Aston University
Birmingham

DOCTORAL THESIS

# Ocular and systemic vascular dysfunction in neurodegenerative disease

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# OCULAR AND SYSTEMIC VASCULAR DYSFUNCTION IN NEURODEGENERATIVE DISEASE

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**ASTON UNIVERSITY**
December 2011

**Aston University**
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**2011**

# Thesis Summary

The important role played by vascular factors in the pathogenesis of neurodegenerative disease has been increasingly realised over recent years. The nature and impact of ocular and systemic vascular dysfunction in the pathogenesis of comparable neurodegenerative diseases such as glaucoma and Alzheimer's disease (AD) has however never been fully explored. The aim of this thesis was therefore to investigate the presence of macro- and micro-vascular alterations in both glaucoma and AD and to explore the relationships between these two chronic, slowly progressive neurodegenerative diseases.

The principle sections and findings of this work were:

**1. Is the eye a window to the brain? Retinal vascular dysfunction in Alzheimer's disease**

- Mild newly diagnosed AD patients demonstrated ocular vascular dysfunction, in the form of an altered retinal vascular response to flicker light, which correlated with their degree of cognitive impairment.

**2. Ocular and systemic vascular abnormalities in newly diagnosed normal tension glaucoma (NTG) patients**

- NTG patients demonstrated an altered retinal arterial constriction response to flicker light along with an increased systemic arterial stiffness and carotid artery intima-media thickness (IMT). These findings were not replicated by healthy age matched controls.

**3. Ocular vascular dysregulation in AD compares to both POAG and NTG**

- AD patients demonstrated altered retinal arterial reactivity to flicker light which was comparable to that of POAG patients and altered baseline venous reactivity which was comparable to that of NTG patients. Neither alteration was replicated by healthy controls.

**4. POAG and NTG: two separate diseases or one continuous entity? The vascular perspective**

- POAG and NTG patients demonstrated comparable alterations in nocturnal systolic blood pressure (SBP) variability, ocular perfusion pressure, retinal vascular reactivity, systemic arterial stiffness and carotid IMT.
- Nocturnal SBP variability was found to correlate with both retinal artery baseline diameter fluctuation and carotid IMT across the glaucoma groups.

**Keywords**: Neurodegeneration, Glaucoma, Alzheimer's disease, Retinal Vessel Reactivity, Vascular dysregulation

# Acknowledgments

I would like to thank my supervisor, Dr Doina Gherghel, for her expertise and guidance throughout this thesis. I would additionally like to gratefully acknowledge Aston University as the funding body for this research via the clinical demonstrator programme.

I would like to thank all of the staff at Heartlands hospital, Birmingham Midland Eye Centre and The Barberry who supported and assisted me in my patient recruitment, in particular Mr Velota Sung, Mr Anil Negi and Ms Jan Wright. I would additionally like to extend my sincere thanks to all the patients and subjects who kindly gave up their time to make this research possible and to my colleagues, Lu Qin and Sunni Patel, for their assistance with data collection and blood analyses at times when one pair of hands was just not enough

My thanks also go to my friends and colleagues in the Aston University Optometry department for their continued support and encouragement, in particular to Olivia Hunt for her clinic and timetabling support and to my dear office buddy Olivia Howells for keeping me going, plying me with sweets and always being there to either lend an ear or offer a distraction.

Finally my special thanks go to the friends who encouraged me into starting this whole process and to my family for their unfailing support, guidance and encouragement throughout.

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# Abbreviations

| | |
|---|---|
| ABPM | Ambulatory blood pressure monitoring |
| ACE-R | Addenbrooke's Cognitive Examination Revised |
| AD | Alzheimer's disease |
| AIx | Augmentation index |
| ANS | Autonomic nervous system |
| ARMD | Age related macular degeneration |
| Aß | Amyloid-beta |
| BBB | Blood-brain barrier |
| BDF | Baseline diameter fluctuation |
| BFR | Baseline corrected flicker response |
| BMI | Body mass index |
| BP | Blood pressure |
| CBF | Cerebral blood flow |
| CCD | Charged coupling device |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| CRA | Central retinal artery |
| CRV | Central retinal vein |
| CSF | Cerebral spinal fluid |
| DA | Dilation amplitude |
| DBP | Diastolic blood pressure |
| DSM-IV-TR | Diagnostic and Statistical Manual of Mental Disorders, VIth edition |
| DVA | Dynamic retinal vessel analysis |
| ECG | Electrocardiogram |
| EDCF | Endothelial derived constricting factor |
| EDRF | Endothelial derived relaxing factor |
| EDVF | Endothelial derived vasoactive factors |
| ELM | External limiting membrane |
| eNOS | Endothelial nitric oxide synthase |
| ET-1 | Endothelin-1 |
| ET-2 | Endothelin-2 |
| ET-3 | Endothelin-3 |
| FMD | Flow mediated dilation |
| GON | Glaucomatous optic neuropathy |

| | |
|---|---|
| GSH | Reduced glutathione |
| GSSG | Oxidised glutathione |
| GTN | Nitroglycerin |
| $H^+$ | Hydrogen |
| HDL-C | High density lipoprotein cholesterol |
| HF | High frequency |
| HR | Heart rate |
| HRV | Heart rate variability |
| HTG | High tension glaucoma |
| ICA | Internal carotid artery |
| ILM | Inner limiting membrane |
| IMT | Intima-media thickness |
| iNOS | Inducible nitric oxide synthase |
| IOP | Intraocular pressure |
| IQR | Inter-quartile range |
| $K^+$ | Potassium |
| LDL-C | Low density lipoprotein cholesterol |
| LF | Low frequency |
| LF/HF | Low frequency/high frequency ratio |
| LGN | Lateral geniculate nucleus |
| LPCAs | Long posterior ciliary arteries |
| MABP | Mean arterial blood pressure |
| MC | Maximum constriction |
| MC% | Percentage constriction |
| MCI | Mild cognitive impairment |
| MD | Maximum diameter |
| MD% | Percentage dilation |
| MMP-9 | Metaalloproteinase-9 |
| MMSE | Mini mental state examination |
| MRI | Magnetic resonance imaging |
| NFL | Nerve fibre layer |
| NINCDS-ADRDA | National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders working group |
| NMD | Nitroglycerin mediated dilation |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| $NO_2^-$ | Nitrite |

| | |
|---|---|
| NOS | Nitric oxide synthase |
| NRR | Neuro-retinal rim |
| NTG | Normal tension glaucoma |
| NVU | Neurovascular unit |
| $O_2$ | Oxygen |
| OA | Ophthalmic artery |
| OAG | Open angle glaucoma |
| OBF | Ocular blood flow |
| OHT | Ocular hypertension |
| ONH | Optic Nerve Head |
| OPP | Ocular perfusion pressure |
| PCAs | Posterior ciliary arteries |
| $pCO_2$ | Partial carbon dioxide pressure |
| PET | Positron emission tomography |
| PNS | Parasympathetic nervous system |
| $pO_2$ | Partial oxygen pressure |
| POAG | Primary open angle glaucoma |
| PVD | Primary vascular dysregulation syndrome |
| PWA | Pulse wave analysis |
| RBC | Red blood cell |
| RGC | Retinal ganglion cell |
| RNFL | Retinal nerve fibre layer |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPCs | Radial peripapillary capillaries |
| RPE | Retinal Pigment Epithelium |
| RRI | Recurrent reperfusion injury |
| RT | Reaction time |
| SA node | Sinoatrial node |
| SBP | Systolic blood pressure |
| SDRA | Sequential and diameter response analysis |
| $Slope_{AC}$ | Arterial constriction slope |
| $Slope_{AD}$ | Arterial dilation slope |
| $Slope_{VC}$ | Venous constriction slope |
| $Slope_{VD}$ | Venous dilation slope |
| SNS | Sympathetic nervous system |
| SOV | Superior ophthalmic vein |

| | |
|---|---|
| SPCAs | Short posterior ciliary arteries |
| SVD | Secondary vascular dysregulation syndrome |
| TG | Triglycerides |
| t-GSH | Total glutathione |
| TM | Trabecular meshwork |
| tMC | time to maximum constriction |
| UM | Units of measurement |
| VaD | Vascular dementia |
| VF | Visual field |
| vSMCs | Vascular smooth muscle cells |
| vWf | von Willebrand factor |

# 1. Introduction

The term neurodegeneration refers to a progressive loss of structure and function in the neurons of the central and/or peripheral nervous system and encompasses a large range of different disease conditions. One of the most significant risk factors for neurodegeneration is advancing age and as life expectancies increase so does its prevalence, making it a global issue of increasing concern [1]. Discovering the triggers which promote neurodegeneration in the elderly and others and discovering the pathological mechanisms by which neurodegenerative disease develops is therefore currently a research area of intense interest. One of the most recognised locations for neurodegeneration within the central nervous system (CNS) is the brain, where it is commonly affiliated with the development of cognitive impairments such as Alzheimer's disease (AD). AD is a prevalent neurodegenerative disease of poorly understood aetiology which affects over 35 million people worldwide [2]. One of the major factors limiting our aetiological understanding of the neurodegenerative processes in AD and other cerebral diseases is the notorious difficulty associated with assessing and visualising the brain and cerebral neurons directly. As a result researchers are naturally starting to look to other more accessible regions of the CNS in order to gain a potential insight into the alterations which may be occurring at the cerebral level. Of particular interest in this regard is the eye and the neurons of the retina and optic nerve head (ONH), which also form part of the CNS and have been found to exhibit a number of features comparable to that of the cerebral unit [3]. Indeed the ONH itself is recognised as an important site of neurodegeneration, whereby the progressive degeneration of ONH neurons is strongly affiliated with the development of the ocular disease glaucoma. Interestingly tentative associations have previously been made between the occurrence of ocular neurodegeneration in the form of glaucoma and cerebral neurodegeneration in the form of AD. This in combination with the multiple similarities previously identified in the structure and functioning of the

ocular and cerebral units has raised the question of whether these two neurodegenerative diseases may share a common underlying aetiology. This thesis aims to explore this possibility and to additionally address the question of whether functional assessment at the ocular level could prove effective as an indirect measure of cerebral function and hence whether the eye could be effectively used as a 'window to the brain' in neurodegenerative disease. Of particular interest to this thesis is the involvement of vascular factors in the aetiology of both AD and glaucoma. Indeed one of the most prominent features shared by the ocular and cerebral units is the nature of their vascular supply, with the ocular and cerebral microcirculations in particular demonstrating a large number of anatomical and physiological similarities [4]. Furthermore, although the involvement of vascular factors in the aetiology of both AD and glaucoma individually has been increasingly realised, the nature of this involvement is still somewhat uncertain and many questions remain. As such this thesis additionally aims to explore the presence and aetiological relevance of vascular disorders at both the ocular and systemic level in glaucoma and AD in order to try and enhance our understanding of the pathological mechanisms involved in these neurodegenerative diseases individually. To provide a basis for the research conducted in this thesis the following sections will outline the background and current aetiological thinking, firstly for the development of glaucoma and secondly for the development of AD, with particular emphasis on the role of vascular factors. The relevant ocular, cerebral and cardiovascular anatomy and physiology will also be discussed, followed by an outline of the current literature associating these two neurodegenerative diseases.

## 1.1 Primary Open Angle Glaucoma

Primary open angle glaucoma (POAG) is a chronic, slowly progressive optic neuropathy characterised by progressive visual field loss and a distinctive excavation of the ONH [5]. It is one of the leading causes of blindness in the world, affecting over 66 million people worldwide and accounting for approximately 13% of those on the blind register in England and Wales [6]. Its origin is strongly linked to the presence of elevated intraocular pressure (IOP) [7, 8], but despite this IOP is no longer included in the definition of glaucoma as its involvement with the disease process has been shown to be inconsistent.

The first description of the term glaucoma (or 'glaucosis' in Greek) is accredited to Hippocrates in approximately 400 B.C [9]. It was thought for many centuries that the 'hardness of the eyeball' first identified as glaucoma resulted from some form of vitreous abnormality and it wasn't until the mid-nineteenth century, following the development of the ophthalmoscope, that the now characteristic features of abnormal cupping of the ONH, increased 'eye tension' and visual loss were first linked [9]. A differentiation between different forms of glaucoma, namely acute, chronic and secondary, all of which were associated with increased IOP, was first made by von Graefe in 1854 [9]. This was followed by the proposal that there may also be IOP-independent causes of glaucomatous optic neuropathy (GON) by Jaeger in 1858 [10] and that both mechanical and vascular factors may be involved in the development of glaucoma by Smith in 1886 [11]. The suggestion that atrophy and excavation of the ONH occurring in the absence of elevated IOP should be considered a distinct form of glaucoma was subsequently made by Schnabel in 1892, leading to the categorisation of what is now commonly referred to as normal tension glaucoma (NTG). Finally, following the development of gonioscopic devices in the early 20$^{th}$ century, which allowed the anterior chamber drainage angle to be observed, a further

differentiation between open angle and closed angle glaucoma was made, leading ultimately to the adoption of a simple and broad classification for glaucoma in 1954 [9] which still forms the basis for glaucoma classification in the present day (Figure 1.1).

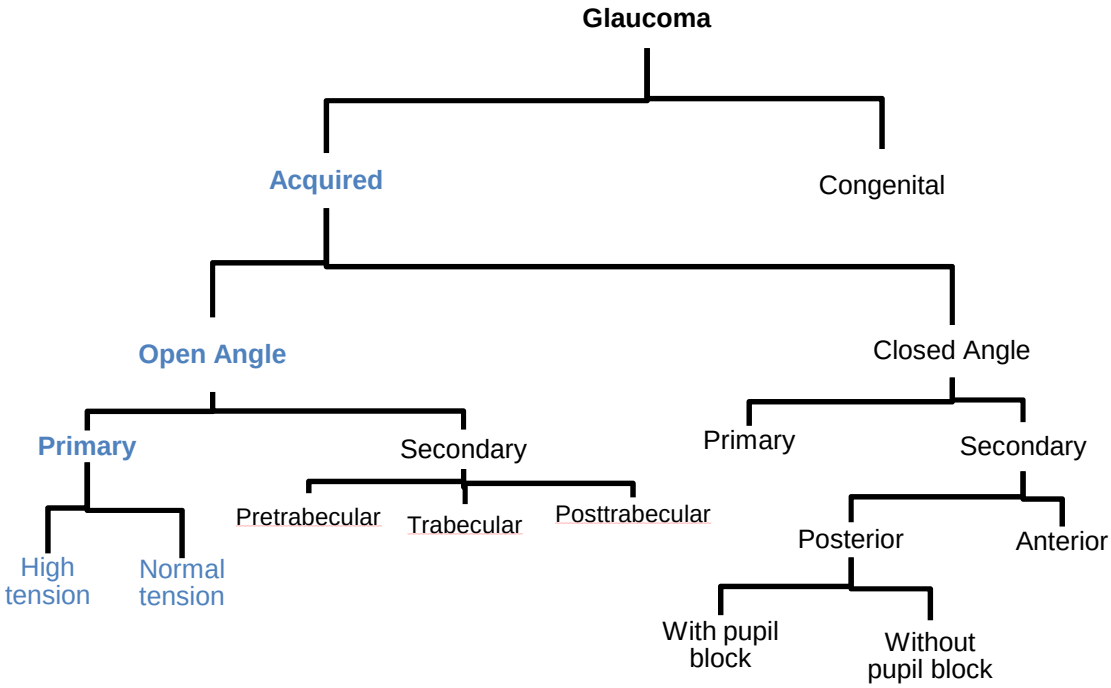



**Figure 1.1: Classification of glaucoma**

Open angle glaucoma (OAG) is the most prevalent type of glaucoma and forms the focus of this research. It is commonly divided into two subcategories, namely high tension or primary open angle glaucoma (HTG/POAG), in which IOP is elevated (>21 mmHg) and NTG, in which IOP falls within the normal range (10-21 mmHg on diurnal testing). The distinctions between these two subcategories of OAG however have become somewhat blurred over recent years and this is discussed further in section 1.1.2. Nevertheless, as it is still currently common practice in the recent literature to come across the terms POAG and NTG being referred to individually, for the purpose of this thesis, the term POAG will be used to refer to the development of GON in the

presence of raised IOP and NTG, which accounts for approximately 1/3 of all open angle glaucoma cases, will be considered separately. The only exception to this will be when citing previous research in which no distinction between patients with regard to their IOP level was made. In these cases the term ‘glaucoma’ is used to describe participants and will indicate a non-specific diagnosis of open angle glaucoma.

### 1.1.1 Risk factors for glaucomatous damage

Both POAG and NTG are asymptomatic until the late stages of the disease by which point significant visual field loss has already occurred. The necessity for early detection and management of the disease is therefore clear however this is hindered by our still relatively poor understanding of the pathogenesis of glaucoma, despite extensive research in the area. Multiple risk factors have been implicated in the development of glaucoma, the most acknowledged of which include advancing age [12], elevated IOP [13], positive family history [14] and African descent [15, 16]. At the ocular level the presence of myopia [17, 18], a large ONH [19] and/or a thinner central corneal thickness [15, 20, 21] have also been recognised to increase the risk of GON development as well as, hypertension [22], hypotension [23, 24], reduced OBF [25], vasospasm [26], oxidative stress [27, 28] and cardiovascular disease history [24] at the systemic level. With regard to NTG specifically, the occurrence of female gender [29], Japanese ethnicity [30] and optic disc haemorrhages [31] are factors additionally identified as increasing the risk of its development. Such differences in the risk factors associated with POAG and NTG suggests that these two forms of OAG may represent distinct clinical entities, each with their own pathogenesis; however the relationship between them is not this clear cut, as discussed in the following section.

### 1.1.2 POAG vs. NTG

Although the separation of OAG into two distinct clinical entities on the basis of IOP has been common practice for many years, a large number of overlaps between the

pathogenesis and features of both POAG and NTG have been identified more recently, throwing this concept of a distinct separation into dispute. Indeed it has even recently been suggested that the terms POAG and NTG should in fact be abolished, along with the 'arbitrary' 21mmHg IOP cut off value and that glaucoma should instead be considered as a disease continuum across which IOP and pressure-independent risk factors coexist with a varying degree of influence [32-35]. In support of this, factors more traditionally associated with POAG, such as elevated IOP, have been firmly established as part of the pathogenetic process in NTG [36-38] and similarly vascular alterations and other IOP independent factors, more traditionally linked to NTG, have also been linked to POAG [39-43]. On the contrary however, there are still a number of studies which describe subtle but important differences in both the structural and functional ONH changes, as well as the vascular risk between POAG and NTG patients [31, 44-47]. Indeed NTG patients have previously been demonstrated to show a greater degree of inferotemporal NRR thinning, notching and disc haemorrhaging in comparison to POAG patients [48, 49], as well as deeper, steeper sided and more central visual field (VF) defects [50, 51]. Furthermore, with regard to vascular risk, stronger associations between the presence of vascular factors such as vasospasm, vascular dysregulation and hypotension have been made with NTG in comparison to POAG [26, 42, 52-54] and additionally NTG patients have also been demonstrated to show a greater frequency of related conditions such as migraine and Raynaud's phenomenon [55]. The possibility that POAG and NTG may still represent two distinct clinical entities can therefore not be ruled out, however much of the literature is conflicting and whilst the similarities and differences discussed above have been identified by some studies, others have found no such relationships, making it difficult to draw any firm conclusions [32, 44-46]. Further investigation into the relationship between POAG and NTG and a thorough evaluation of their validity as distinct clinical entities would be beneficial in enhancing our understanding, diagnosis and future

management of these two conditions. A summary of the most commonly identified similarities and differences between POAG and NTG is given in table 1.1.

| | POAG | NTG |
|---|---|---|
| **Onset and Symptoms** | Gradual, asymptomatic | Gradual, asymptomatic |
| **Clinical Profiles** | Non-specific | - Lower body weight<br>- Detail-orientated and health conscious |
| **Risk factors** | - Age<br>- IOP*<br>- Gender (female)<br>- Family history<br>- African Descent<br>- Myopia<br>- Thinner corneal thickness<br>- Hypertension*<br>- Hypotension<br>- Altered OBF<br>- Vasospasm/Vascular dysregulation<br>- Oxidative stress<br>- Cardiovascular disease history* | - Age<br>- IOP<br>- Gender (female)*<br>- Family history<br>- African Descent<br>- Japanese descent*<br>- Myopia<br>- Thinner corneal thickness<br>- Hypertension<br>- Hypotension*<br>- Altered OBF*<br>- Vasospasm/Vascular dysregulation*<br>- Oxidative stress<br>- Cardiovascular disease history |
| **Structural ONH Change** | - Progressive enlargement and deepening of the optic cup in conjunction with thinning of NRR<br>- Deep cupping | - Progressive enlargement and deepening of the optic cup<br>- Inferotemporal NRR thinning predominantly<br>- Disc haemorrhages |
| **Visual Field Defects** | - Arcuate scotomas<br>- Paracentral locations involved at later stages of field loss | - Deep, steep, paracentral defects |
| **Nature of Progression** | - More pronounced<br>- Correlates with level of IOP | - Less pronounced or even non-progressive |

**Table 1.1: Comparison between POAG and NTG (*Indicates where a risk factor is more predominant)**

All in all, it is clear from the wide range of both ocular and systemic risk factors implicated in the development of both forms of OAG that it is a disease of multifactorial origin, the end stage of which is known to involve the apoptotic loss of retinal ganglion cells (RGC), tissue remodelling and excavation of the ONH. The

mechanisms by which these multiple risk factors go on to contribute towards the classic glaucomatous ONH changes in both forms of the disease is however currently uncertain, with a number of different aetiological theories having been proposed. Gaining a better understanding of the exact pathophysiology of glaucoma could lead to huge advances in its diagnosis and treatment and significantly improve the prognosis for affected individuals. The current knowledge surrounding the aetiology of glaucoma and the involvement of both mechanical and vascular factors will now be discussed in detail, along with the anatomy and physiology of the ocular structures, which is necessary to aid the interpretation of this thesis.

## 1.2 Anatomy and Physiology of the Retina, Optic Nerve and Ocular Circulation

### 1.2.1 The Retina

The vertebrate retina is a complex multilayered structure consisting of five types of neuronal cells (ganglion, bipolar, amacrine, horizontal cells and photoreceptors) and one type of glial cell (Muller) inter-dispersed by synaptic connections [56]. Its anatomical structure is depicted in figure 1.2.

The inner most layer, closest to the vitreous body is termed the internal limiting membrane (ILM) and is formed from Muller cell end plates. These cells extend vertically through the retina from the ILM to the external limiting membrane (ELM) [56] and act to maintain homeostasis and provide support and protection for the neurons. The ganglion cells lie immediately behind the ILM with their axons making up the retinal nerve fibre layer (NFL), and their nuclei forming the so called ganglion cell layer itself. Two other cellular layers are also present in the retina, namely the inner nuclear layer and the outer nuclear layer. The inner nuclear layer contains the nuclei

and cell bodies of the bipolar cells, the amacrine cells and, along its outer margin, the horizontal cells. The outer nuclear layer on the other hand contains the cell bodies of the photoreceptors [56]. Separating these two neural layers are two plexiform layers of synaptic connections; namely the inner plexiform layer and the outer plexiform layer [56]. The ELM separates the photoreceptor layer from the outer nuclear layer and in a similar way to the ILM is formed from Muller cell end plates [56]. Finally the retinal pigment epithelium (RPE) forms the outermost retinal layer, separating the neurosensory retina from the underlying choroid and providing support for the photoreceptors.

Ultimately, the RGCs are the means by which the retinal information is finally transferred to the relay stations in the brain for integration and processing [57]. This transfer of information is achieved by convergence of the RGC axons from across the inner retinal margin, onto the ONH, and along the optic nerve [56] and only occurs following a series of complex interactions between the retinal photoreceptors (rods and cones) and the retinal neurons (bipolar, amacrine and horizontal cells). A full account of this is beyond the scope of this thesis but can be found in 'Webvision: The Organization of the Retina and Visual System [Internet] [58]. In short, the two types of retinal photoreceptors (rods and cones), which contain visual pigments (rhodopsin and opsin respectively), detect quanta of light and transfer information regarding this light signal to the outer plexiform layer [59]. At the level of the outer plexiform layer the photoreceptor processes synapse with the horizontal and bipolar cells [60] and this information ultimately reaches the ganglion cells, via the inner plexiform layer through either the vertical pathways (photoreceptors – bipolar cells – ganglion cells) or the lateral pathways (photoreceptor – horizontal cells – amacrine cells – ganglion cells) [57].

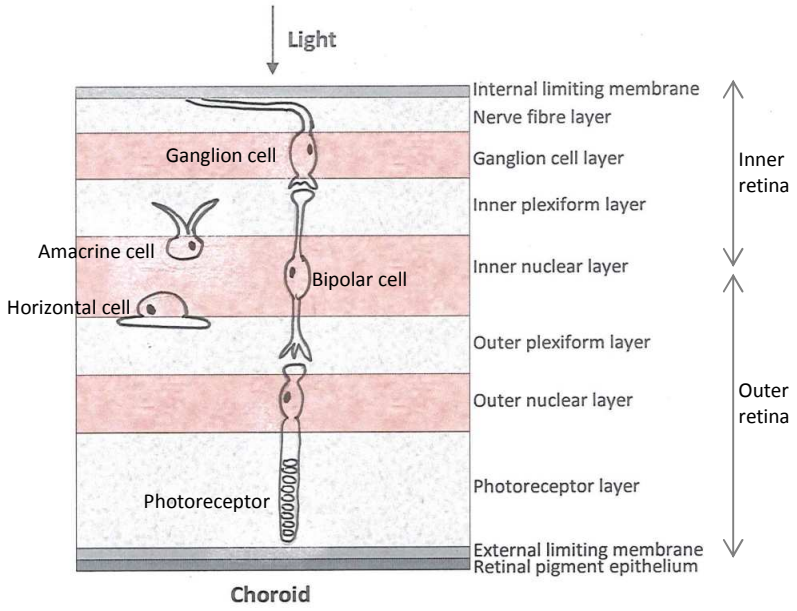



**Figure 1.2: Diagrammatic representation of the human retina**

### 1.2.2 The Optic Nerve

The optic nerve or second cranial nerve is part of the CNS with a pathway extending from the retinal level to the primary visual centres in the brain. It is commonly subdivided into four segments termed the intraocular, intraorbital, canalicular and intracranial segments [61]. The intraocular segment of the optic nerve is commonly referred to as the head and the anatomy and physiology of this portion of the optic nerve is particularly relevant with regard to the development of optic neuropathies such as glaucoma.

The ONH arises when the intraorbital portion of the optic nerve pierces the sclera, choroid and outer retinal layers and it is located approximately 4mm nasal to the posterior pole of the globe [61]. The ONH itself can be further subdivided into three sections, termed the surface nerve fibre layer, the prelaminar region and the lamina cribrosa region (figure 1.3), with the remainder of the optic nerve being then termed the retrolaminar portion [62]. The subdivisions of the ONH are outlined below:

- The surface NFL: a layer of compact nerve fibres formed from the convergence of the retinal nerves across the entire retinal surface towards the ONH before they bend to run backward along the optic nerve itself [62]. It is the most anterior layer of the ONH and it is covered by the ILM of the retina.
- The prelaminar layer: lies immediately behind the surface NFL and consists of nerve fibres arranged in bundles and surrounded by glial tissue septa [62]. Within the glial septa are capillaries and between the bundles any loose glial tissue form trabelculae [62]. The network of glial cells provides support, protection and nutrition to the optic nerve fibres in this portion of the nerve [63]. The prelaminar layer is closely connected to the lamina cribrosa at its base and separated at its edge from the adjacent retina and choroid by a further layer of glial tissue [62].
- The lamina cribrosa: a band of dense compact connective tissue which bridges across the entire thickness of the optic nerve. It is lamellar in structure, being made up of alternating sheets of connective and glial tissue and containing many oval or rounded openings which are lined by glial cells and allow the transmission of the nerve fibre bundles through the lamina layer. It also has a central opening allowing the transmission of the central retinal vessels [62]. These openings or pores are larger in the superior and inferior sections compared to the nasal and temporal sections of the ONH [64]. Each nerve fibre bundle is surrounded by a continuous glial membrane which separates it from the adjacent connective tissue and provides support, protection and nutrition. Capillaries, which lie within the fibrous septa, form a dense capillary plexus making the lamina region a highly vascular section of the ONH [65].
- The retrolaminar portion: encompasses the intraorbital, canalicular and intracranial sections and is enclosed by dura, arachnoid and pia [62]. In the intraorbital region the nerve fibres become myelinated and their bundles lie in polygonal spaces formed by the connective tissue septa. The septa contain blood vessels and are attached to the pia in the periphery, the connective tissue

envelope of the central retinal vessels centrally and the lamina cribrosa anteriorly [62]. From here the nerve fibres continue posteriorly through the canalicular and intracranial segments of the optic nerve to the primary visual centres in the brain.

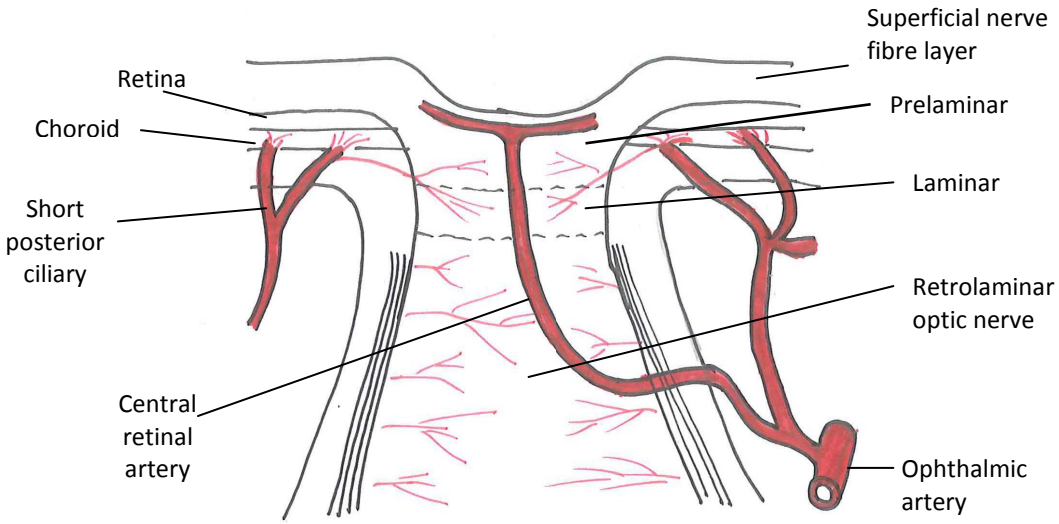


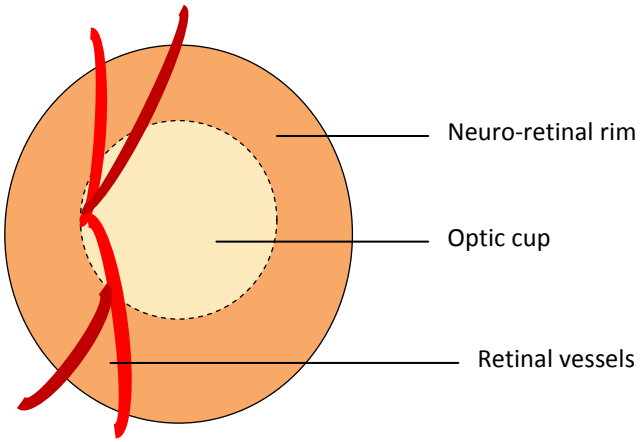

**Figure 1.3: Diagrammatic representation of the optic nerve head and its vasculature**

### 1.2.2.1 The Healthy Optic Nerve Head on Clinical Examination

As well as the structural profile of the ONH it is important to also consider its clinical picture visible on examination. Indeed on clinical examination the ONH is vertically oval in form with a central depression, referred to as the cup, surrounded by a rim of neural tissue, termed the neuroretinal rim (NRR) (figure 1.4) [66, 67].

In the healthy ONH the NRR is well perfused and orange/pink in colour. It has a characteristic physiological shape, defined by the 'ISNT rule', whereby its broadness decreases from being maximum in the inferior region, followed by the superior region, the nasal region and finally reaching its narrowest point in the temporal region of the ONH [66]. The size of the ONH itself can show great physiological variation between individuals, with its area having been shown to range from 0.80 $mm^2$ to 6.00 $mm^2$ in a normal white population [66, 68, 69]. The central cup is an area devoid of neural tissue and is usually horizontally oval and paler in colour compared to the NRR [66]. Its size

and depth can also show great inter-individual variation and it is commonly graded in terms of cup-to-disc ratio [66]. In the healthy ONH the area of the optic disc and the optic cup are correlated with each other, so larger optic discs are associated with larger optic cups [67, 69] and larger optic cups are associated with greater depths [67]. Also visible in the central portion of the healthy ONH is the entrance/exit points of the CRA, CRV and their branches. Variations away from this healthy clinical picture can be indicative of optic neuropathy and the relevance of this with regard to glaucomatous nerve damage is discussed later in section 1.3.




Healthy optic nerve head

**Figure 1.4: Diagrammatic representation of the healthy optic nerve head**

## 1.2.3 The Ocular Circulation

### 1.2.3.1 The Retrobulbar Vessels

The blood supply to the ocular tissues primarily arises from the internal carotid artery, which branches to form the ophthalmic artery (OA) and subsequently the central retinal artery (CRA) and posterior ciliary arteries (PCAs) (figure 1.5). The OA, CRA and PCAs are collectively referred to as the retrobulbar vessels and their anatomy is as follows:

### a) Ophthalmic Artery (OA)

The OA is the first major branch of the internal carotid artery, arising, in most cases, just after the internal carotid leaves the cavernous sinus. From here it travels anteriorly, passing within the optic canal, usually inferolaterally to the optic nerve, until it enters the orbit at its apex [70]. Numerous branches arise from the OA along its pathway to the orbit and supply not only the orbit itself but also some structures in the face, nose and meninges [71]. Anatomical studies have revealed multiple inter-individual variations in the site of origin of the OA itself, its branches and the course it follows, therefore it is common practice to discuss its anatomy and pathway in terms of that most commonly observed. The most relevant branches arising from the OA with regard to the ocular circulation are the CRA and PCAs.

### a) Central Retinal Artery (CRA)

The CRA is one of the first branches of the OA. It penetrates the optic nerve approximately 8mm posterior to the globe and travels anteriorly along the centre of the optic nerve before ultimately dividing into four major branches which supply the inner retinal layers [72] (see section 1.2.3.2).

### b) Posterior ciliary arteries (PCAs)

The PCA circulation is the major source of the blood supply to the ocular structures and ONH. One to five posterior ciliary arteries branch from the OA at a point distal to the origin of the CRA. They travel forward along the optic nerve and further divide into multiple branches which pierce the sclera, usually laterally or medially to the optic nerve [73]. These branches are of two types, termed short and long PCAs. The short PCAs (SPCAs), of which there can be 10-20, are also of two types, paraoptic and distal. Paraoptic SPCAs enter the sclera close to the optic nerve and contribute to the blood supply of the ONH, peripapillary choroid and the circle of Zinn and Haller, as well as sending recurrent branches to the retrolaminar ONH plial vascular plexus [73]. The distal SPCAs enter the sclera a short distance away from the ONH and run radially towards the equator. Each distal SPCA supplies a sector of the choroid

extending from the posterior pole to the equator and then further subdivides into smaller choroidal arterioles which ultimately supply a lobule of choriocapillaries [73]. The choriocapillaries exist as a single network of continuous capillaries located directly beneath the RPE, which demonstrate a segmental distribution and supply the outer portion of the retina extending from the RPE to the outer part of the inner nuclear layer [74, 75]. The long PCAs (LPCAs), of which there are two (medial and lateral), enter the sclera in the horizontal plane on the medial and lateral sides and run radially towards the iris. Each LPCA supplies a sector of the peripheral choroid as well as a small section of the ciliary body and iris [73]. The choroidal circulation accounts for 85% of the total ocular blood flow and is characterised by very high flow and low oxygen extraction [76, 77]. This high flow is facilitated by low resistance of the choroidal capillaries [78] and unlike the retinal capillaries, the choriocapillaries are fenestrated, demonstrate less autoregulation and have a rich autonomic innervation [79-82].

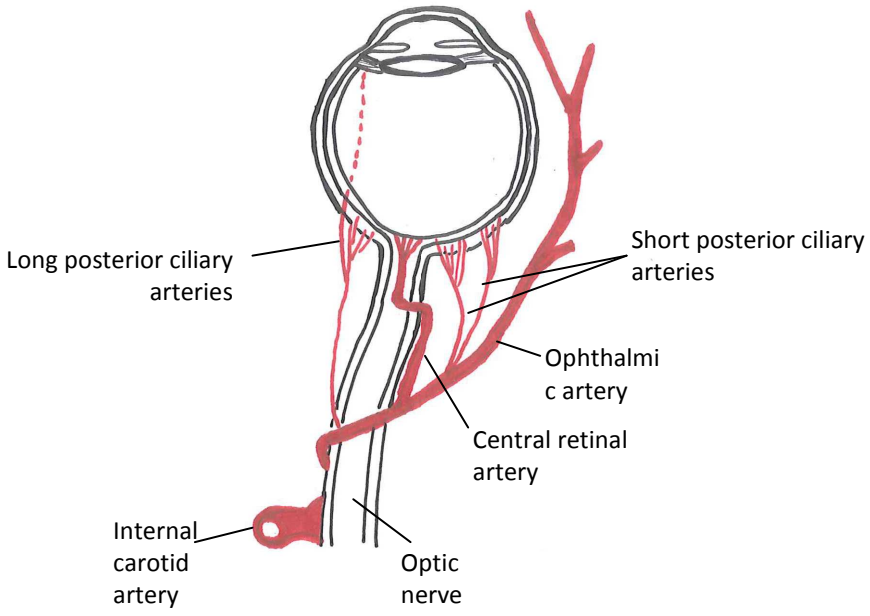



**Figure 1.5: Diagrammatic representation of the retrobulbar circulation**

### 1.2.3.2 Retinal Circulation

Normal retinal function requires a stable blood supply that does not interfere with the optics of the system but still provides adequate nutrient delivery and temperature control. The major source of blood supply to the inner retina (extending from NFL to inner section of inner nuclear layer) is the CRA, whereas the outer portion of the retina (extending from outer section of inner nuclear layer to RPE) receives its blood supply from the SPCAs, via the choriocapillaries [75].

The CRA, on leaving the optic nerve, divides into four major branches which lie in the nerve fibre and ganglion cell layers and run from the posterior pole to the periphery, supplying the whole inner retina [75]. Some arterioles branch further to form side arms and supply a complex retinal capillary network. The retinal capillary network is split into three main sections, termed the radial peripapillary capillaries (RPCs), the inner capillaries and the outer capillaries [75]. The RPCs lie within the inner part of the nerve fibre layer and run along the paths of the major CRA branches. The inner capillaries lie in the nerve fibre and ganglion cell layer, underlying the RPCs and form a complex capillary inner plexus. Finally the outer capillaries lie in the inner plexiform layer and inner nuclear layer and run to the border of the outer plexiform layer [75]. This capillary network extends throughout the length of the retina, with the only exception being in the central macula region where a capillary free zone exists parafoveally [75].
The outer portion of the retina, extending from the RPE to the outer section of the inner nuclear layer, receives its blood supply from the choriocapillaries which form a continuous single network and lie in the inner most layer of the choroid [75, 83].

Drainage of the retinal circulation is achieved via the central retinal vein (CRV), which travels centrally within the optic nerve, alongside the CRA, before exiting the nerve and ultimately draining into either the superior ophthalmic vein (SOV), other intraorbital venous branches or directly into the cavernous sinus [61]. Drainage of the

choroidal circulation and hence the outer portion of the retina is via the vorticose veins.

On the whole, retinal circulation is characterised by a low level of flow and high level of oxygen extraction [78, 84]. It is autoregulated, allowing blood supply to be adjusted to metabolic demand, but it receives no autonomic innervation [85-87]. Tight junctions exist between the capillary endothelial cells forming a blood-retinal barrier similar to the blood-brain barrier of the CNS. The RPE, which is considered the most posterior layer of the retina, forms an outer blood-retinal barrier which complements the inner blood retinal barrier formed by the retinal capillaries and prevents the passage of all but essential metabolites from the bloodstream to the retinal tissues [78].

#### 1.2.3.3 ONH Circulation

The ONH is a highly vascular structure which receives its primary blood supply from the SPCAs, either directly or via the peripapillary choroid. If present, contributions are also made from the intrascleral circle of Zinn and Haller [64].

The nature of the blood supply varies slightly between the different portions of the ONH as outlined below:

- Surface NFL: mainly supplied by the retinal arterioles and hence the CRA, in a manner continuous with the peripapillary retina [88]. The temporal portion may be supplied by the PCA circulation from the underlying prelaminar region and if a cilioretinal artery is present then it will also contribute in the corresponding area [64].
- Prelaminar region: mainly supplied by centripetal branches from the peripapillary choroidal vessels, particularly on the temporal side, however some of the blood supply may also come from the vessels located in the

lamina region [88]. Blood supply is sectorial, in a similar way to the choroid, but no contribution is made by the CRA or the peripapillary choriocapillaries [64].

- Lamina cribrosa region: supplied by centripetal branches arising either directly from the SPCAs or from the circle of Zinn and Haller, if present. The vessels form a dense capillary plexus making it a highly vascular region of the ONH [64, 88] however there is no contribution from the CRA.
- Retrolaminar portion: has a centripetal vascular system formed primarily by recurrent pial branches arising from the peripapillary choroid and the circle of Zinn and Haller. Additional pial branches may also arise from the CRA or other orbital arteries. This network of precapillaries and capillaries run centripetally within the connective tissue septa [64].

Venous drainage of the ONH is primarily via the central retinal vein however in the prelaminar region drainage can also occur via the peripapillary choroidal veins (vorticose veins) [64].

The circulation of the optic nerve resembles the cerebral circulation and that of the rest of the CNS, in that, on the whole, tight junctions exist between vascular endothelial cells creating a blood-brain barrier (BBB) [89]. This barrier prevents the passage of all but essential metabolites from the blood into the tissues, protecting them from the effects of foreign, potentially damaging, substances that could be present in the bloodstream as well as the effects of hormones or neurotransmitters that may be active in the rest of the body [90]. The prelaminar portion of the ONH, however, has been to found to lack the BBB properties typical of the rest of the CNS and optic nerve, indicating that the vessels in the prelaminar region may demonstrate different permeability characteristics to that of the other regions of the ONH [89]. These permeability differences could be important with regard to regulation of blood flow

and disease pathology in this region due to the possibility of diffusion of vasoactive substances from the choroidal circulation into the ONH.

The ONH receives no direct autonomic innervation [91, 92], however its blood flow is autoregulated [87, 93, 94]. This capacity for autoregulation is however considered to be less efficient than in the retina, but more efficient than in the choroid [95].

#### 1.2.3.4 Venous Drainage

Anatomical studies reveal the orbital venous system to be complex and highly variable [70, 96]. The SOV is generally accepted as the main route for venous drainage from the orbit. It is formed from the union of the supraorbital and angular veins just posterior to the trochlea and travels posteriorly, following the course of the OA, before ultimately leaving the orbit through the superior orbital fissure and draining into the cavernous sinus [96]. Multiple venous tributaries drain into the SOV including the superior vortex, lacrimal, muscular, inferior orbital and CRV [70].

The CRV is the main source of venous drainage from the retinal circulation. It travels centrally within the optic nerve, exiting 2mm behind the CRA entry point before continuing to run posteriorly below it. The CRV then either drains into the SOV, into other intraorbital venous branches or directly into the cavernous sinus [61].

The vorticose veins, of which there are normally four, are the main source of venous drainage for the uvea. Each vorticose vein (superior and inferior lateral and medial) drains the corresponding quadrant of the choroid as well as the corresponding quadrant of the iris and ciliary body [83]. The superior medial and lateral vorticose veins ultimately drain into the SOV and the inferior lateral and medial vorticose veins drain into the inferior ophthalmic venous plexus [96]. The inferior ophthalmic venous plexus is the main component of the inferior orbital venous system. It runs posteriorly, close to

the orbital floor, and joins either the SOV or drains directly into the cavernous sinus. It receives contributing veins from the lower lid, lacrimal sac, inferior rectus and oblique muscles and the sclera (inferior vortex veins) [70, 96].

#### 1.2.3.5 Assessment of Ocular Blood Flow

A variety of different techniques are available for the assessment of ocular blood flow (OBF) however no single technique is able to provide all the relevant information about the ocular vasculature in one reading as each tends to be designed to measure a specific aspect of ocular perfusion from one specific ocular vascular bed. The technique selected for the assessment of OBF in any research therefore needs to correspond with the vascular bed of interest. Indeed OBF can be assessed at the level of the retina, ONH, choroid or retrobulbar vessels and can either be measured directly or indirectly in terms of blood flow velocity, pulsatile OBF or vessel diameter changes. More recently enhanced techniques such as dynamic retinal vessel analysis (DVA), which enables retinal vessel reactivity to be assessed and retinal oximetry, which provides a direct assessment of retinal vessel metabolism, have also been introduced [97 98]. An overview of the techniques currently available for the assessment of OBF is given in table 1.2.

| Method | Vascular bed and measurement | Advantages | Disadvantages |
|---|---|---|---|
| Color Doppler Imaging (CDI) | Retrobulbar vessels (OA, CRA, CRV, PCA)<br><br>Blood flow velocity | Non-invasive, high reproducibility, quick | Poor inter-observer variability, retrobulbar assessment only, low resolution for smaller vessels |
| Pulsatile OBF (POBF)<br><br>(Langham OBF system; Laser interferometry) | Choroid<br><br>Pulsatile OBF via IOP pulse wave or interferometry | Simple, non-invasive | Provides an approximation only and influenced by IOP and gender |
| Laser Doppler flowmetry (LDF) | Choriod, ONH (depending on wavelength used)<br><br>Capillary blood flow | Direct assessment of blood flow, non-invasive | Restricted to a small measurement area, exact volume of tissue measured unclear, inter-individual comparisons poor |
| Laser Doppler velocimetry (LDV) | Retina<br><br>Blood flow velocity | Simple, quick, quantitative, | Single vessel only |
| Heidelberg Retina Flowmeter (HRF)<br><br>(= a scanning laser Doppler flowmeter) | Retina, ONH<br><br>Capillary blood flow | Non-invasive, does not require dilation, quick, direct assessment of OBF | Requires clear media and good fixation. Very sensitive to illumination and measurement window changes, limited reproducibility |
| Dynamic Retinal vessel analysis (DVA) | Retinal vessels (>60μm)<br><br>Retinal vascular diameter and reactivity | Non-invasive, high reproducibility, low variability | Requires clear media, good fixation and pupil dilation. More aimed at assessing retinal vascular function than OBF |
| Fluorescein angiography (in conjunction with scanning laser ophthalmoscope) | Retina<br><br>Blood flow velocity | Provides useful information on ocular perfusion | Debatable correlation of passage of time of dye to OBF. Invasive. Difficult to quantify |
| Indocyanine green angiography (in conjunction with scanning laser ophthalmoscope) | Choroid<br><br>Blood flow velocity | Provides useful information on ocular perfusion | Debatable correlation of passage of time of dye to OBF. Invasive. Difficult to quantify |
| Laser speckle analysis | Retina, ONH<br><br>Blood velocity | Provides overall map of retinal blood flow if used with scanning. Non-invasive | Not direct assessment of OBF |
| Blue field entoptic technique | Foveal retina | Allows assessment of foveal perfusion, non-invasive | Dependent on patient cooperation, subjective, limited to foveal area, large inter-individual variation |
| Retinal Oximetry | Retinal vessels | Allows assessment of retinal metabolism | Risk of influence by external factors e.g light |

**Table 1.2: Overview of techniques available for assessment of ocular blood flow**

## 1.3 Glaucomatous Optic Neuropathy

Whilst glaucomatous damage to the visual system has been demonstrated to involve pathological alterations in numerous areas, including the RGC bodies [99, 100], photoreceptors [101, 102], lateral geniculate nuclei (LGN) [103, 104] and visual cortex [104], it is the lamina cribrosa of ONH which is considered the principal site of RGC axon insult [105-107]. Indeed profound alterations within the prelaminar, laminar and peripapillary sclera tissues of the ONH have been identified by animal studies in the earliest detectable stages of experimental glaucoma [108-110]. Such alterations, combined with RGC loss, contribute towards the characteristic enlargement and deepening of the optic cup and thinning of the NRR observed clinically in GON (figure 1.6) [111, 112]. Indeed prelaminar thinning, posterior deformation of the lamina cribrosa and excavation of the ONH have been identified as key features of GON [111, 113]. NRR thinning, indicative of nerve fibre loss, is unique to glaucomatous nerve damage and has a predilection for the inferotemporal and superotemporal regions of the ONH, extending to involve the temporal region as the nerve damage progresses before finally affecting the nasal region in advanced glaucoma [114, 115]. Visual field and retinal NFL defects, characteristic of GON, develop in correspondence to the NRR thinning and subsequent excavation of the ONH [67, 116, 117].

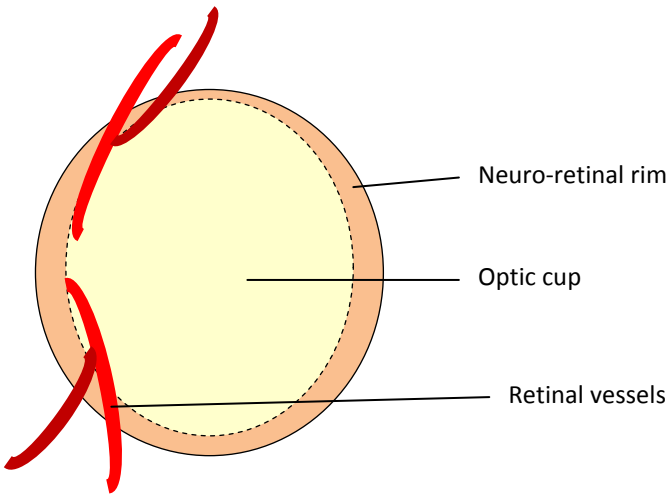



**Figure 1.6: Diagrammatic representation of the optic nerve head in glaucoma**

Whilst the clinical picture and features of GON are fairly well evidenced, the causes of the primary insults which trigger the cascade of events leading up to its development are less clear and have subsequently become a key focus of current glaucoma research.

The evidence surrounding the potential role of elevated IOP in the developmental process will now be outlined in brief, followed by a detailed discussion of the vascular concept of glaucoma.

### 1.3.1 The Role of Intraocular Pressure

Traditionally the management of glaucoma has focused on the therapeutic reduction of IOP to below a certain target level with the aim of limiting the progression of ONH damage and visual field loss [118]. There is no doubt that IOP is a relevant pathogenetic factor in the development of GON and since the introduction of the Schiotz tonometer in 1905, followed by the Goldmann applanation tonometer in 1954 [9], it has been the focus of the majority of diagnostic examinations and considered the only modifiable risk factor of glaucoma.

By definition IOP is a mechanical entity, referring to the normal force per unit area exerted by the intraocular fluids on the tissues that contain them [119]. The normal range of IOP is defined as between 10-21 mmHg [118] and rather than being stable it exists in a state of constant flux, varying with cardiac and respiratory cycles and being influenced by factors such as posture and diet [120]. Numerous studies have explored the normal circadian variations in IOP that occur over a 24 hour period [121-123] and whilst it was initially believed that IOP levels were at their highest in the morning, lower later in the afternoon and at their lowest at night [120], other studies have suggested that IOP levels may in fact reach their highest levels nocturnally [124-126], regardless of whether measurements are being taken in the sitting or supine position

[127]. Deviations away from this normal circadian rhythm have been demonstrated in those diagnosed with glaucoma and it is possible that abnormal fluctuations in IOP could increase the risk of GON development [128-130]. This has led to the suggestion that 24 hour measurement of IOP may be beneficial in at risk patients[130, 131].

By its very nature IOP exerts force on the lamina cribrosa, astroglia and axons of the ONH and when IOP is increased so is the strength of this mechanical force [132]. As a result, the mechanical theory of glaucoma development proposes that in susceptible individuals abnormally elevated IOP damages the ONH by placing high levels of stress and strain on its tissues, ultimately leading to deformation of the cribriform plates of the lamina cribrosa and glial cell activation, followed by compression of the optic nerve fibre bundles and nerve fibre damage [109, 111, 133]. Indeed support for this theory comes from multiple studies which have demonstrated GON development in response to experimentally increased levels of IOP which progresses according to severity and duration of elevated IOP exposure [8, 134, 135]. The overall susceptibility of the ocular structures to the effects of IOP however appears to vary between individuals as a function of the individual eye's anatomy and composition [111, 119] and such variation is perhaps demonstrated by the fairly high occurrence of the condition 'ocular hypertension' (OHT), whereby elevated IOP exists in the absence of GON and by the occurrence of NTG and of progressive glaucoma, in which nerve damage continues to progress despite therapeutically lowered IOP. Therefore, whilst there is undoubtedly a lot of evidence for the role of IOP in the pathogenesis of glaucoma [8] and for the benefits of IOP reduction in the treatment of management of glaucoma [36-38] it is clear that alternative or additional causative factors need to also be considered with regard to the development of GON in the majority of cases. The most researched of these factors is the involvement of vascular abnormalities.

Disturbed vascular function is a concept which has long been recognised with regard to GON, with authors as early as 1925 proposing an alternative vascular theory of glaucoma development along with the aetiological involvement of microcirculatory disturbances and vascular dysregulation [136, 137]. The role of both ocular and systemic vascular factors in the development of GON has been subsequently explored by numerous researchers; however our understanding of the complex interactions between these and the many other factors implicated in the aetiology of the disease is still incomplete.

It is possible that rather than existing as two separate entities the so called mechanical and vascular theories of glaucoma could be intertwined and act synergistically to produce glaucoma, a concept first proposed by Flammer in 1985 [138]. Indeed elevated IOP can potentially influence ocular haemodynamics by subsequently raising venous pressure at the exit point of the eye and lowering the OPP (see section 1.4.1) [139]. Furthermore large diurnal fluctuations in IOP could potentially alter the quality and stability of the blood supply to the ONH and also contribute to GON development [140]. Alternatively it has been hypothesised that even if the primary insult of the ONH were not mechanical in origin, IOP related stress and strain could still contribute towards its final deformation [111]. The vascular theory of glaucoma forms the focus of this thesis and will now be explored in detail.

### 1.3.2 The Vascular Theory

The concept that the quality of the blood supply to the ONH is altered in glaucoma is fairly well established and increasing amounts of evidence suggest that vascular insufficiency at the ONH plays an important role in GON development [95, 140-142]. It is hypothesised that an alteration in blood supply, which could result from the direct or indirect actions of a combination of risk factors, contributes to GON development through ischemic and hypoxic insult of the ONH tissues, including the RGC axons,

astrocytes, glial cells, pericytes and the central retinal vessels [140]. It is further hypothesised that the ischemic/hypoxic insult of the ONH tissues could produce its damaging effects through mechanisms of recurrent reperfusion injury (RRI) and oxidative stress [141]. RRI is central to the current vascular theory of glaucoma and refers to the damage to a tissue caused when blood supply returns after a period of ischemia [143]. This can lead to increased production of harmful ROS, which, if not combated by antioxidant activity, can potentially damage the ONH cellular components, leading to apoptotic loss of RGC and their axons. This in combination with astrocyte activation and an increased production of vasoactive agents such as ET-1, is thought to contribute to the development of GON [144]. The concept of RRI and oxidative stress with regard to GON development is discussed further in section 1.10.3.2.

Confirming these vascular hypotheses and determining the combination of factors which contribute towards the reduction in the quality of the blood supply to the ONH in glaucoma is one of the main focuses of this thesis and current glaucoma research as a whole. Of particular interest is the exploration of the role that vascular factors play in NTG and progressive glaucoma development, where IOP involvement is less prominent. At the current stage aetiological roles for both ocular and systemic vascular abnormalities have been identified in the development of GON. These abnormalities extend from reductions in blood flow, through to disturbances in vascular regulation and increases in vessel rigidity at both levels. In addition systemic dysregulations such as peripheral vasospasm and endothelial and ANS dysfunctions, as well as high levels of oxidative stress, have been advocated to play an important part in the development of glaucoma. Alongside this alterations in factors such as BP and HR, which can influence overall vascular physiology, have also been implicated however many questions still remain. A summary of how these many ocular and systemic vascular abnormalities may link together in the development of GON is

given in section 1.11 at the end of this chapter (figure 1.16). The physiology of OBF along with the current evidence surrounding the involvement and interactions of each of these factors will now be explored in detail.

## 1.4 Ocular Blood Flow

The overall blood flowing through the body is determined by the cardiac output, which itself depends upon stroke volume and heart rate. The distribution of this cardiac output between vascular beds varies according to the hemodynamic situation of the body and is influenced by systemic factors such as the ANS and circulating hormones [78]. Within each vascular bed local factors strive to regulate the supplied blood flow to meet local needs. At the ocular level this regulation of blood flow has been identified as a multi-factorial and complex process [95], dependant on both ocular perfusion pressure (OPP) and vascular resistance. Indeed the rate of blood flow through any vascular bed is known to be directly proportional to the pressure gradient and inversely proportional to resistance [145].

### 1.4.1 Ocular perfusion pressure

OPP refers to the force of the blood flow through the intraocular vessels and is defined as the difference between the arterial and venous pressure. As a direct measure of arterial and venous pressure is not easily obtained from the retinal circulation, estimations have to be made in order to determine OPP. Experimentation has revealed that, due to the drop in BP between the heart and the OA, retinal arterial pressure can be estimated as 2/3 of the mean arterial pressure (MABP) and venous pressure can be taken to be approximately equal to the level of IOP [78, 146], as a result OPP can be calculated as shown in equations 1.1 and 1.2

$$OPP = \frac{2}{3}\, MABP - \, IOP$$

OPP = Ocular perfusion pressure

MABP = Mean arterial blood pressure

IOP = Intraocular pressure

**Equation 1.1: Calculation of ocular perfusion pressure**

**Where:**

$$MABP = \frac{2}{3}\, DBP + \frac{1}{3} SBP$$

MABP = mean arterial blood pressure

DBP = Diastolic blood pressure

SBP = Systolic blood pressure

**Equation 1.2: Calculation of mean arterial blood pressure**

The relationship between blood flow and OPP in the eye is complex and as the equation above demonstrates both a lowering of MABP and an increase in IOP could potentially reduce OPP.

### 1.4.2 Vascular resistance

Vascular resistance, in addition to OPP, also plays a role in determining the amount of blood that flows through a vascular bed and itself is determined by blood viscosity, vessel length and vessel diameter [78]. In the retinal vascular bed, due to constant capillary perfusion and a lack of precapillary sphincters, alterations in vessel length are not thought to play an important part in the regulation of OBF [78, 147]. Furthermore, whilst alterations in blood viscosity have been found to substantially influence retinal blood flow [148], such alterations to not tend to occur without the presence of underlying pathology, such as sickle cell anaemia and other blood disorders [149], therefore the role that blood viscosity plays in the regulation of OBF in health is also not considered

significant. Vessel diameter on the other hand plays a major role in determining retinal vascular resistance and OBF. Vascular resistance is known to be inversely proportional to the fourth power of the radius of a blood vessel, hence only small changes in vessel diameter can significantly influence vascular resistance, with narrowing of the vessel diameter increasing resistance and reducing blood flow and widening of vessel diameter, reducing resistance and increasing blood flow. Reduced/unstable OBF could therefore occur, not only as a result of reduced OPP, but also as a result of an increase in vascular resistance due to abnormal alterations in vessel diameter, which themselves could occur as a result of either structural changes in the vessel wall or the presence of a functional dysregulation [95].

Under normal physiological conditions the retinal vasculature exists in a state of partial constriction, referred to as the basal vascular tone. This state of partial constriction is one from which vessel diameter can be readily modified in response to hemodynamic alterations, such as a drop in OPP, to ensure maintenance of a constant blood supply to tissues and organs by a mechanism referred to as autoregulation [150]. Autoregulation is an important mechanism which acts in response to alterations in perfusion pressure through modification of vascular resistance. The autoregulation of blood flow will now be discussed in detail.

### 1.4.3 Autoregulation

Autoregulation refers to the ability of the cardiovascular system to modify vascular resistance in order to allow a constant blood supply to be maintained despite variations in perfusion pressure [151]. It operates to ensure tissues and organs receive an adequate blood supply despite variations in hemodynamic conditions and has been demonstrated in both the retinal [86, 152] and ONH circulation [94] as well as, to a lesser extent, in the choroidal circulation [81].

The most common method of evaluating autoregulatory function is through assessing the response of the ocular and systemic vasculature to provocation. This provocation can take the form of posture changes, artificial lowering of IOP, cold provocation, hand grip testing, flicker light stimulation or induced hypoxia and hypercapnia. Such provocations put the vascular system under stress and should evoke an autoregulatory response which allows maintenance of normal ocular perfusion, a failure to observe this autoregulatory response is indicative of disturbed autoregulation.

Although the exact mechanisms underlying autoregulation are still unclear, metabolic, myogenic, neurogenic and humoral factors are all known to trigger autoregulatory responses in the ocular circulation, as are endothelial derived vasoactive agents [151, 153], as summarised in figure 1.7. These autoregulatory triggers and their responses are outlined in the following sections

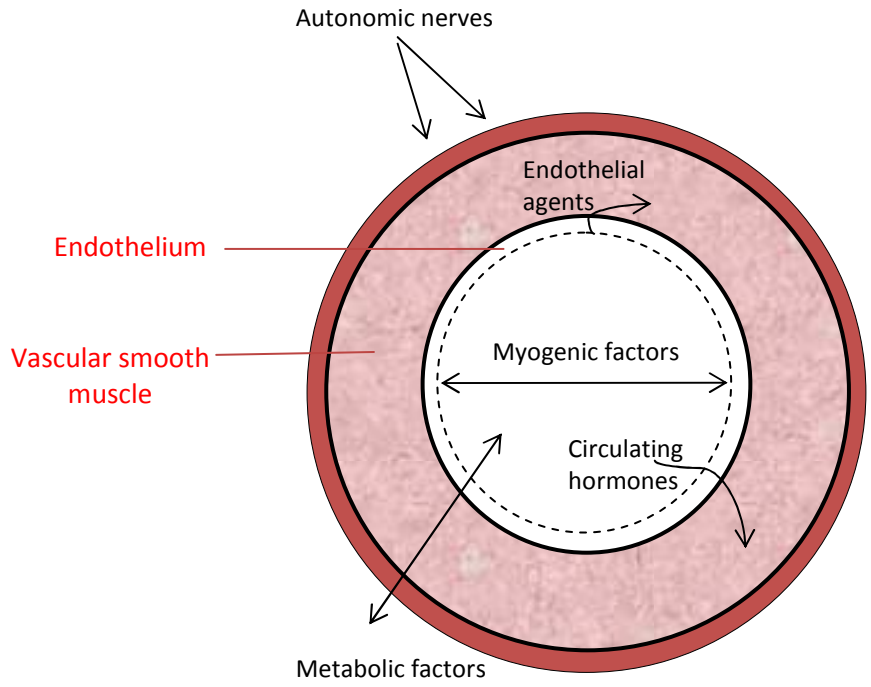



**Figure 1.7: Summary of the factors which trigger autoregulation of blood flow in the ocular circulation**

### 1.4.3.1 Metabolic autoregulation

Metabolic autoregulation refers to the regulation of blood flow in accordance with tissue metabolite concentration. A tight coupling mechanism is thought to exist between tissue metabolism and ocular perfusion [151], with alterations in the local concentrations of metabolites including oxygen ($O_2$) and carbon dioxide ($CO_2$) [154, 155], potassium ($K^+$), hydrogen ($H^+$) [156] and adenosine [157] having been shown to influence ocular vascular tone.

Partial oxygen pressure ($pO_2$) has been identified as one of the main driving forces of metabolic autoregulation [158, 159]. Under conditions of systemic hyperoxia and hypoxia autoregulatory mechanisms act to maintain retinal and ONH $O_2$ at constant levels. Hyperoxic conditions trigger retinal arteriolar vasoconstrictions, reducing retinal blood flow and $pO_2$ [154, 160] and hypoxic conditions trigger retinal arteriolar vasodilations, increasing retinal blood flow [161] and allowing normalisation of $pO_2$. The hemodynamic response of the retinal vasculature to hyperoxia is thought to be mediated by endothelin [162], whereas the hypoxia-induced vasodilation of the retinal vasculature is thought to involve endothelial derived prostaglandins and/or adenosine [163-166]. The choroidal vasculature, in comparison, has been demonstrated to show little or no alterations in response to changes in blood oxygenation [167, 168].

Under conditions of hypercapnia, in which the partial pressure of $CO_2$ ($pCO_2$) is increased, metabolic autoregulatory mechanisms have been demonstrated to function in the retinal, ONH and choroidal circulation bringing about a vasodilatory response of the vasculature which results in an increase in blood flow and $pO_2$ [167, 169, 170]. The exact mechanism behind hypercapnia induced vasodilation is still the subject of debate, however it is thought that interactions between nitric oxide (NO) and endothelial derived prostaglandins play an important role [171].

### 1.4.3.2 Myogenic autoregulation

Myogenic autoregulation refers to the regulation of blood flow in response to alterations in systemic BP and allows a constant blood flow to be maintained despite variations in BP [151]. The myogenic response was first described by Bayliss in 1902 and is characterised by a decrease in vessel diameter following an increase in transmural pressure [172]. Its primary function in the body is to maintain the Starling equilibrium for capillary fluid exchange; however it is unclear whether this is also its primary function in the eye [173]. Myogenic autoregulation is on the whole considered to be mechanically independent of the endothelium and intrinsic to the vascular smooth muscle cells (vSMCs), whereby stretching of the vessel wall is thought to lead to depolarisation of the vSMC membrane and vascular constriction [174]. In the cerebral and renal circulation however myogenic induced vasoconstriction has also been suggested to be at least partly mediated by endothelial factors [175]. Whilst myogenic regulation has been demonstrated in the ONH and retina [176] it is unclear whether myogenic mechanisms are also involved in the regulation of choroidal blood flow [173].

### 1.4.3.3 Neurogenic control

The eye has a rich autonomic innervation however this only extends to the uvea, PCAs and the extraocular portion of the CRA [177-179] and does not include the retina and prelaminar portion of the ONH [179, 180]. Neuronal regulatory mechanisms are therefore suggested to play a key a role in the regulation of choroidal blood flow but have little effect on retinal or ONH blood flow [181, 182], despite alpha and beta-adrenergic receptors having been identified in the retinal vessels [183, 184]. Sympathetic stimulation of the choroid, via sympathetic nerves originating from the superior cervical ganglion, triggers constriction of the choroidal blood vessels and increases choroidal vascular resistance, reducing blood flow [80, 182]. It has been suggested that this vasoconstriction response of the uveal vasculature may function to protect the eye against overperfusion during periods of increased HR or BP [185] and

indeed there is evidence demonstrating that under conditions of exercise, where sympathetic activity is increased, a regulatory vasoconstriction response of the choroidal vasculature allows maintenance of a constant blood flow in the face of an increase in OPP [81, 186]. Numerous agents have been implicated as mediators of this neuronal regulation including acetylcholine [187], noradrenaline [187], vasoactive intestinal polypeptide [188], substance P [189] and NO [190], however the exact role played by each of these agents in ocular circulation physiology is still unclear.

Parasympathetic nerves reach the eye through the oculomotor nerve, facial nerve and through the ophthalmic and maxillary divisions of the trigeminal nerve [78, 178] and there is evidence to suggest that parasympathetic innervation can stimulate a vasodilation response in the choroidal vasculature and increase blood flow. This evidence is variable however, as whilst intracranial stimulation of the facial nerve has been demonstrated to cause significant vasodilation in the choroid [80, 191], electrical stimulations of parasympathetic nerve fibres of the ciliary ganglion, although inducing intense miosis, have not been found to notably change the uveal vascular resistance [182]. The role of parasympathetic innervation in ocular neurogenic regulation is therefore uncertain.

#### 1.4.3.4 Humoral Control

Humural control refers to the potential regulatory influence of numerous vasoactive agents present in the circulating blood which, through either direct interaction with the vascular smooth muscle cells (vSMCs) and pericytes or through mediation of endothelial cells [192], could influence OBF. Angiotensin and catecholamines for example, which are both circulating hormones, have been suggested to influence retinal and choroidal circulation, however the evidence is variable. Indeed whilst angiotensin-II receptor binding sites have been identified in ocular tissue [193], suggesting a role for the renin-angiotensin-system in OBF regulation [194], the majority

of data seems to suggest that in human healthy subjects this system does not play a major regulatory role [173, 195]. Furthermore a similar uncertainty surrounds the role of catecholamines in OBF regulation after both a decrease [196] and an increase [197] in retinal perfusion following administration of adrenergic drugs has been demonstrated. Therefore whilst the potential influence of circulating and local hormones should not be discarded the evidence suggests that they are unlikely to have a major impact on the regulation of the choroidal and retinal blood flow. Furthermore the presence of the BBB prevents direct contact between the circulating blood and the retinal and ONH vSMCs indicating that the role of circulating hormones in the regulation of ONH blood flow in particular may be even less; however some diffusion of molecules from the choroidal vasculature may occur in the prelaminar region of the ONH due to the structural differences in this region (see section 1.2.3.3).

#### 1.4.3.5 Endothelial dependent regulation of vascular tone

The vascular endothelium is an important mediator of vascular tone, releasing vasoactive agents both under basal conditions and in response to various chemical and mechanical stimuli. These vasoactive agents are commonly referred to as endothelial derived constricting factors (EDCF) and endothelial derived relaxing factors (EDRF) and they play an important role in the regulation of OBF [156]. The endothelium and its regulatory roles are discussed in more detail in the following section.

## 1.5 The Endothelium

The vascular endothelium plays a critical role in the regulation of blood flow at both the ocular and cerebral level and as such a dysfunction of the vascular endothelium is commonly implicated as a possible causative factor in vascular disease [198, 199].

### 1.5.1 Background

The endothelium is a highly specialised monolayer of cells which lines the inner surface of all of the blood vessels in the circulatory system (figure 1.8) [200]. Its strategic anatomical position between the blood components and the vSMCs and pericytes places it in an ideal position to monitor and regulate vascular homeostasis [199] in response to mechanical (e.g. shear stress), chemical (e.g. oxygen tension) and biological stimuli (hormones and vasoactive agents). [201]

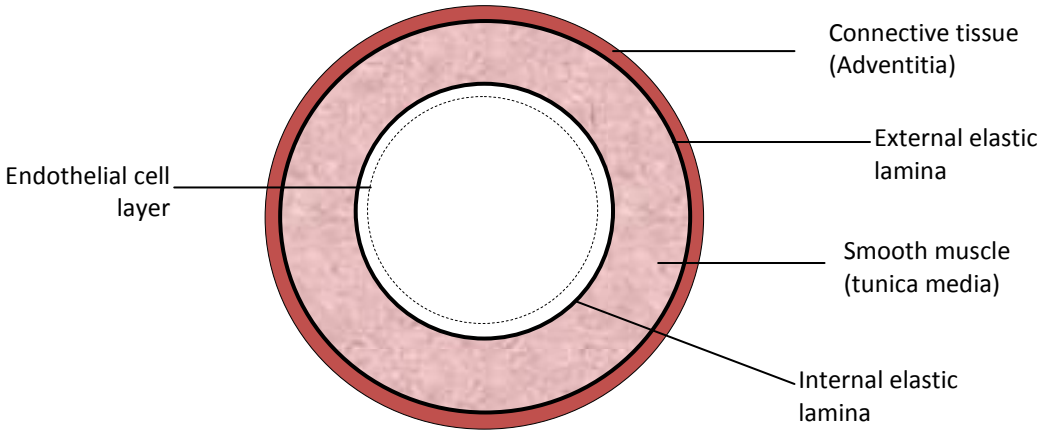



**Figure 1.8 Diagrammatic representation of the structure of the vascular wall and the location of the vascular endothelium**

The most critical role of the endothelium is as an active regulator of vascular tone [202-205] however its other functions include inhibition of vSMC proliferation, regulation of inflammation, thrombosis and platelet aggregation, angiogenesis and control of vascular permeability [199, 201, 206]. The endothelium exerts its functional roles through the release of a variety of regulatory agents following stimulation. Its critical role as an active regulator of vascular tone is achieved through the release of endothelial derived vasoactive factors (EDVF) which act on the underlying SMCs to bring about either vasodilation or vasoconstriction of the vessel wall (table 1.3). Of these EDVFs, nitric oxide (NO) has been identified as the most potent vasodilator and endothelin-1 (ET-1) as the most potent vasoconstrictor [207-209] (see sections 1.5.2 and 1.5.3)

| | |
|---|---|
| **Endothelial derived relaxing factors (EDRF)** | Nitric oxide (NO), prostacyclin ($PGI_2$), endothelial derived hyperpolarising factor (EDHF), adenosine triphosphate (ATP), substance P, acetylcholine, C-type natriuretic factor |
| **Endothelial derived constricting factors (EDCF)** | Endothelin-1 (ET-1), angiotensin II, thromboxane $A_2$, prostaglandin $H_2$, superoxide anions, ATP |

**Table 1.3: Summary of the vasoactive agents released by the vascular endothelium**

### 1.5.2 Nitric oxide (NO)

NO was first recognised in 1980 by Furchgott and Zawadski as the primary vasodilating agent released by the vascular endothelium [202]. It is synthesised during the oxidation of the amino acid L-arginine to L-citrulline within the endothelial cell membrane, by the enzyme endothelial nitric oxide synthase (eNOS) [210]. Two other isoforms of nitric oxide synthase (NOS) also contribute to NO production, namely neuronal NOS (nNOS) which is found in some neurons of the central and peripheral nervous system [211] and inducible NOS (iNOS) which is only expressed after a challenge by immunological or inflammatory stimuli [212]. All three isoforms of NOS have been identified in the ocular circulation [213-215].

There is a constant basal production of NO by the endothelium, which contributes to the maintenance of optimum vascular tone [207, 216]. However, on stimulation of the endothelial cell membrane by chemical, mechanical or biological factors, NO production is increased [217-219]. This increase in production is achieved via activation of both a calcium/calmodulin complex and the NOS enzyme, following an influx of calcium across the cell membrane [210]. Diffusion of NO from the endothelium into the neighbouring SMCs ultimately brings about a vasodilatory response of the vessel wall through stimulation of the guanylate cyclise enzyme and increased production of cyclic guanine monophosphate (cGMP) [220, 221] (figure 1.9).

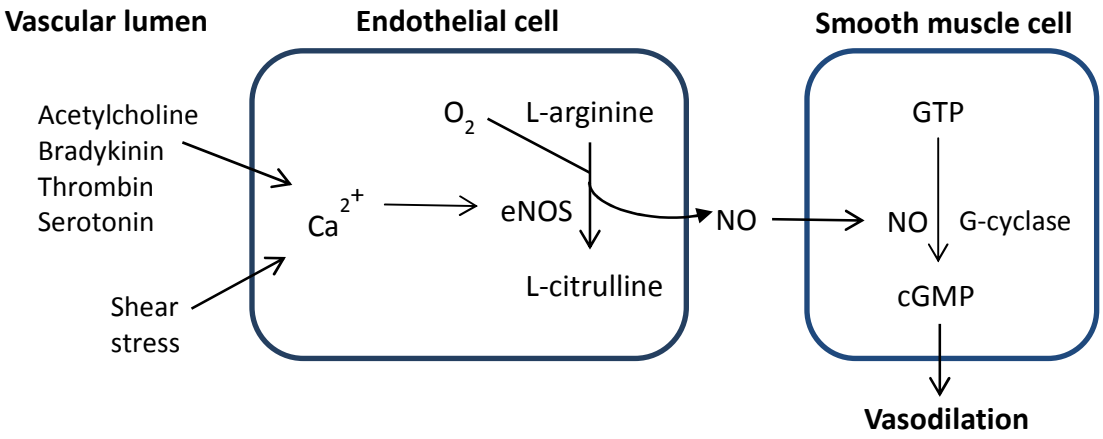



**Figure 1.9: Mechanism of endothelial nitric oxide (NO) synthesis. $Ca^{2+}$: calcium ions; eNOS: endothelial NO-synthase; $O_2$: oxygen; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate**

NO plays an important role in the regulation of physiological functions in the cardiovascular system and the central and peripheral nervous systems. In addition to its vasodilatory role its range of functions extend to include anti-platelet, antithrombotic, anti-proliferative and anti-atherosclerotic actions [222]. At the ocular level, NO has been identified as a physiological mediator in not only the retinal circulation but also the OA, PCA and the choroid [222, 223]. Furthermore NO has also been suggested to play a functional role in the conjunctival vasculature, the cornea, the lens epithelium, the ciliary body and the neural retina [224]. The NO mediated vasodilatory response is well evidenced in the ocular circulation and the important role played by NO in both the regulation of OBF and the maintenance of basal tone is well recognised [207, 225-227]. As such any disturbance in the production or release of NO by the endothelium can have a significant effect on the ocular haemodynamics. Indeed disturbances of the l-arginine/NO system have already been identified in numerous ocular diseases including diabetic retinopathy, glaucoma and retinopathy of prematurity [198, 222, 228].

### 1.5.3 Endothelin-1 (ET-1)

ET-1 is the most important vasoconstrictive factor released by the endothelium and is active not only in the eye but throughout the rest of the body [229, 230]. It is synthesised from the pre-propeptide big endothelin via an endothelin-converting enzyme dependent pathway in response to physiological stimuli such as hypoxia, ischemia and shear stress or in response to circulating substances such as thrombin, angiotensin II and cytokines [230, 231] (figure 1.13). The majority of its secretion occurs abluminally however there is also an element of intraluminal secretion which leads to a certain concentration of ET-1 in circulating blood [232, 233]. Two other isoforms of endothelin also exist, namely endothelin-2 (ET-2) and endothelin-3 (ET-3), each of which are derived from different genes and different cell types, however their roles are more poorly understood and less studied in comparison to ET-1 [229, 230].

The biological effects of ET-1 are governed by two different endothelin receptor subtypes, $ET_A$ and $ET_B$. ET-1 has a higher affinity for $ET_A$ receptors which are located primarily in vascular SMCs and are the main mediator of its potent vasoconstrictive effect [234, 235]. $ET_B$ receptors, which are non-selective towards the endothelin subtypes, are located primarily on endothelial cells, neurons and glia however they can also be found in vascular SMCs where they contribute toward the endothelin induced vasoconstrictive response [236]. Although at high concentrations ET-1 is known to bring about its potent and sustained vasoconstrictive effect, at lower concentrations it has in fact been demonstrated to produce a vasodilatory response [237, 238]. This vasodilatory response is mediated via the release of NO and/or prostacyclin following stimulation of the $ET_B$ receptors located on the endothelial cells [239] (figure 1.10). Under physiological conditions the net effect of ET-1 therefore depends upon the balance between the expression of $ET_A$ receptors in vSMCs and $ET_B$ receptors on endothelial cells. In pathological states however an upregulation of $ET_B$ receptors on vSMCs and a possible downregulation of $ET_B$ receptors on

endothelial cells always results in a predominant ET-1 induced vasoconstrictive effect [231, 240].

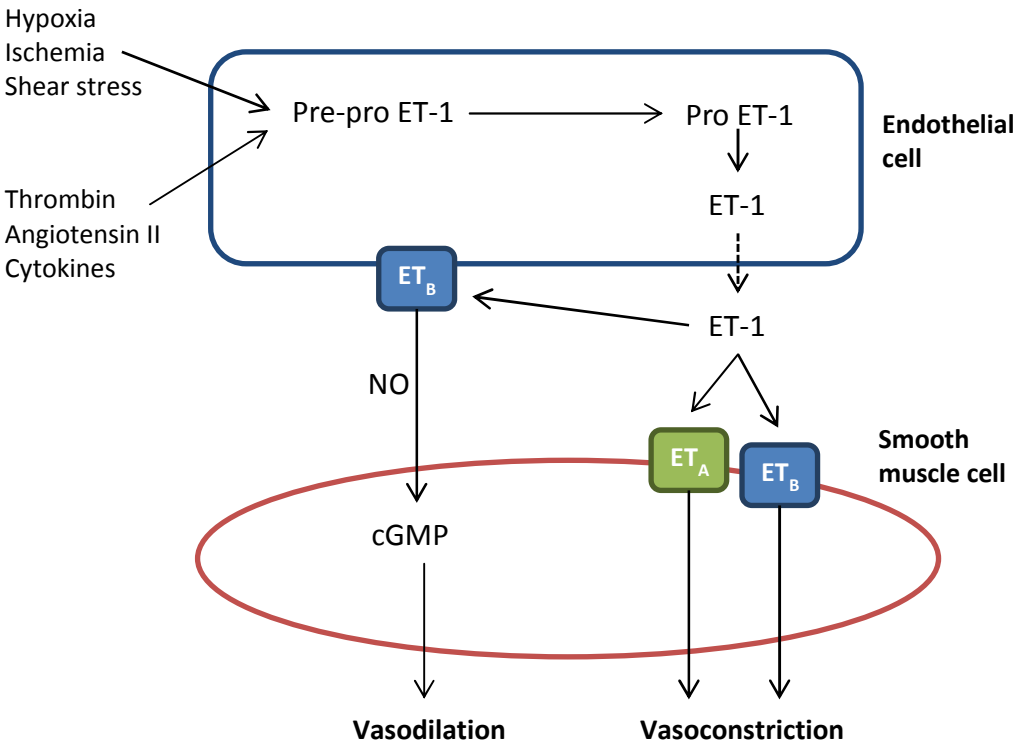



**Figure 1.10: Mechanism of Endothelin-1 (ET-1) synthesis. $ET_A$: endothelin type-A receptors; $ET_B$: endothelin type-B receptors; cGMP: cyclic guanosine monophosphate**

The functions of ET-1 extend from the regulation of vascular tone and local blood flow to neuronal support and signalling as well as the proliferation and migration of vSMCs [230, 241, 242]. Clinically endothelin has been associated with many pathological conditions including hypertension, congestive heart failure and coronary artery disease [243-245]. At the ocular level the endothelin system is thought to play a significant role in both normal and pathological processing, with $ET_A$ receptors having been identified in the retinal and choroidal vasculature as well as the iris and $ET_B$ receptors having been identified in the retinal neurons, glia and the lamina cribrosa of the ONH [246-248]. Indeed multiple studies looking at the alterations in OBF which occur on administration of systemic ET-1 have revealed a dose-dependent reduction in choroidal, retinal and ONH blood flow when ET-1 levels are increased [249-251]. Furthermore the role of ET-1 in the regulation of retinal blood flow [252] and choroidal

blood flow has also been confirmed, through for example, studies exploring the effect of $ET_A$ receptor antagonist infusion on choroidal blood flow during isometric exercise [253]. Due to its prominent role in the regulation of OBF, dysfunctions in ET-1 activity could potentially be implicated in the development of any ocular disease with an ischemic effect, such as glaucoma, via disturbances in autoregulation or vasospasm [254].

### 1.5.4 Endothelial dysfunction

Endothelial dysfunction refers to an altered ability of endothelial cells to perform their normal physiological functions [255, 256]. Its occurrence has been linked to the presence of established cardiovascular risk factors, such as hypertension, smoking, hypercholesterolemia, diabetes mellitus and obesity, chronic infections such as herpes viruses and cytomegalovirus, aging, cardiovascular diseases such as chronic renal failure, congestive heart disease and coronary artery disease as well as environmental factors such as hypoxia, oxidants, drugs and nutrition [231, 257-259]. In the presence of endothelial dysfunction the vasculature exists in a pro-inflammatory and pro-thrombotic state accompanied by increased atherosclerotic plaque formation, increased vascular tone and reduced vasodilatory responsiveness [201, 256]. These features are the result of a pathological alteration in the balance of mediators produced by the endothelium, characterised by:

- A decreased biosynthesis and/or bioavailability of NO - potentially resulting from either deficient NOS levels, increased levels of NOS inhibitors such as asymmetric dimethylarginine, apoptotic loss of NOS containing cells or rapid inactivation of NO after its release [198, 201]
- Increased production of vasoconstrictors such as ET-1 and angiotensin II
- An excess of oxidants

Endothelial dysfunction is known to be present at the earliest stages of a disease process and in addition to having been identified as an important early event in the pathogenesis of atherosclerosis [256] it has also been recognised as a preceding or predictive factor for the development of future cardiovascular disease [260-262] and in the development of glaucoma [198]. Early assessment of endothelial function in the peripheral and/or coronary circulation of at risk individuals could therefore provide important prognostic information about the risk of future disease [256, 263]. This, in addition to the fact that endothelial dysfunction has been shown to be modifiable in its early stages, has led to it becoming a research topic of great interest.

The precise cause of endothelial dysfunction is currently unclear however oxidative stress and the production of ROS is one of the main aetiological factors implicated to play a central role in its development and is a common denominator in the majority of the above mentioned associated conditions [257, 264]. Indeed increased production of ROS in the vascular system has been found to affect both the synthesis and activity of NO [265] and to promote the contraction of vSMCs [266]. Strategies aimed at reducing cardiovascular risk, such as smoking cessation and physical exercise as well as treatment regimes involving lipid-lowering therapy, angiotensin converting enzyme inhibitors and antioxidants have all shown to be effective at improving endothelial dysfunction to differing degrees [231, 267, 268].

### 1.5.5 Assessment of endothelial function

The ability to reliably detect and assess endothelial dysfunction is important with regard to the diagnosis, understanding and treatment of neurodegenerative diseases such as glaucoma and AD. The methods available can generally be separated into three categories, the first being the determination of the presence of soluble circulating endothelial markers such as NO, ET-1 and von Willebrand factor (vWf), the productions of which are known to be disrupted in the presence of endothelial

dysfunction, the second being the functional measurement of endothelial dependent vascular tone at focal sites of circulation [269] and the third being measurement of morphological and mechanical characteristics of the vascular wall, which can be altered in the presence of endothelial dysfunction. A summary of these methods is given in table 1.4.

The initial invasive methods of assessing endothelial function, such as quantitative coronary angiography and strain gauge plethysmography, have now largely been superseded by the introduction of non-invasive techniques such as brachial artery flow mediated dilation (FMD). Brachial artery FMD is considered the gold standard method of systemic endothelial function assessment in clinical practice and has therefore been the technique of choice in this thesis. Its principles are discussed in more detail in Chapter 3: Subjects and Methods. As a supplement to this technique an analysis of the circulating endothelial marker, vWf, was also made in this thesis.

vWF is a large glycoprotein which is synthesised exclusively by vascular endothelial cells and circulates in the human plasma at a concentration of approx 10 µm/mL [270]. It plays an important role in mediating platelet adhesion to damaged arterial walls [271] and its production is known to be increased in the presence of damaged endothelial cells [272, 273]. Indeed elevated levels of vWF have been identified in a number of different conditions known to be associated with endothelial dysfunction, such as atherosclerosis [272], diabetes [274], hypertension [275], cerebrovascular disease [276] and myocardial infarction [277]. Furthermore correlations have been identified between impaired FMD, the gold standard technique for assessment of systemic endothelial dysfunction and increased levels of circulating vWF [278, 279]. It is therefore recognised as a useful biomarker for the presence of endothelial dysfunction [270], however it is important to note that some questions still remain around whether elevated levels of vWf can be considered a true indicator of impaired endothelial dysfunction in all

cases [280]. This is largely due to its activity as an acute phase reactant, whereby its plasma levels may be increased in the presence of clinical conditions, such as infection or injury, which are not associated with endothelial dysfunction [280, 281]. In the absence of other acute phase markers, such as C-reactive protein however, elevated vWF levels can be more reliably attributed to endothelial dysfunction [282].

| Technique | Advantages | Disadvantages |
|---|---|---|
| **Circulating Markers** | | |
| Endothelin-1 (ET-1)<br>Von Willebrand factor (vWf)<br>Asymmetric dimethylarginine (ADMA)<br>Tissue type plasminogen activator<br>Plasminogen activator inhibitor-1<br>Intracellular adhesion molecules<br>Vascular cell adhesion molecules<br>E-selectin<br>P-selectin | Decreased bioavailability can be one of earliest detectable signs<br><br>Relatively simple technique | Can be difficult to distinguish between release via normal endothelial stimulation and endothelial damage<br><br>Large variations can occur day to day based on diet etc |
| **Nitric oxide (NO) production assays** | | |
| Urine NO concentration ($NO_3^-$)<br>Urine cGMP | Early indicator of endothelial dysfunction | Heavily affected by dietary habits |
| **Functional tests** | | |
| **Systemic**<br>– Coronary angiography | Sensitive indicator of endothelial function | Invasive<br>Poor repeatability and reproducibility |
| – Forearm venous occlusion plethysmography | | |
| – Coronary positron emission tomography | Non-invasive | |
| – Brachial artery ultrasonography (Flow mediated dilation, FMD) | Non-invasive<br>Repeatable<br>Sensitive indicator of endothelial function | Requires training and good control of testing environment to ensure reliability |
| **Ocular**<br>– Dynamic vessel analysis (DVA) | Non-invasive simple procedure. Good repeatability | Affected by high refractive error, hazy media and unstable fixation |
| **Mechanical and morphological vascular wall assessment** | | |
| – Pulse wave analysis<br>– Pulse contour analysis<br>– Pulse amplitude tonometry<br>– Intima-media thickness (IMT) | Non-invasive, repeatable, simple techniques | More indirect method of endothelial dysfunction compared to other techniques – less sensitive |

**Table 1.4: Methods of assessing endothelial function** [283, 284]

## 1.6 Ocular Blood Flow and Glaucoma

Evidence of reduced OBF, compromised circulation and increased vascular rigidity has been discovered by multiple studies at the level of the ONH [285-289], retina [25, 290-295], choroid [294, 296-298] and retrobulbar vessels [299-302] in patients with glaucoma and these blood flow reductions have been found to be prevalent in NTG and progressive patients [294, 303] and to correlate with deteriorations in visual function [289, 304, 305]. Furthermore multiple population based studies have linked lower baseline OPP to increased prevalence of GON [22, 306-308], with Tielsch et al [306] for example finding that those with perfusion pressures of less than 30 mmHg have a six times greater risk of developing POAG than those with perfusion pressures of more than 50 mmHg and a number of other longitudinal studies demonstrating baseline perfusion pressure as not only an important risk factor for the development of GON but also for the progression of the disease [15, 23, 24]. A full summary of these studies is given in appendix 1 and on the back of these findings reduced OBF is now considered an important IOP independent risk factor for the development of glaucoma. Concern was initially raised in the literature however about the possibility that the reduced blood flow detected in glaucoma patients, rather than being causative, could simply occur as a secondary effect to the loss of RGCs and reduction in OBF demand which occurs with the development of GON [95]. Multiple studies have however gone on to demonstrate reduced blood flow in glaucoma patients prior to the development of ONH damage [25, 286, 309] and further studies have linked reduced blood flow to disease progression [302], indicating a primary causative role. Furthermore, moving away from the eye, an increased occurrence of ischemic lesions of the ear [310], heart [311] and brain [312, 313] as well as reduced blood flow in the carotid arteries [314] and peripheral capillaries [26, 315, 316] have also been identified in glaucoma patients. The co-existence of such systemic blood flow alterations is an important observation as it would not be

expected if the reduced blood flow demonstrated in glaucoma patients was simply occurring as a consequence of the nerve damage.

The cause of the blood flow alterations detected in glaucoma patients has been the subject of debate. Rather than a sustained reduction in blood flow evidence suggests that the development of GON is more closely linked to unstable or fluctuating OBF [317]. Indeed the finding that conditions known to cause chronic reductions in blood flow, such as arteriosclerosis, are only weakly related to the development of GON [22, 318] and that other conditions also known to cause a chronic reduction in blood flow, such as multiple sclerosis, often lead to atrophy but not excavation of the ONH, provide support for this theory [233, 319]. Unstable OBF has subsequently become one of the most researched hypotheses of glaucoma development. The primary cause of this unstable blood flow is thought to be a dysfunctional autoregulatory mechanism. Indeed in the presence of disturbed autoregulation the ONH is susceptible to changes in OPP, IOP and BP, as well as to increases in local metabolic demand (see section 1.4). In basic terms, if blood supply is insufficiently regulated then the ONH and ocular structures are at risk of ischemic damage, RRI and subsequent RGC loss in periods when demand for blood and oxygen is high. The concept of disturbed vascular autoregulation in glaucoma will now be explored in more detail.

### 1.6.1 Autoregulatory Dysfunction

When confronted with an alteration in OPP, a change in vascular resistance or an increase in metabolic demand, autoregulatory mechanisms should act to adjust blood supply to ensure the needs of the ocular tissues are still met. If these autoregulatory mechanisms are defective however then the ocular tissues are at risk of exposure to ischemic episodes and RRI whenever metabolic demand is increased or blood flow parameters are altered. Defective autoregulation, through its contribution to unstable blood flow, could therefore potentially be linked to RGC loss, excavation of the ONH

and the GON development. Indeed multiple studies have provided evidence of disturbed autoregulation in glaucoma patients, not only at the ocular level [39, 320-322] but also at the systemic level [323, 324] and this disturbance has been found to be particularly noticeable in those with NTG [325, 326] and progressive glaucoma [327, 328].

The mechanisms by which OBF autoregulation may become defective are unclear, however it is important to consider that, in addition to the mechanism itself being defective, defects in the OBF autoregulation could also potentially occur if the capacity of normal autoregulation is exceeded. Indeed the capacity of autoregulation is not infinite and can only function within a certain range of OPPs [87, 139, 156]. If this range is exceeded, for example when IOP is exceedingly high (> 30 mmHg) or BP is exceedingly low (see equation 1.1), even a normally functioning autoregulatory mechanism would be unable to act to normalise blood flow and blood supply would be insufficient [87, 329]. With regard to glaucoma, autoregulatory dysfunction has been identified in both NTG and progressive cases where IOP is considered within the normal range [294, 303]. It is therefore considered more likely that in glaucoma patients, rather than the capacity of a normally functioning autoregulatory system having been exceeded, the autoregulatory mechanism itself may be defective in the majority of cases [139, 330]. Indeed exploring the causes, mechanisms and implications of such autoregulatory dysfunctions is now one of the main focuses of glaucoma research. As mentioned previously autoregulation of OBF is a complex process involving numerous different systems including the ANS (neurogenic control), systemic BP (myogenic control) circulating hormones (humoral control) and the endothelium (see section 1.4.3) and underlying abnormalities such as endothelial dysfunction, vasospastic syndrome and ANS dysfunctions have been implicated to play a role in the development of autoregulatory abnormalities in glaucoma [331]. Research is however ongoing and many questions still remain. These possible causes of defective autoregulation in glaucoma are summarised in figure 1.11 and the

background of these mechanisms with regard to the development of GON will now be discussed.

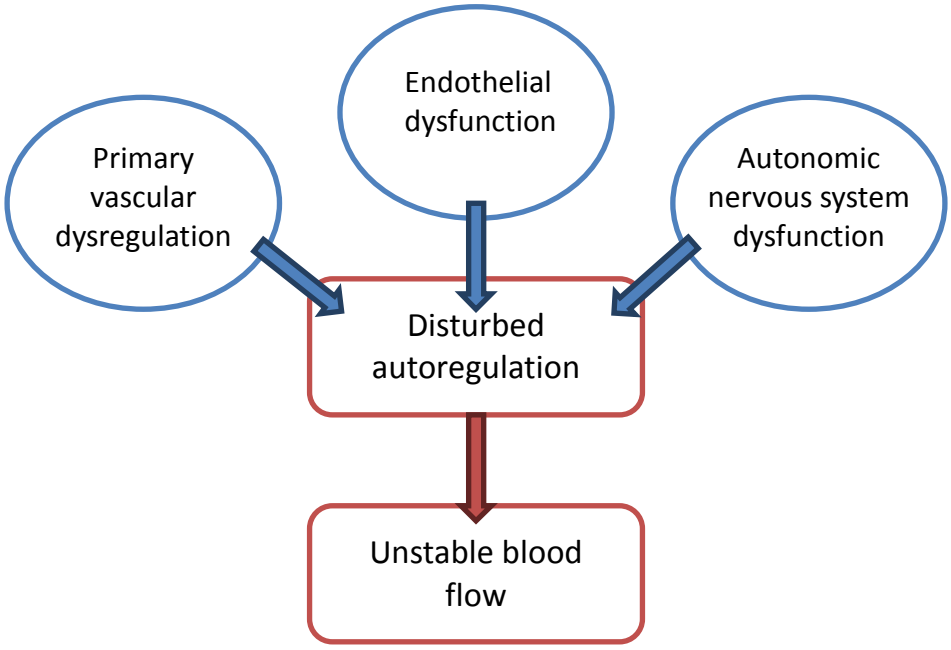



**Figure 1.11 Summary of the potential contributing factors to disturbed autoregulation and hence unstable ocular blood flow in glaucoma**

### 1.6.2 Vascular dysregulation syndrome

Vascular dysregulation is defined as an inappropriate constriction or inadequate dilation of the microcirculation when stimulated, combined with simultaneous dilations of the arteries or veins of neighbouring tissues [233]. It was a term first introduced in the context of glaucoma by Flammer in 1994, superseding the use of the term vasospastic syndrome, which it was decided did not encompass the full nature of the condition despite vasospasms still being considered responsible for inducing the majority of the associated symptoms [332].

Vascular dysregulation syndrome refers to the presence of a 'global vascular dysregulation' which affects many organs simultaneously or sequentially, including the heart, brain, fingers and eye [233, 333]. These affected regions are subjected to

episodes of local vasospasm and/or disturbed autoregulation often simultaneously [334]. This syndrome occurs in contrast to local dysregulations of blood flow which can develop in isolated regions in individuals as a result of pathological conditions such as endothelial or ANS dysfunction. Vascular dysregulation syndrome can be classified into two subcategories, namely primary vascular dysregulation (PVD) and secondary vascular regulation (SVD), with PVD being most relevant with regard to the development of GON.

#### 1.6.2.1 Primary Vascular Dysregulation syndrome (PVD)

PVD refers to the occurrence of vascular dysregulation in the absence of any underlying disease [333]. Individuals with PVD are usually otherwise healthy and do not require treatment [333]. Under baseline conditions these individuals can hardly be distinguished from others as PVD syndrome has little influence on baseline blood flow, however under conditions of stress, such as cold, psychological or mechanical stress, there is an inborn tendency for the vascular systems of these individuals to respond differently, showing a more frequent and intense vasospastic response which can result in reduced blood flow [233, 333]. This difference in response is thought to be due to a disturbance of the normal autoregulatory mechanisms in these individuals [332].

PVD occurs more frequently in women [335] and in those with low body mass index, type A personalities [333] and a Japanese ethnicity [336, 337]. Individuals with PVD syndrome have a tendency towards cold hands and feet [333], slower sleep onset times [338], low BP, particularly at night and when young [339] and a reduced feeling of thirst (drinking because they know they have to rather than because of thirst) [340]. They have also been shown to have a good sense of smell [141] and suffer more frequently from migraine than non-PVD individuals, however this is not a well established relationship [333]. Some individuals also show an altered sensitivity to drugs, such as

an increased response to calcium channel blockers and systemic beta blockers, possibly related to the lack of an ABC transporter protein [341]. Generally, all of these symptoms will first manifest in puberty and then decrease with age, showing a marked reduction after the menopause in women, but a possible increase again if they are treated with oestrogen-replacement therapy [141].

There is a large body of evidence associating PVD syndrome with the ocular circulation, with the first links being made in the early 1980's when diffuse or glaucomatous-like visual field defects, which fluctuated markedly and could spontaneously disappear, were noted in PVD sufferers [342, 343]. This occurrence was at the time referred to as ocular vasospastic syndrome and was the first indicator that the eye may be affected by what is now referred to as vascular dysregulation syndrome [344]. Further to this Gherghel et al [334], using Color Doppler imaging, found that individuals with vasospasm had decreased mean OPP and an increased resistivity index in the CRA compared to non-vasospastic individuals, providing evidence of both involvement of the retinal circulation and disturbed autoregulation in PVD sufferers. Indeed a number of ocular diseases have now been linked to the occurrence of PVD including, central serous chorioretinopathy [345], venous and arterial occlusions at a younger age [346], anterior ischemic optic neuropathy [347] and Susac syndrome [348]. Of relevance here however is the large body of evidence linking PVD syndrome to the development of GON, as discussed in the following section.

#### 1.6.2.2 PVD and glaucoma

The first associations, made between the presence of PVD and the development of glaucoma, arose from the finding that a minority of patients with the so called ocular vasospastic syndrome went on to develop GON [333]. The possibility that PVD and glaucoma could share a common underlying mechanism was suggested on the back of multiple studies demonstrating disturbed autoregulation and altered blood flow in

PVD sufferers, similar to that found in glaucoma patients. Indeed there is now a large body of evidence supporting the role of PVD in glaucoma development, particularly with regard to NTG and an equally large body of evidence demonstrating increased tendency towards vasospasm in glaucoma patients. The role of PVD in the development of NTG is supported by the finding that NTG and PVD share a number of similarities, for example both occur more frequently in women [29, 335] and those of Japanese ethnicity [30, 337]. Furthermore NTG patients suffer more frequently from migraine [55] as do individuals with PVD [349] and they suffer more commonly from silent myocardial ischemia [311, 350], of which PVD syndrome is a cause [351]. Additionally, the occurrence of flame haemorrhages at the optic disc, which develop more commonly in NTG [31] has been linked to the presence of PVD [352]. Other studies have identified direct signs of vasospasm or vascular dysregulation in NTG patients, for example, the presence of peripheral vasospasm in NTG patients, using nail fold capillaroscopy and cold provocation has been demonstrated on numerous occasions [26, 353, 354]. Furthermore, findings such as raised levels of ET-1, a potent endothelial derived vasoconstrictor [355-357], and of associations between NTG and the presence of migraine and Raynaud's phenomenon [55], also highlight the potential role of vasospasm in the development of NTG. Similarly, indirect evidence of vasospasm in NTG patients has been found by studies assessing vascular and functional responses to breathing the vasodilator $CO_2$, [358, 359] whereby it is hypothesised that significant increases in OBF parameters, recorded in response to $CO_2$, are suggestive of the vasculature having been in an initial vasospastic state.

The presence of PVD and vasospasm have also additionally been linked to the development of POAG, for example Goldberg et al [459] using the Hettinger Hand Vibration Test and Butt et al [361], both found signs of vasospastic tendencies in NTG and POAG patients, although the pattern of disturbance was slightly different between the two. Furthermore Rankin et al [301] found evidence of increased mean

resistivity index and decreased mean flow velocity in the OA and CRA of patients with POAG which were similar to those found in NTG patients. Other studies looking specifically at POAG patients have found that they show larger diurnal fluctuations in OBF parameters compared to controls, indicating unstable ocular perfusion and supporting the hypothesis that there may be an underlying vascular dysregulation in these patients as well [40]. Additionally, Hosking et al [362] assessed BP and blood flow parameter changes to induced hyperoxia and hypercapnia and found that POAG patients showed a larger increase in blood flow velocity in response to hypercapnia, a vasodilator, compared to controls but no significant change in blood flow in response to hyperoxia, a vasoconstrictor, to which controls showed a decrease in blood flow, again suggestive that there may be an initial state of vasospasm in POAG patients similar to that mentioned previously with regard to NTG.

It is possible that, in addition to disturbing blood flow regulation and contributing to ischemic damage, PVD may also render the eye more susceptible to changes in IOP. Indeed Schulzer et al [363] identified a distinct group of patients with vasospastic tendencies, which included both NTG and POAG patients, in whom a high positive correlation was found between mean deviation index of field severity and highest IOP. No such association was found in the other group who did not show vasospastic tendencies. Furthermore Hafez et al [364] found that vasospastic POAG and OHT patients showed the greatest improvement in NRR blood flow after sustained IOP reduction, compared to non-vasospastic patients and healthy controls, suggesting a closer relationship between OBF parameters and IOP level in patients with PVD syndrome compared to those without. Finally, Gugleta et al [328] found that progressive glaucoma patients who exhibited a vasospastic response which decreased choroidal blood flow by at least 10%, had lower IOPs compared to those with a milder vasospastic response, possibly suggesting that those with a greater degree of

vasospasm are more susceptible to IOP and therefore more likely to progress despite IOP being normalised.

On the basis of this plentiful evidence it has been suggested that PVD syndrome should be considered an independent risk factor for the development of glaucoma and its presence could potentially interfere with OBF either through the manifestation of disturbed autoregulation, or through the combined effects of hypotension, which is more prevalent in PVD, and reduced OPP [95]. A clinical diagnosis of PVD is largely based on history and symptoms and as such enquiries aimed at determining its presence, such as the occurrence of cold hands and feet or delayed sleep onset, are now recommended as routine practice in glaucoma [233]. A more reliable diagnosis of PVD can be made using nail fold capillaroscopy and cold provocation, whereby capillary blood flow is measured in the nail fold area before, during and after cooling to -15$^{o}$C and a reduced baseline blood flow velocity or a prolonged flow stop after cold provocation is considered indicative of PVD [349, 354, 365].

#### 1.6.2.3 Secondary Vascular Dysregulation

Secondary vascular dysregulation refers to the presence of a local or systemic vascular dysregulation which has developed secondary to an underlying disease, such as multiple sclerosis, rheumatoid arthritis or giant cell arteritis [141, 233]. Under conditions of stress or on stimulation, the vasculature of those with SVD exhibits a vasospastic response. The exact mechanism by which the underlying disease evokes this secondary vasospastic response is unclear, however a marked increase in levels of circulating ET-1, a potent vasoconstrictor released by the vascular endothelium, in response to inflammatory stress, has been identified in the majority of cases [233, 333].

SVD tends to cause a more or less constant reduction in baseline blood flow and has little effect on autoregulation, making it less relevant to glaucoma development [141]. At

an ocular level the effects can range from minimal to mild visual field reduction and a pale ONH [233]. As SVD syndrome does not interfere with autoregulation it is considered only a minor risk factor for the development of glaucoma [366] in comparison to PVD.

### 1.6.3 Endothelial dysfunction

Endothelial dysfunction could potentially occur in isolation or in combination with PVD and/or ANS dysfunction in glaucoma patients [367]. The concept and features of endothelial dysfunction were discussed in section 1.5.4 and there is now a fairly large body of evidence indicating the involvement of both ocular and systemic endothelial dysfunction in the dysregulation of blood flow and pathogenesis of both NTG and POAG. This endothelial dysfunction commonly takes the form of an imbalance in NO and ET-1 production, leading to reduced vasodilation, increased vasoconstriction and disturbed autoregulatory mechanisms in response to increased demand. The observation of both altered systemic and ocular endothelial dysfunction in glaucoma emphasises that, rather than just being a local ocular phenomenon, glaucoma may actually form part of a more global vascular dysfunction. The evidence linking endothelial dysfunction at both the systemic and ocular level to the development of glaucoma will now be discussed.

#### 1.6.3.1 Ocular Endothelial Dysfunction and glaucoma

Direct assessment of endothelial dysfunction at the ocular level is more challenging than that at the systemic level as the ocular vasculature is less accessible. Nevertheless, multiple studies have found evidence of reduced NO [368 369] and increased ET-1 levels [357, 370, 371], characteristic of endothelial dysfunction, in the aqueous humour of both NTG and POAG patients, however as well as relating to the vascular endothelium, these findings could also be indicative of endothelial

dysfunction in the cells lining the trabecular meshwork and Schlemm's canal. Stronger support for the presence of ocular vascular endothelial dysfunction in glaucoma therefore comes from functional studies. With regard to NO, abnormal OBF responses to systemic NOS inhibition, suggestive of reduced constitutive NOS at the ocular vascular level have been identified in POAG patients [372]. Furthermore abnormal or reduced neurovascular coupling responses of the retinal and ONH vasculature have been identified in early glaucoma patients following exposure to flicker light stimulation via dynamic retinal vessel analysis (DVA) [41, 373]. The concept of DVA, flicker light stimulation is discussed in more detail in Chapter 3: Subjects and Methods.

With regard to ET-1, in addition to raised levels in the aqueous humour, chronic ET-1 mediated ischemia of the ONH has been shown to induce glaucoma-like optic neuropathy in animals [374-376] suggesting a causative role. Furthermore the high sensitivity of the ocular circulation to changes in local ET-1 concentration has been demonstrated by the finding that intravenous application of ET-1 reduces pulsatile blood flow in the choroid and ONH at doses which show no effect on systemic haemodynamics [251].

The ability to assess the ocular endothelium is improving through the development of techniques such as DVA, however due to the wide variety of procedures available and the easy accessibility of the systemic circulation more plentiful evidence exists for the presence of endothelial dysfunction at the systemic level in glaucoma patients.

#### 1.6.3.2 Systemic Endothelial Dysfunction and glaucoma

A variety of different methodologies have demonstrated impaired NO activity and increased ET-1 activity along with reduced vasodilation and increased vasoconstriction responses, characteristic of endothelial dysfunction, in the systemic

vasculature of glaucoma patients. Considering NO first, reduced plasma levels of NO, cGMP and nitrite ($NO_2^-$), have been demonstrated in both POAG [377] and NTG patients [378]. Furthermore a higher plasma C-reactive protein level, a marker of reduced eNOS activity [379], has also been demonstrated in NTG patients [380], however this finding should be considered with caution as increased or normalised systemic levels of such markers have been demonstrated in glaucoma patients by other studies [381]. In addition to this biological evidence, functional assessment of the system.ic endothelium in glaucoma patients has also revealed evidence of NO mediated dysfunction. Indeed using venous occlusion plethysmography, a reduced vasodilation response in the forearm to both acetylcholine [382] and $ET_A$ receptor antagonism has been identified in NTG patients [383, 384]. Furthermore reduced brachial artery flow mediated dilation responses, indicative of impaired NO mediated vasodilation, have also been demonstrated in NTG patients [52] as well as POAG patients [385], although possibly to a lesser extent in this second group [42]. (These measurement techniques were introduced in section 1.5.5 and are discussed further in Chapter 3: Subjects and Methods, section 3.3.4)

With regard to ET-1, again from a biological point of view multiple studies have found increased levels in the plasma of NTG patients [355, 356] and progressive glaucoma patients [386]. However, the majority of studies report no difference in plasma ET-1 levels between POAG and healthy controls [357, 370, 387-389]. This suggests systemic vasoconstriction or vasospastic tendencies play less of a role in the development of POAG in comparison to NTG and progressive cases. Indeed, a further illustration of ET-1 abnormalities in NTG patients comes from functional testing, whereby abnormal changes in plasma ET-1 levels, indicative of disturbed autoregulatory mechanisms and systemic endothelial dysfunction have been identified in response to posture changes [390] and following peripheral cooling in NTG patients [391], suggesting abnormal ET-1 mediated vasoreactivity . This also ties in with the findings of higher occurrence

of vasospastic related conditions such as migraine [55], Raynaud's phenomenon and myocardial ischemia [311, 350] in NTG patients.

Plentiful evidence currently exists therefore for the presence of endothelial dysfunction at the systemic level in glaucoma patients. In addition to the above findings which relate to NO and ET-1, plasma levels of circulating von Willibrand factor (vWf), an established marker of endothelial damage, have been found to be abnormal in both POAG and NTG patients providing further evidence for the presence of systemic endothelial dysfunction in glaucoma [392]. Additionally, genetic studies have found evidence of polymorphisms in the eNOS gene in some familial POAG patients [393-395] and in the $ET_A$ receptor gene of some NTG patients [396, 397] suggesting there a possible genetic involvement in the development of endothelial dysfunction in certain cases.

#### 1.6.3.3 Summary of the role of endothelial dysfunction in glaucoma

A combined state of impaired vasodilation and increased vasoconstriction in the ocular and/or systemic vasculature of patients with glaucoma, as a result of endothelial dysfunction, is therefore hypothesised to play a key role in GON development through its significant impact on OBF regulation. Whether endothelial dysfunction occurs in isolation or in combination with other factors such as PVD or ANS dysfunction is currently unclear and little research has been conducted into the occurrence of simultaneous dysfunction of the ocular and systemic circulation in individual patients. Exploring the associations of endothelial dysfunction and determining whether a global dysfunction, affecting both the systemic macrovasculature and the ocular microvasculature, exists in glaucoma patients could lead to significant advances in our understanding of the development of the disease.

### 1.6.4 The Autonomic Nervous System (ANS)

The hemodynamic situation of the body is supervised and influenced by the ANS via constant regulation and control of heart rate (HR) and blood pressure (BP), amongst other factors [398, 399]. As such a dysfunction of the ANS system can impact on the regulation of blood flow in tissues in the eye and throughout the rest of the body and potentially play a part in the development of GON.

#### 1.6.4.1 Background

The ANS itself forms part of the efferent division of the peripheral nervous system and is split into two systems, namely the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS), both of which innervate smooth muscle, cardiac muscle and glands [145, 400].

The SNS promotes responses that prepare the body for strenuous physical activity in emergency or stressful situations ('fight or flight'). The release of adrenergic agents, such as noradrenaline, from the postganglionic fibres of the SNS, results in an increased HR and force of contraction, vasoconstriction, pupil dilation, stimulation of sweat glands and a breakdown of glycogen and fat stores [400]. The PNS on the other hand dominates under resting, relaxed, non-threatening circumstances, with the release of acetylcholine from the postganglionic fibres of the PNS leading to a decrease in HR and force of contraction, pupil constriction and constriction of the bronchioles [400].

Under normal conditions both systems are partially active, giving rise to a basal sympathetic/parasympathetic tone. This tone can be involuntarily modified according to hemodynamic needs, allowing one system to dominate over the other so that the required response can be brought about [400]. Most organs or tissues throughout the

body have both SNS and PNS innervations which are under reciprocal control; the exception to this is innervated blood vessels which only have sympathetic innervation.

#### 1.6.4.2 Role of the ANS in Regulating Cardiac Rhythm

Among the hemodynamic factors regulated by the ANS is the cardiac rhythm. Rhythmical atrial and ventricular contractions pump blood out of the heart and into the systemic and pulmonary circulations as part of the normal cardiac cycle [145]. Both electrical and mechanical events play a role in producing these rhythmical contractions.

##### Electrical events

Contraction of the cardiac muscle (myocardium) is initiated by spontaneous, electrical impulses which originate from the so called cardiac pacemaker cells. The sinoatrial node (SA node) is the primary pacemaker cell, located in the right atrial wall. It undergoes spontaneous depolarisation approximately 70 times per minute and brings about atrial contraction. The electrical impulse generated by the SA node is conducted via the internodal conduction pathway to the atrioventricular node (AV node), the secondary pacemaker cell. Subsequent conduction of the electrical impulse from the AV node to the ventricle walls, via the bundle of His and purkinje fibres, ultimately leads to ventricular contraction [145]. The SA node receives extensive autonomic innervation from both the SNS and PNS meaning HR can be regulated by the ANS. Parasympathetic innervation of SA node initiates a rapid response which leads to a decrease in HR, whereas sympathetic innervation acts over a longer time course, bringing about increased HR and contractility of the cardiac tissue [398]. At any given moment the overall HR is therefore determined by the balance between the PNS and SNS and under resting conditions it is the PNS which dominates, generating an average resting HR of 70 beats per minute [145].

### Mechanical events

The cardiac cycle begins with ventricular contraction, a phase referred to as systole. As the ventricles contract the pressure inside them rises rapidly and once it exceeds that inside the aorta and pulmonary artery, blood is ejected from the ventricle into the systemic and pulmonary circulations. The pressure subsequently decreases and the ventricles refill with blood, a phase referred to as diastole [145]. The cycle then repeats itself.

## 1.6.4.3 Heart Rate Variability (HRV)

Under normal resting conditions the sinusal rhythm is highly irregular, varying as a result of changes in physiological parameters such as respiration, BP, body temperature, metabolic rate, hormone levels and sleep cycles [401]. This high irregularity is evident when HR is examined beat by beat and is termed HRV. As the SA node, whose activity determines HR, is densely innervated by both the SNS and PNS, HRV reflects the modulating effect of the ANS on the intrinsic firing rate of the cardiac pacemaker cells [398]. As such assessment of HRV can provide an indirect assessment of the autonomic control of the heart and a change in HRV pattern is recognised as an early sensitive indicator of compromised health [398]. Indeed a low HRV, suggestive of a poor adaptability of the ANS, has been correlated with increased mortality [402]. HRV is therefore commonly used for the investigation of normal physiology and pathological conditions and can be assessed using either a frequency domain or time domain analysis, as outlined below.

### Frequency domain analysis

This technique involves spectral analysis of the arterial pulse wave in order to evaluate the predominance of the sympathetic and parasympathetic divisions of the ANS and their effects on HR [403]. In normal individuals cyclic variations in HR occur in association with respiration and BP fluctuations. Respiratory related cyclic variations

are mediated by the PNS and occur at a high frequency (HF) (0.2-0.4 Hz) [398, 404]. Conversely, cyclic variations related to fluctuations in BP and the subsequent changes in baroreceptor activity, typically occur at low frequency (LF) (0.0-0.04 Hz) and are mediated by the SNS [405]. As well as considering these parameters individually the dynamics of the HRV signal and the sympathovagal balance of ANS function, can also be assessed through the evaluation of the LF/HF ratio [398, 406].

#### Time domain analysis

This technique involves the analysis of an individual's electrocardiogram (ECG) profile and the subsequent determination of the average normal-to-normal heart beat interval (mean NN interval). This is then converted into a measure of HRV via simple mathematical evaluation [398, 407].

### 1.6.4.4 Role of ANS in Regulating Blood Pressure

Alongside modifying HR and influencing cardiac output, the ANS also plays a role in regulating MABP (see equation 1.2). MABP is influenced by both cardiac output and total peripheral resistance, with an increased cardiac output and/or increased peripheral resistance leading to the elevation of MABP. Baroreceptors located within the circulatory system constantly monitor MABP and if any deviation away from normal is detected a series of reflex responses, including stimulation of the ANS, are initiated [145]. Subsequent autonomic innervation of the heart, arterioles and veins then leads to either adjustment of cardiac output or modification of peripheral resistance in order to normalise MABP accordingly. A detected increase in MABP, for example, leads to a decrease in sympathetic activity and an increase in parasympathetic activity and hence normalisation of MABP through a reduction of HR and cardiac output and an increase in peripheral vasodilation. A fall in MABP leads to the opposite effect.

Both BP and HR follow a normal circadian rhythm which is dependent on the ANS [145, 408]. ANS activity itself is also thought to follow a circadian rhythm, with sympathetic activity having been shown to be lower at night, along with HR and BP, however this can also vary according to sleep cycles [401]. Through its influence on HR and BP a dysfunction or chronic imbalance of ANS activity could therefore significantly impact the regulation of blood flow not only in the heart, brain and/or peripheral vasculature, but also in the ocular circulation, due to the close proximity of the eye to the heart and brain via the carotid artery [409]. The relevance of this with regard to the development of GON is discussed in the following section

#### 1.6.4.5 Autonomic Nervous System Dysfunction and Glaucoma

Due to the important role that the ANS plays in the maintenance of blood flow physiology and the regulation of variables such as HR and BP, it is clear that a dysfunction of the ANS could have significant adverse hemodynamic effects. With regard to GON development both systemic parasympathetic and sympathetic neuropathies have been reported in those with POAG [43, 410-412] and those with NTG [410, 413-415] using a variety of different assessment techniques.

Studies monitoring 24 hour BP and HRV have demonstrated greater low frequency (LF) values in NTG patients [413] and greater LF values and LF/HF (low frequency/high frequency) ratio in POAG patients [43], both diurnally and nocturnally, suggestive of a high sympathetic tone. Furthermore abnormalities suggestive of altered systemic ANS function have been linked to abnormal OBF regulation in POAG patients using cold pressor testing [416] and on this basis it could be hypothesised that disturbed ANS function may contribute to the disturbed regulation of OBF and subsequently to the development of GON.

When considered as a whole the most recent evidence indicates the presence of a heightened sympathetic activity and suppressed parasympathetic activity in glaucoma patients; however some studies have found parasympathetic activity to also be increased. The exact nature of ANS dysfunction in glaucoma is therefore still unclear and the decision on whether it should be considered an independent risk factor for the disease or whether it simply occurs in coexistence with other factors has not been made. As mentioned in previous sections it is only the choroid and retrobulbar vessels which receive autonomic innervation at the ocular level, however multiple studies have demonstrated impaired regulation and reduced blood flow in both the retinal and ONH vessels as well. It is therefore likely that, if ANS dysfunction does play a role in the development of GON, it does not act in isolation. Links have been made in cardiovascular research between the coexistence of impaired ANS function and impaired endothelial function and it is possible that, due to the links between GON and endothelial dysfunction, similar dual impairments could be occurring in glaucoma patients [367].

#### 1.6.4.6 ANS and endothelial dysfunction

Under normal conditions vascular tone is maintained by both the ANS and the endothelium, which work together in opposition. The release of vasodilating factors from the endothelium is balanced with the release of vasoconstricting factors from the sympathetic nerve terminals [367] and these vasoactive factors act together on the vascular smooth muscle cells to maintain vascular tone.

The ANS and the endothelium are not completely separate systems. Endothelial cells possess receptors for both SNS and PNS neurotransmitters and therefore ANS activity can directly influence the endothelium [218, 417]. As well as this direct influence, the ANS can also influence the endothelium indirectly through the release of neurotransmitters from its nerve terminals which influence the release of vasoactive

factors from the endothelium [418]. Conversely the endothelium can also directly influence ANS function, with the increased release of NO from the endothelium being found to inhibit the vasoconstricting effects of the SNS [418, 419] and the increased release of ET-1 from the endothelium being found to increase the vasoconstricting effects of the SNS [420]. Both the ANS and the endothelium therefore interact with each other under normal conditions to ensure optimum vascular tone is achieved.

It is possible that, due to this close working relationship between the ANS and the vascular endothelium, if a dysfunction occurs in one system then the other system may also be affected. A number of diseases, including diabetes, cardiovascular disease, hypertension and congestive heart failure, have been associated with both abnormalities of ANS regulation [421-423] and abnormalities of endothelial function [424-426]. Whether a dysfunction in one system may have driven a dysfunction in the other, or whether both systems have developed a dysfunction independently as part of the disease process, for example due to the effects of factors such as oxidative stress, aging or insulin resistance, is difficult to determine however.

In patients with congestive heart failure, studies have found that endothelin levels correlate negatively with some measures of HRV [312] and in patients with diabetes associations have been found between lower HRV and higher levels of von Willibrand factor, a marker of endothelial dysfunction [427]. No such data has been collected for patients with glaucoma to date and it is therefore unknown whether similar coexisting dysfunctions are occurring in these patients. Determining this would provide important information that could assist in determining the aetiology of GON.

## 1.7 The Role of Systemic Blood Pressure

Variations in systemic BP are among the vascular risk factors implicated in the development of GON [428]. Regulation of BP is globally controlled by the ANS and follows a normal circadian rhythm [408]. Due to close relationship between BP, IOP and OPP (equation 1.1), abnormal fluctuations in BP can significantly impact the ocular circulation and in the presence of disturbed autoregulation, due to, for example, PVD syndrome, endothelial dysfunction and/or ANS dysfunction (see section 1.6), the impact of abnormal BP would be increased. Both hypertension and hypotension have been linked to the development of GON and the evidence surrounding their involvement along with an outline of the normal systemic BP physiology is given in the following sections.

### 1.7.1 Systemic Blood Pressure - Background

BP, in basic terms, defines the force exerted by the blood against the vessel wall and is determined by both the volume of blood in the vessel and the distensibility of the vessel wall. It is commonly described in terms of systolic (SBP) and diastolic (DBP) blood pressure, with systolic being the highest measured pressure, corresponding to ventricular contraction and diastolic being the lowest measured pressure, corresponding to ventricular relaxation and refilling. It is measured in milligrams of mercury (mmHg) from the brachial artery in the upper arm using a sphygmomanometer. The most recent World Health Organisation (WHO) and International Society of Hypertension guidelines, which are based on the 1999 publication for the classification of hypertension, are outlined in table 1.5.

| Category | Systolic (mmHg) | Diastolic (mmHg) |
|---|---|---|
| Optimal | <120 | <80 |
| Normal | 120-129 | 80-84 |
| High Normal | 130-139 | 85-89 |
| Hypertension: | | |
| Grade 1 (mild) | 140-159 | 90-99 |
| Grade 2 (moderate) | 150-179 | 100-109 |
| Grade 3 (severe) | ≥ 180 | ≥ 110 |
| Isolated systolic hypertension | ≥ 140 | < 90 |

**Table 1.5 Classification of hypertension**
**(World Health Organisation & International society of Hypertension)**

Another term used to describe BP is 'mean arterial blood pressure' (MABP), which was defined in equation 1.2. MABP defines the average BP over a single cardiac cycle and is constantly monitored and regulated by the body through control of cardiac output and total peripheral resistance. The regulation of MABP by the ANS was discussed in section 1.6.4.4.

#### 1.7.1.1 Normal Circadian Rhythm

BP follows a distinctive circadian rhythm characterised by a decline in both SBP and DBP during sleep, reaching their lowest point between 2.00 am and 4.00 am, followed by a transient spike in arterial pressure in the early morning, soon after waking, which corresponds with the peak occurrence of cardiovascular incidents [429-431].

As indicated by this circadian rhythm, it is normal to observe a physiological dip in BP nocturnally. This dip is related to the reduced need for oxygen and nutrients in peripheral tissues during sleep and is brought about by a number of factors including, the normal decline in sympathetic nervous system activity over night, translocation of blood to the peripheral circulation, resulting in a lower venous return, and the effects of the recumbent posture taken on during sleep. The nocturnal dip in BP is traditionally categorised as being either a physiological dip, a non-dip or an excessive

dip [429, 432, 433]. A drop in BP of around 10-20% of the average daytime level is considered physiological and occurs in approximately 2/3 of the healthy population [434]. Of the remaining population, those with a nocturnal dip in BP of less than 10% are classified as non-dippers and those with a nocturnal dip in BP of greater than 20% are classified as extreme dippers [433].

Non-dippers tend to have an increased risk of cardiovascular morbidity, myocardial ischemia and cerebrovascular damage such as stroke, haemorrhages and thrombosis [435, 436]. This is likely to be due to their sustained exposure to high BP levels over the 24 hour period compared to physiological dippers and extreme dippers [434]. Extreme dippers on the other hand tend to have an increased risk of ischemic conditions such as nocturnal myocardial infarction and silent cerebrovascular damage, as significantly reduced BP levels can have a significant effect on perfusion levels.

In order to fully assess an individual's BP profile (or dipping status), 24 hour ambulatory BP monitoring (ABPM) is required. The superiority of 24 hour ABPM in comparison to conventional methods of BP assessment has been well demonstrated with regard to enhanced prediction of cardiovascular mortality and ability to overcome the so called 'white coat syndrome', whereby BP readings are recorded artificially higher in a clinical setting [437-440]. The use of ABPM to evaluate BP in this thesis is discussed further in Chapter 3: Subjects and Methods, section 3.3.4.5.

### 1.7.2 Hypertension and glaucoma

Whilst the presence of hypertension has been associated with the development of GON, the evidence is somewhat variable. Some studies have identified hypertension as a potential risk factor for both development and progression of POAG [441, 442], especially if poorly controlled [443]. Furthermore other studies have found that patients

with POAG in particular, tend to have slightly higher BP than controls [444-448] suggesting a possible causative link. In contrast to these findings however a number of other studies have found either no association between hypertension and glaucoma [307, 449], or a positive association, whereby hypertension has been suggested to have a protective against the development of glaucoma. Leske et al [23], for example, found that those with systemic hypertension at baseline had half the relative risk of developing glaucoma after four years compared to those without hypertension and similarly Leske et al [24] found that those with higher SBP at baseline had a significantly lower risk of disease progression and these findings were then also confirmed by Leske et al [15].

It is clear that the evidence linking hypertension and glaucoma is therefore very variable, with both positive and negative associations having been identified. On the whole, the literature suggests that hypertension should therefore not be considered to be a significant risk factor in the development and progression of glaucoma in the majority of patients. However, on saying that, with regard to circadian rhythm, there is evidence to suggest an association between progression of glaucoma and having an absent or reduced nocturnal dip in BP (less than 10%) i.e. having sustained exposure to high BP levels nocturnally may increase the risk of developing glaucoma [433, 450-452]. Tokunaga et al [433] hypothesised that this may be associated with impaired microcirculation around the ONH, in the form of increased peripheral resistance and impaired blood flow, as a result of the increased BP and an increased production of free radicals or other toxic substances, however more research would be needed to confirm this.

### 1.7.3 Hypertension and IOP

There is some evidence to suggest that a positive correlation exists between systolic and diastolic BP and level of IOP introducing the possibility that hypertension may

contribute to the development of POAG through the elevation of IOP [22, 306, 453, 454]. The magnitude of change in IOP with increasing BP however is very small, with a 10mmHg increase in SBP leading to only around a 0.2 to 0.44 mmHg increase in IOP [455]. Nevertheless theories to explain the associations between BP, IOP and the development of POAG have been put forward, relating to the activities of the ANS and the renin-angiotensin system, both of which have dual control of BP and IOP regulation and to alterations in choroidal volume which can occur in response to elevated BP and impact on IOP [401, 456-458]. Regardless of this, due to the weak relationship, the role that elevated BP plays in increasing IOP is considered unlikely to make a significant contribution to the disease process.

### 1.7.4 Hypotension and glaucoma

The evidence linking hypotension and glaucoma is more consistent and the majority of studies suggest that hypotension, particularly nocturnal hypotension should be considered an important risk factor for the development and progression of glaucoma, particularly with regard to NTG. The Early Manifest Glaucoma trial for example identified low SBP as a risk factor for the progression of glaucoma [24] and a number of further studies have found nocturnal BP levels to be significantly lower in both NTG and progressive glaucoma patients [54, 130, 459-462], suggesting a possible causative link. Furthermore, a large number of studies have found associations between large nocturnal dips in BP (>20%) and the progression of glaucoma [313, 433, 450, 463-466], and increased variability in nocturnal BP parameters and the presence of NTG [53, 467, 468].

The mechanisms by which nocturnal hypotension and large nocturnal BP dips may lead to the development and progression of glaucoma is not entirely clear; however it is thought to largely relate to the impact that reduced BP has on OPP. Indeed reduced OPP has been identified as an independent risk factor for the development

of GON (section 1.6) and, as equation 1.1 shows, in the presence of reduced BP, OPP would also be reduced. It could be hypothesised therefore that such a reduction in OPP, if combined with disturbed autoregulation, occurring either as a component of a PVD syndrome, endothelial dysfunction or as a result of disturbed ANS function affecting the normal circadian rhythm, could result in ischemic damage of the ONH and subsequent development of GON in at risk individuals, via RRI. This hypothesis is supported by a study conducted by Gherghel et al [469] which found that glaucoma patients with marked nocturnal dips in BP also had altered retrobulbar blood flow parameters. Furthermore, as IOP also follows a circadian rhythm, with levels being found to be highest nocturnally (see section 1.3.1) a combination of both low nocturnal BP and high nocturnal IOP could further reduce OPP beyond the capacity of the available autoregulatory system and contribute to the development of ischemia and optic nerve damage in susceptible individuals.

An important factor to consider when exploring the presence of large nocturnal dips in BP is the role of antihypertensive medications. Indeed it is proposed that the increased risk of GON development may be associated with, or exacerbated by, the use of antihypertensive medications, which whilst effectively reducing diurnal BP, may lead to nocturnal hypotension [433, 470, 471], however the evidence supporting this association is variable. There are a number of studies, for example, which have demonstrated an association between the use of antihypertensive medications and glaucoma [307, 470, 471], however a number of other studies have found either no association or that antihypertensive medications may actually have a protective effect against the development of glaucoma [23, 448, 472]. The evidence is therefore clearly variable however it is generally considered worthwhile to consider the possible role that antihypertensive medications may be playing on glaucoma, particularly in those patients who are progressing.

## 1.8 The Role of Cardiovascular Risk Factors

The presence of cardiovascular disease and structural changes to the vascular wall have been variably linked to the development and presence of glaucoma and could potentially contribute to the alterations in vascular function and OBF regulation observed in glaucoma patients. The following sections outline the anatomy and physiology of the cardiovascular system and go on to discuss the role that systemic arterial stiffness and cardiovascular disease may play in the development or progression of glaucoma.

### 1.8.1 Physiology of the Cardiovascular System

The principle function of the cardiovascular system is to maintain adequate blood flow to all tissues, ensuring their oxygen and nutrient demands are met and waste products removed. Blood flows through the cardiovascular system primarily as a result of the pressure produced by the contraction of the heart ventricles, referred to as the systemic BP [473]. Both systemic BP and HR are constantly regulated and controlled by the ANS in accordance with the hemodynamic needs of the body. The background and relevance of systemic BP and the ANS with regard to cardiovascular physiology is outlined in the following sections.

### 1.8.2 Anatomy of the Cardiovascular System

The cardiovascular system is composed of the heart and circulatory blood vessels and plays a primary role in the transport of materials to and from all regions of the body, including the eye and brain. On leaving the heart, the blood is distributed between two principle cardiovascular pathways, namely the pulmonary circuit and the systemic circuit [473]. An overview of these circuits with relevance to this thesis is given in the following sections.

#### 1.8.2.1 The Heart

The human heart resembles a double pump system consisting of four chambers, namely the right and left atria and the right and left ventricles (figure 1.12). The atria function primarily as reservoirs, collecting blood returning in the veins and feeding it into the ventricles. The ventricles are the major muscular pumps of the heart, ejecting blood from the heart and into the circulatory system. Each ventricle serves as a pump for a specific cardiovascular pathway. The right ventricle pumps deoxygenated blood received back from the systemic circulation into the pulmonary circuit. Following the removal of carbon dioxide and the addition of oxygen, the left ventricle then receives the oxygenated blood from the pulmonary veins, via the left atrium, and pumps this blood into the systemic circuit via the aorta [473].

#### 1.8.2.2 Coronary arteries

The blood supply to the metabolically active cardiac muscle, which lines the wall of the heart, is provided by the coronary arteries. The left coronary artery originates on the left side of the aorta and separates into three branches which supply much of the anterior wall of the heart and most of the left ventricle. The right coronary artery originates on the right side of the aorta and separates into two main branches which supply most of the wall of the right ventricle. The coronary arterial blood supply to the heart muscle can be described as an ‘end circulation’ as it represents the only source of blood supply to the myocardium, as such any blockage or dysfunction of the coronary arteries, for example through atherosclerosis, poses a critical threat to heart function, and could potentially lead to heart attack and/or myocardial infarction [474].

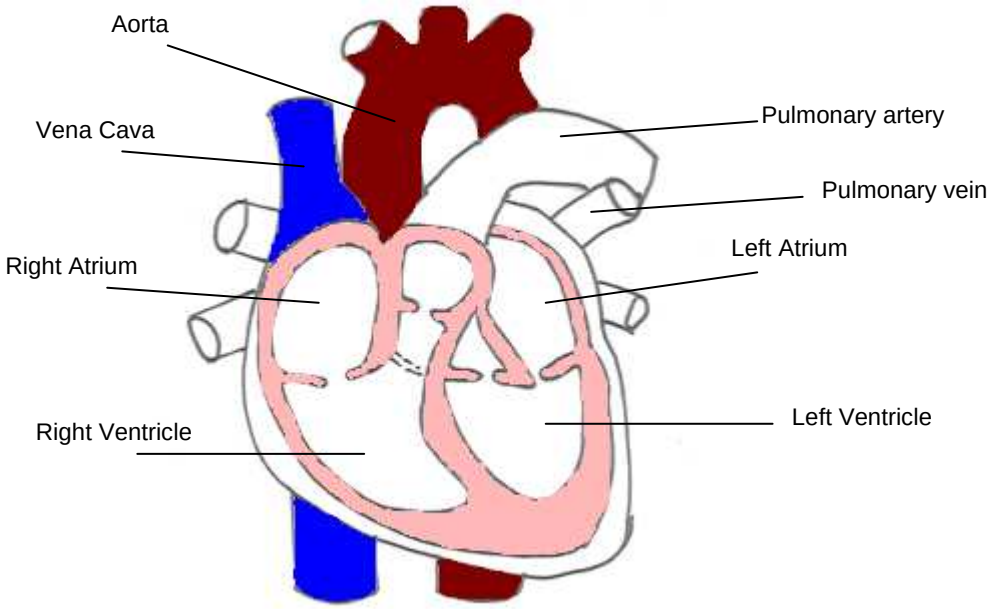



**Figure 1.12: Diagrammatic representation of the heart**

### 1.8.2.3 Systemic Circulation: Carotid, Brachial and Radial Arteries

The systemic circulation refers to the flow of blood from the left ventricle of the heart to the tissues of the body and back to the right atrium. All of the arteries which make up the systemic circulation branch from the aorta, which itself can be considered in three parts, namely the ascending aorta, the aortic arch and the descending aorta. The blood supply to the head and neck, which ultimately supplies the vascular networks of the eye and brain, originate from the aortic arch. Of primary interest to this thesis are the right and left common carotid arteries. The right common carotid artery originates from the first branch of the aortic arch, referred to as the brachiocephalic artery, whereas the left common carotid artery branches directly from the aortic arch itself. Both the right and left common carotid arteries extend superiorly within the corresponding parts of the neck before branching to form the right and left, internal and external carotid arteries [473] (figure 1.13).

Other systemic arterial branches referred to in this thesis include the brachial artery and the radial artery. The brachial arteries are located in the upper arm on both sides

and are the common site for BP measurement. They arise from the subclavian artery, which itself arises either directly or indirectly from the aortic arch. The brachial artery branches at the elbow to form the radial artery which supplies the forearm and the hand and is common site for taking a pulse.

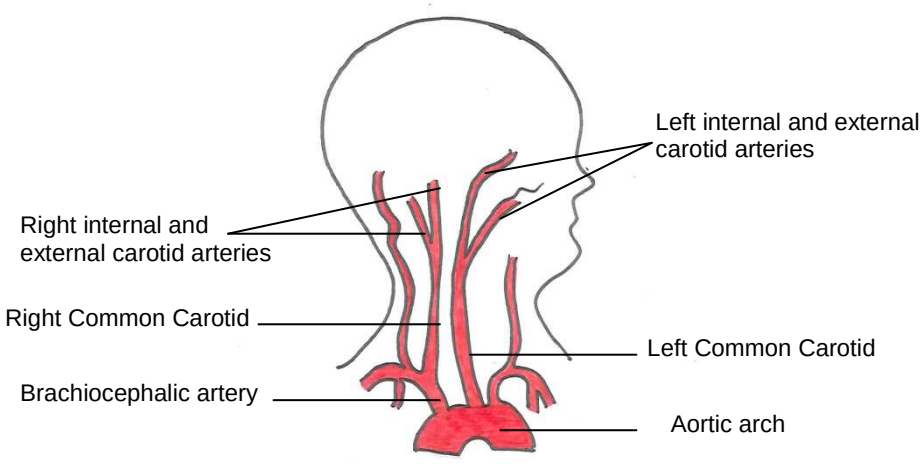



**Figure 1.13 Diagrammatic representation of the arteries of the neck**

### 1.8.2.4 Structure of the Arterial Walls

On leaving the heart the blood flows from the aorta, into the systemic arteries, which then repeatedly branch throughout the body forming progressively smaller arterioles and capillaries. As the vessels branch the structure of the arterial wall undergoes a gradual transition from high elasticity, low smooth muscle cell (SMC) content to high SMC content and low elasticity. The walls of the arteries and arterioles are composed of three layers referred to as the tunica intima, the tunica media and the tunica adventitia, as depicted in figure 1.14. The tunica intima is collective name for the innermost layers of the vessel wall, encompassing the endothelium and connective tissue, the tunica media is the middle and thickest layer and the tunica adventitia is the outermost layer [473].

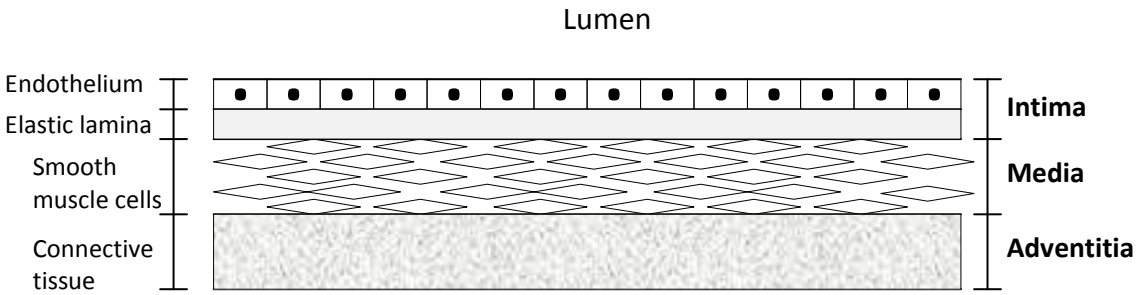



**Figure 1.14 Diagrammatic representation of the artery wall**

The structure of the smallest vessels, referred to as the capillaries, is somewhat different from that of the arteries and arterioles in that their wall consists only of a thin layer of endothelial cells, accompanied by an underlying basement membrane. This structure facilitates the exchange of materials between the capillaries and surrounding tissues.

### 1.8.2.5 Atherosclerosis

The arterial walls of the circulatory system undergo changes as they age, the most significant of which is a loss of elasticity. This loss of elasticity or 'hardening of the arteries' is termed arteriosclerosis and primarily effects the large elastic arteries such as the aorta and carotid arteries. A specific form of arteriosclerosis, termed atherosclerosis, refers to the hardening of the artery wall as a result of the deposition of fatty materials and plaque formation on the inner surface of the wall. This deposition leads to narrowing of the arterial wall, increasing resistance to blood flow and impairing blood circulation [473]. It also increases the risk of vessel occlusion through thrombus formation. The severity and rate at which atherosclerotic changes develop has been linked to factors such as lack of exercise, obesity, smoking and high cholesterol diets but may also have a genetic influence [475]. The detection of atherosclerotic vessel changes and the adjustment of modifiable risk factors is recommended in the treatment and prevention of cardiovascular disease. A summary

of the techniques available for detecting and/or monitoring atherosclerotic changes is given in table 1.6

| Method | Advantages | Disadvantages |
|---|---|---|
| Angiography | Original technique<br><br>Allows an assessment of artery stenosis | Detects only severe narrowing not the underlying atherosclerotic disease<br>Invasive |
| Stress testing | Allows a general assessment of physical condition of patient | Detects only severe narrowing not the underlying atherosclerotic disease<br>Invasive |
| **Anatomic methods**: | | |
| • Coronary Calcium scoring by CT<br><br>• Carotid intima-media thickness measurement by ultrasound<br><br>• Intravascular ultrasound | Directly measure aspects of the actual atherosclerotic disease process itself<br><br>Allow detection of atherosclerotic changes before patient becomes symptomatic<br><br>Allow tracking of disease progression | More expensive<br><br>Can be invasive |
| **Physiological methods**: | | |
| • Lipoprotein subclass analysis<br><br>• HbA1c analysis<br><br>• C-Reactive protein analysis (hsCRP)<br><br>• Homocysteine analysis | Cheap<br><br>Safe<br><br>Modification of abnormal parameters can slow progression | Don't allow the state of the disease to be quantified<br><br>Don't allow progression of disease to be tracked |

**Table 1.6: Overview of the techniques available for the detection of atherosclerosis**

### 1.8.3 Cardiovascular risk and Glaucoma

There is evidence to suggest a higher incidence of cardiovascular disorders such as coronary artery disease, cardiac arrhythmias, atrial fibrillation, congestive heart failure and hemodynamic crisis in NTG patients compared to the average population [142, 350, 444, 476]; however this is not confirmed by all studies [477, 478]. Furthermore POAG patients have been identified as having a higher cardiovascular risk than control patients [446] and cardiovascular disease history has been identified as a risk factor for POAG

progression [24]. Additionally an increased cardiovascular mortality has been described in those previously diagnosed with open angle glaucoma [479].

Whilst these findings obviously support the role that systemic vascular disease may play in the development of glaucoma, other studies directly assessing arterial stiffness or the presence of arteriosclerosis and its risk factors in those diagnosed with glaucoma have given more mixed results, with some finding strong associations [307, 480-483] and others finding no association at all [318, 484, 485]. Furthermore risk factors classically associated with cardiovascular disease and arteriosclerosis, such as obesity, smoking and hypercholesterolemia have not been found to show any strong links to the development of GON [485-487]. The role that systemic arterial stiffness and cardiovascular disease may play in the development or progression of glaucoma is therefore still uncertain.

Increased arterial stiffness, an independent predictor of cardiovascular disease and stroke [488], can arise as a result of an age related breakdown of the elastin structures in the arterial walls, damage to the endothelium/smooth muscle system or an increase in MABP. Interestingly these are all factors that have been individually associated with GON development. Furthermore endothelial dysfunction has also been identified as the first detectable change in an atherosclerotic vessel so it is not unreasonable to hypothesise that, due to the fairly strong links between glaucoma and endothelial dysfunction; vessel wall changes such as atherosclerosis could contribute towards vascular dysfunction and GON development in certain individuals. Interestingly a recent study by Oettli et al [295] was able to identify increased arterial stiffness at the retinal level in NTG patients and found this correlated with level of glaucomatous damage, suggesting a possible role for both ocular and systemic arterial wall changes in the development of GON.

The possibility that cardiovascular disease and arterial stiffness may contribute towards the development of GON can therefore not be ruled out however further research is needed to elicit the exact nature of their association. Systemic arterial stiffness and cardiovascular risk can be assessed by means of central pulse pressure, ultrasound measurement of distensibility and compliance, pulse wave velocity assessment, pulse wave analysis (PWA) techniques and carotid artery intima-media thickness (IMT) measurement [489]. The most commonly used indices of arterial stiffness are detailed in table 1.7. Both PWA and IMT were conducted in this thesis and are discussed further in Chapter 3: Subjects and Methods

| **Indices of arterial stiffness** | **Definition** |
|---|---|
| Arterial distensibility | Relative diameter or area change for a pressure increment |
| Arterial compliance | Absolute diameter or area change for a given pressure step at fixed vessel length |
| Elastic Modulus | The pressure step required for 100% stretch from resting diameter at fixed vessel length |
| Pulse wave velocity (PWV) | Speed of travel of pulse along an arterial segment |
| Pressure augmentation | Increase in aortic or carotid pressure after the peak of blood flow in the vessel |
| Young's Modulus | Elastic modulus per unit area; pressure step per square cm required for 100% stretch from resting length |

**Table 1.7: Overview of commonly used indices of arterial stiffness [490]**

## 1.9 Oxidative stress and the concept of reperfusion injury

Regardless of the cause, the final common pathway in the development of GON remains the apoptotic loss of RGCs, tissue remodelling and excavation of the ONH. Increasing evidence suggests that oxidative stress, in combination with the more extensively researched causative factors discussed in previous sections, could also be a contributing factor in the development of GON.

### 1.9.1 Background

In simple terms, oxidative stress refers to an imbalance between the production of oxidants and antioxidants, in favour of the former, with the potential for damage [491]. In biochemical terms 'oxidation' is a harmful process which refers to the loss of electrons and/or gain of oxygen by a molecule and is brought about by the action of so called 'oxidising agents' or 'oxidants' [492]. Oxidants take the form of either free radicals, which are usually highly reactive and have one or more unpaired electrons, or non-radical species and can be derived from either oxygen (reactive oxygen species, ROS) or nitrogen (reactive nitrogen species, RNS), as outlined in table 1.8 [493]

| **Reactive Oxygen Species** | **Features** |
|---|---|
| Singlet Oxygen ($^1O_2$) | Oxygen molecule in its excited state. Generated through photodynamic reactions [494] |
| Superoxide anion ($O_2^-$) | Generated continuously by normal cellular processes from the reduction of $O_2$. Converted to hydrogen peroxide by superoxide dismutase enzyme [493] |
| Hydrogen Peroxide ($H_2O_2$) and the hydroxyl radical ($OH^-$) | $H_2O_2$ only weak oxidising agent, but forms the more potent $OH^-$ on crossing cell membranes and reacting with copper/iron ions [493] |
| **Reactive Nitrogen Species** | **Features** |
| Nitric oxide (NO) and Peroxynitrite ($ONOO^-$) | NO is a free radical and reacts strongly with superoxide ($O_2^-$) to form $ONOO^-$. NO has both oxidant and antioxidant properties. [495] |

**Table 1.8: Summary of common reactive oxygen and reactive nitrogen species**

ROS are potentially harmful and can be generated as a by-product of normal $O_2$ metabolism by a cell. Therefore, although $O_2$ is essential for maintenance of normal life in aerobic organisms, it also has the potential to cause harm [491]. The eye, due to its unique constant exposure to light, atmospheric oxygen, environmental chemicals and physical abrasion is particularly susceptible to ROS formation [366]. Under optimal conditions the rate and magnitude of ROS formation is balanced by the rate of ROS elimination through the action of antioxidants and minimal tissue damage occurs [492]. Such antioxidants act by significantly delaying or preventing the oxidation of a substrate and form part of the body's natural defence mechanism against the action of ROS [491]. They take a variety of different forms and can be categorised as either enzymatic or non-enzymatic. These are summarised in table 1.9.

| **Enzymatic antioxidants** | **Features** |
|---|---|
| Superoxide Dismutase (SOD) | Scavenges the superoxide anion ($O_2^-$): catalyses the dismutation of $O_2^-$ to $H_2O_2$ and water [496] |
| Catalase (CAT) | Important role in removing $H_2O_2$: catalyses $H_2O_2$ to water and oxygen [497] |
| Glutathione Peroxidase (GPX) | Important role in removing $H_2O_2$: reduces $H_2O_2$ to water by oxidising glutathione (GSH). Oxidised from of glutathione (GSSG) is then catalysed by glutathione reductase [498] |
| **Non-enzymatic antioxidants** | **Features** |
| Glutathione (GSH) | Strong antioxidant properties - donates electrons readily converting it to its oxidised from GSSG. GSH/GSSG ratio of a cell is good indicator of cellular redox balance [499, 500] |
| Heat shock proteins | Produced by cells in response to stress. Regulate the function of other proteins [501] |
| Transferrins | Bind free iron or metal irons in forms that will not stimulate free radical reactions [502] |
| Haptoglobins | Haemoglobin-binding proteins – decrease effectiveness of lipid peroxidation [503] |

**Table 1.9 Summary of enzymatic and non-enzymatic antioxidants**

If the oxidant/antioxidant balance is disturbed so that the level of oxidants exceeds the antioxidant capacity a state of oxidative stress is reached. The body is able to respond to a small level of oxidative stress by increasing its defence and repair mechanisms [504]. However under conditions of sustained oxidative stress or excessive

ROS production the damage induced may exceed the capacity of not only the antioxidants but also the bodies repair mechanisms, leading to more permanent macromolecular damage. Proteins, lipids, sugar residues and DNA are at risk of damage, potentially resulting in growth arrest, growth modulation, cell death and disease formation [493]. Indeed oxidative stress has been implicated as a causative factor in a number of pathological conditions including diabetes [505], atherosclerosis [506], rheumatoid arthritis [507], malignant disease [508], human immunodeficiency virus (HIV) [508] and age-related macular degeneration (AMD) [509] and the bodies capacity for dealing with oxidative stress shows a normal age-related decline [510].

A determination of the presence of oxidative stress can either be made through evaluation of oxidative stress related tissue damage, such as increased DNA breaks and increased proteosome activity, or through the more common method of analysing the circulating plasma oxidant/antioxidant balance which can be disrupted in the presence of oxidative stress. One such antioxidant is glutathione (GSH). GSH is the major low-molecular mass thiol compound in plants and animals and is among the most efficient substances that cells and tissues can use in their defence against oxidative stress [511]. It is a tripeptide consisting of glycine, cysteine and glutamic acid, as depicted in figure 1.15 [512] and is active in the eye. As an antioxidant GSH can prevent the devastating effects of ROS either directly through its oxidisation, or indirectly by maintaining other cellular oxidants in a functional state [513].

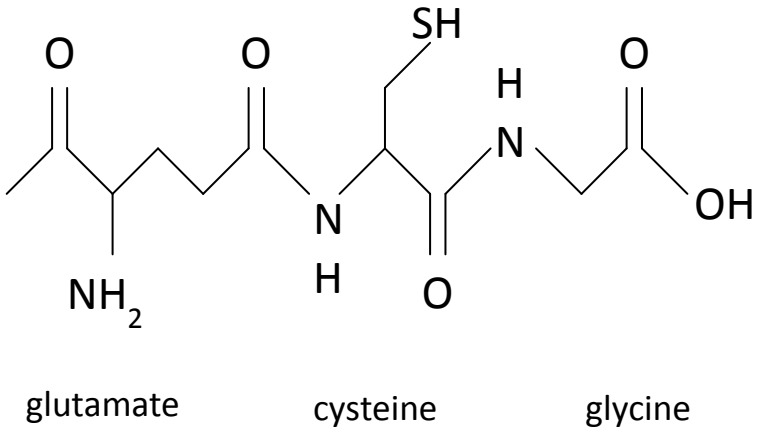



**Figure 1.15: Chemical structure of the tripeptide glutathione**

Glutathione is present in two forms in healthy cells and tissues, namely a reduced form, glutathione (GSH), and an oxidised form, glutathione disulphide (GSSG) and GSH is constantly being broken down to GSSG and re-synthesised [512, 514] (equation 1.3). As such, GSSG, which is formed on oxidation of GSH following its interaction with harmful ROS, accounts for only around 10% of the total glutathione pool of a healthy cell, with GSH accounting for around 90% [514]. Under conditions of oxidative stress however, ROS levels are raised and hence the production of GSSG is increased. As such determining the GSH:GSSG ratio, or glutathione status of a cell is considered a good indicator of cellular redox imbalance and oxidative stress [515], especially as maintaining an optimal GSH:GSSG ratio is critical for cell survival. A reduced GSH:GSSG ratio would be considered indicative of oxidative stress [516]

$$2\,GSH + H_2O_2 \xrightarrow{GPase} GSSG + H_2O$$

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

**Equation 1.3:** GSH: reduced glutathione; $H_2O_2$: hydrogen peroxide (ROS); $H_2O$: water, GSSG: oxidised glutathione; NADPH, $NADP^+$: nicotinamide adenine dinucleotide phosphate. GPase: glutathione peroxidise, GRase: glutathione reductase

### 1.9.2 Oxidative Stress and Endothelial Dysfunction

Over recent years oxidative stress has been increasingly linked to the development of endothelial dysfunction and has been implicated as a causative factor in the pathogenesis of multiple associated diseases, including neurodegeneration and atherosclerosis [517-519]. The predominant pathway by which oxidative stress is thought to promote endothelial dysfunction is through reduction in the bioavailability of the vasodilator NO, resulting primarily from its increased reaction with ROS such as $O_2^-$ under conditions of oxidative stress [519]. Disturbances in the functioning of NOS, or disruptions to the interactions between NO and the vSMCs, both of which are induced by an increased presence of ROS, can however additionally contribute to the reduced bioavailability of NO [519, 520] in oxidative stress. Interestingly the modification or correction of oxidative stress has been shown to alleviate induced endothelial dysfunction [521-523]. As such, the detection and consideration of oxidative stress and the coexistence of endothelial dysfunction is important in the management of associated conditions, including neurodegenerative disease. The mechanisms by which oxidative stress and endothelial dysfunction may contribute to ocular neurodegeneration, in the form of glaucoma, are therefore discussed fully in the following sections.

### 1.9.3 Oxidative stress and glaucoma

The RGCs and axons of the ONH are considered particularly susceptible to the effects of oxidative stress due to their direct exposure to light, their high proportion of polyunsaturated fatty acids and their very high levels of $O_2$ consumption, resulting from their lack of myelin sheaths and high concentration of mitochondria. Indeed multiple studies have found characteristic indicators of oxidative stress in glaucoma patients, including an increased number of DNA breaks [524], an upregulation of ET-1 [386] and metalloproteinase-9 (MMP-9) [525] and increased proteosome activity [526].

Furthermore, low plasma levels of the antioxidant glutathione (GSH) and total glutathione (t-GSH) have been demonstrated in glaucoma patients compared to controls [27] as well as a decreased activity of various other antioxidants and of the total serum antioxidant status [527, 528], indicative of an increased oxidative burden. It is therefore no surprise that oxidative stress has been linked to the development of GON and as well as being implicated in the so called vascular theory of the disease it has also been linked to the development of GON through mechanical means as discussed in the following section.

#### 1.9.3.1 Oxidative stress and IOP

With regard to the mechanical theory, oxidative stress has been implicated to play a causative role in the elevation of IOP and subsequent development of GON. It is proposed that increased levels of oxidative stress in the aqueous humour, occurring as a result of light catalysed reactions, metabolic pathways or inflammation, leads to cellular loss and alterations of glycoprotein structure in the extracellular matrix of the trabecular meshwork (TM) cells. This is followed by an alteration in TM function, impaired aqueous outflow and increased IOP [28, 529, 530]. Increased IOP, as discussed in section 1.9.4, can then contribute to the development of GON through either direct mechanical insult of the optic nerve fibres, inhibition of retrograde neurotrophin support to the RGCs, or alternatively through acting in combination with vascular alterations at the ONH.

Multiple studies have demonstrated depletion of total antioxidant potential and enhanced antioxidant activity in the aqueous humour of glaucoma patients, suggestive of increased oxidative burden [527, 531-534], as well as the presence of oxidative damage in the TM cells [535, 536], which could potentially impede aqueous outflow. Oxidative stress can therefore be considered a potential risk factor for the development of POAG in particular [366].

### 1.9.3.2 Oxidative stress and reperfusion injury

The vascular concept of glaucoma centres around the presence of unstable blood flow and/or altered autoregulation at the ocular and systemic level in susceptible patients. Unstable blood flow is characterised by a repeating cycle of normal then reduced perfusion and could potentially lead to cellular damage through RRI and oxidative stress. RRI refers to the damage to a tissue caused when blood supply returns after a period of ischemia [143]. During ischemia, the absence of oxygen and nutrients from a cell impairs electron transport in the mitochondria, resulting in inefficient energy production and the presence of a number of spare electrons. When perfusion then returns to normal these spare electrons react with the now plentiful supply of $O_2$ molecules, leading to the formation of damaging ROS [537]. Mitochondria are abundant in the RGC axons of the ONH putting it at high risk of damage by ROS and it is hypothesised that, if mild RRI continues over a sustained length of time chronic oxidative stress, endothelial dysfunction, ONH damage and the development of GON would occur, regardless of antioxidant activity [141, 366].

Apoptosis, referring to programmed cell death without necrosis [538], has been strongly implicated as the ulitmate pathway for RGC loss in glaucoma [100, 539, 540]. The exact mechanism by which RRI and oxidative stress may contribute to this process however is still unclear. All cells have a genetically predetermined programme of death that is part of the normal cell life cycle and enables the maintenance of homeostasis and removal of pathologically altered cells. If this programmed cell death is activated inappropriately however, then pathological loss of cells and the development of associated disorders can occur. Both RRI and the subsequent activation of astrocytes are thought to play an important role in initiating inappropriate apoptosis of RGCs in glaucoma [541].

Astrocytes are found in the ONH and provide biochemical support to the RGC axons [541]. They have a high susceptibility to ROS and on becoming activated change appearance and start producing a variety of abnormal molecules, including ET-1 and NO, which can alter the microenvironment of the cell [144]. Diffusion of NO from the astrocytes into the neighbouring RGC axons, where levels of ROS such as $O_2^-$ are high as a consequence of RRI, can lead to the formation of very damaging peroxynitrate ($ONOO^-$) [542]. It is proposed that subsequent diffusion of both $O_2^-$ and $ONOO^-$ within the RGC axons, towards the retina and LGN could then trigger the apoptotic loss of RGCs [141, 543, 544]. In parallel to the loss of RGCs, ONH tissue remodelling is thought to occur as a consequence of not only mechanical force, but also an active biological process including the effects of MMP-9 [141]. Indeed diffusion of ET-1 and MMP-9, which are present in increased concentrations in the bloodstream of glaucoma patients, from the choroid into the surrounding ONH, can result in vasoconstriction and weakening of the BBB [545], with an increased risk of subsequent damage. In addition to via RRI, astrocyte activation may also arise as a direct result of the presence elevated IOP, through either its direct mechanical effect, or through its contribution to the apoptosis of RGC axons via disruption of axoplasmic transport and deprivation of essential growth factors [546, 547]. Additionally raised ET-1 levels, which may occur as a result of factors such as raised IOP, hypoxia or endothelial dysfunction, could also trigger the activation of astrocytes and initiate the apoptosis of RGC and their axons in the presence of oxidative stress [144]

All in all the concept of RRI and oxidative stress, combined with the subsequent activation of astrocytes and apoptotic loss of RGCs is the most widely accepted current pathogenetic concept for the development of GON. Indeed further evidence for the potential role of RRI in the pathogenesis of GON comes from its associations with sleep apnea and reversible shock-like states, both of which are conditions whose pathologies are linked to the presence of RRI [548, 549]. It is the ocular and systemic

vascular mechanisms which contribute to this pathogenesis which is still the subject of debate.

## 1.10 Overall summary of the current pathogenetic concept of glaucoma

From the evidence discussed in the previous sections it is clear that open angle glaucoma is a disease of multifactorial origin and that vascular alterations appear to play a key part in its development. Figure 1.16 summarises the current pathogenetic concept of GON development, the mechanisms thought to be involved and their interactions. Current thinking suggests a synergistic involvement of both mechanical and vascular factors in the aetiology of GON however the extent of involvement and coexistence of the many implicated factors is still uncertain and may vary between individuals

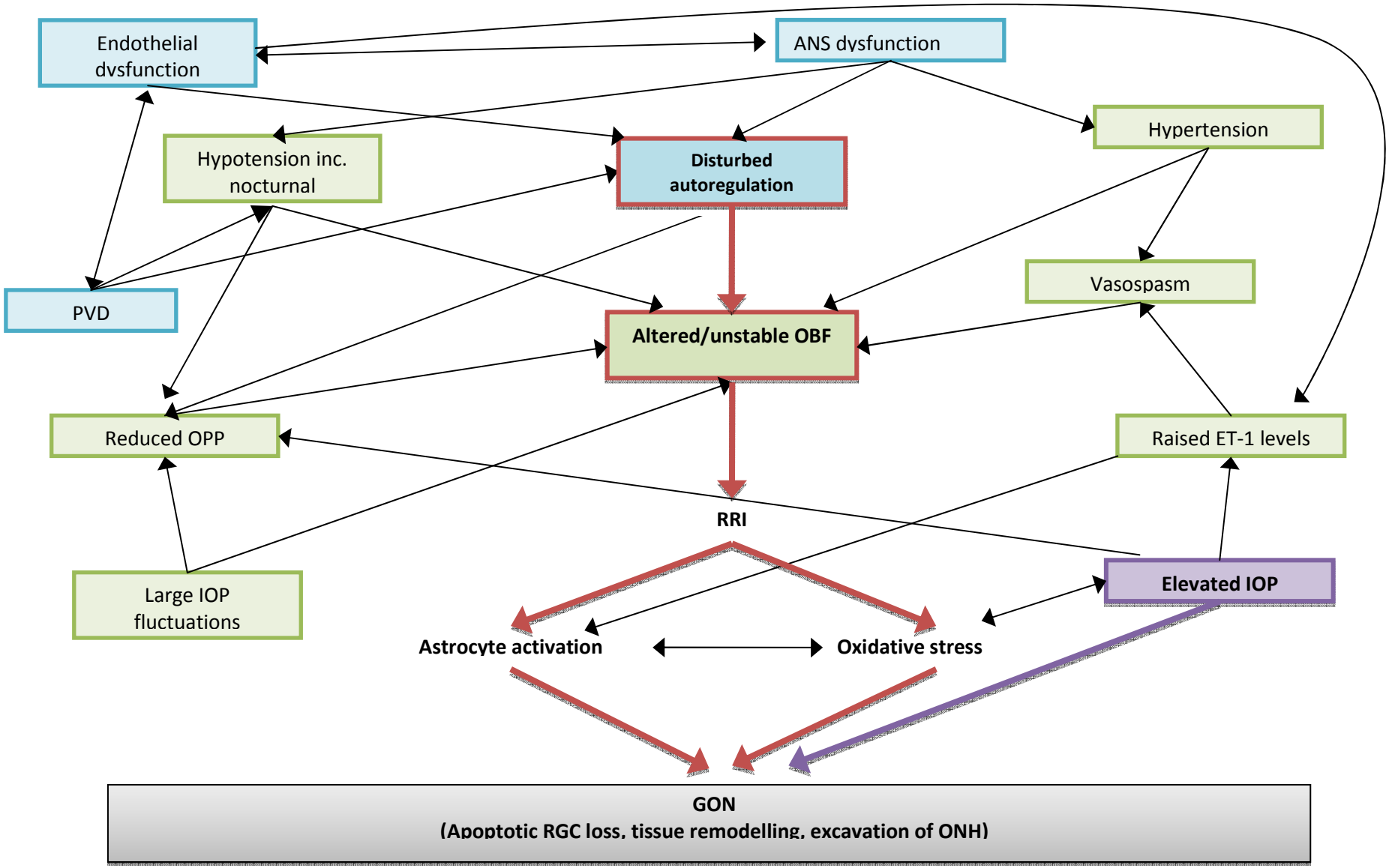



**Figure 1.16 Summary of the current pathogenetic concept of glaucomatous optic neuropathy development**

## 1.11 Cerebral Neurodegeneration: Alzheimer's disease

### 1.11.1 Dementia overview

AD is a form dementia. Dementia describes a progressive decline in cognitive function in a previously unimpaired person, beyond that which is expected through normal aging [550] and is a broad term encompassing a large range of different cognitive impairments [551-553]. The most common form of dementia is AD, which accounts for approximately 80% of all cases and is the main focus of this thesis. An overview of the subcategories of dementia is given in table 1.10

| Dementia Classification | Examples |
|---|---|
| ***Primary degenerative disorders*** (progressive with no associated disease or cause) | Alzheimer's disease<br>Lewy body dementia<br>Pick's disease (fronto-temporal dementia) |
| ***Vascular dementias*** (cerebrovascular disorders with dementia) | Multiple-infarct syndrome (cortical)<br>Small vessel dementia (subcortical ischemic vascular disease)<br>Strategic infarct dementia |
| ***Secondary dementias*** (secondary to other physical disease, infection or injury) | Huntington's chorea related<br>Parkinson's disease related<br>HIV related<br>Creutzfeldt-Jakob disease related |
| ***Reversible/Treatable dementia*** | Normal pressure hydrocephalus related<br>Brain tumour related<br>Hypothyroidism related<br>Vitamin B-12 deficiency (Korsakoff's syndrome)<br>Neurosyphilis related |

**Table 1.10: Overview of the subcategories of dementia**

### 1.11.2 Clinical presentation, diagnosis and treatment of AD

AD is a progressive neurodegenerative disorder, first described by Alois Alzheimer in 1906 [554], which affects over 35 million people worldwide [2] and increases in prevalence after the age of 65 [555]. It is characterised clinically by a gradual decline in cognitive function to include memory impairment, confusion, disorientation, mood swings and in the later stages, speech abnormalities and a gradual loss of bodily function [556, 557]. From

a neuropathological point of view two major hallmark lesions have been identified in the brain of AD sufferers, namely the formation of extracellular beta-amyloid senile plaques and the formation of intracellular neurofibrillary tangles, as a result of abnormal tau protein phosphorylation [558-561]. These classic pathological lesions are thought to contribute towards the characteristic neuronal degeneration and cerebral atrophy which ultimately occurs in the AD brain; however their aetiology is still the subject of debate. A number of different theories for their development have been proposed including, damage through exposure of the CNS to increased mechanical stress [463, 562], inflammation [563], mitochondrial dysfunction [564] and oxidative stress [565]. Of particular interest in this thesis however is the involvement of vascular factors, which have also been strongly hypothesised to play an aetiological role in AD [566].

#### 1.11.2.1 Diagnosis

AD predominantly develops sporadically, with only 2.5% of all cases having being deemed to be of genetic origin [567, 568]. The need for early and accurate diagnosis of the condition is therefore well recognised, particularly with regard to ensuring optimum treatment, care and prognosis for sufferers, however it still remains a challenge. A definitive diagnosis of AD can only currently be made post-mortem by the histopathological confirmation of the presence of senile plaques and neurofibrillary tangles in the brain. This is in accordance with the National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders (NINCDS-ADRDA) working group, which, along with the Diagnostic and Statistical Manual of Mental Disorders, VIth edition (DSM-IV-TR), forms the basis of AD diagnosis in research [569].

The NINCDS-ADRDA, first proposed in 1984, is among the most used diagnostic criteria for AD [570]. It allows the diagnosis of definitive, probable, possible or unlikely AD, as outlined in table 1.11. A diagnosis of probable AD is the maximum that can be made

clinically and requires the presence of progressive memory impairment with involvement of at least one other cognitive domain and the lack of other systemic or brain diseases that may account for the cognitive deficits [570]. The DSM-IV-TR criteria, published in 2000, also requires the presence of both memory impairment and a disorder in at least one other cognitive domain for the probable diagnosis of AD; however it additionally requires that these features also interfere with social function or activities of daily living [569, 571].

Whilst both the NINCDS-ADRDA and DSM-IV-TR have been validated against gold standards and have demonstrated high levels of sensitivity and specificity in distinguishing those with AD from those without dementia [572, 573], significant advances in the scientific knowledge and understanding of pathogenic events in the disease over recent years has lead to the proposal of revised diagnostic criteria which consider the presence of biomarkers of AD and centre around the detection of early memory impairment [569]. Current biomarkers include an abnormal cerebral spinal fluid (CSF) beta-amyloid and tau protein profile, structural brain changes on magnetic resonance imaging (MRI), hypometabolism or hypoperfusion changes on positron emission tomography (PET) scanning and the presence of pathogenic gene mutations [557, 574]. Determining the presence of biomarkers allows in vivo biological evidence of AD pathology to be gained, however the methods by which they are obtained are often invasive so development of alternative non-invasive techniques would be beneficial.

A large degree of variation exists in the presentation and course of AD between individuals and furthermore the disease is thought to exist in an undiagnosed state for an indeterminate period of time before its symptoms become apparent [575]. Enhancing the ability to detect AD in its earliest stages has therefore become a research area of intense interest over the last few years due to its obvious benefits with regard to patient prognosis and management. As such several different stages of AD or memory

impairment have now been defined in order to document the progression of the disease. These are outlined in table 1.12 and are variably referred to as mild cognitive impairment (MCI), preclinical AD, prodromal AD and finally AD dementia, which itself can be further separated into mild, moderate and advanced AD [569]. It is recommended that such further separation of AD dementia into differing degrees of cognitive impairment is made using formal standardised cognitive tests such as the Mini Mental State Examination (MMSE) [576, 577]. This is a simple, widely used and validated test which takes only 10 minutes to complete and provides a global assessment of cognitive function. Out of a maximum score of 30, those achieving 25 or higher are generally considered as normal and those scoring less than 10 are considered to have a severe impairment [577]. A score of around 19-24 is generally considered to indicate mild AD, however as the MMSE result has been shown to be influenced by factors such as age, ethnicity and education, it is recommended that adjustments are made where necessary to account for these factors [578, 579].

| Diagnosis | Features |
|---|---|
| **Probable AD** | Clinical diagnosis:<br>• Dementia established by clinical examination and documented by the Mini-Mental Test (MMSE) or similar and confirmed by neurophysical tests<br>• Deficits in two or more areas of cognition<br>• Progressive worsening of memory and cognitive decline<br>• Normal level of consciousness<br>• Onset between ages 40-90<br>• No other possible medical or neurological explanation<br>Supported by presence of:<br>• Progressive aphasia, apraxia and agnosia; impaired activities of daily living; family history of similar disorder; brain atrophy on CT/MRI; normal CSF<br>Other consistent clinical features:<br>• Plateaus in the course of progression<br>• Associated symptoms, such as depression, insomnia, incontinence, illusions, weight loss, emotional or physical outbursts<br>• Seizures in advanced stages<br>• CT normal for age<br>Exclusion factors:<br>• Acute onset<br>• Focal neurological findings<br>• Seizures or gait disorders at early stages of disease |
| **Possible AD** | • The presence of dementia with an atypical onset or course that occurs in the absence of any other medical/neuropsychiatric explanation<br>• Dementia in the presence of another brain or systemic disease not considered to be the cause of the dementia<br>• Gradually progressive severe cognitive deficit in the absence of other identifiable cause (research purposes only) |
| **Definite AD** | • All the criteria of 'probable AD' plus histopathological evidence from biopsy or autopsy |

**Table 1.11: NINDS-ADRDA Diagnostic criteria for AD [570]**

| Stage | Description |
|---|---|
| **Mild Cognitive Impairment (MCI)** | • Subjective memory and/or cognitive symptoms<br>• Objective memory and/or cognitive symptoms<br>• Normal, unaffected activities of daily living<br>• Do not meet criteria for dementia or AD |
| **Preclinical AD** | • Long asymptomatic period between the first brain lesion and the first appearance of symptoms<br>• Later go on to fulfil diagnostic criteria for AD |
| **Prodromal AD** | • Symptomatic pre-dementia phase<br>• Often included within MCI category<br>• Symptoms not yet severe enough to meet AD criteria |
| **AD dementia** | • Symptoms and features sufficient to meet accepted diagnostic criteria for dementia and AD<br>• Progresses from mild to moderate and then advanced as symptoms worsen |

**Table 1.12: Clinical stages of Alzheimer's disease** [569]

As mentioned previously a number of different theories have been proposed for the aetiology of the classic pathological brain lesions associated with AD. Of primary interest in this thesis is the role that vascular factors may play in the development of AD and the links between the ocular and cerebral vasculature. The following sections will discuss the cerebrovascular anatomy along with the current evidence surrounding vascular involvement in AD at both the ocular and systemic level.

## 1.12 Anatomy and Physiology of the Cerebral Vasculature

The cerebral circulation can be broadly separated into the macro-vascular system and the microvascular system. These will now be discussed in turn.

### 1.12.1 Macrovasculature

The arterial blood supply to the brain is received is via two major vessels, the internal carotid artery (ICA) and the vertebral artery. These arteries enter the skull at the base of the brain and branch dorsally, spreading over the surface of the cerebrum in the subarachnoid space above the pia mater. The ICA is primarily responsible for the anterior circulation of the brain and gives rise to the anterior and middle cerebral arteries, which supply 80% of the blood that reaches the cerebral hemispheres [580]. The vertebral arteries, on the other hand, fuse within the cranium to form the basilar artery which is primarily responsible for supplying the posterior circulation of the brain including the midbrain, cerebellum and brainstem as well as a small proportion of the cerebral hemispheres [580].

The anterior and posterior circulatory regions do not act independently of each other and are interconnected by a series of communicating arteries which create the so called circle of Willis at the base of the brain [581]. This network of vessels consists of the right and left ICAs, anterior cerebral arteries, posterior cerebral arteries, posterior communicating arteries and the anterior communicating artery (figure 1.17). This inter-communication between the anterior and posterior regions is advantageous as if blood flow becomes insufficient in either area it is able to be redistributed, via the communicating arteries of the circle of Willis, according to requirement [580].

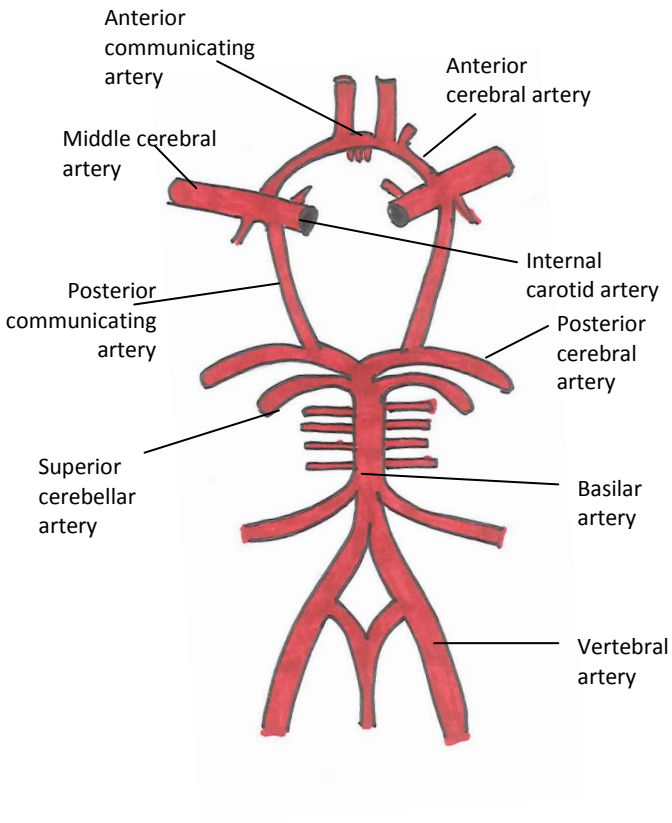



**Figure 1.17: Diagrammatic representation of the cerebral macrovasculature**

The cerebral macro-circulation is crucial in the conductance of blood flow to the various regions of the brain. The cerebral microcirculation then acts to regulate local blood flow and vascular tone within these regions.

### 1.12.2 Microvasculature

The cerebral microvasculature consists of a dense three-dimensional network of fine capillaries which act to maintain the BBB and sustain continuous oxygen, nutrient, electrolyte and waste product transfer between the blood and the cerebral tissues [580]. The density of the capillary network is not uniform throughout the cerebrum and has been shown to be greater in areas of increased metabolic activity, in the grey matter compared to the white matter and in sensory and association centres compared to motor centres [580, 582, 583].

The cerebral capillaries have a specific structure which enables them to fulfil the crucial role of maintaining normal BBB function (figure 1.18). In a similar way to in the ONH (see section 1.2.3.3), the cerebral BBB prevents the passage of all but essential metabolites from the blood into the cerebral tissues, protecting them from the effects of foreign, potentially damaging substances that could be present in the bloodstream as well as the effects of hormones or neurotransmitters that may be active in the rest of the body [90].

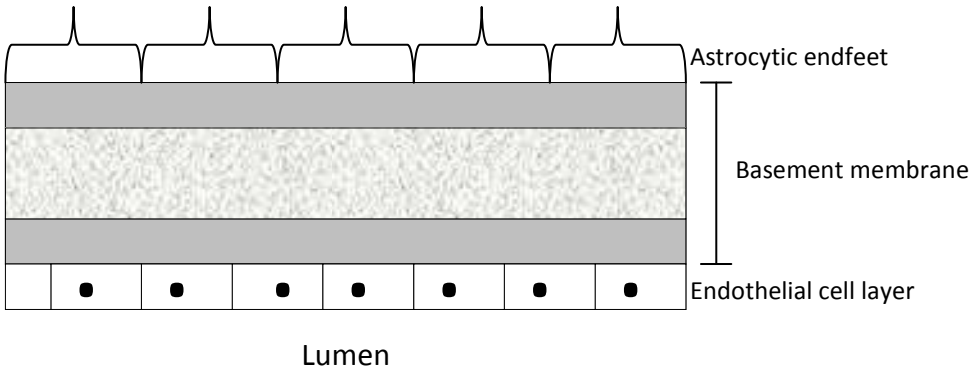



**Figure 1.18 Diagrammatic representation of the structure of the cerebral capillaries**

A key feature of the cerebral capillary structure is the tight junctions that exist between the vascular endothelial cells. These form the basis of the BBB by preventing the free passage of metabolites from the blood stream into the cerebral tissues. Additionally the lack of endothelial cell fenestrations and the high level of mitochondria within the endothelial cells also assist in maintaining the structure of the BBB and the functioning of the specific BBB transport proteins, as does the basement membrane surrounding the endothelial cells. The basement membrane is lamellar in structure and has also been identified to provide physical support to the microvessels, control cellular migration, influence endothelial function, promote cell adhesion and protect the brain against circulating proteins [584]. Another key feature of the cerebral capillary structure is the astrocytic processes which are apposed to the abluminal surface. These act as a link

between synaptic activity and the cerebrovascular cells and play a key role in the maintenance of BBB function [585] and in the regulation of vascular tone [586, 587].

### 1.12.3 Regulation of cerebral blood flow

As discussed in section 1.4 the blood flow through any microvascular bed depends on both perfusion pressure and vascular resistance and the maintenance of normal tissue structure and function depends upon a continuous and well regulated blood supply. The cerebral blood flow (CBF), in a similar way to OBF, is regulated according to changes in perfusion pressure (autoregulation) and also to changes in neural activity (neurovascular coupling) so that blood supply can always meet demand [588].

#### 1.12.3.1 Autoregulation

Autoregulation, which was discussed in section 1.4.3, refers to the ability of the cardiovascular system to modify vascular resistance in order to allow a constant blood supply to maintained despite variations in perfusion pressure [151]. It was first demonstrated in the cerebral circulation by Fog in the 1930s [589, 590] and later established by Lassen in 1959 [591]. Whilst the exact mechanisms underlying cerebral autoregulation are still unclear it is known to be triggered by metabolic, myogenic, neurogenic and to a lesser extent, humoral factors, as well as by endothelial derived vasoactive agents [588, 592, 593]. These triggers were discussed in detail in section 1.4.3 and act on the cerebral vasculature in a similar manner to that discussed with regard to the ocular circulation. Worth noting is the fact that the cerebral vessels, in a similar way to the choroidal vessels and unlike the retinal vessels, have a rich autonomic innervation which allows maintenance of blood flow in the presence of altered BP or HR. Sympathetic stimulation, although having little effect on regulation of blood flow under normal conditions, attenuates the increase in blood flow observed when BP is raised [594, 595] and parasympathetic stimulation acts to increase cerebral blood flow when required [596].

### 1.12.3.2 Neurovascular Coupling

The other means by which CBF is regulated is via neurovascular coupling mechanisms. Neurovascular coupling refers to the ability to regulate blood flow according to changes in neural function and was first identified in the cerebral circulation in the late 1800s [597, 598]. The normal neurovascular coupling response at the cerebral level is characterised by the rapid dilation of arterioles and capillaries in a restricted brain region in response to a local episode of increased neural activity [599]. This dilation response leads to a rapid increase in blood flow and oxygen supply to the active brain regions, ensuring the increased metabolic demand is met. The exact mechanisms which drive the neurovascular coupling response are still unclear, however it is thought to involve a complex interplay between neurons, astrocytes and vascular cells (endothelial cells, pericytes and vascular smooth muscle cells) [600, 601], with the vasodilator nitric oxide (NO) having been identified as one of the important mediators of the response [602, 603]. The key components of the neurovascular coupling response, namely neurons, astrocytes and vascular cells are commonly referred to collectively as the 'neurovascular unit' (NVU) and they are not only involved in the regulation of blood flow but also work to maintain the homeostasis of the cerebral microenvironment through controlling BBB exchange and contributing to immune surveillance [604]. As well as having been recognised at the cerebral level, neurovascular coupling has also been demonstrated at the ocular level in both the retinal and ONH circulations[605-607], whereby an increase in retinal neuronal activity stimulates the dilation of the retinal arteries and capillaries, increasing blood to flow to the excited regions. Further similarities in the functioning of the ocular and cerebral microcirculations are discussed in the following section.

### 1.12.4 The Vascular Theory of Alzheimer's disease

As discussed previously the formation of extracellular beta-amyloid senile plaques and intracellular neurofibrillary tangles, as a result of abnormal tau protein phosphorylation, are considered the hallmark lesions of AD [558-561]. However whilst there is clear evidence that these features contribute towards the degeneration of neurons and synapses in the AD brain, the mechanisms which trigger their development is currently unclear. The potential role played by vascular factors and microvascular dysfunction in the development of such features has long been recognised and increasing amounts of evidence for a vascular aetiology of AD has accumulated over recent years. Indeed the presence of traditional vascular risk factors such as obesity [608], smoking [609], hypertension [610], hypercholesterolemia [611], diabetes [612, 613] and alcohol consumption [614], in both mid-life and in the elderly, as well as the occurrence of vascular diseases, such as heart disease, atherosclerosis, stroke and transient ischemic attacks [615, 616], have all been identified as established associates of AD. The so called vascular theory of AD development, first introduced by de la Torre et al [566], proposes that the presence of such cardiovascular risk factors could contribute to the development of AD through triggering either dysregulation of CBF and exposure of the cerebral tissues to repeated ischemic episodes, the development of chronic brain hypoperfusion [566, 617, 618], a disturbance of BBB function or a direct alteration in beta-amyloid regulation and function [619-621]. Indeed, in support of this, numerous studies have found evidence of reduced CBF and glucose metabolism in AD patients [622-625], as well as disturbed cerebral autoregulation [567, 604, 617, 620] and altered neurovascular coupling mechanisms [567, 626-630]. Additionally impaired vascular function has been identified at the systemic level in AD patients [631] along with the presence of endothelial dysfunction and disturbances of the BBB [619, 632]. Links have also been made between cerebrovascular dysfunction and the abnormal regulation of beta-amyloid, a key feature of AD [604] and furthermore the distribution of beta-amyloid deposits across the cerebral tissues, has been shown to map the pattern of

microvascular damage in AD [633]. The relationship between beta-amyloid and microvascular dysfunction however is not straight forward as it is possible for either of them to drive the development of the other. The accumulation of beta-amyloid, for example, is known to have a detrimental effect on the cerebrovasculature and to act as a potent vasoconstrictor; if occurring first it could therefore potentially contribute towards the development of cerebrovascular dysfunction [620]. The initial presence of cerebrovascular dysfunction or ischemia on the other hand could promote the accumulation of beta-amyloid and, through disruption of the BBB, impair its regulation and removal from the cerebral tissues, contributing towards beta-amyloid plaque formation [620]. Both mechanisms would lead to the development of AD pathology and recent evidence suggests their relationship is likely to be synergistic [604].

Further evidence in support of cerebrovascular dysfunction in AD comes from studies looking at structural alterations in the cerebral microvasculature in AD patients, which could disrupt the cerebral microenvironment homeostasis and promote neuronal dysfunction [604]. Indeed decreased microvascular density, increased tortuousity, basement membrane thickening, degenerative wall changes and the accumulation of beta-amyloid has been evidenced in the cerebral arterioles and capillaries of those with AD [580, 621, 634], along with the presence of atherosclerotic plaques in the cerebral macrovasculature [635, 636].

The concept that a dysregulation of CBF, in the form of either disturbed autoregulation or neurovascular coupling, may be associated with the development of AD is therefore well evidenced. It has been proposed that the resultant exposure of the cerebral tissues to repeated ischemic/hypoxic episodes, if sustained over a long period of time, could potentially lead to neurodegeneration and the development of AD-type brain lesions, through the mechanism of RRI, astrocyte activation and exposure to oxidative stress [617], in a similar manner to that discussed with regard to glaucoma (section 1.9.13). Indeed

oxidative stress induced tissue damage in the form of lipid, nucleic acid and DNA modifications has previously been demonstrated in the cerebrovasculature of those with AD and also linked to the cerebral accumulation and dysfunction of beta-amyloid, a key feature of the disease [565, 619, 620, 637, 638]. Interestingly the development of cerebrovascular dysregulation, if present, is thought to occur as an early event in the pathogenesis of AD, preceding symptoms and the characteristic progressive decline in cognitive function [632, 639]. The detection of such dysregulation at the earliest stages in at risk individuals could therefore be critical in the early diagnosis and management of the disease [640].

Overall the evidence linking vascular dysfunction and cardiovascular risk factors to the development of AD is fairly strong. Indeed, on the back of recent evidence, questions have been raised as to whether AD should even still be considered as a primarily neurodegenerative disorder or in fact should be considered a primary vascular disorder [630]. Despite its apparently strong vascular component however it is important that AD is still distinguished from vascular dementia (VaD), the second most common type of dementia accounting for around 15% of cases, in comparison to AD's 75-80% [641, 642]. For clarity an overview of VaD and its characteristics in comparison to AD is given in the following section.

#### 1.12.4.1 Alzheimer's disease vs. Vascular dementia

VaD can be defined as a cognitive impairment resulting from cerebrovascular disease and ischemic and/or haemorrhagic brain injury [643]. Where the hallmark characteristic of AD is the formation of extracellular beta-amyloid senile plaques and intracellular neurofibrillary tangles [558-561], the hallmark characteristics of VaD is ischemic damage and the presence of multiple infarctions due to cerebral vessel occlusion [644].

The relationship between AD and VaD has become complex due to the increasing amounts of evidence for vascular involvement in AD and the considerable overlap in the symptoms and pathophysiology of the two conditions. Indeed, it has recently been suggested that rather than considering these conditions as two separate entities they may in fact represent a dementia continuum, extending from pure AD through to pure VaD, with a 'mixed' component separating the two extremes [645, 646]. Nevertheless and despite their similarities there are a number of distinctions that can be made between AD and VaD and this is particularly important for research purposes. These similarities and differences are summarised in table 1.13.

| | Alzheimer's disease (AD) | Vascular Dementia (VaD) |
|---|---|---|
| **Onset** | Gradual, often present for many years before diagnosis | Abrupt |
| **Risk factors** | - Stroke<br>- Hypertension<br>- Heart disease<br>- Diabetes<br>- Hyperlipidemia<br>- Atherosclerosis<br>- Smoking<br>- Obesity<br>- Alcohol<br>- Age<br>- Education | - Stroke<br>- Hypertension<br>- Heart disease<br>- Diabetes<br>- Hyperlipidemia<br>- Atherosclerosis<br>- Smoking<br>- Obesity<br>- Alcohol<br>- Age<br>- Education |
| **Characteristic features / hallmark lesions** | - Beta-amyloid plaques<br>- Neurofibrillary tangles | - Primary damage is ischemic<br>- Physical and imaging evidence of cerebrovascular disease |
| **Clinical signs/symptoms** | - Cognitive decline and functional deterioration<br>- Memory and language deficits more common | - Cognitive decline and functional deterioration<br>- Attention, planning and speed of mental processing deficits more common<br>- Earlier and more severe mood/personality changes |
| **Diagnostic criteria** | NINDS-ADRDA<br><br>(National Institute of Neurological Disorders and Stroke – Alzheimer's disease and related disorders association) | NINDS/AIRENS<br>(National Institute of Neurological Disorders and Stroke / Association Internationale pour la Recherche et l'Enseignement en Neurosciences) |
| **Progression / Course** | - Predictable pattern of cognitive impairment<br>- Predictable spread of cortical neuronal death<br>- Predictable rate of cognitive decline | - Fluctuating and variable<br>- Unpredictable<br>- Shorter life expectancy than AD but similar rate of decline |

**Table 1.13 Features of Alzheimer's disease vs. Vascular dementia**

### 1.12.5 Cerebral and Ocular Microcirculations: Associations

The cerebral and ocular microcirculations share a large number of similarities in their anatomy, physiology and embryology, with both forming part of the CNS [4]. The tissues they supply, namely the brain and retina, are both highly metabolically active and rely upon the integrity of their respective microcirculations for maintenance of normal function. Blood flow is actively regulated at both sites via the mechanisms of autoregulation and neurovascular coupling [647] and maintenance of both normal brain and retinal tissue function is reliant on the integrity of the BBB, or blood-retinal-barrier as it is commonly referred to at the ocular level [648]. As discussed in section 1.12.2, the cerebral capillaries have a characteristic structure which allows formation and maintenance of the BBB and this structure is replicated in the inner retinal capillaries. Additionally, both the cerebral and retinal microcirculations have been shown to undergo similar changes with aging, whereby a decrease in both CBF and OBF is observed along with disturbances in the structural integrity of the microvessels [4, 580, 649]. These plentiful similarities between the anatomy and physiology of the cerebral and ocular circulations have inevitably led to the cerebral vasculature being considered in ocular disease and the ocular circulation being considered in cerebral disease.

### 1.12.6 The Concept of using the 'Eye as a Window to the Brain'

With the increasing amount of evidence linking vascular dysfunction to AD, the development of new or enhanced methods of detecting such vascular dysfunctions in the earliest stages of the disease process would be beneficial with regard to its diagnosis and management. It is with this in mind that AD research has expanded to look, not only at the cerebral vasculature, but also at the significantly more accessible ocular vasculature. Indeed the concept of using the 'eye as a window to the brain' has been increasingly recognised and explored [3] and it is proposed that through imaging and

analysis of the easily visible retinal vessels, information regarding the state and functioning of the cerebral vessels could be obtained non-invasively and diagnosis and understanding of cerebral diseases such as AD could be enhanced.

The relevance of the retinal circulation in cerebral disease is highlighted by the presence of retinal microvascular abnormalities, including arterial narrowing, arteriosclerosis and the presence of retinal exudates or micro-aneurysms, in various forms of cognitive impairment [650-652]. With regard to AD in particular, alterations in numerous aspects of ocular function, including visual changes, structural retinal vessel changes and OBF alterations, have been identified, highlighting the probable involvement of the ocular circulation in the AD disease process [3]. Indeed difficulty with reading and finding objects, colour recognition problems, abnormalities in visual attention, memory, depth and motor perception, as well as reduced contrast sensitivity have been previously identified in AD patients [653-655] in the earliest stages of the disease, often before diagnosis has been firmly established, but these are primarily been linked to higher visual cortex changes rather than changes at the ocular level. The exception to this is reduced contrast sensitivity which has also been linked with alterations in the retinal ganglion cells and RNFL [653, 656, 657]. At the retinal level, multiple studies have demonstrated structural and functional changes in AD patients including RGC degeneration [427, 658], a reduction in RNFL thickness and macular thickness [659, 660], RGC beta-amyloid deposition [661, 662], narrowed retinal blood vessels and reduced retinal blood flow [663]. Furthermore increased cupping and reduced NRR volume and area has also identified at the ONH in AD patients [664-666] and away from the retina, beta-amyloid deposition has been identified in both the intraocular lens and aqueous humour [3, 667].

Ocular involvement in AD is therefore fairly well evidenced and assessment of the retrograde loss of retinal and ONH nerve fibre layer tissue has even been proposed as a potential early biomarker of AD [668]. The importance of considering the ocular circulation

in AD is supported further by the associations found between AD and the presence of ocular diseases such as glaucoma and AMD [669-671]. Indeed the presence of beta-amyloid has even been identified in the drusen of patients with AMD [672]. Of relevance to this thesis is the possible aetiological associations between AD and glaucoma, this is discussed in more detail in the following section.

### 1.12.7 Alzheimer's disease and glaucoma

The possibility that AD and glaucoma may share a common underlying mechanism has been increasingly realised over recent years to the extent that glaucoma has been suggested as an ocular form of AD and AD as a cerebral form of glaucoma [671, 673]. The two conditions have obvious associations in that they are both chronic neurodegenerative diseases, strongly related to aging, which involve the loss of nerve cells by apoptosis. The end stages of both conditions are fairly well evidenced, with RGC loss and ONH excavation being the key feature of glaucoma and the formation of extracellular beta-amyloid senile plaques and intracellular neurofibrillary tangles being the hallmark cerebral lesions of AD. The aetiological mechanisms which lead to the development of these characteristic lesions is currently uncertain for both diseases, however vascular dysregulation and oxidative stress has been strongly linked to both [141, 565] (see sections 1.9 and 1.12.4).

Evidence for an association between AD and both POAG and NTG comes from epidemiological, pathological and vascular studies. Firstly from an epidemiological point of view, multiple studies have demonstrated an increased rate of occurrence and progression of OAG (POAG or NTG) in patients diagnosed with AD [400, 436, 449, 674] and an increased prevalence of glaucoma in those with cognitive impairment or dementia [435, 675]. Other studies, however have failed to demonstrate such an increased rate of POAG and NTG in those with AD [426, 676], making it difficult to draw any firm conclusions. The differences between these studies however could be accounted for by their differing

nature of evaluation, with the studies finding no increased rate being of a retrospective and register-based nature, which is a less favoured method of evaluation and has received some criticism [677]. Secondly, from a pathological point of view, classic lesions commonly associated with AD have been reported at the ocular level in glaucoma patients and vice versa. Decreased levels of beta-amyloid and increased levels of tau-protein, a feature commonly demonstrated in the CSF of AD patients, has for example been demonstrated in the vitreous humour of glaucoma patients [423]. Furthermore, in glaucoma patients with unregulated IOP, an increased presence of abnormal tau protein molecules has been shown in the posterior retina [678] and in experimental glaucoma an increased aggregation of beta-amyloid and the presence of amyloid precursor proteins has been demonstrated in the RGC and ONH of rat eyes [419]. On the other hand, reduced retinal perfusion and increased ONH cupping, characteristic of glaucoma, has been identified at the ocular level in AD patients [663, 665]. This discovery of coexisting lesions in AD and glaucoma patients provides more direct support for the concept that both diseases may share a common underlying pathology. The nature of this common underlying pathology is unclear; however due to the strong vascular links outlined in both conditions individually and the similarities in the ocular and cerebral microcirculation, it is highly possible that this shared pathology may be vascular in origin. Indeed, evidence of increased cerebral small vessel ischemia [313] and AD-like perfusion patterns [468], have been demonstrated in NTG patients and evidence of disturbed cerebral autoregulation, in association with altered sympathetic innervation or endothelial function, has been found in both POAG and NTG patients [323]. As both ischemia and cerebrovascular dysfunction have been previously linked to the development of AD, these finding suggest that microvascular abnormalities present at the ocular level in glaucoma patients could potentially extend to the cerebral level and be associated with the development of AD.

Aside from vascular factors, other aetiological theories linking AD and OAG have also been proposed relating, for example, to decreased cerebrospinal fluid pressure in both conditions [679], raised intracranial pressure in association with raised IOP [562] or the presence of common genetic risk factors [680, 681].

All in all the associations between AD and glaucoma, although evident, are still surrounded by uncertainty. Establishing the nature of their relationship could be beneficial to the early diagnosis, treatment and pathological understanding of both conditions. Furthermore, exploring the relationship between the cerebral and retinal microcirculation and the concept of using the 'eye as a window to the brain' could offer new methods of assessment and diagnosis in AD. Both of these issues are addressed in this thesis

# 2. Research Rationale

Central to both current glaucoma and AD research is the consideration that, aside from the local ocular or cerebral circulations, multiple additional systemic sites may also be involved in the aetiology of these diseases and that a generalised vascular dysfunction, affecting the circulatory system as a whole, may exist in diagnosed individuals. Exactly how such vascular alterations at multiple sites may inter-relate and their impact on the neurodegenerative disease process is however still poorly understood. Elucidating these inter-relations in both AD and glaucoma individually and establishing their relative impact in different subcategories of these diseases, such as POAG and NTG, could not only enhance our understanding of disease aetiology but also open up the possibility for new and alternative diagnostic and therapeutic avenues.

As well as the interrelationships between vascular alterations at multiple sites in AD and glaucoma individually, the interrelationships between these two neurodegenerative diseases themselves, along with the potential benefits of utilising more accessible regions of the CNS, such as the ocular circulation, to gain an insight into the pathological mechanisms which may be occurring at the cerebral level, have been increasingly realised over recent years. Such utilisation of the ocular circulation could significantly aid the assessment and diagnosis of vascular alterations in AD and enhance our understanding and management of the disease. Furthermore if the exact nature of the relationship between AD and glaucoma could be established, the relevance of considering each disease process in the presence of the other could be highlighted and the diagnosis, management and aetiological understanding of both conditions could potentially be enhanced. At the present date, direct evidence of vascular dysfunction at the ocular level, comparable to that at the cerebral level is yet to be demonstrated and although a number of similarities between AD and glaucoma have been acknowledged in recent years, the exact association between these two

neurodegenerative diseases is still surrounded by uncertainty. Furthermore the concept of whether AD and glaucoma share a common underlying aetiology, comparable in both POAG and NTG, has not been fully explored. Through the development of new technologies aimed specifically at assessing ocular vascular function, such as dynamic retinal vessel analysis (DVA), there is however now an increased potential for this.

Taking all of this into consideration, the principle purpose of this research was to fully establish and explore the presence of vascular alterations at both the ocular and systemic level in AD, POAG and NTG patients and to evaluate the potential impact that these vascular alterations may have, both on these disease processes individually and on the inter-relationships between them. In line with this the overall aims of this research are as outlined in the following section.

## 2.1 Aims

- To investigate the presence and impact of ocular and systemic vascular alterations in AD and to explore the concept of using the ‘eye as a window to the brain’
- To investigate the presence and impact of ocular and systemic vascular alterations in NTG
- To investigate the possibility of a shared vascular aetiology, involving both the ocular and systemic circulations, in AD and both POAG and NTG.
- To compare and contrast vascular alterations at both the ocular and systemic level in POAG and NTG and to explore their validity as distinct clinical entities

# 3. Subjects and Methods

This chapter outlines the recruitment procedure, inclusion and exclusion criteria for the AD, POAG, NTG and control participants involved in this research. It then goes on to outline the study protocol and investigative techniques used throughout this thesis for the assessment of ocular and systemic vascular function. All disease participants recruited for this research were newly diagnosed and previously untreated. This ensured any vascular alterations identified were less likely to have occurred as a secondary effect of the disease process and could be more reliably attributed to the development or pathogenesis of the disease itself.

## 3.1 Patient recruitment

### 3.1.1 Recruitment of mild newly diagnosed AD patients

Successive newly diagnosed mild AD patients were recruited from the Birmingham and Solihull Mental Health NHS Trust (BSMHT, UK) by a team of dementia specialists. Posters advertising the study were displayed in the clinic waiting areas along with information leaflets and flyers. The dementia team were fully briefed on the study protocol, inclusion and exclusion criteria.

#### 3.1.1.1 AD inclusion criteria

Only those patients diagnosed with AD in accordance with the NINCDS-ADRDA and DSM-IV-TR criteria for diagnosis (see section 1.10.2) were considered for this study [569, 682]. Of these diagnosed patients only those classified as having mild AD, based on a MMSE score of between 18 and 24, were included in the final study. Following the identification of a suitable participant by the dementia team, the study was discussed with them and a detailed information booklet provided. 24 hours later the potential participants were contacted by the author to further discuss the study, answer any questions and confirm enrolment in the study for those who were willing.

### 3.1.2 Recruitment of newly diagnosed POAG patients

Successive, early stage, newly diagnosed and previously untreated POAG patients were recruited from the glaucoma clinics at both the Heart of England and Sandwell and West Birmingham NHS Trusts. Posters advertising the study were displayed in the clinic waiting areas along with information leaflets and flyers. The diagnosis of early POAG was made by a team of ophthalmologists working under the supervision of two glaucoma consultants. Following diagnosis suitable participants were provided with detailed information about the study by the author and allowed at least 24 hours to consider their enrolment.

#### 3.1.2.1 POAG inclusion criteria

Only those patients identified as having glaucomatous cupping of the optic disc, normal open anterior chamber angles and visual field (VF) defects consistent with the diagnosis of early glaucoma using program 24-2 of the Humphrey visual field analyser (HFA:Zeiss-Humphrey, San Leandro, CA) were included. An early glaucomatous VF defect was defined as a mean deviation (MD) score of ≥ -6.00dB along with either a glaucoma hemifield test outside normal limits and/or a corrected pattern standard deviation (CPSD) with p-value<0.05 [683]. Only reliable VF plots, with <20% fixation losses and <33% false positive and false negative responses, were considered. Classification as POAG was based on an IOP measurements consistently above 21 mmHg on diurnal testing (measurements every 2 hours over an 8 hour period) with applanation tonometry. All patients were newly diagnosed and had no current or previous treatment with IOP lowering drops.

### 3.1.3 Recruitment of newly diagnosed NTG patients

Successive, early stage, newly diagnosed and previously untreated NTG patients were recruited from the glaucoma clinics at both the Heart of England and Sandwell and West Birmingham NHS Trusts. Posters advertising the study were displayed in the clinic

waiting areas along with information leaflets and flyers. The diagnosis of early NTG was made by a team of ophthalmologists working under the supervision of two glaucoma consultants. Following diagnosis suitable participants were provided with detailed information about the study by the author and allowed at least 24 hours to consider their enrolment.

#### 3.1.3.1 NTG inclusion criteria

Only those patients identified as having glaucomatous cupping of the optic disc, normal open anterior chamber angles and visual field (VF) defects consistent with the diagnosis of early glaucoma using program 24-2 of the Humphrey visual field analyser (HFA:Zeiss-Humphrey, San Leandro, CA) were included. An early glaucomatous VF defect was defined as a mean deviation (MD) score of ≥ -6.00dB along with either a glaucoma hemifield test outside normal limits and/or a corrected pattern standard deviation (CPSD) with p-value<0.05 [683]. Only reliable VF plots, with <20% fixation losses and <33% false positive and false negative responses, were considered. Classification as NTG was based on an IOP measurement consistently less than or equal to 21 mmHg on diurnal testing (measurements every 2 hours over an 8 hour period) with applanation tonometry. All patients were newly diagnosed and had no current or previous treatment with IOP lowering drops.

### 3.1.4 Recruitment of healthy controls

Age-matched healthy controls were recruited by inviting the participation of patients' spouses, same-generation relatives and friends, as well as through promotion of the study at the Aston University Health Clinics via posters, information leaflets and flyers displayed in the waiting areas.

#### 3.1.4.1 Healthy control inclusion criteria

All healthy controls were screened for ocular disease and dementia, using the Addenbrooke's Cognitive Examination-Revised (ACE-R; appendix 2) [684], before inclusion in the study. Only healthy individuals with a normal ocular examination (fundus assessment, IOP measurement and visual fields) and an ACE-R score of at least 88 were then included in the study [684].

### 3.1.5 Exclusion criteria for all groups

Patients with closed iridocorneal angles, evidence of secondary glaucoma, pseudoexfoliation, pigmentary dispersion, history of intraocular surgery or any form of retinal or neuro-ophthalmological disease that could result in visual field defects were excluded from the study. AD, POAG, NTG and healthy participants were excluded if they were smokers or had a positive diagnosis of cardio- or cerebro-vascular disease, (coronary artery disease - CAD, heart failure, arrhythmia, stroke, transient ischaemic attacks), peripheral vascular disease, severe dyslipidaemia (defined as plasma triglycerides>6.00mmol/L or cholesterol levels>7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment. Participants were screened for ocular disease and were excluded from the study if they had a refractive error of more than ±3DS and more than ±1DC, IOP>24 mmHg, cataract or any other media opacities, as well as if they had a history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system. Well controlled systemic hypertension was neither an inclusion nor exclusion criteria; however any individuals taking other medications which could potentially influence vascular function were excluded.

## 3.2 Ethical approval

Prior to the study ethical approval was received from South Birmingham local research ethics committee, Heart of England and Sandwell and West Birmingham NHS Research Ethics Committees as well as the Aston University Life and Health Sciences Ethics Committee. Written informed consent was received from all subjects before entry into the study and all procedures were designed and conducted in accordance with the tenets of the Declaration of Helsinki.

## 3.3 Methods

The investigative techniques used throughout this thesis were carefully selected to ensure the most accurate and reliable assessment of vascular function at both the ocular and systemic level could be obtained. A summary of these techniques is given in table 3.1. All techniques were performed by the author with the exception of the blood analyses for von Willebrand factor and glutathione status, which were performed by Dr Lu Qin, an experienced lab technician. Prior to commencing recruitment the author was trained in the use of each technique by experienced colleagues and conducted a series of preliminary examinations on at least 15 volunteers to ensure an adequate level of competency was achieved. Across this series of preliminary examinations the importance of careful patient instruction, optimal patient adjustment and optimal examiner positioning was realised and the measurement procedures adjusted accordingly.

| Technique | Purpose |
|---|---|
| **Ocular level** | |
| Dynamic retinal vessel analysis (DVA) | Assessment of ocular vascular function |
| **Systemic level** | |
| Flow mediated dilation (FMD) | Systemic endothelial dysfunction |
| Ambulatory blood pressure monitoring (ABPM) | 24 hour blood pressure profile |
| 24 hour Heart rate variability assessment (HRV) | Autonomic nervous system assessment |
| Pulse wave analysis (PWA) | Systemic arterial stiffness |
| Intima-media thickness measurement (IMT) | Cardiovascular risk / atherosclerosis |
| **Blood analysis** | |
| Von Willebrand factor (vWf) | Systemic endothelial function |
| Glucose, Cholesterol, Triglycerides | Cardiovascular risk |
| Glutathione status (GSH vs. GSSG) | Oxidative stress |

**Table 3.1 An overview of the investigative techniques conducted in this thesis**

### 3.3.1 Experimental Protocol

All measurements were performed between 8 am and 11 am following a 12 hour overnight fast, which included no alcohol or caffeine. All procedures were conducted in a consistent order outlined below and detailed in the following sections:

1. Suitable participant identified, approached and provided with the study information pack
2. Procedures and risks explained, concerns addressed
3. Consent form read, understood, completed and signed by participant
4. Preliminary assessments:
    - Demographic questionnaire
    - Addenbrooke's Cognitive Examination-Revised (ACE-R) questionnaire
    - Ocular assessment (Indirect ophthalmoscopy and visual fields)
    - IOP measurement
    - Height and weight measurement
    - Baseline BP readings
5. 1% Tropicamide inserted into randomly selected eye
6. Fasting venous blood sample obtained by venepuncture
7. 24 hour BP and HRV monitor fitted (POAG, NTG, Controls only)
8. Assessment of retinal vessel reactivity (DVA)
9. Pulse wave analysis (POAG, NTG, Controls only)
10. Intima-media thickness measurement (POAG, NTG, Controls only)
11. Assessment of systemic endothelial function (FMD)
12. Final BP measurement

### 3.3.2 Preliminary Assessments

Demographic data regarding age, gender, ethnicity and current medication were collected by means of a short questionnaire. In order to ensure our healthy control and glaucoma patients were not suffering from any undiagnosed cognitive impairments which could have affected the reliability of our results, participants from these two groups were also screened for dementia prior to inclusion in the study using the ACE-R test (appendix 2) [684]. In accordance with guidelines only healthy individuals with ACE-R score of at least 88 were included in the study [684].

IOP was measured using Goldman applanation tonometry following instillation of 0.4% oxybuprocaine hydrochloride and fluorescein. Visual field analysis was conducted using the SITA 24-2 program of the Humphrey visual field analyser and the fundus was assessed using indirect ophthalmoscopy. Weight and height were recorded and BMI calculated (equation 3.7, section 3.3.5.4). EDTA blood samples were obtained from the antecubital fossa vein of all participants and were tested immediately for fasting triglycerides (TGs), glucose and total and HDL cholesterol, using a Reflotron Desktop Analyser (Roche Diagnostics, UK) and later analysed for vWf and oxidative stress. SBP and DBP were measured using an automatic BP monitor (UA-767, A&D Co. Ltd, UK).

### 3.3.3 Assessment of Ocular Vascular Function: Dynamic retinal vessel analysis (DVA)

As discussed in section 1.9.5, the presence of reduced/unstable blood flow in the retina, choroid, ONH and retrobulbar vessels of patients with glaucoma is already fairly well established; however the mechanisms by which the blood flow becomes reduced / unstable are less established. With regard to this thesis therefore, techniques aimed at assessing ocular vascular function, as opposed to simply measuring OBF, were of much greater interest. The dynamic retinal vessel analyser (DVA) is currently the most widely

used method for assessing retinal microvascular reactivity in health and disease and is considered to be a sensitive indicator of retinal microvascular function [685, 686]; it has therefore been the technique of choice for assessing ocular vascular parameters in this thesis. DVA allows the dynamic behaviour of the retinal vessels to be assessed through the continuous online measurement of vessel diameter changes in response to provocation [687]. As discussed in section 1.4, adjustments in vascular diameter and hence adjustments in vascular resistance play an important role in the local autoregulation of OBF. As disturbed autoregulation has been identified as a key risk factor in the development of glaucoma and AD, (see sections 1.9.7 and 1.10.3), assessment of the alterations in retinal vessel diameter in response to provocation, by means of DVA, can provide useful diagnostic information about autoregulatory function in such individuals.

#### 3.3.3.1 Basic principles

The setup of the dynamic retinal vessel analyser device (DVA, IMEDOS, Germany) consists of a fundus camera (FF450, Zeiss Jena, Germany), a charged coupling device (CCD) camera, a high resolution video recorder, a real time monitor and a personal computer with analysis software, as illustrated in figure 3.1.

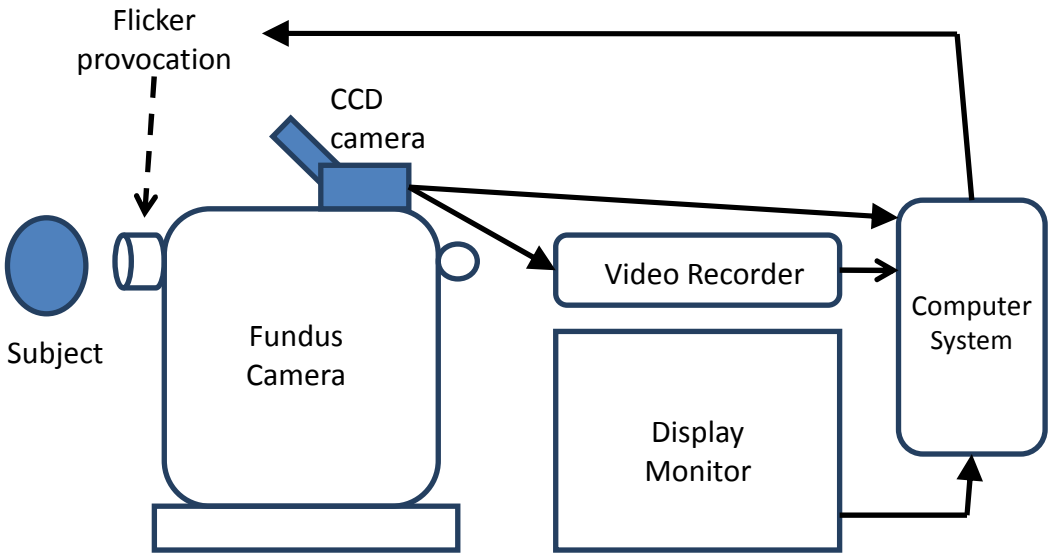



**Figure 3.1 Diagrammatic representation of the dynamic retinal vessel analyser set up**

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

- The illumination light of the fundus camera enters the eye through a dilated pupil and is reflected by the different layers of the retina and retinal vessels.
- It is then delivered via the observation pathway to the CCD camera
- The brightness profile data is then analysed by the computer system and simultaneously recorded by a high quality video recorder so that off-line analysis can be conducted at a later date if necessary.

To ensure optimal alignment and set up the operator can observe an image of the fundus on the computer display (figure 3.2) and to ensure optimal contrast for vessel visualisation a green filter is inserted into the illumination pathway of the fundus camera. Furthermore, the device is equipped with a series of adaptive algorithms to compensate for any disruption of the vessel brightness profile, by the presence of either shadowing structures from the background or reflections from the vessel surface. It also has the capability to correct for slight eye movements during assessment and can continuously monitor image quality, according to image contrast and then automatically remove any inadequate measurements from the analysis [687, 688].

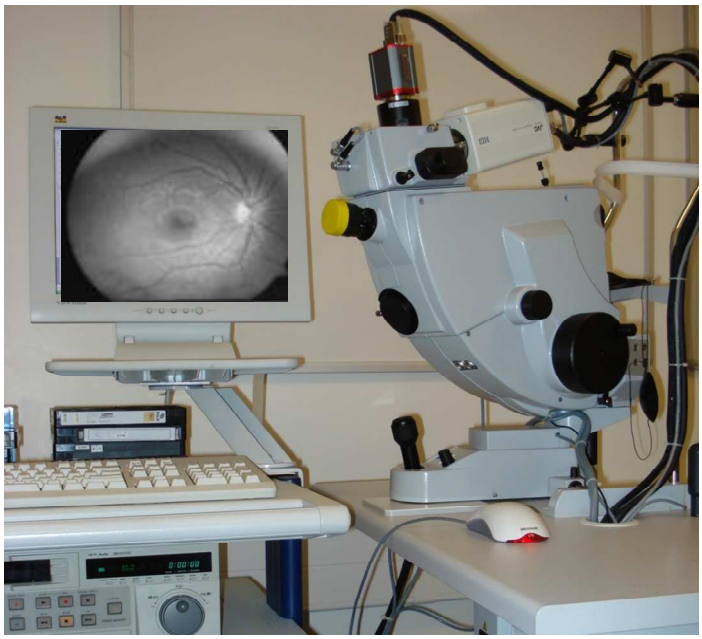


**Figure 3.2 Dynamic retinal vessel analyser (DVA)**

The technical specifications of the DVA are summarised in table 3.2 [687]. The resolution of the device is such that accurate measurements cannot be achieved from vessels with diameters < 90 µm and the temporal resolution of the device is 40 ms, such that 25 video frames are captured per second (i.e. sampling rate = 25Hz). The image field or camera angle should be set at $30^0$ and a clear fundus image, with good contrast and even illumination should be obtained through a fully dilated pupil. All size related measurements are expressed in 'units of measurement' (UM), whereby 1 UM is equivalent to 1 µm in a normal emmetropic eye [687].

| Parameter | Value |
|---|---|
| Measurement range | 90 µm |
| Measurement resolution | < 1 µm |
| Temporal resolution | ≥ 40 ms |
| Image field angle | $30^o$ |
| Measuring time | 350 seconds ( but can be up to 10 mins) |
| Maximum length of vessel segment | 3 mm |
| Spatial resolution (along vessel segment) | 180 µm |
| Measuring sensitivity | 1 MU/1 µm |

**Table 3.2: Technical specification of the DVA [687]**

Over the last few years DVA has become a widely used method for assessment of retinal vascular function however it does have some limitations which are summarised below, along with its relative advantages:

#### 3.3.3.2 Advantages

- Non-invasive
- Continuous recording of vessel diameter allows quantification of the effects of provocation with high time resolution
- Vessel segments and different retinal vessels can be investigated simultaneously
- High reproducibility [689]
- Low variability [690]

#### 3.3.3.3 Limitations

- Reliant on patient having clear media to gain an image of sufficient quality
- Reliant on good patient fixation over 350 second testing period
- Requires full pupil dilation with Tropicamide 1% only (to avoid adverse vascular effects)
- Assumes the eye has no refractive error
- Doesn't measure absolute retinal vessel size, instead uses standardised or relative units

As mentioned previously in order to assess the dynamic behaviour and autoregulatory capacity of the retinal vessels the vessels need to be provoked. The DVA can be operated in conjunction with a variety of provocation devices including suction cup IOP enhancement, pure oxygen breathing, inhalation of $CO_2$ and flicker light stimulation [687]. For the purposes of this thesis flicker light stimulation has been used to assess dynamic retinal vessel reactivity and this will be discussed in more detail in the following section.

### 3.3.3.4 Flicker light stimulation

Flicker light can be considered the most natural provocation method for assessing dynamic retinal vessel behaviour and has the advantage of stimulating the retina exclusively, without the involvement of any other vascular bed. The normal vascular response to flickering light has been widely studied [686, 691] and there is plentiful evidence to indicate that, under normal circumstances flicker stimulation should lead to an increase in vessel diameter, retinal blood flow and ONH blood flow in humans [692].

Flicker light can be defined as illumination which alternates in brightness or colour at a frequency of approx 1-50 Hz [691]. Electrophysiological studies have shown that the maximum sensitivity of the human visual system to flicker stimulation is obtained with a flicker frequency of between 10-20 Hz [685]. The DVA device used throughout this thesis was equipped to generate flickering light at a sampling rate of 12.5 Hz via an optoelectronic shutter placed in the optical pathway of the camera. A sampling rate of 12.5 Hz lies within the optimum flicker frequency range and has been shown to provide appropriate retinal stimulation by numerous studies [685, 691, 693].

The measurement protocol used to assess retinal microvascular reactivity to flicker light in this thesis is in accordance with that introduced by Nagel et al [686]. This protocol is widely used and recommended [688] and is outlined in the following section.

### 3.3.3.5 Measurement protocol

- The fundus camera is positioned so as to obtain a uniformly illuminated fundus image through the fully dilated pupil without unwanted reflections and the brightness is adjusted to ensure optimal contrast
- The patient's fixation is directed, with the use of a fixation needle, so that the measurement area of interest lies in the centre of fundus picture

- A region of interest is defined on the retina by the user selecting a rectangular area on the real time monitor which outlines the region to be studied (usually inferior to ONH).
- From within this area a section of the inferior temporal retinal artery and a section of the inferior temporal retinal vein, located approximately 1.5 disc diameters from the ONH and approx 0.5-1 disc diameters in length and a reasonable distance apart, are selected for analysis and monitoring (figure 3.3).

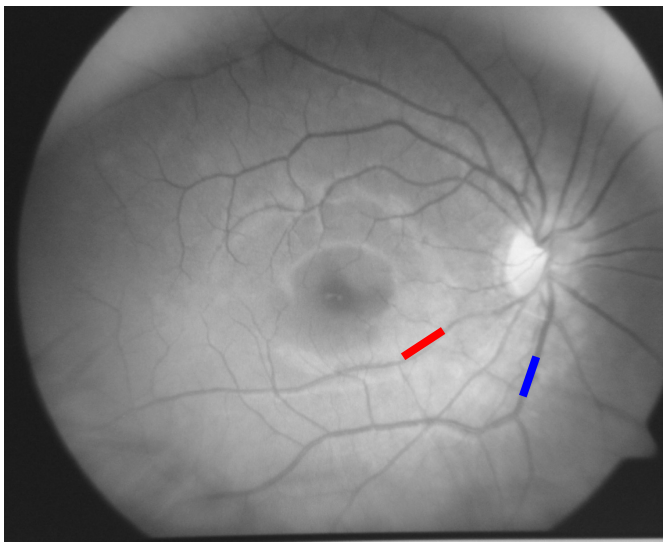


**Figure 3.3 Example of retinal vessel selection prior to dynamic retinal vessel analysis**

- Measurement then starts automatically and vessel diameters are continuously calculated along the length of the selected vessels over a 350 second testing period. This consists of 50 seconds of baseline measurements under still illumination (25Hz), followed by 3 cycles of 20 second flicker stimulation (optoelectronically generated at 12.5 Hz) each interrupted by 80 seconds of still illumination (recovery), as outlined in figure 3.4.

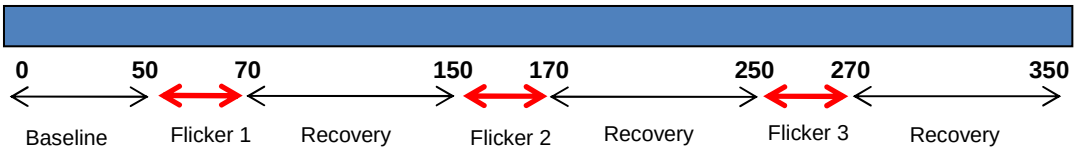



**Figure 3.4 Breakdown of the 350 second DVA testing period**

All measurements are performed in a quiet, temperature-controlled room (22$^{o}$C) following full dilation of one unselected eye (1% tropicamide, Chauvin Pharmaceuticals Ltd).

The use of a 350 second testing period with three sequential flicker cycles was initially introduced so that an averaged vessel response could be calculated and analysed to ensure stable results could be obtained over a testing period of tolerable length [686, 694]. More recently however new techniques of analysis have been introduced which also consider the vessel responses to each flicker cycle individually as well as the overall average [695]. This is discussed in more detail in section 3.3.3.8.

### 3.3.3.6 Normal vascular response

The normal retinal vessel response profile to flicker light stimulation by the DVA is illustrated in figure 3.5. Previous studies have shown that the maximum vessel response to flickering light typically occurs within 20 seconds [693]. After this time period only small increases in diameter occur. Once the flicker stimulation ends, dilation ceases immediately but rather than simply returning to baseline, the baseline is usually overshot and a vasoconstriction occurs. This overshoot has been found to start within approximately 6-10 seconds following the end of the flicker period, reaching its minimum diameter between 10-40 seconds following the end of flicker [691, 693]. The vessel diameter then returns to its baseline level. Both retinal arteries and veins respond to flicker light stimulation, however arteries tend to show a more pronounced diameter change in

comparison to veins and whilst arteries start to react immediately veins have been shown to have approximately a 5 second delay before a response is seen [685, 693].

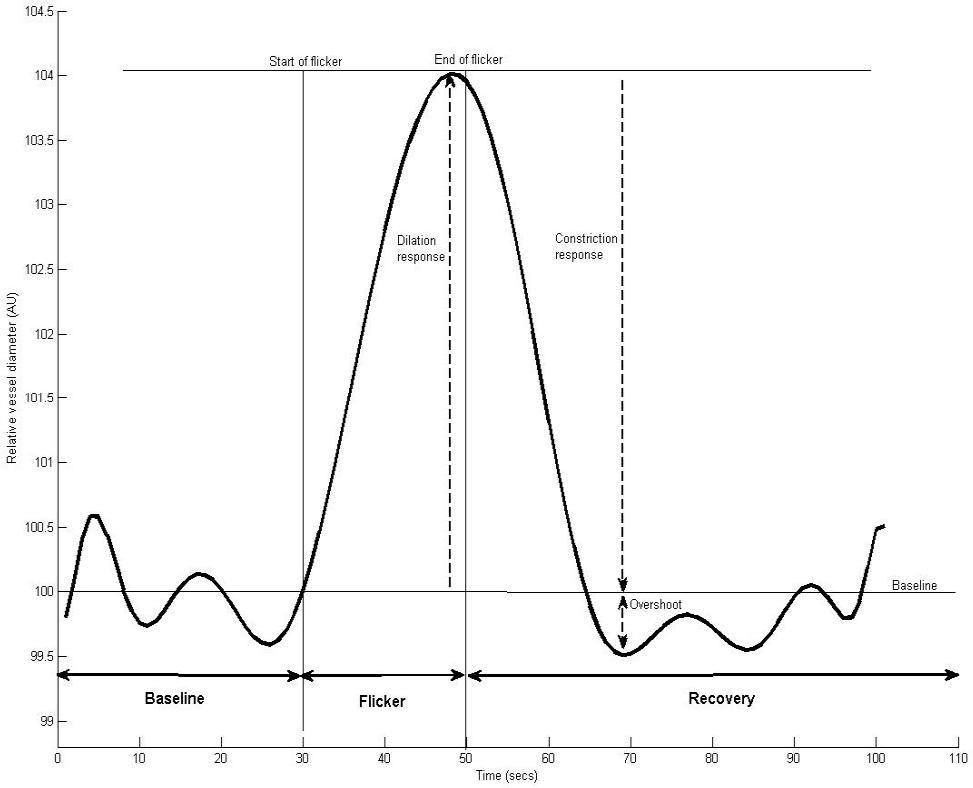



**Figure 3.5 Diagrammatic representation of a typical retinal vessel response to flicker light on dynamic retinal vessel analysis**

Deviation away from this normal vascular response profile can be indicative of vascular disease and indeed numerous studies have already provided evidence of altered vascular response to flickering light in both ocular and systemic disease, including POAG [41], ARMD [696], diabetes [697-699] and hypertension [686]. In order to understand the implications of an impaired or altered vascular response to flicker light with regard to the development of disease, it is necessary to try and understand the mechanisms by which flicker light provokes the retinal vessels.

### 3.3.3.7 Reaction mechanism

Flicker light stimulation increases the neural demand of the retina, which, under normal circumstances should trigger a neurovascular coupling response of the retinal microvasculature, resulting in vasodilation [692] (see section 1.5.3.2) . In general terms an increase in the metabolic rate of the photoreceptors, following stimulation by flickering light, is thought to trigger the release of NO from the retinal vascular endothelium, bringing about an increase in vessel diameter followed by an increase in blood supply to meet the increased demand [226]. An altered vascular response to flicker light therefore could be indicative of impaired autoregulatory mechanisms and/or endothelial dysfunction in the form of reduced bioavailability of NO (see section 1.6). However, due to the complexity of the neurovascular coupling response, it is highly likely that other causative factors such as altered ET-1 levels, altered astrocyte activity or changes in the basal tonus of the vessels could also play a role in altering the retinal vascular response to flicker. The role of the these alternative factors may be particularly relevant when considering the vascular constriction response following flicker [696].

### 3.3.3.8 Data analysis

Of primary interest when analysing the retinal vascular response profile to flicker light is the percentage dilation of the vessel in response to the stimulus and the time scale across which this happens, along with the percentage constriction or overshoot of the vessel following cessation of flicker and again the time scale across which this happens. In this thesis such analysis of the retinal vascular response was conducted using elements of a newly defined method of DVA analysis, termed ‘Sequential and Diameter Response Analysis’ (SDRA) in conjunction with our own novel vascular profile imaging methods. The inbuilt software of the DVA device itself does have the capability to provide an analysis of the retinal vascular response to flicker light, however this has been identified to have a number of shortfalls and the need to move away from this traditional inbuilt software analysis and to evaluate the retinal vascular response profile

in more detail has been increasingly realised by numerous authors in recent years [688, 695, 696]. Indeed SDRA, first introduced by Heitmar et al [695], was primarily designed to overcome the shortfalls of the inbuilt software analysis programme. An overview of this inbuilt DVA analysis and its limitations, along with a summary of SDRA and the novel imaging analysis methods used in this thesis is given in the following sections.

#### 3.3.3.9 Previous methods of analysis

The inbuilt DVA software calculates the vascular dilation response to flicker light by averaging all three of the stimulation cycles and then taking the average diameter from the last +/- 3 seconds of flicker stimulation as the maximum diameter response to flicker (i.e average diameter reached between 17-23 seconds from start of flicker taken as maximum diameter response). The shortfalls of this method largely arise due to its incorporation of both time and diameter responses and its inaccurate assumptions about the nature of the vascular response [695]. Indeed subjects who reach their maximum dilation outside the 17-23 second window would have their maximum dilatory response underestimated by this technique and furthermore by averaging the results from all three flicker cycles differences in the reaction pattern or time course of each individual cycle cannot be determined [695]. Additionally this method of analysis does not take into consideration baseline fluctuation in vessel diameter (BDF), a parameter first highlighted as important in DVA analysis by Nagel et al [686]. BDF refers to the spontaneous variations in vessel diameter, which occur under normal resting conditions, as a result of vascular tone and arterial pulsation and are superimposed on the vascular response profile [686]. In order to account for the influence of BDF, Nagel et al [686] introduced the concept of baseline corrected flicker response (BFR), where BDF is accounted for by subtracting it from the dilation amplitude (DA) of the vascular response, as shown in equations 3.1 and 3.2.

$$BFR = DA - BDF$$

BFR = Baseline corrected flicker response

DA = Dilation amplitude

BDF = Baseline diameter fluctuation

**Equation 3.1 Baseline corrected flicker response**

Where:

$$DA = MD - MC$$

DA = dilation amplitude

MD = maximum dilation

MC = maximum constriction

**Equation 3.2 Dilation amplitude**

#### 3.3.3.10 Sequential and Diameter Response Analysis (SDRA)

SDRA has the advantage of utilising the raw data set generated by the DVA device and allowing each individual flicker cycle to be considered separately. This enables a more accurate assessment of dynamic vessel response to be obtained and enables the inclusion of the parameters BDF, BFR and DA on top of the standard percentage dilation and constriction response parameters. Additionally it allows the time taken to reach maximum dilation and the time taken to reach maximum constriction to be determined for both the artery and vein for each individual flicker cycle. A summary of the SDRA parameters and how they relate to the vascular response profile is illustrated in figure 3.6 and summarised in table 3.4.

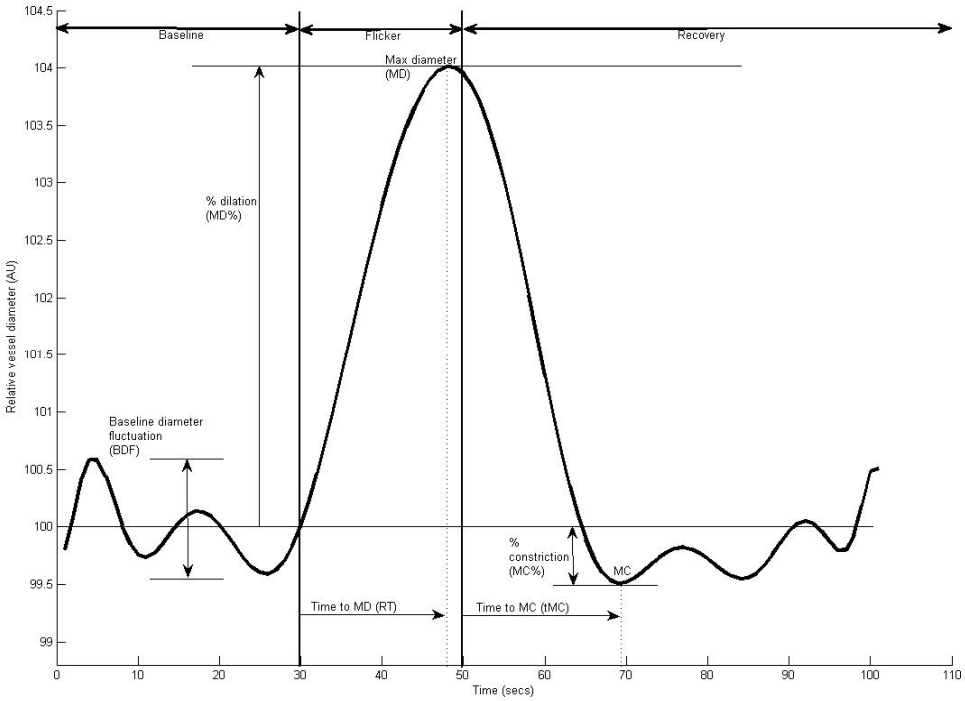



**Figure 3.6 Diagrammatic representation of the parameters calculated from the dynamic retinal vessel analysis (DVA) response profile**

| Parameter | Acronym | Explanation |
|---|---|---|
| Baseline diameter fluctuation | BDF | Calculated as the maximum range in vessel diameter during first 30 seconds of baseline readings (i.e. Difference between max diameter and min diameter at baseline) |
| Percentage dilation | MD% | Calculated as the percentage change in vessel diameter from baseline to maximum following onset of flicker |
| Baseline corrected flicker response | BFR | Percentage change in vessel diameter after taking into consideration the baseline diameter fluctuation (equation 3.1) |
| Reaction time | RT | Time taken to reach maximum diameter from the onset of flicker |
| Percentage constriction | MC% | Percentage constriction of vessel diameter below baseline following cessation of flicker. Calculated as smallest vessel diameter reached following cessation of flicker subtracted from average baseline diameter |
| Constriction time | tMC | Time taken to reach the point of maximum constriction following cessation of flicker |
| Dilation amplitude | DA | Amplitude of the dilation response, calculated as the difference in diameter between the maximum and minimum points (equation 3.2) |

**Table 3.3 Summary of the parameters which can be calculated and in used for analysis of the dynamic retinal vessel response profile**

It is important to note that analysis of individual flicker cycles is reliant on a full data set having been obtained from the participant on each subsequent cycle. If this is not achieved, for example due to poor patient fixation, loss of concentration or excessive blinking, calculating and analysing the average data set using SDRA is considered more reliable.

Overall the SDRA method has been validated and shown to be a sensitive measure of the vascular response to flicker light with good coefficients of variation [695]. Furthermore it is able to overcome a number of the shortfalls of the inbuilt RVA analysis software. It has therefore been the analysis method of choice for this thesis; however we have taken it one step further and using the principles of SDRA have developed an additional way of imaging the retinal vascular profile to allow further aspects of the vascular response to be explored.

#### 3.3.3.11 Novel Analysis

Whilst SDRA overcomes many of the limitations of the inbuilt RVA software analysis it still does not allow visualisation of the entire dynamic retinal vessel response profile. Furthermore, it has been suggested that, in addition to the parameters illustrated in figure 3.6 and summarised in table 3.3, evaluation of the slope of both the dilation and constriction responses to flicker light could give additional important information about the state of the retinal microvasculature in health and disease. In order to address this we have developed a new method of analysing and interpreting the retinal vascular response to flickering light using Matlab (MATLAB R2010a; MathWorks Inc., Natick, MA). Our method expands on the SDRA methodology by extracting the raw response data and applying a statistical polynomial regression algorithm, implemented using the `polyfit` and `polyval` functions of the Matlab high-level programming language (MATLAB R2010a; MathWorks Inc., Natick, MA). Dr Aniko Ekart, an experienced

mathematician and Matlab user assisted the author with the construction of the statistical algorithm used here.

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 $\sum_{i=1}^{T} (y_i - p(t_i))^2$ is minimized.

This involves solving the system of equations

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

If we denote $t_i^{n-j+1} = v_{ij}$ then $V = (v_{ij})$ is the Vandermonde matrix and the least squares problem to be solved can be written as $Vp = y$ where the vectors $p = \begin{pmatrix} p_1 \\ \vdots \\ p_{n+1} \end{pmatrix}$ and

$$y = \begin{pmatrix} y_1 \\ \vdots \\ y_T \end{pmatrix}$$

The `polyval` function was used to calculate 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 the averaged data for each individual patient. The degree of the polynomial, $n$, is an adjustable parameter. We applied $n = 15$, as this value provided the closest fit polynomials on our data points.

As well as the original SDRA parameters of baseline diameter fluctuation (BDF), maximum dilation (MD), maximum constriction (MC), reaction time (RT), dilation amplitude (DA) and baseline corrected flicker response (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 and compared between study groups. The slope may be an important parameter as it describes the interaction between the change in vessel diameter and the rate at which this change occurs, both of which are parameters that have shown to be altered in disease states individually. Dilation and constriction slope for both the arteries and veins were calculated as shown in equation 3.3.

$$Dilation\ slope = \frac{MD\text{ - }Av\ Baseline}{RT} \qquad Constriction\ slope = \frac{MC - MD}{tMDMC}$$

Where: MD = maximum dilation
RT = reaction time
MC = maximum constriction
tMDMC = time between MD and MC

**Equation 3.3 Dilation and constriction slope**

### 3.3.4 Assessment of systemic parameters

#### 3.3.4.1 Endothelial function: Flow mediated dilation (FMD)

Determination of the presence of endothelial dysfunction at the systemic level in neurodegenerative disease could provide important information about the involvement of macro-vascular function in the pathogenesis of the disease.

FMD is considered the gold standard technique for assessing systemic endothelial function [199]. It is a well-established technique that has been widely used in clinical research to assess peripheral vascular function and cardiovascular disease risk factors [700, 701]. Its main advantage over other techniques is that it is non-invasive and by nature of this, allows repeated measurements to be taken either sequentially or over time with a high level of patient acceptance [52]. Its only disadvantage is that it requires practice to master the technique and carefully controlled experimental conditions are required to ensure reproducibility and reliability of results [52, 702].

FMD refers to the dilation of a vessel in response to increased blood flow. A sudden increase in blood flow is known to exert a shear stress stimulus on the vessel wall which, under normal circumstances, triggers the release of the vasodilator NO from the endothelium leading to an increase in vessel diameter to accommodate the increased blood flow. A failure to observe this vascular dilation response to increased flow is considered indicative of endothelial dysfunction and hence important information about the functioning of the endothelium can be gained by assessing the dilation response of a systemic artery to an experimentally produced increase in blood flow in FMD.

#### 3.3.4.2 Protocol

Measurement of endothelium dependent FMD in this thesis has been conducted in accordance with the guidelines for assessment published by Corretti et al [702].

The procedure involves ultrasound imaging of the brachial artery of the upper arm, with an ultrasound probe positioned in the longitudinal plane, 5-10cm above the antecubital fossa, using a 2D colour Doppler ultrasound system (CDI).

Prior to all FMD assessments conducted in this thesis, patients were required to undergo a 12 hour fast due to the potential influence that food intake and caffeine can have on flow mediated vascular reactivity [702]. Furthermore, as temperature can also influence vascular reactivity all procedures were conducted in a quiet, dark, temperature controlled room at 22$^{O}$C. The measurement procedure is summarised as follows:

- The patient is positioned supine and allowed to rest in this position for 10 minutes prior to first scan.
- Their arm is then positioned comfortably in an extended position and an ultrasound image of the brachial artery is obtained from the upper right arm
- A segment of the artery with clear anterior and posterior intimal-lumen interfaces is selected on the imaging screen to ensure optimum recording of vessel diameter changes (figure 3.7)

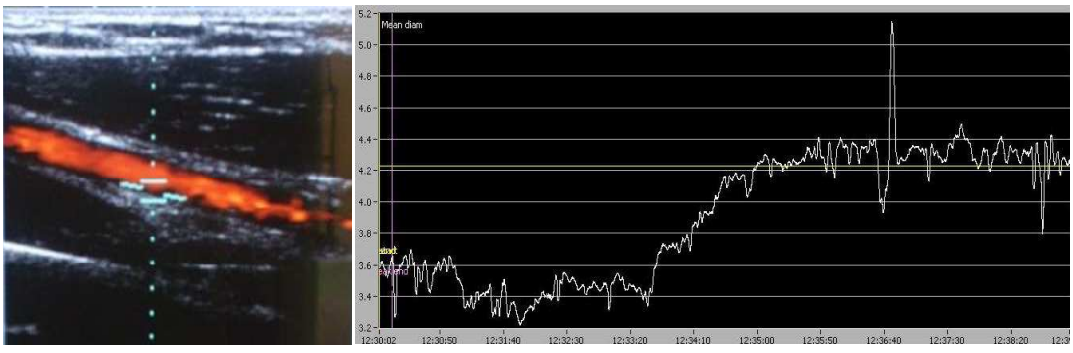



**Figure 3.7 Imaging screen in FMD with brachial artery segment highlighted**

- 2 minutes of baseline vessel diameter readings are taken from the brachial artery
- A 5.6 inch wide BP cuff (sphygmomanometer) positioned at the forearm is then inflated to 50mmHg above the patients recorded SBP and is kept inflated for 5

minutes. The inflated cuff effectively occludes the blood flow through the brachial artery, inducing hypoxia and causing dilation of downstream resistance vessels.

- After 5 minutes the cuff is rapidly deflated and the brachial artery is imaged for a further 2 minutes to assess the hyperaemic response. (figure 3.8)

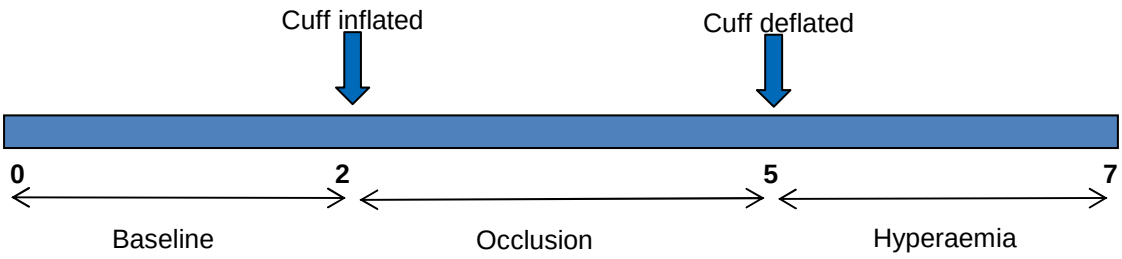



**Figure 3.8 Breakdown of the 7 minute FMD examination protocol**

On deflation of the cuff, the brachial artery is no longer occluded and there is a sudden increase of blood flow through it (hyperaemia). As mentioned previously this increased blood flow produces a shear stress stimulus on the vessel wall which should trigger the endothelium to release NO, possibly in combination with other vasodilators, and an increase in vessel diameter should be seen. A failure to observe a good vasodilation response would indicate systemic endothelial dysfunction.

Following FMD assessment and after a 10 minute rest period, the procedure is repeated using a sublingual tablet of nitroglycerin (GTN 0.3mg) in place of the sphygmomanometer. GTN is converted by the body into NO and this then acts directly on the vascular smooth muscle cells to bring about vasodilation, independent of the endothelium. GTN is therefore used to confirm whether any impaired dilation response detected by FMD can definitely be attributed to a dysfunctional endothelium and is not simply the result of a vascular smooth muscle dysfunction. It would be expected that, even in the presence of endothelial dysfunction, nitroglycerin mediated vasodilation (NMD) should be unchanged. If NMD were significantly impaired to the same degree as

FMD it would suggest the vascular smooth muscle is not functioning properly and any impaired FMD responses could not be convincingly attributed to endothelial dysfunction.

#### 3.3.4.3 Analysis

Flow mediated dilation is calculated as the percentage change in brachial artery diameter in response to hyperaemia (equation 3.4). Baseline vessel diameter is taken as the average diameter from the first 2 minutes of brachial artery ultrasound imaging and the vessel diameter following hyperaemia is taken as the maximum diameter reached in the 2 minutes following cuff deflation.

$$FMD = \frac{VD\ hyperaemia - Baseline\ VD}{Baseline\ VD}\ x\ 100$$

FMD = Flow mediated dilation
VD = vessel diameter

**Equation 3.4 FMD dilation response**

Previous studies have suggested that normal values for FMD should be considered to be between a 5-15% increase in brachial artery diameter and a 5-6 fold increase in blood flow on release of the BP cuff. A dilation response of between 0-5% indicates an impaired flow mediated dilation response [703, 704].

Nitroglycerin mediated dilation is calculated in the same way but as the percentage change in brachial artery diameter in response to nitroglycerin (equation 3.5).

$$NMD = \frac{VD\ nitroglycerine - Baseline\ VD}{Baseline\ VD}\ x\ 100$$

NMD = Nitroglycerine mediated dilation
VD = vessel diameter

**Equation 3.5 NMD dilation response**

#### 3.3.4.4 Circulating markers – von Willebrand factor

vWF levels were determined in this thesis via the analysis of fasting venous blood samples obtained by the author. The analysis was conducted by an experienced lab technician (Dr Lu Qin) according to the methodology outlined below:

- Following the collection of fasted venous blood samples into citrate tubes, the samples were centrifuged at 3000rpm for 15 minutes and the supernatant was aliqouted and stored in a -80$^0$C freezer.
- The citrated plasma was then thawed and analysed for vWf levels using a standardised ELISA kit which was optomised according to previously established methods [705, 706] and conducted as follows:
    1. A microtitre plate was coated with 100µl of dilated primary antiserum solution (30µl in 20.5ml coating buffer at pH 9.6) at room temperature and then refrigerated for a minimum of 60 minutes to over night
    2. The microplate was washed 4 times with 250µl of wash buffer per well before 100 µl of substrate was added with working strength detection antibody dilutant and incubated for 60 minutes at room temperature
    3. The microplate was then washed 3 times with wash buffer before 100µl of secondary antiserum was added and incubated at room temperature for 45 minutes
    4. The microplate was then washed again for a final 3 times and 100µl of substrate was added and then incubated at room temperature for 20 minutes
    5. The enzymatic reaction was then stopped by adding 50µl of hydrosulphuric acid
    6. The absorbance of the solution was then immediately read on a microwell plate reader set at 492nm

### 3.3.4.5 Ambulatory blood pressure monitoring (ABPM)

ABPM was the technique of choice for the assessment of systemic BP in this thesis and was conducted using the Cardiotens-01 device which is a computer operated ambulatory BP and ECG monitor (Cardiotens-01, PMS Instruments, Maidenhead, UK). This device has been validated in accordance with recommended protocols and has been widely used in previous clinical studies [707-710]. Its set up is such that a blood pressure cuff is positioned around the upper left arm and connected to a personal monitoring device worn around the waist, via a fibre optic cable. The device was programmed prior to use using the BP monitoring software Cardiovisions 1.7.2 (PMS Instruments, Maidenhead, UK) and customised to each individual, with regard to sleep and wake times and measurement intervals, amongst other factors. Measurements of BP are obtained automatically using an oscillometric method and in the event of a faulty reading the device is programmed to re-inflate a second time in order to avoid missed data points. For the purposes of this thesis the device was programmed to take readings at intervals of 15 minutes during daytime and intervals of 30 minute nocturnally. Patients were advised to carry out their normal daily activities and to complete a diary outlining any periods of unusual exertion or changes in activity for consideration when evaluating their BP variations. After the 24 hour period the BP data was downloaded and analysed using the 'Medibase' software program (Meditech, version 1.42). Maximum and minimum, diurnal and nocturnal, SBP, DBP and MABP were recorded and the mean nocturnal dip in BP calculated (equation 3.6). Furthermore the short-term variability in SBP was determined for both the diurnal and nocturnal periods through calculation of the average coefficient of variation for each group (equation 3.7).

$$Nocturnal\ BP\ dip = (Diurnal\ MABP - Nocturnal\ MABP) \times 100$$

Where: MABP = mean arterial blood pressure, calculated according to equation 1.2

**Equation 3.6 Nocturnal blood pressure dip**

$$Coefficient\ of\ variation = \frac{Standard\ deviation}{Mean}\ x\ 100$$

**Equation 3.7 Coefficient of variation**

### 3.3.4.6 Autonomic Nervous system assessment – Heart rate variability

Assessment of ANS function commonly falls into two categories, the first being the evaluation of BP and HR responses to provocative stimuli designed to test baroreflex sensitivity and the second being the analysis of systemic BP, resting HR and HRV over a 24 hour period [399]. For the purposes of this thesis ANS function was assessed using 24 hour ECG monitoring and a frequency domain analysis of HRV using the Cardiotens-01 device and 'Medibase' software introduced in the previous section (3.3.4.5). This device records continuous real time beat-to-beat ECG analysis via two independent channels following the precise placement of electrodes on the patient's chest in the positions illustrated in figure 3.9. The electrodes are connected via a fibre optic cable to the personal monitoring device worn around the patient's waist.

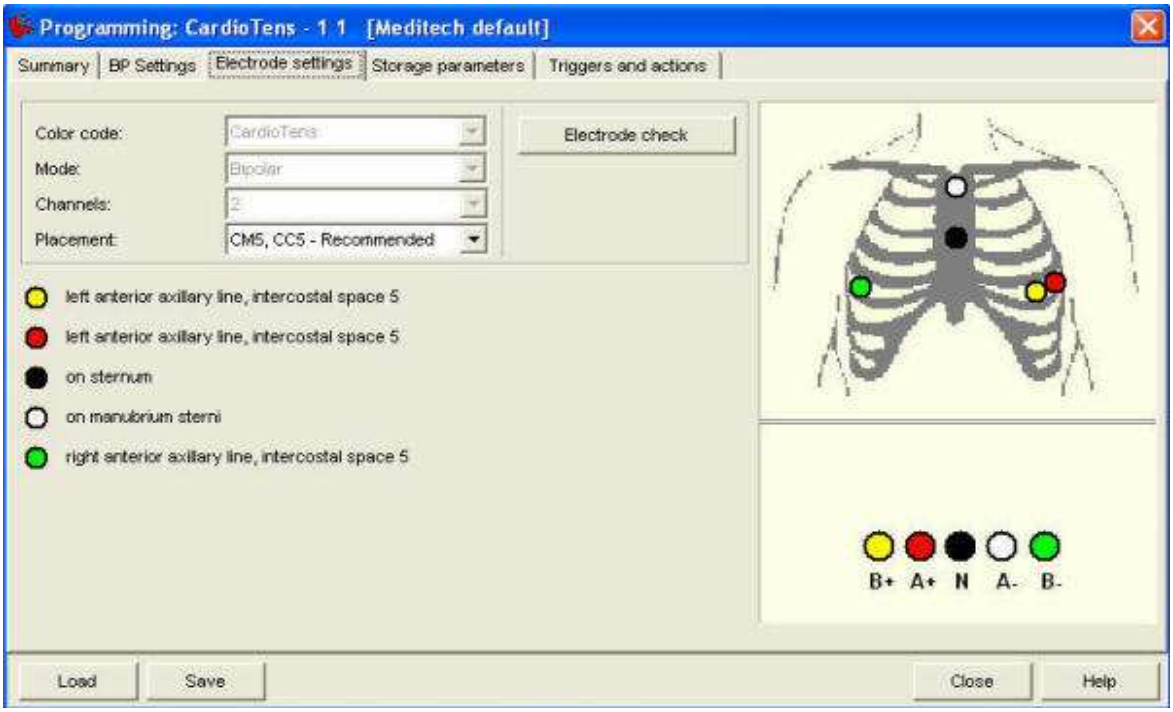



**Figure 3.9 Diagrammatic representation of the electrode positioning for 24 hour ECG recording with the Cardiotens-01 device**

Following 24 hours of ECG recordings the data can be downloaded from the device and a full frequency domain HRV analysis performed by the 'Medibase' software using a series of validated algorithms. An assessment of HRV and ANS function can then be made through analysis of the LF and HF values and the LF/HF ratio (see section 1.7.2), recorded during the diurnal (active) phase, nocturnal (passive) phase and over the entire 24 hour period.

### 3.3.5 Assessment of Cardiovascular risk

As discussed in section 1.9.12 there is a possible role for increased ocular and systemic vascular stiffness and the presence of cardiovascular disorders in the development of GON, however the current evidence is variable. In order to explore this relationship further an assessment of systemic arterial stiffness, atherosclerotic vessel changes and cardiovascular risk has been made in this thesis using pulse wave analysis, intima-media thickness measurement, calculation of Framingham risk score, BMI and routine blood analysis as outlined in the following sections.

#### 3.3.5.1 Assessment of arterial stiffness: Pulse wave analysis (PWA)

The method of choice for the assessment of arterial stiffness in this thesis was pulse wave analysis (PWA) which was performed using the SphygmoCor device in accordance with a well-established protocol [711, 712]. This is a simple, non-invasive, validated device which allows an assessment of arterial stiffness to be made with high repeatability [711-713]. The procedure involves the placement of a high fidelity pressure sensor on the outside of the skin overlying the radial artery at the wrist which generates a signal representative of the intravascular pulse (figure 3.10). From this signal the SphygmoCor device generates a peripheral pressure waveform and then converts it, using measured values of brachial SBP and DBP, into a central aortic pressure wave from which arterial stiffness parameters, such as augmentation index (AIx) can be

calculated by the software. The SphygmoCor software also generates quality control indices and therefore, to ensure reliability of measurements, in this thesis only those readings obtained with an operator index of greater than 80 were accepted.

**Figure 3.10: SphygmoCor device set up**

The principle of PWA is therefore the generation and analysis of the central aortic pressure waveform, the profile of which can provide important information about the stiffness of the systemic vasculature. This aortic pressure waveform is derived from two separate components, namely a forward pressure wave created by ventricular contraction and a backward or reflected pressure wave created by the reflection of the incident wave back from bifurcations and peripheral vascular beds.

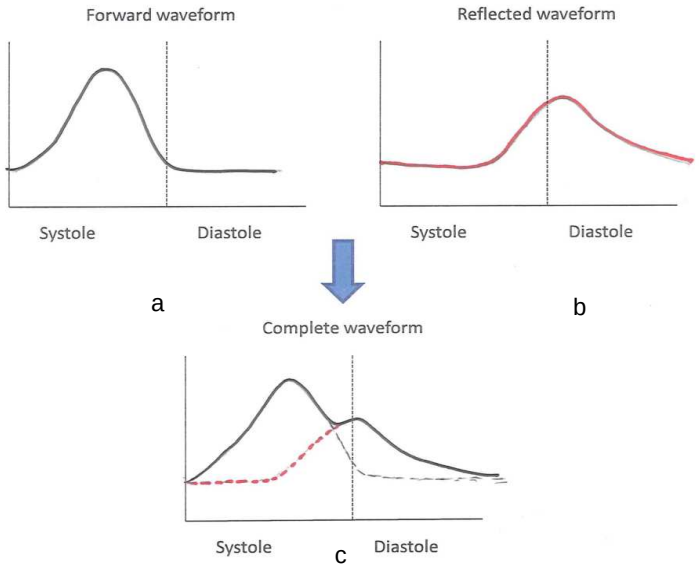



**Figure 3.11 a,b,c: forward aortic waveform, reflected waveform, complete aortic waveform**

In elastic vessels the reflected wave returns to the aortic root in diastole, supplementing coronary perfusion in this phase and creating the waveform profile illustrated in figure 3.11c. In stiff vessels the reflected wave can return earlier and therefore in systole, causing augmentation of systolic pressure and a decrease in diastolic pressure, along with decreased coronary perfusion, as illustrated in figure 3.12b.

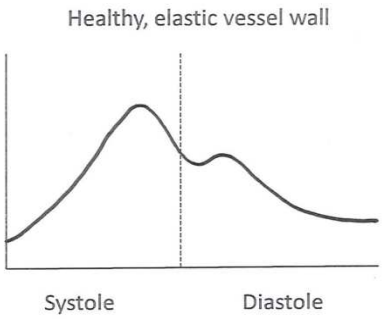



Figure 3.12a: Normal aortic pulse waveform: smooth ascending limb, notch in descending limb just after systole due to wave reflection.

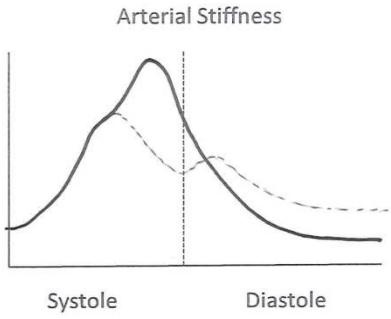



Figure 3.12b: Aortic waveform in presence of arterial stiffness: increased systolic peak, shoulder in ascending limb indicating early return of reflected wave, steeper descending limb with loss of notch

**Figure 3.12 a: Normal aortic pulse waveform; b: Aortic waveform in presence of arterial stiffness**

The AIx parameter generated by the SphygmoCor was used as the marker for systemic arterial stiffness in this study. AIx is a widely used research parameter and is considered a sensitive indicator of arterial stiffness [712]. It indicates the amount by which the aortic pressure is increased by the peripheral reflection of blood flow and it is given as a percentage value corrected for a HR of 75 beats per minute [714] (figure 3.13). AIx increases with increasing arterial stiffness due to an increased speed of reflection and has been shown to vary with both age and gender, being greater in females and older persons [715].

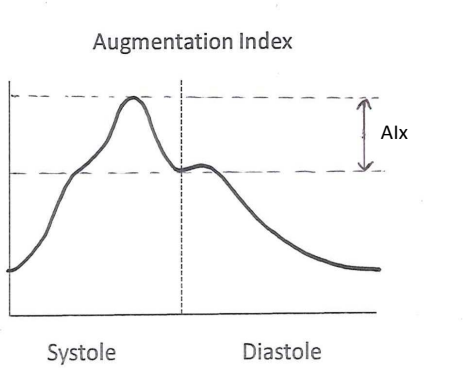



**Figure 3.13: Diagrammatic representation of the aortic pressure waveform and augmentation index**

Normal or reference values for AIx in healthy individuals have been reported for numerous different ethnic groups. The reference values given by the SphygmoCor group for healthy individuals according to age are shown in table 3.4.

| Age | Mean (%) | Lower 5% CI (%) | Upper 5% CI (%) |
|---|---|---|---|
| 20 | -4.67 | -23.27 | 16.87 |
| 30 | 3.03 | -15.57 | 24.57 |
| 40 | 10.73 | -7.87 | 32.27 |
| 50 | 18.43 | -0.17 | 39.97 |
| 60 | 26.13 | 7.53 | 47.67 |
| 70 | 33.83 | 15.23 | 55.37 |
| 80 | 41.53 | 22.93 | 63.07 |

**Table 3.4: AIx Reference values for healthy individuals provided by SphygmoCor**

One potential limitation of PWA stems from the suggestion that the estimation of the central pressure waveform alone may be insufficient to accurately quantify the magnitude of pressure waveform reflection and therefore the true accuracy and reliability of any results generated through PWA may be limited [716]. However, the majority of reports indicate that PWA and AIx can be considered good indicators of arterial stiffness and they are amongst the most widely used assessment parameters [711].

#### 3.3.5.2 Intima-media thickness (IMT) measurement

Measurement of carotid artery IMT allowed a simple and non-invasive assessment of early arterial wall changes to be made in this thesis. IMT is a parameter widely used in clinical research and has been shown to provide a direct measure of carotid arteriosclerosis and an indirect measure of generalised atherosclerosis [717]. Furthermore it has been demonstrated to offer predictive value with regard to future cardiovascular complications [718] and has shown a close relationship with the presence of cardiovascular risk factors [719-721] and calculated risk scores such as the Framingham risk score [722] (see section 3.3.5.3).

Measurement of IMT (figure 3.14; 3.15) was conducted in this thesis using a well-established protocol [717, 723]. Patients were positioned supine with their head turned away from the measurement site and their neck slightly extended. High resolution B-mode ultrasonography (Siemens; Acuson Sequoia, UK) was used to obtain an image of the right common carotid artery at the level of the carotid bifurcation. IMT measurements were then taken from the central region of the inferior wall of the artery, using the inbuilt software calliper system, at a site proximal to the artery bifurcation. The presence of any arteriosclerotic plaques along the vessel wall was also noted and measured. A normal IMT measurement is considered to be anything below 0.1 cm [717].

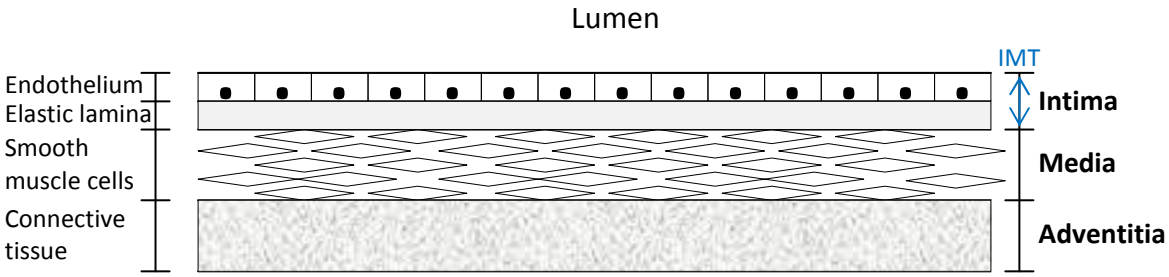



**Figure 3.14 Diagrammatic representation of the carotid artery wall**

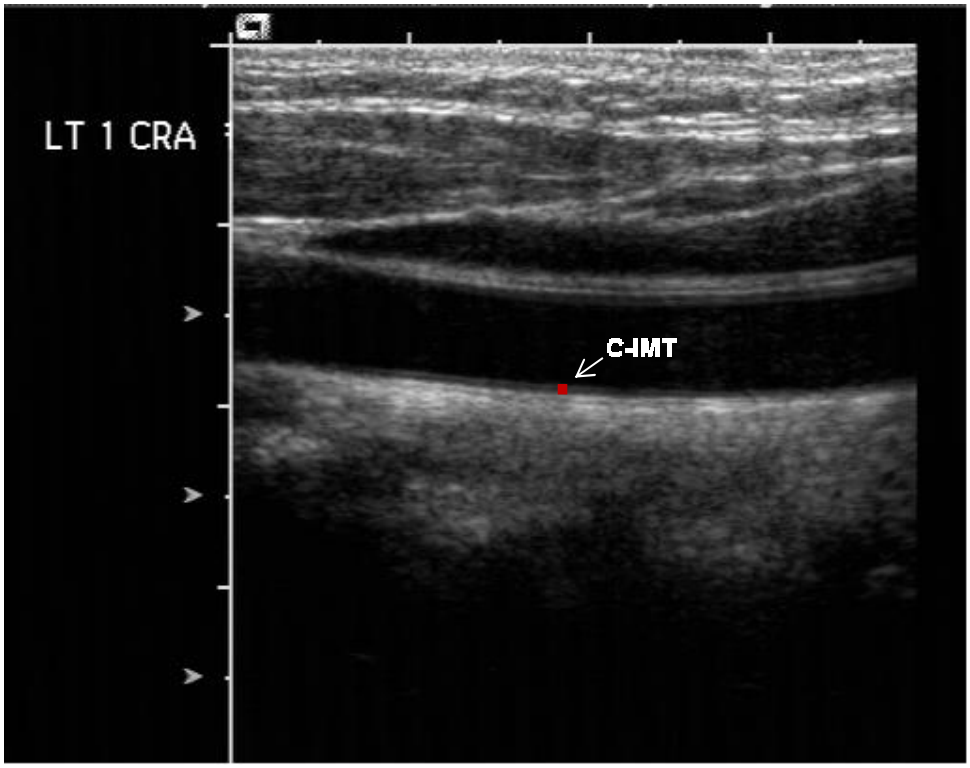



**Figure 3.15 Ultrasound image indicating carotid artery intima-media thickness measurement site. C-IMT: carotid-intima media thickness**

Ultrasound IMT measurement is generally considered a non-invasive and safe technique that is low cost and offers highly reproducible results. Questions have been raised about the possible subjective nature of the measurements and the risk of high between-observer variability, with the potential benefits of alternative automated analysing systems having been discussed [723, 724]. However the within-observer variability in measurements has been shown to be small and hence manual IMT measurement is

still considered a very valid technique [723]. All IMT measurements in this thesis were performed by a single operator.

Research has suggested that IMT can be influenced by age, systolic BP and weight. These are parameters that therefore all need to be controlled when considering the results. Furthermore relationships have been identified between IMT and FMD, with multiple studies suggesting an inverse correlation between the two, in that a greater IMT is associated with a lower FMD result [725]. This may be unsurprising as endothelial dysfunction has been shown to be an early occurrence in the development of atherosclerosis, of which IMT is a measure. Other studies have shown no correlation between the two measures suggesting that maybe they represent different stages of the atherosclerotic process, with endothelial dysfunction occurring in the earlier stages and increased IMT developing after more prolonged exposure to risk factors [726]. Nevertheless determining the presence of any correlations between IMT and FMD in this thesis could provide a further insight into the role of arterial wall changes in GON.

#### 3.3.5.3 Framingham risk score

Determination of cardiovascular risk is often made on the basis of lifestyle and history questionnaires which aim to determine the presence of factors such as obesity, hypercholesterolemia, diabetes and smoking. A more quantitative assessment however can be made through the calculation of risk scores such as the Framingham risk score. The Framingham risk score provides an estimate of the 10 year absolute risk of an individual developing coronary heart disease [727]. It is sex specific, taking into account age, total cholesterol, HDL cholesterol, BP, diabetes and smoking and has been shown to be validated for use across different ethnic groups [728]. The reference table on which the calculation is based is given in appendix 3. A Framingham risk score was calculated for all study participants in this thesis to provide an additional quantified measure of cardiovascular risk.

### 3.3.5.4 Body mass index (BMI) and blood analysis

In addition to the above procedures a further assessment of cardiovascular risk was made through the recording of both weight and height measurements for all participants, followed by the calculation of body mass index (BMI) (equation 3.8)

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

**Equation 3.8: BMI**

Furthermore fasting EDTA blood samples were obtained from the antecubital fossa vein of all participants and tested immediately for fasting triglycerides (TGs), glucose levels and total and HDL cholesterol, using a Reflotron Desktop Analyser (Roche Diagnostics, UK).

## 3.3.6 Evaluation of Oxidative Stress

The potential role of oxidative stress in the development of neurodegenerative diseases such as glaucoma and AD was discussed in previous sections (see sections 1.10 and 1.12.4). An evaluation of oxidative stress was obtained in this thesis through the determination of circulating levels of the antioxidant glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) from fasting venous blood samples obtained by the author. The analysis was conducted by an experienced lab technician (Dr Lu Qin) according to protocols optimised in house according to previously reported and validated methods [729]. This methodology is outlined below:

- Within 10 minutes of collection, 30μl of the fasting venous EDTA blood sample. combined with 33.3μl of sulfosalicylic acid and 936.7μl of sodium phosphate stock buffer solution, was centrifuged at 15000rpm for 5 minutes
- 150μl of the supernatant was then aliqouted and cooled to -80$^{o}$C for later analysis

- The analysis of glutathione levels is then determined using an enzymatic reaction created by:
    1. Adding 150µl of daily buffer to 50µl of DTNB solution in each microwell
    2. Then adding 25µl of the prepared plasma samle to each well and incubating this at 37$^{o}$C for 3 minutes
    3. 25µl of GSR solution is then added to the previous mixture and read using a microplate reader set at 410nm.

Following determination of the plasma GSH and GSSG levels the redox status or GSH:GSSG ratio was additionally calculated, along with the total plasma glutathione level (total-GSH, t-GSH) (see equation 3.9) [512, 729].

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

**Equation 3.9: Calculation of total-GSH**

# 4. Is the eye a window to the brain? Retinal vascular dysfunction in Alzheimer's disease

## 4.1 Abstract

**Purpose:** To assess ocular and systemic vascular function and its relationship to the degree of cognitive deficit in newly diagnosed AD patients without overt systemic vascular disease.

**Methods**: Retinal vascular reactivity to flickering light was assessed in 10 AD patients and 28 age-matched healthy individuals by means of Dynamic Vessel Analysis (DVA). Systemic vascular function was assessed in the same patients by means of flow mediated dilation (FMD). Mini-mental state examination (MMSE) and Addenbrooke's Cognitive Examination-Revised (ACE-R), as well as BP measurements and blood analyses for lipid metabolism markers were also performed in all cases.

**Results**: AD patients demonstrated differences in their vascular reaction times to three repeated cycles of flicker exposure at the retinal arterial level compared with healthy controls ($p=0.038$, $p=0.049$ and $p=0.028$ respectively), which correlated with their degree of cognitive impairment at the time of the test ( $R=-0.782$, $p=0.013$). Additionally AD patients demonstrated an increased venous baseline diameter fluctuation prior to the onset of flicker in comparison to controls ($p=0.001$). No significant differences in systemic endothelial function were found between groups ($p>0.05$).

**Conclusions**: Signs of microvascular dysfunction measured at the retinal level correlate with the degree of the cognitive decline at the time of diagnosis in mild AD patients. It is possible that this method of screening could offer valuable information about risk for future vascular complications as well as progressive cognitive decline these patients.

## 4.2 Introduction

In addition to other risk factors such as mechanical stress [463, 562], genetics [568] and oxidative stress [565], vascular disease leading to chronic brain hypo-perfusion [566, 617, 618] and more recently cerebral vascular dysregulation have also been implicated in the development of AD [619, 630-632]. As such the assessment of cerebral vascular function is considered to be of increasing importance in regard to the early diagnosis and management of AD; however, due to the notorious difficulties associated with directly assessing and visualising the vasculature in this region the ability to do this non-invasively is currently limited.

One concept being increasingly explored is the possibility of using the eye as a "window to the brain" [3]. This is a particularly attractive concept as the retinal and brain vessels are known to share a large number of embryological and anatomical similarities [4] and alterations in retinal structure that replicate cortical neurodegeneration [3, 659, 663], alterations in retinal blood flow [663] and deposits of amyloid-ß (Aß) [730] have all previously been demonstrated at the ocular level in AD patients. Additionally, the co-existence of either increased risk for, or clinical evident, cardiovascular disease (CVD) has previously been shown to result in a poor cognitive prognosis in AD [731]. All of these observations lead to the suggestion that through the assessment of retinal vasculature function information could potentially be gained not only on future systemic vascular risk in AD patients but also on cerebral vascular changes which may impact on cognitive prognosis. Indeed it has already previously been demonstrated that by assessing retinal vascular responses to flickering light early signs of vascular dysfunction in apparently healthy individuals with various degrees of risk for CVD can be identified [710, 732]. This study therefore aims to explore, through the use of DVA, if anomalies in vascular function are evident at the retinal level in mild newly diagnosed AD patients and whether

they can be linked to their degree of cognitive deficit and/or their degree of systemic vascular dysfunction, as measured by FMD.

## 4.3 Aims

The aim of this study was to identify if anomalies in vascular function are evident at the retinal level in mild newly diagnosed AD patients and whether any such anomalies can be linked to the degree of cognitive deficit and/or the presence of coexisting systemic vascular impairments in these patients.

## 4.4 Hypothesis

Disturbed vascular function will be present at the ocular level in mild newly diagnosed AD patients and will correlate with their degree of cognitive impairment and/or their degree of systemic vascular dysfunction.

## 4.5 Subjects and Methods

Mild newly diagnosed AD patients and healthy age matched controls were recruited for this study. The recruitment details, inclusion and exclusion criteria for these patients was detailed in section 3.1. The investigative procedures performed in this study are outlined below and were conducted in accordance with the protocols detailed in section 3.2.

1. Preliminary tests
2. Fasting venous blood sample obtained
3. Blood pressure measurement
4. Assessment of retinal vessel reactivity (DVA)
5. Assessment of systemic endothelial function (FMD)

## 4.6 Statistical analysis

The Kolmogorov-Smirnov test was used to determine the distribution of the data. As normality could not be confirmed in all cases the differences between groups were assessed using either the Student t-test for independent variables or the Mann-Whitney-U test as appropriate and presented as either mean ± SD or median (IQR) accordingly. Two factor repeated-measures ANOVA was used to compare the retinal reactivity responses across each flicker cycle and between groups, with log transformations being made where necessary. A Pearson's linear correlation analysis assessed the relationship between the level of cognitive impairment (MMSE score) and the vascular response. Multivariate analysis was performed to determine the influence of age, BMI, BP and the circulating markers on the measured variables; however no significant influences were found. P-values of less than 0.05 were considered significant, except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons. All analyses were performed using Statistica, version 6.0, Statsoft, Tusla, OK.

## 4.7 Power calculations

With regard to DVA, new measurement parameters and novel analysis methods were used in this study in patient groups which have not previously been examined with these techniques. Power calculation was therefore based on the results of previous studies which share the most similar protocols to that of the present research and was conducted using the computer based programme, GPower 3 [733]. A retinal vessel reactivity response to flicker light of 6% with a standard deviation of 2.5% is considered normal on the basis of previous research and around a 50% alteration in this response has been shown to be clinically significant [686]. Furthermore, with regard to FMD, a brachial artery dilation response of 8% with a standard deviation of 3% is considered normal on the basis of previous AD research and a 40% reduction in this response has

been shown to be clinically significant [631]. Analysis by t-tests for independent samples, as well as two factor repeated measures ANOVA was required in this study. Taking this into consideration, it was calculated that, in order to provide a statistical power of 80% at an α level of 0.05 a sample size of between 10-18 per group would be required (10 DVA t-test, 18 DVA within groups ANOVA, 18 DVA within/between ANOVA, 12 FMD t-test). The aim was therefore to recruit at least 18 patients in each study group.

## 4.8 Results

A total of 12 AD patients and 34 healthy controls were screened for inclusion in the present study however, in order to ensure all participants were matched on critical factors such as age and hypertensive status, all those under the age of 50, over the age of 75 or with a MBP of greater than 115 mmHg had to be excluded from analysis. Additionally, following the careful review of the obtained images, any patients who exhibited poor or incomplete results were also excluded meaning 10 mild newly diagnosed AD patients and 28 healthy controls were included in the final analysis. These numbers obviously fall below the intended target of 18 in the AD group, however statistical significance was still observed. The difficulties faced with recruitment of this category of patients is discussed in section 4.11

### 4.8.1 Baseline values

There were no significant differences in age, systemic BP, BMI, TGs, glucose, HDL cholesterol levels and total cholesterol levels between the two groups (all $p>0.05$). Furthermore there were no significant differences in IOP, MABP, OPP or Framingham risk score between groups (all $p>0.05$, table 4.1). The number of subjects with well controlled high BP was also proportionally similar in both groups (AD: n=3 and Controls: n=9, $p>0.05$; Chi-square test)

| | AD | Controls | P-value |
|---|---|---|---|
| **N** | 10 | 28 | - |
| **Gender** | 5F:5M | 11F:17M | - |
| **Age (years)** | 62.50±8.07 | 57.89±7.25 | 0.103 |
| **SBP (mmHg)** | 141.70±14.21 | 131.21±17.67 | 0.100 |
| **DBP (mmHg)** | 80.30±7.51 | 78.71±10.53 | 0.665 |
| **BMI** | 27.61±5.80 | 27.58±4.80 | 0.989 |
| **Glucose** | 4.40±1.44 | 4.99±0.96 | 0.163 |
| **Triglycerides** | 1.28±0.60 | 1.18±0.36 | 0.542 |
| **HDL-C (mmol/L)** | 1.33±0.25 | 1.16±0.29 | 0.121 |
| **Total-C (mmol/L)** | 4.77±0.64 | 4.66±0.82 | 0.708 |
| **IOP (mmHg)** | 16.50±2.12 | 18.00±2.62 | 0.470 |
| **MBP (mmHg)** | 100.77±7.67 | 96.21±11.92 | 0.269 |
| **OPP** | 84.96±9.46 | 80.26±12.48 | 0.299 |
| **Fram Risk Score** | 10.67±3.28 | 9.84±5.96 | 0.697 |
| **MMSE score** | 23.60 ± 3.57 | - | - |

Table 4.1: Summary of the baseline characteristics of the study groups. P<0.05 is considered a significant difference. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; Total C: Total cholesterol; IOP: Intraocular pressure; MMSE: mini mental state examination. The presented SBP, DBP and IOP values are the baseline readings taken on the morning of the study and do not represent the 24 hour or diurnal averages. OPP was subsequently calculated using these baseline values.

### 4.8.2 Systemic endothelial dysfunction

No significant differences were found between groups with regard to brachial artery FMD or NMD (p>0.05, table 4.2). Furthermore no significant differences in circulating vWF levels were identified between groups

| | AD | Controls | p-value |
|---|---|---|---|
| FMD (%) | 10.97±11.28 | 11.04±8.53 | 0.985 |
| NMD (%) | 25.95 (13.66-39.74) | 20.80 (18.85-26.38) | 0.894 |
| vWF | 118.34±64.28 | 120.95±52.23 | 0.920 |

Table 4.2: Systemic endothelial function. Data presented as mean ± SD or mean (IQR) depending on distribution. P<0.05 (*) is considered significant. FMD: flow mediated dilation; NMD: nitroglycerine mediated dilation; vWF: von Willebrand factor.

### 4.8.3 Dynamic retinal vessel analysis

For ease of interpretation the DVA response profile was considered in two parts, the first being the dilation response (baseline to maximum dilation) and the second being the

constriction response (maximum dilation to maximum constriction). Each flicker cycle was analysed individually using traditional SDRA analysis with the artery and vein being considered separately. The dynamic nature or slope of the retinal vascular response profiles were fully explored using the polynomial fitted curves generated via our novel computational analysis (MatLab R2010a; MathWorks Inc., Natick, MA) (see section 3.3.3.11).

**Arterial Response**

With regard to the arterial dilation response no significant differences in baseline diameter fluctuation (BDF), maximum diameter (MD), reaction time (RT) or baseline corrected flicker response (BFR) were found between groups on average ($p>0.01$, table 4.3). On consideration of each flicker cycle individually however the arterial RT was found to be significantly longer in AD patients compared to controls on both the 1$^{st}$ and 3$^{rd}$ flicker cycles ($p=0.039$, $p=0.028$ respectively) and significantly shorter on the 2$^{nd}$ flicker cycle ($p=0.049$) (table 4.4). Furthermore although no within groups differences were found in RT on progressing from flicker 1 to flicker 3, the nature of this progression was however found to differ between groups, with AD patients showing a reduction in RT on going from flicker 1 to 2 and healthy controls showing a contrasting increase ($p=0.007$, table 4.4). With regard to the dynamic nature of the arterial dilation response profile, evaluated using our novel matlab analysis, no significant difference in arterial dilation slope ($Slope_{AD}$) was however found between study groups ($p>0.05$, table 4.5).

With regard to the second part of the dynamic response curve, no significant differences were found in the arterial constriction response (MC%) or the arterial constriction response time (tMC) between groups ($p>0.01$, table 4.3). Furthermore on consideration of the dynamic nature of the arterial constriction response profile no significant differences in constriction slope ($Slope_{AC}$) were found between groups ($p>0.05$, table 4.5).

| ARTERY | AD | Controls | p-value |
|---|---|---|---|
| BDF | 5.44±2.11 | 4.86±1.77 | 0.420 |
| MD (%) | 5.78±3.25 | 5.56±2.02 | 0.811 |
| RT | 21.83 (16.33-31.33) | 17.83 (15.50-24.33) | 0.273 |
| BFR | 4.88 (3.18-7.35) | 4.66 (3.72-5.76) | 0.529 |
| MC (%) | -3.29±1.56 | -2.59±1.62 | 0.267 |
| tMC | 32.63±10.24 | 28.07±8.51 | 0.101 |

Table 4.3: Arterial vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). Data presented as mean ± SD or mean (IQR) depending on distribution. P<0.01 (*) is considered as significant.BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; RT: reaction time BFR: baseline corrected flicker response MC(%): percentage constriction below baseline; tMC: constriction time.

| ARTERY | AD | Controls | p-value | Within/Between groups ANOVA |
|---|---|---|---|---|
| **RT** | | | | |
| Flicker 1 | 29.30±16.61 | 18.59±12.21 | 0.039* | 0.007* |
| Flicker 2 | 16.30±11.48 | 23.68±9.26 | 0.049* | |
| Flicker 3 | 27.89±17.62 | 17.07±10.43 | 0.028* | |
| **Within group ANOVA** | 0.093 | 0.059 | | |

Table 4.4: Arterial vascular function parameters by flicker cycle. P<0.05 (*) is considered as significant on two factor repeated measures ANOVA. RT: reaction time.

| DYNAMIC RESPONSE | AD | Controls | p-value |
|---|---|---|---|
| **Arteries** | | | |
| Slope $_{AD}$ | 0.265±0.221 | 0.278±0.153 | 0.853 |
| Slope $_{AC}$ | -0.194±0.070 | -0.187±0.113 | 0.848 |

Table 4.5: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{AD}$: slope of arterial dilation; Slope $_{AC}$: slope of arterial constriction

### Venous Response

With regard to the venous dilation response no significant differences in maximum diameter (MD), reaction time (RT) or baseline corrected flicker response (BFR) were found between groups (p>0.01, table 4.6). The venous baseline diameter fluctuation (BDF) however was found to be significantly greater in our AD patients compared to controls both on average (p=0.001) and across each individual flicker cycle (F1: p=0.013; F2: p=0.004; F3: p=0.014, table 4.7). On consideration of the dynamic nature of the venous dilation response profile, evaluated using our novel matlab analysis, no

significant difference in the venous dilation slope was found between study groups (p>0.05, table 4.8).

With regard to the second part of the dynamic response curve, no significant differences were found in the venous constriction response (MC%) or constriction time (tMC) between groups (p>0.01, table 4.6). Furthermore on analysis of the dynamic nature of the venous constriction response no significant differences in the venous constriction slope ($Slope_{VC}$) were found between groups on average (p>0.05, table 4.8).

| VEIN | AD | Controls | p-value |
|---|---|---|---|
| BDF | 5.83 (5.10-8.34) | 3.24 (2.04-4.03) | 0.001* |
| MD (%) | 6.12±3.14 | 5.18±2.69 | 0.352 |
| RT | 20.33 (18.67-24.50) | 20.00 (18.67-24.00) | 0.688 |
| BFR | 1.84 (-1.01-6.34) | 3.18 (1.54-4.66) | 0.265 |
| MC (%) | -2.61±2.13 | -1.99±1.34 | 0.341 |
| tMC | 29.74±6.20 | 33.98±8.05 | 0.348 |

Table 4.6: Venous vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). Data presented as mean ± SD or mean (IQR) depending on distribution. P<0.01 (*) is considered as significant.BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; BFR: baseline corrected flicker response MC(%): percentage constriction below baseline.

| VEIN | AD | Controls | p-value | Within/Between groups ANOVA |
|---|---|---|---|---|
| **BDF** | | | | |
| Flicker 1 | 6.78±3.15 | 3.48±2.00 | 0.013* | 0.961 |
| Flicker 2 | 6.07±3.45 | 2.92±1.13 | 0.004* | |
| Flicker 3 | 7.08±4.21 | 3.59±2.52 | 0.014* | |
| **Within groups ANOVA** | 0.820 | 0.295 | | |

Table 4.7: Venous vascular function parameters by flicker cycle. P<0.05 (*) is considered as significant on two factor repeated measures ANOVA. BDF: baseline diameter fluctuation.

| DYNAMIC RESPONSE | AD | Controls | t-test p-value |
|---|---|---|---|
| **Veins** | | | |
| Slope $_{VD}$ | 0.295±0.172 | 0.218±0.124 | 0.214 |
| Slope $_{VC}$ | -0.204±0.193 | -0.164±0.122 | 0.451 |

Table 4.8: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{VD}$: slope of venous dilation; Slope $_{VC}$: slope of venous constriction.

### 4.8.4 Correlations

No significant correlations were found between the retinal vascular reactivity parameters (BDF, MD%, RT, MC%, tMC, DA, BFR, Slope) and the systemic vascular parameters (FMD) in either group (P>0.05). Interestingly however a significant correlation was found between the degree of cognitive impairment in our AD patients, as indicated by MMSE score, and the arterial RT on the 1st flicker cycle (R=-0.782, p=0.013, figure 4.1)

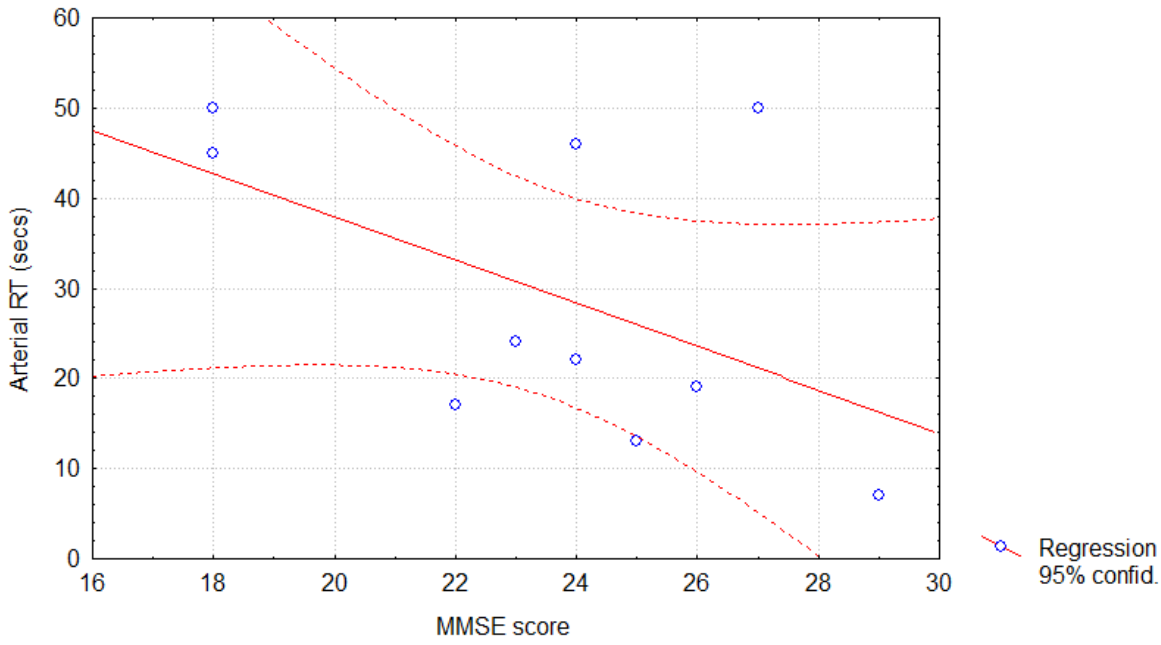



**Figure 4.1 Correlation between arterial reaction time (RT) on the 1st flicker cycle and MMSE score**

## 4.9 Discussion

### 4.9.1 Main findings

This study has revealed differences in the response of the retinal vasculature to flicker light between mild newly diagnosed AD patients and healthy age matched controls. Newly diagnosed AD patients took significantly longer to reach the point of maximum arterial dilation on two out of the three occasions in which their retinal vessels were challenged by flickering light and this alteration in response was found to correlate with their degree of cognitive impairment. Furthermore AD patients demonstrated a consistently increased fluctuation in venous baseline diameter prior to the onset of flicker across all three cycles in comparison to healthy controls. No significant differences however were found in systemic endothelial dysfunction between groups.

### 4.9.2 Systemic endothelial function

No significant differences in systemic vascular endothelial function, as measured by FMD, were found between groups in this study. This is in contradiction to previous research by Dede et al (2007) who demonstrated impaired brachial artery FMD in a group of diagnosed AD patients compared to healthy controls [631]. Such contradiction in findings between previous research and our present study could partly be accounted for by differences in the measurement protocol and/or differences in the severity of the AD patients assessed. Our present study included only mild newly diagnosed AD patients and therefore it could be hypothesised that, as endothelial dysfunction is known to occur much earlier at the microvascular level than at the macrovascular level in a disease process [734], signs of systemic endothelial dysfunction, although not yet detectable in our newly diagnosed AD patients on FMD, could become more apparent as the disease progresses. In line with this Dede et al [631] found that the degree of endothelial dysfunction as indicated by FMD correlated with the severity of cognitive impairment in their AD patients, with endothelial dysfunction getting worse as the severity of the

disease increased. Further research however would be required to confirm this hypothesis before any firm conclusions can be drawn. Alternatively, it is possible that the small sample size of AD patients included in this study did not provide sufficient power to detect a difference in FMD between groups. Indeed on power analysis it was identified that a sample size of 12 would be required to provide 80% power at an α level of 0.05, however only 10 AD participants were able to be recruited. Post-hoc analysis, conducted using G-power 3, reveals the sample sizes achieved in this study were in fact only able to provide a power of 69%. This could potentially explain these non-significant findings.

### 4.9.3 Retinal vessel reactivity

A large body of evidence exists linking AD to the presence of cerebral vascular dysfunction and highlighting the involvement of the ocular circulation in the AD disease process [3, 567, 617, 620, 621, 668, 669]. Furthermore, the easy access to the neural and vascular tissue at the retinal level, as well as the many anatomical and physiological similarities shared by the ocular and cerebral microcirculation, makes the retina an ideal screening target in cerebrovascular disease. The use of techniques such as the DVA method used in the present study, which are aimed at assessing retinal microvascular function, could therefore not only offer information regarding general microvascular function in AD patients but also offer an assessment of the potential risk for future decline and development of systemic vascular disease. In the present study, DVA analysis revealed how our newly diagnosed mild AD patients without manifest systemic vascular disease took significantly longer to reach the point of maximum dilation on two out of the three occasions in which their retinal vessels were challenged by flickering light. These results indicate that some form of microvascular dysfunction, detectable at the retinal level, does indeed appear to exist in AD patients; the cause of this vascular dysfunction, however, can only be hypothesised at this point.

The retinal vascular response to flicker light occurs due to an increase in retinal metabolic demand and is predominantly a neurovascular coupling driven response [226, 685, 686, 691, 692]. It could therefore be hypothesised that the altered retinal vessel reactivity demonstrated in our AD patients is indicative of a disturbed neurovascular coupling mechanism, possibly related to endothelial dysfunction, a decreased bioavailability of the vasodilator NO or an alteration in the activity of astrocytes in these patients, all of which are known to be key mediators of the neurovascular response [600]. Interestingly, both disturbed neurovascular coupling and dysfunction of the vascular endothelium have been previously linked to the aetiology of AD [631, 632, 735, 736], as have alterations in astrocyte activity [737-739] and in NO production/release [565, 740]. Such alterations in the production/release of NO could additionally be attributed to either cholinergic receptor degeneration in AD [741] and the subsequent reduction in acetylcholine mediated NO release, or to Aß deposition and the subsequent impairment of neuronal NO production. Indeed acetylcholine receptor stimulation and subsequent release of NO has been previously identified to play a role in the retinal vasodilation response to flicker light in rabbits [742, 743] and the accumulation of Aß, a key feature of AD, has been previously identified to reduce NO production in retinal neuronal cultures [730], adding plausibility to this theory.

Aside from disturbed neurovascular coupling mechanisms it could alternatively be hypothesised that factors such as relative arterial inertia due to either increased vascular stiffness or vasospasm, similar to that documented in AD and cognitively impaired patients at the cerebral level [636, 744, 745] may also contribute to the observed abnormalities in retinal vascular function. Such an alternative hypothesis could also partly explain the dissimilarities in reaction time observed in our AD patients on consecutive flicker cycles, in that the presence of limited arterial elasticity could theoretically lead to an incomplete baseline recovery after the initial stimulation cycle, reducing the time taken to then reach the point of maximum dilation on the subsequent flicker cycle. Exhaustion of vasoactive

mediator reserves, such as that of NO, which is a feature commonly associated with vascular endothelial dysfunction [201], could then theoretically override this and prolong the reaction time again on the final cycle. Further research however would be required to validate these assumptions.

With regard to the venous dynamic retinal vessel response profile our AD patients demonstrated an increased fluctuation in baseline vessel diameter across all three cycles, prior to the onset of flicker, which was not replicated by our healthy controls. Consideration of baseline diameter fluctuation (BDF) was first recommended by Nagel et al [686] as a way of taking into account the effect of the spontaneous variations in vessel diameter that occur under normal resting conditions on the observed response of the vasculature to flicker light stimulation, however it is a parameter which is not commonly reported in the literature and which has, to date, mainly been considered in regard to the retinal arteries, where it has been tentatively linked to vascular disturbance in the form of instability or increased variation in vascular tone or rigidity [695, 746-748]. As such the cause and relevance of increased BDF in the venous circulation is currently unclear. 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 and instigating a regulatory contribution passively in response to increased blood flow [685]. Interestingly increased retinal venous diameters have been previously linked to impaired cerebral blood flow and have been suggested as a marker of both retinal and cerebral ischemia and hypoxia [652, 749-751]. The finding here is somewhat different as fluctuations in diameter have been assessed dynamically as opposed to vessel diameter measurements being taken statically from photographs. Nevertheless, as both ischemia and hypoxia have been well linked to the development of AD, it could be hypothesised that the increased fluctuation in baseline diameter observed in these patients could be reflective of early hypoxic changes and perhaps an

increased risk of future damage. Further investigation however would be necessary to confirm these hypotheses.

### 4.9.4 Vascular function vs. Cognitive impairment

A significant positive correlation was found between the abnormal retinal arterial reaction to flickering light and the level of cognitive impairment in AD patients, suggesting a possible link may exist between the degree of vascular dysfunction and disease severity even in the early stages of the disease process. This is supported by previous research in which vascular dysregulation at the cerebral level has been shown to become more pronounced with increasing severity of AD [629] and by the finding of a correlation between cognition and the geometry of the retinal vessels in elderly people after correcting for age, visual acuity and apolipoprotein E status [752]. The possibility that the degree of vascular dysfunction at the retinal level could be a sensitive predictor of cognitive decline in mild AD patients emphasises the important role that vascular factors might play in the aetiology of the disease. In addition, it is tempting to propose that examining the function of retinal microvasculature could predict future cognitive decline in patients suffering from AD; nevertheless, more research is necessary to validate our presumption.

## 4.10 Conclusion

This study demonstrates for the first time that abnormalities in retinal vascular dysfunction, potentially indicative of a disturbed neurovascular coupling response, may be present in mild newly diagnosed AD patients and that the extent of these abnormalities may be a sensitive indicator of the degree of cognitive impairment. More research however would be necessary to validate our assumptions.

## 4.11 Limitations

One factor potentially limiting the conclusions which can be drawn from this study is the small sample size of AD patients. The strict inclusion/exclusion criteria were necessary to avoid any possible unwanted influences on the measured vascular parameters; however they also made patient recruitment a challenge. Regular contact was always maintained with the dementia team in order to try and maximise the recruitment of suitable participants and additionally all members of the team received a thorough briefing on the study protocol, inclusion and exclusion criteria. Although significant differences in DVA parameters were found, as mentioned previously, it is possible that the small sample size of AD patients and subsequent lack of statistical power may have limited the conclusions which can be drawn for other parameters, especially FMD.
The results of this study do however go some way to providing a positive indicator that vascular dysfunction is a factor involved in the pathogenesis of AD and offer a relevant and adequate platform for further research in this area. Future research to include more patients with various degrees of cognitive impairment would be beneficial to validate these findings.

# 5. Ocular and Systemic Vascular Abnormalities in Newly-Diagnosed Normal Tension Glaucoma Patients

## 5.1 Abstract

**Purpose**: To investigate signs of ocular and systemic vascular abnormalities in newly diagnosed and previously untreated normal tension glaucoma patients (NTG).

**Methods**: Retinal vascular reactivity to flickering light was assessed in 19 NTG and 28 healthy age-matched controls by means of dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). Using a newly developed computational model, the entire dynamic vascular response profile to flicker light was imaged and used for analysis. Assessments of systemic endothelial function (FMD), carotid intima-media thickness (IMT) and pulse wave analysis (PWA) were conducted on all participants, along with blood analyses for von Willebrand factor (vWf), glucose and lipid metabolism markers.

**Results**: Patients with NTG demonstrated an increased carotid IMT ($p=0.015$) and an elevated PWA augmentation index ($p=0.017$) in comparison to normal controls, as well as an altered retinal arterial vascular response profile and a significantly steeper retinal arterial constriction slope ($slope_{AC}$) following flicker light stimulation ($p=0.031$). No significant differences in systemic endothelial function were identified between groups ($p>0.05$)

**Conclusions**: Newly diagnosed and untreated NTG patients showed signs of subclinical vascular abnormalities at both ocular and systemic levels. Due to the importance of circulation-related pathologies in the aetiology and progression of this type of glaucoma early detection of such abnormalities and the introduction of disease modifying interventions could prove beneficial for disease prognosis.

## 5.2 Introduction

Although abnormal intraocular pressure (IOP) is still considered a crucial risk factor for the development of normal tension glaucoma (NTG) [36, 37], other local culprits including ocular microvascular dysregulation [325] have been implicated in its aetiology. Moreover, systemic vascular pathologies, such as peripheral vasospasm [26, 353], endothelial [42, 384] and autonomic nervous system (ANS) dysfunctions [413, 414] as well as high levels of oxidative stress [753] have also been reported in NTG patients, along with a high incidence of systemic cerebrovascular and/or cardiovascular disease (CVD) [24, 323, 444, 468, 477, 478, 754]. The impact and inter-relationships of such vascular alterations, which appear to affect both the macro- and micro-vascular beds, is however still poorly understood and many questions still remain around the exact pathogenesis of glaucomatous optic neuropathy (GON).

A common link between micro- and macro-vascular pathologies is endothelial dysfunction [255] and the assessment of this type of dysfunction across different vascular beds has been shown to be a good predictor of future vascular risk [263, 755, 756]. Interestingly, the presence of endothelial dysfunction has previously been reported in the systemic macro-circulation of NTG patients [42] however, in the course of vascular disease development, the microcirculation is thought to be affected much earlier than the macro-circulation [734]. Dynamic retinal vessel analysis (DVA) allows an assessment of the retinal microvascular response to stress and using this technique, a diminished venous dilation response to flickering light has previously been reported in early primary open angle glaucoma (POAG) [41]. No such studies have however yet been carried out in NTG patients. Additionally the majority of currently published DVA analyses focus almost solely on the dilatory response of the retinal vasculature to flickering light however, in order to completely understand the mechanisms behind vascular dysfunction in ocular disease, as already recommended by previous studies [688, 696], the

entire profile of the retinal vascular response, including vessel recovery, should be considered. With this in mind the present study aimed, through the use of a new computational analysis of the complete DVA response profile, to study both the retinal dilation and constriction responses to flickering light in newly diagnosed and untreated NTG patients and to explore the coexistence of both micro- and macro-vascular abnormalities in these patients through the simultaneous evaluation of systemic macro-vascular function markers. The assessment of vascular function in NTG patients is of particular interest as IOP is thought to play a less prominent role in the NTG disease process and hence diagnosis and treatment of the condition more commonly looks to IOP-independent causes, such as vascular alterations, whose roles are yet to be fully clarified.

## 5.3 Aims

The aim of this study was to assess ocular microvascular reactivity, in the form of both the retinal dilation and constriction responses to flickering light in otherwise healthy, newly diagnosed and untreated NTG patients and to fully explore the ‘sick eye in a sick body’ concept of glaucoma, through assessment of systemic endothelial function by means of FMD, systemic arterial stiffness and carotid artery IMT.

## 5.4 Hypothesis

Newly diagnosed and previously untreated NTG patients demonstrate altered retinal vessel reactivity to flicker light stimulation in conjunction with signs of vascular dysfunction at the systemic level.

## 5.5 Subjects and Methods

Newly diagnosed and previously untreated NTG patients and healthy age matched controls were recruited for this study. The recruitment details, inclusion and exclusion criteria for these patients was detailed in section 3.1. The investigative procedures performed in this study are outlined below and were conducted in accordance with the protocols outlined in section 3.2:

1. Preliminary tests
2. Fasting venous blood sample obtained
3. BP measurement
4. Assessment of retinal vessel reactivity (DVA)
5. Pulse wave analysis (PWA)
6. Intima-media thickness (IMT) measurement
7. Assessment of systemic endothelial function (FMD)

## 5.6 Statistical Analysis

The Kolmogorov-Smirnov test was used to determine the distribution of the data. As normality could not be confirmed in all cases the differences between groups were assessed using either the Student t-test for independent variables or Mann-Whitney-U test as appropriate and reported as either mean±SD or mean(IQR) accordingly. The only exception to this was with regard to IMT, where multiple regression analysis revealed systolic BP to be an influencing factor and hence a one-way ANCOVA, followed by Tukey's post-hoc analysis, was subsequently conducted. Correlations were determined according to Pearson's method or Spearman's rank method as appropriate. P-values of less than 0.05 were considered significant, except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons and minimise bias towards type I errors. All analyses were performed using Statistica, version 6.0, Statsoft, Tusla, OK.

## 5.7 Power calculations

With regard to DVA, new measurement parameters and novel analysis methods were used in this study in patient groups which have not previously been examined with these techniques. Power calculation was therefore based on the results of previous studies which share the most similar protocols to that of the present research and was conducted using the computer based programme, GPower 3 [733]. A retinal vessel reactivity response of 6% with a standard deviation of 2.5% is considered normal on the basis of previous research and around a 50% alteration in this response has been shown to be clinically significant [686]. With regard to FMD, a brachial artery dilation response of 7.5% with a standard deviation of 2.3% is considered normal on the basis of previous glaucoma research and around a 40% alteration in this response has been shown to be clinically significant [42]. With regard to PWA AIx and IMT similar deductions to that above, regarding clinical significance, were made on the back of previous research [482, 715, 726]. Analysis by t-tests for independent samples was required in this study. Taking this into consideration, it was calculated that, in order to provide a statistical power of 80% at an α level of 0.05, a sample size of between 7-22 per group would be required (10 DVA, 7 FMD, 22 PWA, 9 IMT). The aim was to therefore recruit at least 22 participants in each study group.

## 5.8 Results

A total of 25 NTG patients and 34 healthy controls were screened for inclusion in the present study however, in order to ensure all participants were matched on critical factors such as age and hypertensive status, all those under the age of 45, over the age of 75 or with a MBP of greater than 115 mmHg had to be excluded from analysis. Additionally, following the careful review of the obtained images, any patients who exhibited poor or incomplete results were also excluded and 19 NTG patients (11 female, 8 male) and 28 healthy age matched controls (12 female, 16 male) were

included in the final analysis. The number of NTG patients ultimately fell slightly below the optimum sample size suggested by power calculation; however post-hoc analysis using GPower 3 revealed the obtained sample sizes were still able to provide a statistical power of 82%.

### 5.8.1 Baseline values

There were no significant differences in age, systemic BP, BMI, triglycerides, glucose, HDL cholesterol levels and total cholesterol levels between the two groups (all $p>0.05$). Furthermore there were no significant differences in IOP, MABP, OPP or Framingham risk score between groups (all $p>0.05$) (Table 5.1). The number of subjects with well controlled high BP was also similar in both groups (NTG: n=5 and Controls: n=9, $p>0.05$; Chi-square test).

| | NTG | Controls | P-value |
|---|---|---|---|
| **N** | 19 | 28 | - |
| **Gender** | 11F:8M | 12F:16M | - |
| **Age (years)** | 60.16±12.13 | 56.82±7.54 | 0.251 |
| **SBP (mmHg)** | 130.06±15.37 | 127.00±18.52 | 0.563 |
| **DBP (mmHg)** | 78.39±11.14 | 76.39±10.91 | 0.551 |
| **BMI** | 27.92±4.20 | 26.81±4.22 | 0.407 |
| **Glucose** | 5.08±0.86 | 4.93±0.94 | 0.594 |
| **Triglycerides** | 1.04±0.31 | 1.12±0.36 | 0.403 |
| **HDL-C (mmol/L)** | 1.12±0.29 | 1.18±0.32 | 0.556 |
| **Total-C (mmol/L)** | 4.58±1.04 | 4.75±0.86 | 0.560 |
| **IOP (mmHg)** | 17.40±1.80 | 16.71±2.36 | 0.460 |
| **MBP (mmHg)** | 95.61±10.93 | 93.26±13.00 | 0.529 |
| **OPP** | 76.09±12.66 | 80.85±12.87 | 0.223 |
| **Fram Risk Score** | 9.67±7.50 | 8.31±5,24 | 0.499 |

Table 5.1: Summary of the baseline characteristics of the study groups. $P<0.05$ is considered a significant difference. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; Total C: Total cholesterol; IOP: Intraocular pressure. The presented SBP, DBP and IOP values are the baseline readings taken on the morning of the study and do not represent the 24 hour or diurnal averages. OPP was subsequently calculated using these baseline values.

### 5.8.2 Systemic vascular parameters

#### 5.8.2.1 Pulse wave analysis

Pulse wave analysis AIx was found to be significantly greater in newly diagnosed NTG patients compared to healthy age matched controls (p=0.017) (Table 5.2).

#### 5.8.2.2 Intima-media thickness measurement

Carotid artery IMT was found to be significantly higher in NTG patients compared to controls (p=0.015) (Table 5.2). Multiple regression analysis revealed significant positive correlations between IMT and SBP (p=0.035); therefore, the given p-value was calculated using ANCOVA correcting for the effect of SBP.

| | NTG | Controls | p-value |
|---|---|---|---|
| PWA: AIx (%) | 26.06 ± 11.25 | 17.18 ± 9.32 | 0.017* |
| IMT (cm) | 0.064 ± 0.015 | 0.042 ± 0.223 | 0.015* |

Table 5.2: Systemic macro-vascular parameters. P<0.05 (*) is considered significant. PWA:AIx: pulse wave analysis: augmentation index; IMT: intima-media thickness.

#### 5.8.2.3 Systemic endothelial function

No significant differences were found between groups with regard to brachial artery FMD or NMD. (p>0.05, table 5.3). Furthermore no significant differences in circulation vWF levels were identified between groups (P>0.05).

| | NTG | Controls | p-value |
|---|---|---|---|
| FMD (%) | 11.01±6.97 | 12.55 ± 9.21 | 0.669 |
| NMD (%) | 25.89 (15.12-52.96) | 22.66 (19.69-29.63) | 0.665 |
| vWF | 118.17±63.46 | 126.58±55.35 | 0.696 |

Table 5.3: Systemic endothelial function. P<0.05 (*) is considered significant. Data presented as mean ± SD or mean (IQR) depending on distribution. FMD: flow mediated dilation; NMD: nitroglycerine mediated dilation; vWF: von Willebrand factor.

### 5.8.3 Dynamic retinal vessel analysis

For ease of interpretation, the dynamic retinal vessel profile curve was 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). The principle results are given based on the average of the 3 flicker cycles with the artery and vein being considered separately. Although each flicker cycle was also analysed individually this analysis did not yield any additional information and has therefore not been presented. The dynamic nature of the retinal vascular response profiles were fully explored using the polynomial fitted curves generated via our novel computational analysis (MatLab R2010a; MathWorks Inc., Natick, MA). These curves are illustrated in figures 5.1 and 5.2 for the artery and vein respectively.

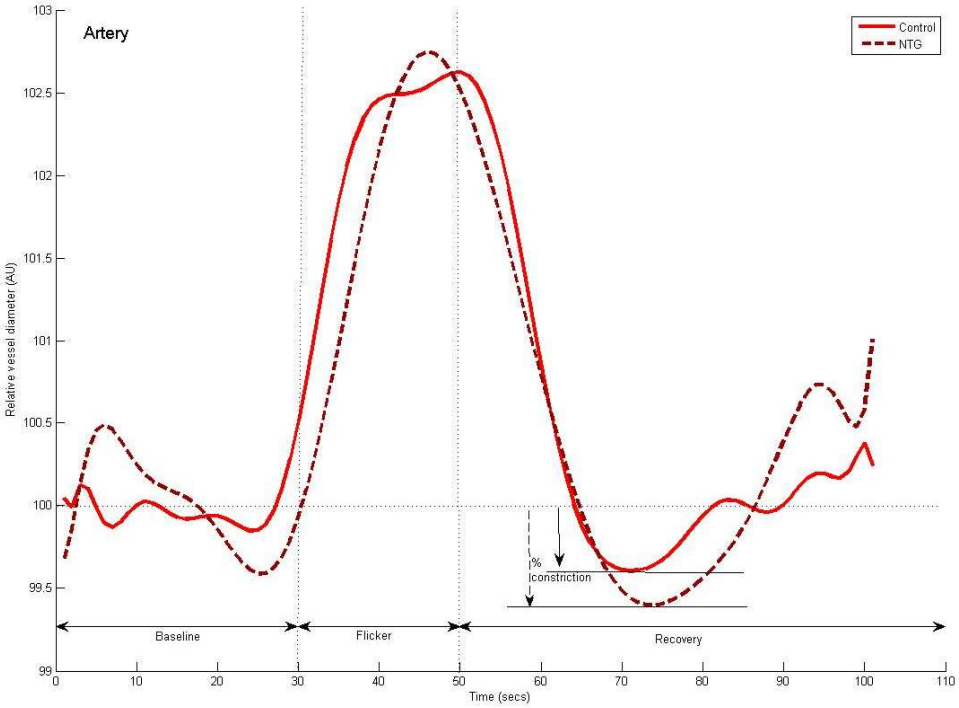



Figure 5.1: Averaged arterial response profile for NTG patients and healthy controls generated through Matlab. Demonstrates the significantly steeper arterial constriction slope found in NTG patients and the apparently greater percentage constriction response below baseline

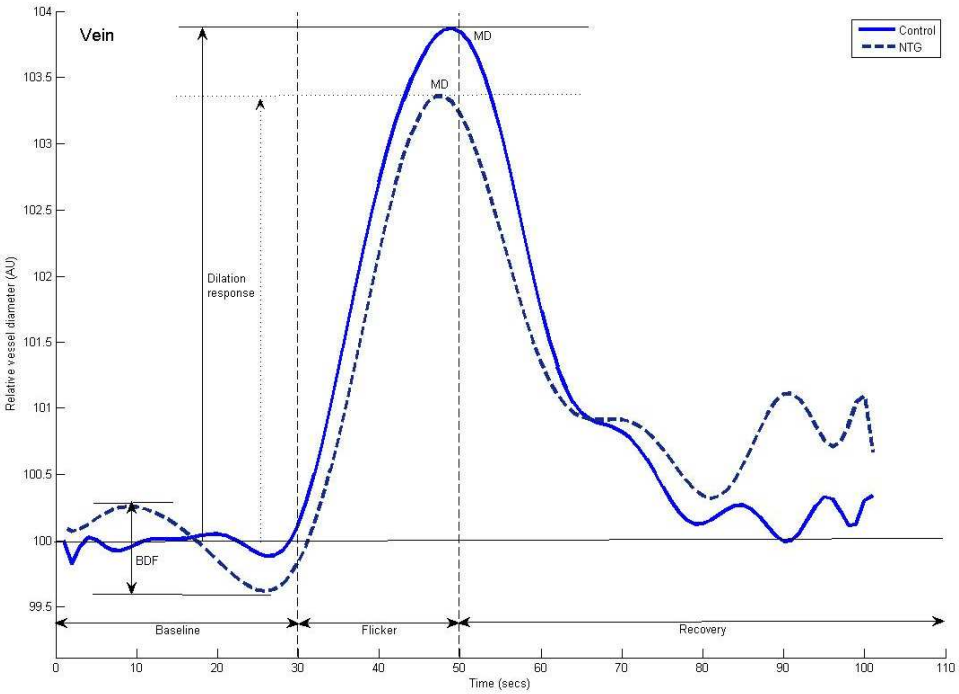



Figure 5.2: Averaged venous response profile for NTG patients and healthy controls generated through Matlab.

**Arterial response**

Following the adoption of a stricter p value of less than 0.01 to account for the effects of multiple comparisons, no significant differences in the maximum arterial dilation (MD%), reaction time (RT), arterial baseline corrected flicker response (BFR) or the baseline diameter fluctuation (BDF) was found between study groups (all p>0.01, table 5.4). Furthermore with regard to the second part of the arterial dynamic profile curve, which looks at the activity of the retinal vessels following dilation and on cessation of flicker, again following the adoption of a stricter p-value of 0.01, no significant differences in the arterial constriction response (MC%) or constriction time (tMC) was also found between groups (p=0.028, p=0.462 respectively, table 5.4)

On consideration of the dynamic nature of the arterial dilation response, evaluated using our novel matlab analysis however, despite no significant differences being found between groups with regard to slope (p>0.05, table 5.5), the arterial dilation profile did

appear to follow a different path in NTG patients compared to controls, with healthy controls demonstrating a 'two humped' arterial dilation response which was absent in our NTG patients (figure 5.2). Furthermore the arterial constriction slope ($Slope_{AC}$) was found to be significantly steeper in NTG patients compared to healthy controls on consideration of the dynamic nature of the arterial constriction profile, (p=0.031, table 5.5).

| ARTERY | NTG | Controls | p-value |
|---|---|---|---|
| BDF | 7.21 (4.50-9.08) | 4.66 (3.51-5.45) | 0.018 |
| MD (%) | 4.28 (2.80-6.31) | 3.70 (2.29-4.44) | 0.138 |
| RT | 21.09 ± 8.67 | 18.59 ± 5.79 | 0.256 |
| BFR | -0.04±2.41 | 0.50±1.95 | 0.423 |
| MC (%) | -2.64 ± 1.61 | -1.56 ± 1.46 | 0.028 |
| tMC | 34.99 ± 8.30 | 32.99 ± 8.92 | 0.462 |

Table 5.4: Arterial vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). Data presented as mean ± SD or mean (IQR) depending on distribution. P<0.01 is considered as significant (*).BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; BFR: baseline corrected flicker response MC(%): percentage constriction below baseline.

| DYNAMIC RESPONSE | NTG | Controls | p-value |
|---|---|---|---|
| **Arteries** | | | |
| Slope $_{AD}$ | 0.300 ± 0.159 | 0.250 ± 0.149 | 0.294 |
| Slope $_{AC}$ | -0.236 (-0.449—0.158) | -0.153 (-0.207—0.110) | 0.031* |

Table 5.5: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. Data presented as mean ± SD or mean (IQR) depending on distribution. P< 0.05 (*) is considered significant. Slope $_{AD}$: slope of arterial dilation; Slope $_{AC}$: slope of arterial constriction

**Venous response**

Following the adoption of a stricter p value of less than 0.01 to account for the effects of multiple comparisons, no significant differences were found between groups with regard to venous MD%, RT, BFR or BDF (p>0.01, table 5.6). Furthermore, with regard to the second part of the dynamic response curve, no significant differences were found in the venous constriction response (MC%) or the constriction response time between groups (p>0.01, table 5.6)

On consideration of the dynamic nature of the venous response profile, evaluated using our novel matlab analysis, no significant difference in the venous dilation slope or the venous constriction slope ($Slope_{VD}$) was found between groups ($p>0.05$, table 5.7).

| VEIN | NTG | Controls | t-test p-value |
|---|---|---|---|
| BDF | 4.55 ± 1.72 | 3.36 ± 1.38 | 0.019 |
| MD (%) | 4.38 ± 2.16 | 4.15 ± 2.33 | 0.753 |
| RT | 22.63 ± 7.12 | 19.99 ± 4.08 | 0.128 |
| BFR | -0.04 ± 2.56 | 1.72 ± 2.36 | 0.026 |
| MC (%) | -0.61 ± 0.86 | -0.93 ± 1.04 | 0.326 |
| tMC | 39.31 ± 6.93 | 37.73 ± 9.57 | 0.575 |

Table 5.6: Venous vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). P<0.01 (*) is considered as significant.BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; BFR: baseline corrected flicker response MC(%): percentage constriction below baseline.

| DYNAMIC RESPONSE | NTG | Controls | t-test p-value |
|---|---|---|---|
| **Veins** | | | |
| Slope $_{VD}$ | 0.235 ± 0.132 | 0.225 ± 0.133 | 0.816 |
| Slope $_{VC}$ | -0.147 ± 0.090 | -0.164 ± 0.124 | 0.630 |

Table 5.7: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{VD}$: slope of venous dilation; Slope $_{VC}$: slope of venous constriction.

#### 5.8.3.1 Correlations

No significant correlations were found between the retinal vascular reactivity parameters (BDF, MD, MC, DA and BFR, Slope) and the systemic vascular parameters (AIx, IMT and FMD) in either of our groups (all $p>0.05$).

## 5.9 Discussion

### 5.9.1 Main findings

Using a novel computational model, our results reveal that newly diagnosed, previously untreated NTG patients with no overt clinical signs of systemic vascular disease show an altered retinal arterial response profile to flicker light along with a significantly steeper retinal arterial constriction response following cessation of flicker. Moreover, subclinical signs of systemic arterial stiffness along with increased carotid artery IMT are consistently exhibited by our NTG patients but not by our age-matched controls.

### 5.9.2 Systemic macro-vascular alterations in NTG

Both the presence of systemic vascular disease and alterations in systemic vascular function have been well linked with the development of glaucoma, particularly NTG [42, 311, 446, 757, 758]. Nevertheless, previous research into the role of systemic measures, such as arterial stiffness in the aetiology of glaucoma have given inconsistent results with some showing a strong association [481-483] and others showing no association at all [318, 485]. Such variability in results could be partly accounted for by differences in the inclusion/exclusion of patients suffering from already diagnosed systemic vascular disease in these studies, especially as arterial stiffness is a measure of vascular function [759]. In this study we have demonstrated increased arterial stiffness and IMT in our NTG patients. Patients with well controlled hypertension were included in this study, however a similar number of subjects with such status were included in both the NTG and control group. The present report therefore simply shows that newly diagnosed NTG patients present with stronger signs of systemic vascular pathology, which could have possibly contributed to the onset of the ocular disease. In light of this it could be suggested that screening for such abnormalities in at risk or newly diagnosed individuals and addressing them through appropriate interventions could be beneficial with regard to disease prognosis.

With regard to macrovascular function, no significant differences in systemic endothelial function were found between NTG patients and controls in this study on brachial artery FMD or on the evaluation of the circulating endothelial marker, vWf. These findings are in contrary to the plentiful evidence in the literature indicating the involvement of systemic endothelial dysfunction in the glaucomatous disease process and to the findings of Su et al [52] who demonstrated impaired FMD in NTG patients. Such contradiction in findings between previous research and our present study could partly be accounted for by differences in both the measurement protocol and the patient inclusion criteria used, particularly as it is unclear whether the NTG patients included by Su et al were newly diagnosed and untreated or at a more advanced stage of the disease process. With this is mind it could be hypothesised that, as endothelial dysfunction is known to occur much earlier at the microvascular level than at the macrovascular level in a disease process [734], signs of systemic endothelial dysfunction, although not yet detectable in our newly diagnosed NTG patients on FMD, could become more apparent as the disease progresses. Further research however would be required to confirm this hypothesis and it is worth considering that there are studies which have previously indicated the presence of systemic endothelial dysfunction even in newly diagnosed NTG patients using alternative and now less favoured methods of assessment such as venous occlusion plethysmography [382, 384], however, these studies whilst conclusive, did involve very small sample sizes and it is unclear to what extent coexisting systemic disease was considered.

The states of the macro- and microcirculation are known to be closely related [760-762] and in addition to systemic vascular abnormalities, NTG has also been associated with ocular microcirculatory changes. Indeed, Oettli et al [295] recently demonstrated an increased retinal vessel rigidity in untreated NTG patients that correlated with the level of glaucomatous damage. Our present results also demonstrate that in addition to

systemic vascular abnormalities, newly diagnosed and untreated NTG patients show signs of abnormal vascular function at the retinal vessel level.

### 5.9.3 Retinal microvascular reactivity in NTG

Considering the arterial dynamic retinal vessel profile first, no differences in dilation response were found between groups in this study; however the dilation profile did appear to follow a different path in NTG patients compared to controls. As illustrated in figure 5.2, our healthy controls demonstrated a 'two humped' arterial dilation response that is consistent with that previously demonstrated by Lanzl et al in a sample of young healthy volunteers [763] and to a lesser extent in a sample of older healthy volunteers [696]. Our patients fall between the age ranges included in the above research and whilst the 'two humped' aspect was present in our healthy control patients it was absent in our age-matched NTG patients. Lanzl et al [696] propose that such a 'two humped' profile is, amongst other factors, reflective of two separate systems that contribute to arterial dilation, the first being a fast onset, short duration system, mediated by endothelial NO-synthase and free NO and the second being a slow onset, long duration system which represents the summation of dilation and constriction factors. As no differences were found in age, maximum dilation or the time taken to reach maximum dilation between our groups it is difficult to determine which of these systems, if any, may be affected in our NTG patients and lead to the loss of this 'two humped' pattern. It could be hypothesised that whilst there is sufficient NO production/availability to induce a dilatory response in the retinal arteries in our NTG patients initially, this NO is either of short supply or is rapidly deactivated after release, leading to a loss of the second dilatory phase and a predominance of vasoconstrictive factors such as ET-1. In line with this assumption, our newly diagnosed NTG patients demonstrated a steeper arterial constriction slope compared to our healthy age matched controls on cessation of flicker. Slope calculation considers the interaction between the percentage change in vessel diameter and the rate at which this change occurs. The relevance of a steeper arterial

constriction slope, as demonstrated in our NTG patients, is currently unclear as very few previous studies looking at vascular reaction to flickering light have investigated the constriction responses in detail. One factor which could contribute towards such a steeper arterial constriction slope is an enhanced percentage constriction response below baseline following cessation of flicker. Re-establishment of baseline diameter following stimulation is part of a complex inherent biological control process and a certain degree of overshoot in vascular diameter below baseline is expected even in healthy individuals following flicker light exposure [685, 693]. Interestingly, in a similar way to our newly diagnosed NTG patients, a greater overshoot in arterial vessel diameter below baseline has previously been noted by Gugleta et al [746] in individuals with PVD, a condition known to be associated with both vascular dysfunction and NTG [26, 300, 334, 353, 360, 383]. With this in mind we could therefore propose that the steeper constriction response demonstrated in our NTG patients could be indicative of some form of vascular dysfunction. The cause of this vascular dysfunction, however, can only be hypothesised at this point. One potential contributing factor, as mentioned previously, could be a predominance of vasoconstrictive factors such as ET-1, the levels of which, although not measured here, have previously been shown to be increased in NTG patients and vasospastic patients [340, 355, 356 391]. Alternatively an abnormal proliferation of astrocytes, which are known to be key mediators of the neurovascular coupling response [600] could also contribute to these findings. Indeed abnormal astrocyte proliferation has been previously linked to both the development of glaucoma [144] and to the re-establishment of vasomotor tone following neuronal stimulation [764, 765]. Further investigations however would be needed to validate these hypotheses.

With regard to the venous dynamic retinal vessel profile to flicker light stimulation, prior to correction for multiple comparisons a significantly reduced venous baseline corrected flicker response (BFR) was demonstrated in our newly diagnosed previously untreated NTG patients, which reinforces previous findings by Garhofer et al [41] who identified an

impaired retinal venous dilation response to flicker in early POAG patients. Similarly, baseline diameter fluctuation (BDF) was found to be significantly greater in both arteries and veins of those with NTG, however again this significance was lost on correction for multiple comparisons. Despite this there is evidence to suggest that increased BDF could be a relevant indicator of vascular disturbance [695, 746], perhaps indicating instability or increased variation in vascular tone or rigidity [747, 748] and therefore it may be a parameter worth exploring further in future research, especially as increased arterial flow pulsations, associated with aging and arterial stiffening have been linked with the occurrence of microvascular disease [766, 767].

### 5.9.4 Ocular microvasculature vs. Systemic macrovasculature

No correlations were found between the subclinical signs of systemic vascular disease and the retinal vascular response to flickering light in NTG patients; however, due to the complexity of comparing parameters from two different vascular beds using different measuring techniques this may not be surprising. Interestingly, this finding is in concordance with that of a number of recent studies which have similarly demonstrated no direct correlation between anomalies identified at the macro- and micro-vascular levels in various disease states [710, 768, 769]. This supports the view that, whilst the states of these two systems may be closely related, they may in fact act independently of each other with regard to the rate and pathophysiological mechanism of their vascular dysfunction development. The possibility that each may drive or influence the other however cannot be excluded [770] and this requires further investigation.

## 5.10 Conclusion

In conclusion this study demonstrates the coexistence of static macro-vascular abnormalities and functional retinal microvascular abnormalities in newly diagnosed and

previously untreated NTG patients, highlighting the importance of considering multi-level circulation-related pathologies in the development of this type of glaucoma.

## 5.11 Limitations

Only a moderately sized cohort of NTG patients could be recruited for this study which could potentially limit the statistical power of the analysis and the conclusions which can be drawn from the presented data. The overall limitations of the presented research are discussed further in section 8.3.

# 6. Ocular Vascular Dysregulation in AD compares to both POAG and NTG

## 6.1 Abstract

**Purpose:** To investigate and compare vascular function at both the ocular and systemic level in newly diagnosed mild AD patients, newly diagnosed normal tension glaucoma (NTG) patients and newly diagnosed primary open angle glaucoma (POAG) patients.

**Methods:** Retinal vessel reactivity to flickering light was assessed in 10 AD, 19 POAG, 19 NTG and 20 healthy age matched control patients by means of dynamic retinal vessel analysis (DVA, IMEDOS, GmbH, Jena, Germany). Systemic vascular endothelial function was additionally assessed in all patients by means of brachial artery flow mediated dilation and all patients underwent BP measurements and blood analysis for glucose and lipid metabolism markers.

**Results:** AD patients demonstrated altered arterial retinal vessel reactivity to flicker light stimulation which was comparable to that of POAG patients ($p=0.013$) and altered baseline venous reactivity which was comparable to that of NTG patients ($p=0.001$). Neither were replicated in healthy controls. No significant differences in systemic endothelial function were identified between groups ($p>0.05$).

**Conclusion:** AD patients demonstrate similar signs of retinal vascular dysfunction to both POAG and NTG patients at the early stages of their disease process, providing support for the concept of a common underlying vascular aetiology in both conditions and highlighting the need to consider ocular health in AD and cerebral health in glaucoma.

## 6.2 Introduction

The possibility that AD and glaucoma may share a common underlying vascular aetiology has been increasingly realised over recent years. This stems not only from their obvious similarities as chronic neurodegenerative diseases associated with aging but also from the results of numerous studies which have identified alterations in cerebral perfusion and dynamic cerebral autoregulation, more commonly associated with AD, in both POAG [323] and NTG patients [313, 323, 468]. Moreover reduced retinal perfusion and increased ONH cupping, more characteristic of glaucoma; have also been demonstrated in AD patients [663, 665]. Despite these apparent associations however there is currently no research which directly explores and compares the nature and coexistence of vascular function abnormalities in both AD and glaucoma patients simultaneously. Interestingly, in Chapters 4 and 5 of this thesis, evidence of vascular alteration was identified in both newly diagnosed AD patients and newly diagnosed NTG patients, individually, in comparison to healthy controls. In order to expand on these findings, this study aims, through the simple, non-invasive evaluation of both dynamic retinal vessel function and systemic vascular endothelial function, to investigate whether such ocular and systemic vascular alterations coexist in newly diagnosed glaucoma and AD patients and to explore the similarities and differences between them. Furthermore, through consideration of two separate categories of glaucoma patients, namely POAG and NTG, this study additionally aims to determine whether any vascular alterations identified in AD patients are more closely related to those of patients diagnosed with POAG or to those of patients diagnosed with NTG. Increasing our understanding of the nature of vascular dysfunction in all of these conditions, if present, could provide an important insight into their disease aetiology and lead to better awareness and understanding of their potential coexistence.

## 6.3 Aims

The aim of this study is to investigate whether ocular and systemic vascular alterations coexist in newly diagnosed POAG, NTG and AD patients and to explore the similarities and differences between them. Furthermore, this study aims to determine whether vascular alterations in AD, if identified, related to both POAG and NTG.

## 6.4 Hypothesis

Newly diagnosed mild AD patients will demonstrate signs of vascular dysfunction at the ocular and systemic level which are comparable to that also demonstrated by newly diagnosed POAG patients and/or NTG patients.

## 6.5 Subjects and Methods

Newly diagnosed and previously untreated NTG and POAG patients, mild newly diagnosed AD patients and healthy age matched controls were recruited for this study. The recruitment details, inclusion and exclusion criteria for these patients was detailed in section 3.1. The investigative procedures performed in this study are outlined below and were conducted in accordance with the protocols outlined in section 3.2:

1. Preliminary tests
2. Fasting venous blood sample obtained
3. Blood pressure measurement
4. Assessment of retinal vessel reactivity (DVA)
5. Assessment of systemic endothelial function (FMD)

## 6.6 Statistical Analysis

All data were reported as mean ± standard deviation. The Kolmogorov-Smirnov test was used to determine the distribution of the data. Multivariate analysis was performed to

determine the influence of age, BMI, BP and circulating markers on the measured variables. Differences between groups were subsequently assessed using one-way ANOVA or ANCOVA, as appropriate, followed by Tukey's post hoc analysis. Two factor repeated-measures ANOVA was used to compare the retinal reactivity responses across each flicker cycle. In cases where the normality of the data could not be confirmed log transformations were made. Correlations between the ocular and systemic parameters were explored using either Pearson's linear correlation or Spearman's rank method as appropriate. P-values of less than 0.05 were considered significant, except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons and minimise bias towards type I errors. All analyses were performed using Satistica, version 6.0, Statsoft, Tusla, OK.

## 6.7 Power calculations

With regard to DVA, new measurement parameters and novel analysis methods were used in this study in patient groups which have not previously been examined with these techniques. Power calculation would normally be based on the results of previous studies which share the most similar protocols to that of the present research, however due to the nature of the statistical analysis required in this study, namely one-way ANOVA (or ANCOVA) and two factor repeated measures ANOVA and the uniqueness of the comparisons being made, appropriate previous research was not available and the power calculations were therefore ultimately made based on a number of assumptions. Firstly a large effect size of 0.40 was selected from Cohen's standardised effect sizes [771], along with a Pearson's correlation coefficient among repeated measures of 0.5 and a non-sphericity correction of 1. The power calculation was made using the computer based programme, GPower 3 [733] and it was calculated that, in order to provide a statistical power of 80% at an α level of 0.05 a sample size of between 4-19 per group would be required (19 one-way ANOVA, 4 within groups ANOVA, 13 between groups

ANOVA, 5 within/between groups ANOVA). The aim was therefore to recruit at least 19 patients per group in this study.

## 6.8 Results

A total of 12 AD patients, 20 POAG patients, 25 NTG patients and 34 healthy controls were screened for inclusion in the present study however, in order to ensure all participants were matched on critical factors such as age and hypertensive status, all those under the age of 48, over the age of 75 or with a MBP of greater than 110 mmHg had to be excluded from analysis. Additionally, following the careful review of the obtained images, any patients who exhibited poor or incomplete results were also excluded meaning a total of 10 mild newly diagnosed AD patients, 19 POAG patients, 19 NTG patients and 20 healthy controls were recruited for this study. These numbers obviously fall below the target of 19 per group with regard to AD patients however on analysis of the results statistical differences between AD, glaucoma and control groups were obtained inferring sufficient power was still achieved. The difficulties associated with recruitment of this group of participants was discussed in section 4.11

### 6.8.1 Baseline values

There were no significant differences in age, systemic BP, BMI, triglycerides, glucose, HDL cholesterol levels and total cholesterol levels between the four groups (all $p>0.05$, table 6.1). Furthermore there were no significant differences in MBP or Framingham risk score ($P>0.05$, table 6.1) and the number of subjects with well controlled high BP was proportionally similar between groups (AD: n=3; POAG: n=3; NTG: n=5; Controls: n=6; Chi-square test). As expected IOP was found to be significantly greater in our POAG patients in comparison to all other groups ($p<0.001$, table 6.1) and consequently OPP was also found to be lower in our POAG patients but only significantly so with regard to AD patients and controls ($p=0.017$, table 6.1).

| | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANOVA p-value | Post-hoc |
|---|---|---|---|---|---|---|
| **N** | 10 | 19 | 19 | 20 | - | - |
| **Gender** | 5F:5M | 9F:10M | 11F:8M | 8F:12M | - | - |
| **Age (years)** | 62.50±8.07 | 65.26±9.52 | 60.16±12.13 | 58.00±4.32 | 0.143 | - |
| **SBP (mmHg)** | 141.70±14.21 | 136.32±15.42 | 130.06±15.37 | 131.70±17.90 | 0.278 | - |
| **DBP (mmHg)** | 80.30±7.51 | 79.11±9.80 | 78.39±11.14 | 79.70±9.39 | 0.952 | - |
| **BMI** | 27.61±5.80 | 27.71±5.00 | 27.92±4.20 | 27.56±4.67 | 0.997 | - |
| **Glucose** | 4.40±1.44 | 4.53±0.97 | 5.08±0.86 | 4.87±1.02 | 0.307 | - |
| **Triglycerides** | 1.28±0.60 | 1.22±0.50 | 1.04±0.31 | 1.17±0.40 | 0.488 | - |
| **HDL-C (mmol/L)** | 1.33±0.25 | 1.17±0.26 | 1.12±0.29 | 1.14±0.32 | 0.315 | - |
| **Total-C (mmol/L)** | 4.77±0.64 | 4.29±0.78 | 4.58±1.04 | 4.73±0.67 | 0.376 | - |
| **IOP (mmHg)** | 16.50±2.12 | 23.94±3.00 | 17.40±1.80 | 17.20±2.68 | 0.000* | 2>1,3,4 |
| **MBP (mmHg)** | 100.77±7.67 | 99.29±11.35 | 95.61±10.93 | 97.03±11.67 | 0.637 | - |
| **OPP** | 84.96±9.46 | 47.12±16.93 | 76.09±12.66 | 82.65±12.06 | 0.002* | 2<1,4 |
| **Fram Risk Score** | 10.67±3.28 | 11.18±5.56 | 9.67±7.50 | 10.11±5.20 | 0.921 | - |

Table 6.1: Summary of the baseline characteristics of the study groups. P<0.05 is considered a significant difference. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; Total C: Total cholesterol; IOP: Intraocular pressure. The presented SBP, DBP and IOP values are the baseline readings taken on the morning of the study and do not represent the 24 hour or diurnal averages. OPP was subsequently calculated using these baseline values.

### 6.8.2 *Systemic endothelial function*

No significant differences were found between groups with regard to brachial artery FMD or NMD (p>0.05, table 6.2). Furthermore no significant differences in circulating vWF levels were identified between groups (p>0.05)

| | POAG | NTG | AD | Controls | ANOVA p-value | Post-hoc |
|---|---|---|---|---|---|---|
| FMD (%) | 5.82±5.64 | 11.01±6.97 | 8.54±9.19 | 10.27±7.64 | 0.235 | - |
| NMD (%) | 15.30±12.44 | 29.09±17.83 | 24.74±16.42 | 23.76±7.63 | 0.258 | - |
| vWF | 149.09±50.93 | 96.39±39.05 | 118.34±64.28 | 108.60±49.23 | 0.496 | |

Table 6.2: Systemic endothelial function. P<0.05 (*) is considered significant. FMD: flow mediated dilation; NMD: nitroglycerine mediated dilation; vWF: von Willebrand factor.

### 6.8.3 Dynamic retinal vessel analysis

For ease of interpretation, the dynamic retinal vessel profile curve was 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). Each flicker cycle was analysed individually using traditional SDRA analysis and the artery and vein were considered separately. The dynamic nature or slope of the retinal vascular response profiles were fully explored using the polynomial fitted curves generated via our novel computational analysis (MatLab R2010a; MathWorks Inc., Natick, MA).

#### 6.8.3.1 Arterial response

**Dilation**

With regard to the first part of dynamic profile curve, following the adoption of a stricter p value of 0.01 to correct for the effects of multiple comparisons no significant differences were found in the average arterial baseline diameter, maximum diameter (MD%), reaction time (RT) or baseline corrected flicker response (BFR) between all four study groups (all $p>0.01$, table 6.3). When considering each flicker cycle individually however using two factor repeated measures ANOVA, the arterial RT was found to be significantly longer on the final flicker cycle (F3) in both AD and POAG patients in comparison to healthy controls ($p=0.016$, table 6.4). Furthermore the sequential changes in the RT of the retinal arteries on progressing from flicker 1 to flicker 3 was found to vary significantly between groups ($p=0.007$, table 6.4), with healthy controls showing a significant decrease in RT on going from F2 to F3 ($p=0.011$, table 6.4) which was not replicated by any of the other groups, where in fact an increase was observed. On consideration of the dynamic nature of the arterial dilation response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope ($p>0.05$, table 6.5).

### Constriction

With regard to the second part of the arterial dynamic profile curve, which looks at the activity of the retinal vessels following dilation and on cessation of flicker, again following the adoption of a stricter p value of 0.01 to account for the effects of multiple comparisons, no significant differences in maximum arterial constriction (MC%) or the time taken to reach maximum constriction (tMC) were found between groups (p>0.01, table 6.3). Furthermore, on consideration of the dynamic nature of the arterial constriction response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 6.5).

| ARTERY Average data | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANOVA P-value | Post-hoc |
|---|---|---|---|---|---|---|
| BDF | 5.44±2.11 | 7.67±4.17 | 7.85±4.18 | 4.74±1.94 | 0.012 | - |
| MD (%) | 5.78±3.25 | 5.90±3.65 | 7.27±3.02 | 5.19±2.19 | 0.188 | - |
| RT | 24.04±11.74 | 21.59±5.96 | 23.20±8.23 | 20.48±6.77 | 0.568 | - |
| BFR | 3.46±3.79 | 2.60±3.91 | 2.84±2.97 | 2.80±1.89 | 0.943 | - |
| MC (%) | -3.29±1.56 | -3.85±3.72 | -3.85±1.93 | -2.55±1.85 | 0.210 | - |
| tMC (secs) | 32.63±10.24 | 30.31±10.05 | 28.09±6.96 | 27.08±7.68 | 0.752 | - |

Table 6.3: Arterial vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). P<0.01 is considered as significant (*).BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; RT: reaction time BFR: baseline corrected flicker response MC(%): percentage constriction below baseline; tMC: time taken to reach maximum constriction.

| ARTERY | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANOVA P-value | Post-hoc | Interaction p-value |
|---|---|---|---|---|---|---|---|
| **RT** | | | | | | | |
| Flicker 1 | 29.30±16.61 | 19.42±14.17 | 24.94±14.46 | 20.05±12.07 | 0.236 | | |
| Flicker 2 | 16.30±11.48 | 21.72±12.11 | 21.82±15.80 | 25.85±9.00 | 0.260 | | |
| Flicker 3 | 27.89±17.62 | 26.40±10.82 | 23.94±8.74 | 15.55±11.07 | 0.016* | 1,2>4 | |
| **Within groups ANOVA** | 0.093 | 0.314 | 0.787 | 0.011* (F2-F3) | | | 0.007* |

Table 6.4: Arterial vascular function parameters by flicker cycle. P<0.05 (*) is considered as significant on repeated measures ANOVA. RT: reaction time.

| DYNAMIC RESPONSE | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANVOA p-value | Post-hoc |
|---|---|---|---|---|---|---|
| **Arteries** | | | | | | |
| Slope $_{AD}$ | 0.265±0.221 | 0.271±0.450 | 0.300±0.159 | 0.273±0.169 | 0.985 | - |
| Slope $_{AC}$ | -0.194±0.070 | -0.269±0.154 | -0.282±0.158 | -0.170±0.095 | 0.055 | - |

Table 6.5: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{AD}$: slope of arterial dilation; Slope $_{AC}$: slope of arterial constriction;

#### 6.8.3.2 Venous response

**Dilation**

With regard to the first part of the venous dynamic profile curve, no significant differences were found in the average venous baseline diameter, maximum diameter (MD%), reaction time (RT) or baseline corrected flicker response (BFR) between all four study groups (all p>0.01, table 6.6). Venous baseline diameter fluctuation (BDF) however was found to be significantly greater on average in both our AD and NTG patients in comparison to our healthy controls (p=0.001, table 6.6). On consideration of the dynamic nature of the venous dilation response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 6.7).

**Constriction**

With regard to the second part of the venous dynamic profile curve, which looks at the activity of the retinal vessels following dilation and on cessation of flicker, no significant differences in maximum constriction (MC%) or the time taken to reach maximum venous constriction (tMC) were found between groups (all p>0.01, table 6.6). Furthermore, on consideration of the dynamic nature of the venous constriction response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 6.7).

| VEIN | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANOVA p-value | Post-hoc |
|---|---|---|---|---|---|---|
| BDF | 6.44±2.67 | 4.98±2.44 | 5.29±1.99 | 3.27±1.56 | 0.002* | 1,3>4 |
| MD (%) | 6.12±3.14 | 5.35±1.99 | 5.94±3.03 | 5.13±2.86 | 0.720 | - |
| RT | 22.67±9.39 | 19.94±4.39 | 21.69±8.37 | 20.48±3.68 | 0.756 | - |
| BFR | 2.16±4.03 | 2.81±2.56 | 2.72±2.92 | 3.30±2.28 | 0.765 | - |
| MC (%) | -2.61±2.13 | -2.60±2.46 | -2.09±2.09 | -1.77±1.40 | 0.572 | - |
| tMC (secs) | 29.74±6.20 | 34.25±8.65 | 31.63±12.99 | 34.25±9.40 | 0.664 | - |

Table 6.6: Venous vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). P<0.01 is considered as significant (*).BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; RT: reaction time BFR: baseline corrected flicker response MC(%): percentage constriction below baseline; tMC: time taken to reach maximum constriction.

| DYNAMIC RESPONSE | AD (1) | POAG (2) | NTG (3) | Controls (4) | ANVOA p-value | Post-hoc |
|---|---|---|---|---|---|---|
| **Veins** | | | | | | |
| Slope $_{VD}$ | 0.295±0.172 | 0.229±0.094 | 0.219±0.141 | 0.222±0.132 | 0.518 | - |
| Slope $_{VC}$ | -0.204±0.193 | -0.154±0.103 | -0.160±0.10 | -0.164±0.137 | 0.281 | - |

Table 6.7: Dynamic characteristics of the retinal vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant; Slope $_{VD}$: slope of venous dilation; Slope $_{VC}$: slope of venous constriction.

### 6.8.4 Correlations

No significant correlations were found between the retinal vessel reactivity parameters and the systemic parameters (FMD, blood analyses) between groups (all P>0.05).

## 6.9 Discussion

### 6.9.1 Main findings

This study has revealed for the first time evidence of altered reactivity to flicker light in the retinal arteries of both newly diagnosed, previously untreated POAG patients and newly diagnosed mild AD patients which is of a similar nature in both conditions and is not replicated by healthy control patients. The time taken for the retinal arteries to reach the point of maximum dilation following the onset of flicker light stimulation was found to be significantly greater in both AD and POAG patients in comparison to healthy controls on the final flicker cycle. Furthermore the sequential changes in the reaction time of the retinal arteries on progressing from flicker 1 to flicker 3 was found to vary significantly between groups with healthy controls showing a significant reduction in reaction time on heading into the final flicker cycle which was not replicated by any of the other groups. This study has also revealed evidence of altered baseline retinal venous activity which is of a similar nature in both our newly diagnosed and previously untreated NTG patients and our newly diagnosed mild AD patients and is again not replicated by healthy controls. No significant differences were identified between groups with regard to the systemic vascular parameters.

### 6.9.2 Systemic endothelial function

No significant differences in systemic vascular endothelial function, as measured by FMD, were found between all four groups in this study. This result indicates that the functioning of the systemic vascular endothelium is comparable between newly diagnosed glaucoma patients, mild AD patients and healthy controls. A comparison of this kind between all of these groups simultaneously has not previously been made however on an individual basis disturbed systemic endothelial function has previously been identified in both POAG [42, 385] and NTG patients [52] compared to healthy controls and also in AD patients compared to healthy controls [631]. The possibility therefore that

vascular dysfunction at the systemic level may play a role in their disease aetiologies individually cannot be ruled out. All of our participants were newly diagnosed and at the very earliest stages of their disease process. It could be hypothesised therefore that measureable signs of systemic macrovascular dysfunction were simply not yet detectable in these patients at the point of investigation. Indeed it has previously been demonstrated that endothelial dysfunction affects the microvasculature at an earlier stage in a disease process in comparison to the macrovasculature [734] . Further research and follow-up would be required however in order to confirm this hypothesis.

### 6.9.3 Retinal vessel reactivity

With regard to retinal artery reactivity our mild AD patients and our newly diagnosed POAG patients took comparably and significantly longer to reach the point of maximum arterial dilation following the onset of flicker light compared to our healthy controls and NTG patients on the final stimulation cycle. A prolonged reaction time to flicker light is an established parameter which has been shown to indicate the presence of some form of vascular dysfunction at the retinal level [695, 710, 732]. The present results suggest that this vascular dysfunction appears to be shared by both our AD and POAG patients however its exact aetiology can only be hypothesised at this point. As the retinal vascular response to flickering light occurs due to an increase in retinal metabolic demand and is predominantly a neurovascular coupling driven response [226, 685, 686, 691, 692], it could be hypothesised that the altered retinal vessel reactivity demonstrated here may be indicative of a similar disturbance of neurovascular coupling in AD and POAG patients which is exacerbated by repeated flicker stimulation. The variability in the progression of the arterial reaction time between groups over successive flicker cycles and the significant involvement of the final flicker cycle could further suggest that exhaustive factors, such as a progressive depletion of NO reserves, may play a role in the altered vascular reactivity observed here. Indeed altered NO activity and bioavailability has been previously described in both AD [772, 773] and POAG patients [368, 369, 377] and has also

previously been linked to alterations in retinal vessel reactivity to flicker light [41, 226]. The presence of a common endothelial dysfunction in AD and POAG, oxidative stress or a common alteration in astrocyte activity, all of which have been previously demonstrated to varying degrees in these disease processes individually [42, 144, 198, 631, 632, 739] and are known to be key mediators of the neurovascular response [600], could potentially explain these exhaustive alterations. Due to the complexity of the neurovascular coupling response however, before any firm conclusions can be made regarding the exact nature of the observed vascular dysfunction in our AD and POAG patients', further investigation would be required to validate all of these hypotheses.

On consideration of the retinal venous reactivity, our newly diagnosed mild AD patients demonstrated increased fluctuations in baseline vessel diameter on average, prior to the onset of flicker, which were comparable to that of our newly diagnosed NTG patients and were not replicated by healthy controls or POAG patients. Consideration of baseline diameter fluctuation (BDF) was first recommended by Nagel et al [686] as a way of taking into account the effect of the spontaneous variations in vessel diameter that occur under normal resting conditions on the observed response of the vasculature to flicker light stimulation, however it is a parameter which is not commonly reported in the literature and which has, to date, mainly been considered in regard to the retinal arteries, where it has been tentatively linked to vascular disturbance in the form of instability or increased variation in vascular tone or rigidity [695, 746-748]. As such the cause and relevance of increased BDF in the venous circulation is currently unclear. 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 and instigating a regulatory contribution passively in response to increased blood flow [685]. Interestingly increased retinal venous diameters have been previously linked to impaired cerebral blood flow and have been suggested as a marker of both retinal and cerebral ischemia and hypoxia [652, 749-751]. The finding here is somewhat different as fluctuations in

diameter have been assessed dynamically as opposed to vessel diameter measurements being taken statically from photographs. Nevertheless as both ischemia and hypoxia have been well linked to the development of AD and NTG, it could be hypothesised that the increased fluctuation in baseline diameter observed in these patients could be reflective of early hypoxic changes and a common increased risk of future damage in these patients.

### 6.9.4 Retinal vascular dysfunction in AD compares to both POAG and NTG

The concept that AD and glaucoma may share a common underlying vascular aetiology has been increasingly realised over recent years and evidence of vascular dysfunction, related to either disturbed autoregulation or disturbed neurovascular coupling mechanisms, has been previously demonstrated at the cerebral level in both conditions [323, 567, 620, 626] and at the ocular level in both POAG and NTG patients [39, 41, 320]. Until now however the presence of dynamic vascular dysfunction at the ocular level has not been assessed in AD patients and directly compared to that of glaucoma patients and healthy controls. The present study addresses this and provides support for not only a shared vascular dysfunction in AD and glaucoma patients at the earliest stages of their disease process but also for the presence of vascular dysfunction in both POAG and NTG. Interestingly the elements of ocular vascular dysfunction which we have demonstrated in our AD patients appear to relate to that of both POAG and NTG patients in a slightly different manner. Indeed with regard to retinal artery reactivity AD patients demonstrated a dysfunction that was similar in nature to that displayed by our POAG patients and not replicated by our healthy controls or NTG patients, whereas with regard to the retinal venous reactivity, AD patients demonstrated baseline disturbances that were of a similar nature to that displayed in our NTG patients and were again not replicated by the other two groups. The key feature of both POAG and NTG is RGC loss and excavation of the

ONH [109, 113]. Vascular factors have been implicated in the aetiology of both conditions [141, 320, 325] and both have been previously linked to AD [676, 677]. It has been recently suggested that rather than being divided into two separate entities, glaucoma should instead be considered as a disease continuum, extending from pure POAG at one end, in which raised IOP plays a predominate role, to pure NTG at the other end, in which IOP-independent or vascular factors play a more predominate role [32-34]. This allows for a certain degree of overlap between the two extremes where a 'mixed' aetiology may exist. In a similar manner, due to the increasing evidence of vascular involvement in the AD process, AD dementia has also been described as a disease continuum extending from pure AD at one end to VaD at the other end, again with a mixed component separating the two extremes [645, 646]. VaD is a condition which bares similarities to AD dementia but has an established primary vascular cause such as stroke [643]. Increasing evidence suggests that the majority of patients may in fact lie somewhere between the two extremes in both the glaucoma and AD continuums and exhibit a mixed aetiology with important vascular elements. This may in part explain the findings here of vascular dysfunction in AD patients which relates to that of both our POAG and NTG patients. Further research investigating vascular dysfunction in AD and glaucoma patients however is still required to elicit the exact nature of the relationship between the two conditions. The need for consideration of glaucoma and ocular health in AD and the need for consideration of AD in those diagnosed with all forms of glaucoma has nevertheless been highlighted by this study.

## 6.10 Conclusion

In conclusion this study demonstrates for the first time that retinal vascular reactivity to flickering light is altered in a similar manner in both AD and glaucoma patients providing support for a common underlying vascular aetiology in both conditions. Furthermore this study has identified differences in the nature of these retinal vascular reactivity

disturbances between AD and POAG patients and AD and NTG patients, however further investigation is required in order to determine the significance of these findings.

## 6.11 Limitations

Only a small sample size of AD patients and a moderate cohort of glaucoma patients could be recruited for this study. This could potentially limit the statistical power and conclusions which can be drawn from the presented data. The overall limitations of the presented research are discussed further in section 8.3.

# 7. Primary open angle glaucoma and Normal tension glaucoma: two separate diseases or one continuous entity? The vascular perspective

## 7.1 Abstract

**Purpose:** To compare and contrast the presence of ocular and systemic vascular function across multiple levels in newly diagnosed and previously untreated primary open angle glaucoma (POAG) and normal tension glaucoma (NTG) patients and to evaluate the validity of these conditions as distinct clinical entities.

**Methods:** Systemic vascular function was assessed in 19 POAG patients, 19 NTG patients and 20 healthy controls by means of 24 hour ambulatory blood pressure (ABPM), 24 hour heart rate variability (HRV) assessment, pulse wave analysis (PWA), carotid intima-media thickness (IMT) and flow mediated dilation (FMD). Ocular vascular function was assessed by means of retinal vascular reactivity to flicker light using dynamic retinal vessel analysis (DVA, IMEDOS, GmbH, Jena, Germany). All patients additionally underwent blood analysis for oxidative stress.

**Results:** When compared to normal controls both POAG and NTG patients exhibited increased nocturnal systolic blood pressure (SBP) variability (p=0.011); systemic arterial stiffness (p=0.015), carotid IMT (p=0.040) and a comparably reduced OPP (p=0.001). Furthermore, both groups of glaucoma patients also exhibited a significantly steeper retinal arterial constriction slope following cessation of flicker (p=0.007) and an increased fluctuation in arterial and venous baseline diameter (p=0.008; p=0.010) when compared to healthy controls. No significant difference in FMD, HRV or oxidative stress parameters were identified between groups.

**Conclusion:** POAG and NTG patients demonstrate multiple comparable alterations in both ocular and systemic vascular function at the early stages of their disease process. This highlights not only the importance of considering vascular factors in both conditions, but also the need to perhaps become less rigid in our separation of the two conditions into distinct clinical entities when considering vascular risk.

## 7.2 Introduction

The separation of OAG into two distinct clinical entities on the basis of IOP has been common practice for many years, with GON in the presence of IOP over 21 mmHg having long been described as POAG [774] and all other cases having been described as either low tension glaucoma (LTG) or NTG [775]. Over recent years however, additional risk factors for both of these forms of OAG have been identified and the concept of POAG and NTG as distinct clinical entities has been questioned. Indeed features more traditionally linked to NTG development, such as ocular and systemic vascular dysfunctions, have been similarly demonstrated in POAG patients [39-43] and the effects of IOP reduction on disease progression has been similarly demonstrated in NTG patients [36-38]. Consequently recent research has recommended the abolishment of the terms POAG and NTG, along with the 'arbitrary' 21mmHg cut off value [34]. Furthermore it has been suggested instead that OAG should be considered a disease continuum in which the aetiology of the disease extends from being predominantly IOP dependent at one end (pure POAG), to predominantly IOP-independent at the other end (pure NTG), with the involvement of vascular factors increasing as the predominance of IOP decreases and a large area of overlap existing between the two extremes [35]. Nevertheless, a number of subtle but important differences have however previously been described between POAG and NTG patients in regard to both their structural and functional ONH changes [31, 44-46] as well as to their vascular risk [47]. Indeed NTG patients show stronger vasospastic tendencies [26], greater systemic endothelial dysfunction [42, 52] and stronger links to the presence of hypotension [53, 54] in comparison to POAG patients. The possibility that these two conditions do in fact represent different clinical entities with different mechanisms of ONH damage can therefore not be ruled out and such a division is currently considered important with regard to both clinical diagnosis and management of the disease. Further investigation is therefore required into, not only the similarities and differences between POAG and NTG, but also into the aetiological role

and interactions of IOP and vascular factors in each condition. In Chapter 5 of this thesis abnormalities in both retinal vascular function and systemic vascular parameters were identified in our newly diagnosed NTG patients, highlighting not only vascular involvement in NTG but also the fact that a glaucomatous eye may represent a 'sick eye in a sick body'. Furthermore in Chapter 6 of this thesis, NTG patients were found to exhibit comparable alterations in retinal vascular function to that of AD patients, which differed in nature to that identified in POAG and AD patients. Expanding on these findings and exploring whether POAG patients exhibit similar or differing vascular abnormalities in comparison to NTG patients across an even broader range of ocular and systemic vascular parameters than used in previous chapters could not only allow the concept of a mixed aetiology and a glaucomatous disease continuum to be explored but could also allow further exploration of the 'sick eye in a sick body concept' in glaucoma. As such this study aims to explore, compare and contrast vascular function at the ocular level and at numerous systemic levels in NTG and POAG patients in comparison to healthy controls. Determining which vascular features, if any, are shared by both POAG and NTG patients and which features, if any, could potentially distinguish the two conditions could prove beneficial for enhancing disease prognosis, understanding and management in the future.

## 7.3 Aims

To compare and contrast the presence of vascular dysfunction at the ocular level in the form of retinal vascular dysfunction and at the systemic level in the form of systemic endothelial dysfunction, arterial stiffness, IMT, HRV, ambulatory blood pressure and the presence of oxidative stress, in POAG and NTG patients in comparison to healthy controls. Furthermore to evaluate the concept of a glaucomatous disease continuum and the concept that glaucoma represents a sick eye in a sick body.

## 7.4 Hypothesis

Evidence of ocular and systemic vascular dysfunction will be evident in both POAG and NTG patients but not healthy controls. The extent of vascular involvement will be more pronounced in NTG patients.

## 7.5 Subjects and Methods

Newly diagnosed and previously untreated NTG patients and POAG patients, along with healthy age matched controls were recruited for this study. The recruitment details, inclusion and exclusion criteria for these patients was detailed in section 3.1. The investigative procedures performed in this study are outlined below and were conducted in accordance with the protocols outlined in section 3.2:

1. Preliminary tests
2. Fasting venous blood sample obtained
3. 24 hour blood pressure and heart rate variability monitor fitted
4. Assessment of retinal vessel reactivity (DVA)
5. Pulse wave analysis
6. Intima-media thickness measurement
7. Assessment of systemic endothelial function (FMD)

## 7.6 Statistical Analysis

All data were reported as mean ± standard deviation. The Kolmogorov-Smirnov test was used to determine the distribution of the data. Multivariate analysis was performed to determine the influence of age, BMI, BP and circulating markers on the measured variables. Differences between groups were subsequently assessed using one-way ANOVA or ANCOVA, as appropriate, followed by Tukey's post hoc analysis. In cases where the normality of the data could not be confirmed log transformations were made. Correlations between the ocular and systemic parameters were explored using either

Pearson's linear correlation or Spearman's rank method as appropriate. P-values of less than 0.05 were considered significant, except in certain cases where a stricter p-value of less than 0.01 was adopted in order to correct for multiple comparisons and minimise bias towards type II errors. All analyses were performed using Satistica, version 6.0, Statsoft, Tusla, OK

## 7.7 Power calculations

With regard to DVA, new measurement parameters and novel analysis methods were used in this study in patient groups which have not previously been examined with these techniques. Power calculation would normally be based on the results of previous studies which share the most similar protocols to that of the present research, however due to the nature of the statistical analysis required in this study, namely one-way ANOVA (or ANCOVA) and the uniqueness of the comparisons being made between the three groups, appropriate previous research was not available. The power calculation for this study was therefore made on the basis of Cohen's standardised effect sizes [771], whereby a large effect size of 0.40 was selected. The calculation was conducted using the computer based programme, GPower 3 [733] and it was revealed that in order to provide a statistical power of 80% at a α level of 0.05 a sample size of 22 per group would be required. The aim was therefore to recruit at least 22 patients per group in this study.

## 7.8 Results

A total of 20 POAG patients, 25 NTG patients and 34 healthy controls were screened for inclusion in the present study however, in order to ensure all participants were matched on critical factors such as age and hypertensive status, all those under the age of 45, over the age of 75 or with a MBP of greater than 115 mmHg had to be excluded from analysis. Additionally, following the careful review of the obtained images, any patients

who exhibited poor or incomplete results were also excluded meaning 19 POAG patients (9 female, 10 male), 19 NTG patients (12 female, 7 male) and 20 healthy age matched controls (9 female, 11 male) were included in the final analysis. These sample sizes fall slightly short of the target of 22 recommended by power calculation however statistical significance was still achieved at a power level of 76%.

### 7.8.1 Baseline values

There were no significant differences in age, systemic BP, BMI, triglycerides, glucose, HDL cholesterol levels, total cholesterol levels and Framingham risk score between the three groups (all $p>0.05$, table 7.1). Furthermore the number of subjects with well controlled high BP was proportionally similar between groups (POAG: n=4; NTG: n=5; Controls: n=6, $p>0.05$). As expected IOP was found to be significantly greater in our POAG patients in comparison to NTG and controls ($p<0.001$, table 7.1). OPP on the other hand was found to be significantly lower in both our POAG and NTG patients in comparison to healthy controls ($p=0.001$, table 7.1).

| | POAG(1) | NTG(2) | Controls(3) | ANOVA P-value | Post-hoc |
|---|---|---|---|---|---|
| **N** | 19 | 19 | 20 | - | - |
| **Gender** | 9F:10M | 11F:8M | 9F:11M | - | - |
| **Age (years)** | 65.26±9.52 | 60.16±12.13 | 60.65±4.20 | 0.174 | - |
| **SBP (mmHg)** | 136.32±15.42 | 130.06±15.37 | 130.00±19.13 | 0.418 | - |
| **DBP (mmHg)** | 79.11±9.80 | 78.39±11.14 | 78.15±10.03 | 0.956 | - |
| **BMI** | 27.71±5.00 | 27.92±4.20 | 26.86±3.79 | 0.757 | - |
| **Glucose** | 4.53±0.97 | 5.08±0.86 | 4.84±1.03 | 0.270 | - |
| **Triglycerides** | 1.22±0.50 | 1.04±0.31 | 1.06±0.31 | 0.320 | - |
| **HDL-C (mmol/L)** | 1.17±0.26 | 1.12±0.29 | 1.18±0.35 | 0.840 | - |
| **Total-C (mmol/L)** | 4.29±0.78 | 4.58±1.04 | 4.76±0.74 | 0.293 | - |
| **IOP (mmHg)** | 23.94±3.00 | 17.40±1.80 | 17.20±2.68 | <0.001* | 1>2,3 |
| **MBP (mmHg)** | 99.29±11.35 | 95.61±10.93 | 96.14±13.09 | 0.594 | - |
| **OPP** | 47.12±16.93 | 46.24±7.84 | 72.77±18.01 | 0.001* | 1,2<3 |
| **Fram Risk Score** | 11.18±5.56 | 9.67±7.50 | 10.22±5.14 | 0.824 | - |
| **MD** | -1.58±0.54 | -2.18±3.22 | - | 0.545 | - |

Table 7.1: Summary of the baseline characteristics of the study groups. P<0.05 is considered a significant difference. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; Total C: Total cholesterol; IOP: Intraocular pressure; MD: mean deviation. The presented SBP, DBP and IOP values are the baseline readings taken on the morning of the study and do not represent the 24 hour or diurnal averages. OPP was subsequently calculated using these baseline values.

### 7.8.2 Ambulatory blood pressure

No significant difference in SBP, DBP or MBP was found between groups across the diurnal, nocturnal or 24 hour measurement periods of this study (p>0.05, table 7.2). Furthermore no significant difference in the percentage nocturnal dip in BP (p>0.05, table 7.2) or in the number of non-dippers (less than 10% nocturnal dip in MBP), physiological dippers (between 10-20% nocturnal dip in MBP) and over dippers (greater than 20% nocturnal dip in MBP) between groups, were found in this study. A significantly higher variability in the nocturnal SBP measurements however was

identified in both our POAG and NTG patients in comparison to healthy controls (p=0.011, table 7.3).

| | POAG(1) | NTG(2) | Controls(3) | ANOVA P-value | Tukeys |
|---|---|---|---|---|---|
| 24hr SBP | 128.00±20.29 | 120.60±11.11 | 119.23±9.38 | 0.241 | - |
| 24 hr DBP | 71.64±11.75 | 67.00±9.49 | 69.23±7.95 | 0.458 | - |
| 24 hr MBP | 90.43±13.63 | 84.87±8.57 | 85.90±8.16 | 0.330 | - |
| DSBP | 133.20±18.86 | 127.19±12.86 | 125.31±11.87 | 0.343 | - |
| DDBP | 77.93±12.49 | 71.69±10.91 | 73.69±9.91 | 0.300 | - |
| DMBP | 96.36±13.66 | 90.19±10.11 | 90.90±10.32 | 0.286 | - |
| NSBP | 115.00±17.34 | 111.71±9.62 | 107.69±7.42 | 0.326 | - |
| NDBP | 62.09±11.31 | 60.07±7.57 | 61.31±6.12 | 0.832 | - |
| NMBP | 79.73±12.74 | 77.29±6.99 | 76.77±6.31 | 0.691 | - |
| Dip (%) | 13.43±9.89 | 13.40±7.10 | 15.04±6.87 | 0.835 | - |

Table 7.2: Ambulatory blood pressure parameters: SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; D- : diurnal; N- : nocturnal. P values <0.05 are considered significant

| Coefficients of variation | POAG(1) | NTG(2) | Controls(3) | ANOVA p-value | Tukeys |
|---|---|---|---|---|---|
| 24 hr SBP (%) | 12.44±2.95 | 12.82±2.31 | 11.99±3.15 | 0.746 | - |
| DSBP (%) | 10.96±2.27 | 11.27±3.10 | 10.02±2.59 | 0.464 | - |
| NSBP (%) | 13.08±2.68 | 12.54±3.77 | 9.12±3.40 | 0.011* | 1,2>3 |

Table 7.3: Coefficient of variation for systolic blood pressure across 24 hours, diurnally and nocturnally. SBP: systolic blood pressure; D- : diurnal-; N- : nocturnal-. P-values <0.05 (*) are considered significant

### 7.8.3 Heart rate variability

No significant differences in LF, HF or LF:HF were found between groups during the diurnal, nocturnal or 24 hour measurement period (all p>0.05, table 7.4)

| | POAG(1) | NTG(2) | Controls(3) | ANOVA P-value | Post-hoc |
|---|---|---|---|---|---|
| 24hr LF | 57.15±18.77 | 63.81±10.01 | 63.30±11.27 | 0.714 | - |
| 24hr HF | 37.46±16.55 | 31.56±7.12 | 32.80±11.82 | 0.640 | - |
| 24hr LF:HF | 2.12±1.63 | 2.18±0.78 | 2.23±0.99 | 0.709 | - |
| DLF | 56.92±18.67 | 63.00±11.25 | 65.20±12.57 | 0.359 | - |
| DHF | 36.67±15.89 | 31.82±8.21 | 28.20±8.07 | 0.360 | - |
| DLF:HF | 2.20±1.97 | 2.17±0.81 | 2.53±1.00 | 0.773 | - |
| NLF | 54.42±19.92 | 65.31±10.06 | 60.67±15.54 | 0.366 | - |
| NHF | 41.08±18.21 | 31.13±7.99 | 36.78±15.17 | 0.298 | - |
| NLF:HF | 1.93±1.61 | 2.34±1.03 | 2.16±1.43 | 0.717 | - |

Table 7.4: Heart rate variability parameters: LF: low frequency; HF: high frequency; LF/HF: low to high frequency ratio; D- : diurnal; N- : nocturnal. P<0.05 is considered a significant difference

### 7.8.4 Pulse wave analysis

Following correction for DBP, identified as an influence factor on multivariate analysis, pulse wave analysis augmentation index (AIx) was found to be significantly greater in both our POAG and NTG patients compared to healthy age matched controls (p=0.015) (Table 7.5).

| | POAG(1) | NTG(2) | Controls(3) | ANCOVA P-value | Post-hoc |
|---|---|---|---|---|---|
| PWA: AIx | 27.88±8.20 | 26.06±11.25 | 16.00±10.66 | 0.015* | 1,2>3 |

Table 7.5: Systemic arterial stiffness. P<0.05 (*) is considered significant. PWA:AIx: pulse wave analysis: augmentation index

### 7.8.5 Intima-media thickness measurement

Following correction for age and BMI, IMT was found to be increased in both our NTG and POAG patients compared to controls (p=0.040, table 7.6).

| | POAG(1) | NTG(2) | Controls(3) | ANCOVA P-value | Tukeys post-hoc test |
|---|---|---|---|---|---|
| IMT (cm) | 0.063±0.014 | 0.064±0.015 | 0.042±0.028 | 0.040* | 1,2>3 |

Table 7.6: Carotid artery intima-media thickness. P<0.05 (*) is considered significant; IMT: intima-media thickness

### 7.8.6 Systemic endothelial function

No significant differences were found between groups with regard to brachial artery FMD or nitroglycerine-mediated dilation (NMD) ($p>0.05$, table 7.7). Furthermore no significant differences in circulating vWF levels were identified between groups ($p>0.05$)

| | POAG(1) | NTG(2) | Controls(3) | ANOVA P-value | Tukeys |
|---|---|---|---|---|---|
| FMD (%) | 6.38±8.66 | 11.01±6.97 | 13.35±8.71 | 0.190 | - |
| NMD (%) | 14.80±7.58 | 25.04±15.61 | 25.07±7.59 | 0.093 | - |
| vWF | 141.03±46.30 | 118.17±63.46 | 107.85±51.20 | 0.414 | - |

Table 7.7: Systemic endothelial function. $P<0.05$ (*) is considered significant. FMD: flow mediated dilation; NMD: nitroglycerine mediated dilation; vWF: von Willebrand factor

### 7.8.7 Dynamic retinal vessel analysis

For ease of interpretation, the dynamic retinal vessel profile curve was 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). The principle results are given based on the average of the 3 flicker cycles with the artery and vein being considered separately. Although each flicker cycle was also analysed individually this analysis did not yield any additional information and has therefore not been presented. The dynamic nature of the retinal vascular response profiles were fully explored using the polynomial fitted curves generated via our novel computational analysis (MatLab R2010a; MathWorks Inc., Natick, MA).

#### 7.8.7.1 Arterial Response

**Dilation**

With regard to the first part of dynamic profile curve, no significant differences were found in the average arterial baseline diameter, maximum diameter (MD%), reaction time (RT) or baseline corrected flicker response (BFR) between all four study groups (all

p>0.01, table 7.8). The arterial baseline diameter fluctuation (BDF) however was found to be significantly greater in both POAG and NTG patients compared to controls (p=0.008, table 7.8). On consideration of the dynamic nature of the arterial dilation response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 7.9).

**Constriction**

With regard to the second part of the arterial profile curve, which looks at the activity of the retinal vessels following dilation and on cessation of flicker, no significant differences in maximum constriction (MC%) or the time taken to reach maximum arterial constriction after dilation (tMC) were found between groups (all p>0.01, table 7.8). On consideration of the dynamic nature of the arterial constriction response however, a significantly steeper arterial constriction was demonstrated in both our POAG and NTG patients in comparison to our healthy controls (p=0.007, table 7.9).

| ARTERY | POAG(1) | NTG(2) | Controls(3) | ANOVA P-value | Tukey's |
|---|---|---|---|---|---|
| BDF | 7.28±3.18 | 7.47±3.84 | 4.55±1.90 | 0.008* | 1,2>3 |
| MD (%) | 4.07±3.24 | 4.80±2.40 | 3.30±1.41 | 0.196 | - |
| RT | 24.93±9.16 | 21.09±8.67 | 18.47±6.35 | 0.061 | - |
| BFR | -0.02±4.61 | -0.04±2.41 | 0.05±2.01 | 0.997 | - |
| MC (%) | -2.72±3.18 | -2.64±1.61 | -1.30±1.43 | 0.101 | - |
| tMC (secs) | 36.55±5.24 | 34.99±8.30 | 33.46±8.52 | 0.463 | |

Table 7.8: Arterial vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). P<0.01 is considered as significant (*).BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; RT: reaction time BFR: baseline corrected flicker response MC(%): percentage constriction below baseline; tMC: time taken to reach maximum constriction.

| DYNAMIC RESPONSE | POAG (1) | NTG (2) | Controls (3) | ANOVA P-value | Tukey's |
|---|---|---|---|---|---|
| **Arteries** | | | | | |
| Slope $_{AD}$ | 0.249±0.402 | 0.300±0.159 | 0.261±0.173 | 0.844 | - |
| Slope $_{AC}$ | -0.257±0.144 | -0.282±0.158 | -0.150±0.075 | 0.007* | 1,2>3 |

Table 7.9: Dynamic characteristics of the arterial vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{AD}$: slope of arterial dilation; Slope $_{AC}$: slope of arterial constriction;

### 7.8.7.2 Venous response

#### Dilation

With regard to the first part of the venous dynamic profile curve, no significant differences were found in the average venous baseline diameter, MD%, RT, or BFR between all three study groups (all p>0.01, table 7.10). The venous BDF however was found to be significantly greater in both our POAG and NTG patients in comparison to our healthy controls (p=0.015, table 7.10). On consideration of the dynamic nature of the venous dilation response however, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 7.11).

#### Constriction

With regard to the second part of the venous dynamic profile curve, which looks at the activity of the retinal vessels following dilation and on cessation of flicker, no significant differences in MC% or the time taken to reach maximum venous constriction after dilation (tMC) were found between groups (all p>0.01, table 7.10). Furthermore, on consideration of the dynamic nature of the venous constriction response, evaluated using our novel matlab analysis, no significant differences were found between groups with regard to slope (p>0.05, table 7.11).

| VEIN | POAG (1) | NTG (2) | Controls (3) | ANOVA P-value | Tukey's |
|---|---|---|---|---|---|
| BDF | 4.84±2.28 | 5.07±2.15 | 3.29±1.41 | 0.010* | 1,2>3 |
| MD (%) | 4.04±1.43 | 4.12±2.32 | 3.99±2.47 | 0.982 | - |
| RT | 20.89±7.98 | 23.47±7.65 | 18.55±2.22 | 0.081 | - |
| BFR | 0.59±1.44 | -0.10±2.62 | 1.79±2.25 | 0.033 | - |
| MC (%) | -1.38±1.57 | -0.85±1.57 | -1.09±1.18 | 0.543 | - |
| tMC (secs) | 39.08±7.93 | 39.02±6.80 | 36.29±9.34 | 0.489 | - |

Table 7.10: Venous vascular function parameters determined using dynamic retinal vessel analysis (DVA, IMEDOS GmbH, Jena, Germany). P<0.01 is considered as significant (*).BDF: baseline diameter fluctuation; MD(%): percentage change in diameter from baseline to maximum; RT: reaction time BFR: baseline corrected flicker response MC(%): percentage constriction below baseline; tMC: time taken to reach maximum constriction

| DYNAMIC RESPONSE | POAG (1) | NTG (2) | Controls (3) | ANOVA P-value | Tukey's |
|---|---|---|---|---|---|
| **Veins** | | | | | |
| Slope $_{VD}$ | 0.261±0.200 | 0.219±0.141 | 0.225±0.140 | 0.705 | - |
| Slope $_{VC}$ | -0.161±0.094 | -0.160±0.10 | -0.167±0.133 | 0.982 | - |

Table 7.11: Dynamic characteristics of the arterial vascular response profiles determined using our novel computational model. P< 0.05 (*) is considered significant. Slope $_{VD}$: slope of venous dilation; Slope $_{VC}$: slope of venous constriction;

### 7.8.8 Oxidative stress markers

No significant differences in GSH, GSSG or total-GSH levels were found between groups in this study. Furthermore the redox balance (ratio of GSH:GSSG) was also found to be comparable between groups (all p>0.05, table 7.12)

| | POAG(1) | NTG(2) | Controls(3) | ANCOVA P-value | Tukey's |
|---|---|---|---|---|---|
| **GSSG** | 38.98±21.99 | 37.59±23.88 | 61.89±46.34 | 0.169 | - |
| **GSH** | 574.89±217.27 | 623.09±269.65 | 687.67±393.90 | 0.644 | - |
| **tGSH** | 652.85±247.44 | 698.26±280.42 | 836.41±448.38 | 0.361 | - |
| **GSH:GSSG** | 17.34±7.12 | 20.43±10.81 | 13.93±8.53 | 0.159 | - |

Table 7.12: Oxidative stress analysis. GSSG: oxidised glutathione, GSH: reduced glutathione; tGSH: total glutathione; GSH:GSSG: redox balance. Calculated using ANCOVA, correcting for SBP, DBP and BMI.

### 7.8.9 Correlations

A significant positive correlation was identified between nocturnal SBP variability and right IMT in POAG patients only (R= 0.74, p<0.001, figure 7.1) and between nocturnal SBP variability and retinal arterial BDF in NTG patients only (R=0.71, p=0.001, figure 7.2). No other correlations between our systemic and ocular parameters could be found.

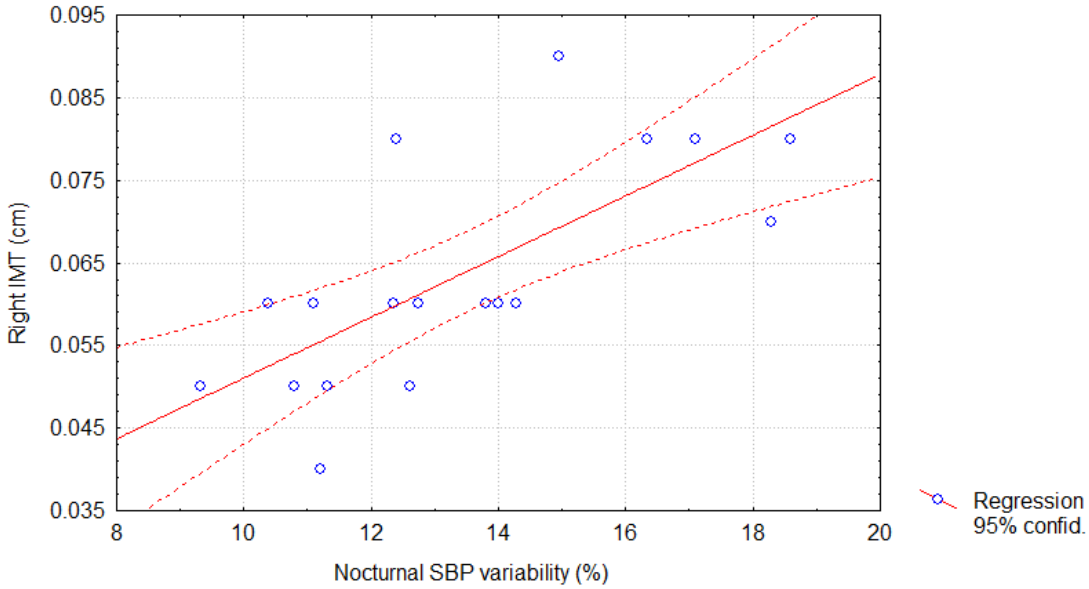



**Figure 7.1 Nocturnal systolic blood pressure variability vs. intima-media thickness in POAG patients**

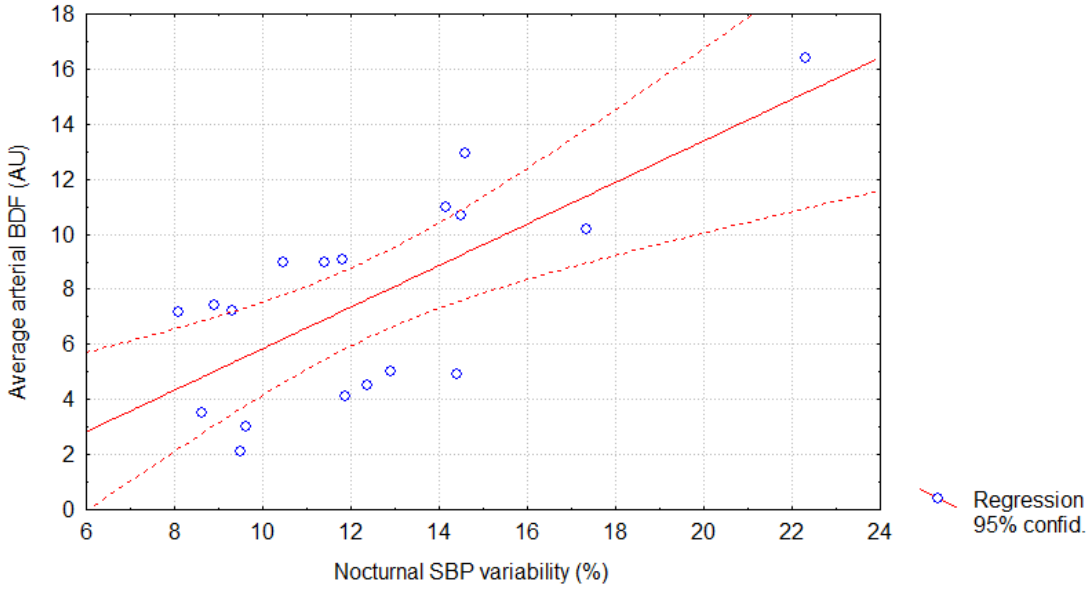



**Figure 7.2. Nocturnal systolic blood pressure variability vs. retinal arterial baseline diameter fluctuation in NTG patients**

## 7.9 Discussion

### 7.9.1 Main findings

This study reveals evidence of systemic and ocular vascular abnormalities which are comparable in both newly diagnosed and previously untreated POAG and NTG patients and are not replicated by healthy age matched controls. At the systemic level ambulatory BP abnormalities, in the form of increased variability in nocturnal SBP, along with comparably reduced OPP, increased systemic arterial stiffness and increased IMT were identified in both groups of glaucoma patients. Furthermore, at the ocular level, signs of retinal vascular dysfunction, in the form of a significantly steeper retinal arterial constriction slope following cessation of flicker and an increased fluctuation in arterial and venous baseline diameter, was identified in both our POAG and NTG patients and was again not replicated in the healthy control group. No significant differences in HRV, systemic endothelial dysfunction or glutathione levels were found between groups.

### 7.9.2 Systemic vascular parameters

#### 7.9.2.1 Ambulatory blood pressure assessment

Systemic BP follows a normal circadian rhythm and is constantly regulated by the ANS through modifications in cardiac output and total peripheral resistance [408]. At the ocular level a close relationship exists between BP, IOP and OPP. As such the evaluation of ambulatory BP in patients with or at risk of ocular diseases such as glaucoma can not only give an insight into the presence of modifiable cardiovascular risk factors such as abnormal BP, but can also give an indication of the presence of local risk for ischemia. In the present study no significant differences in the average diurnal and nocturnal blood pressure parameters (SBP, DBP, MBP), or the nocturnal dipping status, were found between POAG, NTG and healthy control subjects following 24 hour BP assessment. This finding is consistent with that of a number of other studies that have assessed 24

hour BP in both newly diagnosed and more established glaucoma patients and have found no significant differences between groups [43, 414, 709, 776]. Indeed, in retrospect, such a comparable finding may not be surprising with regard to this study as very strict inclusion criteria was imposed on participants and there was a necessity for the groups to be not only age matched but also disease matched. It cannot be denied however that many other studies have been able to identify associations between abnormal systemic BP and the presence of GON. Indeed, whilst the evidence surrounding hypertension has a tendency to be somewhat variable and inconsistent [307, 441-443, 449], the presence of hypotension, in particular nocturnal hypotension, has been more widely linked to the occurrence and progression of glaucoma [24], especially NTG[54, 130, 459-462], as has the presence of large nocturnal dips in BP (>20%)[313, 433, 450, 463-466]. An increased variability/fluctuation in nocturnal SBP, as well as a reduced OPP at the time of testing, was however identified in both our POAG and NTG patients in comparison to healthy controls. Increased variability in BP is a parameter widely recognised in cardiovascular and hypertension research as a signal of increased risk for end organ damage [777] and over recent years its occurrence has also been increasingly considered in regard to other vascular diseases, including glaucoma [53, 467, 468]. Indeed, in line with our findings here, previous studies have also identified increased nocturnal variability of BP in both NTG patients [53] and those with focal ischemic glaucoma [460], as well as in those with progressive NTG [451] following 24 hour ABPM and in those with POAG during the daytime using a slightly different technique [778]. Whilst an association does therefore appear to exist between increased BP variability and GON, the exact mechanisms by which such increased variability, over the nocturnal period in particular, may relate to the development of GON is currently unclear. It could be hypothesised that, due to the close relationship between BP and OPP, an increased variability in BP may subsequently lead to an increased variability or fluctuation in OPP, which may have particular effect nocturnally when OPP is already physiologically reduced and the ONH is more vulnerable. Indeed strong links have previously been made between the development of

GON, nocturnal hypotension [24, 433, 450] and fluctuating or reduced OPP [15, 779-781], in that the risk of ischemia/reperfusion injury at the ONH and the subsequent risk of GON development is thought to be increased in the presence of these features, particularly if occurring in combination with autoregulatory dysfunction [141]. In order to validate this hypothesis calculation of 24 hour OPP would ideally be needed; however in this study this information is not available as 24 hour IOP measurements were not taken. At the time of assessment however, OPP was found to be significantly reduced in both our POAG and NTG patients in comparison to healthy controls suggesting perfusion related vascular alterations are likely to be playing a part in the pathogenesis of both conditions. The relative contribution of IOP to this reduced OPP however is likely to vary between our glaucoma groups and as such consideration of the 24 hour IOP and its fluctuations, which themselves have been previously linked to GON development [129, 130], would also be beneficial.

#### 7.9.2.2 Arterial stiffness, IMT and systemic endothelial function

In conjunction with increased nocturnal SBP variability, a comparably increased systemic arterial stiffness and carotid artery IMT was identified in both our POAG and NTG patients in comparison to our healthy controls. Arterial stiffness is considered an independent predictor of cardiovascular disease [488] and increased IMT has been suggested as an indirect measure of generalised atherosclerosis and cardiovascular risk [782]. The presence of cardiovascular disease and structural vascular wall changes has been variably linked to the presence of GON over recent years, with a number of studies having identified strong associations in both POAG [307, 481, 482] and NTG patients [480, 483] individually and a number of further studies having revealed no such associations [318, 484, 485, 487]. The variability in results between these previous studies could in part be accounted for by differences in the inclusion/exclusion of patients suffering from already diagnosed systemic vascular disease, especially as arterial stiffness is a known

measure of vascular function [759]. As most glaucoma patients seen in day-to-day practice suffer from a large variety of vascular pathologies, a careful selection of only those free from such diseases could bias results towards very rare occurrences. Consequently, the present study included POAG and NTG patients with well controlled hypertension, along with a similar number of subjects with such status in the control group. All groups were additionally matched on age and functional loss. In light of this and on the basis of our findings here, it could be hypothesised that systemic vascular wall changes may in some way contribute to, or signal a higher risk for, the development of GON in the early stages of the disease, regardless of the level of IOP. The mechanisms surrounding these associations however are currently unclear but could perhaps relate to the development of endothelial dysfunction or increased MABP, both of which have been widely linked to the presence of arterial stiffness and atherosclerosis [488] as well as to the presence of vascular dysfunction and the development of GON [198, 488]. On exploration however, no direct correlations could be found in this study between arterial stiffness, IMT and systemic endothelial function, however as systemic endothelial dysfunction, measured by FMD, was actually found to be comparable between groups here this finding may not be surprising. Interestingly however a significant positive correlation between nocturnal SBP variability and IMT was identified in POAG patients in this study. Such a relationship between these two parameters has also previously been reported in cardiovascular research [783, 784] and is thought to relate to the increased stress on the vascular wall imposed by increased fluctuations in BP which can result in structural alterations in the medial layer of the carotid artery [783]. This finding provides support for the concept of a generalised systemic vascular dysfunction in glaucoma patients, however no such correlations were found in our NTG patients perhaps suggesting different mechanisms may be occurring here. Further investigation would be required to clarify this issue.

#### 7.9.2.3 Heart rate variability (HRV) and the Autonomic nervous system (ANS)

The ANS supervises and influences the hemodynamic situation of the body through its constant regulation of HR and BP and plays a vital role in blood flow physiology [399]. Through the assessment of HRV an indirect measure of the autonomic control of the heart and hence the state of the ANS can be gained. In the presence of ANS dysfunction blood perfusion can be either altered or impaired in multiple vascular beds throughout the body, including in the eye. Its assessment and detection in those with ocular diseases with known vascular associations, such as glaucoma, is therefore of significant interest. No significant differences in ANS parameters (LF, HF, LF:HF) where identified between POAG, NTG and healthy controls in this study following 24 hour HRV assessment. Previous studies, using similar techniques, have however been able to identify HRV alterations suggestive of a high sympathetic tone in both newly diagnosed POAG patients [43] and NTG patients [413], but those findings could not be replicated here. Stricter inclusion criteria with regard to cardiovascular disease were imposed on this study compared to that of previous studies, which could perhaps explain these findings, however before any firm conclusions can be drawn further investigation into the role of ANS in the development and progression of GON is required. Intriguingly there is evidence to suggest that the extent of any ANS dysfunction increases with glaucoma severity and progression, particularly in NTG patients [413, 414], the possibility that the ANS may become involved later in the disease process of our newly diagnosed glaucoma patients can therefore not be ruled out, however again further follow up would be required to confirm this.

Interesting links have previously been made in cardiovascular research between the presence of ANS dysfunction and the presence of endothelial dysfunction in regard to the abnormal regulation of vascular tone [785, 786], whereby a dysfunction in one system has been suggested to drive a dysfunction in the other. No such research however has

previously been conducted in glaucoma patients, although both parameters have been individually linked to the disease. In the present study, across all three study groups, no correlations were found between our ANS parameters (LF, HF and LF:HF) and our systemic endothelial function parameters (FMD), which themselves were all found to be comparable between groups. A larger scale study, perhaps looking at patients with varying degrees of, or progressive, POAG and NTG may help to explore the potential relationship between the ANS and the endothelium further and determine whether those associations identified in cardiovascular disease are evident in glaucoma patients at any stage of the disease process. Such a discovery would contribute towards an enhanced understanding of the pathological process involved in the development of GON.

### 7.9.3 Retinal vascular function

With regard to retinal vascular function both our POAG and NTG patients demonstrated a comparably steeper retinal arterial constriction slope following the cessation of flicker, as well as an increased arterial and venous baseline diameter fluctuation (BDF) in comparison to healthy controls. Slope is an important parameter which allows an evaluation of the dynamic nature of the vascular constriction profile and is influenced not only by the percentage constriction in vessel diameter below baseline following cessation of flicker but also by the time scale across which this happens. As discussed in chapter 5 the relevance of such a steeper arterial constriction slope in glaucoma patients is currently unclear as very few previous studies looking at vascular reaction to flickering light have investigated the constriction responses in detail. Nevertheless, due to the links which have previously been hypothesised between an increased overshoot in vessel diameter below baseline following cessation of flicker, a parameter known to influence constriction slope, and the presence of PVD syndrome [746], as well as between altered astrocyte activity and re-establishment of vasomotor tone [764, 765], it is not unreasonable to hypothesise that the steeper retinal arterial constriction slope identified

here signals the presence of retinal vascular dysfunction in both our POAG and NTG patients. It is interesting that a steeper arterial constriction slope was identified in both groups of glaucoma patients in comparison to controls, especially as some of the conditions to which we have theoretically linked this finding, namely PVD and raised ET-1 levels, have been more frequently related to NTG patients in the literature [26, 356]. As such it could be hypothesised that similar conditions to those more commonly exhibited in our NTG patients are also evident in the early stages of POAG, however in order to determine whether this is in fact the case additional information, for example on the presence of PVD symptoms, such as cold hands and feet, exploration of nail fold capillary perfusion and determination of ET-1 levels in both groups of patients would be required.

In addition to the steeper retinal arterial constriction slope, comparably increased fluctuations in baseline arterial and venous diameter, prior to the onset of flicker were also identified in both our POAG and NTG patients in comparison to healthy controls. BDF, as discussed in previous chapters, is a parameter, which although recognised is not commonly reported in the literature. Its consideration was first recommended by Nagel et al [686] as a way of taking into account the effect of spontaneous variations in vessel diameter, which occur under normal resting conditions, on the observed response of the vasculature to flicker light, however it is a parameter which is not commonly reported in the literature. As such the relevance of these findings with regard to GON development is currently unclear. Tentative links have been made in previous studies between the occurrence of increased arterial diameter fluctuations and the presence of vascular disturbance in both smokers and vasospastic subjects [695, 746-748] and between increased retinal venous diameters and the presence of retinal ischemia and hypoxia [749, 751]. On this basis it could be hypothesised that the increased BDF identified in both our POAG and NTG patients in this study is a further indicator that common alterations in retinal vascular function may exist in these individuals, perhaps

related to an increased variation in vascular tone or rigidity or the presence of ischemic and hypoxic changes. Interestingly a significantly positive correlation was found between arterial BDF and nocturnal BP variability in our NTG patients only. Variations in BP are known to influence baseline retinal vessel diameter even under normal resting conditions and this correlation confirms that in NTG patients in particular this influence is significant and may require consideration. Indeed on correcting for the effect of nocturnal BP variability on arterial BDF using ANCOVA the significant difference between NTG patients and controls is lost however the significance still remains between POAG patients and healthy controls. This not only highlights the importance of BP variability in NTG but also perhaps suggests that other factors separate to BP fluctuation may be affecting the stability of the arterial baseline diameter in POAG patients.

### 7.9.4 Oxidative stress

Oxidative stress has previously been implicated in the pathophysiology of both POAG and NTG. In this study however no significant differences in the circulating levels of the antioxidant glutathione could be found between groups (GSH, GSSG, GSH:GSSG). Such a direct comparison of oxidative stress status between POAG, NTG and controls has not previously been made, however there a number of studies which have been able to demonstrate evidence of oxidative stress in these glaucoma groups individually, in the form of increased DNA breaks [524], altered ET-1 and proteosome activity [386, 526] and decreased antioxidant activity [527, 528]. Of particular relevance is a study by Gherghel et al [787] which was able to identify low plasma levels of GSH and t-GSH in newly diagnosed POAG patients, suggestive of an increased oxidative burden. The patients included in this above study were slightly older than that of the present study which, due to the strong links between oxidative stress and age, could partly explain the difference in results; however it is difficult to draw any firm conclusions. Indeed, although no

significant differences in circulating glutathione levels were identified between groups in this study, the possibility that evidence of oxidative stress may be present in other forms in our POAG and NTG patients or be present in amounts which are not yet detectable systemically, cannot be ruled out. Further investigation may therefore be required in order to determine the exact associations between GON and oxidative stress.

### 7.9.5 POAG vs. NTG

The aim of this study was to compare and contrast the presence of vascular dysfunction at the ocular level and multiple systemic levels in POAG and NTG patients and to evaluate the validity of the concept that glaucoma may exist as a disease continuum and represent a sick eye in a sick body. This is the first time that direct comparisons such as these have been made between POAG and NTG patients across so many ocular and systemic parameters simultaneously, making it difficult to directly compare our findings to that of previous studies. Nevertheless we have identified evidence of altered retinal vascular reactivity, altered ocular perfusion, altered nocturnal BP variability and signs of systemic vascular pathology in both POAG and NTG patients, which are comparable and not replicated by healthy controls. Such findings suggest that a considerable overlap may in fact exist in the aetiology of both POAG and NTG, especially in the early stages of the disease. The finding of so many comparable parameters between our two groups of newly diagnosed glaucoma patients is perhaps somewhat surprising, especially as, on the basis of previous research, it was hypothesised that the evidence of vascular dysfunction would be more pronounced in our NTG patients. The relative influence of IOP on the results obtained in this study is difficult to determine and the possibility that the mechanisms by which these multiple vascular parameters become similarly altered could still vary between glaucoma groups cannot be ruled out. Regardless of this it does appear from our findings that the consideration of OAG as a disease continuum as opposed to as two separate clinical entities may well be a useful

and valid concept. Indeed we have clearly highlighted the need for consideration of vascular parameters in both POAG and NTG and have blurred the margins between these two forms of OAG.

An additional consideration of this study was to determine whether any further support for the concept that glaucoma should be considered a sick eye in a sick body could be found. Interestingly, in addition to the finding of altered ocular and systemic vascular parameters in our POAG and NTG patients, correlations were also found between increased nocturnal BP variability and both IMT at the systemic level and retinal artery baseline fluctuation at the ocular level. These findings highlight the possibility that a generalised vascular function, in which abnormalities at one level are influencing or being influenced by abnormalities at another level, could be present in glaucoma patients and that glaucoma, may indeed represent a sick eye in a sick body. With this in mind it could be broadly hypothesised that the increased nocturnal BP variability detected in this study, could precipitate, through the imposition of increased stress on the arterial wall, an increase in carotid artery IMT and an alteration in the functioning and reactivity of the retinal vessels in our glaucoma patients, contributing to both an increased cardiovascular risk and the presence of unstable ocular perfusion. Additional alterations in retinal microvascular regulation and astrocyte activity in these patients could then further contribute to these disruptions in retinal vascular reactivity and to the instability of ocular perfusion. The origins of the increased BP variability itself could theoretically be linked to increased arterial stiffness, the presence of which has previously been suggested to interfere with the buffering of BP alterations through reducing the compliance of the vascular wall [788]. Indeed increased systemic arterial stiffness was also identified in our glaucoma patients and although not measured here, increased arterial stiffness at the ocular level has also previously been reported in NTG patients by other studies [295]. If these factors were to combine as hypothesised and create an unstable ocular perfusion, the mechanisms by which they may then go on to

produce GON is currently uncertain, however it would not be unreasonable to hypothesise the involvement of recurrent ischemia/reperfusion injury. Interestingly however no differences in oxidative stress, a key component of ischemia/reperfusion injury or in ANS activity and systemic endothelial function, both of which are commonly linked to such vascular alterations, were found between groups in this study. Further investigation may therefore be required to elicit the exact role of these parameters in the aetiology of GON and to expand on the above mentioned hypothesis of generalised vascular dysfunction in glaucoma. Indeed it is possible that these parameters may become altered at a later stage of the disease process or may be already altered at a degree not yet detectable at the investigated level, for example in the microvasculature rather than the macrovasculature with regard to endothelial dysfunction in particular. As such their involvement although unclear cannot be ruled out completely.

## 7.10 Conclusion

In conclusion this study has demonstrated multiple comparable alterations in both ocular and systemic vascular function between POAG and NTG patients, which not only highlights the importance of considering vascular factors in the aetiology and treatment of both conditions, but also highlights the need to become less rigid in our separation of the two conditions into distinct clinical entities when considering vascular risk. Furthermore additional support for the concept that a generalised vascular dysfunction exists in glaucoma patients and that glaucoma should be considered a sick eye in a sick body has been provided.

## 7.11 Limitations

One factor not explored in this study was the precise nature of the structural ONH changes between our glaucoma groups, so it is unclear whether differences were present in this regard. Furthermore, considering the presented mean IOP values for

each group (table 7.1), it is possible that a certain degree of overlap may exist between the reported glaucoma categories, which could perhaps account for the large number of comparable findings between the POAG and NTG patients in this study. The categorising of patients into groups in this study based on clinical IOP levels above or below 21mmHg is however reflective of current practice. It would be interesting to determine through further research whether the similarities between POAG and NTG identified here remain consistent as the disease develops or whether they become less comparable with progression and also whether they remain in POAG patients with higher IOP levels (26 mmHg or over). This would be important not only from an aetiological point of view but also clinically as different treatment methods may be required at later stages of the disease process.

# 8. Summary and Conclusions

## 8.1 Summary

Evaluating the importance of ocular and systemic vascular risk factors in the aetiology of both glaucoma and AD has been a research area of interest for some time and the scientific literature relating to this has been reviewed in Chapter 1 of this thesis. It is still unclear however how hemodynamic disturbances, affecting multiple vascular beds, may interrelate to cause neuronal degeneration at the ocular and/or cerebral levels in glaucoma and AD and how valid the assessment of vascular function at the ocular level may be as an indicator of dysfunction at the cerebral level. Elucidating or validating these relationships could not only enhance our aetiological understanding of ocular and cerebral neurodegenerative disease but could also potentially open up new diagnostic or therapeutic avenues for both glaucoma and AD. As such this thesis has been concerned with investigating the presence, impact and interactions of ocular and systemic vascular alterations in POAG, NTG and AD patients and how they may relate to the pathogenesis of these neurodegenerative diseases. Furthermore this thesis has explored the concept of using the eye as a window to the brain in AD patients as well the validity of POAG and NTG as distinct clinical entities and the validity of considering glaucoma as a 'sick eye in a sick body'.

In summary the findings of this work were:

### 8.1.1 Is the eye a window to the brain? Ocular and systemic vascular dysfunction in Alzheimer's disease

Cerebral vascular dysregulation has previously been suggested to play a role in the development and progression of AD however the direct assessment of the cerebral vasculature is notoriously difficult. Furthermore questions still remain around exactly

how alterations in vascular function may lead to neuronal degeneration in AD. The aim of this study was therefore to determine for the first time whether vascular dysregulation could be detected at the ocular level in mild newly diagnosed AD patients using novel analysis methods and whether the degree of any dysregulation discovered could relate to either the degree of cognitive impairment or the presence of systemic vascular dysfunction in these patients. Following dynamic retinal vascular analysis, alterations in the retinal arterial response to flicker light, in the form of an increased arterial RT on two out of the three occasions in which the vessels were stimulated, along with alterations in baseline venous diameter, were detected in our mild AD patients. Furthermore this alteration in retinal arterial RT was found to correlate positively with degree of cognitive impairment in these patients. No significant differences in systemic endothelial function were however identified between groups. The exact cause of the retinal vascular alterations identified in this study can only be hypothesised at this point but nevertheless they provide support for a vascular aetiology in AD and, through the identification of retinal vascular dysfunction in mild AD patients for the first time, highlight the involvement of the ocular circulation in the disease process. Furthermore this study firmly introduces the idea that the functioning of the ocular circulation, as assessed by DVA, may be a useful marker for determining cognitive prognosis in AD patients and has provided a good basis for further exploration into vascular dysfunction in AD.

### 8.1.2 Ocular and systemic vascular abnormalities in newly diagnosed normal tension glaucoma patients

NTG has been widely linked to the presence of ocular and systemic vascular alterations however very few studies have simultaneously explored and directly compared the presence of ocular and systemic vascular alterations in these patients and many questions still remain around exactly how these alterations may relate to RGC loss and GON development. As such the aim of this study was to investigate the presence of

cardiovascular risk factors and alterations in vascular function at both the ocular and systemic level in NTG patients, using novel investigative techniques, and to evaluate the concept that glaucoma may be considered a ‘sick eye in a sick body’. It was hypothesised that, on the back of recent evidence highlighting the involvement of both the ocular and systemic circulations in NTG, our newly diagnosed and previously untreated NTG patients would demonstrate altered retinal vascular reactivity to flicker light in conjunction with signs of systemic vascular dysfunction. On investigation our NTG patients were indeed found to exhibit increased systemic arterial stiffness and carotid artery IMT in conjunction with alterations in retinal arterial reactivity to flicker light stimulation, in the form of an altered dynamic response profile and a steeper constriction slope following cessation of flicker. No direct correlations however could be found between the systemic and ocular vascular parameters explored in this study, and furthermore no significant differences in systemic endothelial function could be identified between groups. These findings indicate that subclinical signs of systemic vascular disease and disturbances in ocular vascular reactivity appear to be present in NTG patients at the earliest stages of the disease process and could potentially be contributing to the disease aetiology. The importance of considering systemic vascular factors in the diagnosis and management of NTG has therefore been highlighted and, furthermore, through the utilisation of novel analysis methods in conjunction with DVA assessment, a contribution to the aetiological understanding of vascular dysfunction at the ocular level in NTG has also been made. Additionally, although not conclusive, this research goes someway to reaffirming the concept that glaucoma may be considered ‘a sick eye in a sick body’. Further exploration into this concept, using a wider range of systemic investigation techniques, was subsequently conducted in chapter 7 of this thesis.

### 8.1.3 Ocular Vascular Dysregulation in AD compares to both POAG and NTG

Having established the presence of retinal vascular dysfunction in both AD and NTG patients individually, an investigation into whether the nature of this vascular dysfunction is comparable between these groups of patients was the next logical step. A number of associations have previously been made between the presence and aetiology of these two neurodegenerative diseases; however the exact nature of their relationship is still uncertain. As such the aim of this study was to investigate for the first time, using novel techniques, the coexistence of vascular alterations at both the ocular and systemic level, in AD and both POAG and NTG patients, with the hypothesis that, on the back of recent evidence highlighting the potential aetiological similarities between these conditions, comparable alterations in vascular function would be found. On investigation our mild newly diagnosed AD patients were found to demonstrate an altered retinal arterial vessel reactivity to flicker light which was comparable to that of our POAG patients and an alteration in baseline venous reactivity which was comparable to that of our NTG patients; however no differences in systemic endothelial function were identified between groups. These findings go some way to provide support for the concept of a common underlying microvascular aetiology in AD and glaucoma and highlight the need to consider ocular health in AD and cognitive health in glaucoma. Interestingly, the similarities in the nature of the retinal vascular alterations identified in our AD and glaucoma patients appear to differ depending on whether POAG or NTG is being considered, this is a novel finding which would benefit from further investigation in order to clarify its significance. It also provokes questions about the similarities and differences which may exist in ocular and systemic vascular dysfunction between POAG and NTG patients themselves, which was subsequently explored in the following chapter.

### 8.1.4 Primary open angle glaucoma and Normal tension glaucoma: two separate diseases or one continuous entity? The vascular perspective

Although more traditionally associated with NTG, vascular alterations at both the ocular and systemic level have also previously been identified in POAG patients and in a similar manner the benefits of IOP reduction, more traditionally linked with POAG, have also been demonstrated in NTG patients. Overlaps such as these has lead to the questioning of whether these two forms of glaucoma should still be considered as distinct clinical entities or whether glaucoma may in fact represent a disease continuum with no distinct borders of separation. As such the aim of this study was to compare and contrast the presence of ocular and systemic vascular alterations at multiple levels in both newly diagnosed and previously untreated POAG and NTG patients and to not only evaluate the validity of these two conditions as distinct clinical entities but to also further explore the concept that glaucoma may represent a 'sick eye in a sick body'. It was hypothesised that, on the back of recent evidence, ocular and systemic vascular dysfunction would be present in both POAG and NTG patients but the extent of this vascular involvement would be greater in NTG patients. On investigation however comparative alterations in retinal vessel reactivity, OPP, nocturnal SBP variability and both systemic arterial stiffness and carotid IMT were found in our POAG and NTG patients. Furthermore significant correlations between systemic BP variability, IMT and retinal arterial baseline diameter fluctuation were found across the glaucoma groups. No significant differences in HRV, systemic endothelial function or oxidative stress parameters were however found between groups. This is the first time that direct comparisons between so many ocular and systemic parameters have been made in POAG and NTG patients. The inter-relationships identified between the investigated ocular and systemic parameters certainly provide support for the concept of a generalised vascular dysfunction in glaucoma and for the concept that glaucoma may

represent a ‘sick eye in a sick body’. Furthermore, although the finding of so many comparable vascular alterations between both POAG and NTG patients was perhaps surprising, it highlights the importance of considering the role of vascular factors in both forms of glaucoma and goes some to way to supporting the concept that glaucoma should be considered as a disease continuum rather than a disease with distinct clinical forms.

## 8.2 Conclusions

The aims of this work were:

### 8.2.1 To investigate the presence and impact of ocular and systemic vascular alterations in AD and to explore the concept of using the ‘eye as a window to the brain’

The findings of this work were:

- An altered retinal vascular response to flicker light stimulation, indicative of ocular vascular dysfunction, was identified on DVA analysis in mild newly diagnosed AD patients
- The degree of this retinal vascular dysfunction was found to correlate with the degree of cognitive impairment in these AD patients
- No significant differences in systemic endothelial dysfunction were identified between groups
- Support for the concept of using the ‘eye as a window to the brain’ for the diagnosis and screening of cognitive impairment was provided

### 8.2.2 To investigate the presence and impact of ocular and systemic vascular alterations in NTG

The findings of this work were:

- An altered retinal arterial vascular constriction response following flicker light stimulation, indicative of ocular vascular dysfunction, was identified on DVA analysis in newly diagnosed NTG patients
- Signs of subclinical vascular pathology in the form of increased systemic arterial stiffness and carotid artery IMT were also identified in these NTG patients
- No significant differences in systemic endothelial function were identified between groups

### 8.2.3 To investigate the possibility of a shared vascular aetiology, involving both the ocular and systemic circulations, in AD and both POAG and NTG.

The findings of this work were:

- AD and POAG patients demonstrated a common alteration in retinal arterial function in the form of a prolonged reaction time to flickering light
- AD and NTG patients demonstrated a common alteration in retinal venous function in the form of an increased baseline fluctuation in venous diameter
- No significant differences in systemic endothelial function were identified between groups
- The possibility that AD and glaucoma may share a common underlying microvascular aetiology was highlighted, along with possibility that the nature of this shared aetiology may differ between POAG and NTG patients

### 8.2.4 To compare and contrast vascular alterations at both the ocular and systemic level in POAG and NTG and to explore their validity as distinct clinical entities

The findings of this work were:

- POAG and NTG patients demonstrated comparable alterations in nocturnal SBP variability, OPP, retinal vascular reactivity, systemic arterial stiffness and carotid artery IMT
- Correlations were identified between nocturnal SBP variability and both retinal artery baseline diameter fluctuation and carotid IMT, highlighting the possibility that a generalised vascular dysfunction, affecting multiple vascular beds may be present in OAG patients and providing support for the concept that glaucoma may be considered a 'sick eye in a sick body'
- On the back of these finding the validity of POAG and NTG as distinct clinical entities can be questioned and the concept that glaucoma may represent a 'sick eye in a sick body' can be somewhat reaffirmed.

## 8.3 Overall Limitations

The studies outlined in this thesis are subject to a number of potential limitations. Firstly, only a small sample of AD patients and a moderate sample of glaucoma patients could be recruited. This, along with the cross-sectional design, potentially limits the conclusions which can be drawn from the presented results as well as the statistical power of the analysis. The reason for this limited sample of patients was the very strict patient inclusion/exclusion criteria, which were necessary to avoid any possible unwanted influences on the measured vascular parameters, but unavoidably limited the number of suitable study patients that could be recruited.

Secondly, with regard to the glaucoma patients, objective data such as the precise nature of the structural ONH changes and VF defects was not recorded and used in analysis. Such parameters were however considered by the clinicians in the diagnosis of POAG and NTG and were used indirectly to group the POAG and NTG patients. Inclusion of such objective data could have potentially allowed clearer distinctions between the glaucoma groups involved in this study to be made and enhanced the interpretation of results.

Finally multiple parameters were assessed and analysed simultaneously in AD, glaucoma and healthy control patients in this thesis. There is a risk when comparing multiple parameters that the rate of type 1 errors (false positives) will be increased. On statistical advice, a correction for multiple comparisons was therefore made in this thesis when comparing multiple sequential parameters on DVA analysis, whereby the p value for significance was reduced from p=0.05 to p=0.01. As the majority of other investigative techniques included in this thesis had only single output parameters a correction for multiple comparisons was not deemed necessary in those cases. It is however still possible that an increased family-wise error rate could have influenced the significant findings of this thesis, especially when reviewing the results of all of the investigative techniques as a whole. Significant parameters where the p-value is close to the 0.05 boundary should therefore be considered with caution.

## 8.4 Clinical implications

### 8.4.1 Implications with regard to AD

The need to consider the impact of vascular dysregulation and the role of vascular risk factors in the aetiology, diagnosis and management of AD has been highlighted. Furthermore the potential for utilising the easily accessible retinal vasculature to gain an insight into cerebral vascular alterations and/or cognitive risk, via DVA analysis, has been demonstrated. Finally the need to consider ocular health in AD patients, in the form of both POAG and NTG, has also been emphasised. On the basis of these findings and despite the need for further research and expansion of the study, it is recommended that the evaluation and assessment of ocular vascular function, ocular health and the presence of vascular risk factors should be considered in both the diagnosis and monitoring of AD, particularly in the early and pre-clinical stages of the disease.

### 8.4.2 Implications with regard to glaucoma

The potential role of both ocular and systemic vascular factors in the aetiology of POAG and NTG has been highlighted. Furthermore the coexistence of vascular alterations at multiple sites throughout the body and the impact of these alterations on the ocular circulation have been demonstrated in glaucoma patients. As such the extensive screening and assessment of both ocular vascular function and systemic vascular risk factors, to include 24 hour ABPM and arterial stiffness assessment, is recommended in the routine diagnosis and management of both of these groups of glaucoma patients and may be particularly relevant in cases where the disease is progressing.

### 8.4.3 Implications with regard to assessment of ocular vascular function

A number of different ocular and systemic vascular techniques were conducted in the thesis. With regard to ocular vascular function and DVA in particular, through the utilisation of SDRA and the introduction of our novel Matlab imaging analysis, the dynamic nature of the retinal vascular response profile to flicker light was able to been explored fully for the first time. The importance of considering the full vascular profile, including constriction response, slope and reaction times was highlighted by the significant differences found in these parameters in this thesis, which would otherwise have been missed. It is therefore recommended that such analysis should be conducted in all future studies utilising DVA.

## 8.5 Future Directions

A number of new questions have arisen from the data presented in the major chapters of this thesis from which the following avenues of future research are worth highlighting in particular:

### 8.5.1 Expansion of preliminary data

Despite statistically significant results being demonstrated in this thesis with the sample sizes obtained, expansion of the four main presented studies to include a larger cohort of patients would enhance the validity and application of our findings. Furthermore expansion of our blood analyses to include the endothelial marker ET-1 could offer additional information on the state of the vascular endothelium and vessel tonus, which could in turn offer additional information of both aetiological and clinical relevance with regard to neurodegenerative disease.

### 8.5.2 Assessment of retinal vascular dysfunction in mild cognitive impairment (MCI)

As well as expanding the presented research to include a larger sample of mild AD patients, it would be interesting to determine at what stage in the disease process such vascular alterations become apparent and whether their detection and management can impact on disease prognosis. Of particular interest in this regard would be the assessment of retinal vascular function in MCI patients. These patients are recognised as having memory impairment greater than that expected through aging alone and are considered to be at an increased risk for AD development. Indeed around 10-15% of MCI patients are thought to convert to AD annually, in comparison to only 1-2% of the normal elderly population . Assessment of retinal vascular function in MCI patients could not only provide an insight into whether vascular alterations are present at this mild level of cognitive impairment but could also, if conducted as part of a longitudinal study, provide an insight into whether the presence of retinal vascular dysfunction may predict or increase their risk of progression to AD. Furthermore a comparison between the nature of retinal vascular dysfunction in MCI patients and in those with mild and/or more advanced AD could provide an insight into how such vascular dysfunctions may contribute to neurodegenerative disease development and/or progression.

### 8.5.3 Impact of ocular and systemic vascular dysfunction on the progression of POAG and NTG

The presence of comparable alterations in ocular and systemic vascular dysfunction has been identified in mild newly diagnosed POAG and NTG patients in this thesis. As well as expanding on the aetiological relevance of these findings, future work assessing, through means of longitudinal study, whether the presence of such vascular alterations and risk factors at the earliest stages of the disease process affect the speed or severity

of disease progression and/or whether modification of these parameters, where possible, aids disease management would be beneficial in determining their clinical relevance. Furthermore the assessment of both ocular and systemic vascular function in glaucoma patients who are progressing despite maximum IOP therapy would also be of interest as it is widely thought that vascular or IOP-independent factors play a greater role in disease aetiology in such individuals. Indeed the assessment of ocular and systemic vascular function in progressive glaucoma patients not only has the potential to provide an insight into the vascular factors linked to this progression but could also open up new treatment and management options for these individuals without having to resort to surgery.

### 8.5.4 Development of dynamic retinal vessel analysis as a tool for the assessment of neurodegenerative disease

A database of normative values for retinal artery and venous function as assessed by DVA is not currently available. As such, although it has strong research implications, the viability of DVA as a diagnostic tool in neurodegenerative disease is currently limited. Future work to establish both a normative DVA database, across a range of ages and ethnic groups, using the parameters presented in this thesis and a disease related DVA database, across a variety of disease stages, is therefore needed.

### 8.5.5 Expansion of flow mediated dilation analysis

Current analysis of the systemic endothelial response to FMD is limited, with the percentage dilation response being the only parameter considered. Expansion of this analysis, to include parameters such as response and recovery times, dilation and constriction slopes and baseline diameter fluctuation, which are currently more commonly associated with DVA, could enhance the evaluation of systemic endothelial function and make comparisons between our ocular (DVA) and systemic vascular

parameters (FMD) more reliable. The development of new software programs and algorithms, capable of coping with large amounts of data, would be necessary for this to be conducted successfully.

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# 10. Appendices

1. Summary of Ocular Blood Flow Studies in Glaucoma

2. Addenbrooke's Cognitive Examination-Revised (ACE-R)

3. Framingham Risk Score Calculation Chart

4. Conference Presentation: Is the eye a window to the mind? Retinal vascular reactivity as a marker for endothelial function in Alzheimer's disease. EVER 2010; Acta Ophthalmologica, Suppl 2010

| Study | Participants | Site and technique of measurement | Findings |
|---|---|---|---|
| | | **Retrobulbar** | |
| Plange et al (2003) | 29 NTG / 29 Controls | CRA and SPCA<br>Color Doppler Imaging and Fluorescein angiograms of ONH | ↓ blood flow parameters and ↑ resistance in most vessels, along with larger ONH filling defects in NTG patients |
| Butt et al (1995) | 34 LTG / 17 Controls | CRA and OA<br>Color Doppler Imaging | ↓ BF and ↑ resistance in CRA and OA of LTG patients |
| Nasemann et al (1994) | 8 NTG / 8 Controls | CRA and OA<br>Fluorescence perfusion scintigraphy | ↓ blood flow velocity in OA and CRA |
| Yamazaki et al (1997) | 16 NTG, prog VF<br>15 NTG stable VF<br>14 HTG, prog VF<br>14 HTG, stable VF | SPCA, CRA and OA<br>Color Doppler imaging | ↓ blood flow velocity and ↑ resistance in CRA and SPCA of NTG patients with progressive visual fields |
| Rankin et al (1995) | 52 POAG<br>24 NTG<br>28 Controls | CRA and SPCA<br>Color Doppler imaging | ↓ blood flow velocity and ↑ resistance in CRA and SPCA of both NTG and POAG patients |
| Rojanapongpun et al (1993) | 60 POAG<br>42 NTG<br>35 Controls | OA<br>Transcranial Doppler ultrasound (2MHz) | ↓ blood flow velocity in OA of both POAG and NTG patients |
| Gallassi et al (2003) | 44 POAG over 7 years | OA<br>Color Doppler imaging and VF analysis | ↓ blood flow velocity and ↑ resistance in patients with deteriorating visual fields |
| Satilmis et al (2003) | 20 progressive POAG over approx 4 years | CRA<br>Color Doppler imaging and VF analysis | ↓ blood flow velocity in CRA correlates with visual field progression rate |
| Zeitz et al (2006) | 114 NTG / 40 Controls | CRA, SPCA and OA<br>Color Doppler imaging | ↓ blood flow velocity in the CRA and SPCA of progressing glaucoma patients |
| Schumann et al (2000) | 20 progressive POAG | CRA and OA<br>Color Doppler imaging and VF analysis | ↓ blood flow velocity in OA and ↑ resistance in CRA. Interocular differences in VF progression correlate with interocular differences in blood flow parameters. |
| Januleviciene et al (2008) | 30 POAG / 30 Controls | CRA, OA and SPCA<br>Color Doppler Imaging<br>RNFL thickness using scanning laser polarimetry | ↓ blood flow velocity correlated with thinner RNFL in POAG patients. |
| | | **Optic nerve head** | |
| Michelson et al (1998) | 91 POAG / 44 Controls | Juxtapapillary retina and NRR<br>Scanning laser Doppler flowmetry<br>Analysis of visual fields | ↓ ONH and juxtapapillary retina blood flow in POAG with no field defect, borderline field defect and advanced disease |

| | | | |
|---|---|---|---|
| Plitz-Seymour et al (2001) | 21 POAG suspects<br>22 POAG<br>15 Controls | ONH – 4 quadrants, cup and foveola<br>Laser Doppler flowmetry | Similar levels of ↓ blood flow in the cup, superotemporal and inferotemporal NRR of suspect POAG and POAG patients |
| Lam et al (2005) | 16 glauc with asymmetry between eyes<br>20 glauc with asymmetry superior to inferior | Superotemporal and inferotemporal NRR and cup<br>Laser Doppler flowmetry | Reduction in blood flow greater in the eye with worse damage and the hemifield of the disc with greater damage |
| Fontana et al (1998) | 95 NTG / Controls | Pulsatile ocular blood flow | POBF ↓ in NTG patients with and without field loss and greater ↓ in eye with field defect compared to that with no field defect. |
| Adam et al (1980) | 171 POAG, OHT and Controls | Filling defects in the rim, wall and floor of cup.<br>Optic disc fluorescein angiograms | Greater % of filling defects in the wall of the cup in glaucoma patients and ↑ with degree of field loss |
| Grunwald et al (1998) | 19 POAG / 15 Controls | ONH – 4 quadrants and cup and foveola<br>Laser Doppler flowmetry | ↓ in blood flow in inferotemporal and superotemporal NRR in POAG. Lower blood flow in those with more advanced VF defects. |
| Findl et al (2000) | 90 POAG / 61 Controls | Cup and NRR<br>Scanning laser Doppler flowmetry<br>Fundus pulsation amplitude – cup and macula | ↓ blood flow and pulsation amplitude in cup, NRR and macula of POAG patients and correlation with degree of field defect |
| Michelson et al (1996) | 43 POAG / 43 Controls | NRR and juxtapapillary retina<br>Scanning laser Doppler flowmetry | ↓ blood flow at both the NRR and juxtapapillary retina in POAG |
| Sato et al (2006) | 54 NTG | Superior and inferior NRR<br>Heidelberg retina flowmetry | Region NRR with greatest reduction in blood flow corresponded with region of visual field defect |
| | | **Retina** | |
| Mitchell et al (2005) | 59 POAG<br>163 OHT<br>3065 Controls | Stereo optic disc photography – analysis of retinal vessel diameter | Significantly narrower retinal arteriolar diameters in POAG compared to OHT and controls |
| Rader et al (1994) | 226 POAG/NTG<br>206 Controls / OHT | Analysis of retinal vessel diameter | Vessels more constricted closer to the disc compared to downstream in POAG/NTG patients and correlation with degree and site of ON damage |
| Arend et al (2002) | 36 NTG<br>31 Controls | Arteriovenous passage (AVP) time and peripapillary arterial and venous diameters.<br>Digital scanning laser fluorescein | Prolonged AVP time in NTG patients but no differences in vessel diameters compared to controls |

| | | | |
|---|---|---|---|
| | | angiograms | |
| Duijm et al | 45 POAG<br>43 NTG<br>11 OHT<br>20 Controls | Retinal arteriovenous passage time.<br>Video fluorescein angiograms | Retinal AVP prolonged in POAG compared to other groups |
| Berisha et al (2008) | 12 Early POAG<br>8 Controls | Inferotemporal retinal artery blood flow parameters - Canon laser Doppler blood flow instrument<br>Peripapillary RNFL thickness - OCT | ↓ blood flow and speed and thinner RNFL in POAG patients. Negative correlation between blood flow and RNFL thickness in POAG |
| Logan et al (2004) | 58 POAG<br>76 NTG<br>38 Controls | Retinal blood flow – Heidelberg retinal flowmetry<br>Structural damage of ONH – Heidelberg retinal tomography | ↓ retinal blood flow in both NTG and POAG.<br>ONHs with abnormal segments had lower corresponding blood flow parameters.<br>Glaucoma patients with normal ONH segments had ↓ blood flow compared to controls with normal ONH segments. |
| Wolf et al (1993) | 51 OAG<br>Controls | Retinal parameters including AVP time<br>Video fluorescein angiograms | Prolonged AVP time, ↓ dye velocity, increased plasma viscosity in POAG |
| | | **Choroid** | |
| Duijm et al (1997) | 45 POAG<br>43 NTG<br>11 OHT<br>20 Controls | Choroidal blood refreshment time<br>Video fluorescein angiograms | Slower choroidal circulation in NTG compared to other groups |
| Yin et al (1997) | 25 POAG<br>5 Optic Atrophy<br>18 Controls | Choroidal filling time – fluorescein angiography<br>Choroidal thickness – light microscopy | Thinner choroids and delayed choroidal perfusion in POAG patients |
| Cellini et al (1996) | 15 POAG | Choroid, OA and SPCA<br>Color Doppler Imaging | ↓ blood flow velocity and ↑ resistance in the SPCA and choroid in POAG |
| Fuchsjager-Mayrl et al (2004) | 140 POAG/OHT<br>102 Controls | Temporal NRR and cup – scanning laser Doppler flowmetry<br>Choroidal blood flow – laser interferometry | ↓ ONH and choroidal blood flow in POAG/OHT patients and an abnormal association between BP and ocular perfusion |
| Kerr et al (1998) | 10 POAG<br>14 OHT | Temporal NRR and cup and peripapillary retina – scanning laser Doppler flowmetry and pulsatile ocular blood flow | ↓ blood flow at NRR, lamina cribrosa and choroid in POAG |

# Appendix 2: Addenbrooke's Cognitive Examination-Revised (ACE-R)

## ADDENBROOKE'S COGNITIVE EXAMINATION - ACE-R

*Final Revised Version A (2005)*

Name :
Date of birth :
Hospital no. :

*Addressograph*

Date of testing:..... /..... /.....
Tester's name: .....
Age at leaving full-time education: .....
Occupation: .....
Handedness: .....

### ORIENTATION

| | | | | | | |
|---|---|---|---|---|---|---|
| Ask: What is the | Day | Date | Month | Year | Season | [Score 0-5] |
| Ask: Which | Building | Floor | Town | County | Country | [Score 0-5] |

### REGISTRATION

Tell: 'I'm going to give you three words and i'd like you to repeat after me: lemon, key and ball'. After subject repeats, say 'Try to remember them because i'm going to ask you later'. Score only the first trial (repeat 3 times if necessary).

Register number of trials .....

[Score 0-3]

### ATTENTION & CONCENTRATION

Ask the subject: ' could you take 7 away from a 100? After the subject responds, ask him or her to take away another 7 to a total of 5 subtractions. If subject make a mistake, carry on and check the subsequent answer (i.e. 93, 84, 77, 70, 63 -score 4)

Stop after five subtractions (93, 86, 79, 72, 65). ..... ..... ..... ..... .....

[Score 0-5]
(for the best performed task)

Ask: 'could you please spell **WORLD** for me? Then ask him/her to spell it backwards:

..... ..... ..... ..... .....

### MEMORY - Recall

Ask: 'Which 3 words did I ask you to repeat and remember?'

..... ..... .....

[Score 0-3]

### MEMORY - Anterograde Memory

Tell: ' I'm going to give you a name and address and I'd like you to repeat after me. We'll be doing that 3 times, so you have a chance to learn it. I'll be asking you later'

Score only the third trial

[Score 0-7]

| | 1st Trial | 2nd Trial | 3rd Trial |
|---|---|---|---|
| Harry Barnes | ..... ..... | ..... ..... | ..... ..... |
| 73 Orchard Close | ..... ..... ..... | ..... ..... ..... | ..... ..... ..... |
| Kingsbridge | ..... | ..... | ..... |
| Devon | ..... | ..... | ..... |

### MEMORY - Retrograde Memory

[Score 0 -4]

- Name of current Prime Minister .....
- Name of the woman who was Prime Minister .....
- Name of the USA president .....
- Name of the USA president who was assassinated in the 1960's .....

ATTENTION&ORIENTATION MEMORY

## VERBAL FLUENCY - Letter 'P' and animals

➢ **Letters**

Say: 'I'm going to give you a letter of the alphabet and I'd like you to generate as many words as you can beginning with that letter, but not names of people or places. Are you ready? You've got a minute and the letter is P'

[Score 0 - 7] ☐

| | |
|---|---|
| ≥17 | 7 |
| 14-17 | 6 |
| 11-13 | 5 |
| 8-10 | 4 |
| 6-7 | 3 |
| 4-5 | 2 |
| 2-3 | 1 |
| <2 | 0 |
| total | correct |

➢ **Animals**

Say: 'Now can you name as many animals as possible, beginning with any letter?

[Score 0 - 7] ☐

| | |
|---|---|
| ≥21 | 7 |
| 17-21 | 6 |
| 14-16 | 5 |
| 11-13 | 4 |
| 9-10 | 3 |
| 7-8 | 2 |
| 5-6 | 1 |
| <5 | 0 |
| total | correct |

## LANGUAGE - Comprehension

➢ Show written instruction

[Score 0-1] ☐ ☐

**Close your eyes**

➢ 3 stage command:
'Take the paper in your right hand. Fold the paper in half. Put the paper on the floor'

[Score 0-3] ☐ ☐

## LANGUAGE - Writing

➢ Ask the subject to make up a sentence and write it in the space below:
Score 1 if sentence contains a subject and a verb (see guide for examples)

[Score 0-1] ☐ ☐

## LANGUAGE - Repetition

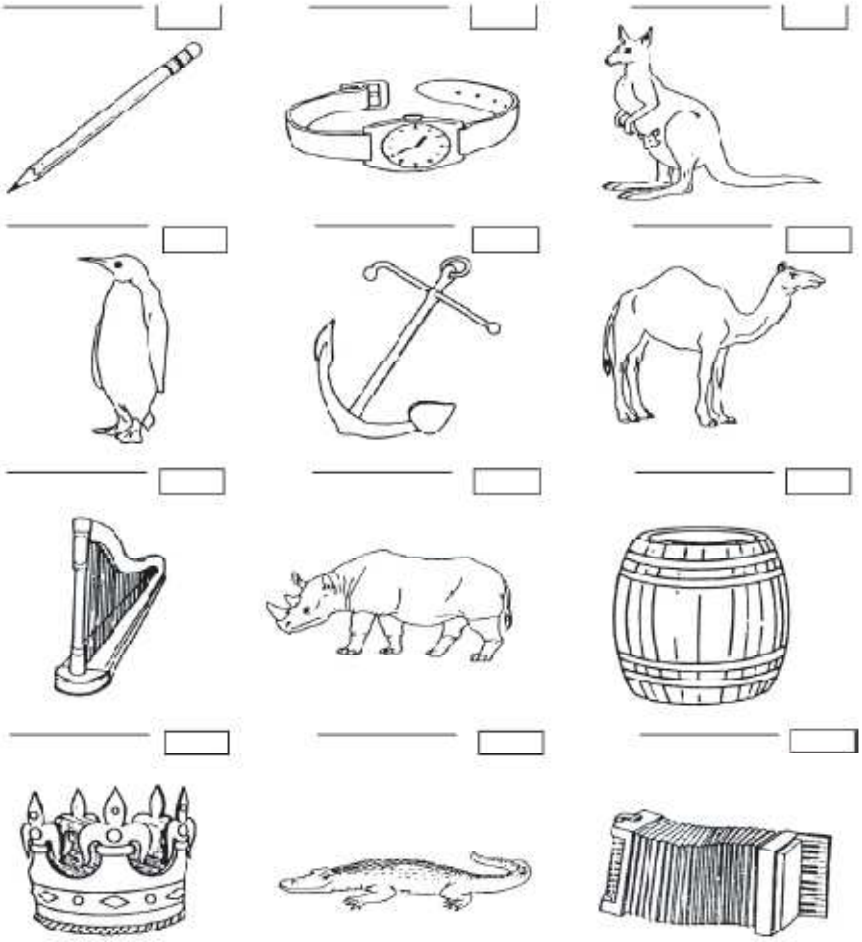
➢ Ask the subject to repeat:' **hippopotamus'; 'eccentricity; 'unintelligible'; 'statistician'** [Score 0-2]
Score 2 if all correct; 1 if 3 correct; 0 if 2 or less.

➢ Ask the subject to repeat: **'Above, beyond and below'** [Score 0-1]

➢ Ask the subject to repeat: **'No ifs, ands or buts'** [Score 0-1]

## LANGUAGE - Naming

➢ Ask the subject to name the following pictures: [Score 0-2] pencil + watch



[Score 0-10]

## LANGUAGE - Comprehension

➢ Using the pictures above, ask the subject to: [Score 0-4]

- Point to the one which is associated with the monarchy _____
- Point to the one which is a marsupial _____
- Point to the one which is found in the Antarctic _____
- Point to the one which has a nautical connection _____

LANGUAGE

## LANGUAGE - Reading

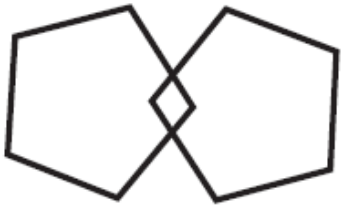
➢ Ask the subject to read the following words: [Score 1 only if all correct] [Score 0-1]

sew
pint
soot
dough
height

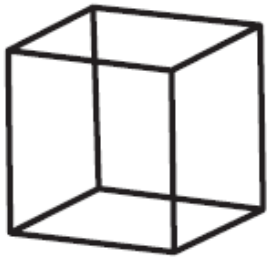
LANGUAGE

## VISUOSPATIAL ABILITIES

➢ Overlapping pentagons: Ask the subject to copy this diagram: [Score 0-1]



➢ Wire cube : Ask the subject to copy this drawing (for scoring, see instructions guide) [Score 0-2]



➢ Clock: Ask the subject to draw a clock face with numbers and the hands at ten past five. (for scoring see instruction guide: circle = 1, numbers = 2, hands = 2 if all correct) [Score 0-5]

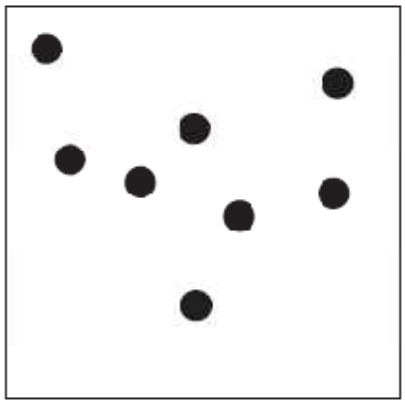
VISUOSPATIAL

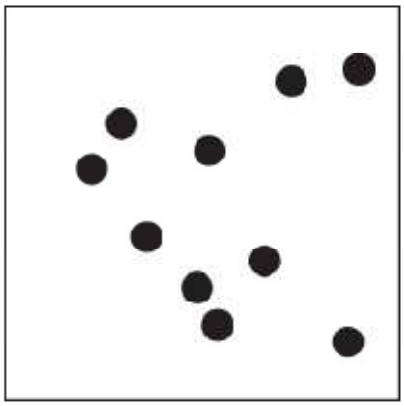
## PERCEPTUAL ABILITIES

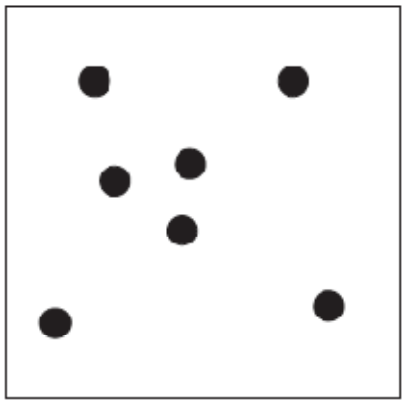
VISUOSPATIAL

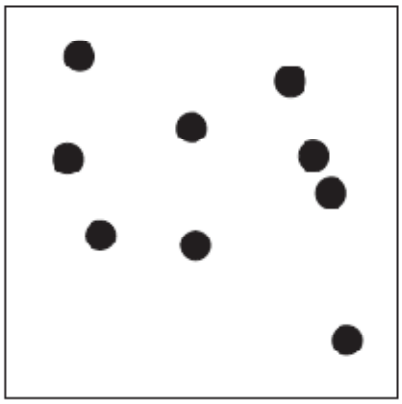
➢ Ask the subject to count the dots without pointing them

[Score 0-4]





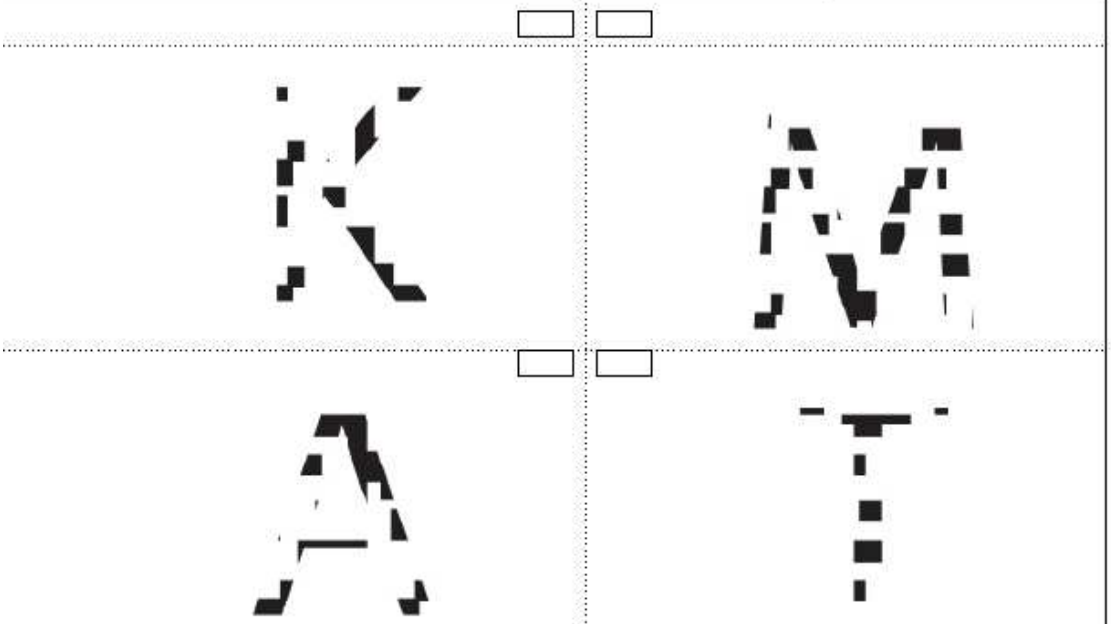




ADDENBROOKE'S COGNITIVE EXAMINATION - ACE-R *Final Revised Version A (2005)*

## PERCEPTUAL ABILITIES

➢ Ask the subject to identify the letters [Score 0-4]



## RECALL

➢ Ask "Now tell me what you remember of that name and address we were repeating at the beginning"

Harry Barnes
73 Orchard Close
Kingsbridge
Devon

[Score 0-7]

## RECOGNITION

➢ This test should be done if subject failed to recall one or more items. If all items were recalled, skip the test and score 5. If only part is recalled start by ticking items recalled in the shadowed column on the right hand side. Then test not recalled items by telling "ok, I'll give you some hints: was the name X, Y or Z?" and so on. Each recognised item scores one point which is added to the point gained by recalling.

[Score 0-5]

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Jerry Barnes | | Harry Barnes | | Harry Bradford | | recalled | |
| 37 | | 73 | | 76 | | recalled | |
| Orchard Place | | Oak Close | | Orchard Close | | recalled | |
| Oakhampton | | Kingsbridge | | Dartington | | recalled | |
| Devon | | Dorset | | Somerset | | recalled | |

## General Scores

| | |
|---|---|
| MMSE | /30 |
| ACE-R | /100 |

## Subscores

| | |
|---|---|
| Attention and Orientation | /18 |
| Memory | /26 |
| Fluency | /14 |
| Language | /26 |
| Visuospatial | /16 |

VISUOSPATIAL MEMORY SCORE

Normative values based on 63 controls aged 52-75 and 142 dementia patients aged 46-86

Cut-off <88 gives 94% senstivity and 89% specificity for dementia
Cut-off <82 gives 84% sensitivity and 100% specificity for dementia

# FRAMINGHAM RISK SCORE to predict 10 year ABSOLUTE RISK of CHD EVENT

## WEST HERTFORDSHIRE CARDIOLOGY

**This risk assessment only applies to assessment for PRIMARY PREVENTION of CHD, in people who do not have evidence of established vascular disease.**
Patients who ***already*** have evidence of vascular disease usually have a >20% risk of further events of over 10 years, and require vigorous **SECONDARY PREVENTION.**
People with a Family History of premature vascular disease and some Asians are at higher risk than predicted; Southern Europeans may have a lower risk in relation to standard risk factors.

**STEP 1: Add scores by sex for Age, Total Cholesterol, HDL-Cholesterol, BP, Diabetes and Smoking.** (If HDL unknown, assume 1.1 in Males, 1.4 in Females)

| Age | M | F |
|---|---|---|
| 30-34 | -1 | -9 |
| 35-39 | 0 | -4 |
| 40-44 | 1 | 0 |
| 45-49 | 2 | 3 |
| 50-54 | 3 | 6 |
| 55-59 | 4 | 7 |
| 60-64 | 5 | 8 |
| 65-69 | 6 | 8 |
| 70-74 | 7 | 8 |

| Total Cholesterol | M | F |
|---|---|---|
| < 4.1 | -3 | -2 |
| 4.1 – 5.1 | 0 | 0 |
| 5.2 – 6.2 | 1 | 1 |
| 6.3 – 7.1 | 2 | 1 |
| ≥7.2 | 3 | 3 |

| HDL Cholesterol | M | F |
|---|---|---|
| < 0.9 | 2 | 5 |
| 0.9 - 1.16 | 1 | 2 |
| 1.17 - 1.29 | 0 | 1 |
| 1.30 - 1.55 | 0 | 0 |
| ≥1.56 | -2 | -3 |

| Systolic BP | Diastolic BP | | | | |
|---|---|---|---|---|---|
| Male | <80 | 80-84 | 85-89 | 90-99 | ≥100 |
| <120 | 0 | 0 | 1 | 2 | 3 |
| 120-129 | 0 | 0 | 1 | 2 | 3 |
| 130-139 | 1 | 1 | 1 | 2 | 3 |
| 140-159 | 2 | 2 | 2 | 2 | 3 |
| ≥160 | 3 | 3 | 3 | 3 | 3 |
| Female | <80 | 80-84 | 85-89 | 90-99 | ≥100 |
| <120 | -3 | 0 | 0 | 2 | 3 |
| 120-129 | 0 | 0 | 0 | 2 | 3 |
| 130-139 | 0 | 0 | 0 | 2 | 3 |
| 140-159 | 2 | 2 | 2 | 2 | 3 |
| ≥160 | 3 | 3 | 3 | 3 | 3 |

If Systolic and Diastolic BP fall into different categories, use score from higher category

| Diabetes | M | F |
|---|---|---|
| No | 0 | 0 |
| Yes | 2 | 4 |

| Smoking | M | F |
|---|---|---|
| No | 0 | 0 |
| Yes | 2 | 2 |

| Categorisation of 10 year Risk of CHD Event | |
|---|---|
| Very Low risk | < 10% |
| Low risk | < 15% |
| Moderate risk | 15 20% |
| High risk | > 20% |

**STEP 2: Use total score to determine Predicted 10 year Absolute Risk of CHD Event (Coronary Death, Myocardial Infarction, Angina) by sex**

| Total Score | ≤-2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 3 | 14 | 15 | 16 | ≥17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 year Risk: Male | | <2% | 3% | 3% | 4% | 5% | 7% | 8% | 10% | 13% | 16% | 20% | 25% | 31% | 37% | 45% | ≥53% | ≥53% | ≥53% | ≥53% |
| 10 year Risk: Female | <1% | 2% | 2% | 2% | 3% | 3% | 4% | 4% | 5% | 6% | 7% | 8% | 10% | 11% | 13% | 15% | 18% | 20% | 24% | ≥27% |

**STEP 3: Compare Predicted 10 year Absolute Risk with "Average" and "Ideal" 10 year Risks, to give Relative Risks**

| Age | 30 - 34 | 35 - 39 | 40 - 44 | 45 - 49 | 50 - 54 | 55 - 59 | 60 - 64 | 65 - 69 | 70 - 74 |
|---|---|---|---|---|---|---|---|---|---|
| "Average" Male | 3% | 5% | 7% | 11% | 14% | 16% | 21% | 25% | 30% |
| "Ideal" Male | 2% | 3% | 4% | 4% | 6% | 7% | 9% | 11% | 14% |
| "Average" Female | < 1% | < 1% | 2% | 5% | 8% | 12% | 12% | 13% | 14% |
| "Ideal" Female | < 1% | 1% | 2% | 3% | 5% | 7% | 8% | 8% | 8% |

| "Ideal" risk represents |
|---|
| Total Cholesterol = 4.1 - 5.1 |
| HDL = 1.2 (Male), 1.4 (Female) |
| BP < 120/80 |
| No Diabetes, Non Smoker |

People with an absolute risk of ≥30% should be considered for treatment: with a Statin to achieve a Total Cholesterol <5 and/or LDL cholesterol <3
People with an absolute risk of ≥15% should be considered for treatment with anti-hypertensives to achieve a BP ideally ≤140/90

# Appendix 4: Conference Presentation

**Is the eye a window to the mind? Retinal vascular reactivity as a marker for endothelial function in Alzheimer's disease.**

Stephanie Mroczkowska, Alexandra Benavente-Perez, Sunni Patel, Lu Qin, Doina Gherghel

School of Life & Health Sciences, Aston University, Birmingham, B4 7ET, UK

**Aim:** To assess the retinal and systemic vascular function in patients diagnosed with mild Alzheimer's disease in comparison to healthy age matched controls.

**Methods:** Nine newly diagnosed mild AD patients (MMSE score 18- 24) and 23 healthy age-matched controls without any cognitive dysfunction (ACE-R score ≥ 88) were recruited for the study. Retinal vessel reactivity was assessed using the retinal vessel analyser (RVA, IMEDOS, Germany). From these recordings the time taken to reach maximum dilation (RT), was determined for each individual flicker cycle. Systemic vascular function was assessed using flow mediated dilation (FMD) technique at the brachial artery level (Siemens; Acuson Sequoia, UK). Intraocular pressure (IOP) and systemic blood pressure (BP) were also recorded for each participant and OPP was then calculated.

**Results:** There were no significant differences in age, mean BP, IOP or OPP between the two study groups ($p>0.05$). The retinal arterial RT to flicker light stimulation was found to be significantly longer in AD patients as compared to healthy control for both the first ($p= 0.01$) and the third ($p=0.049$) flicker cycles. In addition, the RT measured at the chosen vein level was significantly longer in AD patients compared to controls for the first ($p=0.046$) and second ($p=0.043$) flicker cycles. No significant differences were found in the brachial arterial diameter between the two groups ($p>0.05$).

**Conclusion:** In patients suffering from AD, the prolonged retinal vessel RT to flicker provocation could represent an early sign of vascular dysfunction evident at the microvascular level.

**Awarded 'Best paper in section' – 500 euro travel grant**

# Is the eye a window to the mind? Retinal vascular reactivity as a marker for endothelial function in Alzheimer's disease

S. Mroczkowska[1] , A. Benavente-Perez[2], S.Patel[1], L Qin[1], P. Bentham[3], D. Gherghel[1]

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Aston University Birmingham

Birmingham and Solihull NHS

Alzheimer's Research Trust

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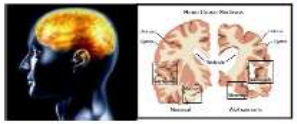
## Introduction

**Alzheimer's disease (AD)**

- Progressive neurodegenerative disorder
- Gradual decline in cognitive function
- Most common form of dementia in elderly
- Affects over 35 million people worldwide



**The Aetiological Puzzle of AD**

- Exact aetiology still unclear despite extensive research
- Early diagnosis and treatment remains difficult
- Multiple theories proposed, including exposure to increased mechanical stress, genetics, oxidative stress and vascular factors

**Vascular Theory**

- Centres around the concept that the presence of cardiovascular risk factors and subsequent chronic brain hypo-perfusion contributes to AD development in some cases [1]
- Vascular dysregulation in form of impaired autoregulation and endothelial dysfunction also recently implicated as an early development in AD [2]

**Q. Could early detection of signs for vascular dysregulation lead to improved diagnosis and management of AD patients?**

**Q. Could the eye be used as a window to the mind?**

**Reasoning:**

- Difficult to assess cerebral vasculature directly
- Increasing evidence of ocular involvement in AD :
  - Neurodegenerative changes at the retinal level in AD patients that replicate those found at the cortical level
  - Abnormal retinal blood flow in AD patients
  - Links between AD and ocular conditions such as glaucoma and ARMD

**Hypothesis**

As the retinal and cerebral vessels share many similarities it could be hypothesised that through direct observation of retinal vascular function in AD patients, an insight into the changes occurring at the cerebral level could be gained.

## Aims

- To identify for the first time whether abnormalities of retinal vascular function are evident in AD patients.
- To establish a possible link between measures of retinal vascular and systemic endothelial function in AD patients

## Implications

- Identification of potential ocular vascular biomarkers for AD
- Better aetiological understanding of the condition
- Improved diagnosis
- Improved methods for disease monitoring

## Methods

**Participants:**

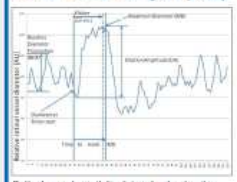
- 9 newly diagnosed mild AD patients – recruited from Birmingham and Solihull Mental Health Trust , MMSE score between 18-24. Screened to ensure no coexisting ocular disease.
- 25 healthy age matched controls – recruited by volunteer participation, ACE-R score of 88+. Screened to ensure no coexisting ocular disease
- No coexisting vascular problems or medications known to affect blood flow or endothelial function.

**Measurements:**

- Fasting venous blood sample (analysis of triglycerides, HDL cholesterol, total cholesterol, glucose)
- Dynamic Retinal Vessel analysis (DVA; IMEDOS GmbH,Jena) – assessment of vascular function at ocular level
- Flow mediated dilation (Siemens; Acuson Sequoia, UK) – assessment of systemic endothelial function
- Blood pressure (BP), intraocular pressure (IOP), height and weight measurements for BMI calculation

| | AD | Controls | P-value |
|---|---|---|---|
| Mean Age (years) | 62.88 ± 8.46 | 56.84 ± 7.88 | 0.061 |
| Gender | 5M : 4F | 12M : 13F | - |
| BMI | 28.42 ± 5.75 | 27.86 ± 4.83 | 0.799 |
| SBP (mmHg) | 143.56 ± 13.72 | 131.08 ± 18.31 | 0.072 |
| DBP (mmHg) | 80.89 ± 7.72 | 78.52 ± 9.67 | 0.513 |
| OPP (mmHg) | 86.52 ± 8.56 | 80.28 ± 13.02 | 0.206 |

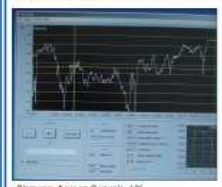
### Retinal Vessel Analysis (RVA)



Retinal vessel reactivity determined using the newly defined method of Sequential and Diameter Response Analysis (SDRA) [4]

- Continuous retinal vessel diameter measurement
- Monitor arterial and venous responses to flicker stimulation
- 50 seconds baseline, 20 seconds flicker, 60 seconds recovery x3.[3]
- Total of 3 flicker cycles
- Calculate time taken to reach maximum dilation (RT) for each flicker cycle for artery and vein (SDRA method).

### Flow mediated dilation (FMD)

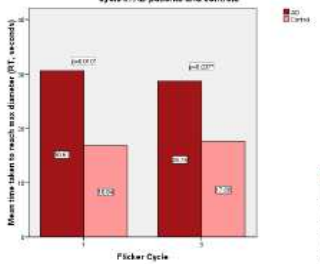


Siemens, Acuson Sequoia, UK

- Ultrasound imaging of brachial artery
- Gold standard for systemic endothelial function assessment [5]
- 2 minutes baseline measurements
- Inflate BP cuff around wrist to 50 mmHg above SBP for 5 minutes
- Release cuff
- 2 minutes recovery measurements
- Calculate maximum vessel diameter (MD) and time taken to reach MD following cuff release

## Results

Time taken to reach maximum arterial diameter on the first and third flicker cycle in AD patients and controls




- No significant differences in age, BP, BMI or fasting blood parameters (triglycerides, HDL cholesterol, total cholesterol, glucose) ( (p>0.05);
- AD patients took significantly longer to reach maximum dilation on the first and third arterial flicker cycles compared to controls (p=0.010; p=0.037);
- AD patients took significantly longer to reach maximum dilation on the second venous flicker cycle compared to controls (p=0.049);
- No significant differences found in FMD between groups (p>0.05).

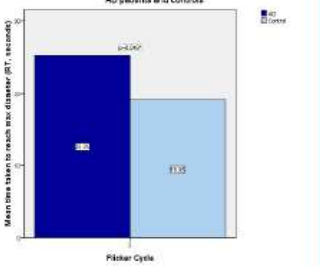
RVA - ARTERY RT (secs)

| | AD patients | Controls | p-value |
|---|---|---|---|
| Flicker 1 | 30.67 ± 17.01 | 16.94 ± 11.43 | 0.010* |
| Flicker 2 | 16.89 ± 12.02 | 22.24 ± 8.61 | 0.160 |
| Flicker 3 | 26.75 ± 18.14 | 17.50 ± 10.42 | 0.037* |
| Average | 25.44 ± 12.07 | 18.89 ± 6.75 | 0.054 |

RVA - VEIN RT (secs)

| | AD patients | Controls | p-value |
|---|---|---|---|
| Flicker 1 | 23.12 ± 6.90 | 20.17 ± 6.51 | 0.281 |
| Flicker 2 | 25.25 ± 13.11 | 15.96 ± 4.32 | * 0.049 |
| Flicker 3 | 21.00 ± 10.57 | 19.88 ± 5.78 | 0.719 |
| Average | 22.57 ± 9.39 | 19.07 ± 4.21 | 0.259 |

Time taken to reach maximum venous diameter on the second flicker cycle for AD patients and controls




## Conclusions

- Prolonged RT to flicker stimulation found in AD patients could represent an early sign of vascular dysfunction evident at the micro-vascular level in these patients;
- Could be attributed to abnormal levels of NO, abnormal neurovascular coupling mechanism, vascular rigidity, vasospasm or hypoxia;
- No significant differences found in FMD responses between groups could be attributed to vascular dysfunction occurring earlier at the micro-vascular level than at the macro-vascular level

## References

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None of the authors named above has declared any conflicts of interest which may arise from being named as an author on this poster

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