition, baseline corrected flicker response (BFR), a parameter which indicates the overall dilation response of the vessels to flicker after correcting for the fluctuations in baseline diameter which occur with arterial pulse (Nagel et al., 2004), was also calculated by subtracting the baseline diameter fluctuation (BDF) from the difference between the maximum diameter and minimum diameter during the baseline recording. In addition, the original SDRA parameters maximum dilation (MD), maximum constriction (MC), reaction time (RT) to reach the MD to flicker, dilation amplitude (DA), BDF (Figure 2.11), BFR, the polynomial fitted curves allowed the nature

of the dynamic response profile and the slope of the vascular dilation and constriction responses to be calculated. The slope may be an important parameter to describe the endothelium-related changes in vessel diameter and the rate at which this change occurs, both of which parameters have shown to be altered in disease states individually (Lanzl et al., 2011).

For the purpose of this thesis, the term of *Dilation Slope* which defined as the diameter changes of maximum dilation diameter minus the average baseline against to the time used to reach MD; and *Constriction Slope* which defined as the diameter changes of maximum constriction diameter subtracts the maximum dilation diameter against to the time used between the two points, were employed. The arterial and venous slopes for both dilation and constriction were calculated according to the Equation 2.3.

$$\textit{Dilation Slope} = \frac{\textit{MD – average Baseline}}{\textit{RT}}$$

$$\textit{Constriction Slope} = \frac{\textit{MC - MD}}{\textit{tMDMC}}$$

Equation 2.3: The calculation of dilation slope and constriction slope. MD: maximum dilation; MC: maximum constriction; RT: reaction time to reach maximum dilation; tMDMC: time from the points of MD to MC.

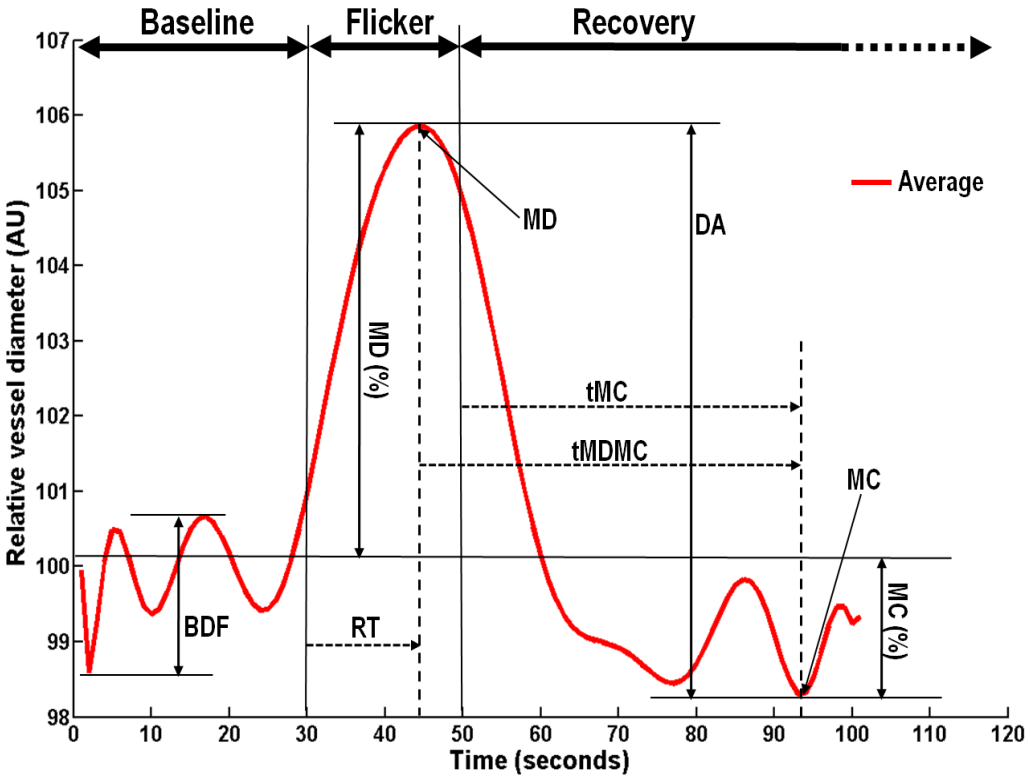


Figure 2.11: Typical average dynamic retinal vessel response profile, generated through Matlab, displaying parameters calculated and used in analysis. MD: maximum dilation; MC: maximum constriction; RT: reaction time to reach maximum dilation; BDF: baseline diameter fluctuation; DA: dilation amplitude is defined as the difference between the maximum dilatation and subsequent constriction after flicker stimulation; MD(%): percentage change in diameter from baseline to MD; MC(%): percentage change in diameter from MC to baseline diameter; tMC: taken time to MC after the cessation of the flicker; tMDMC: time from the points of MD to MC. Baseline corrected flicker response (BFR) is calculated as DA-BDF.

2.5 Other ocular assessments

2.5.1 Intraocular pressure (IOP) measurement

As the design of the study did not allow the participants to be seated at a slit-lamp, IOP was measured using “The Pulsair EasyEye” (Keeler Ltd., Clewer Hill Road, Windsor, Berkshire, SL4 4AA) before and after instillation of tropicamide 1.0%, a topical anaesthetic. The Pulsair EasyEye is an easy to use, non-contract, portable handheld instrument that provides a speed and accuracy measurement of IOP with the accuracy comparable to the Goldmann tonometry. The Pulsair EasyEye automatically fires a gentle puff of air at the cornea when the hand unit is correctly aligned with the tested eye, and an average of IOP was recorded by taking four independent readings.

2.5.2 Macular pigment optical density (MPOD) measurement

MPOD was determined using the MPS 9000 (also known as the M:Pod and the QuantifEYE, Topcon,Topcon House, Kennet Side, Bone Lane, Newbury, Berkshire, RG14 5PX, UK) (Figure 2.12). This device adopts a novel approach to measurement of MPOD by heterochromatic flicker photometry, in which subjects respond to the appearance of flicker as the alternation rate is decreased at 6 Hz per sec from a starting level of 60 Hz (van der Veen et al., 2009). As this is above the critical flicker fusion frequency for the test conditions, subjects do not perceive any flicker initially. A sequence of blue-green ratios is used and these are inverse-yoked to ensure that overall luminance remains constant. The device determines each observer’s sensitivity to flicker prior to the main part of the test.

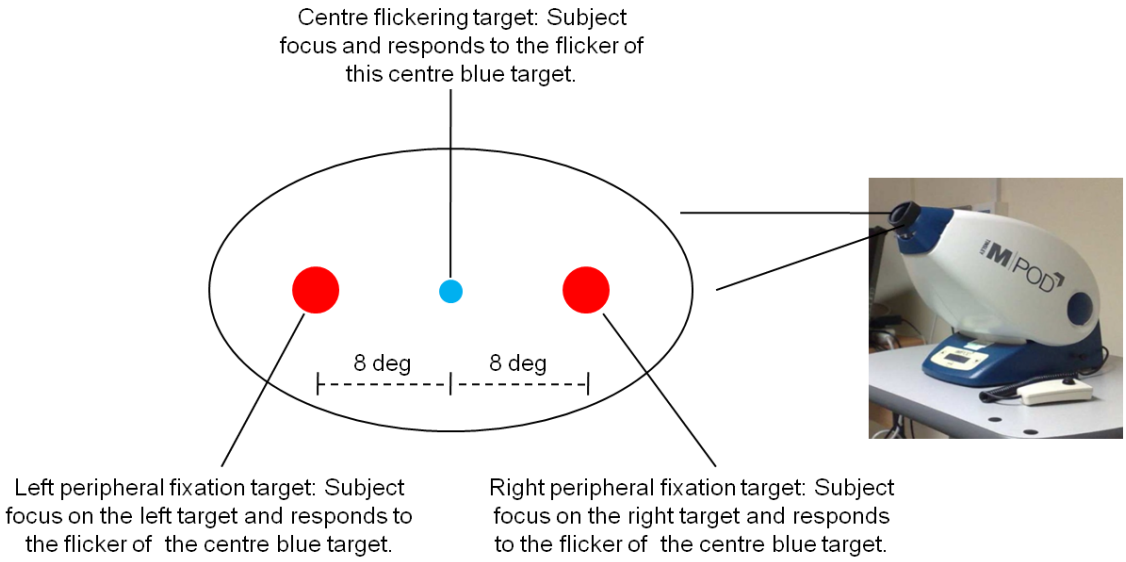


Figure 2.12: The device (MPS 9000) for the measurement of macular pigment optical density.

The eye not being tested was occluded and participants wore their habitual refractive correction (a trial frame and lenses were used when necessary). The central target is a 1° circular stimulus composed of blue (465 nm) and green (530 nm) LEDs. For the foveal (central) test, the observer looked directly at the stimulus whilst the alternation rate between the blue and green was ramped down from 60 Hz. At the point when they first detected flicker, the observer pressed a response button and this plotted a point on a graph that was visible to the operator on a computer screen. Once the flicker had been perceived, the process started again. The first five responses were used to ascertain the flicker sensitivity of the subject. The observer is asked to respond to a series of green-blue ratios until a V-shaped curve is plotted on the computer screen. The minimum point on the curve corresponds to equiluminance of the blue and green lights (Figure 2.13). The process was then repeated for the peripheral test, where the subject's gaze was directed to a larger red target, 8° eccentric from the central spot. The difference between the central and peripheral minima is used by an internal algorithm to calculate the MPOD.

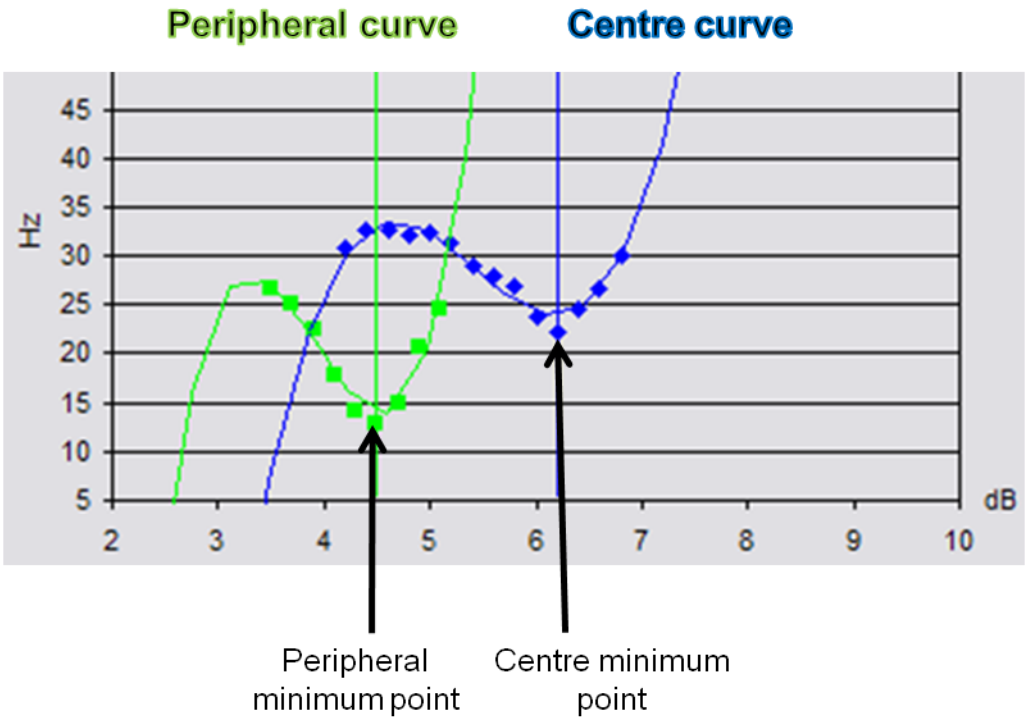


Figure 2.13: The measured curves on a computer screen. Showing the minimum points of peripheral and centre of the retina, which were used to calculate the macular pigment optical density.

2.5.3 Fundus photography

Colour fundus photograph were obtained from each participant, at a 30 degree angle and centred on the macular and optic nerve head (ONH), using the inbuilt Zeiss FF450 fundus camera (FF450 plus, Zeiss GmbH, Jena, Germany) of the DVA (IMEDOS Systems UG, Germany). The photograph was taken in a quiet, temperature-controlled room (22^{o}C) after full dilation of one unselected eye (1% tropicamide, Chauvin Pharmaceuticals Ltd). Great care was taken to ensure good contrast and sufficient illumination of the images obtained. The obtained photographs were evaluated for grading of AMD patients using a modification of the Wisconsin AMD grading system (Cheung et al., 2007a;Cheung et al., 2007b), which will be described in Section 4.4.1.2.

2.6 Blood assessments

2.6.1 Blood collection and store

For the purpose of the present thesis, venous blood were obtained from the antecubital fossa vein, and analysed in the house. The materials used for the blood collection is listed in Table 2.1. The obtained blood samples were processed immediately for storage according to each protocol described below. For glutathione, vWF and ET-1 assays, the obtained blood samples were stored in the -70 degrees Celsius freezer and stable at least for two months (Gherghel et al., 2005;Jones et al., 1998), and these assays were performed every month approximately depended on the number of obtained blood samples.

Materials	**Supplier**	**Purpose**
Safety Blood Collection Set with a butterfly needle attached	Greiner Bio-One Ltd, catalogue number 450091.	Blood collection
EDTA K3 pre- coated plastic tube	Greiner Bio-One Ltd, catalogue number 456036.	Fasting glucose , TG, Choesterol, HDL, GSH, GSSG and ET-1 anaylsis
3.2% buffered citrate pre-coated plastic tube	Greiner Bio-One Ltd, catalogue number 454325.	vWF analysis

Table 2.1: Materials were used for blood collection and sampling.

2.6.2 Blood sampling and assessment for CVD risk- glucose, triglycerides and cholesterol

Fasting glucose, triglycerides (TG), total cholesterol (CHOL) and high-density lipoprotein cholesterol (HDL-C) were measured using a Reflotron Desktop Analyser (Roche Diagnostics, UK), and various Reflotron test strips used were listed in the Table 2.2.

	Supplier	**Catalogue Number**
Glucose strip	Inverness Medical UK Limited, Cheshire, SK7 5BW	278006
Triglycerides strip	Inverness Medical UK Limited, Cheshire, SK7 5BW	278009
Total cholesterol strip	Inverness Medical UK Limited, Cheshire, SK7 5BW	278010
High-density lipoprotein cholesterol strip	Inverness Medical UK Limited, Cheshire, SK7 5BW	278020

Table 2.2: The test strips were used for glucose, triglycerides, total and high-density lipoprotein cholesterol using a Reflotron Desktop Analyser (Roche Diagnostic, UK).

In this step, 30 µl fresh EDTA (ethylenediamide tetra-acetic acid) blood were transferred to a glucose test strip and TG test strip, separately. The fasting glucose and TG levels were calculated automatically by the Reflotron Desktop Analyser. The remaining fresh EDTA blood were centrifuged at 35000 rpm for fifteen minutes, and 30 µl EDTA plasma were transferred to a total cholesterol strip and a HDL-C strip separately, and then the CHOL and HDL-C were calculated automatically. The low-density lipoprotein cholesterol

(LDL-C) was calculated by the Friedewald Formula (Friedewald et al., 1972;Warnick et al., 1990) (Equation 2.4):

LDL-C = CHOL - HDL - 1/5TG

- **LDL-C:** low-density lipoprotein cholesterol
- **CHOL:** total cholesterol
- **HDL-C:** high-density lipoprotein cholesterol
- **TG:** triglycerides

Equation 2.4: The calculation of low-density lipoprotein cholesterol according to the Friedewald Formula.

2.6.3 Assessment of oxidative stress - glutathione

Glutathione (GSH) is a tripeptide consisting of glycine, cysteine and glutamic acid. It is an antioxidant which protects cells to be damaged by free radicals and toxic compounds. It is an important intracellular antioxidant that can detoxify ROS. Since, oxidative stress can lead to excessive production of ROS, loss of antioxidant defenses or both; result in GSH depletion. GSH is oxidized to GSSG and recycled back by glutathione reductase. Therefore, the ratio of GSH to GSSG is often used to assess exposure of cells to oxidative stress (Schulz et al., 2000;Vaziri et al., 2000).

2.6.3.1 Blood sampling for the total GSH and GSSG assays

Seven millilitres of blood was collected in EDTA (ethylenediamide tetra-acetic acid) tubes (to prevent oxidation) (Anderson, 1996) by venipuncture to the antecubital vein. Thirty microliters of blood was then transferred into centrifuge tubes for the initial processing. The GSH was released from the blood cells by protein precipitation and cellular disruption achieved by addition of 33.3 μl of 5-sulphosalicylic acid (SSA), 1 g/ml within 10 minutes from the blood collection (Anderson and Meister, 1980). Each sample was then diluted

with 936.7 μl stock buffer (sodium phosphate, pH 7.5) and the content of each tube was mixed rapidly in a centrifuge, at 13000 rpm for five minutes. 150 μl of supernatant were then collected into clean centrifuge tubes and immediately cooled at –70 degrees Celsius. Samples stored at this temperature are stable for at least two months and can be transported on dry ice without deterioration (Jones et al., 1998). In my hands, GSH loss is less than 5% over this time period. The total GSH and GSSG were then analysed according to a protocol described in next.

2.6.3.2 Total GSH assay

The total GSH levels (t-GSH) were assessed by a glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling procedure (Anderson, 1996;Gherghel et al., 2005). The reagents used for this assay are listed in Table 2.3.

The GSH standards were prepared 0 to 80 μM in 20 μM increments using 10 mM GSH solution, and contained the 33.3 μl of SSA and 966 μl stock buffer. The standards contained the same final concentrations of SSA as for the samples. To each well of a 96-well plate, 150 μl of daily buffer, 50 μl of DTNB solution and 25 μl standards or blood samples were added in triplicate, and the plate was incubated at 37^{o}C for three minutes. Finally, 25 μl glutathione reductase (GSR) solution was added to each previous mixture, and read the plate immediately was read at 410 nm using a 96-well plate reader (BioTek Ltd; model: EL800), and then read again at 1, 2, 5, 10 minutes. Changes in optical density (OD) at different time points, expressed against concentration of GSH standard, and a most linear standard curve of total GSH was then generated using a linear regression program (Microsoft Excel® - Figure 2.14).

Reagents	Contents
Stock buffer	125mM sodium phosphate (Sigma catalogue number S0876-500 g), 6.3mM disodium EDTA (Sigma catalogue number ED-2SS 100g), PH7.5
Daily buffer	3mg NADPH (Sigma catalogue number N1630-50mg) into 10ml stock buffer
DTNB solution	9.6mg DTNB (Sigma catalogue number D8130-1g) into 4ml stock buffer
SSA solution	1g SSA (Sigma catalogue number 247006-500MG) into 1ml distil water
GSH solution	30.7mg GSH (Sigma catalogue number G4251-5G) into 1ml distil water
Glutathione reductase (GSR) solution	Make up to 20U/ml in stock buffer. GSR is from Sigma, catalogue number G3664-500UN
GSSG solution	30.7mg GSSG (Sigma catalogue number 150568-500MG) into 0.5ml distil water
TEA	Triethanolamine, reagent grade, 98% (Sigma catalogue number T58300)
2-VP	2-vinyl pyridine (Sigma catalogue number 132292-100ML)

Table 2.3: The reagents were used in the GSH and GSSH assay. EDTA: ethylenediamide tetra-acetic acid; NADPH: β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate. DTNB: 5.5 dithiobis-2-nitrobenzoic acid; SSA: 5-sulphosalicylic acid; GSH: glutathione in its reduced form; GSSG: glutathione in its oxidized form; TEA: triethanolamine; 2-VP: 2-vinyl pyridine.

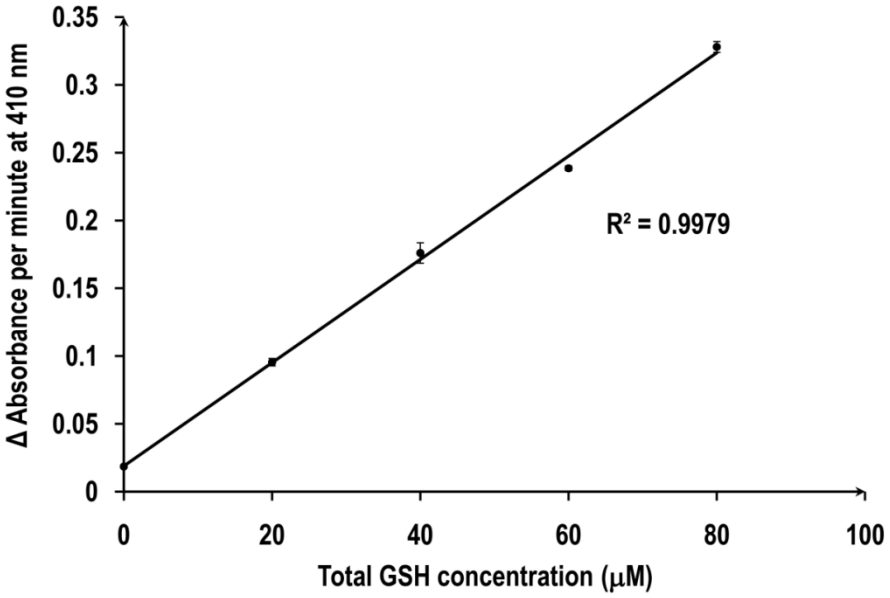


Figure 2.14: A standard curve for total GSH assay.

2.6.3.3 GSSG assay

The GSSG levels were assessed using a glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling procedure (Anderson, 1996;Gherghel et al., 2005). The reagents used in this assay were those already described in Table 2.3 and, in addition, triethanolamine (TEA) and 2-vinyl pyridine (2-VP). TEA prevents a local high pH and oxidation while 2-VP is used for derivitization GSH.

A GSSG standard curve from 0 to 10 µM, in 1 µM increments was prepared, and contained the 33.3 µl of SSA and 966 µl stock buffer. The standards contained the same final concentrations of SSA as for the samples. 100 µl of standards and samples were transferred into separate centrifuge tubes and 2 µl 2-VP was added to each tube. TEA was then used to adjust the pH of the standards/samples to pH7.5. The assay was carried

out as for GSH assay described above. Finally, a most linear standard curve of GSSG was then generated using a linear regression program (Microsoft Excel® - Figure 2.15).

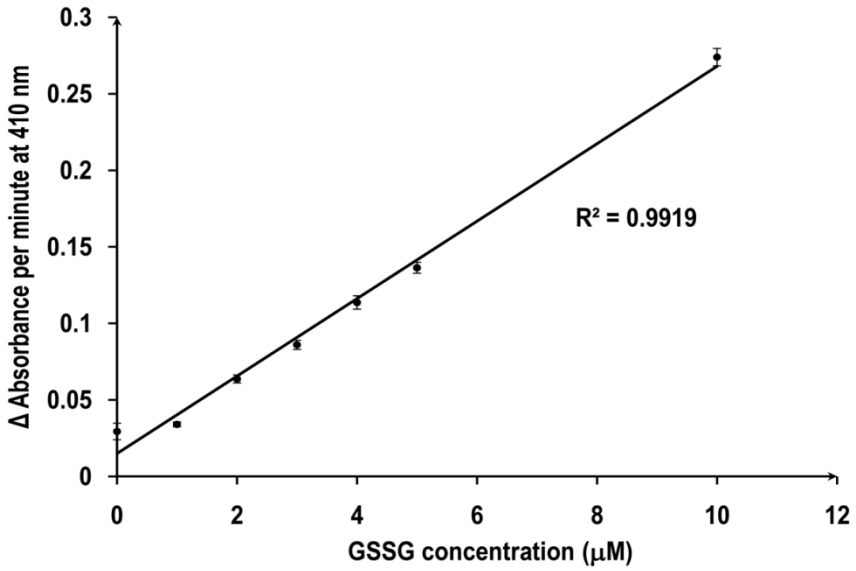


Figure 2.15: A standard curve for GSSG assay.

The GSH (Equation 2.5) and the redox index (Equation 2.6) were calculated in accordance with the equations (Rossi et al., 2002).

$$GSH = t\text{-}GSH - (2 \times GSSG)$$

- GSH: glutathione in its reduced form
- t-GSH: the point of maximum dilation
- GSSH: glutathione in its oxidized form

Equation 2.5: The calculation of the levels of reduced form glutathione.

Redox index = GSH/ GSSG

- GSH: glutathione in its reduced form
- GSSH: glutathione in its oxidized form

Equation 2.6: The calculation of the redox index.

2.6.4 Assessment of von Willebrand factor (vWF)

vWF is an important glycoprotein (released and stored in the endothelial cells but also platelets) involved in cardiovascular physiology as it is associated with platelet aggregation and adhesion. The primary function of vWF is to bind other proteins (factor VIII in its inactive form) to be activated by thrombin so that it can bind to collagen or platelets when exposed to vessel damage and shear stress. (Constans and Conri, 2006;Vlot et al., 1998). Therefore, vWF is considered a gold standard in the measurement of endothelial impairments and endothelial dysfunction and has prognostic value in those with heart disease, peripheral vascular disease and inflammatory vascular disease (Vischer, 2006).

2.6.4.1 Blood sampling for the vWF assay

Three millilitres of blood was collected in citrate vacuettes tubes by venipuncture to the antecubital vein, and was mixed rapidly in a centrifuge at 35000 rpm for fifteen minutes. The supernatant were then collected into clean cryogenic vials and immediately cooled at –70 degrees Celsius for further batch analysis by an enzyme-linked immunosorbent assay (ELISA) according to a protocol described in next.

2.6.4.2 vWF assay

A method ELISA was used for assessing the level of plasma vWF using commercial antisera from Danish company Dako. The method used in this thesis was developed and described by Dr. Blann (Blann, 1992). A similar method was performed in house by the author. The reagents used and the procedure of method for vWF assay were described in Table 2.4 and Figure 2.16. A standard curve was generated using a liner regression program (Microsoft Excel®- Figure 2.17).

2.6.5 Assessment of endothelin-1 (ET-1)

ET-1 is mainly generated by vascular endothelial cells, which has vasoactive properties that affect both systemic and peripheral circulation (Angerio and Kot, 1997). It has been demonstrated that ET-1 plasma levels are significantly increased in patients suffering from systemic vascular disease (Lerman et al., 1991;Videm et al., 2007), such CHD and symptomatic atherosclerosis. Moreover, as ET-1 has been shown to be involved in the systemic vascular diseases mechanisms and might alter the macro- and/or micro-vascular system, as well as ocular circulation as a part of micro-vascular circulation that might be influenced. Wollensak et al. has suggested that raised level of ET-1 is particular importance in a variety of ocular diseases including diabetes mellitus, hypertension and optic neuritis (Wollensak et al., 1998). According those studies, increased ET-1 may indicate the pathophysiology of the endothelial dysfunction, and therefore, ET-1 may act a potential indicator for endothelial function.

1. Reagents used for the vWF assay

The reagents used in the assay were listed in the Table 2.4.

Reagents	Contents	Method
Coating Buffer	Sodium carbonate (Na_2CO_3, Sigma catalogue number S1641); Sodium hydrogen carbonate ($NaHCO_3$, Sigma catalogue number S5761).	Add 795 mg sodium carbonate and 1465 mg sodium hydrogen carbonate to 500 ml distilled water. Place on rotary mixer with magnetic stir bar. The salts may take five minutes to dissolve at room temperature. It is generally not necessary to check the pH of your buffer as this recipe is very stable. Store at 4 degree Celsius.
Primary Antiserum	Rabbit anti-human vWf polyclonal antiserum (DakoCytomation, their catalogue number is A0082).	Store at 4 degree Celsius.
Microtitre plates	Immulon 2, flat-bottomed 96 well high binding microtitre plates (Thermo Fisher Scientific, catalogue number 3455).	Store at room temperature
Wash buffer	Phosphate buffer saline tablets (Sigma catalogue number P4417); Tween 20 (Sigma catalogue number P1379); and distilled water.	Add 5 tablets and 0.5 ml Tween 20 (use a 200-1000 microlitre micropiptette) into one litre of distilled water. Place on rotainixer with stir bar, wait until tablets have dissolved. Store at room temperature.
Secondary Antiserum	Rabbit anti-human vWf polyclonal antiserum conjugated to horse radish peroxidase (HRP) (DakoCytomation-Inverness Medical UK Limited, their catalogue number P022602).	Store at 4 degree Celsius.
Citrate Phosphate Buffer	Citric acid (anhydrous) ($C_3H_4OH(COOH)_3$, Sigma catalogue number C0759); Sodium hydrogen phosphate (anhydrous) (Na_2HPO_4, Sigma catalogue number S0876); and distilled water.	Citrate phosphate buffer- add 3.65 g citric acid and 4.73 g sodium hydrogen phosphate to 500 ml distilled water, it should solublise immediately. Check pH to be about 5.3, and store this buffer at 4 degree Celsius.
Substrate	Ortho-phenylene diamine (OPD, Sigma catalogue number P8287); Hydrogen peroxide (Sigma catalogue number H1009)	Add one OPD tablet and 10 ml hydrogen peroxide into 20 ml citrate phosphate buffer. The OPD tablet should dissolve in a few minutes, mixing those in a big plastic tube (ensure good mixing). This substrate was prepared just before used.
Stop Solution	1 mol/L hydrochloric acid. No need to dilute. Sigma catalogue number 318949.	Store at room temperature

Table 2.4: The reagents used in vWF assay.

2. The procedure of vWF assay

The procedure of vWF assay is described briefly in a flow chart (Figure 2.16).

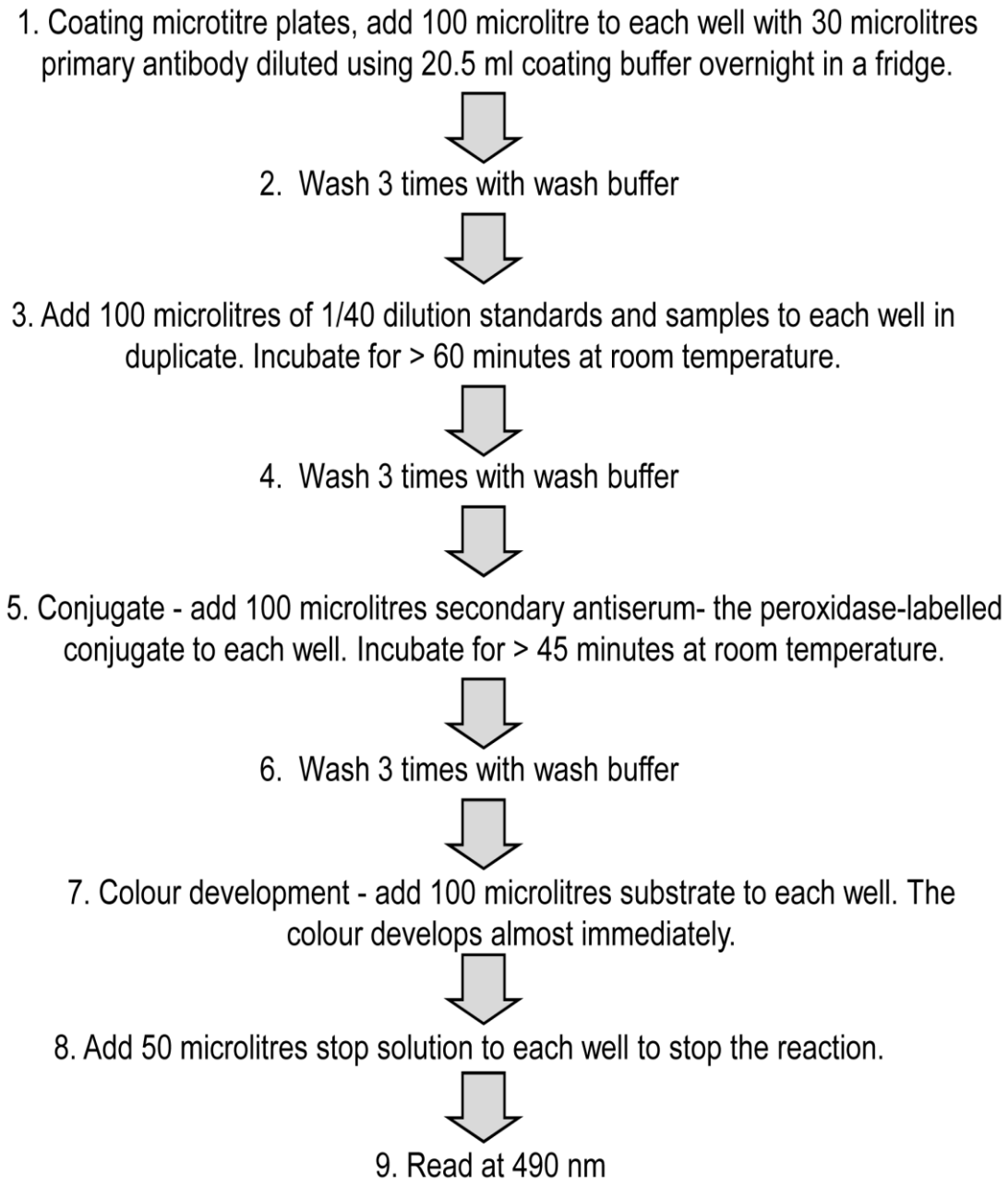


Figure 2.16: The procedure of vWF assay in brief.

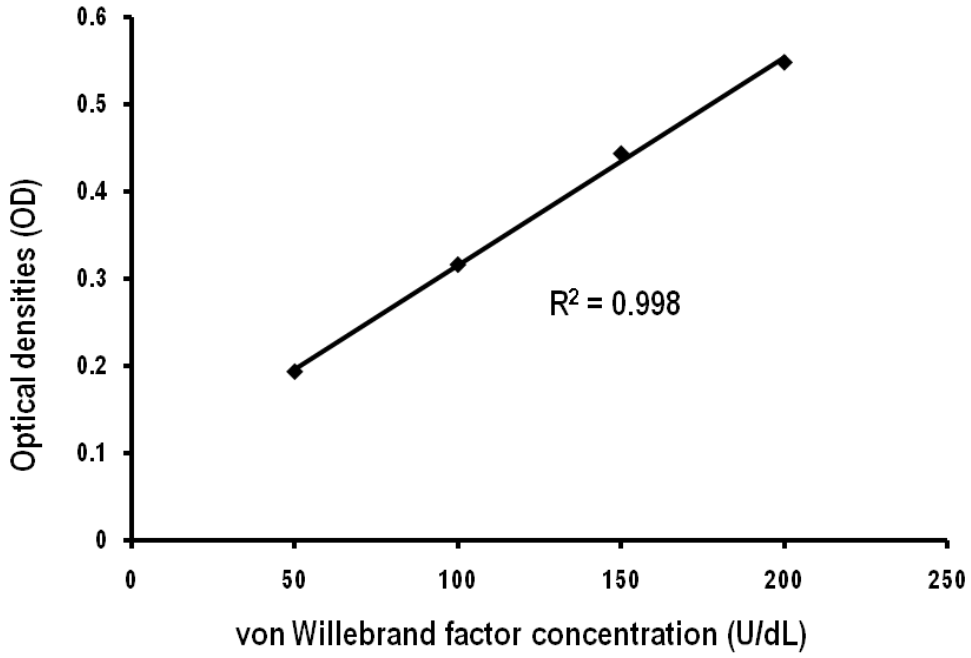


Figure 2.17: A standard curve for von Willebrand factor assay.

2.6.5.1 Blood sampling for the ET-1 assay

Six millilitres of blood was collected in EDTA vacuettes tubes by venipuncture to the antecubital vein, and was mixed rapidly in a centrifuge at 35000 rpm for fifteen minutes. The supernatant were then collected into clean cryogenic vials and immediately cooled at –70 degrees Celsius for further batch analysis by ELISA according to a protocol described in below.

2.6.5.2 ET-1 assay

The plasma levels of ET-1 were determined by ELISA. This assay was performed by using the human ET-1 commercial kits (R&D Systems Europe Ltd, 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, catalogue number QET00B) according to the manufacturer's protocol. Standards and plasma samples were added to each

monoclonal ET-1 antibody pre-coated well and incubated for 1.5 hours on a horizontal orbital microplate shaker at room temperature, and any ET-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol (a chemiluminescence) substrate solution was added to the wells and light is produced in proportion to the amount of ET-1 bound in each well. A microplate luminometer (Berthold Technologies (UK) Ltd; model: Orion II Microplate Luminometer) is used to measure the intensity of the light produced. Read the values of the intensity of the light expressed against the concentration of ET-1 standards, and a standard curve of plasma ET-1 was then generated using a linear regression program (Microsoft Excel®- Figure 2.18).

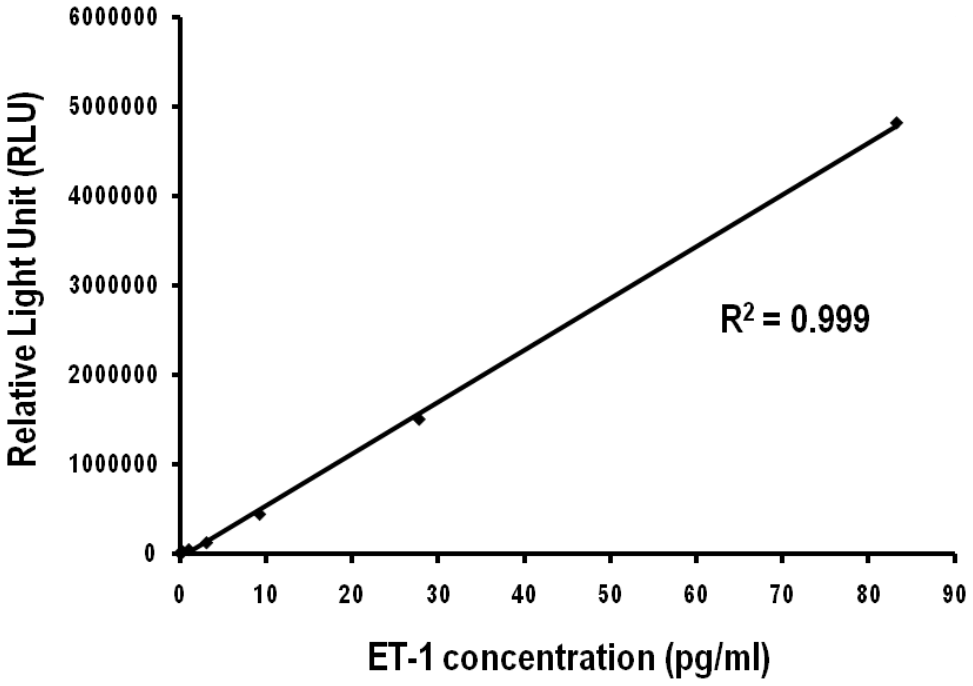


Figure 2.18: A standard curve for Endothelin-1 assay.

Chapter 3

Macular pigment optical density is related to blood glutathione levels in healthy individuals

The publication of this work is presented in Appendix 2.1

3.1 Abstract

Purpose: To assess the relationship between MPOD and blood markers for antioxidant defence in otherwise healthy volunteers.

Methods: 47 healthy volunteers were subjected to blood analysis to detect the levels of circulating GSH and GSSG. The level of MPOD was measured using heterochromatic flicker photometry. Systemic BP parameters, HR and BMI as well as plasma levels of CHOL, HDL-C and LDL-C cholesterol and TG were also determined.

Results: A simple correlation model revealed that the level of MPOD correlated significantly and positively with both GSH ($p<0.001$) and t-GSH ($p<0.001$) levels but not with those of GSSG ($p>0.05$). Age, gender, systemic BP parameters, HR, BMI and plasma levels of cholesterol and TG did not have any influence on either MPOD or glutathione levels (all $p>0.05$). Furthermore, a forward stepwise multiple regression analysis showed MPOD to have a significantly and independent correlation with GSH levels ($\beta=0.63$, $p<0.001$).

Conclusions: There is a positive correlation between local and systemic antioxidant defence mechanisms in otherwise healthy, elderly individuals.

3.2 Introduction

Oxidative stress represents a harmful state defined by the presence of pathological levels of ROS relative to the antioxidant defence, have been reported to play a role in the etiology of AMD (Beatty et al., 2000;Cai et al., 2000;Khandhadia and Lotery, 2010). In healthy subjects, local retinal protection against free radicals is aided by the presence of the MP, which is comprised of the carotenoid lutein and its isomers, zeaxanthin (Bone et

al., 1985) and meso-zeaxanthin (Khachik et al., 2002). It has been suggested that these xanthophylls play a similar role in humans as in plants, that is, as antioxidants and screeners of high-energy blue light (Krinsky, 2002). In this way, MP may prevent light-initiated oxidative damage to the retina and therefore protect against subsequent AMD (Hammond B.R et al., 1998). Indeed, the absorbance spectrum of MP peaks at 460 nm and it is thought to act as a broadband filter, reducing the sensitivity of the macular region to short wavelength light which is most damaging in the 440 to 460 nm range (Pease et al., 1987;Reading and Weale, 1974). In addition, the fact that lutein and zeaxanthin have been found in higher concentration in the rod outer segments of the perifoveal retina than the peripheral retina, lends support to their proposed protective role against AMD (Rapp et al., 2000). These carotenoids are able to quench singlet oxygen (a potent oxidant) (Krinsky and NI, 1979), scavenge ROS (Di Mascio et al., 1989), limit peroxidation of membrane phospholipids (Lim et al., 1992), and reduce lipofuscin formation (Sundelin and Nilsson, 2001).

In addition to local retinal damage, high levels of oxidative stress also induces vascular changes that confer a background for circulatory disturbances in the systemic macro- and microcirculation that are also present in AMD patients (Lip et al., 2001;Zaliuniene et al., 2008). Indeed, it has been demonstrated that in addition to ocular vascular complications, patients suffering from AMD are at higher risk for developing CHD and stroke (Wong et al., 2006;Wong et al., 2007); moreover, they also are more likely to have a decreased survival rate comparing to the general age-matched population (Hyman et al., 2000). It has been shown that AMD patients demonstrate lower circulating glutathione, a major low-molecular weight antioxidant peptide (Samiec et al., 1998); in addition to a direct vascular effect this deficiency could also result in low bioavailability of the vasodilatory molecule, NO (Evereklioglu et al., 2003) and endothelial dysfunction, a condition known to precede both metabolic and cardiovascular diseases but also ocular circulatory disturbances.

3.3 Hypothesis

In fact, dietary intake of lutein and zeaxanthin can increase the level of MP with possible positive effect on AMD prevention and prognosis (Bhosale et al., 2007;Wenzel et al., 2007). Additionally, in taking carotenoid rich food has a positive influence on systemic antioxidant status in healthy individuals (Upritchard et al., 2003). Therefore, I hypothesize that the local antioxidant defence is associated with the status of systemic antioxidant defence in healthy elderly volunteers.

3.4 Aims

The aim of the present study was to examine the relationship between local and systemic antioxidant defence markers in elderly population without ocular and/or systemic diseases.

3.5 Subjects and Methods

3.5.1 Recruitment of participants

Healthy subjects were recruited by advertising at Aston University, Birmingham, UK. The participants had to be free from any form of vascular and heart disease.

3.5.2 Exclusion criteria

The details of exclusion criterion were described in Section 2.1.1.

3.5.3 Ethical approval

Ethical approval was sought from the local ethics committee, and written informed consent was received from all participants prior to enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

3.5.4 Investigations

3.5.4.1 Subjects preparation

Subjects were instructed to fast after 9pm on the evenings before being tested. On the morning of the test, subjects were requested to have only a light breakfast such as simple toast. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits or fruit juice (Jones et al., 1992). In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least two hours before the visit.

All participants were required to complete a questionnaire on their general health, physical activity, alcohol consumption, daily intake of fruit and vegetables, as well as of other nutrients. Height and weight of the participants were measured and the BMI (weight/height^2) was then calculated.

3.5.4.2 BP measurement

BP parameters were measured in each subject in the morning between 8 and 9 am with a BP monitor (UA-779, A7D Instruments Ltd., Oxford, UK). The details of measurement were described in the Section 2.3.1.

3.5.4.3 Blood sampling and analyses

All blood samples were obtained between 9am and 10am in the same day of the visit. Fasting TG, CHOL and HDL-C were measured automatically using a Reflotron Desktop Analyser (Roche Diagnostics, UK) and described detailed in the Section 2.6.2.

Total GSH assay: The total blood GSH sample was prepared using the EDTA blood with SSA (1g/ml) to minimizes artefactual sample oxidation and removes interfering protein thiols, and details of sample preparation have been offered in the Section 2.6.3.1. The total GSH assay were performed using a glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling assay, which has been already described in the Section 2.6.3.2.

GSSG assay: The preparation of blood GSSG sample was same as total blood GSH sample, and details of sample preparation have been offered in the Section 2.6.3.1. The GSSG assay was also assessed using the glutathione reductase-DTNB recycling assay. In addition, TEA and 2-VP were added in this assay to prevent a local high PH and oxidation. More details of this assay have been offered in the Section 2.6.3.3.

The GSH levels [t-GSH - (2 x GSSG)] and the redox index (defined as the GSH/ GSSG ratio) were then calculated.

3.5.4.4 MPOD measurement

MPOD was determined using the MPS 9000 (also known as the M:Pod and the QuantifEYE, Topcon, Topcon House, Kennet Side, Bone Lane, Newbury, Berkshire, RG14 5PX, UK) in each participant. Detailed description of this device and its measurement has been offered in the Section 2.5.2.

3.5.5 Statistical analysis

The statistical analysis was performed using Statistica® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. Data are expressed as mean with standard deviation (SD). A Pearson's correlation test and a forward stepwise multiple regression analysis were performed to test the relationship between the measured variables. P-values of less than 0.05 were considered statistically significant.

3.6 Results

3.6.1 Sample

Sixty-one healthy subjects with similar dietary habits were selected for the inclusion in the present study. However, after the evaluation of the fundus photographs and eliminating those with potential macular changes, only 47 healthy subjects (29 women and 18 men) were included in the final analysis. The characteristics of the study participants are given in the Table 3.1.

Age (years)	Gender (F/M)	SBP (mmHg)	DBP (mmHg)	BMI (kg/m^2)	Total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)	TG (mmol/L)
50 (9)	29/18	118 (13)	73 (9)	25.20 (4.15)	4.46 (0.82)	1.29 (0.33)	2.61 (0.80)	1.23 (0.62)

Table 3.1: The characteristics of the study participants. F: female; M: male; SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; CHOL: total cholesterol; HDL-C: high-density lipoprotein; LDL-C: low-density lipoprotein; TG: triglycerides.

3.6.2 Measured blood lipids, oxidative stress and MPOD parameters

Table 3.2 shows the measured anthropometric and vascular parameters determined by gender. Men exhibited higher SBP (p=0.018), DBP (p=0.01), MBP (p=0.09) and lower HDL-C levels (p=0.002) than women. However, MPOD and systemic oxidative markers were similar between the two genders (all p>0.05).

	Female	Male	P-value
Age (years)	49 (9)	50 (8)	>0.05
MPOD	0.46 (0.18)	0.47 (0.12)	>0.05
SBP (mmHg)	115 (12)	124 (12)	0.018
DBP (mmHg)	70 (9)	77 (8)	0.010
MBP (mmHg)	88 (9)	92 (9)	0.009
HR (bpm)	72 (7)	69 (8)	>0.05
BMI (kg/m^2)	24.48 (4.06)	26.37 (4.13)	>0.05
TG (mmol/L)	1.16 (0.65)	1.35 (0.58)	>0.05
CHOL (mmol/L)	4.52 (0.69)	4.37 (1.00)	>0.05
HDL-C (mmol/L)	1.40 (0.29)	1.11 (0.30)	0.002
LDL-C (mmol/L)	2.58 (0.75)	2.65 (0.89)	>0.05
t-GSH (μmol/L)	1177 (564)	1089 (416)	>0.05
GSSG (μmol/L)	88 (44)	78 (51)	>0.05
GSH (μmol/L)	1002 (526)	932 (383)	>0.05
Redox index (GSH:GSSG)	11 (7)	12 (6)	>0.05

Table 3.2: The measured parameters for men and women. MPOD: macular pigment optical density; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; BMI: body mass index; TG: triglycerides; CHOL: total cholesterol; HDL-C: high-density lipoprotein; LDL-C: low-density lipoprotein; GSSG: oxidized glutathione; GSH: reduced glutathione; t-GSH: total GSH.

3.6.3 Correlation

A simple correlation model revealed that the level of MPOD correlated significantly and positively with both blood GSH ($r=0.64$; $p<0.001$) and t-GSH ($r=0.63$, $p<0.001$) levels but not with those of GSSG ($p>0.05$). Age, systemic BP parameters, BMI and plasma levels of cholesterol and TG did not have any influence on either MPOD or blood glutathione levels (all $p>0.05$). A stepwise multiple regression analysis revealed MPOD levels to be independently, significantly and positively correlated with the blood GSH levels ($\beta=0.64$, $r^2=0.413$, $p<0.001$, Figure 3.1).

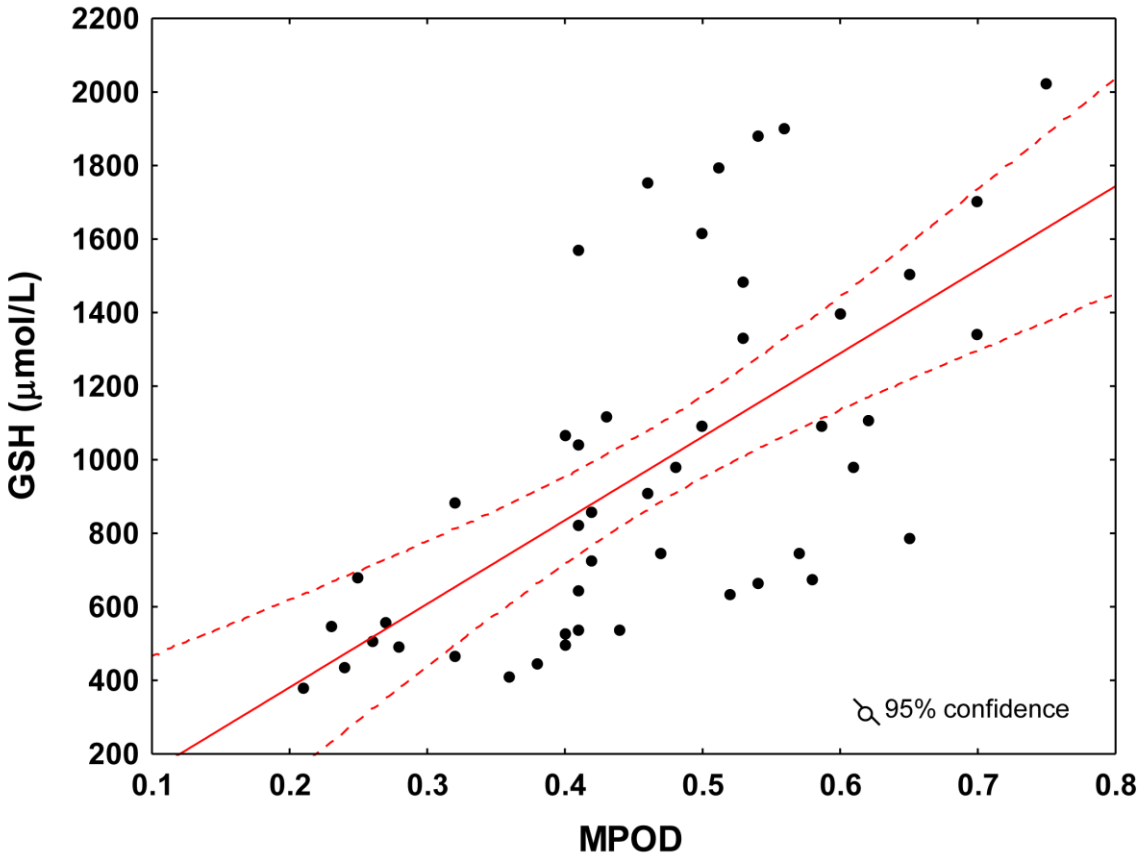


Figure 3.1: The correlation between the levels of MPOD and GSH in the study population ($p<0.001$). MPOD: macular pigment optical density; GSH: reduced glutathione.

3.7 Discussions

3.7.1 Main finding

The present study assessed the MPOD, circulating GSH and GSSG levels in the elderly healthy population. The results demonstrated that MPOD correlated with the circulating GSH level in older, health individuals.

3.7.2 Relationship between MPOD and systemic oxidative stress

At the retinal level, oxidative stress results in degeneration and death of the RPE and photoreceptors (Liang and Godley, 2003). As these retinal structures are not able to regenerate after such insult, protective mechanisms have developed to ensure a minimal local effect of free radicals (Davies and Morland, 2004). Indeed, MP is present in the rod outer segments and RPE and its specific spectral absorption and the presence of lutein and zeaxanthin have enabled it with strong, protective antioxidant properties. There are several methods for measuring the level of MP, including various subjective psychophysical and objective optical techniques (Davies and Morland, 2004). One of these subjective methods is represented by the heterochromatic flicker photometry that involves the calculation of MPOD based on the luminance ratio of short wavelength blue light presented in the central retina (where is assumed to be partially absorbed by the MP) compared to that presented at a more peripheral retinal point, where MP levels are assumed to be minimal (Bartlett et al., 2010). This method offers a good measure of the MPOD levels and is widely available in the practice. By using such method, it has shown for the first time an independent, significant and positive relationship between MPOD and blood GSH levels. At this stage more research is necessary to provide better knowledge of the exact mechanisms responsible; nevertheless, few hypotheses can be still proposed.

It has been reported that dietary intake of carotenoids had an influence not only on the level of MP (Bhosale et al., 2007;Wenzel et al., 2007) but also on the systemic circulating antioxidant markers (Upritchard et al., 2003). In addition, exogenous supply of GSH protects the RPE against oxidative damage (Sternberg et al., 1993). Although the individuals included in the present study did not receive either a special diet rich in carotenoids nor GSH or other antioxidant supplementation, the above mentioned results simply show that a relationship between local and systemic protective mechanisms, such as that found by this study could exist. Consequently, although novel, the results should not represent a surprise. Other mechanisms can also be speculated. Melatonin, a neurohormone that is secreted by retinal and the pineal gland, has an influence on the RPE and controls the amount of light reaching the photoreceptors; in addition, it also acts as potent antioxidant at both ocular and systemic level and, in such capacity, it has been advocated to reduce the risk for pathologies associated with high oxidative damage such as AMD (Yi et al., 2005) and cardiovascular disease (Reiter et al., 2010). In addition, melatonin also activates other antioxidant defences including glutathione peroxidase (GPx), an enzyme that uses GSH as a substrate to eliminate ROS (Bendich, 1990;Pompella et al., 2003). The melatonin production could be affected by ageing as the pupil diameter and the light absorption through the crystalline lens changes with age progression (Charman, 2003;Skene and Arendt, 2006;Turner and Mainster, 2008). Consequently, ageing contributes to an abnormal melatonin production and in addition decreases antioxidant defence, which in turn will result in accelerated ageing processes with various effects throughout the human body, including the eye.

Although measuring melatonin levels was not an aim in the present research, it could be still hypothesized that a link between ocular and systemic antioxidant mechanisms could also be established indirectly via melatonin. This hypothesis should, however, be tested in various age groups. In addition, other mechanisms are most certainly involved and should be further researched using more complex analyses. The role of antioxidants proved to

have a link to both macular pigment and circulating GSH levels should also be researched. Nevertheless, as previously emphasized the positive correlation between the levels of MPOD and GSH level demonstrated in this study's selected sample probably only shows that in healthy individuals, the antioxidant defence mechanisms present at various levels act in the same direction to protect the body against harmful effects of ROS. However and maybe most importantly, this research points towards the necessity of studying various normal relationships between ocular and systemic protective mechanisms against diseases with multiple etiologies and complications, such as AMD. In this way we could understand better results reported after various pathological changes have already occurred. Reducing the risk for AMD is important and strengthening bodily natural mechanisms that are at their best in healthy individuals seems to be one of the possible approaches.

Although some studies report gender differences in plasma GSH levels (Flagg et al., 1993), others did not confirm it in either plasma or blood GSH (Michelet et al., 1995). In agreement with the later studies I also could not find any difference between men and women with respect to blood glutathione levels. It is possible that various methods used for glutathione assay are responsible for this lack in results consistency. There is no general agreement to what method is best for analyzing circulating glutathione. In the present study I used a validated method for measuring blood levels of GSH and GSSG (Anderson, 1996;Gherghel et al., 2005;Griffith, 1980) and known to minimize variability from more complicated sample preparation steps associated with methods measuring plasma GSH (Michelet et al., 1995). In addition, in order to avoid variations and GSH loss I have paid particular attention to blood collection, initial processing and storage. Blood samples were collected at the same time (between 9am and 10am) and processed in the same way and time interval from collection in all individuals. Moreover, incorporation of GSR in our assay confers it specificity to glutathione.

3.7.3 Conclusion

In conclusion, this study has demonstrated for the first time that in older, healthy, non-smoking individuals MPOD correlates with the whole blood levels of GSH. As assays for measuring systemic levels of antioxidant molecules are complex and need specialized laboratories, it is tempting to propose that a simple MPOD assessment could be used as surrogate indicator of the individual's systemic capacity of dealing with the damaging effect of free radicals and, consequently, of their risk for developing chronic ocular and/or systemic pathologies. Nevertheless, this assumption should be verified in larger studies and clinical significance of association such as the one reported by this present research should be carefully analyzed.

3.7.4 Study limitations

As previously emphasized, this present research included only patients over the age of 40 that were carefully selected to exclude macular changes or other ocular pathologies as well as systemic chronic disease and various therapeutic interventions. I have also excluded smokers and individuals taking any antioxidant supplements. This careful selection has limited the number of individuals included in the final analysis. It is possible that the relationship between MPOD and circulating glutathione level is different not only in various age groups but also in individuals with additional risk factors for AMD and/or vascular disease. Moreover, individuals having a diet either lacking or very rich in carotenoids or receiving various supplements as well as patients suffering from AMD with or without vascular complications may display very different results. More studies to include larger and more various populations to cover the many possible confounds that have been either missed or intentionally avoided in the present study are warranted.

Chapter 4

Macro- and microcirculation abnormalities and high oxidative stress level in patients with early age-related macular degeneration and without overt cardiovascular disease

4.1 Abstract

Purpose: To investigate signs of systemic and retinal vascular dysfunction and their relationship to circulatory markers of oxidative stress in patients diagnosed with early AMD.

Methods: All the participants were underwent BP, C-IMT and AIx measurements. Retinal vascular reactivity was assessed by means of DVA using a modified protocol. Blood analyses were conducted to detect the level of blood GSH and GSSG, as well as for plasma levels of CHOL, HDL-C, LDL-C and TG.

Results: The AMD patients showed significantly higher LDL-C (p=0.033), C-IMT (p=0.029) and AIx (p=0.042) than the age-matched controls. In addition, they demonstrated greater venous reaction time (RT) (p=0.026) as well as a shallower slope of arterial dilation ($Slope_{AD}$) (p=0.005). Blood analyses also revealed that AMD patients exhibited higher levels of blood GSSG (p=0.024) and lower redox index (p=0.043) than the healthy controls. Venous RT parameter correlated positively with blood GSSG levels (r=0.58, p=0.038) in AMD subjects, but not in the controls (p>0.05).

Conclusions: Even in the absence of overt systemic vascular disease, in patients diagnosed with early AMD there are potential signs of retinal vascular function impairment that correlates with high levels of systemic oxidative stress.

4.2 Introduction

Systemic risk factors suggested for AMD, a blinding disease affecting millions of people worldwide, include smoking (Chakravarthy et al., 2010), increased BMI (Adams et al., 2011), and poor nutrition (Cho et al., 2001). In addition, high levels of oxidative stress (Cai

et al., 2000;Khandhadia and Lotery, 2010), chronic inflammation (Nagineni et al., 2012), and endothelial dysfunction (Lip et al., 2001) also involved in the pathogenesis of this disease. These later risk factors confer a background for functional and structural circulatory disturbances in both the macro- and micro-circulation in subjects at risk and are shared by other serious vascular pathologies, such as CVD. Indeed, data from the Rotterdam Eye (Vingerling et al., 1995), Beaver Dam Eye (Klein et al., 1993) and Cardiovascular Health Studies (Klein et al., 2003) showed that AMD is associated with subclinical signs of CVD. In addition, the Atherosclerosis Risk in Communities (ARIC) Study found that patients suffering from AMD are at higher risk than age-matched healthy controls for developing overt pathologies, such as CHD and stroke (Wong et al., 2006;Wong et al., 2007), all leading to a decreased survival rate (Tan et al., 2008). By screening more than 1.5 million subjects over 65 years of age, Liao et al. (2008) also found that independent of demographic factors and comorbidity, the risk of incident stroke was 20-30% higher in AMD patients (representing 10.6% of the total) compared to controls (Liao et al., 2008).

In an attempt to comprehensively characterise the risk for CVD in AMD patients, measures such as BP, peripheral arterial stiffness (McCarty et al., 2008;Sato et al., 2006a), and C-IMT (Klein et al., 2007;Sato et al., 2006a;Zaliuniene et al., 2008) have all been employed in isolation or in combination with various blood analyses. Nevertheless, the results reported by the above studies are not consistent, possibly due to various confounding factors, such as smoking, existing chronic diseases and systemic medications which effect vascular health. In addition, these studies used techniques that cannot be easily performed in primary care settings and, therefore, are not useful as screening methods. As a positive AMD diagnosis may, independently of other risk factors, predict future occurrence of stroke or CHD (Tan et al., 2007;Tan et al., 2008), early screening of AMD patients for such pathologies could, however, represent an important

step to allow disease prevention and disease modifying interventions at both ocular and systemic levels.

Vascular endothelial dysfunction is known to precede both metabolic and cardiovascular diseases (De Vriese et al., 2000;Yoshinaga et al., 2011). By using the flow mediated dilation (FMD) technique, a standard method of measuring systemic endothelial function (Donald et al., 2006), Zaliuniene et al. (2008) did not find any difference in brachial artery function between patients with AMD and those with cataract (Zaliuniene et al., 2008). Endothelial dysfunction however, is known to occur much earlier at the microvascular than the macrovascular level (Gates et al., 2009) and, consequently several tests including retinal vascular function have been developed to demonstrate the presence of such microcirculatory abnormalities in patients with various diseases including AMD. Indeed, by using DVA, Lanzl et al. demonstrated abnormal retinal arterial and venous reaction to flickering light in patients suffering from exudative AMD and systemic hypertension (Lanzl et al., 2011). As the measured changes could have been induced by the already present ocular and systemic vascular pathologies, the question remains, however, if similar changes are also evident and measurable in AMD patients at a much earlier stage. It has been already validated that the usefulness of retinal microvascular function as a measure of early vascular changes that signal the risk for future CVD in asymptomatic individuals (Heitmar et al., 2010;Patel et al., 2011), therefore, finding an answer to this question seems achievable.

4.3 Aim

This longitudinal prospective study investigates the retinal vascular function using a novel analysis and its relationship to systemic indicators for risk of general vascular dysfunction in otherwise healthy individuals diagnosed with early AMD.

4.4 Subjects and methods

4.4.1 Recruitment of untreated AMD patients and healthy controls

AMD patients and age-matched healthy subjects, recruited by advertisement at the Optometry Clinic, Aston University, Birmingham, UK, were considered for inclusion in this prospective study.

4.4.1.1 Inclusion criteria

Subjects with signs of early AMD were recruited for the AMD group. Age- matched healthy subjects were enrolled into control group.

4.4.1.2 Early AMD assessment

Retinal photographs were taken using a digital camera (FF450 plus, Zeiss GmbH, Jena, Germany). Signs of AMD were evaluated prior to inclusion in the study using the Wisconsin AMD grading system (Cheung et al., 2007a;Cheung et al., 2007b). Soft drusen was defined as those having a diameter larger than 63 μm. Retinal pigmentary changes associated with AMD were defined as present or absent. Early AMD was defined as the presence of either soft drusen alone, retinal pigmentary changes alone, or a combination of soft drusen with increased retinal pigment, retinal depigmentation in the absence of late AMD, or both (Cheung et al., 2007a;Cheung et al., 2007b). Patients with late AMD, defined as the presence of exudative AMD or pure geographic atrophy (Cheung et al., 2007a;Cheung et al., 2007b) were excluded from being included in the study. Only one eye per subject was included in the analysis. If both eyes had changes consistent with AMD, the eye with the more advanced changes was used to categorise the patients.

4.4.1.3 Exclusion criteria

Subjects were excluded if they were smokers, and if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system. The more details of exclusion criterion were described in Section 2.1.1.

4.4.2 Ethical approval

Ethical approval was sought from the local ethics committee, and written informed consent was received from all participants prior to enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

4.4.3 Investigations

4.4.3.1 Subjects preparation

Subjects were instructed to fast after 9pm on the evenings before being tested. On the morning of the test, subjects were requested to have only a light breakfast such as simple toast. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits or fruit juice (Jones et al., 1992). In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least two hours before the visit. All participants were required to complete a questionnaire on their general health, daily diet, physical activity and alcohol consumption.

4.4.3.2 General assessment for the presence of cardiovascular risk factors

1. General Investigations

All the participants underwent standardized anthropometric measurements for height and weight (used then to calculate BMI= weight/height^2). SBP and DBP were measured in each

subject in the morning between 8 and 9 am with a BP monitor (UA-779, A7D Instruments Ltd., Oxford, UK). The details of measurement were offered in the Section 2.3.1.

Blood samples were obtained in the morning between 9 am and 10 am, as well as fasting plasma glucose, TG, CHOL and HDL-C were measured using standard routine laboratory techniques using the Reflotron Desktop Analyser (Roche Diagnostics, UK), and described detailed in the Section 2.6.2. The Framingham risk score of CHD (10- year risk) was also calculated from the above values according to Framingham Heart Study (Wilson et al., 1998).

2. Carotid Intima-media thickness (C-IMT) measurement

C-IMT, a thickness between tunica intima and tunica media of a carotid artery, was measured for all participants through analysis of ultrasound images taken from the left common carotid artery (Siemens; Acuson Sequoia, UK). A normal C-IMT was considered to be less than 1mm (Simon et al., 2002). The details of assessment were offered in Section 2.3.3.

3. Assessment of arterial stiffness

An assessment of arterial stiffness was obtained using the SphygmoCor device (AtCor Medical /PWV Medical Pty Ltd, Australia) and pulse wave analysis. AIx, a parameter indicating the amount by which the aortic pressure is increased by the peripheral reflection of the blood flow and it is given as a percentage value corrected for a heart rate of 75 beats per minute (Skinner et al., 2011), was used as a measure of arterial stiffness. The SphygmoCor software generates quality control indices and therefore, to ensure reliability of measurements, only those readings obtained with an operator index of greater than 80% were accepted. The details of assessment were described in Section 2.3.4.

4.4.3.3 Retinal vascular function measurements

Retinal vessel reactivity was measured with the DVA (IMEDOS GmbH, Jena, Germany) using an already established protocol. Briefly, this protocol allows continuous measurement of retinal vessel diameter over a 350 seconds time period, which consists of 50 seconds of baseline measurements under still illumination (25Hz), followed by 3 cycles of 20 seconds flicker stimulation (optoelectronically generated at 12.5 Hz) each interrupted by 80 seconds of still illumination (recovery). All measurements were performed in a quiet, temperature-controlled room (22^{o}C) following full dilation of one unselected eye (1% tropicamide, Chauvin Pharmaceuticals Ltd). The details of assessment were described in Section 2.4.1. In order to fully explore the nature of the entire dynamic response profile of the retinal microvasculature to flicker light stimulation, a novel analysis was employed in this study, and have already described in details in Section 2.4.2.

4.4.3.4 Assessments for circulating t-GSH and GSSG

Glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling assay was used to determine the t-GSH and GSSG concentration, and further calculate to the GSH levels [t-GSH – (2 x GSSG)] and the redox index (defined as the GSH/ GSSG ratio). The details of the assay were offered in Section 2.6.3.

4.4.4 Statistics

All statistical analyses were performed using the software package Statistica version 6.0 (Statsoft, UK). Distributions of continuous variables were determined by the Shapiro-Wilks test because of small sample sizes. Differences between the groups were assessed using either the t-test for normally distributed as mean with SD, or the Mann-Whitney-U test for non-normally distributed as median with inter-quartile range (IQR). Data were correlated

using Pearson's method (if normally distributed) or Spearman's method (if non-normally distributed). A p-value of <0.05 was considered as statistically significant.

4.5 Results

4.5.1 Sample

Eighteen AMD patients were selected for the inclusion in the present study. However, after the evaluation of the fundus photographs and eliminating those with potential advanced macular changes, only 14 AMD patients (2 men and 12 women) were included in the final analysis. In addition, 14 age-matched healthy subjects (4 men and 10 women) were also included in the present research.

The baseline characteristics of both study groups are presented in the Table 4.1. The AMD patients showed significant higher LDL-C (p=0.033), C-IMT (p=0.029) and AIx (p=0.042) in comparison to the healthy controls.

4.5.2 Dynamic retinal vessel analysis (DVA)

For ease of interpretation, the dynamic retinal vessel profile curve is considered in two parts, the first part being the dilation response (baseline to maximum dilation) and the second part being the constriction response (maximum dilation to maximum constriction). All results are given based on the average of the 3 flicker cycles and have been calculated from the fitted degree 20 polynomial curves generated by a program written in Matlab. No significant differences were found between groups for arterial and venous BDF, MD, MC, DA, BFR and arterial RT (all p>0.05, Table 4.2); however, AMD patients demonstrated a significantly longer venous RT than the age-matched healthy controls (26.33 vs. 20.67 seconds, p=0.026, Figure 4.1).

	AMD [n=14]	Controls [n=14]	p-value
Age [years]	59 (8)	57 (5)	>0.05
SBP [mmHg]	122 (16)	119 (14)	>0.05
DBP [mmHg]	74 (11)	76 (10)	>0.05
HR [bpm]	69 (8)	65 (8)	>0.05
BMI [kg/m2]	25.50 (3.49)	24.90 (2.96)	>0.05
TG [mmol/L]	1.18 (0.60)	1.07 (0.54)	>0.05
CHOL [mmol/L]	4.56 (0.83)	4.02 (0.69)	>0.05
HDL-C [mmol/L]	1.32 (0.37)	1.32 (0.39)	>0.05
LDL-C [mmol/L]	2.62 (0.77)	2.03 (0.63)	0.033
Framingham risk score [%]	6 (3 - 7)	4 (3 - 7)	>0.05
C-IMT [mm]	0.78 (0.01)	0.66 (0.01)	0.029
AIx [%]	33 (26 - 37)	26 (20 - 28)	0.042

Table 4.1: Anthropometric profile of the study population. $p<0.05$ is considered a significant difference. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; BMI: body mass index; TG: Triglycerides; CHOL: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Framingham risk score: a risk score to predict 10 year absolute risk of CHD event; C-IMT: Intima-media thickness of the carotid artery; AIx: augmentation index, was defined as the ΔP (was the maximum systolic pressure minus pressure at the inflection point) divided by pulse pressure and expressed as a percentage.

	AMD [n=14]	Controls [n=14]	p-value
Arteries			
BDF	5.78 (4.09 - 8.71)	7.48 (5.94 - 9.22)	>0.05
MD [%]	4.93 (3.27 - 6.18)	5.15 (4.13 - 6.85)	>0.05
RT [seconds]	23.00 (20.33 - 32.33)	18.17 (17.17 - 26.83)	>0.05
MC [%]	-2.17 (-3.44 - -1.34)	-2.43 (-3.74 - -1.58)	>0.05
DA	6.68 (5.33 - 8.01)	7.58 (7.32 - 9.01)	>0.05
BFR	0.59 (-0.45 - 2.27)	1.19 (-2.38 - 2.93)	>0.05
Veins			
BDF	5.27 (4.18 - 7.45)	6.05 (4.68 - 8.82)	>0.05
MD [%]	5.01 (4.11 - 7.34)	5.27 (4.16 - 7.71)	>0.05
RT [seconds]	26.33 (23.33 - 30.67)	20.67 (17.33 - 25)	0.026
MC [%]	-0.82 (-1.36 - -0.19)	-1.13 (-1.93 - -0.72)	>0.05
DA	6.15 (5.24 - 9.85)	8.64 (6.88 - 10.48)	>0.05
BFR	1.96 (-0.30 - 3.51)	2.20 (-1.61 - 3.87)	>0.05

Table 4.2: Vascular function parameters on ocular level determined using the dynamic vessel analysis (DVA). $p<0.05$ is considered significant. BDF: baseline diameter fluctuation; MD (%): percentage change in diameter from baseline to maximum dilation; RT: reaction time to reach maximum dilation; MC (%): percentage change in diameter from maximum constriction to baseline diameter; DA: dilation amplitude is defined as the difference between the maximum dilatation and subsequent constriction after flicker stimulation; BFR: the baseline-corrected flicker response, is calculated as DA-BDF.

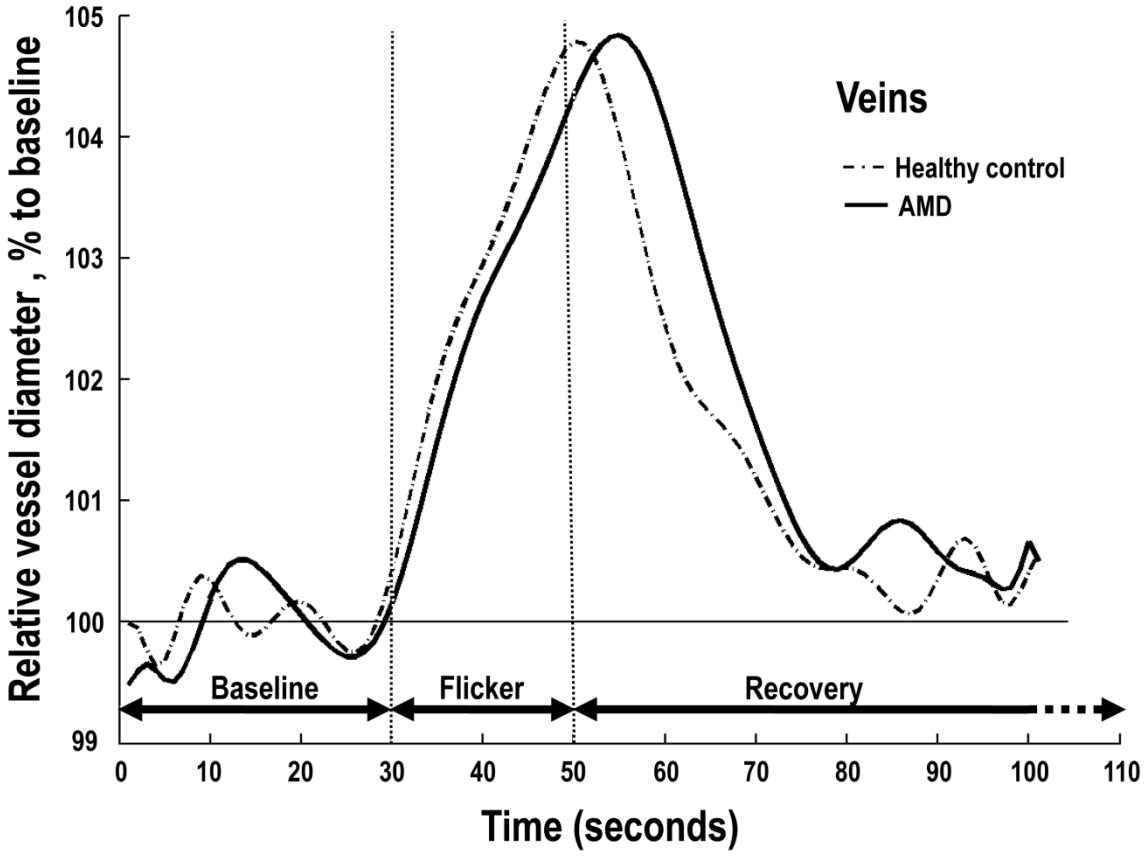


Figure 4.1: Averaged venous response profile for the AMD patients and healthy controls generated through Matlab. Demonstrates the veins took longer time to reach the maximum dilation to response flicker in AMD patients compared to the healthy controls.

The 'Slope' parameters of both the arterial and venous dilation and constriction were calculated based on the extreme values of the fitted polynomials. In the AMD group, the $Slope_{AD}$ was significant shallower than the age-matched healthy controls (0.22 vs. 0.45, p=0.005, Figure 4.2); however, no significant differences were found between the study groups for slope of arterial constriction ($Slope_{AC}$), slope of venous dilation ($Slope_{VD}$) and slope of venous constriction ($Slope_{VC}$) (all p>0.05, Table 4.3).

	AMD [n=14]	**Health [n=14]**	**p-value**
Arteries			
$Slope_{AD}$	0.22 (0.14 - 0.27)	0.45 (0.25 - 0.60)	0.005
$Slope_{AC}$	-0.29 (-0.44 - -0.23)	-0.33 (-0.35 - -0.28)	>0.05
Veins			
$Slope_{VD}$	0.26 (0.16 - 0.36)	0.30 (0.20 - 0.65)	>0.05
$Slope_{VC}$	-0.23 (-0.29 - -0.21)	-0.31(-0.36 - -0.19)	>0.05

Table 4.3: Slope parameters of dynamic retinal vessel analysis. p<0.05 is considered significant. $Slope_{AD}$: slope of arterial dilation, which defined as the diameter changes of maximum dilation diameter minus the diameter measured at the flicker start against to the time used between the two points; $Slope_{AC}$: slope of arterial constriction, which defined as the diameter changes of maximum constriction diameter minus the maximum dilation diameter against to the time used between the two points; $Slope_{VD}$: slope of venous dilation; $Slope_{AC}$: slope of venous constriction.

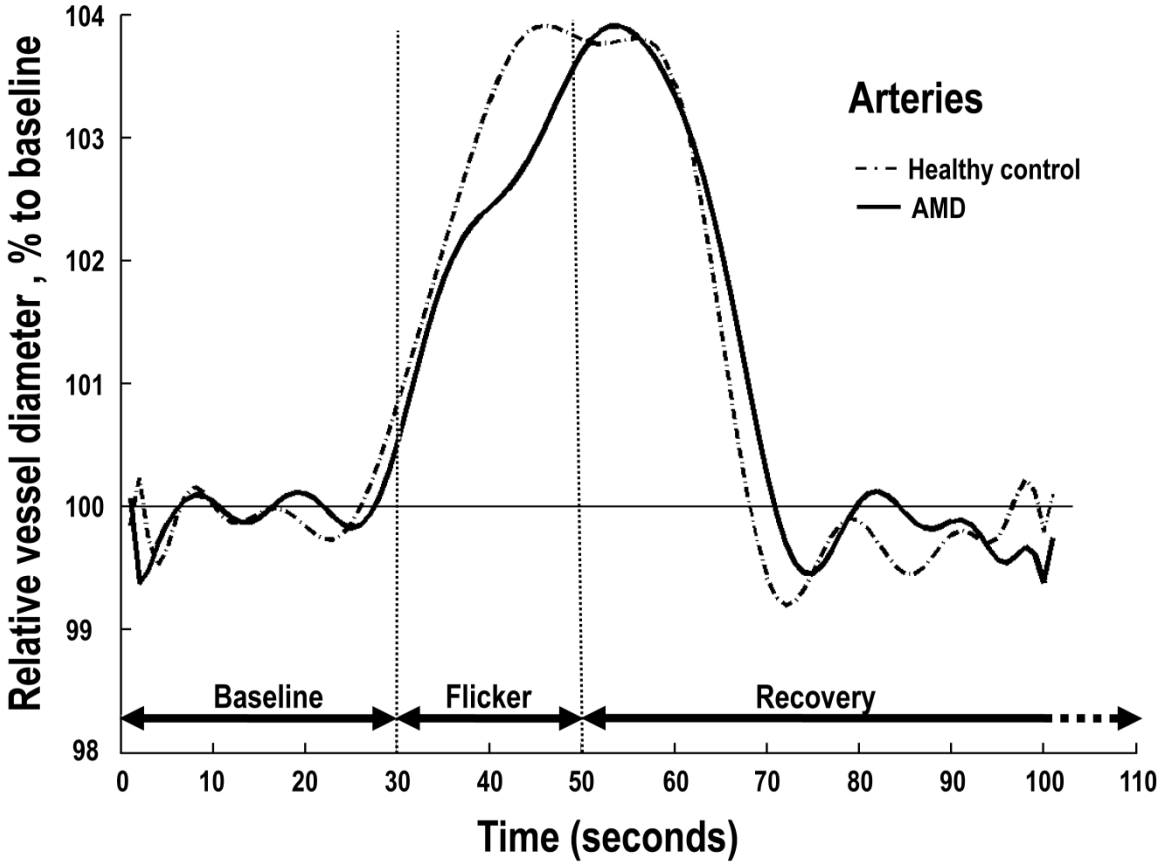


Figure 4.2: Averaged arterial response profile for the AMD patients and healthy controls generated through Matlab. Demonstrates the significant shallower slope of arterial dilation found in AMD patients compared to the healthy controls.

4.5.3 Oxidative stress markers and relationship to vascular function parameters

The results of the blood analyses are displayed in Table 4.4. The AMD patients exhibited statistically significant higher levels of blood GSSG (p=0.024) and lower redox index (p=0.043) than the healthy individuals; however, the GSH levels were comparable (p>0.05).

	AMD [n=14]	Controls [n=14]	p-value
t-GSH [µmol/L]	875 (663 - 1559)	1086 (809 - 1497)	>0.05
GSSG [µmol/L]	113 (64 - 118)	55 (43 - 82)	0.024
GSH [µmol/L]	715 (452 - 1349)	981 (723 - 1397)	>0.05
Redox index [GSH:GSSG]	9 (4 - 14)	17 (10 - 23)	0.043

Table 4.4: Circulatory markers of oxidative stress. p<0.05 is considered significant. GSH: reduced glutathione; t-GSH: total GSH; GSSG: oxidized glutathione.

Blood GSH and GSSG were within the ranges observed in previous studies in subjects of the same age as our patients and normal groups (Rossi et al., 2002;Samiec et al., 1998). There was no relationship between circulating glutathione levels and systemic measures for early vascular disease in either AMD patients or healthy controls (all p>0.05). However, correlations between retinal vascular reactivity parameters and blood markers of oxidative stress showed a significant positive relationship between venous RT and blood GSSG levels (r=0.58, p=0.038, Figure 4.3) in AMD patients. No such relationship was found in the age-matched control group (p>0.05).

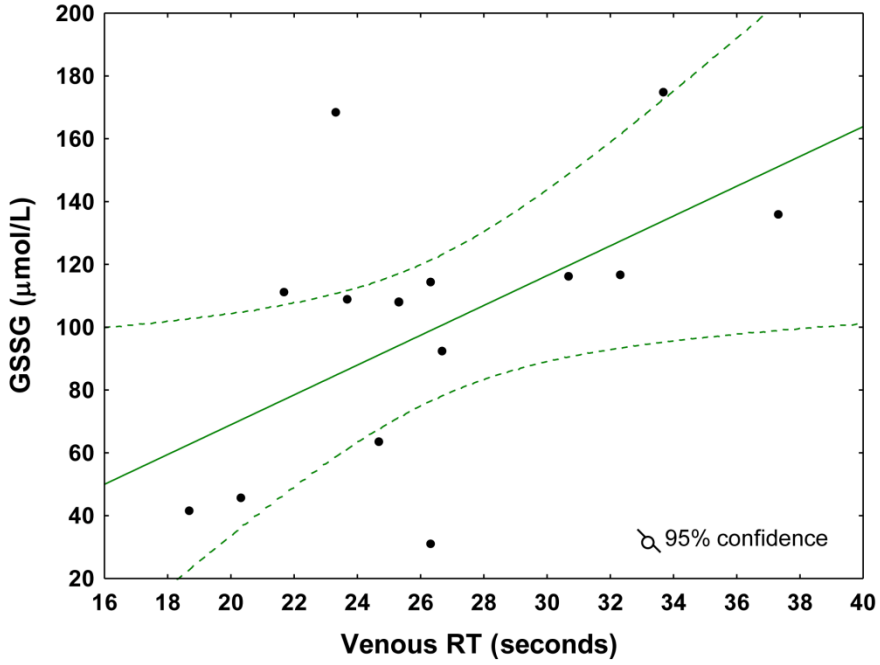


Figure 4.3: The correlation between the oxidized glutathione and the venous reaction time to reach maximum dilation to response flicker in the AMD group (r=0.58, p=0.038). GSSH: oxidized glutathione; Venous RT: the reaction time to reach maximum dilation during stimulation with flickering light on veins.

4.6 Discussions and conclusion

4.6.1 Discussions

The present results demonstrated that patients suffering from early AMD and without overt CVD exhibit signs of vascular abnormalities at different levels and measurable by different validated techniques. In addition, the results showed for the first time a relationship between retinal vascular reaction to flickering light and blood markers of oxidative stress in these patients.

Predicting individuals at risk for CVD is important, particularly in AMD patients known to have higher chance of developing such pathologies. Indeed, similarly to previous research

(Sato et al., 2006a) the present study has shown that AMD patients demonstrate signs of possible atherosclerotic changes at both central (carotid) and peripheral arterial levels. In addition and also supporting previous results (Ikeda et al., 2001;Nowak et al., 2005), I have found that the group of AMD patients demonstrated abnormal lipids metabolism markers compared to age-matched healthy controls. It has been shown previously that high levels of LDL-C, a vasoconstrictor, pro-inflammatory and thrombogenic molecule, inhibits synthesis and release of eNOS, hence resulting in endothelial dysfunction (Rosendorff, 2002) and atherosclerosis. Consequently, the results emphasise once again the importance of screening for systemic vascular pathologies in patients suffering from AMD and more importantly, that early therapeutic interventions to address modifiable risk factors such as high cholesterol should be considered in AMD patients.

The state of the macro- and micro-circulation are known to be closely related (Mitchell et al., 2005;O'Rourke and Safar, 2005;Wong et al., 2001) and in addition to systemic vascular changes, ocular microcirculatory abnormalities have also been reported in patients suffering from AMD (Lanzl et al., 2011;Sato et al., 2006b). By using a new computational model, the author has also demonstrated a significant shallower $Slope_{AD}$ in the AMD patients compared to the age-matched controls, possibly indicating some form of reduced vascular compliance as previously reported in these types of patients (Lanzl et al., 2011;Sato et al., 2006b). In addition, it was also obvious that arterial dilation profile did appear to follow a different path in the AMD patients compared to controls (Figure 4.2), the latter group demonstrating the ‘two humped’ arterial dilation response that is consistent with that reported by previous studies in younger individuals but not in older ones (Lanzl et al., 2011). This particular aspect of the retinal arteries response to the flicker in younger normals was related by Lanzl et al. (2010) to the possible existence of two separate systems contributing to dilation, the first being a fast onset, short duration system, mediated by eNOS and free NO and the second being a slow onset, long duration system which represents the summation of dilation and constriction factors. Our controls

were slightly younger than those included in the above research and the analysis showed that the 'two humped' aspect was still preserved. In addition, the present study has also excluded smokers due to the possible effect on the retinal vascular function (Heitmar et al., 2010) and it is not clear if smoking was an exclusion criterion in the paper by Lanzl et al. (2010). Nevertheless, similar to previous reported in more advanced disease, the 'two humped' shape of the vascular response to flicker was missing in our patients with early AMD. In our AMD patients this pattern was altered, with the first fast onset phase exclusively NO-dependent looking blunted, while the second dilatory phase was unaltered. At this point it is difficult to determine precisely what factors have contributed to this particular aspect especially as no significant differences were found in the arterial MD [%] and RT parameters between the two study groups. Local factors, such as reduced microvascular compliance as well as systemic contributors could all have played a role.

Similarly to Lanzl et al. also showing impaired venous dilation response to flicker in advanced AMD patients (Lanzl et al., 2011), the present research demonstrated a longer venous RT to flickering light at a much earlier stage of the disease. Retinal veins are generally thought to play a more secondary role in retinal autoregulation, perhaps providing a fine tuning of the regulation response following the active reaction of the retinal arteries (Kotliar et al., 2004). It has been proposed that this regulatory contribution of the retinal veins may occur passively in response to increased blood flow; nevertheless, passive diameter changes in response to increased blood flow still require an intact endothelium and a certain degree of vessel elasticity. In addition, the possibility that above reported venous reactivity could occur actively in response to flicker cannot be entirely ruled out (Kotliar et al., 2004). More research is, however, required in order to understand the relevance of impaired venous dilation response to flicker in AMD patients. This clarification is important since epidemiological studies have linked increased retinal venous calibre with the presence of endothelial dysfunction, as well as other systemic risk

factors for vascular disease (Sun et al., 2009). In addition, other factors that might be involved in the genesis of the above reported alteration should also be researched.

High levels of oxidative stress represent a harmful state defined by the presence of pathological levels of ROS relative to the antioxidant defence. Glutathione (GSH, L-γ-glutamyl-L-cysteinylglycine), a tripeptide consisting of glycine, cysteine and glutamic acid, prevents the effects of ROS either directly as an antioxidant or indirectly, by maintaining other cellular antioxidants in a functional state (Bendich, 1990;Pompella et al., 2003). With either ageing or pathology, glutathione oxidation effects vital cell functions such as proliferation rate (Abate et al., 1990;Huh et al., 2006) and apoptosis (Malorni et al., 1993;Sykes et al., 2007) leading to accelerated ageing (Rebrin and Sohal, 2008) and increased risk for various pathologies, including CVD and AMD (Ballatori et al., 2009). Previous research demonstrated the importance of oxidative stress markers as predictors of systemic vascular dysfunction in asymptomatic individuals (Ashfaq et al., 2006;Ashfaq et al., 2008) and similar to other studies (Javadzadeh et al., 2010), this present work also reported abnormal circulatory levels of oxidation markers in the patients suffering from AMD. In my samples, I have determined high levels of GSSG possibly resulting from GSH overuse; however, the two groups demonstrated similar GSH levels. One explanation could be that the GSSG resulting from the reaction between GSH and ROS was rapidly reduced back to GSH, thus completing the redox cycle and rebuilding GSH levels (Wang and Ballatori, 1998); nevertheless, this mechanism was somehow faulty, higher level of GSSG still remaining in the circulation and possibly contributing to some of the observed vascular dysfunctions (Vallance et al., 1989;Vallance and Chan, 2001). In addition, it is also possible that the otherwise healthy AMD patients could have retained the ability for rapid *de novo* glutathione synthesis, therefore keeping the GSH levels normal; however, all the GSH produced this way could have been used in oxidant generation and its regeneration from the disulfide form was impaired resulting in the observed major shift in GSH/GSSG ratio (Rebrin and Sohal, 2008). Further investigation would be needed, however, to validate all the above assumptions.

Although a deficit in local or systemic NO due to high levels of oxidative stress could result in altered vascular function, to date no study has linked circulating thiols to retinal vascular reactivity and this study is the first to do so in patients with AMD, a disease in which both local (Bhutto et al., 2010) and plasma (Totan et al., 2001) NO levels have been found to be altered. In my recent study (Qin et al., 2011a) demonstrated that in older, healthy subjects, the level of macular pigment, which is known to have local strong, protective antioxidant properties, correlated with blood levels of glutathione, possibly indicating that in healthy individuals, the antioxidant defence mechanisms present at various levels act in the same direction to protect the body against harmful effects of free radicals. Oxidative stress seems, therefore, a common link between ocular and systemic changes that occur simultaneously in patients at risk or suffering from AMD. As both oxidative stress and endothelial dysfunction are modifiable variables, the effect of specific therapeutic interventions to improve or reduce either or both should be better researched in this type of patients (Zhang and Osborne, 2006).

4.6.2 Conclusion

In conclusion, this present study demonstrates for the first time that patients suffering from AMD with no subclinical signs of systemic vascular disease, show signs of abnormal retinal vascular function that could be due, amongst other things, to the harmful effect of free radicals and oxidation and that should be addressed by preventive measures with positive effect at both ocular and systemic level.

Chapter 5

Relationship between retinal vascular reactivity and circulatory markers of endothelial function and cardiovascular risk in ageing healthy individuals

5.1 Abstract

Purpose: To assess the relationship between retinal microvascular function and circulatory markers of endothelial dysfunction and cardiovascular risk in otherwise healthy individuals.

Methods: Fifty-one healthy subjects were subjected to blood analyses to detect the level of circulatory GSH, GSSG, ET-1, and vWF. Fasting glucose as well as cholesterols and TG levels were also determined. Retinal vascular function was assessed by means of DVA using a novel computational analysis.

Results: Men exhibited higher DBP (p=0.021) as well as Framingham risk score (p=0.013), and lower HDL-C levels (p=0.002) and AIx (p=0.006) than women. A simple correlation model revealed that $Slope_{AC}$ positively correlated with CHOL (r=0.29, p=0.049), LDL-C (r=0.35, p=0.014), ET-1 (r=0.31, p=0.033) and Framingham risk score (r=0.35, p=0.015); additionlly, $Slope_{AD}$ positively correlated with SBP and redox index (r=0.32, p=0.026 and r=0.32, p=0.027, respectively); but the similar relationships were not found in the retinal venous parameters (all p>0.05). Age had a positive effect on the level of ET-1, vWF and $Slope_{AC}$ (r=0.61, p<0.001; r=0.37, p=0.010; and r=0.35, p=0.017, respectively), but not on other measured parameters (all p>0.05). Furthermore, a forward stepwise multiple regression analysis revealed that the $Slope_{AC}$ significantly and positively correlated with the level of ET-1 and LDL-C (β=0.30, p=0.029; and β=0.35, p=0.014, respectively); and $Slope_{AD}$ positively correlated with redox index (β=0.32, p=0.027).

Conclusion: In otherwise healthy individuals, the microvascular function as assessed at the retinal level is in direct relationship with established circulation markers for endothelial dysfunction/CVD risk.

5.2 Introduction

Although systemic factors such as hypertension, hyperlipidemia, diabetes, smoking and ageing are still considered as the main risk factors for the development of CVD (Gerszten and Wang, 2008;Greenland et al., 2003;Khot et al., 2003), oxidative stress and endothelial dysfunction are also thought to contribute to the onset and progression of this type of pathology, possibly due to dysregulation of vascular tone and thrombogenicity and inflammation induced by these changes (Lerman and Zeiher, 2005;Lusis, 2000;Staels, 2002;Thomas, 2011). vWF is a large glycoprotein produced by endothelial cells, and represents a powerful biomarker for endothelial dysfunction, via mediating platelet adhesion to injured endothelium which is the first step in thrombus formation (Frankel et al., 2008;Smith et al., 2010). Additionally, ET-1 is released by endothelial cells, causing massive vasoconstriction and increased blood pressure. In addition, ET-1 also stimulates ROS excessive production (oxidative stress) leading to endothelial dysfunction (Kamata et al., 2004;Matsumoto et al., 2008;Piechota et al., 2010). Recent studies suggested that high ET-1 levels have been involved in the pathogenesis of atherosclerotic vascular disease (Dashwood and Tsui, 2002;Miyauchi and Masaki, 1999;Stauffer et al., 2008). Indeed, Freixa et al. demonstrated that high levels of ET-1 were associated with signs of microvascular dysregulation and obstruction (Freixa et al., 2011). Furthermore, abnormal cholesterol levels are also associated with atherosclerosis and CVD risk (Holewijn et al., 2010;Kawamoto et al., 2005), and contribute to endothelial dysfunction and onset of vascular disorders in both the macro- and micro-circulation. In fact, endothelial dysfunction occurs early in the disease process, and it is easily modifiable through proper interventions (Celermajer, 1997); therefore, representing an ideal indicator for assessing risk of future vascular disease (Bacon et al., 2011). Consequently, scanning individuals at risk of CVD for early signs of endothelial dysfunction could contribute towards prevention of disease and associated disabilities for early intervention and prevention for future vascular diseases occurrence including CVD.

Ageing acts as a major risk factor is also considered to be involved in various vascular diseases (Ehrlich et al., 2009;Lee and Oh, 2010;Parmacek and Epstein, 2009). One of the consequences of ageing is a decline in the ability of tissues and organs to respond to different stresses including ischemia, which is a major risk of developing CVD. Indeed, circulating endothelial progenitor cells can home to ischemic tissues and contribute to the angiogenesis (Asahara et al., 1997). Previous researches found that the number and/or the function activities of endothelial progenitor cells have been impaired by ageing (Groleau et al., 2011;Hill et al., 2003;Werner et al., 2005). In addition, a loss of the adaptive response to oxidative stress is one of the major characteristic of ageing, leading to varying degrees of intimal and medial changes of vessels including degeneration of endothelial function, a risk for development of vascular disease (Finkel and Holbrook, 2000;Harman, 2003;Yu and Chung, 2006). This results in systolic hypertension and loss of arterial elasticity (Sawabe, 2010). Moreover, several studies have also been demonstrated that ageing could cause an increase in concentration of ET-1 and vWF even in healthy individuals (Davies et al., 2007;Gill et al., 1987;Maeda et al., 2003). This age-dependent degeneration of macrocirculation has been widely studied; however the microcirculation and its relationship with macrocirculation in elderly healthy individuals are few investigated.

Vascular endothelial dysfunction has been implicated in the development of both metabolic and cardiovascular diseases (De Vriese et al., 2000;Yoshinaga et al., 2011). Endothelial dysfunction is known to occur much earlier at microvascular level than at the macrovascular level (Gariano and Gardner, 2005;Gates et al., 2009), and consequently various tests including retinal vascular function assessment, have been developed and are used to detect such microcirculatory abnormalities at the earliest stage of the disease. Indeed, by using DVA, some studies found a reduced retinal vessel diameter response to a flicker stimulation in patients suffering from diabetes (Garhofer et al., 2004b;Mandecka et al., 2007) and in those diagnosed with glaucoma (Garhofer et al., 2004a). In addition,

Heitmar et al. demonstrated that patients suffering from coronary artery disease (CAD) showed reduced retinal vessel responses to flickering light (Heitmar et al., 2011). The question remains, however, if the similar retinal microcirculatory changes are also obvious and measurable at an early stage of such impaired endothelial function in otherwise healthy individuals but at risk. In the present thesis, it has been already demonstrated that abnormal retinal microcircular function correlated with high levels of systemic oxidative stress in patients diagnosed with AMD and free systemic vascular disease (see Chapter 4); therefore, finding an answer to this question seems achievable.

5.3 Hypothesis

I hypothesised that microvascular function at the retinal level, could be an indicator for systemic vascular/endothelial dysfunction and CVD risk in otherwise healthy individuals without the overt vascular diseases but at risk because of the possible effect of ageing on vascular health.

5.4 Aim

The aim of this study was to examine the relationship between the retinal microvascular function and various circulatory makers of CVD risk in ageing healthy subjects.

5.5 Subjects and Methods

5.5.1 Study Sample

Healthy subjects recruited by advertising at Aston University, Birmingham, UK were considered for inclusion in this prospective study. Ethical approval was sought from the

local ethics committee, and written informed consent was received from all participants prior to enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

5.5.1.1 Exclusion criteria

Exclusion criteria for the healthy participants were described in the Section 2.1.1. Additionally, Individuals younger than 40 years old were excluded from the present study.

5.5.2 Investigations

5.5.2.1 Subjects preparation

Subjects were instructed to fast after 9pm on the evenings before being tested. On the morning of the test, subjects were requested to have only a light breakfast such as simple toast. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits or fruit juice (Jones et al., 1992). In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least two hours before the visit. All participants were required to complete a questionnaire on their general health, daily diet, physical activity and alcohol consumption.

5.5.2.2 General assessment for the presence of cardiovascular risk factors

All subjects were investigated in the morning after an over-night fast. All the participants underwent standardized anthropometric measurements for height and weight (used then to calculate BMI= weight/height^2). Systemic BP was measured in each subject in the morning between 8 and 9 AM with a BP monitor (UA-779, A7D Instruments Ltd., Oxford, UK). The measurement details have been offered in Section 2.3.1.

Blood samples were obtained in the morning between 9 and 10 AM, and fasting plasma glucose, TG, CHOL and HDL-C were measured using standard routine laboratory techniques using the Reflotron Desktop Analyser (Roche Diagnostics, UK) (more details have been described in Section2.6.2). The Framingham risk score was also determined from the above values according to an already published method (Wilson et al., 1998).

C-IMT and PWA/AIx were also measured for each participant, and more details have been offered in Section 2.3.3 and Section 2.3.4.

5.5.2.3 Retinal vascular function measurements

Retinal vessel reactivity was measured with the DVA (IMEDOS GmbH, Jena, Germany) using an already established and recommended protocol (Garhofer et al., 2010;Nagel et al., 2004). Using a newly developed computational model, the entire dynamic vascular response profile to flicker light was imaged and used for analysis. The details of measurement and analysis have been described in Section 2.4.1 and Section 2.4.2.

5.5.2.4 Blood assay for markers of oxidative stress and endothelial dysfunction

1. t-GSH and GSSG assay

Glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling assay was used to determine the t-GSH and GSSG concentration, and further calculate to the GSH levels [t-GSH – (2 x GSSG)] and the redox index (defined as the GSH/ GSSG ratio). The details of the assay were offered in Section 2.6.3.

2. vWF assay

A method ELISA was used for assessing the level of plasma vWF using commercial antisera from Danish company Dako. The procedure of vWF assay was described in Section 2.6.4.

3. ET-1 assay

This assay was performed by using the human ET-1 commercial kits (R&D Systems Europe Ltd, 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, catalogue number QET00B) using an ELISA method according to the manufacturer's protocol. The more details of the procedure were described in Section 2.6.5.

5.5.3 Statistical analysis

All analyses were performed using Statistica® software (StatSoft Inc.; Version 6, USA). Distributions of continuous variables were determined by the Shapiro-Wilks test. Data are expressed for normally distributed as mean with SD or non-normally distributed as median with IQR. A Pearson's method (if normally distributed) or Spearman's method (if non-normally distributed), and forward stepwise regression analysis were performed to test the correlation between the measured variables. A p-value of <0.05 was considered as statistically significant.

5.6 Results

5.6.1 Subjects

Fifty-nine healthy subjects with similar dietary habits were selected for the inclusion in the present study. However, after the evaluation of the fundus photographs and eliminating those with macular changes, only fifty-one non-smoking healthy subjects (age from 40 to

69 years old) were included in the final analysis. Table 5.1 shows the anthropometric and vascular parameters determined by gender. Men exhibited higher DBP (p=0.021) and Framingham risk score (p=0.013), and lower HDL-C (p=0.002) and AIx (p=0.006) than women.

	Female [n=30]	**Male [n=21]**	**p-value**
Age [years]	50 (10)	50 (9)	>0.05
SBP [mmHg]	115 (15)	122 (10)	>0.05
DBP [mmHg]	73 (9)	79 (8)	0.021
HR [bpm]	64 (7)	65 (10)	>0.05
BMI [kg/m2]	25.50 (3.49)	24.95 (3.01)	>0.05
TG [mmol/L]	1.15 (0.64)	1.36 (0.56)	>0.05
CHOL [mmol/L]	4.50 (0.69)	4.40 (1.01)	>0.05
HDL-C [mmol/L]	1.41 (0.29)	1.13 (0.29)	0.002
LDL-C [mmol/L]	2.56 (0.74)	2.65 (0.90)	>0.05
Framingham risk score [%]	4 (3)	6 (3)	0.013
C-IMT [mm]	0.60 (0.15)	0.65 (0.14)	>0.05
AIx [%]	22 (11)	13 (10)	0.006

Table 5.1: The characteristics of the study participants. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; BMI: body mass index; TG: triglycerides; CHOL: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Framingham risk score: a risk score to predict 10 year absolute risk of CHD event; C-IMT: carotid intima-media thickness; AIx: augmentation index.

5.6.2 Age effect on measured vascular functional parameters at both systemic and retinal level

In the study group, age had a positive effect on the levels of ET-1 and vWF as well as $Slope_{AC}$ ($r=0.61$, $p<0.001$, Figure 5.1; $r=0.37$, $p=0.010$, Figure 5.2; and $r=0.35$, $p=0.017$, Figure 5.3, respectively), but not on other measured parameters (all $p>0.05$).

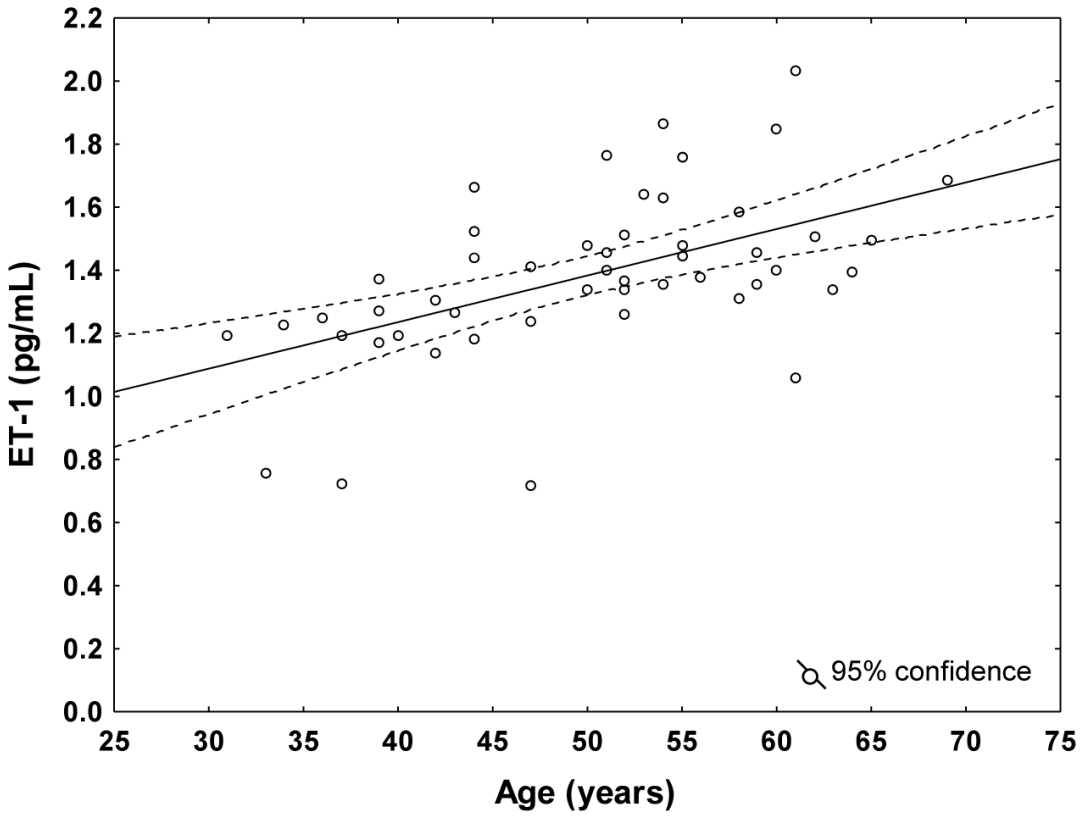


Figure 5.1: Age effect on the plasma level of endthelin-1 (ET-1).

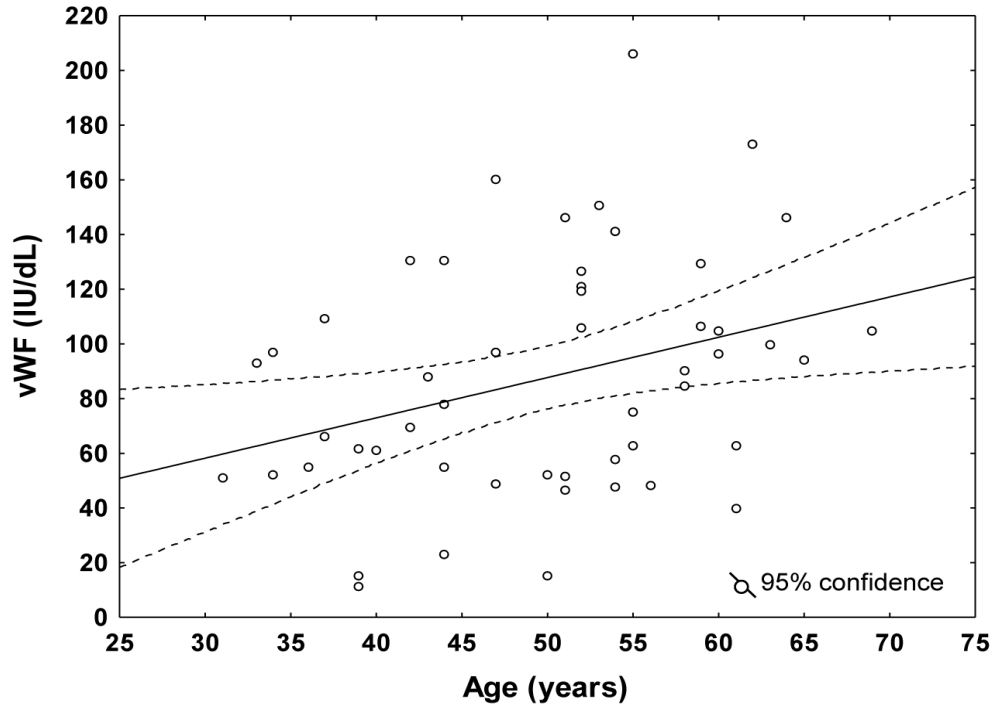


Figure 5.2: Age effect on the plasma level of von Willebrand factor (vWF).

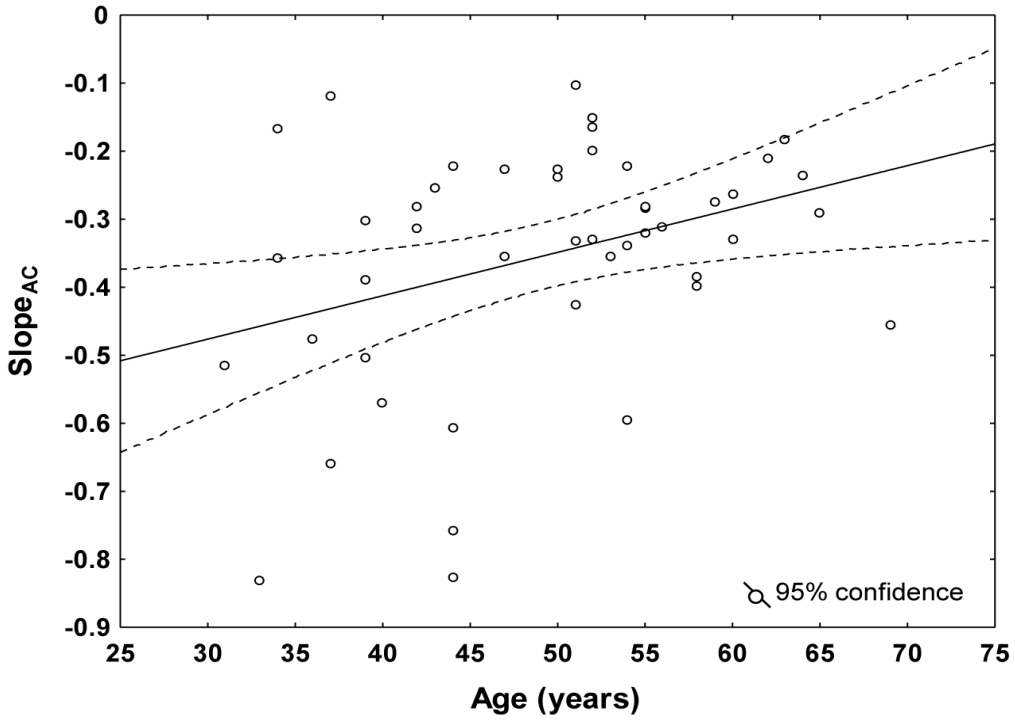


Figure 5.3 Age effect on the slope of retinal arterial constriction. $Slope_{AC}$: slope of arterial constriction.

5.6.3 Measured CVD risk biomarkers and retinal function parameters

In this healthy population, there are no significant differences between female and male participants in theses measured parameters (Table 5.2, all p>0.05).

	Female [n=30]	Male [n=21]	p-value
Biomarkers for CVD risk			
t-GSH [µmol/L]	1206 (576)	991 (462)	>0.05
GSSG [µmol/L]	86 (45)	75 (50)	>0.05
GSH [µmol/L]	1034 (545)	841 (426)	>0.05
Redox index [GSH:GSSG]	12 (10)	11 (7)	>0.05
ET-1 [pg/mL]	1.35 (0.23)	1.42 (0.30)	>0.05
vWF [IU/dL]	84 (47)	92 (37)	>0.05
Parameters of retinal vascular function			
Arteries:			
MD [%]	5.15 (2.26)	5.58 (2.68)	>0.05
MC [%]	−3.38 (2.36)	−3.96 (2.75)	>0.05
RT [seconds]	21 (9)	18 (6)	>0.05
$Slope_{AD}$	0.49 (0.29)	0.51 (0.21)	>0.05
$Slope_{AC}$	−0.37 (0.19)	−0.33 (0.15)	>0.05
Veins:			
MD [%]	5.80 (2.58)	5.45 (2.52)	>0.05
MC [%]	−1.90 (1.32)	−2.89 (2.93)	>0.05
RT [seconds]	22 (5)	20 (4)	>0.05
$Slope_{VD}$	0.28 (0.14)	0.29 (0.14)	>0.05
$Slope_{VC}$	−0.28 (0.12)	−0.27 (0.13)	>0.05

Table 5.2: Vascular function parameters on circulatory level, and ocular level determined using the dynamic vessel analysis. GSH: reduced glutathione; t-GSH: total GSH; GSSG: oxidized glutathione; ET-1: endothelin-1; vWF: von Willebrand factor; MD: maximum dilation; MC: maximum constriction; RT: reaction time to reach maximum dilation; $Slope_{AD}$: slope of arterial dilation; $Slope_{AC}$: slope of arterial constriction; $Slope_{VD}$: slope of venous dilation; $Slope_{VC}$: slope of venous constriction. p<0.05 is considered significant.

5.6.4 Correlation analysis

A simple correlation model revealed that $Slope_{AC}$ positively correlated with the level of CHOL (r=0.29, p=0.049), LDL-C (r=0.35, p=0.014) and ET-1 (r=0.31, p=0.033) as well as Framingham risk score (r=0.35, p=0.015); moreover, $Slope_{AD}$ positively correlated with SBP and redox index (r=0.32, p=0.026; 0.32, p=0.027, respectively); however, the similar relationships were not found in the retinal venous parameters (all p>0.05). A forward stepwise multiple regression analysis (Table 5.3) revealed the $Slope_{AC}$ to be independently, significantly and positively correlated with the level of ET-1 and LDL-C (β=0.30, p=0.029, Figure 5.4 and β=0.35, p=0.014, Figure 5.5 and, respectively); and $Slope_{AD}$ positively correlated with redox index (β=0.32, p=0.027, Figure 5.6).

Y variable	X variable selected	*R*	R^2	R^2 change	*F*	p
Slope $_{AC}$	LDL-C	0.354	0.126	0.126	6.482	0.014
	ET-1	0.465	0.216	0.090	5.069	0.029
Slope $_{AD}$	Redox index	0.322	0.104	0.104	5.22	0.027

Table 5.3: A forward stepwise multiple regression analysis results. It demonstrates LDL-C (R^2 change=0.126) had most important influence on Slope $_{AC}$, and ET-1 slight related to Slope $_{AC}$ (R^2 change=0.090). Slope $_{AD}$ is strongly predicted by redox index (R^2 change=0.104). $Slope_{AC}$: slope of arterial constriction; $Slope_{AD}$: slope of arterial dilation; LDL-C: low-density lipoprotein cholesterol; ET-1: endothelin-1; redox index: GSH/GSSG. p<0.05 is considered significant.

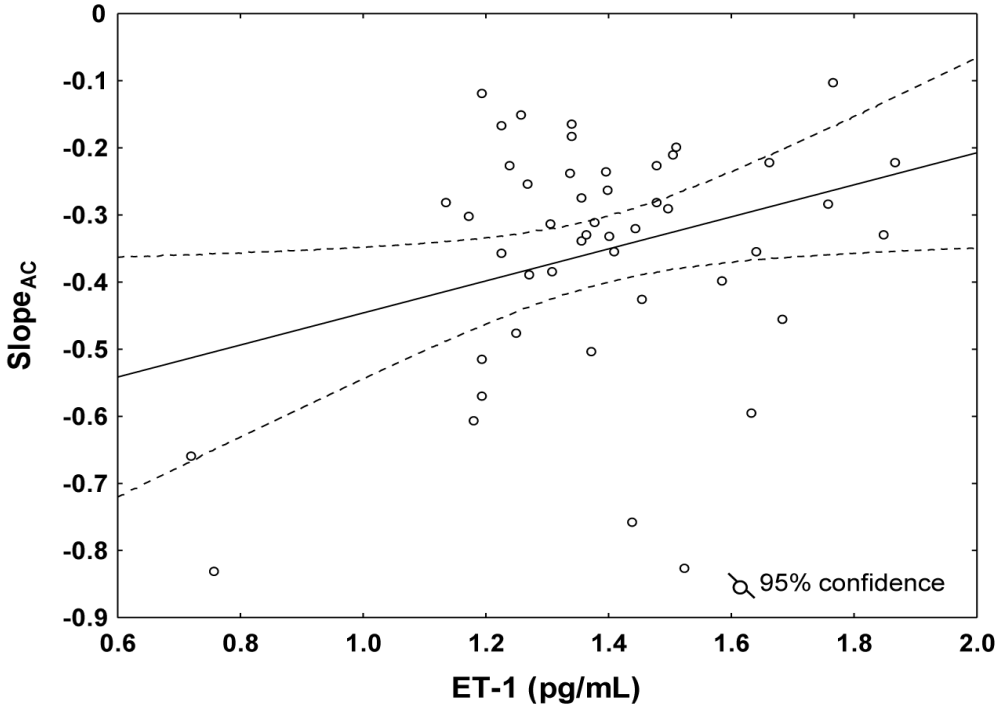


Figure 5.4: The relationship between the slope of retinal arterial constriction and the level of ET-1.

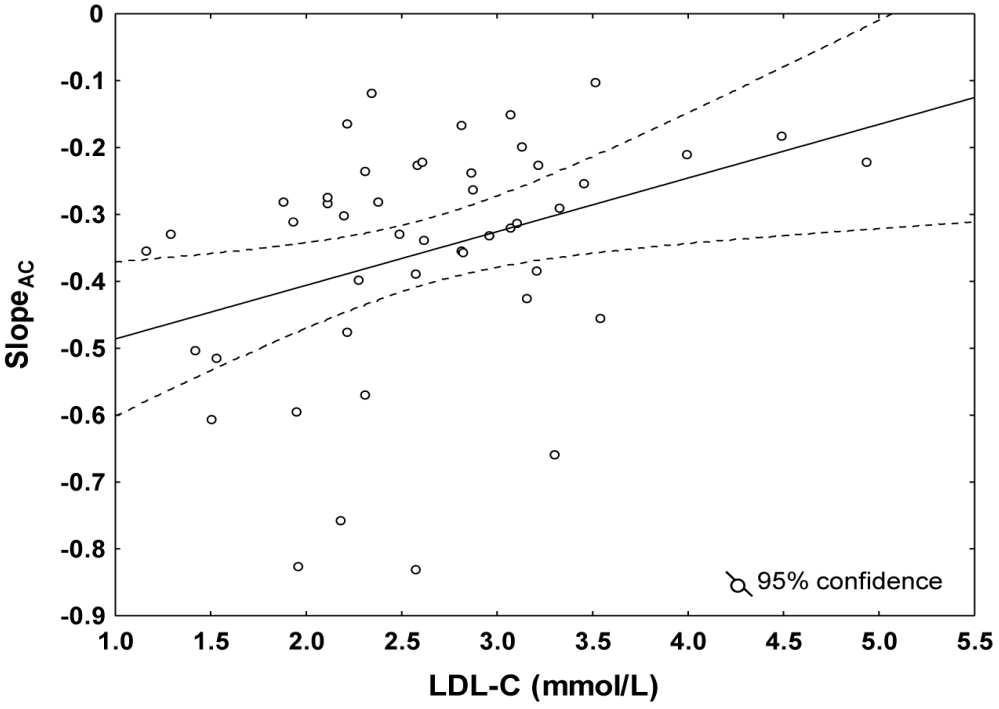


Figure 5.5: The relationship between the slope of retinal arterial constriction and the level of LDL-C.

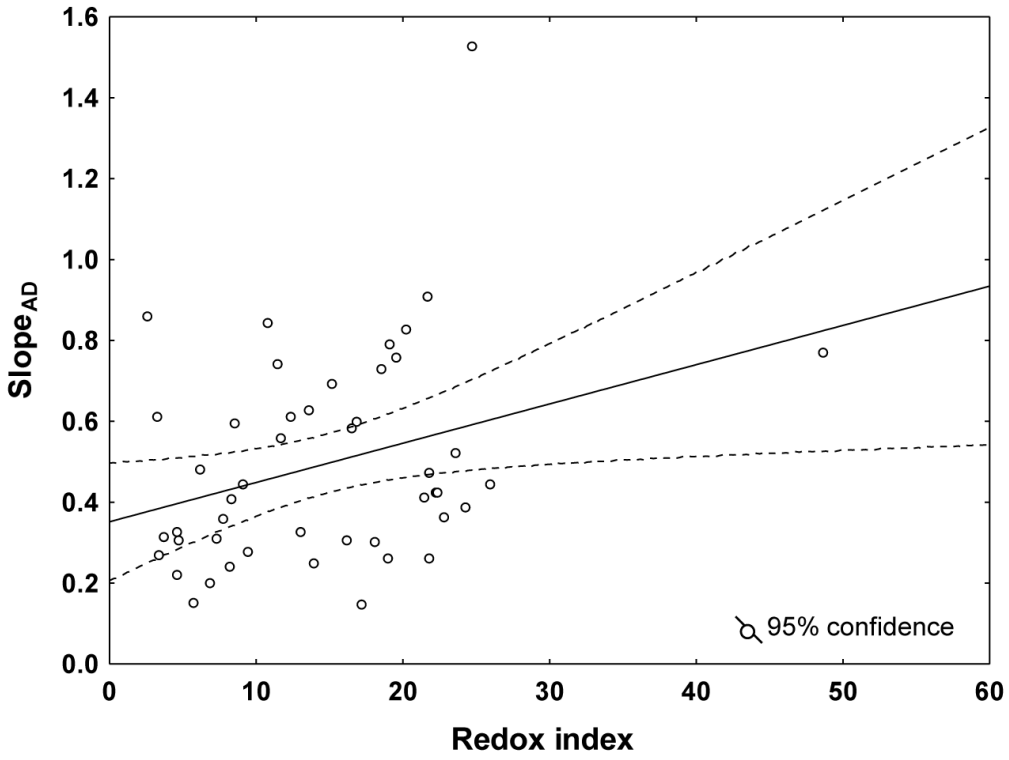


Figure 5.6: The relationship between the slope of retinal arterial dilation and the level of redox index (GSH/GSSG ratio).

5.7 Discussion

5.7.1 Main finding

Using a novel computational model, it was demonstrated that in otherwise healthy, ageing individuals impaired retinal microcirculation function was associated with circulating markers for the risk of CVD.

5.7.2 Age effect on endothelial function

In this study, it showed a positive correlation between age and both ET-1 and vWF. Several studies have been demonstrated that ageing could contribute an increase in concentration of ET-1 and vWF even in healthy individuals (Davies et al., 2007;Gill et al.,

1987;Maeda et al., 2003) and the present results confirm that ageing plays an important role in the occurrence of endothelial dysfunction. In addition, this present results demonstrated that a reduced rate of arterial constriction correlated with increased age. Boehm et al reported a decreased blood flow in the retinal microcirculation with ageing (Boehm et al., 2005). Other researches also shown that reduced blood flow velocities was correlated with advanced age (Greenfield et al., 1995;Groh et al., 1996;Rizzo et al., 1991). Therefore, it is possible that the observed decrease rate of arterial constriction in my subjects, part of a general vascular ageing process that might be attributed to arterial stiffening as well as to increased concentration of vasoconstrictor molecules (such as ET-1), and which results in reduction of perfusion with blood in all tissues and organs including the eye.

5.7.3 Gender influence for CVD risk

It has been previously shown that BP demonstrated gender differences, with men demonstrating higher BP then women (Reckelhoff, 2001). Holland et al. demonstrated that woman had a higher AIx (arterial stiffness indictor) than age-matched men (Holland et al., 2009). In this study, these findings were confirmed in our cohort of healthy individuals. In addition, this present results have also shown that men had higher Framingham risk score and lower AIx compared to age-matched healthy women. The above cited papers do not offer a clear explanation for all these gender-based discrepancies, however, an association between body size and myocardial tissue velocities have been reported and may partly explain it (Peverill et al., 2004).

5.7.4 Retinal microcirculation and CVD risk factors

Among other risk factors, high levels of oxidative stress, abnormal cholesterol as well as abnormal level of plasma markers for endothelial dysfunction such as ET-1 are known to

contribute to dysregulation of vascular tone at multiple levels including the microcirculation (Freixa et al., 2011). In a recent study, it has been also demonstrated that in elderly healthy subjects, the local retinal antioxidant defense mechanisms were related to the systemic antioxidant capacity possibly acting in the same direction to protect the body against the harmful effects of free radicals (Qin et al., 2011a). By using DVA, it demonstrated that in otherwise healthy ageing subjects, signs of microvascular dysregulations measured at the retinal vessels level correlated with established markers of endothelial dysfunction and CVD risk. At this stage more research is necessary to provide better knowledge of the exact mechanisms responsible; nevertheless, few hypotheses still can be proposed.

GSH acts as an antioxidant to prevent the effects of ROS (Bendich, 1990;Pompella et al., 2003). With ageing or pathology, GSH oxidation results in decreased proliferation rate and cellular apoptosis includes endothelial cells (Huh et al., 2006;Sykes et al., 2007), leading to accelerate ageing processes as well as to increased risks for various pathologies (Ballatori et al., 2009). The ratio of GSH to GSSG, so-called 'redox index' represents an indicator for the circulatory oxidative stress status (James et al., 2009;Jones, 2006a;Jones, 2006b). The present results have shown that signs of retinal microcirculation function correlated positively with the redox index. It is possible that high levels of ROS due to ageing, diffuse to the microcirculation and results in a local overproduction of GSSG and consequently breaks the balance of redox status, leading to abnormal vascular reactivity (Feletou et al., 2010;Jin and Loscalzo, 2010;Michiels, 2003;Ogita and Liao, 2004;Schulz et al., 2011).

Another explanation for the observed signs of vascular dysfunction could be that under high oxidative stress condition, LDL-C particles trapped in the local vessel wall become oxidized (oxLDL-C). This oxLDL-C causes a series of reactions leading to endothelial dysfunction (Chen et al., 2007;Cominacini et al., 2001;Sawamura et al., 1997). It has been

reported that oxLDL-C could reduce the expression of eNOS, leading to decrease in the production of NO, increase in expression of ET-1 and adhesion molecules (Itabe et al., 2011;Ou et al., 2010). In turn, the impaired endothelial function could result in reduction in NO production and release and, thereby down-regulate the local retinal vascular tone and function. In addition, oxLDL-C could bind LOX-1, a receptor of oxLDL-C expressed in endothelial cells and smooth muscle cells (Sawamura et al., 1997), to activate NADPH oxidise on cells membrane, leading to rapid increases in intracellular ROS including O_2^- and H_2O_2 (Chen et al., 2007). The increased levels of O_2^- could react with intracellular NO and, consequent resulting in the decrease of local retinal NO release and leading to dysregulation of local retinal vascular tone (Cominacini et al., 2001). Therefore, a slow recovery rate under flicker stimulation may result from this down-regulated local retinal microcirculatory function that was associated with the increased LDL-C level. In this present study, the levels of oxLDL-C were not measured; however, this observation opens new research venues to be explored in the future.

In the subjects of the present work, $Slope_{AC}$ correlated with the circulation levels of ET-1, a potent vasoconstrictor produced primarily in the endothelial cells (Yanagisawa et al., 1988). It is possible that an increased ET-1 production results in platelet adhesion to injured endothelium, arterial thrombogenicity, leading to dysregulation of local retinal vascular tone (Itabe et al., 2011). Additionally, some studies reported that ET-1 can inhibit eNOS activity through up-regulating caveolin-1 pathway which is a negative regulatory protein for eNOS activity, leading to decrease NO production (Kamoun et al., 2006;Karaa et al., 2005;Minshall et al., 2003) and consequently resulting in the local endothelial dysfunction and disrupted vascular tone. Moreover, there is another hypothesis that ET-1 leads to up-regulation of ROS production. Amiri et al. study found that endothelial overproduction of ET-1 could impair NO-dependent vasodilation and increase in vascular ROS production that leading to local retinal microcirculatory disorders (Amiri et al., 2004). If one or all of these mechanisms are responsible for the observed vascular reactivity

abnormalities is unclear at this moment and more research is necessary to fill in this gap in knowledge.

5.7.5 Conclusion

In conclusion, this study has demonstrated for the first time that in healthy, non-smoking, ageing individuals that retinal microcirculatory function correlated with the circulatory marker of endothelial dysfunction and CVD risk. As assays for measuring such as circulatory markers are complex and need specialized laboratories, it is tempting to propose that a simple retinal vascular function assessment could be used as surrogate indicator for the individual's systemic capacity of dealing with the damaging effect of excess LDL-C, ET-1 as well as free radicals and, consequently, for their risk of endothelial dysfunction and future CVD, even in healthy individuals without overt vascular disease.

Chapter 6

Age-related changes in ANS and vascular function in healthy individuals

6.1 Abstract

Purpose: To assess the relationship between ANS and vascular function in middle-aged and elderly healthy subjects.

Methods: Sixty-four healthy subjects underwent ambulatory 24-hour ECG monitoring using Cardiotens-01(PMS Instruments Ltd., Maidenhead, UK). Blood analyses also employed to detect the level of circulatory markers of ET-1 and vWF for endothelial function, along with C-IMT and AIx assessment. Retinal vascular function was assessed by means of DVA using a novel approach, which represent the entire dynamic vascular reactivities response to flicker stimulation.

Results: The healthy elderly group demonstrated significantly different circadian changes in HF ($p=0.011$) than the middle-aged controls. ET-1 ($p<0.001$), vWF ($p=0.025$), C-IMT ($p=0.004$), and AIx ($p=0.004$) were also significantly higher in the elderly group compared to middle-aged controls; moreover, the elderly individuals exhibited a shallow $Slope_{AC}$ ($p=0.047$) than middle-aged subjects. In addition, the $Slope_{AC}$ had a positively correlation with the circadian HF ($p=0.012$) in the elderly group, but no similar relationship found in the middle-aged controls.

Conclusion: Even in the absence of overt vascular pathologies, the elderly healthy individuals showed impaired ANS and vascular function at both macro- and micro-vascular level compared to the middle-aged subjects. In addition, the impaired retinal vascular function correlated with abnormal circadian PSNS activity only in the elderly individuals and these parallel changes could represent the first sign of an increment in the general risk for CVD with ageing.

6.2 Introduction

Ageing is characterized by a progressive dysfunction of cells, tissues and organs that sometimes and in the presence of other risk factors lead to development of vascular diseases, such as CVD and stroke (Karavidas et al., 2010;Lee and Oh, 2010;Shirwany and Zou, 2010). Nevertheless, although there is a general consensus that ageing plays a role in such pathologies, its exact influence on the balance between various factors influencing the vascular homeostasis, such as ANS and endothelial function is still unclear (Harris and Matthews, 2004). It is possible that ageing acts as a disruptor of this carefully maintained equilibrium. Indeed, it has been already demonstrated that senescence has a negative influence on the HRV (Bigger et al., 1996;Corino et al., 2007;Yu et al., 2010). Moreover, ageing is accompanied by a reduction in parasympathetic tone leading to deregulation of ANS controlled function including cardiovascular function, resulting in a higher risk for hypertension (Charkoudian and Rabbitts, 2009;Harris and Matthews, 2004). In addition, it has been shown that ageing associates with a redox imbalance shown as high levels of oxidative stress, inflammation and endothelial dysfunction and, therefore, confers a background for functional and structural disturbances in both macro- and microcirculation in subjects at risk (Lip et al., 2001;Zaliuniene et al., 2008). Indeed, dilation, stiffening, and thickening of the arteries as well as a reduction in cardiac functions are widely reported in ageing-associated changes of the vasculature. Moreover, the endothelium power to counteract the sympathetic-induced vasoconstriction is also diminished in elderly and biochemical markers for endothelial dysfunction also show an increase with ageing (Davies et al., 2007;Davies et al., 2007;Gill et al., 1987;Maeda et al., 2003), all contributing to the increased cardiovascular risk and mortality in these individuals (Harris and Matthews, 2004;Morange et al., 2004;Whincup et al., 2002). All the above evidences show that ageing has indeed a potential disruptive effect on the ANS and endothelial function and, consequently, on the vascular health. Nevertheless, this

effect on retinal vascular function via ANS and endothelial function has never been researched.

6.3 Hypothesis

I hypothesise that the impaired ANS and endothelial function in individuals belonging to different age groups, could act as a fresh insight on the ageing involvement in the development of the first signs of vascular disease.

6.4 Aim

The aim of this study was to assess the both endothelial and ANS function, and their relationship to retinal vascular function in healthy elderly individuals compared to middle-aged healthy subjects.

6.5 Subjects and Methods

6.5.1 Study Sample

Healthy subjects recruited by advertising at Aston University, Birmingham, UK were considered for inclusion in this prospective study. Ethical approval was sought from the local ethics committee, and written informed consent was received from all participants prior to enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

6.5.1.1 Exclusion criteria

Exclusion criteria for the healthy subjects were described in the Section 2.1.1.

6.5.2 Investigations

6.5.2.1 Subjects preparation

Subjects were instructed to fast after 9pm on the evenings before being tested. On the morning of the test, subjects were requested to have only a light breakfast such as simple toast. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits or fruit juice (Jones et al., 1992). In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least two hours before the visit. All participants were required to complete a questionnaire on their general health, daily diet, physical activity and alcohol consumption.

6.5.2.2 General assessment for the presence of cardiovascular risk factors

All subjects were investigated in the morning after an over-night fast. All the participants underwent standardized anthropometric measurements for height and weight then were used to calculate BMI (weight/height^2).

Systemic BP was measured in each subject in the morning between 8 and 9 AM with a BP monitor (UA-779, A7D Instruments Ltd., Oxford, UK). The measurement details have been offered in Section 2.3.1. C-IMT and PWA/AIx have also been investigated, and more details have been offered in Section 2.3.3 and Section 2.3.4.

6.5.2.3 HRV assessment

HRV analysis is a measure of ANS function as described in Section 2.3.2.1 and Section 2.3.2.2. All HRV values were calculated using the Cardiovision 1.7.2 software (PMS Instruments Ltd., Maidenhead, UK). For HRV evaluation, the frequency domain analysis was employed as described earlier in Section 2.3.2.3. Values for LF, HF and ratio of

LF/HF were automatically calculated and obtained for 24 hours, day time (active-period) and night time (passive-period), and then used to calculate circadian changes using the Equation 6.1 according to a previous study (Heitmar et al., 2011).

$$\textit{Circadian change of HRV} = \textit{Active-period HRV} - \textit{Passive-period HRV}$$

Equation 6.1: Calculating the circadian changes of heart rate variability.

6.5.2.4 Blood assay for markers of endothelial dysfunction

1. vWF assay

A method ELISA was used for assessing the level of plasma vWF using commercial antisera from Danish company Dako. The procedure of vWF assay was described in Section 2.6.4.

2. ET-1 assay

This assay was performed by using the human ET-1 commercial kits (R&D Systems Europe Ltd, 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, catalogue number QET00B) using an ELISA method according to the manufacturer's protocol. The more details of the procedure were described in Section 2.6.5.

6.5.2.5 Retinal vascular function measurements

Retinal vessel reactivity was measured with the DVA (IMEDOS GmbH, Jena, Germany) using an already established and recommended protocol (Garhofer et al., 2010;Nagel et al., 2004). Using a newly developed computational model, the entire dynamic vascular response profile to flicker light was imaged and used for analysis. The details of measurement and analysis have been described in Section 2.4.1 and Section 2.4.2.

6.5.3 Statistics

All statistical analyses were performed using the software package Statistica version 6.0 (Statsoft, UK). Distributions of continuous variables were determined by the Shapiro-Wilks test. Data distributed normally are presented as mean with SD and the differences between the groups were assessed using t-test. If non-normally distributed, they represented as median with IQR and groups compared by Mann-Whitney-U test. Data were correlated using Pearson's method (if normally distributed) or Spearman's method (if non-normally distributed). A p-value of <0.05 was considered as statistically significant.

6.6 Results

6.6.1 Sample

64 healthy participants were included the study. There were grouped into middle-aged group (31-54 years old) and elderly group (55-71 years old). The baseline characteristics of both study groups are presented in the Table 6.1. The only difference between the two groups was represented by higher SBP values (122 vs. 112, p=0.008) in the elderly group compared to the middle-aged group.

6.6.2 Parameters of ANS function

Table 6.2 outlines the measured parameters of the autonomic nervous system function. The elderly individuals had significantly different circadian variations in HF (3.5 NU vs. -8.5 NU, p=0.011) compared to middle-aged controls. However, no significant differences were found for other measured parameters of ANS function (all $p>0.05$).

	Elderly [n=31]	Middle-aged [n=33]	p-value
Age [years]	59 (4)	39 (5)	<0.001
SBP [mmHg]	122 (17)	112 (13)	0.008
DBP [mmHg]	77 (10)	73 (11)	>0.05
HR [bpm]	66 (8)	68 (6)	>0.05
BMI [kg/m^2]	25.88 (4.61)	25.16 (3.84)	>0.05

Table 6.1: Anthropometric profile of the study population. p<0.05 is considered significant. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; BMI: body mass index.

	Elderly [n=31]	Middle-aged [n=33]	p-value
LF-24hr [NU]	68 (8)	66 (12)	>0.05
HF-24hr [NU]	29 (7)	32 (11)	>0.05
LF/HF-24hr	2.5 (0.9)	2.5 (1.4)	>0.05
Circadian LF [NU]	4 (-6.5 - 19)	11 (3 - 17)	>0.05
Circadian HF [NU]	3.5 (-7.5 - 13.5)	-8.5 (-16 - -2)	0.011
Circadian LF/HF	0.35 (-0.8 - 1.5)	1.1 (0.3 - 2.1)	>0.05

Table 6.2: Heart rate variability parameters measured using a 24 hours ECG monitor. LF: low frequency; HF: high frequency; NU: normalised units. Circadian LF= day period LF - night period LF; circadian HF= day period HF - night period HF; circadian LF/HF = day period LF/HF - night period LF/HF.

6.6.3 Parameters for systemic vascular function

Table 6.3 showed the elderly group had a significant increment in circulating level of ET-1 ($p<0.001$) and vWF ($p=0.025$), as well as C-IMT ($p=0.004$) and AIx ($p=0.004$) compared to the middle-aged healthy controls.

	Elderly [n=31]	Middle-aged [n=33]	p-value
Blood markers			
ET-1 [pg/mL]	1.45 (0.26)	1.15 (0.32)	<0.001
vWF [IU/dL]	99 (39)	76 (40)	0.025
Vascular tone			
C-IMT [mm]	0.69 (0.17)	0.55 (0.13)	0.004
AIx [%]	26 (8)	15 (12)	0.004

Table 6.3: Measured parameters for systemic vascular function. ET-1: endothelin-1; vWF: von Willebrand factor, a marker for endothelial dysfunction; C-IMT: Intima-media thickness of the carotid artery; AIx: augmentation index, was defined as the ΔP (was the maximum systolic pressure minus pressure at the inflection point) divided by pulse pressure and expressed as a percentage.

6.6.4 Retinal vascular function measured by DVA

The 'Slope' parameters of both the arterial and venous dilation and constriction were calculated based on the extreme values of the fitted polynomials using DVA (Table 6.4). The elderly individuals demonstrated a shallow $Slope_{AC}$ after flicker (-0.29 vs. -0.38, $p=0.047$, Figure 6.1) comparing with middle-aged group, but not in other retinal functional parameters (all $p>0.05$).

	Elderly [n=31]	Middle-aged [n=33]	p-value
Arteries			
$Slope_{AD}$	0.53 (0.47)	0.46 (0.22)	>0.05
$Slope_{AC}$	-0.29 (0.10)	-0.38 (0.21)	0.047
Veins			
$Slope_{VD}$	0.33 (0.20)	0.28 (0.14)	>0.05
$Slope_{VC}$	-0.27 (0.15)	-0.24(0.12)	>0.05

Table 6.4: Vascular function parameters on ocular levels determined by the dynamic vessel analysis. $Slope_{AD}$: slope of arterial dilation; $Slope_{AC}$: slope of arterial constriction; $Slope_{VD}$: slope of venous dilation; $Slope_{AC}$: slope of venous constriction.

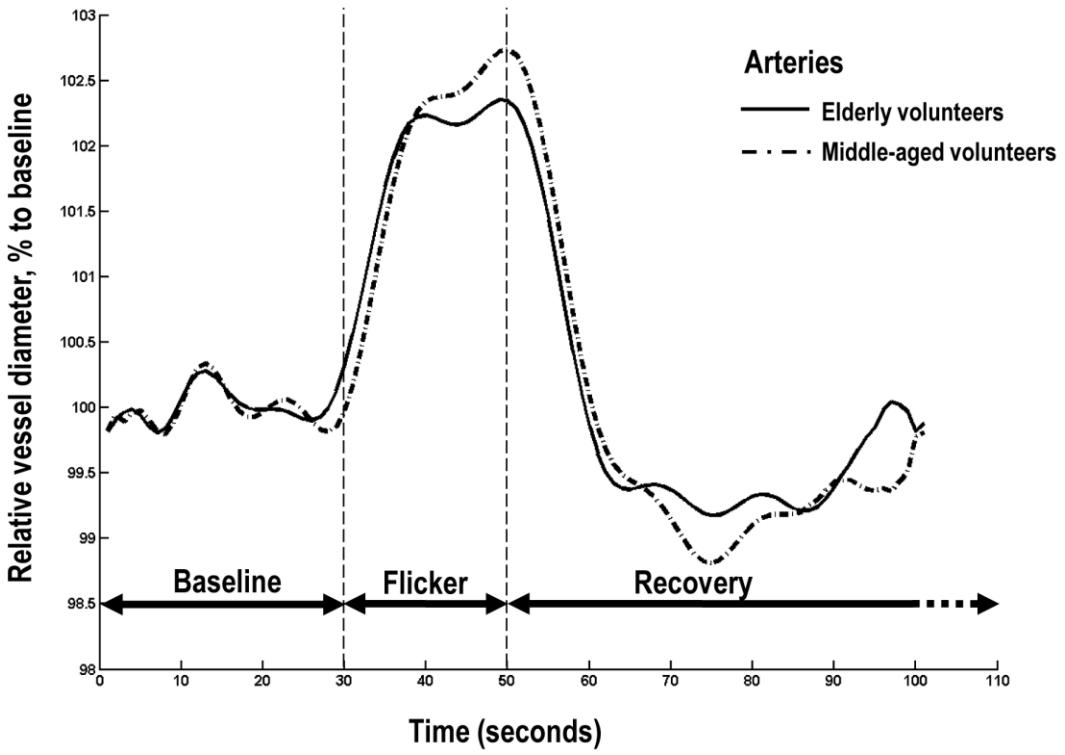


Figure 6.1: Averaged arterial response profile for the elderly and middle-aged subjects generated through Matlab. Demonstrates the significant shallower slope of arterial constriction found in the elderly individuals compared to the middle-aged group.

6.6.5 Correlation

Multiple regression analysis (Table 6.5) revealed that in the elderly group, $Slope_{AC}$ significantly correlated with circadian changes in HF (β=0.725, p=0.012, Figure 6.2). However, in the middle-aged subjects, the indices failed to correlate significantly (p>0.05).

Y variable	X variable selected	*R*	R^2	R^2 change	*F*	p
$Slope_{AC}$	vWF	0.387	0.150	0.150	2.469	0.138
	Circadian HF	0.697	0.485	0.336	8.477	0.012

Table 6.5: A forward stepwise multiple regression analysis results. It demonstrates circadian changes of HF (R^2 change=0.336, p=0.012) had most important influence on $Slope_{AC}$. $Slope_{AC}$: slope of arterial constriction; circadian HF= day period HF - night period HF; HF: high frequency; vWF: von Willebrand factor. p<0.05 is considered significant.

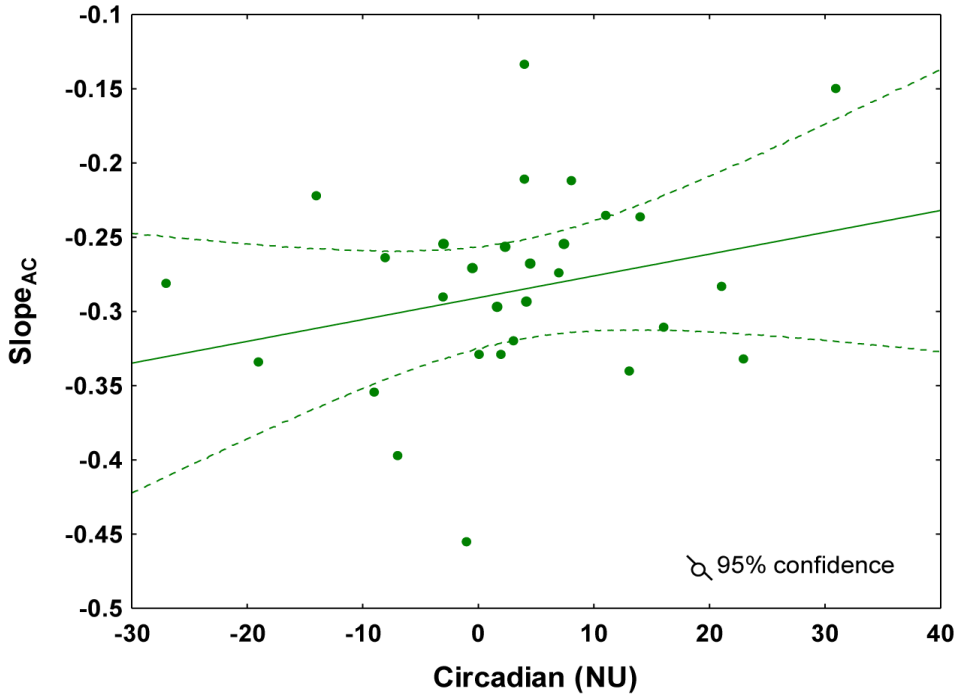


Figure 6.2: The relationship between the slope of retinal arterial constriction and circadian HF in the elderly group. $Slope_{AC}$: slope of arterial constriction; circadian HF: circadian changes of high frequency.

6.7 Discussion

6.7.1 Main finding

Parameters reflecting ANS tone in the healthy elderly were significantly different compared to the healthy middle-aged group. In addition, the elderly group exhibited higher levels of systemic markers for endothelial/vascular dysfunction, and impaired retinal vascular function compared to the middle-aged group. Moreover, the impaired retinal vascular function correlated with ANS disturbance in the healthy elderly individuals.

6.7.2 Autonomic function

The HRV analysis is a reliable non-invasive method for indirect evaluation of ANS function (Bootsma et al., 1994;Pagani et al., 1986). The ratio of the LF to HF components represents a measure of sympathovagal balance (Choi et al., 2006;Pagani and Malliani, 2000). Several studies have demonstrated that elderly individuals exhibited a disturbance in ANS activities compared to young subjects (Choi et al., 2006;Dawson et al., 1999;Yu et al., 2010). In addition, abnormal circadian variations in HRV could indirect reflect an abnormal adaptation to changes in mental and physical activity that occur during the day-to-day life, and this function could be modified by ageing.

Several researches reported that in normal conditions, the PSNS tone is higher during the night (Davy et al., 1998;De Meersman, 1993;Gregoire et al., 1996). Similar these studies, the present study also found a negative value (expected) of circadian HF in the middle-aged subjects from day to night; whereas the elderly group did not show the similar circadian changes in HF that possibly suggested a reduced circadian variation in the parasympathetic activity with ageing. Indeed, previous researches have already shown that the a decline in parasympathetic activity occurs in elderly individuals (Amano et al.,

2001;Antelmi et al., 2004;Bigger et al., 1996;Choi et al., 2006;Corino et al., 2007;Hrushesky et al., 1984;Oida et al., 1999;Yu et al., 2010) and could be responsible for BP alterations (Monahan, 2007). The present results also demonstrated that elderly individuals had higher SBP values than middle-aged controls. It was not able to demonstrate any changes in the SNS with age between the groups. It is possible that the small number of participants included in the present study has acted as a major limitation.

6.7.3 Plasma markers for endothelial function

It has been already demonstrated that vWF acts as an indicator for endothelial dysfunction (Blann and Lip, 1998;Lip et al., 2001). Additionally, ET-1 is described as the most potent vasoconstrictor and plays an important role in endothelial dysfunction (Amiri et al., 2004;Yanagisawa et al., 1988). High levels of vWF and ET-1 have been demonstrated in vascular diseases (Freixa et al., 2011;Heitmar et al., 2011;Kappers et al., 2010), moreover, increased ET-1 has also been found with advanced age (Maeda et al., 2003). Similar to previous research (Maeda et al., 2003), the present results also demonstrated higher circulating levels of ET-1 in the elderly group; moreover, the elderly subjects exhibited higher vWF levels compared to the middle-aged group. Increased ET-1 levels could result in an inhibition of the NO production possibly through down-regulation of the eNOS activity (Minshall et al., 2003), therefore, leading to endothelial dysfunction. An overproduction of ET-1 also results in chronic vasoconstriction, hypoxia and high levels of ROS (Duerrschmidt et al., 2000;Loomis et al., 2005). ROS further react with BH_4 (tetrahydrobiopterin), a cofactor for production of NO (Landmesser et al., 2003;Zheng et al., 2003) leading to further impairment of the eNOS function (eNOS uncoupling). Taken together, increased ET-1 results to ROS over-production, decrease of BH_4 bioavailability and hence eNOS uncoupling, and this cascade of events may play a key role in the age-related endothelial dysfunction that are measurable at various levels including plasma and retinal vessels.

6.7.4 Ocular vascular function

Lanzal et al. recently investigated ocular vascular indices in different age groups, and showed that elderly group exhibited a smaller arterial constriction below baseline after cessation of flickering light compared to young individuals (Lanzl et al., 2011). By using a new computational model, it has been also demonstrated that a decrease rate of arterial constriction following cessation of flicker in the elderly otherwise healthy subjects compared to middle-aged group. This shallow slope of arterial constriction might be related to the impaired basal vascular tone in retina arteries (Lanzl et al., 2011), possible due to higher circulatory levels of ET-1 that negatively influence the retinal endothelial function. In addition, in line with previous research (Lanzl et al., 2011;Mroczkowska et al., 2011;Qin et al., 2011b), the arterial reaction profile measured in both groups did appear to have the expected 'two humped' arterial dilation response profile (Figure 6.1)(Lanzl et al., 2011)(Lanzl et al., 2011). This particular aspect of the retinal arteries response to flickering light was explained by the possible existence of two separate systems responsible for retinal arterial dilation, the first being a fast onset, short duration system which mediated by eNOS and NO and the second being a slow onset, long duration system that represents the summation of dilation and constriction factors (Lanzl et al., 2011).

6.7.5 ANS and vascular function

The effect of ANS to endothelial function has been widely studied. The ANS and the endothelium works together to control the vascular tone through a balance between the releases of vasoconstrictor from sympathetic nerve terminals and vasodilator from endothelial cells (Harris and Matthews, 2004). Harris and Matthews research suggested that a decreased PSNS activity can impair the ability of the ANS to regulate the vascular tone (Harris and Matthews, 2004). This abnormal ANS activity and impaired endothelial

function could contribute to a dysregulation of vascular tone, possible influence on both macro- and micro-vascular levels including retinal vascular function. In addition, previous report suggested that the ANS was more responsible for integrating systemic factors, whereas the endothelium was more responsive to local factors (Burnstock, 1993;Harris and Matthews, 2004). The present results demonstrated that observed the impaired retinal vascular function significantly correlated with the abnormal circadian variation in the PSNS activity in elderly individuals. It is possible that these parallel changes represent the first sign of an increased risk for CVD with ageing.

6.7.6 Conclusion

The healthy elderly subjects showed impaired ANS and vascular function at both macro- and micro-vascular levels compared to the middle-aged group. Additionally, the impaired retinal vascular function was associated with abnormal circadian PSNS activity in elderly individuals and these parallel alterations could represent the first sign of an increment in the general risk for CVD with ageing. At this point, more non-invasive assessment of ANS and ocular function should be needed to complement the diagnosis, risk assessment and monitoring even in healthy elderly individuals as ageing is a vital risk factor for various vascular diseases.

Chapter 7

Summary and Conclusions

7.1 Summary and Conclusions

The importance of vascular risk factors that increase with the ageing process, such as oxidative stress and endothelial dysfunction, has been researched previously in various age-related diseases including AMD, and the relevant scientific literature has been reviewed in Chapter 1 of this thesis. However, it is still unclear when and how haemodynamic disturbances occurring in various ocular and systemic vascular beds act either separately or in association to induce disease. An answer to these questions could open new screening, diagnosis and therapeutic avenues for those at risk. This thesis has been concerned with investigating the presence and impact of ocular and systemic vascular functional and biochemical vascular risk factors in healthy elderly individuals and in AMD patients. Moreover, the possible role played by disruption in the equilibrium between various systems that act to maintain the vascular homeostasis has also been investigated. A separate chapter was dedicated to the relationship between ocular and systemic antioxidative mechanisms in maintaining a healthy status.

In summary, the main findings of this work were:

7.1.1 The relationship between ocular and systemic antioxidant in defense mechanisms in healthy individuals

Oxidative stress is defined by the presence of pathological levels of ROS relative to the antioxidant defence, and it has been reported to play a key role in the aetiology of AMD (Beatty et al., 2000;Khandhadia and Lotery, 2010). In addition, high levels of oxidative stress direct causes low bioavailability of the vasodilatory molecule, NO (Everekliogl u et al., 2003) and endothelial dysfunction (Cai and Harrison, 2000;Nedeljkovic et al., 2003) which is a condition known to precede both metabolic and CVD, as well as ocular

circulatory disturbances (Lip et al., 2001;Zaliuniene et al., 2008). GSH represent one of the most potent factors to prevent oxidative damage to human body. A low level of circulating GSH could cause imbalance of redox status (GSH: GSSG ratio), and have potential harmful effect on various tissues and organs including eyes. At the local level the antioxidant defence is represented by the MP; it consists of lutein, zeaxanthin and meso-zeaxanthin, all potent antioxidants molecules that act against the oxidative damage of high-energy blue light to the retina (Bone et al., 1985;Khachik et al., 2002;Krinsky, 2002) and therefore, protect against subsequent AMD (Hammond B.R et al., 1998).

Although dietary intake of lutein and zeaxabthin could increase the level of MP with positive effects on AMD prevention and prognosis (Bartlett et al., 2010;Davies and Morland, 2004), and carotenoid rich food intake enhance the systemic antioxidant level in healthy individuals (Sternberg et al., 1993), to date no study has investigated whether the local and systemic antioxidant systems are somehow related. Therefore, the aim of this study was to determine if there was a relationship between local and systemic antioxidant defence markers in subjects without either ocular or systemic diseases. In Chapter 3, the results demonstrated that independent of age and gender, there was a positive correlation between MPOD and circulating GSH level in the healthy individuals. As it is complex to measure systemic levels of antioxidant molecules and need specialized laboratories, a simple MPOD assessment could be used as surrogate indicator of the individual's systemic ability of dealing with the harmful effect of free radicals, and consequently to monitor those at risk of developing chronic ocular and/or systemic pathologies.

This study only included the participants over the age of 40 years who are free of macular changes or other ocular pathologies as well as systemic chronic diseases and various therapeutic interventions. Additionally, smokers and individuals taking any antioxidant supplements were also excluded. This careful selection has limited the number of subjects in the final analysis. It is possible that the relationship between MPOD and blood GSH

level is different not only in various age groups but also in individuals with other risk factors for vascular diseases. Further studies may need to be investigated in large and more populations to cover the many possible confounders that have been either missed or intentionally avoided in the present study.

7.1.2 Macro- and micro-vascular abnormalities in patients with early age-related macular degeneration: correlation with blood markers of oxidative stress

Some studies shown that AMD is related with subclinical signs of CVD (Klein et al., 1993;Vingerling et al., 1995). More reports found that patients suffering from AMD are at higher risk than age-matched healthy individuals for developing CHD and stroke (Liao et al., 2008;Wong et al., 2006;Wong et al., 2007). Therefore, early screening of AMD patients for such pathologies could represent an important step of prevention and disease-modifying interventions. It is well known that oxidative stress and endothelial dysfunction have been suggested as early events involved in the pathogenesis of CVD (Lee et al., 2011;Madamanchi et al., 2005;Shechter et al., 2007;Weil et al., 2011), and endothelial dysfunction occur much earlier at the microvascular than the macrovascular level. Consequently, measurements of retinal vascular function have been developed to demonstrate the presence of such microcirculatory abnormalities in patients with already present various diseases including exudative AMD (Gugleta et al., 2011;Lanzl et al., 2011). However, it was never proved if there is an obvious abnormal retinal vascular function that could be measured in early AMD patients. It has been already validated that measures of retinal microvascular function are useful in demonstrating early vascular changes that signal risk for CVD in individuals without overt disease (Heitmar et al., 2010;Patel et al., 2011). The work outlined in this study aimed to assess the retinal

vascular function and its relationship to established systemic factor for CVD risk in early AMD patients without overt systemic pathologies.

The recruitment was limited by the fact that patients diagnosed with early AMD often take antioxidant supplements, and this was an exclusion criterion for my study. Finding healthy elderly individuals was also limiting variable, although this was general problem for the entire study. In addition, I also excluded smokers. However, this very careful selection helped us to remove all the possible biases and to demonstrate that indeed there was an altered vascular response pattern both at retinal and systemic levels in patients suffering from early AMD without overt CVD. Moreover, the present results shown for the first time that a abnormal retinal vascular response to flickering light was related to circulating markers of oxidative stress in these patients. Oxidative stress is a risk factor for developing CVD (Lee et al., 2011;Madamanchi et al., 2005), and contributes to abnormal vascular function. The fact that this extended to the microvessels and we were able to measure it in apparently healthy individuals, is a novelty. This finding could be the first signal for risk of future systemic vascular diseases, and more aggressive interventions to prevent disease should be deployed in these patients.

7.1.3 Relationship between retinal vascular function and circulatory markers of endothelial dysfunction and cardiovascular risk

Among other risk factors, oxidative stress and endothelial dysfunction are also thought to be involved in development of CVD, via dysregulation of vascular tone, growth, thrombogenicity and inflammation (Lerman and Zeiher, 2005;Lusis, 2000;Staels, 2002;Thomas, 2011). Endothelial dysfunction however is known to occur much earlier at microascular level than macrovascular level (Gariano and Gardner, 2005;Gates et al.,

2009), and therefore, many approaches including assessing retinal vascular function, have been developed to investigate such abnormal microcirculation at the earliest stage of vascular disease. Some previous studies have already reported that reduced retinal vascular responses to a flicker stimulation in patients diagnosed with diabetes, glaucoma and CAD (Garhofer et al., 2004a;Garhofer et al., 2004b;Heitmar et al., 2011;Mandecka et al., 2007). This retinal vascular function was demonstrated in patients suffering from overt systemic diseases with already impaired systemic endothelial function, however, if the similar dysregulation of retinal microcirculation exist and measurable at an early process of such endothelial dysfunction in otherwise healthy subjects. By using DVA and novel computational analysis, the present results have shown that retinal vascular function was closely correlated with the oxidative stress, LDL-C and ET-1, established blood markers of CVD risk. These circulating molecules of CVD risk, could have a such early influence on the retinal vascular tone. Early detection of such abnormal retinal vascular function however, could prevent those subjects at risk for future vascular disease.

Only at this stage, more studies need to carry out to provide better knowledge of the exact mechanisms responsible; however, few hypotheses could be proposed. Indeed, with ageing, local high levels of oxidative stress results in apoptosis of endothelial cells, leading down-regulation of local endothelial function and vascular tone (Ballatori et al., 2009;Huh et al., 2006;Jones, 2006a;Sykes et al., 2007). Additionally, under the high oxidative stress, LDL-C is oxidized and form oxLDL-C, cause a decrease in the production of NO, enhance in expression of ET-1, leading to local retinal endothelial dysfunction and deyregulation of vascular tone (Chen et al., 2007;Cominacini et al., 2001;Itabe et al., 2011;Ou et al., 2010;Sawamura et al., 1997). At the moment, it is unclear if one or all of these mechanisms contribute to the observed abnormal vascular reactivity, however, more research is needed to explore it.

7.1.4 Age-associated changes in ANS and vascular function

Ageing is a process that degeneration of cells including endothelial cells over time that could increase the risk for development of vascular diseases such as CVD and stroke (Harman, 2001;Harman, 2006;Karavidas et al., 2010;Kruk et al., 1995;Lee and Oh, 2010;Shirwany and Zou, 2010). It has been already demonstrated that senescence negatively affects the HRV and endothelial function (Charkoudian and Rabbitts, 2009;Corino et al., 2007;Maeda et al., 2003;Morange et al., 2004). This present study demonstrated that the healthy elderly individuals had abnormal circadian changes of PSNS activity, and higher levels of vWF and ET-1 compared to middle-aged subjects. These evidences confirmed again that ageing has a potential disruptive effect on both ANS and endothelial function. Indeed, ANS and the endothelium work together to regulate a proper vascular tone (Harris and Matthews, 2004). Additionally, by using DVA, it firstly demonstrated that the abnormal carcidan changes of PSNS correlated with impaired retinal vascular function in the elderly subjects. These parallel alterations could represent the first sign of increased risk for CVD with ageing.

7.2 Conclusions

The aims of this work were:

7.2.1 To assess the relationship between ocular and systemic anti-oxidative defence in healthy individuals

The main findings of this work were:

- Age, gender and BP parameters had no effect on either MPOD or blood glutathione parameters.

- It has been shown that MPOD independently, significantly and positively correlated with circulatory GSH levels.

7.2.2 To investigate macro- and microcirculation and oxidative stress in early AMD patients without overt systemic disease

The main findings of this work were:

- Patients diagnosed with early AMD exhibit signs of subclinical vascular disese.
- Blood GSSG level was significantly higher in early AMD patients than age-matched controls.
- Patients suffering from early AMD showed abnormal vascular function at retinal level compared to age-matched healthy controls and only in AMD patients this abnormality correlated with blood GSSG levels.

7.2.3 To assess the relationship between ocular vascular function and circulatory markers of endothelial dysfunction and CVD risk

The main findings of this work were:

- Age had a positive effect on ET-1, vWF and slope of retinal arterial constriciton in otherwise healthy individuals.
- Even in otherwise healthy individuals, it has been shown that $Slope_{AC}$ independently, significantly and positively correlated with the level of ET-1 ad LDL-C; additionally, $Slope_{AD}$ independently, significantly and positively correlated with redox index.

7.2.4 To assess age-related changes in ANS and vascular function, and their relationship to retinal vascular function parameters

The main findings of this work were:

- Elderly individuals demonstrated abnormal circadian changes of PSNS activity compared to middle-aged healthy subjects.
- Circulatory levels of ET-1 and vWF, as well as C-IMT and AIx were significant higher in elderly group than middle-aged group.
- Elderly subjects showed the impaired retinal vascular function compared to middle-aged group.
- In the elderly individuals, impaired retinal vascular function independently, significantly and positively correlated with the dysregulation of PSNS activity.

7.3 Clinical implications arising from these results

Retinal vascular function has exhibited a close relationship with circulatory makers of oxidative stress, endothelial dysfunction and CVD risk in otherwise healthy individuals but with various degrees of risk. Assessing retinal vascular function could be a tempting non-invasive approach to monitor potential risk for endothelial dysfunction and CVD risk at a much earlier stage of the disease process when disease-modifying interventions are still possible. In addition, MPOD acts as a non-invasive measurement of retinal oxidative status that positively correlated with circulatory GSH levels; therefore, simple MPOD assessment could be used as surrogate indicator of the systemic capacity of dealing with the damaging effect of free radicals and, consequently, of their risk for developing chronic ocular and/or systemic pathologies. The results of this thesis show that the retinal vascular tone/function could be used as surrogate markers in a number other applications,

such as; mass population screening for vascular diseases, vascular function in other at risk individuals (i.e. advanced age), therapeutic prevention of vascular diseases (i.e. improve life style, exercise, nutritional, medicine), assessment of vascular function in other ocular/vascular disorders (i.e. AMD). This assessment of retinal vasulcar reactivity is a quick and non-invasive technique acts as a screening and preventive measure could be used in everyday clinical setting.

7.4 Future areas of research arising from the present work

Of the number of new questions that this thesis uncovered, three particular avenues of future research are worth highlighting.

7.4.1 The effect on ocular endothelial function in the wet-AMD patients treated with anti-VEGF drugs

Vascular endothelial growth factor (VEGF) represents a major mediator of angiogenesis and a strong vascular permeability factor (Ferrara, 2004), therefore playing a vital role in the pathogenesis of retinal neovascularization and vessel leakage in wet AMD (Ford et al., 2011). Patients suffering from AMD are at higher risk for developing CHD, myocardial infarction and stroke (Liao et al., 2008;Wong et al., 2006;Wong et al., 2007); moreover, they also are more likely to have a increased mortality compared to the age-matched healthy population (Tan et al., 2008). Additionally, some of anti-VEGF treatments could increase the risk for stroke; and this possibility could have important therapeutic implications. Therefore, early identification of those AMD patients at high risk of developing serious cardio and/or cerebro-vascular pathology after receiving anti-VEGF treatments is a priority. The present results of this thesis have already showed that retinal

vascular function measurements could be used to detect early cardiovascular risk. It is possible that by using similar approach to investigate the effect of anti-VEGF drugs used fir AMD treatment on patients' vascular function. In addition, the effect of the anti-VEGF treatment on the vascular healthy could also be monitored, allowing prompt intervention when first signs of risk occur.

7.4.2 Macular pigment as a marker of cardiovascular risk

Among various risk factors such as environmental, nutritional and genetic involved in the aetiology of AMD, high levels of oxidative stress have also been thought to play a role. As already mentioned, in healthy subjects, local retinal protection against free radicals is aided by the presence of MP. In addition to local retinal damage, high levels of oxidative stress also induces vascular changes that confer a background for circulatory disturbances in the systemic macro- and microcirculation that are also present in patients suffering from AMD. Systemically, the body is protected against such effects by a number or circulating substances. In the Chapter 3 of the present thesis, it has been found that the level of MPOD correlates with the circulatory level of antioxidant molecules. However, MPOD is only one way of measuring MP levels. It would be interesting to validate different types of measurements of MP as surrogate indicators of the individual's systemic capacity of dealing with the damaging effect of free radicals and, consequently, of their risk for developing chronic ocular and/or systemic pathologies. In this way, optometrists and ophthalmologists will be equipped with the possibility of screening individuals for vascular health through a simple measurement of the MP and the need of more sophisticated analyses and waiting lists will be thus avoided.

7.4.3 Antioxidant supplementation effect on ocular and systemic vascular parameters in AMD

During the recruitment of early AMD patients for this present thesis, I have found that most of them were taking various antioxidant supplements that included lutein. Indeed, lutein and its isomers, zeaxanthin and meso-zeaxanthin act as antioxidants and a filter of high power blue light (Krinsky, 2002); in addition, they seem to help with improving the visual function in patients suffering from AMD (Richer et al., 2004;Sasamoto et al., 2011). Nevertheless, the results reported after lutein supplementation are inconsistent (Bartlett and Eperjesi, 2008) and more work is necessary to demonstrate the beneficial effect of such supplements at both ocular and systemic level. As antioxidants also claim to improve vascular health, and AMD patients have high oxidative stress and are at higher risk of developing CVD, a prospective, controlled study to assess the effect of antioxidants on the ocular disease prognosis and risk for future vascular pathologies in AMD patients is warranted.

References

Abate, C., Patel, L., Rauscher, F. J.,3rd and Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 249, 1157-1161.

Adams, M. K., Simpson, J. A., Aung, K. Z., Makeyeva, G. A., Giles, G. G., English, D. R., Hopper, J., Guymer, R. H., Baird, P. N. and Robman, L. D. (2011). Abdominal obesity and age-related macular degeneration. *Am. J. Epidemiol.* 173, 1246-1255.

Ahsan, H., Ali, A. and Ali, R. (2003). Oxygen free radicals and systemic autoimmunity. *Clin Exp Immunol* 131, 398-404.

Akerstedt, T. and Froberg, J. E. (1979). Sleep and stressor exposure in relation to circadian rhythms in catecholamine excretion. *Biol. Psychol.* 8, 69-80.

Akselrod, S., Gordon, D., Ubel, F. A., Shannon, D. C., Berger, A. C. and Cohen, R. J. (1981). Power spectrum analysis of heart rate fluctuation: A quantitative probe of beat-to-beat cardiovascular control. *Science* 213, 220-222.

Alfaro, D. V., Liggett, P. E., Mieler, W. F., Quiroz-Mercado, H., Jager, R. D. and Tano, Y. (2005). *Age-Related Macular Degeneration: A Comprehensive Textbook*: Philadelphia, Pa. ; London : Lippincott Williams & Wilkins,.

Algvere, P. V., Marshall, J. and Seregard, S. (2006). Age-related maculopathy and the impact of blue light hazard. *Acta Ophthalmol. Scand.* 84, 4-15.

Alm, A. (1977). The effect of sympathetic stimulation on blood flow through t,e uvea, retina and optic nerve in monkeys (macacca irus). *Exp Eye Res* 25, 19-24.

Alm, A. and Bill, A. (1973). Ocular and optic nerve blood flow at normal and increased intraocular pressures in monkeys (macaca irus): A study with radioactively labelled microspheres including flow determinations in brain and some other tissues. *Exp. Eye Res.* 15, 15-29.

Alquier, T. and Kahn, B. B. (2004). Peripheral signals set the tone for central regulation of metabolism. *Endocrinology* 145, 4022-4024.

Amano, M., Kanda, T., Ue, H. and Moritani, T. (2001). Exercise training and autonomic nervous system activity in obese individuals. *Med. Sci. Sports Exerc.* 33, 1287-1291.

Amiri, F., Virdis, A., Neves, M. F., Iglarz, M., Seidah, N. G., Touyz, R. M., Reudelhuber, T. L. and Schiffrin, E. L. (2004). Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation* 110, 2233-2240.

Anderson, M. E. (1996). Glutathione. In *Free Radicals: A Practical Approach* (ed. N. A. Punchard and F. J. Kelly). Oxford, UK: Oxford University.

Anderson, M. E. and Meister, A. (1980). Dynamic state of glutathione in blood plasma. *J. Biol. Chem.* 255, 9530-9533.

Angerio, A. D. and Kot, P. A. (1997). Endothelin-1: Possible implications in pulmonary vascular disease. *Heart Lung* 26, 299-304; quiz 305-306.

Antelmi, I., de Paula, R. S., Shinzato, A. R., Peres, C. A., Mansur, A. J. and Grupi, C. J. (2004). Influence of age, gender, body mass index, and functional capacity on heart rate variability in a cohort of subjects without heart disease. *Am. J. Cardiol.* 93, 381-385.

Archer, D. B. and Gardiner, T. A. (1981). Electron microscopic features of experimental choroidal neovascularization. *Am J Ophthalmol* 91, 433-457.

Arnal, J. F., Dinh-Xuan, A. T., Pueyo, M., Darblade, B. and Rami, J. (1999). Endothelium-derived nitric oxide and vascular physiology and pathology. *Cell Mol Life Sci* 55, 1078-1087.

Aronson, D., Mittleman, M. A. and Burger, A. J. (2001). Role of endothelin in modulation of heart rate variability in patients with decompensated heart failure. *Pacing Clin. Electrophysiol.* 24, 1607-1615.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964-967.

Ashfaq, S., Abramson, J. L., Jones, D. P., Rhodes, S. D., Weintraub, W. S., Hooper, W. C., Vaccarino, V., Alexander, R. W., Harrison, D. G. and Quyyumi, A. A. (2008). Endothelial function and aminothiol biomarkers of oxidative stress in healthy adults. *Hypertension* 52, 80-85.

Ashfaq, S., Abramson, J. L., Jones, D. P., Rhodes, S. D., Weintraub, W. S., Hooper, W. C., Vaccarino, V., Harrison, D. G. and Quyyumi, A. A. (2006). The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J. Am. Coll. Cardiol.* 47, 1005-1011.

Avogaro, A., Albiero, M., Menegazzo, L., de Kreutzenberg, S. and Fadini, G. P. (2011). Endothelial dysfunction in diabetes: The role of reparatory mechanisms. *Diabetes Care* 34 Suppl 2, S285-290.

Bacic, F., Uematsu, S., McCarron, R. M. and Spatz, M. (1992). Secretion of immunoreactive endothelin-1 by capillary and microvascular endothelium of human brain. *Neurochem. Res.* 17, 699-702.

Bacon, S. L., Lavoie, K. L., Arsenault, A., Dupuis, J., Pilote, L., Laurin, C., Gordon, J., Gautrin, D. and Vadeboncoeur, A. (2011). The research on endothelial function in women and men at risk for cardiovascular disease (REWARD) study: Methodology. *BMC Cardiovasc. Disord.* 11, 50.

Ballatori, N., Krance, S. M., Notenboom, S., Shi, S., Tieu, K. and Hammond, C. L. (2009). Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* 390, 191-214.

Bari, F., Louis, T. M. and Busija, D. W. (1998). Effects of ischemia on cerebral arteriolar dilation to arterial hypoxia in piglets. *Stroke* 29, 222-227; discussion 227-228.

Bartlett, H., Howells, O. and Eperjesi, F. (2010). The role of macular pigment assessment in clinical practice: A review. *Clin Exp Optom* 93, 300-308.

Bartlett, H. E. and Eperjesi, F. (2008). A randomised controlled trial investigating the effect of lutein and antioxidant dietary supplementation on visual function in healthy eyes. *Clin. Nutr.* 27, 218-227.

Bartolomucci, F., De Michele, M., Kozakova, M., Cipriani, F., Polemio, F. and Palombo, C. (2011). Impaired endothelium independent vasodilation in nonobstructive hypertrophic cardiomyopathy. *Am. J. Hypertens.* 24, 750-754.

Baumgart, P. (1991). Circadian rhythm of blood pressure: Internal and external time triggers. *Chronobiol. Int.* 8, 444-450.

Bauser-Heaton, H. D. and Bohlen, H. G. (2007). Cerebral microvascular dilation during hypotension and decreased oxygen tension: A role for nNOS. *Am. J. Physiol. Heart Circ. Physiol.* 293, H2193-2201.

Bayliss, W. M. (1902). On the local reactions of the arterial wall to changes of internal pressure. *J. Physiol.* 28, 220-231.

Beard, K. M., Shangari, N., Wu, B. and O'Brien, P. J. (2003). Metabolism, not autoxidation, plays a role in alpha-oxoaldehyde- and reducing sugar-induced erythrocyte GSH depletion: Relevance for diabetes mellitus. *Mol Cell Biochem* 252, 331-338.

Beatty, S., Koh, H., Phil, M., Henson, D. and Boulton, M. (2000). The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv. Ophthalmol.* 45, 115-134.

Beausang-Linder, M. and Hultcrantz, E. (1980). Early effects of cervical sympathetic stimulation on cerebral, ocular and cochlear blood flow. *Acta Physiol. Scand.* 109, 433-437.

Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* 78, 547-581.

Bendich, A. (1990). Antioxidant micronutrients and immune responses. *Ann. N. Y. Acad. Sci.* 587, 168-180.

Berger, R. D., Saul, J. P. and Cohen, R. J. (1989). Transfer function analysis of autonomic regulation. I. canine atrial rate response. *Am. J. Physiol.* 256, H142-152.

Beutler, E. and Gelbart, T. (1985). Plasma glutathione in health and in patients with malignant disease. *J Lab Clin Med* 105, 581-584.

Bhosale, P., Zhao da, Y. and Bernstein, P. S. (2007). HPLC measurement of ocular carotenoid levels in human donor eyes in the lutein supplementation era. *Invest Ophthalmol Vis Sci* 48, 543-549.

Bhutto, I. A., Baba, T., Merges, C., McLeod, D. S. and Lutty, G. A. (2010). Low nitric oxide synthases (NOSs) in eyes with age-related macular degeneration (AMD). *Exp. Eye Res.* 90, 155-167.

Bigger, J. T.,Jr, Steinman, R. C., Rolnitzky, L. M., Fleiss, J. L., Albrecht, P. and Cohen, R. J. (1996). Power law behavior of RR-interval variability in healthy middle-aged persons, patients with recent acute myocardial infarction, and patients with heart transplants. *Circulation* 93, 2142-2151.

Bill, A., Linder, M. and Linder, J. (1977). The protective role of ocular sympathetic vasomotor nerves in acute arterial hypertension. *Bibl Anat*, 30-35.

Bill, A. and Sperber, G. O. (1990). Control of retinal and choroidal blood flow. *Eye (Lond)* 4 (Pt 2), 319-325.

Bird, A. C., Bressler, N. M., Bressler, S. B., Chisholm, I. H., Coscas, G., Davis, M. D., de Jong, P. T., Klaver, C. C., Klein, B. E. and Klein, R. (1995). An international classification and grading system for age-related maculopathy and age-related macular degeneration. the international ARM epidemiological study group. *Surv Ophthalmol* 39, 367-374.

Blann, A. D. (1992). Von willebrand factor antigen: A radial immunodiffusion method evaluated and compared with an ELISA method. *Med. Lab. Sci.* 49, 141-143.

Blann, A. D. and Lip, G. Y. (1998). The endothelium in atherothrombotic disease: Assessment of function, mechanisms and clinical implications. *Blood Coagul. Fibrinolysis* 9, 297-306.

Blazquez-Medela, A. M., Garcia-Ortiz, L., Gomez-Marcos, M. A., Recio-Rodriguez, J. I., Sanchez-Rodriguez, A., Lopez-Novoa, J. M. and Martinez-Salgado, C. (2010). Increased plasma soluble endoglin levels as an indicator of cardiovascular alterations in hypertensive and diabetic patients. *BMC Med.* 8, 86.

Boehm, A. G., Koeller, A. U. and Pillunat, L. E. (2005). The effect of age on optic nerve head blood flow. *Invest. Ophthalmol. Vis. Sci.* 46, 1291-1295.

Bone, R. A., Landrum, J. T., Hime, G. W., Cains, A. and Zamor, J. (1993). Stereochemistry of the human macular carotenoids. *Invest Ophthalmol Vis Sci* 34, 2033-2040.

Bone, R. A., Landrum, J. T. and Tarsis, S. L. (1985). Preliminary identification of the human macular pigment. *Vision Res* 25, 1531-1535.

Booij, J. C., Baas, D. C., Beisekeeva, J., Gorgels, T. G. and Bergen, A. A. (2010). The dynamic nature of bruch's membrane. *Prog. Retin. Eye Res.* 29, 1-18.

Bootsma, M., Swenne, C. A., Van Bolhuis, H. H., Chang, P. C., Cats, V. M. and Bruschke, A. V. (1994). Heart rate and heart rate variability as indexes of sympathovagal balance. *Am. J. Physiol.* 266, H1565-1571.

Bressler, N. M., Silva, J. C., Bressler, S. B., Fine, S. L. and Green, W. R. (1994). Clinicopathologic correlation of drusen and retinal pigment epithelial abnormalities in age-related macular degeneration. *Retina* 14, 130-142.

Buchi, E. R. (1996). The blood supply to the optic nerve head. In *Ocular Blood Flow* (ed. H. J. Kaiser, J. Flammer and P. Hendrickson), pp. 1-1-8. Basel: Karger.

Buhl, R., Jaffe, H. A., Holroyd, K. J., Wells, F. B., Mastrangeli, A., Saltini, C., Cantin, A. M. and Crystal, R. G. (1989). Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. *Lancet* 2, 1294-1298.

Burnstock, G. (1990). Local mechanisms of blood flow control by perivascular nerves and endothelium. *J Hypertens Suppl* 8, S95-106.

Burnstock, G. (1993). Integration of factors controlling vascular tone. overview. *Anesthesiology* 79, 1368-1380.

Cai, H. and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. *Circ Res* 87, 840-844.

Cai, J., Nelson, K. C., Wu, M., Sternberg, P.,Jr and Jones, D. P. (2000). Oxidative damage and protection of the RPE. *Prog. Retin. Eye Res.* 19, 205-221.

Caprioli, J., Coleman, A. L. and Blood Flow in Glaucoma Discussion. (2010). Blood pressure, perfusion pressure, and glaucoma. *Am. J. Ophthalmol.* 149, 704-712.

Carmody, R. J. and Cotter, T. G. (2001). Signalling apoptosis: A radical approach. *Redox Rep.* 6, 77-90.

Castro, L. and Freeman, B. A. (2001). Reactive oxygen species in human health and disease. *Nutrition* 17, 161, 163-165.

Celermajer, D. S. (1997). Endothelial dysfunction: Does it matter? is it reversible? *J Am Coll Cardiol* 30, 325-333.

Chakravarthy, U., Wong, T. Y., Fletcher, A., Piault, E., Evans, C., Zlateva, G., Buggage, R., Pleil, A. and Mitchell, P. (2010). Clinical risk factors for age-related macular degeneration: A systematic review and meta-analysis. *BMC Ophthalmol.* 10, 31.

Chan, S. L., Capdeville-Atkinson, C. and Atkinson, J. (2008). Red wine polyphenols improve endothelium-dependent dilation in rat cerebral arterioles. *J. Cardiovasc. Pharmacol.* 51, 553-558.

Chang, M. A., Bressler, S. B., Munoz, B. and West, S. K. (2008). Racial differences and other risk factors for incidence and progression of age-related macular degeneration: Salisbury eye evaluation (SEE) project. *Invest Ophthalmol Vis Sci* 49, 2395-2402.

Charkoudian, N. and Rabbitts, J. A. (2009). Sympathetic neural mechanisms in human cardiovascular health and disease. *Mayo Clin. Proc.* 84, 822-830.

Charman, W. N. (2003). Age, lens transmittance, and the possible effects of light on melatonin suppression. *Ophthalmic Physiol Opt* 23, 181-187.

Chen, X. P., Xun, K. L., Wu, Q., Zhang, T. T., Shi, J. S. and Du, G. H. (2007). Oxidized low density lipoprotein receptor-1 mediates oxidized low density lipoprotein-induced apoptosis in human umbilical vein endothelial cells: Role of reactive oxygen species. *Vascul Pharmacol.* 47, 1-9.

Cheung, N., Liao, D., Islam, F. M., Klein, R., Wang, J. J. and Wong, T. Y. (2007a). Is early age-related macular degeneration related to carotid artery stiffness? the atherosclerosis risk in communities study. *Br. J. Ophthalmol.* 91, 430-433.

Cheung, N., Shankar, A., Klein, R., Folsom, A. R., Couper, D. J., Wong, T. Y. and Atherosclerosis Risk in Communities (ARIC) Study Investigators. (2007b). Age-related macular degeneration and cancer mortality in the atherosclerosis risk in communities study. *Arch. Ophthalmol.* 125, 1241-1247.

Cho, E., Hung, S., Willett, W. C., Spiegelman, D., Rimm, E. B., Seddon, J. M., Colditz, G. A. and Hankinson, S. E. (2001). Prospective study of dietary fat and the risk of age-related macular degeneration. *Am. J. Clin. Nutr.* 73, 209-218.

Choi, J. B., Hong, S., Nelesen, R., Bardwell, W. A., Natarajan, L., Schubert, C. and Dimsdale, J. E. (2006). Age and ethnicity differences in short-term heart-rate variability. *Psychosom. Med.* 68, 421-426.

Choudhury, F., Varma, R., McKean-Cowdin, R., Klein, R., Azen, S. P. and Los Angeles Latino Eye Study Group. (2011). Risk factors for four-year incidence and progression of age-related macular degeneration: The los angeles latino eye study. *Am. J. Ophthalmol.* 152, 385-395.

Chow, C. K. (1991). Vitamin E and oxidative stress. *Free Radic Biol Med* 11, 215-232.

Chuang, A. T., Strauss, J. D., Murphy, R. A. and Steers, W. D. (1998). Sildenafil, a type-5 CGMP phosphodiesterase inhibitor, specifically amplifies endogenous cGMP-dependent relaxation in rabbit corpus cavernosum smooth muscle in vitro. *J. Urol.* 160, 257-261.

Ciulla, T. A., Harris, A., Latkany, P., Piper, H. C., Arend, O., Garzozi, H. and Martin, B. (2002). Ocular perfusion abnormalities in diabetes. *Acta Ophthalmol. Scand.* 80, 468-477.

Clemons, T. E., Milton, R. C., Klein, R., Seddon, J. M. and Ferris, F. L.,3rd. (2005). Risk factors for the incidence of advanced age-related macular degeneration in the age-related eye disease study (AREDS) AREDS report no. 19. *Ophthalmology* 112, 533-539.

Cohen, S. M., Olin, K. L., Feuer, W. J., Hjelmeland, L., Keen, C. L. and Morse, L. S. (1994). Low glutathione reductase and peroxidase activity in age-related macular degeneration. *Br J Ophthalmol* 78, 791-794.

Coleman, H. R., Chan, C. C., Ferris, F. L.,3rd and Chew, E. Y. (2008). Age-related macular degeneration. *Lancet* 372, 1835-1845.

Cominacini, L., Rigoni, A., Pasini, A. F., Garbin, U., Davoli, A., Campagnola, M., Pastorino, A. M., Lo Cascio, V. and Sawamura, T. (2001). The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. *J. Biol. Chem.* 276, 13750-13755.

Complications of Age-related Macular Degeneration Prevention Trial (CAPT) Research Group. (2008). Risk factors for choroidal neovascularization and geographic atrophy in the complications of age-related macular degeneration prevention trial. *Ophthalmology* 115, 1474-1479, 1479 e1-6.

Connolly, E. E., Beatty, S., Thurnham, D. I., Loughman, J., Howard, A. N., Stack, J. and Nolan, J. M. (2010). Augmentation of macular pigment following supplementation with all three macular carotenoids: An exploratory study. *Curr. Eye Res.* 35, 335-351.

Constans, J. and Conri, C. (2006). Circulating markers of endothelial function in cardiovascular disease. *Clin Chim Acta* 368, 33-47.

Corino, V. D., Matteucci, M. and Mainardi, L. T. (2007). Analysis of heart rate variability to predict patient age in a healthy population. *Methods Inf. Med.* 46, 191-195.

Cranston, W. I. (1964). Diurnal variation in plasma volume in normal and hypertensive subjects. *Am. Heart J.* 68, 427-428.

Cryer, P. E. (1977). Pheochromocytoma and autonomic dysfunction. *Arch. Intern. Med.* 137, 783-787.

Dashwood, M. R. and Tsui, J. C. (2002). Endothelin-1 and atherosclerosis: Potential complications associated with endothelin-receptor blockade. *Atherosclerosis* 160, 297-304.

Davies, J. A., Collins, P. W., Hathaway, L. S. and Bowen, D. J. (2007). Effect of von willebrand factor Y/C1584 on in vivo protein level and function and interaction with ABO blood group. *Blood* 109, 2840-2846.

Davies, M. G. and Hagen, P. O. (1993). The vascular endothelium. A new horizon. *Ann Surg* 218, 593-609.

Davies, N. P. and Morland, A. B. (2004). Macular pigments: Their characteristics and putative role. *Prog Retin Eye Res* 23, 533-559.

Davies, P. F. and Tripathi, S. C. (1993). Mechanical stress mechanisms and the cell. an endothelial paradigm. *Circ. Res.* 72, 239-245.

Davy, K. P., DeSouza, C. A., Jones, P. P. and Seals, D. R. (1998). Elevated heart rate variability in physically active young and older adult women. *Clin. Sci. (Lond)* 94, 579-584.

Dawson, S. L., Robinson, T. G., Youde, J. H., Martin, A., James, M. A., Weston, P. J., Panerai, R. B. and Potter, J. F. (1999). Older subjects show no age-related decrease in cardiac baroreceptor sensitivity. *Age Ageing* 28, 347-353.

De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., A., G. M.,Jr, Shin, W. S. and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96, 60-68.

de Jong, P. T. (2006). Age-related macular degeneration. *N Engl J Med* 355, 1474-1485.

De Meersman, R. E. (1993). Heart rate variability and aerobic fitness. *Am. Heart J.* 125, 726-731.

De Meersman, R. E. and Stein, P. K. (2007). Vagal modulation and aging. *Biol. Psychol.* 74, 165-173.

De Vriese, A. S., Verbeuren, T. J., Van de Voorde, J., Lameire, N. H. and Vanhoutte, P. M. (2000). Endothelial dysfunction in diabetes. *Br. J. Pharmacol.* 130, 963-974.

Denninger, J. W. and Marletta, M. A. (1999). Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta* 1411, 334-350.

Deutsch, T. A., Read, J. S., Ernest, J. T. and Goldstick, T. K. (1983). Effects of oxygen and carbon dioxide on the retinal vasculature in humans. *Arch. Ophthalmol.* 101, 1278-1280.

Di Mascio, P., Kaiser, S. and Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys* 274, 532-538.

Diedrich, A., Jordan, J., Tank, J., Shannon, J. R., Robertson, R., Luft, F. C., Robertson, D. and Biaggioni, I. (2003). The sympathetic nervous system in hypertension: Assessment by blood pressure variability and ganglionic blockade. *J. Hypertens.* 21, 1677-1686.

Donald, A. E., Charakida, M., Cole, T. J., Friberg, P., Chowienczyk, P. J., Millasseau, S. C., Deanfield, J. E. and Halcox, J. P. (2006). Non-invasive assessment of endothelial function: Which technique? *J. Am. Coll. Cardiol.* 48, 1846-1850.

Donato, A. J., Gano, L. B., Eskurza, I., Silver, A. E., Gates, P. E., Jablonski, K. and Seals, D. R. (2009). Vascular endothelial dysfunction with aging: Endothelin-1 and endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 297, H425-432.

Donoso, L. A., Kim, D., Frost, A., Callahan, A. and Hageman, G. (2006). The role of inflammation in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 51, 137-152.

Dorner, G. T., Garhofer, G., Kiss, B., Polska, E., Polak, K., Riva, C. E. and Schmetterer, L. (2003). Nitric oxide regulates retinal vascular tone in humans. *Am J Physiol Heart Circ Physiol* 285, H631-636.

Duerrschmidt, N., Wippich, N., Goettsch, W., Broemme, H. J. and Morawietz, H. (2000). Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. *Biochem. Biophys. Res. Commun.* 269, 713-717.

Edwards, K. M., Wilson, K. L., Sadja, J., Ziegler, M. G. and Mills, P. J. (2011). Effects on blood pressure and autonomic nervous system function of a 12-week exercise or exercise plus DASH-diet intervention in individuals with elevated blood pressure. *Acta Physiol. (Oxf)* 203, 343-350.

Ehinger, B. (1966). Adrenergic neurons in the retina. *Life Sci* 5, 129-131.

Ehrlich, R., Kheradiya, N. S., Winston, D. M., Moore, D. B., Wirostko, B. and Harris, A. (2009). Age-related ocular vascular changes. *Graefes Arch. Clin. Exp. Ophthalmol.* 247, 583-591.

Eperon, G., Johnson, M. and David, N. J. (1975). The effect of arterial PO2 on relative retinal blood flow in monkeys. *Invest. Ophthalmol.* 14, 342-352.

Ernst, M. E. and Bergus, G. R. (2003). Ambulatory blood pressure monitoring. *South. Med. J.* 96, 563-568.

Esler, M. (1993). Clinical application of noradrenaline spillover methodology: Delineation of regional human sympathetic nervous responses. *Pharmacol. Toxicol.* 73, 243-253.

Evereklioglu, C., Er, H., Doganay, S., Cekmen, M., Turkoz, Y., Otlu, B. and Ozerol, E. (2003). Nitric oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration. *Doc. Ophthalmol.* 106, 129-136.

Ewing, D. J., Campbell, I. W. and Clarke, B. F. (1980). The natural history of diabetic autonomic neuropathy. *Q. J. Med.* 49, 95-108.

Ewing, D. J., Neilson, J. M., Shapiro, C. M., Stewart, J. A. and Reid, W. (1991). Twenty four hour heart rate variability: Effects of posture, sleep, and time of day in healthy controls and comparison with bedside tests of autonomic function in diabetic patients. *Br. Heart J.* 65, 239-244.

Eye Disease Case-Control Study Group. (1993). Antioxidant status and neovascular age-related macular degeneration. *Arch Ophthalmol* 111, 104-109.

Fagius, J. and Wallin, B. G. (1993). Long-term variability and reproducibility of resting human muscle nerve sympathetic activity at rest, as reassessed after a decade. *Clin. Auton. Res.* 3, 201-205.

Falsini, B., Riva, C. E. and Logean, E. (2002). Flicker-evoked changes in human optic nerve blood flow: Relationship with retinal neural activity. *Invest. Ophthalmol. Vis. Sci.* 43, 2309-2316.

Feeney-Burns, L., Hilderbrand, E. S. and Eldridge, S. (1984). Aging human RPE: Morphometric analysis of macular, equatorial, and peripheral cells. *Invest. Ophthalmol. Vis. Sci.* 25, 195-200.

Feletou, M., Cohen, R. A., Vanhoutte, P. M. and Verbeuren, T. J. (2010). TP receptors and oxidative stress hand in hand from endothelial dysfunction to atherosclerosis. *Adv. Pharmacol.* 60, 85-106.

Ferdinandy, P. and Schulz, R. (2003). Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br. J. Pharmacol.* 138, 532-543.

Ferrara, N. (2004). Vascular endothelial growth factor: Basic science and clinical progress. *Endocr. Rev.* 25, 581-611.

Ferrari-Dileo, G. (1988). Beta 1 and beta 2 adrenergic binding sites in bovine retina and retinal blood vessels. *Invest. Ophthalmol. Vis. Sci.* 29, 695-699.

Filosa, J. A. (2010). Vascular tone and neurovascular coupling: Considerations toward an improved in vitro model. *Front. Neuroenergetics* 2, 16.

Fine, S. L., Berger, J. W., Maguire, M. G. and Ho, A. C. (2000). Age-related macular degeneration. *N Engl J Med* 342, 483-492.

Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239-247.

Flagg, E. W., Coates, R. J., Jones, D. P., Eley, J. W., Gunter, E. W., Jackson, B. and Greenberg, R. S. (1993). Plasma total glutathione in humans and its association with demographic and health-related factors. *Br. J. Nutr.* 70, 797-808.

Flapan, A. D., Wright, R. A., Nolan, J., Neilson, J. M. and Ewing, D. J. (1993). Differing patterns of cardiac parasympathetic activity and their evolution in selected patients with a first myocardial infarction. *J. Am. Coll. Cardiol.* 21, 926-931.

Fleming, I., Bauersachs, J. and Busse, R. (1996). Paracrine functions of the coronary vascular endothelium. *Mol Cell Biochem* 157, 137-145.

Ford, K. M., Saint-Geniez, M., Walshe, T., Zahr, A. and D'Amore, P. A. (2011). Expression and role of VEGF in the adult retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 13, 9478-9487.

Forster, B. A., Ferrari-Dileo, G. and Anderson, D. R. (1987). Adrenergic alpha 1 and alpha 2 binding sites are present in bovine retinal blood vessels. *Invest. Ophthalmol. Vis. Sci.* 28, 1741-1746.

Frankel, D. S., Meigs, J. B., Massaro, J. M., Wilson, P. W., O'Donnell, C. J., D'Agostino, R. B. and Tofler, G. H. (2008). Von willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease: The framingham offspring study. *Circulation* 118, 2533-2539.

Freeman, R. (2006). Assessment of cardiovascular autonomic function. *Clin. Neurophysiol.* 117, 716-730.

Freixa, X., Heras, M., Ortiz, J. T., Argiro, S., Guasch, E., Doltra, A., Jimenez, M., Betriu, A. and Masotti, M. (2011). Usefulness of endothelin-1 assessment in acute myocardial infarction. *Rev. Esp. Cardiol.* 64, 105-110.

Friedewald, W. T., Levy, R. I. and Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* 18, 499-502.

Fukuba, Y., Sato, H., Sakiyama, T., Yamaoka Endo, M., Yamada, M., Ueoka, H., Miura, A. and Koga, S. (2009). Autonomic nervous activities assessed by heart rate variability in pre- and post-adolescent japanese. *J. Physiol. Anthropol.* 28, 269-273.

Furchgott, R. F. and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-376.

Gao, H. and Hollyfield, J. G. (1992). Aging of the human retina. differential loss of neurons and retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 33, 1-17.

Garhofer, G., Bek, T., Boehm, A. G., Gherghel, D., Grunwald, J., Jeppesen, P., Kergoat, H., Kotliar, K., Lanzl, I., Lovasik, J. V. et al. (2010). Use of the retinal vessel analyzer in ocular blood flow research. *Acta Ophthalmol.* 88, 717-722.

Garhofer, G., Zawinka, C., Huemer, K. H., Schmetterer, L. and Dorner, G. T. (2003). Flicker light-induced vasodilatation in the human retina: Effect of lactate and changes in mean arterial pressure. *Invest. Ophthalmol. Vis. Sci.* 44, 5309-5314.

Garhofer, G., Zawinka, C., Resch, H., Huemer, K. H., Schmetterer, L. and Dorner, G. T. (2004a). Response of retinal vessel diameters to flicker stimulation in patients with early open angle glaucoma. *J Glaucoma* 13, 340-344.

Garhofer, G., Zawinka, C., Resch, H., Kothy, P., Schmetterer, L. and Dorner, G. T. (2004b). Reduced response of retinal vessel diameters to flicker stimulation in patients with diabetes. *Br J Ophthalmol* 88, 887-891.

Gariano, R. F. and Gardner, T. W. (2005). Retinal angiogenesis in development and disease. *Nature* 438, 960-966.

Gasparyan, A. Y. (2009). The use of carotid artery ultrasonography in different clinical conditions. *Open Cardiovasc. Med. J.* 3, 78-80.

Gates, P. E., Strain, W. D. and Shore, A. C. (2009). Human endothelial function and microvascular ageing. *Exp. Physiol.* 94, 311-316.

Gerszten, R. E. and Wang, T. J. (2008). The search for new cardiovascular biomarkers. *Nature* 451, 949-952.

Gherghel, D., Griffiths, H. R., Hilton, E. J., Cunliffe, I. A. and Hosking, S. L. (2005). Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. *Invest. Ophthalmol. Vis. Sci.* 46, 877-883.

Gherghel, D., Hosking, S. L., Armstrong, R. and Cunliffe, I. A. (2007). Autonomic dysfunction in unselected and untreated primary open angle glaucoma patients: A pilot study. *Ophthalmic Physiol. Opt.* 27, 336-341.

Gherghel, D., Hosking, S. L., Cunliffe, I. A. and Heitmar, R. (2010). Transient cardiac ischaemia and abnormal variations in systemic blood pressure in unselected primary open angle glaucoma patients. *Ophthalmic Physiol. Opt.* 30, 175-181.

Gill, J. C., Endres-Brooks, J., Bauer, P. J., Marks, W. J.,Jr and Montgomery, R. R. (1987). The effect of ABO blood group on the diagnosis of von willebrand disease. *Blood* 69, 1691-1695.

Giraldez, R. R., Panda, A., Xia, Y., Sanders, S. P. and Zweier, J. L. (1997). Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J. Biol. Chem.* 272, 21420-21426.

Goldberger, J. J. (1999). Sympathovagal balance: How should we measure it? *Am J Physiol* 276, H1273-1280.

Gray, H. (1985). *Gray's Anatomy: The Masterclass Edition*. london: chancellor press.

Greenfield, D. S., Heggerick, P. A. and Hedges, T. R.,3rd. (1995). Color doppler imaging of normal orbital vasculature. *Ophthalmology* 102, 1598-1605.

Greenland, P., Abrams, J., Aurigemma, G. P., Bond, M. G., Clark, L. T., Criqui, M. H., Crouse, J. R.,3rd, Friedman, L., Fuster, V., Herrington, D. M. et al. (2000). Prevention conference V: Beyond secondary prevention: Identifying the high-risk patient for primary prevention: Noninvasive tests of atherosclerotic burden: Writing group III. *Circulation* 101, E16-22.

Greenland, P., Knoll, M. D., Stamler, J., Neaton, J. D., Dyer, A. R., Garside, D. B. and Wilson, P. W. (2003). Major risk factors as antecedents of fatal and nonfatal coronary heart disease events. *JAMA* 290, 891-897.

Gregoire, J., Tuck, S., Yamamoto, Y. and Hughson, R. L. (1996). Heart rate variability at rest and exercise: Influence of age, gender, and physical training. *Can. J. Appl. Physiol.* 21, 455-470.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207-212.

Groh, M. J., Michelson, G., Langhans, M. J. and Harazny, J. (1996). Influence of age on retinal and optic nerve head blood circulation. *Ophthalmology* 103, 529-534.

Groleau, J., Dussault, S., Turgeon, J., Haddad, P. and Rivard, A. (2011). Accelerated vascular aging in CuZnSOD-deficient mice: Impact on EPC function and reparative neovascularization. *PLoS One* 6, e23308.

Grossniklaus, H. E. and Green, W. R. (2004). Choroidal neovascularization. *Am J Ophthalmol* 137, 496-503.

Grunwald, J. E., Hariprasad, S. M. and DuPont, J. (1998). Effect of aging on foveolar choroidal circulation. *Arch. Ophthalmol.* 116, 150-154.

Gugleta, K., Kochkorov, A., Waldmann, N., Polunina, A., Katamay, R., Flammer, J. and Orgul, S. (2011). Dynamics of retinal vessel response to flicker light in glaucoma patients and ocular hypertensives. *Graefes Arch. Clin. Exp. Ophthalmol.* 250, 589-594.

Guimaraes, S. and Moura, D. (2001). Vascular adrenoceptors: An update. *Pharmacol. Rev.* 53, 319-356.

Haefliger, I. O., Meyer, P., Flammer, J. and Luscher, T. F. (1994a). The vascular endothelium as a regulator of the ocular circulation: A new concept in ophthalmology? *Surv Ophthalmol* 39, 123-132.

Haefliger, I. O., Zschauer, A. and Anderson, D. R. (1994b). Relaxation of retinal pericyte contractile tone through the nitric oxide-cyclic guanosine monophosphate pathway. *Invest Ophthalmol Vis Sci* 35, 991-997.

Hagbarth, K. E. (1979). Exteroceptive, proprioceptive, and sympathetic activity recorded with microelectrodes from human peripheral nerves. *Mayo Clin. Proc.* 54, 353-365.

Hageman, G. S., Luthert, P. J., Victor Chong, N. H., Johnson, L. V., Anderson, D. H. and Mullins, R. F. (2001). An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res* 20, 705-732.

Hammond B.R, J., Wooten, B. R. and Snodderly, D. M. (1998). Preservation of visual sensitivity of older subjects: Association with macular pigment density. *Invest Ophthalmol Vis Sci* 39, 397-406.

Hammond, B. R.,Jr, Johnson, E. J., Russell, R. M., Krinsky, N. I., Yeum, K. J., Edwards, R. B. and Snodderly, D. M. (1997). Dietary modification of human macular pigment density. *Invest. Ophthalmol. Vis. Sci.* 38, 1795-1801.

Harman, D. (1956). Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298-300.

Harman, D. (1993). Free radical involvement in aging. pathophysiology and therapeutic implications. *Drugs Aging* 3, 60-80.

Harman, D. (2001). Aging: Overview. *Ann. N. Y. Acad. Sci.* 928, 1-21.

Harman, D. (2003). The free radical theory of aging. *Antioxid. Redox Signal.* 5, 557-561.

Harman, D. (2006). Free radical theory of aging: An update: Increasing the functional life span. *Ann. N. Y. Acad. Sci.* 1067, 10-21.

Harris, A., Anderson, D. R., Pillunat, L., Joos, K., Knighton, R. W., Kagemann, L. and Martin, B. J. (1996). Laser doppler flowmetry measurement of changes in human optic nerve head blood flow in response to blood gas perturbations. *J. Glaucoma* 5, 258-265.

Harris, A., Harris, M., Biller, J., Garzozi, H., Zarfty, D., Ciulla, T. A. and Martin, B. (2000). Aging affects the retrobulbar circulation differently in women and men. *Arch. Ophthalmol.* 118, 1076-1080.

Harris, A., Kagemann, L. and Cioffi, G. A. (1998). Assessment of human ocular hemodynamics. *Surv. Ophthalmol.* 42, 509-533.

Harris, K. F. and Matthews, K. A. (2004). Interactions between autonomic nervous system activity and endothelial function: A model for the development of cardiovascular disease. *Psychosom Med* 66, 153-164.

Haveman, M., Perry, J., Salvador-Carulla, L., Walsh, P. N., Kerr, M., Van Schrojenstein Lantman-de Valk, H., Van Hove, G., Berger, D. M., Azema, B., Buono, S. et al. (2011). Ageing and health status in adults with intellectual disabilities: Results of the european POMONA II study. *J. Intellect. Dev. Disabil.* 36, 49-60.

Hayakawa, T., Terashima, M., Kayukawa, Y., Ohta, T. and Okada, T. (1996). Changes in cerebral oxygenation and hemodynamics during obstructive sleep apneas. *Chest* 109, 916-921.

Hayes, R. D., Chang, C. K., Fernandes, A., Broadbent, M., Lee, W., Hotopf, M. and Stewart, R. (2011). Associations between substance use disorder sub-groups, life expectancy and all-cause mortality in a large british specialist mental healthcare service. *Drug Alcohol Depend.* 118, 56-61.

Heitmar, R., Blann, A. D., Cubbidge, R. P., Lip, G. Y. and Gherghel, D. (2010). Continuous retinal vessel diameter measurements: The future in retinal vessel assessment? *Invest. Ophthalmol. Vis. Sci.* 51, 5833-5839.

Heitmar, R., Cubbidge, R., Lip, G., Gherghel, D. and Blann, A. (2011). Altered blood vessel responses in the eye and finger in coronary artery disease. *Invest. Ophthalmol. Vis. Sci.* 52, 6199-6205.

Heitmar, R., Gherghel, D., Armstrong, R., Cubbidge, R. and Hosking, S. (2008). The effect of voluntary fasting and dehydration on flicker-induced retinal vascular dilation in a healthy individual: A case report. *J Med Case Reports* 2, 153.

Hermida, R. C., Ayala, D. E. and Portaluppi, F. (2007). Circadian variation of blood pressure: The basis for the chronotherapy of hypertension. *Adv. Drug Deliv. Rev.* 59, 904-922.

Hetzel, A., Braune, S., Guschlbauer, B., Dohms, K., Prasse, A. and Lucking, C. H. (1999). Multimodality monitoring during passive tilt and valsalva maneuver under hypercapnia. *J. Neuroimaging* 9, 108-112.

Hickey, M. J. (2001). Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. *Clin. Sci. (Lond)* 100, 1-12.

Hicks J, A. G. (1999). A century of change: Trends in UK statistics since 1900. *Social and general statistics section*.

Higashi, Y., Noma, K., Yoshizumi, M. and Kihara, Y. (2009). Endothelial function and oxidative stress in cardiovascular diseases. *Circ J* 73, 411-418.

Hill, J. M., Zalos, G., Halcox, J. P., Schenke, W. H., Waclawiw, M. A., Quyyumi, A. A. and Finkel, T. (2003). Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N. Engl. J. Med.* 348, 593-600.

Hoffman, W. E., Charbel, F. T., Edelman, G. and Abood, C. (1996). Brain tissue response to CO2 in patients with arteriovenous malformation. *J. Cereb. Blood Flow Metab.* 16, 1383-1386.

Hogg, R. E. and Chakravarthy, U. (2006). Visual function and dysfunction in early and late age-related maculopathy. *Prog Retin Eye Res* 25, 249-276.

Holewijn, S., den Heijer, M., Swinkels, D. W., Stalenhoef, A. F. and de Graaf, J. (2010). Apolipoprotein B, non-HDL cholesterol and LDL cholesterol for identifying individuals at increased cardiovascular risk. *J. Intern. Med.* 268, 567-577.

Holland, D. J., Sharman, J. E., Leano, R. L. and Marwick, T. H. (2009). Gender differences in systolic tissue velocity: Role of left ventricular length. *Eur. J. Echocardiogr.* 10, 941-946.

Hollyfield, J. G., Bonilha, V. L., Rayborn, M. E., Yang, X., Shadrach, K. G., Lu, L., Ufret, R. L., Salomon, R. G. and Perez, V. L. (2008). Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat. Med.* 14, 194-198.

Hornig, B., Maier, V. and Drexler, H. (1996). Physical training improves endothelial function in patients with chronic heart failure. *Circulation* 93, 210-214.

Hrushesky, W. J., Fader, D., Schmitt, O. and Gilbertsen, V. (1984). The respiratory sinus arrhythmia: A measure of cardiac age. *Science* 224, 1001-1004.

Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. and Fishman, M. C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75, 1273-1286.

Huh, Y. J., Kim, J. M., Kim, H., Song, H., So, H., Lee, S. Y., Kwon, S. B., Kim, H. J., Kim, H. H., Lee, S. H. et al. (2006). Regulation of osteoclast differentiation by the redox-dependent modulation of nuclear import of transcription factors. *Cell Death Differ.* 13, 1138-1146.

Hyman, L., Schachat, A. P., He, Q. and Leske, M. C. (2000). Hypertension, cardiovascular disease, and age-related macular degeneration. age-related macular degeneration risk factors study group. *Arch Ophthalmol* 118, 351-358.

Iellamo, F., Tesauro, M., Rizza, S., Aquilani, S., Cardillo, C., Iantorno, M., Turriziani, M. and Lauro, R. (2006). Concomitant impairment in endothelial function and neural cardiovascular regulation in offspring of type 2 diabetic subjects. *Hypertension* 48, 418-423.

Ikeda, T., Obayashi, H., Hasegawa, G., Nakamura, N., Yoshikawa, T., Imamura, Y., Koizumi, K. and Kinoshita, S. (2001). Paraoxonase gene polymorphisms and plasma oxidized low-density lipoprotein level as possible risk factors for exudative age-related macular degeneration. *Am. J. Ophthalmol.* 132, 191-195.

Ishizaka, H. and Kuo, L. (1997). Endothelial ATP-sensitive potassium channels mediate coronary microvascular dilation to hyperosmolarity. *Am. J. Physiol.* 273, H104-112.

Itabe, H., Obama, T. and Kato, R. (2011). The dynamics of oxidized LDL during atherogenesis. *J. Lipids* 2011, Epub 418313.

Jager, R. D., Mieler, W. F. and Miller, J. W. (2008). Age-related macular degeneration. *N Engl J Med* 358, 2606-2617.

James, S. J., Rose, S., Melnyk, S., Jernigan, S., Blossom, S., Pavliv, O. and Gaylor, D. W. (2009). Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. *FASEB J.* 23, 2374-2383.

Javadzadeh, A., Ghorbanihaghjo, A., Bahreini, E., Rashtchizadeh, N., Argani, H. and Alizadeh, S. (2010). Plasma oxidized LDL and thiol-containing molecules in patients with exudative age-related macular degeneration. *Mol. Vis.* 16, 2578-2584.

Jin, R. C. and Loscalzo, J. (2010). Vascular nitric oxide: Formation and function. *J. Blood Med.* 2010, 147-162.

Johnson, E. J., Chung, H. Y., Caldarella, S. M. and Snodderly, D. M. (2008). The influence of supplemental lutein and docosahexaenoic acid on serum, lipoproteins, and macular pigmentation. *Am. J. Clin. Nutr.* 87, 1521-1529.

Johnson, P. C. (1986). Autoregulation of blood flow. *Circ. Res.* 59, 483-495.

Jonasson, F., Arnarsson, A., Eiriksdottir, G., Harris, T. B., Launer, L. J., Meuer, S. M., Klein, B. E., Klein, R., Gudnason, V. and Cotch, M. F. (2011). Prevalence of age-related macular degeneration in old persons: Age, Gene/environment susceptibility reykjavik study. *Ophthalmology* 118, 825-830.

Jones, D. P. (2006a). Extracellular redox state: Refining the definition of oxidative stress in aging. *Rejuvenation Res.* 9, 169-181.

Jones, D. P. (2006b). Redefining oxidative stress. *Antioxid. Redox Signal.* 8, 1865-1879.

Jones, D. P., Carlson, J. L., Samiec, P. S., Sternberg, P.,Jr, Mody, V. C.,Jr, Reed, R. L. and Brown, L. A. (1998). Glutathione measurement in human plasma. evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clin. Chim. Acta* 275, 175-184.

Jones, D. P., Coates, R. J., Flagg, E. W., Eley, J. W., Block, G., Greenberg, R. S., Gunter, E. W. and Jackson, B. (1992). Glutathione in foods listed in the national cancer institute's health habits and history food frequency questionnaire. *Nutr Cancer* 17, 57-75.

Junqueira, V. B., Barros, S. B., Chan, S. S., Rodrigues, L., Giavarotti, L., Abud, R. L. and Deucher, G. P. (2004). Aging and oxidative stress. *Mol. Aspects Med.* 25, 5-16.

Kamata, K., Kanie, N., Matsumoto, T. and Kobayashi, T. (2004). Endothelin-1-induced impairment of endothelium-dependent relaxation in aortas isolated from controls and diabetic rats. *J. Cardiovasc. Pharmacol.* 44 Suppl 1, S186-190.

Kamoun, W. S., Karaa, A., Kresge, N., Merkel, S. M., Korneszczuk, K. and Clemens, M. G. (2006). LPS inhibits endothelin-1-induced endothelial NOS activation in hepatic sinusoidal cells through a negative feedback involving caveolin-1. *Hepatology* 43, 182-190.

Kanski, J. J. (2003).*Clinical Ophthalmology*. Oxford: Butterworth-Heinemann.

Kappers, M. H., van Esch, J. H., Sluiter, W., Sleijfer, S., Danser, A. H. and van den Meiracker, A. H. (2010). Hypertension induced by the tyrosine kinase inhibitor sunitinib is associated with increased circulating endothelin-1 levels. *Hypertension* 56, 675-681.

Karaa, A., Kamoun, W. S. and Clemens, M. G. (2005). Oxidative stress disrupts nitric oxide synthase activation in liver endothelial cells. *Free Radic. Biol. Med.* 39, 1320-1331.

Karavidas, A., Lazaros, G., Tsiachris, D. and Pyrgakis, V. (2010). Aging and the cardiovascular system. *Hellenic J. Cardiol.* 51, 421-427.

Kaur, H. and Halliwell, B. (1994). Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett* 350, 9-12.

Kawamoto, R., Oka, Y., Tomita, H. and Kodama, A. (2005). Non-HDL cholesterol as a predictor of carotid atherosclerosis in the elderly. *J. Atheroscler. Thromb.* 12, 143-148.

Kawasaki, Y., Fujikado, T., Hosohata, J., Tano, Y. and Tanaka, Y. (1999). The effect of nitric oxide on the contractile tone of muller cells. *Ophthalmic Res.* 31, 387-391.

Kedzierski, R. M. and Yanagisawa, M. (2001). Endothelin system: The double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* 41, 851-876.

Kelly, D. P. (2011). Cell biology: Ageing theories unified. *Nature* 470, 342-343.

Kerkhof, G. A., Van Dongen, H. P. and Bobbert, A. C. (1998). Absence of endogenous circadian rhythmicity in blood pressure? *Am. J. Hypertens.* 11, 373-377.

Khachik, F., de Moura, F. F., Zhao, D. Y., Aebischer, C. P. and Bernstein, P. S. (2002). Transformations of selected carotenoids in plasma, liver, and ocular tissues of humans and in nonprimate animal models. *Invest Ophthalmol Vis Sci* 43, 3383-3392.

Khandhadia, S. and Lotery, A. (2010). Oxidation and age-related macular degeneration: Insights from molecular biology. *Expert Rev. Mol. Med.* 12, e34.

Khot, U. N., Khot, M. B., Bajzer, C. T., Sapp, S. K., Ohman, E. M., Brener, S. J., Ellis, S. G., Lincoff, A. M. and Topol, E. J. (2003). Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA* 290, 898-904.

Kida, T., Liu, J. H. and Weinreb, R. N. (2008). Effect of aging on nocturnal blood flow in the optic nerve head and macula in healthy human eyes. *J. Glaucoma* 17, 366-371.

Kiel, J. W. (1999). Modulation of choroidal autoregulation in the rabbit. *Exp Eye Res* 69, 413-429.

Kiss, B., Dallinger, S., Findl, O., Rainer, G., Eichler, H. G. and Schmetterer, L. (1999). Acetazolamide-induced cerebral and ocular vasodilation in humans is independent of nitric oxide. *Am J Physiol* 276, R1661-1667.

Kitney, R. I. and Rompleman, O. (1980). *The Study of Heart-Rate Variability*. Oxford: Clarendon Press.

Klein, M. L., Mauldin, W. M. and Stoumbos, V. D. (1994). Heredity and age-related macular degeneration. observations in monozygotic twins. *Arch Ophthalmol* 112, 932-937.

Klein, R., Davis, M. D., Magli, Y. L., Segal, P., Klein, B. E. and Hubbard, L. (1991). The wisconsin age-related maculopathy grading system. *Ophthalmology* 98, 1128-1134.

Klein, R., Deng, Y., Klein, B. E., Hyman, L., Seddon, J., Frank, R. N., Wallace, R. B., Hendrix, S. L., Kuppermann, B. D., Langer, R. D. et al. (2007). Cardiovascular disease, its risk factors and treatment, and age-related macular degeneration: Women's health initiative sight exam ancillary study. *Am J Ophthalmol* 143, 473-483.

Klein, R., Klein, B. E. and Franke, T. (1993). The relationship of cardiovascular disease and its risk factors to age-related maculopathy. the beaver dam eye study. *Ophthalmology* 100, 406-414.

Klein, R., Klein, B. E., Knudtson, M. D., Cotch, M. F., Wong, T. Y., Liu, K., Burke, G. L., Saad, M. F., Jacobs, D. R.,Jr and Sharrett, A. R. (2007). Subclinical atherosclerotic cardiovascular disease and early age-related macular degeneration in a multiracial cohort: The multiethnic study of atherosclerosis. *Arch. Ophthalmol.* 125, 534-543.

Klein, R., Klein, B. E., Knudtson, M. D., Wong, T. Y., Cotch, M. F., Liu, K., Burke, G., Saad, M. F. and R., J. D.,Jr. (2006). Prevalence of age-related macular degeneration in 4 racial/ethnic groups in the multi-ethnic study of atherosclerosis. *Ophthalmology* 113, 373-380.

Klein, R., Klein, B. E. and Linton, K. L. (1992). Prevalence of age-related maculopathy. the beaver dam eye study. *Ophthalmology* 99, 933-943.

Klein, R., Klein, B. E., Marino, E. K., Kuller, L. H., Furberg, C., Burke, G. L. and Hubbard, L. D. (2003). Early age-related maculopathy in the cardiovascular health study. *Ophthalmology* 110, 25-33.

Klein, R., Peto, T., Bird, A. and Vannewkirk, M. R. (2004). The epidemiology of age-related macular degeneration. *Am J Ophthalmol* 137, 486-495.

Knudtson, M. D., Klein, R. and Klein, B. E. (2007). Alcohol consumption and the 15-year cumulative incidence of age-related macular degeneration. *Am J Ophthalmol* 143, 1026-1029.

Kogure, K., Scheinberg, P., Reinmuth, O. M., Fujishima, M. and Busto, R. (1970). Mechanisms of cerebral vasodilatation in hypoxia. *J. Appl. Physiol.* 29, 223-229.

Kontos, H. A., Wei, E. P., Raper, A. J., Rosenblum, W. I., Navari, R. M. and L., P. J.,Jr. (1978). Role of tissue hypoxia in local regulation of cerebral microcirculation. *Am J Physiol* 234, H582-591.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H. and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* 5, 834-842.

Koss, M. C. (1999). Functional role of nitric oxide in regulation of ocular blood flow. *Eur J Pharmacol* 374, 161-174.

Kotliar, K. E., Vilser, W., Nagel, E. and Lanzl, I. M. (2004). Retinal vessel reaction in response to chromatic flickering light. *Graefes Arch. Clin. Exp. Ophthalmol.* 242, 377-392.

Krinsky, N. I. (2002). Possible biologic mechanisms for a protective role of xanthophylls. *J Nutr* 132, 540S-542S.

Krinsky and NI. (1979). Carotenoid protection against oxidation. *Pure & Appi. Chem* 51, 649-660.

Kruk, P. A., Rampino, N. J. and Bohr, V. A. (1995). DNA damage and repair in telomeres: Relation to aging. *Proc. Natl. Acad. Sci. U. S. A.* 92, 258-262.

Kuklinska, A. M., Mroczko, B., Musial, W. J., Sawicki, R., Kaminski, K., Usowicz-Szarynska, M. and Szmitkowski, M. (2010). Endothelial dysfunction and sympathetic nervous system activation in young patients with essential arterial hypertension and without hypercholesterolaemia. *Acta Cardiol.* 65, 535-540.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E. and Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J. Clin. Invest.* 111, 1201-1209.

Landrum, J. T., Bone, R. A., Joa, H., Kilburn, M. D., Moore, L. L. and Sprague, K. E. (1997). A one year study of the macular pigment: The effect of 140 days of a lutein supplement. *Exp. Eye Res.* 65, 57-62.

Lanfranchi, P. A. and Somers, V. K. (2002). Arterial baroreflex function and cardiovascular variability: Interactions and implications. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283, R815-826.

Lanzl, I. M., Seidova, S. F., Maier, M., Lohmann, C., Schmidt-Trucksass, A., Halle, M. and Kotliar, K. E. (2011). Dynamic retinal vessel response to flicker in age-related macular degeneration patients before and after vascular endothelial growth factor inhibitor injection. *Acta Ophthalmol.* 89, 472-479.

Laties, A. M. (1967). Central retinal artery innervation. absence of adrenergic innervation to the intraocular branches. *Arch Ophthalmol* 77, 405-409.

Lecrux, C. and Hamel, E. (2011). The neurovascular unit in brain function and disease. *Acta Physiol. (Oxf)* 203, 47-59.

Lee, H. Y. and Oh, B. H. (2010). Aging and arterial stiffness. *Circ. J.* 74, 2257-2262.

Lee, S., Park, Y., Zuidema, M. Y., Hannink, M. and Zhang, C. (2011). Effects of interventions on oxidative stress and inflammation of cardiovascular diseases. *World J. Cardiol.* 3, 18-24.

Leite-Moreira, A. F. and Bras-Silva, C. (2004). Inotropic effects of ETB receptor stimulation and their modulation by endocardial endothelium, NO, and prostaglandins. *Am. J. Physiol. Heart Circ. Physiol.* 287, H1194-1199.

Lensch, E. and Jost, W. H. (2011). Autonomic disorders in multiple sclerosis. *Autoimmune Dis.* 2011, Epub 803841.

Lerman, A., Edwards, B. S., Hallett, J. W., Heublein, D. M., Sandberg, S. M. and C., B. J.,Jr. (1991). Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med* 325, 997-1001.

Lerman, A. and Zeiher, A. M. (2005). Endothelial function: Cardiac events. *Circulation* 111, 363-368.

Leske, M. C., Wu, S. Y., Hennis, A., Honkanen, R., Nemesure, B. and BESs Study Group. (2008). Risk factors for incident open-angle glaucoma: The barbados eye studies. *Ophthalmology* 115, 85-93.

Leung, I. Y. (2008). Macular pigment: New clinical methods of detection and the role of carotenoids in age-related macular degeneration. *Optometry* 79, 266-272.

Levick, J. R. (1995). Specialization in individual circulations. In *An Introduction to Cardiovascular Physiology*, pp. 231-254. Oxford.: Butterworth Heinemann,.

Levick, J. R. (2000). Overview of the cardiovascular system. In *An Introduction to Cardiovascular Physiology* (ed. J. R. Levick), pp. 1-14. London: Arnold.

Li, J., Zhao, S. P., Li, X. P., Zhuo, Q. C., Gao, M. and Lu, S. K. (1997). Non-invasive detection of endothelial dysfunction in patients with essential hypertension. *Int. J. Cardiol.* 61, 165-169.

Liang, F. Q. and Godley, B. F. (2003). Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: A possible mechanism for RPE aging and age-related macular degeneration. *Exp Eye Res* 76, 397-403.

Liao, D., Mo, J., Duan, Y., Klein, R., Scott, I. U., Huang, K. A. and Zhou, H. (2008). Is age-related macular degeneration associated with stroke among elderly americans? *Open Ophthalmol. J.* 2, 37-42.

Lilly, L. S. (2003). The electrocardiogram. In *Pathophysiology of Heart Disease* (ed. L. S. LILLY), pp. 75-110. Philadelphia: Lippincott Williams & Wilkins.

Lim, B. P., Nagao, A., Terao, J., Tanaka, K., Suzuki, T. and Takama, K. (1992). Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. *Biochim Biophys Acta* 1126, 178-184.

Lip, P. L., Blann, A. D., Hope-Ross, M., Gibson, J. M. and Lip, G. Y. (2001). Age-related macular degeneration is associated with increased vascular endothelial growth factor, hemorheology and endothelial dysfunction. *Ophthalmology* 108, 705-710.

Loomis, E. D., Sullivan, J. C., Osmond, D. A., Pollock, D. M. and Pollock, J. S. (2005). Endothelin mediates superoxide production and vasoconstriction through activation of NADPH oxidase and uncoupled nitric-oxide synthase in the rat aorta. *J. Pharmacol. Exp. Ther.* 315, 1058-1064.

Loot, A. E., Schreiber, J. G., Fisslthaler, B. and Fleming, I. (2009). Angiotensin II impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase. *J. Exp. Med.* 206, 2889-2896.

Lusis, A. J. (2000). Atherosclerosis. *Nature* 407, 233-241.

MacCumber, M. W. and D'Anna, S. A. (1994). Endothelin receptor-binding subtypes in the human retina and choroid. *Arch Ophthalmol* 112, 1231-1235.

Madamanchi, N. R., Vendrov, A. and Runge, M. S. (2005). Oxidative stress and vascular disease. *Arterioscler. Thromb. Vasc. Biol.* 25, 29-38.

Maeda, S., Tanabe, T., Miyauchi, T., Otsuki, T., Sugawara, J., Iemitsu, M., Kuno, S., Ajisaka, R., Yamaguchi, I. and Matsuda, M. (2003). Aerobic exercise training reduces plasma endothelin-1 concentration in older women. *J. Appl. Physiol.* 95, 336-341.

Malliani, A., Pagani, M., Lombardi, F. and Cerutti, S. (1991). Cardiovascular neural regulation explored in the frequency domain. *Circulation* 84, 482-492.

Malorni, W., Rivabene, R., Santini, M. T. and Donelli, G. (1993). N-acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. *FEBS Lett.* 327, 75-78.

Mandecka, A., Dawczynski, J., Blum, M., Muller, N., Kloos, C., Wolf, G., Vilser, W., Hoyer, H. and Muller, U. A. (2007). Influence of flickering light on the retinal vessels in diabetic patients. *Diabetes Care* 30, 3048-3052.

Mann, S., Craig, M. W., Melville, D. I., Balasubramanian, V. and Raftery, E. B. (1979). Physical activity and the circadian rhythm of blood pressure. *Clin. Sci. (Lond)* 57 Suppl 5, 291s-294s.

Mannermaa, E., Vellonen, K. S. and Urtti, A. (2006). Drug transport in corneal epithelium and blood-retina barrier: Emerging role of transporters in ocular pharmacokinetics. *Adv. Drug Deliv. Rev.* 58, 1136-1163.

Mastrodimou, N., Kiagiadaki, F. and Thermos, K. (2008). The role of nitric oxide and cGMP in somatostatin's protection against retinal ischemia. *Invest. Ophthalmol. Vis. Sci.* 49, 342-349.

Matsui, Y., Kario, K., Ishikawa, J., Eguchi, K., Hoshide, S. and Shimada, K. (2004). Reproducibility of arterial stiffness indices (pulse wave velocity and augmentation index) simultaneously assessed by automated pulse wave analysis and their associated risk factors in essential hypertensive patients. *Hypertens Res* 27, 851-857.

Matsumoto, T., Kobayashi, T. and Kamata, K. (2008). Relationships among ET-1, PPARgamma, oxidative stress and endothelial dysfunction in diabetic animals. *J. Smooth Muscle Res.* 44, 41-55.

McCarty, C. A., Dowrick, A., Cameron, J., McGrath, B., Robman, L. D., Dimitrov, P., Tikellis, G., Nicolas, C., McNeil, J. and Guymer, R. (2008). Novel measures of cardiovascular health and its association with prevalence and progression of age-related macular degeneration: The CHARM study. *BMC Ophthalmol.* 8, 25.

McGrath, B. P. and National Blood Pressure Advisory Committee of the National Heart Foundation of Australia. (2002). Ambulatory blood pressure monitoring. *Med. J. Aust.* 176, 588-592.

Metea, M. R. and Newman, E. A. (2006). Glial cells dilate and constrict blood vessels: A mechanism of neurovascular coupling. *J. Neurosci.* 26, 2862-2870.

Michelet, F., Gueguen, R., Leroy, P., Wellman, M., Nicolas, A. and Siest, G. (1995). Blood and plasma glutathione measured in healthy subjects by HPLC: Relation to sex, aging, biological variables, and life habits. *Clin. Chem.* 41, 1509-1517.

Michiels, C. (2003). Endothelial cell functions. *J Cell Physiol* 196, 430-443.

Millar-Craig, M. W., Bishop, C. N. and Raftery, E. B. (1978). Circadian variation of blood-pressure. *Lancet* 1, 795-797.

Minshall, R. D., Sessa, W. C., Stan, R. V., Anderson, R. G. and Malik, A. B. (2003). Caveolin regulation of endothelial function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285, L1179-1183.

Mitchell, G. F., Vita, J. A., Larson, M. G., Parise, H., Keyes, M. J., Warner, E., Vasan, R. S., Levy, D. and Benjamin, E. J. (2005). Cross-sectional relations of peripheral microvascular function, cardiovascular disease risk factors, and aortic stiffness: The framingham heart study. *Circulation* 112, 3722-3728.

Miyauchi, T. and Masaki, T. (1999). Pathophysiology of endothelin in the cardiovascular system. *Annu. Rev. Physiol.* 61, 391-415.

Monahan, K. D. (2007). Effect of aging on baroreflex function in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 293, R3-R12.

Morange, P. E., Simon, C., Alessi, M. C., Luc, G., Arveiler, D., Ferrieres, J., Amouyel, P., Evans, A., Ducimetiere, P., Juhan-Vague, I. et al. (2004). Endothelial cell markers and the risk of coronary heart disease: The prospective epidemiological study of myocardial infarction (PRIME) study. *Circulation* 109, 1343-1348.

Mori, A., Suzuki, S., Sakamoto, K., Nakahara, T. and Ishii, K. (2011). Role of calcium-activated potassium channels in acetylcholine-induced vasodilation of rat retinal arterioles in vivo. *Naunyn Schmiedebergs Arch. Pharmacol.* 383, 27-34.

Mroczkowska, S., Ekart, A., Sung, V., Negi, A., Qin, L., Patel, S. R., Jacob, S., Atkins, C., Benavente-Perez, A. and Gherghel, D. (2011). Reinforcing the paradigm 'A sick eye in a sick body': Macro and micro-vascular abnormalities in newly diagnosed normal tension glaucoma patients. *Br J Ophthalmol.*, Submitted.

Mullner-Eidenbock, A., Rainer, G., Strenn, K. and Zidek, T. (2000). High-altitude retinopathy and retinal vascular dysregulation. *Eye (Lond)* 14 Pt 5, 724-729.

Mulsch, A., Vanin, A., Mordvintcev, P., Hauschildt, S. and Busse, R. (1992). NO accounts completely for the oxygenated nitrogen species generated by enzymic L-arginine oxygenation. *Biochem. J.* 288 (Pt 2), 597-603.

Nagel, E. and Vilser, W. (2004). Flicker observation light induces diameter response in retinal arterioles: A clinical methodological study. *Br J Ophthalmol* 88, 54-56.

Nagel, E., Vilser, W. and Lanzl, I. (2004). Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. *Invest. Ophthalmol. Vis. Sci.* 45, 1486-1492.

Nagineni, C. N., Kommineni, V. K., William, A., Detrick, B. and Hooks, J. J. (2012). Regulation of VEGF expression in human retinal cells by cytokines: Implications for the role of inflammation in age-related macular degeneration. *J. Cell. Physiol.* 227, 116-126.

Nanetti, L., Raffaelli, F., Vignini, A., Perozzi, C., Silvestrini, M., Bartolini, M., Provinciali, L. and Mazzanti, L. (2011). Oxidative stress in ischaemic stroke. *Eur. J. Clin. Invest.* 41, 1318-1322.

Narayanan, N., Leffler, C. W. and Daley, M. L. (2008). Influence of hypercapnic vasodilation on cerebrovascular autoregulation and pial arteriolar bed resistance in piglets. *J. Appl. Physiol.* 105, 152-157.

Nedeljkovic, Z. S., Gokce, N. and Loscalzo, J. (2003). Mechanisms of oxidative stress and vascular dysfunction. *Postgrad Med J* 79, 195-199; quiz 198-200.

Nguyen, T. T., Kreis, A. J., Kawasaki, R., Wang, J. J., Seifert, B. U., Vilser, W., Nagel, E. and Wong, T. Y. (2009). Reproducibility of the retinal vascular response to flicker light in asians. *Curr. Eye Res.* 34, 1082-1088.

Nilsson, S. F. and Bill, A. (1984). Vasoactive intestinal polypeptide (VIP): Effects in the eye and on regional blood flows. *Acta Physiol. Scand.* 121, 385-392.

Nolan, J., Flapan, A. D., Capewell, S., MacDonald, T. M., Neilson, J. M. and Ewing, D. J. (1992). Decreased cardiac parasympathetic activity in chronic heart failure and its relation to left ventricular function. *Br. Heart J.* 67, 482-485.

Nowak, M., Swietochowska, E., Marek, B., Szapska, B., Wielkoszynski, T., Kos-Kudla, B., Karpe, J., Kajdaniuk, D., Sieminska, L., Glogowska-Szelag, J. et al. (2005). Changes in lipid metabolism in women with age-related macular degeneration. *Clin. Exp. Med.* 4, 183-187.

O'Brien, E., Petrie, J., Littler, W., de Swiet, M., Padfield, P. L., Altmanu, D. G., BlandG, M., Coats, A. and Atkins, N. (1993). The british hypertension society protocol for the evaluation of blood pressure measuring device. 11, S43-S62.

O'Brien, E. (2003). Ambulatory blood pressure measurement is indispensable to good clinical practice. *J. Hypertens. Suppl.* 21, S11-8.

O'Brien, I. A., McFadden, J. P. and Corrall, R. J. (1991). The influence of autonomic neuropathy on mortality in insulin-dependent diabetes. *Q. J. Med.* 79, 495-502.

Ogita, H. and Liao, J. (2004). Endothelial function and oxidative stress. *Endothelium* 11, 123-132.

Oida, E., Kannagi, T., Moritani, T. and Yamori, Y. (1999). Aging alteration of cardiac vagosympathetic balance assessed through the tone-entropy analysis. *J. Gerontol. A Biol. Sci. Med. Sci.* 54, M219-224.

Oliver, J. J. and Webb, D. J. (2003). Noninvasive assessment of arterial stiffness and risk of atherosclerotic events. *Arterioscler Thromb Vasc Biol* 23, 554-566.

Organisciak, D. T., Darrow, R. M., Barsalou, L., Darrow, R. A., Kutty, R. K., Kutty, G. and Wiggert, B. (1998). Light history and age-related changes in retinal light damage. *Invest Ophthalmol Vis Sci* 39, 1107-1116.

Orgul, S., Gugleta, K. and Flammer, J. (1999). Physiology of perfusion as it relates to the optic nerve head. *Surv Ophthalmol* 43 Suppl 1, S17-26.

O'Rourke, M. F., Pauca, A. and Jiang, X. J. (2001). Pulse wave analysis. *Br. J. Clin. Pharmacol.* 51, 507-522.

O'Rourke, M. F. and Safar, M. E. (2005). Relationship between aortic stiffening and microvascular disease in brain and kidney: Cause and logic of therapy. *Hypertension* 46, 200-204.

Osborne, N. N., Ugarte, M., Chao, M., Chidlow, G., Bae, J. H., Wood, J. P. and Nash, M. S. (1999). Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv. Ophthalmol.* 43 Suppl 1, S102-S128.

O'Shea, J. C. and Murphy, M. B. (2000). Nocturnal blood pressure dipping: A consequence of diurnal physical activity blipping? *Am. J. Hypertens.* 13, 601-606.

O'Shea, J. G. (1998). Age-related macular degeneration. *Postgrad Med J* 74, 203-207.

Ou, H. C., Song, T. Y., Yeh, Y. C., Huang, C. Y., Yang, S. F., Chiu, T. H., Tsai, K. L., Chen, K. L., Wu, Y. J., Tsai, C. S. et al. (2010). EGCG protects against oxidized LDL-induced endothelial dysfunction by inhibiting LOX-1-mediated signaling. *J. Appl. Physiol.* 108, 1745-1756.

Pagani, M., Lombardi, F., Guzzetti, S., Rimoldi, O., Furlan, R., Pizzinelli, P., Sandrone, G., Malfatto, G., Dell'Orto, S. and Piccaluga, E. (1986). Power spectral analysis of heart rate and arterial pressure variabilities as a marker of sympatho-vagal interaction in man and conscious dog. *Circ Res* 59, 178-193.

Pagani, M. and Malliani, A. (2000). Interpreting oscillations of muscle sympathetic nerve activity and heart rate variability. *J. Hypertens.* 18, 1709-1719.

Palmer, R. M., Ferrige, A. G. and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-526.

Parmacek, M. S. and Epstein, J. A. (2009). Cardiomyocyte renewal. *N. Engl. J. Med.* 361, 86-88.

Patel, S. R., Bellary, S., Qin, L., Gill, P., Taheri, S., Heitmar, R., Gibson, J. M. and Gherghel, D. (2011). Abnormal retinal vascular function and lipid levels in a sample of healthy UK south asians. *Br. J. Ophthalmol.* 95, 1573-1576.

Pease, P. L., Adams, A. J. and Nuccio, E. (1987). Optical density of human macular pigment. *Vision Res* 27, 705-710.

Pernow, J. and Wang, Q. D. (1997). Endothelin in myocardial ischaemia and reperfusion. *Cardiovasc Res* 33, 518-526.

Peverill, R. E., Gelman, J. S., Mottram, P. M., Moir, S., Jankelowitz, C., Bain, J. L. and Donelan, L. (2004). Factors associated with mitral annular systolic and diastolic velocities in healthy adults. *J. Am. Soc. Echocardiogr.* 17, 1146-1154.

Pickering, T. G., Gerin, W. and Schwartz, A. R. (2002). What is the white-coat effect and how should it be measured? *Blood Press. Monit.* 7, 293-300.

Piechota, A., Polanczyk, A. and Goraca, A. (2010). Role of endothelin-1 receptor blockers on hemodynamic parameters and oxidative stress. *Pharmacol. Rep.* 62, 28-34.

Pigozzi, F., Rizzo, M., Fagnani, F., Parisi, A., Spataro, A., Casasco, M. and Borrione, P. (2011). Endothelial (dys)function: The target of physical exercise for prevention and treatment of cardiovascular disease. *J. Sports Med. Phys. Fitness* 51, 260-267.

Polak, K., Schmetterer, L. and Riva, C. E. (2002). Influence of flicker frequency on flicker-induced changes of retinal vessel diameter. *Invest Ophthalmol Vis Sci* 43, 2721-2726.

Pompella, A., Visvikis, A., Paolicchi, A., De Tata, V. and Casini, A. F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* 66, 1499-1503.

Poredos, P. (2004). Intima-media thickness: Indicator of cardiovascular risk and measure of the extent of atherosclerosis. *Vasc. Med.* 9, 46-54.

Porges, S. W. (2007). The polyvagal perspective. *Biol. Psychol.* 74, 116-143.

Pugsley, M. K. and Tabrizchi, R. (2000). The vascular system. an overview of structure and function. *J. Pharmacol. Toxicol. Methods* 44, 333-340.

Pumprla, J., Howorka, K., Groves, D., Chester, M. and Nolan, J. (2002). Functional assessment of heart rate variability: Physiological basis and practical applications. *Int J Cardiol* 84, 1-14.

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A., McNamara, J. O. and and Williams, S. K. (2001). Neuroscience.

Qin, L., Bartlett, H., Griffiths, H. R., Eperjesi, F., Armstrong, R. A. and Gherghel, D. (2011a). Macular pigment optical density is related to blood glutathione levels in healthy individuals. *Invest. Ophthalmol. Vis. Sci.* 52, 5029-5033.

Qin, L., Mroczkowska, S. A., Ekart, A., Patel, S. R., Gibson, J. M., and Gherghel, D. (2011b). Are patients with early age-related macular degeneration at high risk for systemic vascular disease? A pilot investigation. *Invest Ophthalmol Vis Sci.*, Submitted.

Qrgul, S., Meyer, P. and Ciofft, G. A. (1995). Physiology of blood flow regulation and mechanisms involved in optic nerve perfusion. *J. Glaucoma* 4, 427-443.

Ramrattan, R. S., van der Schaft, T. L., Mooy, C. M., de Bruijn, W. C., Mulder, P. G. and de Jong, P. T. (1994). Morphometric analysis of bruch's membrane, the choriocapillaris, and the choroid in aging. *Invest. Ophthalmol. Vis. Sci.* 35, 2857-2864.

Rapenne, T., Moreau, D., Lenfant, F., Boggio, V., Cottin, Y. and Freysz, M. (2000). Could heart rate variability analysis become an early predictor of imminent brain death? A pilot study. *Anesth. Analg.* 91, 329-336.

Rapp, L. M., Maple, S. S. and Choi, J. H. (2000). Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina. *Invest Ophthalmol Vis Sci* 41, 1200-1209.

Ravalico, G., Toffoli, G., Pastori, G., Croce, M. and Calderini, S. (1996). Age-related ocular blood flow changes. *Invest. Ophthalmol. Vis. Sci.* 37, 2645-2650.

Reading, V. M. and Weale, R. A. (1974). Macular pigment and chromatic aberration. *J Opt Soc Am* 64, 231-234.

Rebrin, I. and Sohal, R. S. (2008). Pro-oxidant shift in glutathione redox state during aging. *Adv. Drug Deliv. Rev.* 60, 1545-1552.

Reckelhoff, J. F. (2001). Gender differences in the regulation of blood pressure. *Hypertension* 37, 1199-1208.

Reis, D. J. (1960). Potentiation of the vasoconstrictor action of topical norepinephrine on the human bulbar conjuctival vessels after topical application of certain adrenocortical hormones. *J. Clin. Endocrinol. Metab.* 20, 446-456.

Reiter, R. J., Tan, D. X., Paredes, S. D. and Fuentes-Broto, L. (2010). Beneficial effects of melatonin in cardiovascular disease. *Ann Med* 42, 276-85.

Resch, H., Garhofer, G., Fuchsjager-Mayrl, G., Hommer, A. and Schmetterer, L. (2009). Endothelial dysfunction in glaucoma. *Acta Ophthalmol.* 87, 4-12.

Resink, T. J., Hahn, A. W., Scott-Burden, T., Powell, J., Weber, E. and Buhler, F. R. (1990). Inducible endothelin mRNA expression and peptide secretion in cultured human vascular smooth muscle cells. *Biochem Biophys Res Commun* 168, 1303-1310.

Richer, S., Stiles, W., Statkute, L., Pulido, J., Frankowski, J., Rudy, D., Pei, K., Tsipursky, M. and Nyland, J. (2004). Double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age-related macular degeneration: The veterans LAST study (lutein antioxidant supplementation trial). *Optometry* 75, 216-230.

Riva, C. E., Cranstoun, S. D., Mann, R. M. and Barnes, G. E. (1994). Local choroidal blood flow in the cat by laser doppler flowmetry. *Invest. Ophthalmol. Vis. Sci.* 35, 608-618.

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

Rizzo, J. F.,3rd, Feke, G. T., Goger, D. G., Ogasawara, H. and Weiter, J. J. (1991). Optic nerve head blood speed as a function of age in normal human subjects. *Invest. Ophthalmol. Vis. Sci.* 32, 3263-3272.

Roff, E. J., Harris, A., Chung, H. S., Hosking, S. L., Morrison, A. M., Halter, P. J. and Kagemann, L. (1999). Comprehensive assessment of retinal, choroidal and retrobulbar haemodynamics during blood gas perturbation. *Graefes Arch. Clin. Exp. Ophthalmol.* 237, 984-990.

Rosendorff, C. (2002). Effects of LDL cholesterol on vascular function. *J. Hum. Hypertens.* 16 Suppl 1, S26-S28.

Rossi, R., Milzani, A., Dalle-Donne, I., Giustarini, D., Lusini, L., Colombo, R. and Di Simplicio, P. (2002). Blood glutathione disulfide: In vivo factor or in vitro artifact? *Clin. Chem.* 48, 742-753.

Rubanyi, G. M. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *J. Cardiovasc. Pharmacol.* 22 Suppl 4, S1-14.

Rubanyi, G. M. and Botelho, L. H. (1991). Endothelins. *Faseb J* 5, 2713-20.

Sagaties, M. J., Raviola, G., Schaeffer, S. and Miller, C. (1987). The structural basis of the inner blood-retina barrier in the eye of macaca mulatta. *Invest. Ophthalmol. Vis. Sci.* 28, 2000-2014.

Samiec, P. S., Drews-Botsch, C., Flagg, E. W., Kurtz, J. C., Sternberg, P.,Jr, Reed, R. L. and Jones, D. P. (1998). Glutathione in human plasma: Decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic. Biol. Med.* 24, 699-704.

Sarks, S., Cherepanoff, S., Killingsworth, M. and Sarks, J. (2007). Relationship of basal laminar deposit and membranous debris to the clinical presentation of early age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* 48, 968-977.

Sarks, S. H., Arnold, J. J., Killingsworth, M. C. and Sarks, J. P. (1999). Early drusen formation in the normal and aging eye and their relation to age related maculopathy: A clinicopathological study. *Br. J. Ophthalmol.* 83, 358-368.

Sasamoto, Y., Gomi, F., Sawa, M., Tsujikawa, M. and Nishida, K. (2011). Effect of 1-year lutein supplementation on macular pigment optical density and visual function. *Graefes Arch. Clin. Exp. Ophthalmol.* 249, 1847-1854.

Sato, E., Feke, G. T., Appelbaum, E. Y., Menke, M. N., Trempe, C. L. and McMeel, J. W. (2006a). Association between systemic arterial stiffness and age-related macular degeneration. *Graefes Arch. Clin. Exp. Ophthalmol.* 244, 963-971.

Sato, E., Feke, G. T., Menke, M. N. and Wallace McMeel, J. (2006b). Retinal haemodynamics in patients with age-related macular degeneration. *Eye (Lond)* 20, 697-702.

Sawabe, M. (2010). Vascular aging: From molecular mechanism to clinical significance. *Geriatr. Gerontol. Int.* 10 Suppl 1, S213-220.

Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T. et al. (1997). An endothelial receptor for oxidized low-density lipoprotein. *Nature* 386, 73-77.

Schmetterer, L. and Polak, K. (2001). Role of nitric oxide in the control of ocular blood flow. *Prog Retin Eye Res* 20, 823-847.

Schram, M. T. and Stehouwer, C. D. (2005). Endothelial dysfunction, cellular adhesion molecules and the metabolic syndrome. *Horm. Metab. Res.* 37 Suppl 1, 49-55.

Schulz, E., Gori, T. and Munzel, T. (2011). Oxidative stress and endothelial dysfunction in hypertension. *Hypertens. Res.* 34, 665-673.

Schulz, J. B., Lindenau, J., Seyfried, J. and Dichgans, J. (2000). Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem* 267, 4904-4911.

Seddon, J. M., Ajani, U. A., Sperduto, R. D., Hiller, R., Blair, N., Burton, T. C., Farber, M. D., Gragoudas, E. S., Haller, J. and Miller, D. T. (1994). Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. eye disease case-control study group. *Jama* 272, 1413-1420.

Seddon, J. M., Cote, J., Davis, N. and Rosner, B. (2003a). Progression of age-related macular degeneration: Association with body mass index, waist circumference, and waist-hip ratio. *Arch Ophthalmol* 121, 785-792.

Seddon, J. M., Cote, J. and Rosner, B. (2003b). Progression of age-related macular degeneration: Association with dietary fat, transunsaturated fat, nuts, and fish intake. *Arch Ophthalmol* 121, 1728-1737.

Seddon, M. D., Chowienczyk, P. J., Brett, S. E., Casadei, B. and Shah, A. M. (2008). Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo. *Circulation* 117, 1991-1996.

Shapiro, D. and Goldstein, I. B. (1998). Wrist actigraph measures of physical activity level and ambulatory blood pressure in healthy elderly persons. *Psychophysiology* 35, 305-312.

Shaw, J. A., Chin-Dusting, J. P., Kingwell, B. A. and Dart, A. M. (2001). Diurnal variation in endothelium-dependent vasodilatation is not apparent in coronary artery disease. *Circulation* 103, 806-812.

Shechter, M., Marai, I., Marai, S., Sherer, Y., Sela, B. A., Feinberg, M. S., Rubinstein, A. and Shoenfeld, Y. (2007). The association of endothelial dysfunction and cardiovascular events in healthy subjects and patients with cardiovascular disease. *Isr. Med. Assoc. J.* 9, 271-276.

Shirwany, N. A. and Zou, M. H. (2010). Arterial stiffness: A brief review. *Acta Pharmacol. Sin.* 31, 1267-1276.

Simo, R., Villarroel, M., Corraliza, L., Hernandez, C. and Garcia-Ramirez, M. (2010). The retinal pigment epithelium: Something more than a constituent of the blood-retinal barrier--implications for the pathogenesis of diabetic retinopathy. *J. Biomed. Biotechnol.* 2010, 190724.

Simon, A., Gariepy, J., Chironi, G., Megnien, J. L. and Levenson, J. (2002). Intima-media thickness: A new tool for diagnosis and treatment of cardiovascular risk. *J. Hypertens.* 20, 159-169.

Sims, D. E. (1986). The pericyte--a review. *Tissue Cell* 18, 153-174.

Singh, J. P., Larson, M. G., Tsuji, H., Evans, J. C., O'Donnell, C. J. and Levy, D. (1998). Reduced heart rate variability and new-onset hypertension: Insights into pathogenesis of hypertension: The framingham heart study. *Hypertension* 32, 293-297.

Skene, D. J. and Arendt, J. (2006). Human circadian rhythms: Physiological and therapeutic relevance of light and melatonin. *Ann Clin Biochem* 43, 344-353.

Skinner, S., Barin, E., Gallery, E., Beattie, J. and Kamen, P. (2011).
Pulse wave analysis. In *SphygmoCor Manual: A Clinical Guide*. Australia.

Smith, B. W., Strakova, J., King, J. L., Erdman, J. W. and O'Brien, W. D. (2010). Validated sandwich ELISA for the quantification of von willebrand factor in rabbit plasma. *Biomark Insights* 5, 119-127.

Smith, W., Mitchell, P., Webb, K. and Leeder, S. R. (1999). Dietary antioxidants and age-related maculopathy: The blue mountains eye study. *Ophthalmology* 106, 761-767.

Smolensky, M. H. and Haus, E. (2001). Circadian rhythms and clinical medicine with applications to hypertension. *Am. J. Hypertens.* 14, 280S-290S.

Snell, R. S. and Lemp, M. A. (1998). The orbital blood vessels. In *Clinical Anatomy of the Eye* (ed. R. S. Snell and M. A. Lemp), pp. 277-277-293. Oxford: Blackwell Science.

Somers, V. K., Dyken, M. E., Mark, A. L. and Abboud, F. M. (1993). Sympathetic-nerve activity during sleep in normal subjects. *N. Engl. J. Med.* 328, 303-307.

Stadtman, E. R. (2006). Protein oxidation and aging. *Free Radic. Res.* 40, 1250-1258.

Staels, B. (2002). Cardiovascular biology: A cholesterol tether. *Nature* 417, 699-701.

Stauffer, B. L., Westby, C. M. and DeSouza, C. A. (2008). Endothelin-1, aging and hypertension. *Curr Opin Cardiol* 23, 350-355.

Sternberg, P.,Jr, Davidson, P. C., Jones, D. P., Hagen, T. M., Reed, R. L. and Drews-Botsch, C. (1993). Protection of retinal pigment epithelium from oxidative injury by glutathione and precursors. *Invest. Ophthalmol. Vis. Sci.* 34, 3661-3668.

Stiell, I. G., Hebert, P. C., Weitzman, B. N., Wells, G. A., Raman, S., Stark, R. M., Higginson, L. A., Ahuja, J. and Dickinson, G. E. (1992). High-dose epinephrine in adult cardiac arrest. *N Engl J Med* 327, 1045-1050.

Strauss, O. (2005). The retinal pigment epithelium in visual function. *Physiol. Rev.* 85, 845-881.

Su, W. W., Cheng, S. T., Ho, W. J., Tsay, P. K., Wu, S. C. and Chang, S. H. (2008). Glaucoma is associated with peripheral vascular endothelial dysfunction. *Ophthalmology* 115, 1173-1178.e1.

Sullivan, S. M. and Johnson, P. C. (1981). Effect of oxygen on blood flow autoregulation in cat sartorius muscle. *Am J Physiol* 241, H807-815.

Sun, C., Wang, J. J., Mackey, D. A. and Wong, T. Y. (2009). Retinal vascular caliber: Systemic, environmental, and genetic associations. *Surv. Ophthalmol.* 54, 74-95.

Sun, K., Cai, H., Tezel, T. H., Paik, D., Gaillard, E. R. and Del Priore, L. V. (2007). Bruch's membrane aging decreases phagocytosis of outer segments by retinal pigment epithelium. *Mol. Vis.* 13, 2310-2319.

Sundelin, S. P. and Nilsson, S. E. (2001). Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants. *Free Radic Biol Med* 31, 217-225.

Suzuki, T., Kumazaki, T. and Mitsui, Y. (1993). Endothelin-1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochem Biophys Res Commun* 191, 823-830.

Sykes, M. C., Mowbray, A. L. and Jo, H. (2007). Reversible glutathiolation of caspase-3 by glutaredoxin as a novel redox signaling mechanism in tumor necrosis factor-alpha-induced cell death. *Circ. Res.* 100, 152-154.

Szabo, C., Pacher, P., Zsengeller, Z., Vaslin, A., Komjati, K., Benko, R., Chen, M., Mabley, J. G. and Kollai, M. (2004). Angiotensin II-mediated endothelial dysfunction: Role of poly(ADP-ribose) polymerase activation. *Mol. Med.* 10, 28-35.

Takagi, C., King, G. L., Takagi, H., Lin, Y. W., Clermont, A. C. and Bursell, S. E. (1996). Endothelin-1 action via endothelin receptors is a primary mechanism modulating retinal circulatory response to hyperoxia. *Invest. Ophthalmol. Vis. Sci.* 37, 2099-2109.

Takakuwa, H., Ise, T., Kato, T., Izumiya, Y., Shimizu, K., Yokoyama, H. and Kobayashi, K. I. (2001). Diurnal variation of hemodynamic indices in non-dipper hypertensive patients. *Hypertens. Res.* 24, 195-201.

Tamakoshi, A., Yuzawa, M., Matsui, M., Uyama, M., Fujiwara, N. K. and Ohno, Y. (1997). Smoking and neovascular form of age related macular degeneration in late middle aged males: Findings from a case-control study in japan. research committee on chorioretinal degenerations. *Br J Ophthalmol* 81, 901-904.

Tan, J. S., Mitchell, P., Smith, W. and Wang, J. J. (2007). Cardiovascular risk factors and the long-term incidence of age-related macular degeneration: The blue mountains eye study. *Ophthalmology* 114, 1143-1150.

Tan, J. S., Wang, J. J., Liew, G., Rochtchina, E. and Mitchell, P. (2008). Age-related macular degeneration and mortality from cardiovascular disease or stroke. *Br. J. Ophthalmol.* 92, 509-512.

Thomas, C. (2011). Cardiovascular biology. *Nature* 473, 297.

Thurnham, D. I. (2007). Macular zeaxanthins and lutein -- a review of dietary sources and bioavailability and some relationships with macular pigment optical density and age-related macular disease. *Nutr. Res. Rev.* 20, 163-179.

Totan, Y., Cekic, O., Borazan, M., Uz, E., Sogut, S. and Akyol, O. (2001). Plasma malondialdehyde and nitric oxide levels in age related macular degeneration. *Br. J. Ophthalmol.* 85, 1426-1428.

Touboul, P. J., Hennerici, M. G., Meairs, S., Adams, H., Amarenco, P., Bornstein, N., Csiba, L., Desvarieux, M., Ebrahim, S., Fatar, M. et al. (2007). Mannheim carotid intima-media thickness consensus (2004-2006). an update on behalf of the advisory board of the 3rd and 4th watching the risk symposium, 13th and 15th european stroke conferences, mannheim, germany, 2004, and brussels, belgium, 2006. *Cerebrovasc. Dis.* 23, 75-80.

Trachtman, J. N. (2010). Vision and the hypothalamus. *Optometry* 81, 100-115.

Tsuda, K. (2009). Neuroprotective effects of leptin and nitric oxide against cerebral ischemia. *Stroke* 40, e406; author reply e407.

Tsuji, H., Venditti, F. J.,Jr, Manders, E. S., Evans, J. C., Larson, M. G., Feldman, C. L. and Levy, D. (1994). Reduced heart rate variability and mortality risk in an elderly cohort. the framingham heart study. *Circulation* 90, 878-883.

Turner, P. L. and Mainster, M. A. (2008). Circadian photoreception: Ageing and the eye's important role in systemic health. *Br J Ophthalmol* 92, 1439-1444.

Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M. and Yodoi, J. (2002). Redox control of cell death. *Antioxid. Redox Signal.* 4, 405-414.

Uen, S., Baulmann, J., Dusing, R., Glanzer, K., Vetter, H. and Mengden, T. (2003). ST-segment depression in hypertensive patients is linked to elevations in blood pressure, pulse pressure and double product by 24-h cardiotens monitoring. *J. Hypertens.* 21, 977-983.

Upritchard, J. E., Schuurman, C. R., Wiersma, A., Tijburg, L. B., Coolen, S. A., Rijken, P. J. and Wiseman, S. A. (2003). Spread supplemented with moderate doses of vitamin E and carotenoids reduces lipid peroxidation in healthy, nonsmoking adults. *Am J Clin Nutr* 78, 985-992.

Valencia, E., Hardy, G. and Marin, A. (2002). Glutathione--nutritional and pharmacologic viewpoints: Part VI. *Nutrition* 18, 291-292.

Vallance, P. and Chan, N. (2001). Endothelial function and nitric oxide: Clinical relevance. *Heart* 85, 342-350.

Vallance, P., Collier, J. and Moncada, S. (1989). Nitric oxide synthesised from L-arginine mediates endothelium dependent dilatation in human veins in vivo. *Cardiovasc. Res.* 23, 1053-1057.

van den Meiracker, A. H., Man in 't Veld, A. J., van Eck, H. J., Wenting, G. J. and Schalekamp, M. A. (1988). Determinants of short-term blood pressure variability. effects of bed rest and sensory deprivation in essential hypertension. *Am. J. Hypertens.* 1, 22-26.

van der Veen, R. L., Berendschot, T. T., Hendrikse, F., Carden, D., Makridaki, M. and Murray, I. J. (2009). A new desktop instrument for measuring macular pigment optical density based on a novel technique for setting flicker thresholds. *Ophthalmic Physiol Opt* 29, 127-137.

Van Dongen, H. P., Maislin, G. and Kerkhof, G. A. (2001). Repeated assessment of the endogenous 24-hour profile of blood pressure under constant routine. *Chronobiol. Int.* 18, 85-98.

van Hinsbergh, V. W., Havekes, L., Emeis, J. J., van Corven, E. and Scheffer, M. (1983). Low density lipoprotein metabolism by endothelial cells from human umbilical cord arteries and veins. *Arteriosclerosis* 3, 547-559.

van Leeuwen, R., Boekhoorn, S., Vingerling, J. R., Witteman, J. C., Klaver, C. C., Hofman, A. and de Jong, P. T. (2005). Dietary intake of antioxidants and risk of age-related macular degeneration. *Jama* 294, 3101-3107.

van Zwieten, P. A. and Doods, H. N. (1995). Muscarinic receptors and drugs in cardiovascular medicine. *Cardiovasc. Drugs Ther.* 9, 159-167.

Vaziri, N. D., Wang, X. Q., Oveisi, F. and Rad, B. (2000). Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension* 36, 142-146.

Verdecchia, P., Angeli, F. and Gattobigio, R. (2004). Clinical usefulness of ambulatory blood pressure monitoring. *J. Am. Soc. Nephrol.* 15 Suppl 1, S30-33.

Videla, L. A. and Guerri, C. (1990). Glutathione and alcohol. In *Glutathione: Metabolism and Physiological Functions* (ed. J. Vina), pp. 57-67. Boca Raton: CRC Press.

Videm, V., Wiseth, R., Gunnes, S., Madsen, H. O. and Garred, P. (2007). Multiple inflammatory markers in patients with significant coronary artery disease. *Int J Cardiol* 118, 81-87.

Vingerling, J. R., Dielemans, I., Bots, M. L., Hofman, A., Grobbee, D. E. and de Jong, P. T. (1995). Age-related macular degeneration is associated with atherosclerosis. the rotterdam study. *Am. J. Epidemiol.* 142, 404-409.

Virdis, A., Ghiadoni, L., Giannarelli, C. and Taddei, S. (2010). Endothelial dysfunction and vascular disease in later life. *Maturitas* 67, 20-24.

Vischer, U. M. (2006). Von willebrand factor, endothelial dysfunction, and cardiovascular disease. *J Thromb Haemost* 4, 1186-1193.

Vita, G., Dattola, R., Calabro, R., Manna, L., Venuto, C., Toscano, A., Savica, V. and Bellinghieri, G. (1988). Comparative analysis of autonomic and somatic dysfunction in chronic uraemia. *Eur. Neurol.* 28, 335-340.

Vlot, A. J., Koppelman, S. J., Bouma, B. N. and Sixma, J. J. (1998). Factor VIII and von willebrand factor. *Thromb Haemost* 79, 456-465.

Wald, G. (1945). Human vision and the spectrum. *Science* 101, 653-658.
Wang, L., Cioffi, G. A. and Van Buskirk, E. M. (1998). The vascular pattern of the optic nerve and its potential relevance in glaucoma. *Curr. Opin. Ophthalmol.* 9, 24-29.

Wang, W. and Ballatori, N. (1998). Endogenous glutathione conjugates: Occurrence and biological functions. *Pharmacol. Rev.* 50, 335-356.

Wangsa-Wirawan, N. D. and Linsenmeier, R. A. (2003). Retinal oxygen: Fundamental and clinical aspects. *Arch. Ophthalmol.* 121, 547-557.

Warnick, G. R., Knopp, R. H., Fitzpatrick, V. and Branson, L. (1990). Estimating low-density lipoprotein cholesterol by the friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. *Clin. Chem.* 36, 15-19.

Watson, J. P., Nolan, J. and Elliott, M. W. (1999). Autonomic dysfunction in patients with nocturnal hypoventilation in extrapulmonary restrictive disease. *Eur. Respir. J.* 13, 1097-1102.

Wei, E. P., Kontos, H. A. and Patterson, J. L.,Jr. (1980). Dependence of pial arteriolar response to hypercapnia on vessel size. *Am. J. Physiol.* 238, 697-703.

Weil, B. R., Kushner, E. J., Diehl, K. J., Greiner, J. J., Stauffer, B. L. and Desouza, C. A. (2011). CD31+ T cells, endothelial function and cardiovascular risk. *Heart Lung Circ.* 20, 659-662.

Weiter, J. J., Delori, F. C., Wing, G. L. and Fitch, K. A. (1986). Retinal pigment epithelial lipofuscin and melanin and choroidal melanin in human eyes. *Invest. Ophthalmol. Vis. Sci.* 27, 145-152.

Weiter, J. J., Schachar, R. A. and Ernest, J. T. (1973). Control of intraocular blood flow. II. effects of sympathetic tone. *Invest Ophthalmol* 12, 332-334.

Wenzel, A.,J., Sheehan, J. P., Burke, J. D., Lefsrud, M. G. and Curran-Celentano, J. (2007). Dietary intake and serum concentrations of lutein and zeaxanthin, but not macular pigment optical density, are related in spouses. *Nutrition Research* 27, 462-469.

Werner, N., Kosiol, S., Schiegl, T., Ahlers, P., Walenta, K., Link, A., Bohm, M. and Nickenig, G. (2005). Circulating endothelial progenitor cells and cardiovascular outcomes. *N. Engl. J. Med.* 353, 999-1007.

Whincup, P. H., Danesh, J., Walker, M., Lennon, L., Thomson, A., Appleby, P., Rumley, A. and Lowe, G. D. (2002). Von willebrand factor and coronary heart disease: Prospective study and meta-analysis. *Eur. Heart J.* 23, 1764-1770.

Whitsel, E. A., Raghunathan, T. E., Pearce, R. M., Lin, D., Rautaharju, P. M., Lemaitre, R. and Siscovick, D. S. (2001). RR interval variation, the QT interval index and risk of primary cardiac arrest among patients without clinically recognized heart disease. *Eur. Heart J.* 22, 165-173.

Wiencke, A. K., Nilsson, H., Nielsen, P. J. and Nyborg, N. C. (1994). Nonadrenergic noncholinergic vasodilation in bovine ciliary artery involves CGRP and neurogenic nitric oxide. *Invest. Ophthalmol. Vis. Sci.* 35, 3268-3277.

Wilkinson, I. B., Hall, I. R., MacCallum, H., Mackenzie, I. S., McEniery, C. M., van der Arend, B. J., Shu, Y. E., MacKay, L. S., Webb, D. J. and Cockcroft, J. R. (2002). Pulse-

wave analysis: Clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function. *Arterioscler Thromb Vasc Biol* 22, 147-152.

Wilkinson, I. B., MacCallum, H., Flint, L., Cockcroft, J. R., Newby, D. E. and Webb, D. J. (2000). The influence of heart rate on augmentation index and central arterial pressure in humans. *J Physiol* 525 Pt 1, 263-270.

Williamson, T. H., Lowe, G. D. and Baxter, G. M. (1995). Influence of age, systemic blood pressure, smoking, and blood viscosity on orbital blood velocities. *Br. J. Ophthalmol.* 79, 17-22.

Wilson, P. W., D'Agostino, R. B., Levy, D., Belanger, A. M., Silbershatz, H. and Kannel, W. B. (1998). Prediction of coronary heart disease using risk factor categories. *Circulation* 97, 1837-1847.

Winkler, B. S., Boulton, M. E., Gottsch, J. D. and Sternberg, P. (1999). Oxidative damage and age-related macular degeneration. *Mol Vis* 5, 32.

Wollensak, G., Schaefer, H. E. and Ihling, C. (1998). An immunohistochemical study of endothelin-1 in the human eye. *Curr Eye Res* 17, 541-545.

Wong, T. Y., Klein, R., Klein, B. E., Tielsch, J. M., Hubbard, L. and Nieto, F. J. (2001). Retinal microvascular abnormalities and their relationship with hypertension, cardiovascular disease, and mortality. *Surv Ophthalmol* 46, 59-80.

Wong, T. Y., Klein, R., Sun, C., Mitchell, P., Couper, D. J., Lai, H., Hubbard, L. D., Sharrett, A. R. and Atherosclerosis Risk in Communities Study. (2006). Age-related macular degeneration and risk for stroke. *Ann. Intern. Med.* 145, 98-106.

Wong, T. Y., Tikellis, G., Sun, C., Klein, R., Couper, D. J. and Sharrett, A. R. (2007). Age-related macular degeneration and risk of coronary heart disease: The atherosclerosis risk in communities study. *Ophthalmology* 114, 86-91.

Wood, J., Bellis, M. and Perkins, C. (2005). Life expectancy.

Wu, J., Seregard, S. and Algvere, P. V. (2006). Photochemical damage of the retina. *Surv. Ophthalmol.* 51, 461-481.

Xiao, Z., Zhang, Z., Ranjan, V. and Diamond, S. L. (1997). Shear stress induction of the endothelial nitric oxide synthase gene is calcium-dependent but not calcium-activated. *J Cell Physiol* 171, 205-211.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332, 411-415.

Ye, X. D., Laties, A. M. and Stone, R. A. (1990). Peptidergic innervation of the retinal vasculature and optic nerve head. *Invest Ophthalmol Vis Sci* 31, 1731-1737.

Yi, C., Pan, X., Yan, H., Guo, M. and Pierpaoli, W. (2005). Effects of melatonin in age-related macular degeneration. *Ann N Y Acad Sci* 1057, 384-392.

Yildiz, G. U., Dogan, E. A., Dogan, U., Tokgoz, O. S., Ozdemir, K., Genc, B. O. and Ilhan, N. (2011). Analysis of 24-hour heart rate variations in patients with epilepsy receiving antiepileptic drugs. *Epilepsy Behav.* 20, 349-354.

Yoshinaga, K., Manabe, O. and Tamaki, N. (2011). Assessment of coronary endothelial function using PET. *J. Nucl. Cardiol.* 18, 486-500.

Young, R. W. (1987). Pathophysiology of age-related macular degeneration. *Surv. Ophthalmol.* 31, 291-306.

Young, R. W. (1988). Solar radiation and age-related macular degeneration. *Surv Ophthalmol* 32, 252-269.

Yu, B. P. and Chung, H. Y. (2006). Adaptive mechanisms to oxidative stress during aging. *Mech. Ageing Dev.* 127, 436-443.

Yu, S., Katoh, T., Makino, H., Mimuno, S. and Sato, S. (2010). Age and heart rate variability after soccer games. *Res. Sports Med.* 18, 263-269.

Zalba, G., Fortuno, A., San Jose, G., Moreno, M. U., Beloqui, O. and Diez, J. (2007). Oxidative stress, endothelial dysfunction and cerebrovascular disease. *Cerebrovasc Dis* 24 Suppl 1, 24-29.

Zaliuniene, D., Jasinskas, V., Jurkevicius, R., Gustiene, O. and Zaliunas, R. (2008). Endothelial function, intima-media thickness, and ankle-brachial index in patients with cataract and age-related macular degeneration. *Eur. J. Ophthalmol.* 18, 384-391.

Zarbin, M. A. (2004). Current concepts in the pathogenesis of age-related macular degeneration. *Arch Ophthalmol* 122, 598-614.

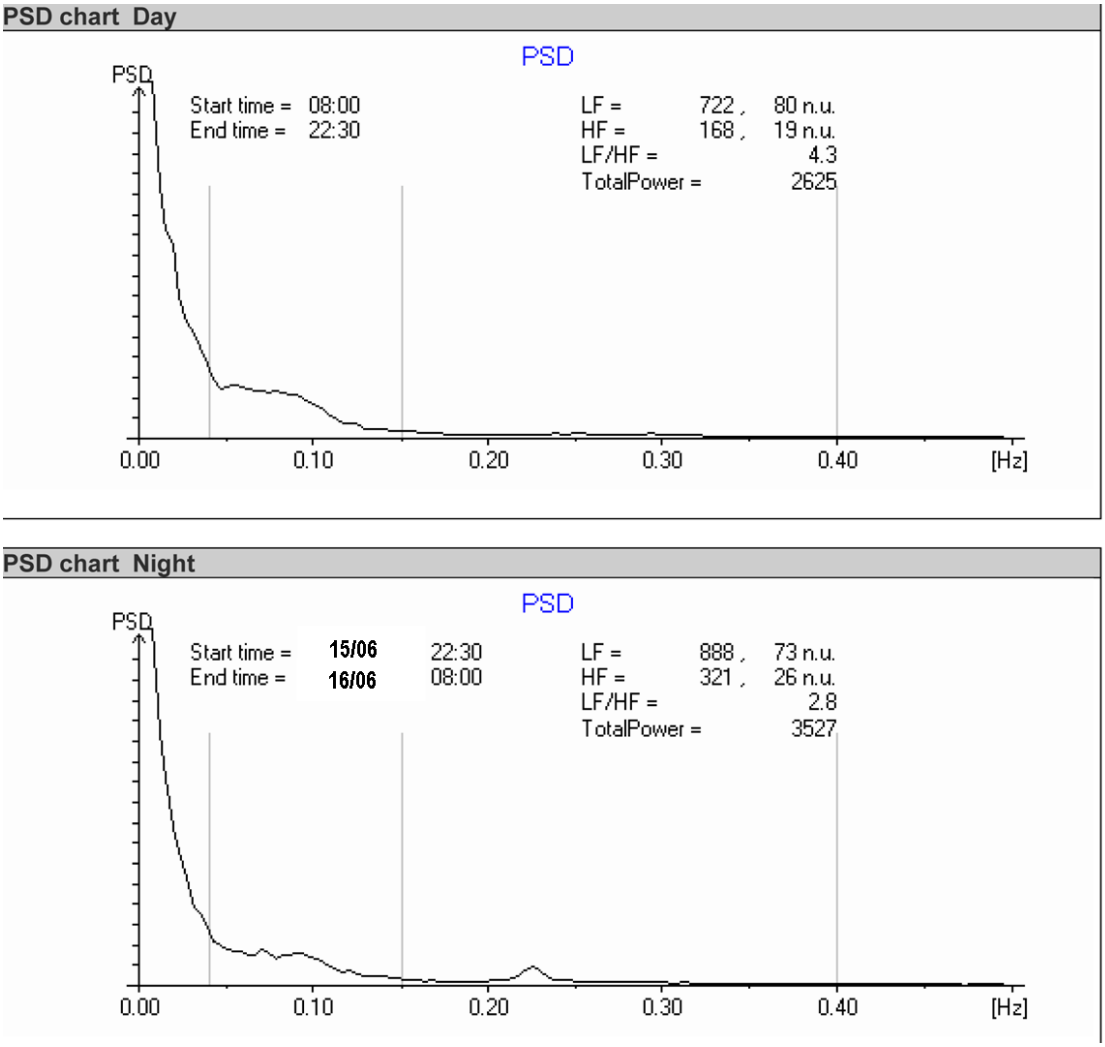
Zeiher, A. M., Krause, T., Schachinger, V., Minners, J. and Moser, E. (1995). Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. *Circulation* 91, 2345-2352.

Zhang, B. and Osborne, N. N. (2006). Oxidative-induced retinal degeneration is attenuated by epigallocatechin gallate. *Brain Res.* 1124, 176-187.

Zheng, J. S., Yang, X. Q., Lookingland, K. J., Fink, G. D., Hesslinger, C., Kapatos, G., Kovesdi, I. and Chen, A. F. (2003). Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation* 108, 1238-1245.

Zieman, S. J., Melenovsky, V. and Kass, D. A. (2005). Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler Thromb Vasc Biol* 25, 932-943.

Appendix 1. A sample of HRV report



LF: low frequency component; HF: high frequency component. Active period refers to Day chart, and passive period refers to Night chart.

Appendix 2: Publication

1. **Lu Qin**, Hannah Bartlett, Helen R. Griffiths, Frank Eperjesi, Richard A. Armstrong, Doina Gherghel. Macular pigment optical density is related to blood glutathione levels in healthy individuals. Invest Ophthalmol Vis Sci. 2011 Aug 24;52(9):5029-33.

2. **Lu Qin**, Stephanie A. Mroczkowska, Aniko Ekart, Sunni R. Patel, Jonathan M. Gibson, Doina Gherghel. Are patients with early age-related macular degeneration at high risk for systemic vascular disease? A pilot investigation. Invest Ophthalmol Vis Sci. Submitted

3. Patel SR, Bellary S, **Qin L**, Gill PS, Taheri S, Heitmar R, Gibson JM, Gherghel D. Abnormal retinal vascular function and lipid levels in a sample of healthy UK South Asians. Br J Ophthalmol. 2011 Nov;95(11):1573-6. Epub 2011 Mar 1.