AUTONOMIC DYSFUNCTION AND SYSTEMIC OXIDATIVE STRESS ASSOCIATED WITH GLAUCOMATOUS OPTIC NEUROPATHY

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Doctor of Philosophy

ASTON UNIVERSITY

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Aston University
Autonomic dysfunction and systemic oxidative stress associated with glaucomatous optic neuropathy
Doina Gherghel
Doctor of Philosophy
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Synopsis

The importance of vascular risk factors in glaucoma pathogenesis has been extensively researched. However, it is still unclear how haemodynamic disturbances occurring in various ocular and systemic vascular beds could interfere with retinal ganglion cells survival, therefore contributing to glaucoma onset and progression. The purpose of the following studies was to investigate the presence and impact of both ocular and systemic vascular dysregulation in POAG pathogenesis and. A possible effect of latanoprost 0.005% on ocular blood flow of patients suffering from POAG was also assessed. There were four principal sections to the work:

1. **Investigation of ocular and systemic vascular risk factors in POAG.**

The principal findings of this work were:

- Glaucoma patients exhibit an anticipatory reaction to the physical stress, similar to subjects at risk for cardiovascular diseases; a blunted BP response and a reduction in ONH blood flow in response to cold provocation was also recorded.
- Silent myocardial ischaemic episodes occurred during peaks in systemic BP and HR.
- Independent of a positive history for cardiovascular diseases, patients suffering from POAG demonstrate a blunt circadian rhythm of the ANS.

2. **Assessment of the relationship between vascular and systemic vascular risk factors in GON.**

The principal findings of this work were:

- POAG patients demonstrate a high sympathetic tonus over a 24-h period.
- POAG patients with lower OBF demonstrate both 24-h systemic BP and HRV abnormalities.
- OBF alterations observed in some glaucoma patients could be either primary or secondary to systemic haemodynamic disturbances and not a consequence of ONH damage.

3. **Assessment of the level of systemic anti-oxidative defence in POAG patients.**

The principal finding of this work was:

- Patients suffering from POAG demonstrated significantly lower GSH and t-GSH levels than normal controls.

4. **Investigation of the effect of treatment with latanoprost 0.005% on visual function and OBF.**

The findings of this work were:

- Treatment with latanoprost 0.005% resulted in a significant decrease in IOP and increase in OPP. VF damage progression has also been stopped.
- Treatment with latanoprost 0.005% resulted in a significant increase in the OBF parameters measured at the ONH and peripapillary retina levels.

Finally, the importance of a clear protocol for managing new POAG cases is highlighted and a clinical conduit is proposed.

Keywords: Ocular blood flow; autonomic nervous system; vascular dysregulation; glutathione, latanoprost

Acknowledgements

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List of Abbreviations

POAG	Primary open-angle glaucoma
VF	Visual field
IOP	Intraocular pressure
VA	Visual acuity
AH	Aqueous humor
TM	Trabecular meshwork
NTG	Normal tension glaucoma
HTG	High tension glaucoma
ONH	Optic nerve head
BP	Systemic blood pressure
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
MBP	Mean blood pressure
HR	Heart rate
ANS	Autonomic nervous system
GON	Glaucomatous optic neuropathy
OA	Ophthalmic artery
CRA	Central retinal artery
PCA	Posterior ciliary artery
ChBF	Choroidal blood flow
NFL	Nerve fibre layer
CRV	Central retinal vein
VIP	Vasoactive intestinal polypeptide
NO	Nitric oxide
ATP	Adenosine triphosphate
CS	Contrast sensitivity
ET	Endothelin
OPP	Ocular perfusion pressure
PP	Pulse pressure
OBF	Ocular blood flow
OBFA	Ocular Blood Flow Analyser

POBF	Pulsatile ocular blood flow
PA	Pulse amplitude
PV	Pulse volume
PR	Pulse rate
HRF	Heidelberg Retina Flowmeter
CDI	Color Doppler imaging
FFT	Fast Fourier transformation
CPT	Cold pressor test
LDF	Laser Doppler flowmetry
FT	Finger temperature
FBF	Finger blood flow
TT	Tympanic temperature
NRR	Neuroretinal rim
CAD	Coronary artery disease
ICA	Internal carotid artery
HRV	Heart rate variability
HF	High frequency
LF	Low frequency
LIE	Longest ischaemic event
TIB	Total ischaemic burden
MD	Mean defect in automated visual field testing
PSD	Pattern standard deviation in automated visual field testing
ROS	Reactive oxygen species
NOS	Nitric oxide syntheses
RNS	Reactive nitrogen species
GSH	Glutathione
GSSG	Glutathione, oxidized form
GST	Glutathione transferase
GPx	Glutathione peroxidase
GSSR	Glutathione reductase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl radical
O_2^-	Superoxide radical
SOD	Superoxide dismutase
CAT	Catalase
EDTA	Ethylene diamine tetra-acetic acid
DTNB	Dithiobis-2-nitrobenzoic acid
TEA	Triethanolamine
2-VP	2- vinyl pyridine
OSA	Obstructive sleep apnoea
AU	Arbitrary units
NU	Normalized units
NS	Non-significant
SD	Standard deviation

Statement of Authenticity

This thesis represents the work of Dr Doina Gherghel during a 3 year research fellowship at the Neuroscience Research Institute, Aston University, Birmingham, UK. All the present studies have been performed with assistance from Mr Ian Cunliffe (Consultant Ophthalmologist, Heartlands and Solihull NHS Trust, Birmingham). The work on oxidative stress was performed with assistance from Dr Emma Hilton who helped with the initial setup for the blood assay method.

The author has no commercial interest in any of the equipment or laboratory methods used in this work.

1. Introduction: Ocular and Systemic Blood Flow in Primary Open-Angle Glaucoma

1.1. Background

Primary open-angle glaucoma (POAG) is a chronic, slowly progressive optic neuropathy, characterized by progressive excavation of the optic nerve head (ONH), with a distinctive pattern of visual field (VF) defects (Van Buskirk and Cioffi, 1992). The disease is multifactorial in origin and is particularly associated with elevated intraocular pressure (IOP) (Krakau, 1981; Bonomi et al, 2001) resulting in the main from reduced drainage of aqueous humour (AH). Despite this, increased IOP is no longer included in the definition of glaucoma since its relationship to the disease is not universal. Clinical studies indicate that other systemic and local factors may be involved in the pathogenesis of the disease.

Glaucoma is generally managed by reducing IOP to a so called "target pressure" with the objective being to prevent further damage to the optic nerve and loss of VF (The European Glaucoma Society, 1998). The finding that about one third of patients develop glaucoma while exhibiting apparently normal IOP, or the fact that a substantial number of cases with POAG continue to progress despite therapeutically lowered IOP, has spurred the search for other categories of risk factors (Gasser and Flammer, 1987; Prunte *et al.,* 1998; Broadway and Drance, 1998; Hayreh, 1999; Bonomi et al, 2000; Ishida *et al.*, 2000; Drance *et al.,* 2001; Pfeiffer *et al.,* 2002; Flammer *et al.,* 2002; Spry *et al.,* 2004; Gherghel *et al.,* 2004*b*).

Both ocular and systemic vascular risk factors have been advocated to play an important part in the pathogenesis of glaucomatous optic neuropathy (GON). The evidence supporting the involvement of this category of risk factors is extensive. On average, patients with glaucoma, especially those suffering from normal-tension glaucoma (NTG) or from progressive high-tension glaucoma (HTG), have slower blood flow velocity in the retina (Wolf *et al.,* 1993; Chung et al, 1999 *b*; Hall et al, 2001; Logan et al, 2004), choroid (Ulrich et al, 1996; Duijm

et al., 1997*a*; Gugleta *et al.,* 2003), and ONH (Michelson et al, 1996*a*; Piltz-Seymour, 1999; Findl *et al.,* 2000; Ciancaglini *et al.,* 2000; Piltz-Seymour et al, 2001; Yaoeda *et al.,* 2003; Hafez et al, 2003. Blood flow is also reduced in the retrobulbar vessels Kaiser *et al.,* 1997; Costa *et al.,* 1994; Nicolela *et al.,* 1996*b*; Yamazaki and Drance, 1997; Piltz-Seymour *et al.,* 2001; Satilmis *et al.,* 2003), carotid arteries (O'Brien *et al.,* 1992), brain (Harris *et al.,* 2003) and in the peripheral capillaries (Gasser *et al.,* 1999*a*; Pache et al., 2003). In addition, it has also been observed that patients with glaucoma frequently suffer from ischaemic lesions in other parts of the body such as the brain (Ong *et al.,* 1995; Stroman *et al.,* 1995; Suzuki *et al.,* 2004), the ear (Susanna and Basseto, 1992), and the heart (Waldmann *et al.,* 1996). Glaucoma-like ONH excavations have also been demonstrated in animals following induced ischaemia (Orgül *et al.,* 1996). This suggests that vascular disturbance can play a primary role in glaucoma pathogenesis (Flammer *et al.,* 1999).

Oxidative stress has also been proposed as a contributing factor in the etiology of glaucomatous optic neuropathy (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,*1996; Izzotti et al., 2003; Ferreira *et al.,* 2004). One possible explanation is the harmful effect of oxidative stress on vascular endothelium (Nedeljovic *et al.,* 2003). Due its crucial role in vascular physiology, any endothelial dysfunction may result in disturbances in systemic blood flow with a direct influence on circulatory homeostasis and vascular motility throughout the body, hence the role in glaucoma pathogenesis.

Among the factors that may influence vascular physiology, variables such as blood pressure (BP), heart rate (HR) (Appenzeller and Orbie, 1997) and anti-oxidative defence (Hardeland *et al.,* 2003) have a circadian rhythm, dependent on the autonomic nervous system (ANS). Chronic imbalances of the ANS could lead to important adverse cardiovascular events including myocardial ischaemia (Korpelainen *et al.,* 1997) as well as to aging and age-related diseases (Ahsan *et al.,* 2003; Ferdinandy and Schulz, 2003). Some of these consequences could affect the eye, either leading to or exacerbating glaucoma in susceptible individuals (Hayreh *et al.,* 1994; Kashiwagi *et al.,* 2000); 3992(Kashiwagi *et al.,* 2001; Gherghel *et al.,* 2001; Gherghel *et al.,* 2004*b*).

Since studies regarding the role of ocular and vascular risk factors in the aetiology of POAG are multiple, a systematic review of the current knowledge is necessary. The information offered in the present chapter will provide the reader with the necessary basic theory to interpret the results of studies of systemic and ocular haemodynamics in POAG patients presented in this thesis.

1.1.1. Primary open-angle glaucoma - Historical review

The first description of "glaukos" (the Greek term for glaucoma) came from Hippocrates (approximately 400 B.C.). He described this disease as the presence of a cloudy blue (colour of the sea) pupil (Nathan, 2000). However, it was later discovered that what was initially described as "glaucoma" actually referred to either cataract or other eye diseases. In 1705, the French surgeon Pierre Bissau, was the first to demonstrate that glaucoma was not a lens disorder; he believed glaucoma to be an abnormality of the vitreous, an opinion shared by many other physicians for the next century (Nathan, 2000).

Although the terms "glaucoma" and "glaucosis" have been used for centuries to describe many eye diseases, historical references also contain clear descriptions of glaucoma itself, especially of the acute cases. For instance, in 1348, the Arabian surgeon Sams-ad-Din, was the first to describe a case characterised by a very hard eyeball, dilated pupil, decreased vision, "dullness of the humours" and hemicrania (Nathan, 2000). Another clear recognition of glaucoma came in the 17th century from a British oculist named Richard Banister; he described a disease called "*gutta serena*" as *"if one feele ... by rubbing upon the eie-lids that the eye be growne more solid and hard than naturally it should be ... the humour growne to any solid or hard substance, it is not possible to be cured."*

Ocular hypertension was not recognised to be an essential feature of glaucoma until about 1840 and then only for the acute and advance forms of the disease. Albrecht von Graefe, a founder of modern ophthalmology, was the first to differentiate between the acute, chronic and secondary glaucomas; he did not,

however, associated optic disc cupping with the presence of either glaucoma or a high IOP (Nathan, 2000). In his opinion, optic disc cupping associated with high IOP was an "amaurosis with excavation of the optic nerve". In 1866 Frans Cornelis Donders, an ophthalmologist from Holland was the first to recognise that cupping of the optic disc in the presence of high IOP represents a form of glaucoma, which he called "simple glaucoma".

In 1858, Jaeger (Jaeger, 1858) proposed that glaucomatous optic neuropathy (GON) might have IOP-independent causes. Smith in 1885 (Smith, 1885) suggested the involvement of both mechanical and vascular factors in the aetiology of glaucoma and Schnabel in 1892 (Schnabel, 1892) showed that atrophy of the optic disc associated with cupping but without a high IOP represented a further form of glaucoma.

The hypothesis that glaucoma may be a problem of vascular dysregulation has been proposed by Magitot in 1925 (Magitot, 1925). In the 1980s, Flammer (Flammer, 1985) proposed that both mechanical and vascular theories might be correct; they hypothesized that the two mechanisms may act synergistically to produce glaucoma. Since then, the theory of a vascular contribution to the pathogenesis of POAG has been explored by various researchers. Continuous progression of understanding the many factors that contribute to the pathogenesis of POAG demonstrate a complex cascade of events that remains to this day incomplete.

For the purpose of interpretation of this thesis, an understanding of the anatomy and physiology of the ocular and systemic circulation and their regulatory mechanisms is required. A summary of these is presented below.

1.2. Anatomy of the ocular vessels

1.2.1. The retrobulbar vascular supply (Figure 1.1)

The retrobulbar ocular vessels are comprised of ophthalmic artery (OA), central retinal artery (CRA) and ciliary arteries. The OA, which represents the first branch of the internal carotid artery (ICA), provides most of the blood supply to

the orbit and to some of the surrounding scalp (Wang *et al.,* 1998). It enters the orbit through the *optic foramen*, below and lateral to the optic nerve and then passes over the nerve to reach the medial wall of the orbit. From this point the OA is situated horizontally, beneath the superior oblique muscle.

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

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

Figure1.1: Retrobulbar blood vessels. Adapted from Snell et. Lamp, (Snell and Lemp, 1998).

1.2.2. Retinal circulation

Retinal vessels are supplied by the CRA, which branches to form four retinal arterioles, one for each quadrant of the retina (Anderson, 1989). These arterioles are situated within the nerve fibre layer (NFL) and give rise to an extensive capillary network that supplies the inner two thirds of the retina.

There are strong resemblances between the retinal and the brain circulations; the principle difference is that the retinal circulation has no autonomic innervation (Bill and Sperber, 1990). The retina is protected from toxic

molecules by a blood-retinal barrier similar to the blood-brain barrier; this is possible due to the presence of tight junctions between retinal capillary endothelial cells, which prevent leakage of proteins, lipid molecules and small water-soluble metabolic substrates. The retinal endothelial cells are surrounded by a single layer of muscular pericytes, which help in altering local vascular resistance (Haefliger and Anderson, 1996).

1.2.3. Blood supply to the optic nerve head

Blood supply to the ONH is illustrated in Figure 1.2. Anatomically, the intraocular portion of the optic nerve is divided into four regions: the superficial NFL, the prelaminar, laminar and retrolaminar regions (Anderson, 1969; Radius and Gonzales, 1981).

- The **superficial NFL** is supplied in principal from capillaries originating from the retinal arteries. It seems that the choroid does not have a contribution to the vascular supply of the superficial NFL region (Leiberman *et al.*,1976).
- The **prelaminar region** represents a small area situated anterior to the lamina cribrosa. This region receives its arterial supply from the short PCAs and from recurrent choroidal arterioles (Ernest, 1976; Olver *et al.*, 1990; Olver, 1990; Onda *et al.*,1995; Hayreh, 1996; Hayreh, 2001)
- The **laminar region** receives its vascular supply from the short PCAs. Ocasionally, the peripapillary choroid may also contribute with small arterioles to the laminar region (Anderson, 1969; Leiberman *et al.*, 1976).
- The **retrolaminar region** is supplied in principal by branches of the pial arteries and by the short PCAs. The CRA also contributes small branches (Olver *et al.*, 1990).

Hayreh (Hayreh, 1989) suggested there is distribution regions between adjacent PCAs in the peripapillary area that act as "watershed zones". These regions are located mainly in the temporal area of the optic nerve. The

existence of these zones could offer an explanation as to why the temporal area of the optic nerve is more susceptible to ischaemic glaucomatous damage than the other regions.

The capillaries supplying the ONH lack blood-brain barrier properties (Hofman *et al.*, 2001); it is possible that some molecules diffuse from the surrounding choroid and influence the ONH circulation (Flammer and Orgül, 1998).

Figure 1.2: Blood supply to the optic nerve. Adapted from Snell et. Lamp, 1998 (Snell and Lemp, 1998)

1.2.4. The venous drainage

The orbit is drained by the superior and inferior ophthalmic veins. The superior ophthalmic vein crosses the optic nerve together with the OA. Each of the two

ophthalmic veins receives two vorticose veins and drain into the cavernous sinus (Snell and Lemp, 1998).

The retinal blood drains via the central retinal vein (CRV), which leaves the eye by piercing the sclera in close vicinity to the CRA. The vein leaves the optic nerve about 10 mm behind the globe and after crossing the subarachnoid space and leaving the dura-arachnoid sheath, drains into the cavernous sinus or enters the superior ophthalmic vein (Hayreh, 1995; Snell and Lemp, 1998).

The venous drainage of the anterior optic nerve also occurs via CRV (Hayreh, 1995). In the peripheral laminar and retrolaminar regions, some optic nerve venous drainage may also occur via pial veins; however, these vessels also drain into the CRV (Onda *et al.,* 1995; (Hayreh, 1996).

1.3. Regulation of ocular blood flow

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

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

1.3.1. Metabolic autoregulation

The metabolic hypothesis of blood flow autoregulation suggests that perfusion and tissue metabolism are tightly coupled. Therefore, any reduction in arterial

blood flow results in an increase of vasodilator metabolites in the affected tissue; this could be the result of either insufficient washout or increase in the production of metabolites, or both (Kontos *et al.,* 1978; Sullivan and Johnson, 1981; Orgül, 1997). The metabolic products that may influence the vascular tone are adenosine, potassium (K^+), carbon dioxide (CO_2), pH and osmolality changes (Orgül, 1997). Moreover, changes in tissue oxygen (O_2) levels can also influence vascular tone (Johnson, 1986).

1.3.1.1. The role of O_2

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

Autoregulatory mechanisms in the inner retina maintain O_2 at constant values during systemic hyperoxia or hypoxia (Orgül *et al.,* 1995*c*). This process is of extreme importance since the intraocular vessels are very sensitive to changes in O_2. Hyperoxia results in a strong vasoconstriction of the inner retinal arterioles, in a manner similar to cerebral circulation (Hickam and Frayser, 1966; Riva *et al.,* 1983; Harris et al., 1996), while hypoxia induces vasodilation of the retinal arterioles similar to that observed in cerebral blood flow studies (Eperon *et al.,* 1975; Kogure *et al.,* 1970). The haemodynamic response to hyperoxia in the retinal circulation is believed to be mediated *via* endothelin (Takagi *et al.,* 1996).

1.3.1.2. The role of CO_2

In the cerebral circulation, decreased arterial blood oxygen saturation is compensated by increased cerebral blood flow (CBF) and consequently the oxygen supply to the brain normally remains unchanged (Hayakawa *et al.,* 1996). Hypercapnia leads to dilation of the cerebral arteries (Wei *et al.,* 1980), decreased cerebral resistance, and increased CBF (Kety and Schmidt, 1948). Changes in blood gas levels influence the retinal and optic nerve blood flow in a

manner similar to that in the cerebral circulation. In the eye, hypercapnia results in increased choroidal, retinal and retrobulbar blood flow (Friedman and Chandra, 1972; Deutsch *et al.,* 1983; Roff *et al.,* 1999; Bayerle-Eder *et al.,* 2000), just as acute hypoxic stress might result in increased ocular haemodynamic parameters (Mullner-Eidenbock *et al.,* 2000).

It has been demonstrated that in animal eyes hypercapnia results in acidosis with resulting changes in metabolism and consequent compromise in visual function (Hiroi *et al.,* 1994). Although one can expect that a high blood flow arising from hypercapnia would result in an improved visual function, (Hosking *et al.,* 2001*b*) showed that hypercapnia results in decreased contrast sensitivity (CS) in untreated glaucoma patients. One explanation for this finding is that in some glaucoma patients, the existence of vascular dysregulation could result in blood flow being redirected away from areas responsible for a good visual function (the so-called "steal phenomenon").

1.3.2. Myogenic autoregulation

By means of myogenic autoregulation, blood flow through different tissues is maintained despite changes in BP (Johnson, 1986; Flammer and Orgül, 1998). The myogenic mechanism is triggered by variation in the transmural pressure during moderate variations in BP, and is achieved by changes in the vascular resistance (Alm and Bill, 1973). The exact mechanism involved in myogenic regulation is still unknown. It seems, however, that extracellular calcium plays an important role; moreover, different endothelial factors could partially mediate the myogenic control of the blood flow (Davies and Hagen, 1993; Davies and Tripathi, 1993).

1.3.3. Neurogenic control of vascular tone

The ANS supplies a large network of vasomotor nerve fibres; these fibres are directed towards the uvea, the PCAs, and the extra-ocular portion of the CRA (Ehinger, 1966; Laties, 1967; Ernest, 1979). Vessels in the retina and prelaminar portion of the optic nerve, however, have no neural innervation

(Laties, 1967; Ye *et al.,* 1990). Consequently, stimulation of the cervical sympathetic chain results in vasoconstriction in the uvea but has no effect on retinal or anterior optic nerve blood flow (Weiter *et al.,* 1973; Bill *et al.,* 1977; Alm, 1977; Gherezghiher *et al.,* 1991).

It seems that the neurogenic control of the ocular circulation is mediated by a multitude of substances such as acetylcholine (Riva *et al.,* 1994*b*), noradrenaline (Riva et al., 1994*b*), substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide (VIP), nitric oxide (NO), neuropeptide Y, and adenosine triphosphate (ATP) (Nilsson and Bill, 1984; Stone *et al.,* 1989; Wienke *et al.,* 1994). The role played by each of these compounds in the physiology of ocular circulation is, however, still unknown.

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

1.3.4. Endothelium-dependent regulation of vascular tone

The vascular endothelium represents a very thin unicellular membrane that forms the luminal surface of all blood vessels. Endothelial cells produce a variety of substances in order to maintain the balance between vasoconstriction and vasodilation. It has been shown that endothelium control subjects the vasomotor tone, play a role in the coagulation pathways, regulate vascular structure, and mediate inflammatory and immunologic responses (Davies and Hagen, 1993). Vasoactive, endothelium-derived factors include prostanoids, NO and NO-containing compounds, smooth muscle cell hyperpolarization

factors, and endothelin. Furthermore, the local rennin-angiotensin system in the vessel wall is also important in vasomotor control (Orgül, 1997).

1.3.4.1. Endothelium-derived vasodilator factor

Production of the so-called endothelium-derived relaxing factor (EDRF) was first described by Furchgott and Zawadzki in 1980 (Furchgott and Zawadzki, 1980). They observed that the vasodilation induced by acetylcholine is dependent on the presence or absence of vascular endothelium. They concluded that the endothelium produces an EDRF, and plays a key role in the effects of acetylcholine on vascular tone. Later, NO was also recognized for its vasodilation properties (Palmer et al., 1987) and its roles in systemic and ocular vascular physiology have been investigated in depth.

Nitric oxide syntheses (NOS) are the enzymes responsible for NO generation. To date, three distinct NOS isoforms have been identified: neuronal NOS (type 1), inducible NOS (type 2) and endothelial NOS (type 3). Under resting conditions, NO synthesis has been mainly attributed to the vascular endothelium and its constitutively active NOS_3.

NO has important roles in the regulation of both systemic haemodynamics and those of individual organs. The roles of NO include:

- Antioxidative and neuroprotective effects by interaction with reactive free radicals (Chiueh, 1999);
- Mediating toxicity after excess glutamate release (Osborne *et al.,* 1999);
- Inhibition of the rolling and adhesion of leukocytes in microvessels (Hickey, 2001);
- Regulation of intestinal motility (Huang *et al.,* 1993), and;
- Role in penile erection (Chuang *et al.,* 1998).

Since NO has such complex and varied roles, any disturbances in its homeostasis could have important consequences. Indeed, it has been shown that systemic NO deficiency results in (Wood, 2003):

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

There has been extensive research into the role of NO on ocular circulation (Haefliger *et al.,* 1994*a*; Haefliger *et al.,* 1994*b*; Kiss *et al.,* 1999; Haefliger *et al.,* 1999; Koss, 1999; Schmetterer and Polak, 2001). In animal studies, NO has been able to modulate the contractile tone of retinal pericytes (Haefliger *et al.,* 1994*b*) and Müller cells (Kawasaki *et al.,* 1999). The human ocular angioarchitecture is, however, different from that of other species. Human experiments indicate that the arterioles and capillaries are in a constant state of vasodilatation, which is maintained by continuous release of NO (Vallance *et al.,* 1989).

In addition to the haemodynamic role, NO has also been shown to induce relaxation of the TM and the ciliary muscle resulting in a decrease in IOP (Wiederholt *et al.,* 1994). Moreover, in glaucomatous eyes, over-expression of NOS_1 in astrocytes and NOS_2 positive cells in the prelaminar region were demonstrated, suggesting excessive NO plays a role in glaucomatous ganglion cell apoptosis, while enhanced staining for NOS_3 is assumed to be a compensatory neuroprotective reaction (Neufeld, 1999).

NO has important physiological functions at both systemic and ocular levels. Any disturbance in the NO balance could, therefore, have dramatic consequences on the progression of glaucoma (Galassi *et al.,* 2004). Whether the high association between glaucoma and systemic vascular diseases suggests a common NO-dependent mechanism or whether the development of

glaucoma is initiated by chronic vascular dysregulation resulting from systemic vascular diseases is, however, still to be determined (Schmetterer and Polak, 2001).

1.3.4.2. Endothelium-derived vasoconstricting factors

Aside their roles in vascular relaxation, endothelial cells also produce potent vasoconsticting substances. The most potent endothelial vasoconstictive factor are endothelins (ETs), a group of 21-amino acid peptides. The endothelins exist in a family of isoforms, ET_1, ET_2, and ET_3, but only ET_1 is synthesized by the endothelial cells (Inoue *et al.,* 1989).

There are two major classes of endothelin receptors, ET_A and ET_B. The ET receptor B has been further subdivided into ET_{B1} and ET_{B2} based on their pharmacological responses (Douglas *et al.,* 1995). In vascular smooth muscle cells, the stimulation of ET_A receptors evokes marked and sustained vasoconstriction, while ET_B could mediate NO production. ET_1 and ET_2 have a similar affinity for the ET_A subtype, whereas ET_3 has much lower affinity for the ET_A receptor than for ET_B.

Endothelins have been found in high concentrations in the iris and ciliary body of rabbit eyes (MacCumber *et al.,* 1991). ET_1 and ET_3 have also been detected in the photoreceptor inner segments and outer plexiform layer of human and rat retinas (Stitt *et al.,* 1996). In human eyes, ET was found primarily in the fibrovascular stroma of the iris, ciliary body and choroid, in the retinal blood vessels, the ciliary and optic nerves, and in the corneal and nonpigmented epithelium (Wollensak *et al.,* 1998). Endothelin-like immunoreactivity was also detected in human and bovine AH (Lepple-Wienhues *et al.,* 1992). This finding could be an indicator of the role of ET_1in aqueous humor dynamics, since ET_1 can contract the ciliary muscle and trabecular meshwork and promote aqueous humor outflow (Erickson-Lamy *et al.,* 1991).

In human retina, ET_A-like receptor-binding sites were found in the neural and glial components of the retina (MacCumber and D'Anna, 1994). Moreover, Stitt et al. (Stitt *et al.,* 1996) found ET_A and ET_B receptors in the choroidal and retinal vessels of human and rat retinas, and Prasanna et al. has showed that ET_A receptors were expressed in human trabecular meshwork, ciliary muscle, and ciliary nonpigmented epithelium.

Both ET_A and ET_B receptors have been identified in the choroid, suggesting that they could be involved in choroidal blood flow regulation (Wollensak *et al.,* 1998; MacCumber and D'Anna, 1994). In the choroid, the interaction between ET_B-mediated NO production and ET_A-mediated vasoconstriction could occur, though choroidal blood vessels appear to be more sensitive to NO than to ET in overcoming resistance (Kiel, 1999).

It has been suggested that ET_1 could play a role in promoting vasospasm and abnormal autoregulation of the retinal microcirculation (Haefliger *et al.,* 1999; Orgül *et al.,* 1996; Gass *et al.,* 1997). This observation together with the fact that patients suffering from NTG demonstrate increased plasma ET_1 levels compared with matched control subjects (Sugiyama *et al.,* 1995; Kaiser *et al.,* 1995) emphasize the role of vascular dysregulation in glaucoma pathogenesis. Numerous reports exploring an ET_1 -induced reduction in retinal blood flow suggest that ET_1 could promote ischaemic damage in the ONH (Ciulla *et al.,* 2000; Toriu *et al.,* 2001).

1.4. Ocular perfusion pressure

The blood flow throughout the body depends on the regional vascular perfusion pressure, which is defined as the difference between the arterial and the venous pressure. The driving force of ocular blood flow is termed the ocular perfusion pressure (OPP) and represents the difference between the pressure in the arteries entering the eye and the pressure in the veins leaving it (Alm, 1998). The pressure in the arteries entering the eye cannot be determined, and as a rule the mean arterial BP (MBP) in the brachial artery is used as a

substitute (Alm, 1998). MBP is defined as the diastolic BP (DBP) plus one-third of the pulse pressure (PP), which is defined as the systolic minus diastolic BP:

$$PP=SBP-DBP$$

- PP= Pulse pressure
- SBP= systolic blood pressure
- DBP= diastolic blood pressure

Equation 1.1: Calculation of pulse pressure

Due to the loss of pressure between the heart and the eye, the pressure in the arteries entering the eye is 35-40 mmHg lower than MBP as determined in the brachial arteries when we stand up (Alm, 1998). The pressure in the veins leaving the eye is almost the same as the IOP; the OPP can therefore be calculated according to the formula (Alm, 1998):

$$OPP = 2/3(MBP - IOP)$$

- OPP= ocular perfusion pressure
- MBP= mean blood pressure
- IOP= intraocular pressure

Equation 1.2: Calculation of ocular perfusion pressure

According to this formula, blood flow is reduced if MBP is reduced or IOP increased; however, this is true unless there is a concomitant change in the vascular resistance. In most tissues, such changes in OPP are compensated for by a change in the vascular resistance, and the blood flow is kept at the same level despite moderate changes in OPP. In the eye, blood flow through the retina is autoregulated, and in a healthy eye retinal blood flow is essentially unchanged up to an IOP of about 35 mmHg (Geijer and Bill, 1979; Riva *et al.,* 1982).

1.5. Ocular vascular dysregulation

Blood flow through tissues and organs is regulated by perfusion pressure and local resistance to flow (Flammer *et al.,* 2002), which in turn is influenced by the diameter of the vessel. Blood flow in the retina and ONH is well regulated (Yao *et al.,* 1991; *et al.,* 1992; Meyer *et al.,* 1995; Riva *et al.,* 1997; Zhu *et al.,* 1997). However, these tissues can become ischaemic and this could happen when either the autoregulatory capacity is exceeded or if regulatory mechanisms are defective (Grünwald *et al.,* 1984; Flammer *et al.,* 1999; Flammer *et al.,* 2001). The former situation is believed to occur when the IOP increases or when the BP is markedly decreased (Flammer *et al.,* 1999). The existence of progressive glaucomatous damage in patients with a mild reduction in or normal OPP, together with the fact that IOP plays an important role even in NTG cases (Cartwright and Anderson, 1988; Crichton *et al.,* 1989; Haefliger and Hitchings, 1990; Araie *et al.,* 1994) is indicative of defective autoregulation as a possible causative mechanism. Defective autoregulation may occur due to either a vasospastic syndrome, characterised by an increase in overall vascular response to challenges such as cold or stress (Saner *et al.,* 1987; Mahler *et al.,* 1989; Flammer *et al.,* 1999; Flammer *et al.,* 2001), atherosclerosis (Buchi, 1996), or damage to autonomic nerve fibres (Miwa *et al.,* 1998). While the role of atherosclerosis in the pathogenesis of glaucomatous optic neuropathy is debatable (Flammer *et al.,* 1999), vasospastic syndrome and autonomic dysfunction could indeed interfere with OBF.

1.6. The vasospastic syndrome

Vasospastic syndrome can be grouped as primary, when it occurs without any underlying disease, or secondary when underlying disorder arises due to a vascular endotheliopathy or an increased level of circulating ET_1 (Nakamura *et al.,* 1999; Flammer *et al.,* 2001; Zuccarello, 2001).

Individuals affected by primary vasospastic syndrome have a tendency towards cold hands and/or feet, low BP (Orgül *et al.,* 1995*b*), a slim build, and slower sleep onset (Pache *et al.,* 2001; Flammer *et al.,* 2001). Identifying primary vasospastic syndrome is of clinical importance because it may be causatively linked to ocular diseases such as POAG (Gasser *et al.,* 1986). Because vasospasm is often combined with simultaneous arterial and venous dilatation in neighbouring vessels, Flammer et al. introduced a new term "vascular dysregulation" used to describe this condition (Flammer, 1998).

1.6.1. Cutaneous blood flow

The observation of parallel changes in peripheral blood flow and VF in vasospastic subjects led to the definition of an entity called „presumed ocular vasospastic syndrome", implying that the regulation of blood flow of various regions of the body surface may show some parallelisms (Flammer *et al.,* 1992; Gasser et al., 1999*a*). Therefore, studying peripheral circulation in patients suffering from glaucoma could offer a better understanding of the OBF disturbances in the context of a systemic vascular dysregulation.

1.6.1.1. Anatomy and physiology

The vasculature of the superficial skin is organized into two horizontal plexi of arterioles and venules; one located just below the papillary dermis (1-1.5 mm below the surface) and one at the dermal-subcutaneous junction (3-5 mm below the surface). The upper (superficial) plexus supplies the individual dermal papillae with nutritive capillary loops and the lower (deep) plexus provides supply to the hair follicles and seat glands (Figure 1.3).

Figure 1.3: Schematic representation of the skin circulation

Skin blood flow has, as a prime function, the regulation of core body temperature. The superficial plexus acts as a large thermal radiator and has a blood-flow range of 1ml/min per 100g of tissue in the cold to 200 ml/min per 100 g tissue in the hot (Levick, 1995). These extremes of flow are possible through the regulation of numerous arteriovenous communications by the ANS. The acral areas (hands and feet) in particular possess abundant direct connections between arterioles and venules; the vessels in these areas are controlled almost exclusively by sympathetic nerve fibers.

The cutaneous vessels react rapidly to any temperature challenge. Local heating of the skin causes dilatation of the arterioles, venules and capillaries, while local cooling causes vasoconstriction.

1.6.1.2. The Cold Pressor Test

The cold pressor test (CPT), represented in its classical form by immersion of one hand in cold water, is a well-known stress test able to induce a reproducible sympathetic activation (Tassorelli *et al.,* 1995). So far it has been used widely in cardiovascular research to determine the cardiac and vascular reactivity to cold in normotensive and hypertensive subjects (Velasco *et al.,* 1997).

Cold provocation results in stimulation of Krausse corpuscles and Ruffini organs; the afferent fibers enter the spinal cord and then generate lateral spinothalamic tract, which travels to the medulla, pons and medium cerebrum. At the medulla level the collaterals reach the so-called pressor area; when stimulated, this area discharges impulses through a sympathetic efferent way towards the vessels and heart. These impulses result in significant increase in systemic BP and in cutaneous vasoconstriction (Velasco *et al.*, 1997) (Figure 1.4).

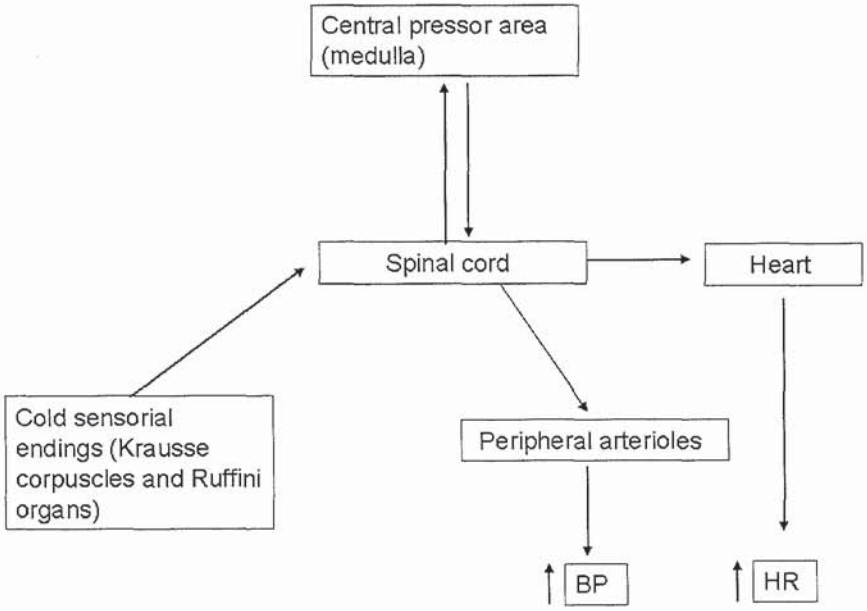


Figure 1.4: Schematic representation of the neural pathway for the cold pressor test. BP: systemic blood pressure; HR: heart rate.

The CPT results in an increased level of plasma catecholamines (Kaul *et al.*, 1973; Johnson *et al.*, 1977). The immediate effect of this response is an increase in systemic BP (Raven *et al.*, 1970; Benetos and Safar, 1991; Weise *et al.*, 1993). Cardiac filling pressure, left ventricular end-diastolic pressure and

volume and stroke volume are also increased (Muza *et al.*, 1988; Voegelaere *et al.*, 1992).

Cold leads to a fast decrease in skin temperature accompanied by a strong vasoconstriction of cutaneous blood vessels, limiting heat transfer to the environment (Johnson and Proppe, 1996; Lafleche *et al.*, 1998) (Figure 1.5).

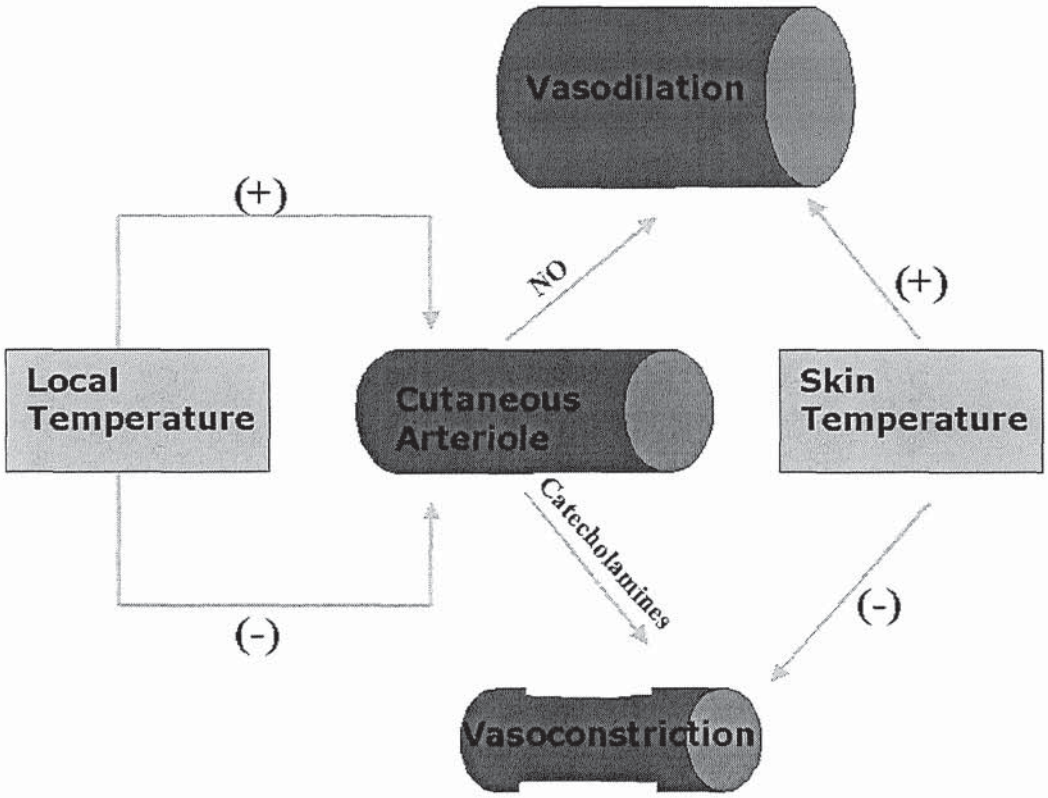


Figure 1.5: Thermoregulatory control of skin blood flow: schematic representation. Adapted from Charkoudian et al., 2003 (Charkoudian, 2003).

Although cold provocation has a dramatic effect on the systemic vasculature, little is known about its consequences on ocular blood flow. Rojanapongpun and Drance (Rojanapongpun and Drance, 1993) and Nicolela et al. (Nicolela *et al.*, 2003) failed to report any blood flow changes in the ophthalmic artery and

retinal circulation of patients suffering from POAG as a response to cold stimulation. Nevertheless, in patients suffering from vascular dysregulation manifested by peripheral vasospasm, VF defects have been reported after cold provocation (Guthauser *et al.,* 1988; Mahler *et al.,* 1989); it has been suggested that this effect could be the result of an ocular circulatory insufficiency. Since vascular dysregulation has been demonstrated to play a role in the pathogenesis of glaucomatous neuropathy (Guthauser *et al.,* 1988; Harris *et al.,* 1994; Pillunat *et al.,* 1994; Flammer *et al.,* 2001; Hosking *et al.,* 2004) we can hypothesize that cold stimulation could result in an abnormal hemodynamic response in normally autoregulated ocular vascular beds.

1.6.1.3. Peripheral Laser Doppler Flowmetry

For the purpose of the present thesis, a summary will be provided of the blood perfusion monitor Perimed Laser Doppler Perfusion Monitor (PF 5010: Perimed, Stockholm, Sweden) that uses a low-power solid-state laser diode as a coherent source of light. The apparatus and the technique have been described in detail by Nilsson et al. (Nilsson *et al.,* 1980*a*; Nilsson *et al.,* 1980*b*). In brief, this device measures microvascular perfusion using a laser beams which, when it strikes an object in motion (such as red blood cells), undergoes a change in light frequency known as the Doppler shift. In the cutaneous LDF, a fibre optic probe is placed against the skin and the beam of monochromatic light is produced from a Helium-Neon laser of a wavelength between 543 nm and 780 nm. Incident laser light is absorbed, transmitted or scattered by underlying skin tissue. Light reflected from moving blood cells is subject to a Doppler shift. The total scattered light consists of the original frequency (reflected from static objects) and slightly shifted frequencies above and below the original (reflected from moving corpuscles such as red blood cells). The light scattered back has its frequency scattered around the original incident frequency. The amount of frequency shift can be calculated according to the following Equation:

$$df = (2\,\pi/\lambda)\,(K_s - K_i) * V$$

- *df:* shift in frequency;
- K_s: wave vector of scattered light;
- K_i wave vector of incident light;
- V: the velocity of the moving object;
- λ: the wavelength of the incident light.

Equation 1.3: Optical Doppler shift calculation.

The reflected light is therefore composed of a spectrum of frequencies each associated with a particular intensity (Bonner and Nossal, 1990). If this spectrum is integrated across the relevant frequencies, a quantity that describes the distance travelled by all the moving particles inside the sample volume per unit time is calculated. Similarly the Doppler shift power spectrum can be integrated to find the mean number of photon collisions with moving particles and this is proportional to the number of moving corpuscles. These quantities, termed "flow" and "volume" form the basis of laser Doppler flowmetry.

Peripheral blood flow measurements are performed by attaching an optic probe to the skin of the terminal phalanx of one of the fingers (usually the middle one) using a purpose-made double-side adhesive tape. The light penetrates the skin to a depth of about 1mm. Some light is absorbed by the tissue and some is reflected back to the probe that contains a photodiode receiver. LDF flow parameter "flow" is recorded in arbitrary units (AU) and displayed on a monitor. The device can be connected to the serial port of an IBM compatible PC with an optoelectronic interface. The final data reading consists on graphical or worksheet-like displays, printouts and statistical analysis for evaluation.

The LDF recordings are influenced by:

a. Position of the probe

Cutaneous blood flow varies greatly not just between regional body areas but also within a regional zone. It has been shown that LDF principally measures blood flow from the superficial dermal plexus and, in particular, from blood originating from the ascending arteriole just below the papillae (Nilsson *et al.,* 1980*a*; Nilsson *et al.,* 1980*b*). As these ascending arterioles are randomly distributed every 1.5 to 7mm across the skin's surface, large temporal and spatial variations in blood flow measurement occur depending on the exact position of the probe. Nevertheless, in a recent study Freccero et al. (Freccero *et al.,* 2003) showed clearly that blood velocities measured at the fingertip were similar to those measured at the forearm level.

b. Skin and ambient temperature

The thermoregulatory control of the skin is important for a good thermal homeostasis of the human body (Charkoudian, 2003). In order to assure this the peripheral blood flow follows every change in human cardiovascular reflexes to different thermal challenges (Peters *et al.,* 2000). In response to heat, skin blood flow can reach 6 to 8 litres/minute while during cold exposure while vasoconstriction in the skin reduces heat loss from the body (Johnson and Proppe, 1996). Even smaller changes in the peripheral skin temperature and reflected by the blood flow circulation can be picked up and recorded by the PF device.

c. Vasomotion

There is evidence that LDF recordings are influenced by vasomotion (Engelhart and Kristensen, 1986; Gniadecki *et al.,* 1992). Vasomotion, or "flow motion", is the constriction and dilation of the pre-capillary arterioles that have a peak cycling frequency of 6-10 cycles per minute (Figure 1.6). There is evidence that vasomotion is partially under sympathetic nervous control as vasomotor frequencies are accentuated during baroreceptor stimulation.

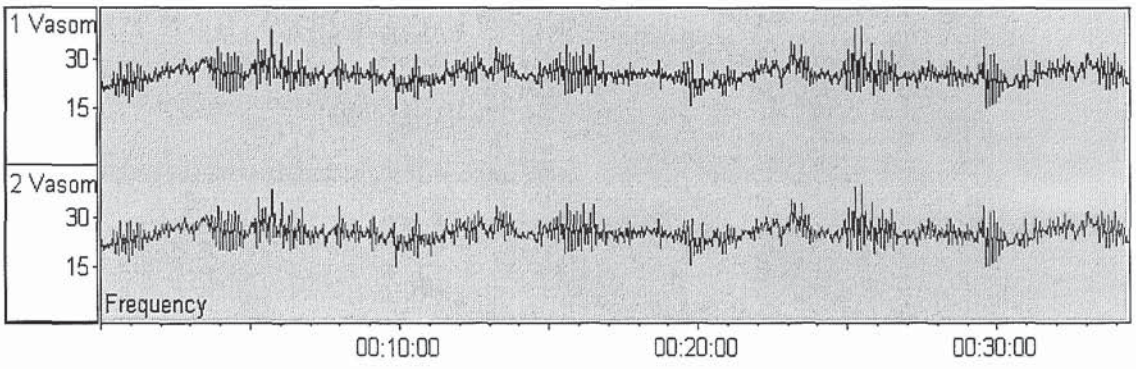


Figure 1.6: Vasomotion recorded using PeriFlux System 5001

d. Seasons

It seems that there are seasonal differences in skin temperature and blood flow, and these differences are significant even if the recordings are made in temperature-controlled laboratory (Gardner-Medwin *et al.,* 2001). These differences are more accentuated in women than in age-matched men. The effect of oestrogen on endothelium-dependent vasodilation (Thompson and Khalil, 2003) could be responsible for this difference.

1.6.2. Vasospasm and ocular blood flow

It has been demonstrated that otherwise healthy, young subjects suffering from peripheral vasospasm have altered blood flow regulation in the retrobulbar vessels (Gherghel *et al.,* 1999). How angiospasm might lead to overt tissue damage is still not well understood. Since the majority of patients with vasospastic diathesis do not develop glaucoma, vasospasms might simply increase the susceptibility of the optic nerve to increased IOP and low BP levels. Therefore, it is conceivable that vasospastic patients with IOP in the high teens or subjects with BP dips might develop overt ischaemic tissue damage.

It has been suggested that vasospastic syndrome could affect ocular blood flow (OBF) in two ways. Firstly, patients suffering from vasospastic syndrome tend to have, on average, lower systemic BP (Flammer *et al.,* 2001; Pache *et al.,*

2003), thus having an increased risk for periods of low perfusion pressure. Secondly glaucoma patients often demonstrate disturbed autoregulation, which might be a manifestation of the primary vasospastic syndrome (Gherghel *et al.,* 2000). Reduced OBF may in some cases be the result of an insufficient adaptation to low perfusion pressure (Gherghel *et al.,* 2000); all these observations seem to point towards a crucial involvement of vascular regulation/dysregulation in the pathogenesis of POAG.

1.6.3. Vasospasm and glaucoma

The relationship between vasospastic syndrome and glaucoma is most interesting. Glaucoma is a disease characterized by progressive excavation of the ONH, and associated characteristic VF defects (Van Buskirk and Cioffi, 1992). Increased IOP is no longer included in the definition of glaucoma, and clinical studies indicate that, although high IOP is a very important risk factor (Krakau, 1981; Bonomi *et al.,* 2001), other factors may be involved. Vascular dysregulation, leading to ocular vasospasm, or impaired autoregulation have been advocated as possible contributing factor in the aetiology of glaucoma (Flammer, 1994; O'Brien, 1998). This vascular dysregulation could make the eye more sensitive to an increase in IOP and to BP fluctuations (Flammer *et al.,* 1999; Flammer *et al.,* 2001). The possible involvement of vascular dysregulation in the pathogenesis of glaucoma could explain some other observations:

- The conversion rate of ocular hypertension to glaucoma is low, indicating that not all patients with high IOPs develop glaucoma (Bengtsson, 1981);
- Glaucomatous neuropathy can occur in the presence of statistically normal IOP; a condition known as NTG;
- Patients suffering from glaucoma, especially from NTG, have a higher plasma concentration of ET_1 (Kaiser *et al.,* 1995; Stokely *et al.,* 2002; Yorio *et al.,* 2002; Nicolela *et al.,* 2003);

- Patients suffering from glaucoma, especially NTG have an increased incidence of optic disc haemorrhages (Sonnsjo *et al.,* 2002);
- NTG is more common in women than in men (Orgül *et al.,* 1995*a*). High oestrogen levels are thought to be responsible for the low tolerance to cold and higher tendency towards vasospasm in women than in men (Tsen *et al.,* 2001). Due to the involvement of vascular risk factors in the aetiology of NTG, this observation could explain the high incidence of this type of glaucoma among women;
- Some glaucoma patients continue to deteriorate despite therapeutically well-controlled IOP. In these patients factors other than IOP seem to play a principal role in the pathogenesis of the disease (Harris *et al.,* 2001; Tezel *et al.,* 2001);
- It has been suggested that glaucoma patients exhibiting an exaggerated nocturnal dip in systemic BP of more than 20% of the diurnal BP values are more prone to VF defect progression than those with a normal dip in systemic blood pressure in the range10%-20% of the diurnal value. Kaiser et al. (Kaiser *et al.,* 1993*b*) showed that the frequency of large blood pressure dips in either progressive open-angle glaucoma or NTG was higher than in POAG patients with stable VF defects or normal control subjects. Other authors have also showed the importance of low nocturnal BP values in patients with both NTG and progressive POAG (Graham *et al.,* 1995; Meyer *et al.,* 1996; Muzyka *et al.,* 1997; Collignon *et al.,* 1998; Graham and Drance, 1999). Since a relationship between vasospastic disorders and systemic hypotension has already been reported (Gasser, 1991) one could hypothesize that low systemic BP is a manifestation of vascular dysregulation with a direct influence on blood flow (Gherghel *et al.,* 2001). There are, however, reports which fail to find a correlation between nocturnal BP dips and glaucoma. Detry et al. (Detry *et al.,* 1996) found a significantly smaller dip in the nocturnal BP in the progressive POAG patients compared to the control non-progressive group. Kashiwagi et al. (Kashiwagi *et al.,* 2001) also reported that the nocturnal BP dip in NTG patients showing progression was significantly smaller than in patients with stable visual field defects. The authors

hypothesized that the reduction in the nocturnal BP dip could be a consequence of some micro damage in the part of the cerebrum involved in regulating the autonomic activity. This hypothesis is yet to be tested.

These evidences suggest that vasospastic syndrome and/or vascular dysregulation should be considered risks factors for GON acting in concert with IOP (Henry *et al.,* 1997; O'Brien, 1998; Flammer *et al.,* 2002).

1.7. Autonomic dysfunction and ocular blood flow

Defective perfusion has been demonstrated to play a role in the appearance and exacerbation of glaucomatous optic neuropathy (Flammer and Orgül, 1998; Flammer *et al.,* 1999; Cioffi, 2001; Flammer, 2001; Flammer *et al.,* 2002; Ben Simon *et al.,* 2003). In addition, patients with glaucoma could suffer from ischemic lesions in other organs and systems dependent on a good local perfusion such as the brain (Stroman *et al.,* 1995), the ear (Susanna and Basseto, 1992), and the heart (Waldmann *et al.,* 1996). The hemodynamic control unit of the body is supervised and influenced by the ANS. Consequently, any autonomic disturbances could have local and systemic pathological circulatory implications relevant to glaucoma pathogenesis. Indeed, POAG and NTG patients have been reported to suffer from sympathetic, and parasympathetic neuropathies (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Kashiwagi *et al.,* 2000; Brown *et al.,* 2002). Nevertheless, further research is needed to prove a direct relationship between glaucoma, associated OBF disturbances and ANS failure.

1.8. The concept of reperfusion damage

Prompt reperfusion of any tissue after transient periods of ischaemia is critical for restoring its normal function. However, this "return of blood flow" can also produce progressive tissue destruction, starting with a marked structural injury of the endothelial cells (Kaeffer *et al.,* 1996). Negovskii (Negovskii, 1962) was

the first to propose that the extent of tissue injury observed following ischaemia might actually reflect damage incurred during reperfusion.

Oxidative stress, defined by the presence of pathological levels of intracellular concentrations of reactive oxygen species (ROS), is the main mechanism for triggering tissue ischaemia-reperfusion damage (Lloberas *et al.,* 2002). The production of free radicals increases markedly during reperfusion (Schempp and Elstner, 1998). An increase in the level of free radicals results in endothelial dysfunction (Nedeljovic *et al.,* 2003) and interferes with the re-uptake of glutamate (Brusini *et al.,* 2000) leading to an increase in extra-cellular glutamate concentration and resulting exocitotoxicity . During exocitotoxicity, the production of NO is also altered. Superoxide anions produced on reperfusion react with NO increasing the damage even further (Haefliger *et al.,* 1999*b*). A perturbed NO level results in both an increased platelet aggregation (Radomski *et al.,* 1987) and rapid adhesion of neutrophils to endothelial cells (Thiagarajan *et al.,* 1997). Decreased NO production may also trigger an acute inflammatory response. The final picture of the reperfusion-induced damage is therefore characterised by the co-existance of vasospasm, inflammation and thrombosis (Laude et al., 2002).

The role of ischaemia-reperfusion injury has been extensively studied in cardiovascular medicine, especially in relation to myocardial infarction. A similar pathological process could also occur in other organs affected by ischaemia, including the eye. In glaucoma patients for example, decreased production of NO could shift the balance between vasoconstricting and vasodilating endothelial active agents (Schmetterer and Polak, 2001). The resultant vascular dysregulation would predispose the ONH to ischaemia and reperfusion damage. Moreover, fluctuation in OBF may also result in repeated, reperfusion injuries (Kashiwagi *et al.,* 2001). Therefore, it has been suggested that all these factors could contribute to GON progression.

1.9. Current techniques for evaluating ocular blood flow

To investigate the role of vascular risk factors in glaucoma's pathogenesis, investigation of blood flow circulation through ocular vessels is of extreme

importance. Today, OBF is investigated using a large variety of techniques. Although all of them have many advantages, none provides all the needed information in one reading (Flammer *et al.,* 2002). The techniques currently available for measuring OBF are outlined in the Table 1.1.

Method	Vascular bed	Parameter
OBF Analyser	Choroid	IOP, pulsatile choroidal blood flow
Laser interferometry	Choroid ONH	Pulsatile OBF
Laser Doppler velocimetry	Retina	Blood velocities in the retinal vessels
Laser Doppler flowmetry	ONH Choroid	Capillary blood flow at the ONH and choroidal level
Blue field entopic technique	Foveal retina	Capillary retinal blood flow
Retinal vessels analyser	Retina	Retinal vascular diameter
Fluorescein angiography	Retina	Retinal blood velocity
Indocyanine green angiography	Choroid	Choroidal blood flow velocity
Heidelberg Retina Flowmeter	ONH Retina	ONH and retinal capillary blood flow
Color Doppler Imaging	Retrobulbar vessels	Retrobulbar blood flow velocities

Table 1.1: Methods for measuring ocular blood flow. OBF: ocular blood flow; IOP: intraocular pressure; ONH: optic nerve head

For the purpose of the present thesis only the OBF Analyser and Heidelberg Retina Flowmeter will be discussed below.

1.9.1. Pulsatile Ocular Blood Flow

The OBF tonometer measures the pulse wave of the rhythmic change in IOP during the cardiac cycle (Langham *et al.,* 1989; Langham, 1994; Kergoat, 1997) (Figure 1.7) .

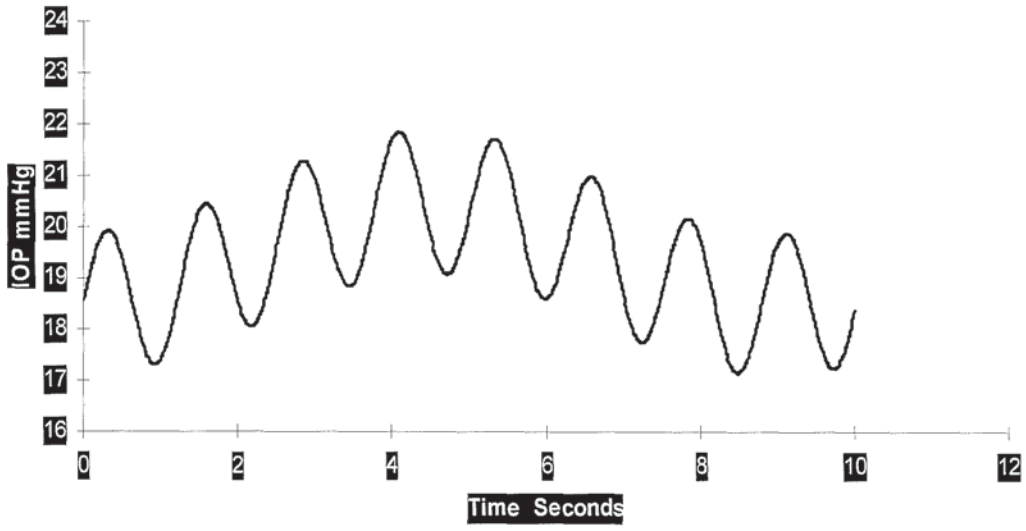


Figure 1.7: Typical intraocular pressure recording over 10 seconds using the OBF tonometer.

The instrument consists of a modified pneumotonometer interfaced with a microcomputer that records the ocular pulse. The amplitude of the IOP pulse wave is used to calculate the change in ocular volume, and thereby to calculate the pulsatile component of OBF.

A number of refinements since the original Langham POBF system have culminated in the presently available instrument: the OBF Blood Flow Analyser (OBFA – Paradigm Medical Industries Inc., Utah, USA). This system measures changes in IOP, which are caused by pulsatile rhythmic filling of the intraocular vessels, with a pneumatic applanation tonometer. Pulse amplitude (PA) represents the maximum change in IOP during the cardiac cycle. Pulse volume (PV) is an estimate of the change in intraocular volume determined using an approximate pressure-volume relationship, known as the derived ocular pulse volume (Langham, 1987). Based on a theoretical model eye, the pulsatile ocular blood flow (POBF) is calculated from the IOP variation with time (Silver and Farell, 1994). This hydrodynamic model is based on the assumption that venous outflow from the eye is non-pulsatile. Moreover, the ocular rigidity, which is used to derive ocular volume changes from changes in IOP, is

assumed to be standard for all the subjects. The calculation of POBF is automatically derived from the five pulses that are most similar to each other in IOP beat-to-beat variation (Schmetterer *et al.,* 1998).

The OBF instrument consists of a probe and a base unit that are connected via plastic piping. The disposable probe tip is of a similar design to the original Perkins' pneumotonometer and has a central channel along which pressurised air, produced from the base unit, flows (Figure 1.8).

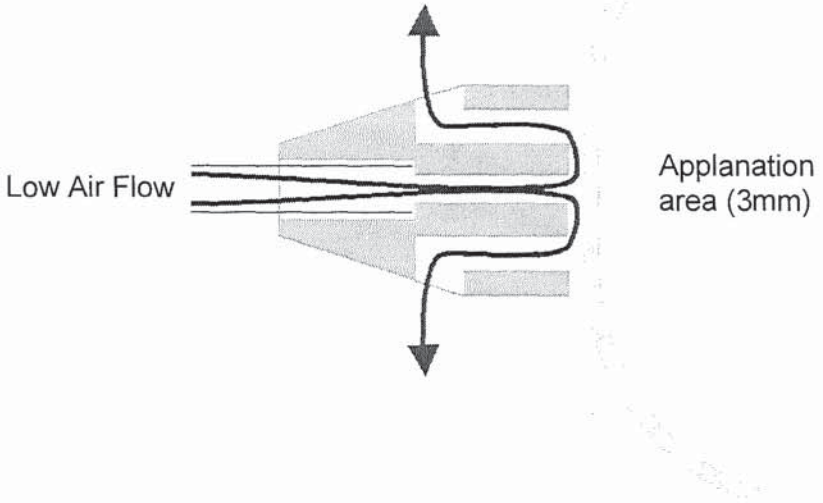


Figure 1.8: The air flowing through the piston enters the tip form the rear and move forward to the eye.

The tip of the probe is capped, but not sealed, with a thin viscoelastic membrane. The OBF tonometer can be used either mounted on a slit-lamp or in a hand-held device. The cornea is applanated and the opposing corneal pressure under the centre of the probe is balanced against the pressurised air produced by the base unit. If the pressurised air exceeds the corneal pressure, the seal between the probe and membrane is broken and excess air escapes into the probe's side channels. The resistance to air flow by the cornea (combined result of IOP and ocular rigidity) will result in varying degrees of back

pressure in the piston chamber. A transducer in the base unit calculates the IOP continuously from the air pressure in the probe.

In order to take a measure the probe is placed against an anaesthetised cornea. The recording of the measurement lasts between 5 and 20 seconds. The instrument searches for 5 similar and satisfactory pulse waveforms; if these are not found within 20 seconds an error message occurs. As well as producing a measure of POBF, the standard output also provides average IOP, PA, PV and pulse rate (PR).

1.9.1.1. Factors influencing pulsatile ocular blood flow

a. Refractive error

It has been demonstrated that myopic eyes have significantly lower PA and POBF values than emmetropic and hyperopic eyes (Shih *et al.,* 1991; Ravalico *et al.,* 1997; Lam *et al.,* 2002). This could be the effect of either a larger ocular or a smaller choroidal volume of myopic eyes that of emmetropic and hyperopic eyes (Cheng *et al.,* 1992). Other factors could also play a role. It is well known that myopic eyes exhibit the following characteristics:

- Changes in scleral fibres' pattern and elasticity, with a more distensible ocular shell producing a smaller pressure change for a given change in volume (Phillips and McBrien, 1995);
- Thinner choroidal beds (as demonstrated by Magnetic Resonance Imaging studies) (Cheng *et al.,* 1992).

b. *Axial length*

Several authors have reported a relationship between the ocular axial length and POBF and PA (James *et al.,* 1991; Ravalico *et al.,* 1997; Shih *et al.,* 1991; Mori et al., 2001). Mori et al. (Mori *et al.,* 2001) have suggested that, in normal subjects as axial length increases, POBF decreases; the authors concluded that the axial length is the major contributory factor to POBF in a normal population.

c. Scleral rigidity

Any factors that stop the tonometer-eye relationship conforming to the ideal Imbert-Fick law (Pressure= Force/Area) could influence the applanation-type tonometry. Factors such as surface tension, corneal thickness and scleral rigidity (Gloster and Perkins, 1963) may indeed have an important effect on measurements taken using OBF tonometer.

There are a number of studies showing that POBF has a positive correlation with scleral rigidity (K), (Kothe, 1994). However as the coefficient of rigidity, defined as the resistance offered by the eye to a change in intra-ocular volume, incorporates the volume of a standard eye, it is unclear how much other individual components, such as scleral elasticity and corneal thickness, affect POBF. Nevertheless, aging has been shown to result in POBF reduction (Ravalico *et al.,* 1996; Mori *et al.,* 2001; Geyer *et al.,* 2003; Lam *et al.,* 2003). Since aging also affects scleral elasticity, it can be concluded that low scleral rigidity results in low POBF readings.

Central corneal thickness (CCT) and corneal curvature are important factors to consider when measuring IOP using OBFA. In a recent study, Gunvant et al. (Gunvant *et al.,* 2004) showed that IOP measured using OBFA increased by 0.048 mmHg per µm increase in CCT and by 1.14 mmHg per mm increase in corneal curvature. Since POBF is calculated from IOP variation with time, it can be hypothesized that CCT and corneal curvature could also have an effect on blood flow readings taken using OBFA.

d. Age

Several studies have shown that POBF decreases with age (Ravalico *et al.,* 1996; Mori *et al.,* 2001; Geyer *et al.,* 2003; Lam *et al.,* 2003). Therefore, age should be considered in all studies that evaluate the haemodynamic aspects of various pathologies affecting the eye.

e. Heart rate

In 1991, Trew et al. (Trew *et al.,* 1991) measured POBF whilst setting the HR of patients with pacemakers at different frequencies. They found that the POBF value was at its highest point at a HR of around 90 beats/minute (bpm). PA, PV and stroke volume measurements were negatively correlated with changes in HR.

A number of investigators have studied the effect of exercise on POBF (Schmidt *et al.,* 1996; (Schmetterer *et al.,* 1998; Lovasik and Kergoat, 2004). In healthy subjects, the normal cardiovascular response to exercise is represented by an increase in both HR and stroke volume (Levick, 1995). After bike exercise Schmidt et al. (Schmidt *et al.,* 1996) found that, despite a high increase in HR, there was no change in PA. In contrast, Lovasik and Kergoat (Lovasik and Kergoat, 2004) found that the duration of the systolic and diastolic phases of the intraocular pulse was shortened, and the PA and PV were reduced after bike exercise. They also found that the POBF increased by 18% compared to the baseline values.

Isometric exercise, such as hand grip, results in significant systemic BP increment. However, Schmetterer et al. (Schmetterer *et al.,* 1998) found no changes in PA or POBF values when subjects underwent isometric exercise.

f. Posture

POBF falls on assuming the supine position (Trew and Smith, 1991*a*; Trew and Smith, 1991b). Initially, this was interpreted as a sign of impoverished blood flow to the eye. However, the majority of the reduction can be explained by the concurrent drop in HR and the increase in non-pulsatile blood flow (Trew and Smith, 1991*a*; Trew and Smith, 1991*b*).

g. Valsalva manoeuvre

The Valsalva manoeuvre represents air expiration against an enforced pressure such as against a closed glottis. During this manoeuvre, the high intrathoracic pressure impedes the return of the venous blood to the heart. This in turn reduces cardiac stroke volume and thus the PA at the eye level. Moreover, POBF also decreases (Schmetterer *et al.,* 1998). This result was

later confirmed by Khan et al. (Khan *et al.,* 2002) who also found that men had a greater drop in POBF during Valsalva manoeuvre than women, possible due to the higher resting heart rate in females.

h. Respiratory CO_2 levels

It has been demonstrated that breathing either 5% CO_2 mixed with room air or 5% CO_2 mixed with 95% O_2 (Carbogen) results in an increase in both the amplitude of the IOP pulse and the POBF measure (Kergoat and Faucher, 1999; Schmetterer *et al.,* 2000). By raising their subjects' end-tindal respiratory CO_2 Level by approximately 15%, Roff et al. (Roff *et al.,* 1999) did not find, however, any changes in POBF.

i. Gender

Gekkieva et al. (Gekkieva *et al.,* 2001) found that compared to men, women showed higher POBF and PA even after correcting for age, refraction, IOP, BP and PR. Centofanti et al. (Centofanti *et al.,* 2000) found that pre-menopausal women have a significant higher POBF and PA than men and post-menopausal women. In addition, the same group found that POBF increases throughout gestation (Centofanti *et al.,* 2002), possible due to an increase in oestrogen which induces endothelial-dependent vasodilatation in several tissues including ocular vessels. How the observed differences in the POBF might influence the preponderance of various ocular diseases in men or women remains to be clarified.

j. Circadian rhythm

A prolonged supine position during sleep may result in a reduction in both ocular perfusion pressure and ocular blood flow due to the combined effects of the IOP rise (Trew and Smith, 1991*a*; Trew and Smith, 1991*b*) and an accompanying fall in systemic BP (Takakuwa *et al.,* 2001). Claridge and Smith (Claridge and Smith, 1994) have demonstrated that nocturnal POBF is unchanged compared to daytime values in normal subjects, ocular hypertensive and glaucoma patients; however, there was a large individual variation with some subjects showing a nocturnal fall in POBF. The authors concluded that these particular individuals may suffer from a disturbed

autoregulation and could be at greater risk of developing glaucomatous damage.

1.9.2. Scanning Laser Doppler Flowmetry

Scanning laser Doppler flowmetry has been introduced in clinical and research practice by Riva et al., in 1972 (Riva *et al.,* 1972) as a method able to measure in a non-invasive way, the perfusion of the retina and the ONH. Scanning laser Doppler flowmetry represents a combination of laser Doppler flowmetry and laser scanning technology.

1.9.2.1. The Doppler effect

The optical Doppler effect represents an alteration to the frequency of light that is emitted or reflected from a moving object. A good approximation of the optical Doppler frequency shift can be derived from the Equation 1.3. Another way to calculate the Doppler shift is given below:

$$df/f = v/c \times \cos a$$

- df: frequency shift
- f: frequency of the light wave
- v: velocity of the moving object
- c: speed of light
- a: the angle between the velocity vector of the moving object and the direction of observation

Equation 1.4: Another way to calculate optical Doppler frequency shift.

The Doppler frequency shift is, therefore, proportional to the velocity of the moving object and depends on the angle of observation (Pillunat *et al.,* 1999).

Due to the movement of red blood cells (RBC) in the retinal vessels, light reflected or scattered by the red blood cells undergoes a Doppler frequency shift. In laser Doppler flowmetry the retinal vessels are illuminated with laser light, some of which is reflected from the vessel walls and the reminder from the moving blood corpuscles. The component of light reflected by the moving RBC undergoes a frequency sift, while the light reflected from surrounding tissue is reflected without frequency change. The two coherent components of the light can interfere, resulting in oscillation of the measured light intensity. The frequency of the intensity oscillation is equal to the Doppler frequency shift. As a result, the Doppler sift of the light frequency is translated to an intensity oscillation with a typical frequency in the range of kHz, i.e., in a measurable range.

1.9.2.2. Measurement technique

The Heidelberg Retina Flowmetry (HRF) technique combines laser Doppler flowmetry with confocal scanning laser tomography (Bohdanecka *et al.,* 1998; Lietz *et al.,* 1998; Chauhan and Smith, 1997; Griesser *et al.,* 1999). The method is non-invasive and results are rapidly obtained. It requires clear media and good fixation. The laser source is a diode laser of wavelength 780nm (infrared) and optical power of 180(W). The parallel laser beam is focused on the retinal surface by an emetropic eye. The direction of the laser beam entering the eye is periodically changed in two orthogonal directions by means of two oscillating mirrors, so that a two-dimensional region of the retina is scanned line by line. The scan field is 10° wide and 2.5° high, corresponding to a size of 2.88 mm x 0.72 mm. In order to obtain the frequencies of the variations of reflected light intensity, the measured temporal intensity signal is subjected to a Fast Fourier Transform (FFT) algorithm to produce the desired Doppler-shift power spectrum. Doppler frequencies between 125 Hz and 2000 Hz are sampled. Frequencies below 125 Hz are excluded so as to minimize low-frequency artefacts such as breathing. Before displaying the perfusion values an average background intensity noise value is subtracted from each pixel. Finally the scanned retinal area can be displayed as a two-dimensional

perfusion map (Figure 1.9). The brightness of each pixel is coded for flow, volume or velocity; the brighter the pixel, the higher the relative perfusion value.

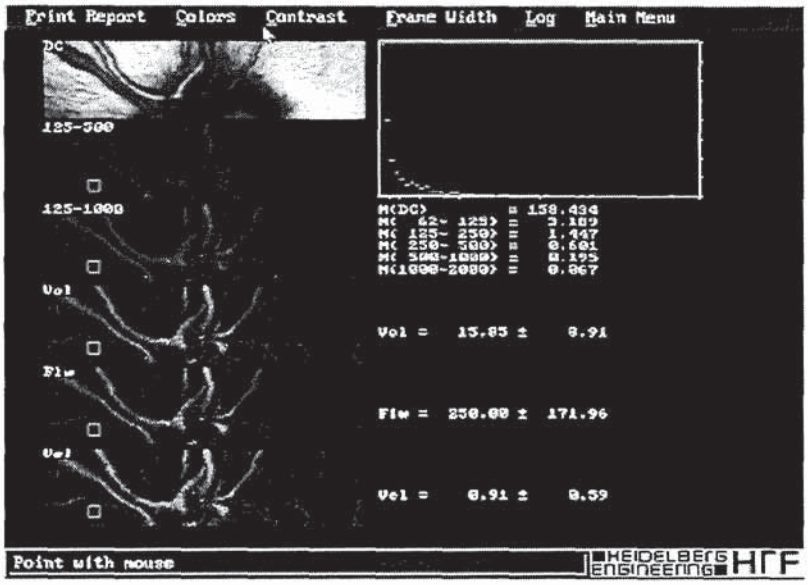


Figure 1.9: Perfusion map as obtained using the HRF instrument. Vol: volume; Flw: flow; Vel: velocity.

Due to the upper frequency limit on the FFT (2000 Hz), high blood cell velocities in the main retinal vessels cannot be accurately measured. A perfusion window, that avoids the larger vascular tree, is therefore chosen for the measurement from the map. The range of window sizes varies from 10 x 10 pixels up to 50 X 50 pixels, with 10 x 10 being the most commonly used.

1.9.2.3. Factors affecting HRF measurement

a. Cardiac cycle

The relatively long image capture time of 1.6 seconds can cause temporal artefacts such as eye movements and pulsatile perfusion changes associated with each heart beat. Sullivan et al. (Sullivan *et al.,* 1999) showed how the alternating light and dark horizontal bands on an HRF perfusion map were

associated with the peaks and troughs of the cardiac cycle. Although the authors concluded that this variation, which accounted for up to 30% to 50% difference, was probably due to capillary perfusion change they could not exclude that ocular movements (fundus pulsation) were partially responsible.

b. Camera-eye distance

Kagemann et al., (Kagemann *et al.,* 1998) found that increasing the camera-eye distance to 5 cm significantly reduced the correlation between individual flow values and when increased to 7 cm the correlation was lost all together. The present recommended camera-eye distance is 1.5 cm.

c. Background illumination

Tsang et al (Tsang *et al.,* 1999) found that HRF measurements were significantly influenced by background intensities. The investigators believed that the HRF's own background noise correction software is responsible for this, with velocities being suppressed in bright backgrounds. The authors state this error with the HRF leads to the following requirements when taking the measurements:

- When performing repeated examinations of a single normal eye, it is imperative to align the image perfectly from one eye examination to the next;
- The illumination must be set consistently to the same level between images when examining the same eye.

d. Measurement frame

Small measurements windows such as 4 x 4 pixels are associated with poor reproducibility (Nicolela *et al.,* 1996c). Later, an automatic full field perfusion image analyser (AFFPIA) software, which removes image artefacts and larger vessels to produce a whole image perfusion index and a measure of capillary perfusion, has been developed (Michelson *et al.,* 1998; Kagemann *et al.,* 1998). In this technique, the examined retinal area has a size of 2.7 mm x 0.7 mm. It has a resolution of 256 points x 64 lines. One sample is taken from each point

of the line. Each line is scanned 128 times with a repetition rate of 4000 Hz, leading to an intensity matrix of 256 points x 64 lines x 128 times. The collected intensity data of each retinal point of measurement are then analysed by a discrete FFT, thus calculating the frequency shift for each point of measurement by which the blood flow of each pixel can be computed. From the calculated data a two dimensional colour map of the retinal/optic nerve head perfusion is created. The authors believe that this technique may remove some of the variation induced by the cardiac cycle and operator decision-making.

1.10. The effect of antiglaucoma therapy on ocular blood flow

1.10.1. Background

Besides the role of vascular factors in glaucoma's pathogenesis, investigation in the effects of antiglaucoma drugs on the various ocular vascular beds seem of interest. Although a large number of studies are dealing with the effect of currently available antiglaucoma drugs on OBF, only few report results in glaucoma patients (Costa *et al.,* 2003). From the data available at the moment only few drugs stand out as possible candidates for improving OBF in glaucoma, along to their IOP-lowering properties:

- **Carbonic anhydrase inhibitors (CAIs).** Dorzolamide hydrochloride 2% has been proven to accelerate arterial-venous passage time (AVP) in NTG patients (Harris *et al.,* 1999; Harris *et al.,* 2000) as well as to improve the end diastolic velocity (EDV) parameter and resistivity index (RI) in the OA in both normal control subjects and glaucoma patients (Martinez *et al.,* 1999). In addition, treatment with dorzolamide 2% also improved blood flow velocity in the PCAs has (Galassi *et al.,* 2002) of patients suffering from POAG.
- **Beta-blockers.** It has been hypothesized that betaxolol could improve OBF by avoiding non-selective beta-blockade-induced vasoconstriction and by a possible Ca^{2+} -channel blocker activity (Costa *et al.,* 2003). Therefore, a number of studies investigated the effect of this frog on ocular haemodynamics in both glaucoma and normal control subjects.

Although several authors reported improvements in both retinal (Arend *et al.,* 1998; Yoshida *et al.,* 1998) and ONH blood flow (Tamaki *et al.,* 1999), the results in glaucoma patients are less promising. Harris et al. could not find any change in AVP time or CDI parameters in the OA and CRA following 1-month period treatment with betaxolol. Nevertheless, other investigators did find an improvement in retrobulbar blood velocities promoted by betaxolol (Evans *et al.,* 1999*a*; Evans *et al.,* 1999*b*).

- **Prostaglandin analogues.** Unoprostone isopropyl represents a derivate of a prostaglandin metabolite. This derivate classified as a docosanoid (i.e., has 22 carbon atoms and a keto group at C-15), while latanoprost and primary prostaglandins are eicosanoids (i.e., have 20 carbon atoms and a hydroxyl group at C-15). Sponsel et al. (Sponsel *et al.,* 2002) found that POBF increased with about 16% in POAG patients treated with unoprostone for 1 month. However, no effect has been reported on the choroidal and ONH blood flow (Beano *et al.,* 2001).

Glaucoma patients included in the present research have been treated with latanoprost 0.005% (Xalatan®). Current knowledge on the effect of latanoprost on OBF is presented below.

1.10.2. The effect of latanoprost on ocular blood flow

Latanoprost represents a newly developed prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$)-related FP receptor agonist substance (Resul *et al.,* 1997). Latanoprost lowers IOP by increasing uveoscleral outflow. While the effect on IOP of this drug is well researched, its benefits on OBF are less documented.

1.10.2.1. Latanoprost influence on ocular blood flow: animal studies

In monkey eyes a single dose of 6µg/g of latanoprost did not change the blood flow (as measured with radioactively labelled microspheres) anywhere in the eye with the exception of the anterior sclera (Stjernschantz *et al.,* 1999). Experiments conducted in cats (Stjernschantz *et al.,* 2000) and rabbits (Astin *et*

al., 1994) also showed that latanoprost causes anterior segment vasodilation. Later, Ishii et al. (Ishii *et al.,* 2001) demonstrated that treatment with latanoprost results in increased ONH blood flow in rabbits and monkeys.

1.10.2.2. Latanoprost influence on ocular blood flow: normal human eyes

The most frequently reported result after treatment with latanoprost is that of increased POBF (Geyer *et al.,* 2001; Sponsel *et al.,* 2002). The reported effects on ONH and retinal blood flow are, however, contradictory. Although some authors reported that both a single dose of latanoprost and a 7-day course increase blood velocity at the ONH (Tamaki *et al.,* 2001; Ishii *et al.,* 2001), others failed to find a significant change in ONH or peripapillary blood flow after a single dose of the drug (Seong *et al.,* 1999). It is, however, important to emphasize that the two groups of authors used different methods for the OBF assessment. While Tamaki et al. and Ishii et al. used a laser speckle analyser, Seong et al. used the HRF technique. It is possible that the different results were simply due to differences in the OBF measurement techniques.

1.10.2.3. Latanoprost influence on ocular blood flow: glaucoma patients

Nicolela et al. (Nicolela *et al.,* 1996*a*) was the first to report that treatment with latanoprost did not affect blood flow velocities measured at the retrobulbar vessels level of POAG patients. As for the normal control subjects, the most significant effect on OBF reported after treatment with latanoprost 0.005% is an increase in POBF (Vetrugno *et al.,* 1998; McKibbin and Menage, 1999; Georgopoulos *et al.,* 2002). Liu et al. also reported that latanoprost 0.005% increases POBF after 4 weeks of treatment; however, after correcting for changes in IOP, the increase became insignificant. To date no study reported beneficial effects on ONH and retinal blood flow after treatment with latanoprost 0.005% in glaucoma patients.

1.11. The cardiovascular system

The present research concentrates on both ocular and systemic vascular risk factors for GON. Among factors that influence OBF, BP and cardiovascular activity in general are of great importance. Therefore, an understanding of the cardiovascular anatomy and physiology is, in this context, necessary.

The circulation of blood in the human body is a concept introduced relatively late in the Western world, in the1600s by William Harvey, an English physiologist. He proved experimentally that blood is impelled mechanically by a "pump-like" heart; he also measured the amount of blood in the circulatory system.

The human heart consists of four chambers: the left and right atria and the left and right ventricle. The right atrium receives deoxygenate blood which is then pumped into the right ventricle and then into the pulmonary artery and the lungs. Oxygenated blood then returns to the heart, into the left atria from where is pumped into the left ventricle, aorta, and systemic circulation. Approximately 80 cm^3 of blood is pumped out of the heart in each of its beats, making a total of approximately 5.5 litres of blood every minute (Levick, 2000).

1.11.1. Cardiac cycle

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

1.11.1.1. Autonomic control of cardiac rhythm

The heart is innervated by both the sympathetic and parasympathetic divisions of the ANS; HR monitoring is thus a reflection of their modulating effect on the cardiac pacemaker cells (Goldberger, 1999). During the day, there is an augmentation of sympathetic tone, while vagal (parasympathetic) tone is highest during resting conditions (Goldberger, 1999). These changes in cardiac control occur on all time scales as shown by studies performed on cosmonauts

during orbital flight (Ivanov *et al.,* 1999). Sympathetic activation results in an increase in HR, conduction system velocity and contractility (Greenwood *et al.,* 1997), while parasympathetic innervation slows the HR via the direct activation of acetylcholine (Pumprla *et al.,* 2002).

1.11.1.2. Electrical events

The cardiac muscle (myocardium) undergoes spontaneous and rhythmic contractions generated by a specialised collection of muscle fibres with oscillatory properties, which comprise the sino-atrial node (SAN) (Levick, 2000). In the normal human adult heart, the SAN generates oscillations of approximately 70 beats per minute. From SAN, the electrical excitation spreads over the atria and when it reaches the junction between the atria and the ventricles, it excites another electrical centre named the atrio-ventricular node (AVN). The ventricular contraction is the result of transmission of the excitatory signal along a bundle of modified cardiac fibres known as the Purkinje bundle (Levick, 2000).

1.11.1.3. Mechanical events

The term *systole* refers to ventricular contraction, while *diastole* refers to ventricular relaxation and filling. As the ventricles start to contract, the pressure inside them rises rapidly and soon exceeds that inside the aorta and pulmonary artery, allowing the blood to be ejected into the systemic and pulmonary circulations (Levick, 2000). After that, the pressure decreases and the ventricles are filled with blood; the cycle then repeats itself.

1.11.2. The electrocardiogram

The cardiac activity can be measured and its recording is known as the electrocardiogram (ECG or EKG). The ECG provides information about both cardiac structure and function.

1.11.2.1. Sequence of normal cardiac activation

Each heart beat is represented on the ECG by three major deflections (Levick, 2000) (Figure 1.10):

- The *P wave*, the first positive wave, represents atrial contraction (depolarisation of the atria)
- The *QRS complex* represents the contraction (depolarisation) of the ventricles/ventricular muscle cells. This complex can always be subdivided into individual components (Lilly, 2003*a*): Q wave, which is defined as the first downward deflection of the QRS complex; R wave, which represents the first upward deflection (whether or not a Q wave is present); and S wave, which is defined as any downward deflection following the R wave.
- The *T wave* represents ventricular repolarization

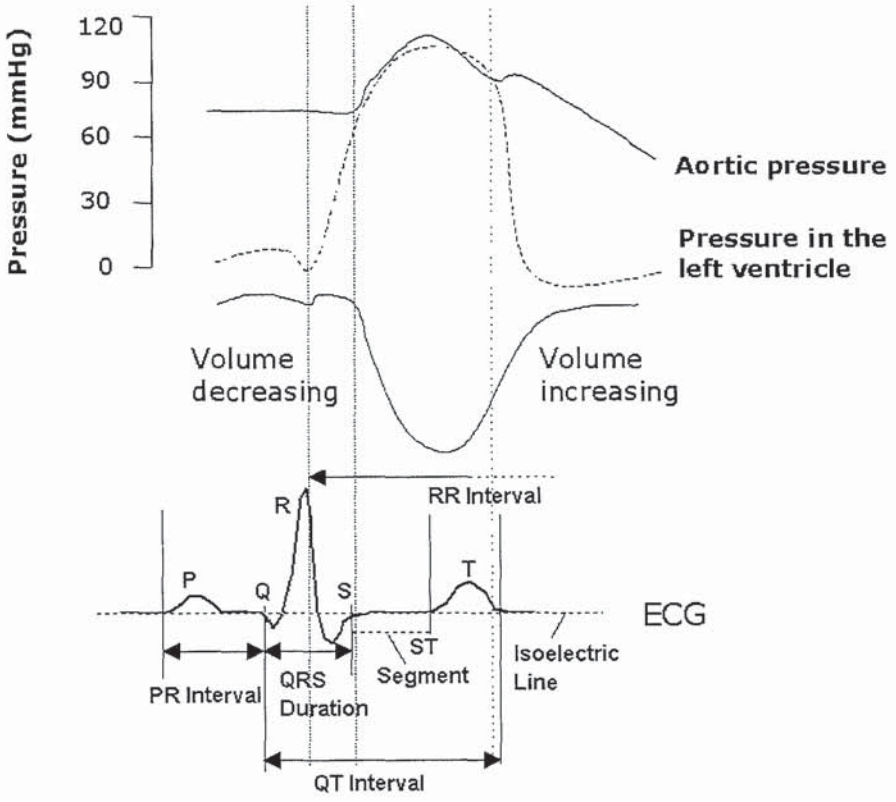


Figure 1.10: Variation of cardiac pressure and volume during the cardiac cycle (above) and the resultant ECG recording (below).

1.11.2.2. Technical considerations

ECG is recorded on either a paper or computer monitor. The ECG graph is divided into lines situated 1 mm apart in both the horizontal and vertical directions. The voltage is measured on the vertical axis and is expressed in millivolts (mV). Each 1 mm line separation represents 0.1 mV. The time is measured on the horizontal axis; each 1 mm line separation represents 0.04 seconds (Figure 1.11).

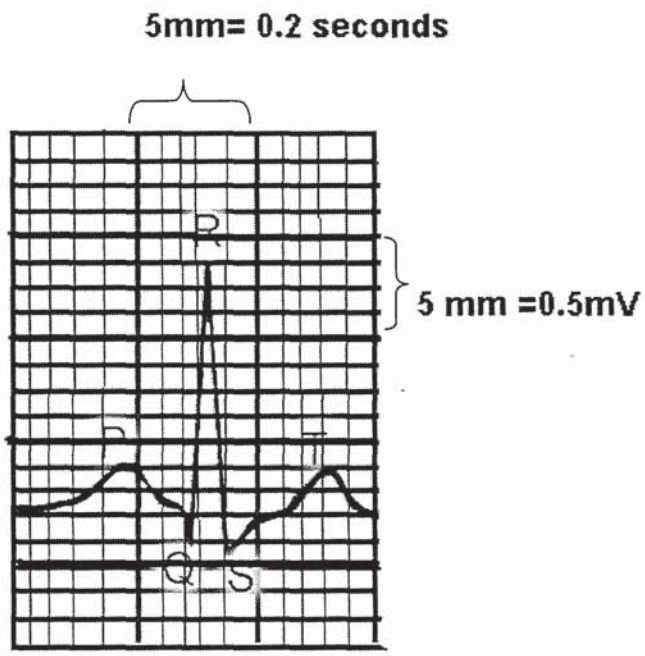


Figure 1.11: ECG strip. Each 1 mm on the vertical axis represents 0.1 mV and each 1mm on the horizontal axis represents 0.04 seconds.

1.11.2.3. ECG analysis

ECG is altered whenever there is a cardiac abnormality or disease. Therefore, a careful analysis is crucial for detection of pre-clinical and/or clinical conditions.

The ECG analysis includes (Lilly, 2003*a*):

- Heart rate (HR), which is calculated according to one of the formulas:

$$HR\ (beats/minute) = \frac{25mm/sec \times 60\ sec/min}{Number\ of\ mm\ between\ beats};\ or$$

$$HR\ (beats/minute) = \frac{1500}{Number\ of\ small\ boxes\ between\ 2\ consecutive\ beats}$$

- HR: heart rate

Equation 1.5: Calculation of heart rate on the electrocardiogram strip.

- Heart rhythm. If the HR is between 60 and 100 beats/minute we have a normal sinus rhythm.
- Major deflections and their abnormalities.
- Intervals (PR, QRS, ST). Normal PR=0.12-0.20 sec.; QRS<0.10 sec; QT< ½ X R-R interval.
- ST-segment and T wave abnormalities. These occur whenever there is a difference between myocardial O_2 demand and supply (myocardial ischaemia) and are represented by:
    - ST depressions, in subendocardial ischaemia and metabolic abnormalities;
    - ST elevations, in cardiac infarct and pericarditis (inflammation of the pericardium).

1.11.3. Myocardial ischaemia

Myocardium receives its blood flow via the coronary arteries. Any blood flow impairment to the heart muscle results is ischaemia. Myocardial ischaemia is characterised by both O_2 deprivation and accumulation of toxic metabolites.

1.11.3.1. Coronary arteries

Coronary arteries supply the heart muscle with O_2 and nutrients. There are two main coronary arteries: left coronary artery, with two major branches (left circumflex and left anterior descending); and right coronary artery, with one major branch (posterior descending, Figure 1.12). These arteries also send perforating branches into the ventricular muscle, where they form anastomosies in the walls of all cardiac chambers. The venous blood is drained via the coronary veins, which follow a distribution similar to the main coronary arteries.

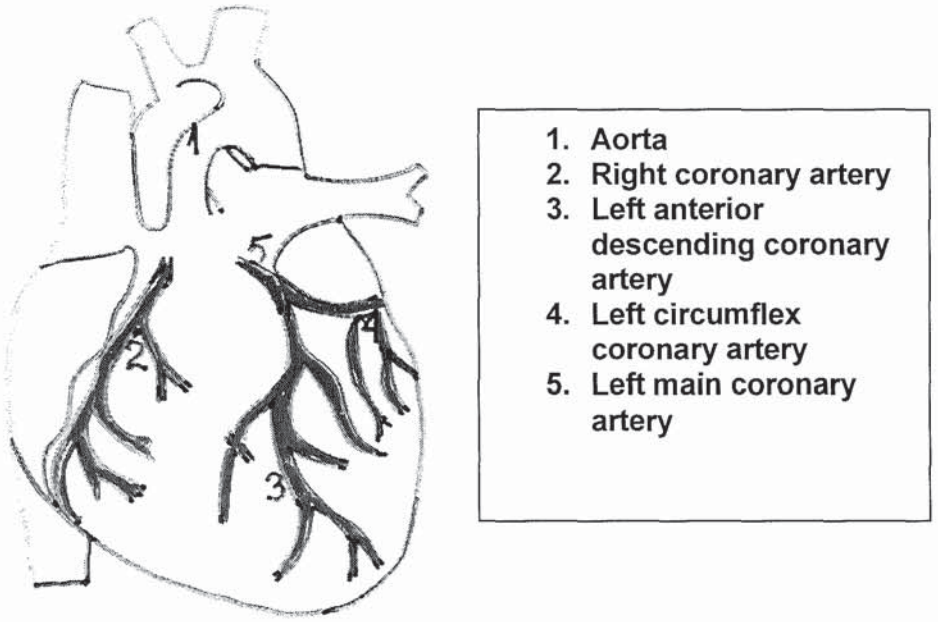


Figure 1.12: Coronary arteries.

The blood flow thorough the coronary arteries can be calculated according to the formula:

$$Q = P/R$$

- Q: blood flow
- P: vessel's perfusion pressure
- R: vascular resistance

Equation 1.6: Calculation of coronary blood flow

The predominance of coronary blood flow occurs during cardiac diastole, as opposed to other arterial systems in which the blood perfusion occurs during cardiac systole (Gupta *et al.,* 2003).

1.11.3.2. Regulation of coronary blood flow

The blood flow through the coronary arteries is regulated by many factors. These are outlined in Table 1.2.

Factors that could interfere with this complex regulation could result in myocardial ischaemia. These factors are represented by:

- Aging (Toro *et al.,* 2002);
- Menopause (Low *et al.,* 2002);
- Cigarette smoking (Dal Palu and Zamboni, 1990);
- Hypertension (Wilson, 1997);
- Diabetes mellitus (Jeemendy, 2003);
- Family history of coronary artery disease (CAD) (Lauer, 1999);
- Dyslipidemia (Shirai, 2004).

Type of regulation	Description
Metabolic	• Myocardial O_2 consumption • Adenosine (coronary dilator) • Lactate, acetate, H^+, CO_2
Endothelial factors	• EDRF • ET_1 • Prostacyclin
Neural factors	• Sympathetic nervous system • Parasympathetic nervous system
Extravascular compressive forces	• Systole stops blood flow through the coronary arteries • Diastole facilitated coronary blood flow
Transmural distribution of myocardial blood flow	• Systole obstructs the blood flow in the subendocardium more than in subepicardium

Table 1.2: Regulation of coronary blood flow. O_2: oxygen, H^+: hydrogen ion, CO_2: carbon dioxide; EDRF: endothelium-derived relaxing factor, ET_1: endothelin 1.

1.11.3.3. Myocardial ischaemia: clinical presentation

Myocardial ischaemia could present with various clinical pictures. These and their description are outlined in Table 1.3.

For the purpose of the present thesis, silent myocardial ischaemia will be discussed in detail below.

Condition	Description
Myocardial infarction	Necrosis of the heart muscle due to prolonged cessation of the blood supply
Stable angina	Chronic chest pain (angina pectoris) due to myocardial ischaemia and provoked by physical or emotional stress. It disappears after few minutes and is not associated with permanent myocardial damage
Variant angina	Angina pectoris occurring at rest, due to coronary spasm
Unstable angina	Episodes of angina pectoris with pattern of increasing frequency and duration. If untreated, progresses to myocardial infarction
Silent myocardial ischaemia	Asymptomatic episodes of myocardial ischaemia, which can be detected only by ECG analysis or other laboratory tests

Table 1.3: Clinical presentations of myocardial ischaemia. Adapted from Gupta et al. 2003 (Gupta *et al.*, 2003).

1.11.3.4. Silent myocardial ischaemia

Silent myocardial ischaemia is characterised by asymptomatic ischaemic episodes that can be detected by either ambulatory ECG recording or exercise stress testing (Gupta *et al.*, 2003). The reason why these episodes are asymptomatic and not associated with chest pain like the typical *agina pectoris* attacks has not been elucidated. Abnormal central processing of the pain signals could be involved in the pathophysiology of this entity (Rosen *et al.*, 1996). This observation is further supported by the fact that silent myocardial ischaemia is more common in diabetes mellitus, a disease associated with autonomic neuropathy (Vinik *et al.*, 2003).

Although the mechanism behind the occurrence of this type of myocardial ischaemia is still to be elucidated, it is true that repeated episodes could result

in irreversible myocardial changes (Hess *et al.,* 1988). Therefore, screening among both general population and subjects at risk is necessary.

1.11.3.5. Electrocardiogram recording in silent myocardial ischaemia

The screening for silent myocardial ischaemia is performed by using ambulatory ECG. The characteristic ECG sign for silent myocardial ischaemia is represented by ST-segment depression (Figure 1.13). These episodes are of 0.1 millivolt (mV) or more, last longer than 30 seconds, and occur with the same circadian rhythm as the myocardial infarction; therefore, it could be concluded that silent myocardial ischaemia is causally related to serious cardiac events (Cohn *et al.,* 2003).

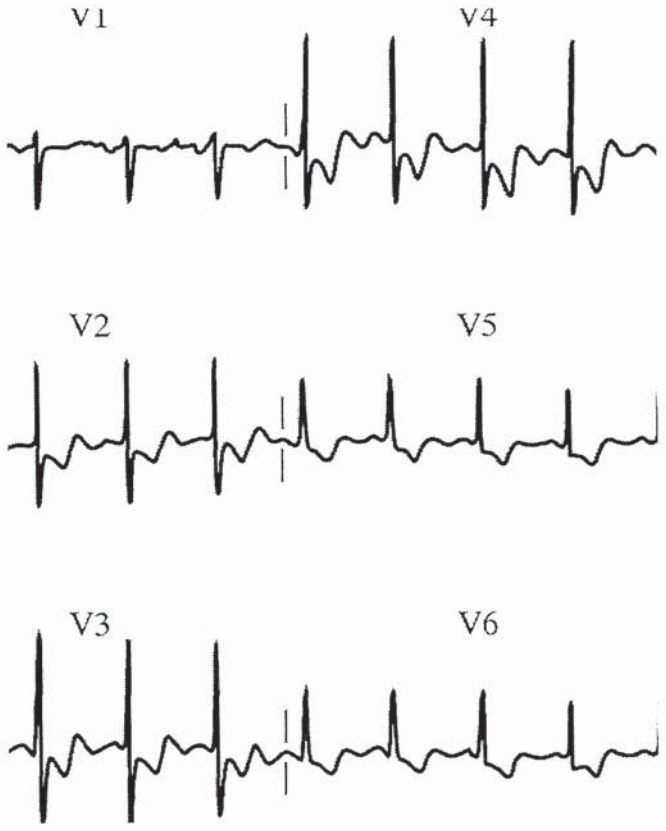


Figure 1.13: ST-segment depression in the leads V2-V6.

1.11.3.6. Silent myocardial ischaemia and glaucoma

Glaucoma, especially NTG, is significantly associated with the occurrence of silent myocardial ischaemia (Kaiser *et al.,* 1993*a*; Waldmann *et al.,* 1996).

Since the ECG changes associated with asymptomatic myocardial ischaemia occurred mainly during the night while the patients were asleep, the authors concluded that the major cause were not atherosclerotic changes, but functional vascular dysregulation. Indeed, vascular dysregulation has been implicated in the aetiology of both glaucoma (Flammer, 1994; O'Brien, 1998; Chung *et al.,* 1999 *a*; Anderson, 1999; Gherghel *et al.,* 1999; Gherghel *et al.,* 2000; Hosking *et al.,* 2004) and myocardial ischaemia (Gorlin, 1983; Motoyama *et al.,* 1997; Momose *et al.,* 2002). Nevertheless, the question on why the myocardial ischaemic episodes are asymptomatic in glaucoma patients, still remains. Since POAG has been associated with various ANS dysfunctions (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003), the existence of an autonomic neuropathy in these patients could result in a defective processing of pain signals.

1.11.4. Heart rate variability

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

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

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

respiratory disorders (Watson *et al.,* 1999) and neurological diseases (Rapenne *et al.,* 2000);

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

HRV can be assessed either by using a frequency-domain or a time-domain analysis. The first method consists of a spectral analysis of the arterial pulse wave, which is used to analyze the predominance of the sympathetic and parasympathetic divisions of the ANS and their effect on HR%(Kitney and Rompleman, 1980). In normal individuals cyclic changes in HR occur in association with respiration and this high-frequency (HF) (0.2-0.4 Hz) cyclical HRV is mediated by the parasympathetic nervous system (Pagani *et al.,* 1986). Conversely, cyclic variations due to changes in BP result from changes in baroreceptor activity and are typically low-frequency (LF) (0.0-0.04 Hz) in nature, being mediated via the sympathetic nervous system (Sleight *et al.,* 1995) (Figure 1.14, a and b).

The LF/HF ratio represents the dynamic of the HRV signal and has been used to measure the sympathovagal balance of the ANS function (Scholz *et al.,* 1997). The time-domain analysis uses the average normal-to-normal heart beat (mean NN interval) to measure the HRV. It is simple to use and according to Goldberg (Goldberger, 1999), satisfies all the criteria for an accurate measurement of HRV and ANS assessment. Other practical tests for the autonomic activity are listed in the Table 1.4.

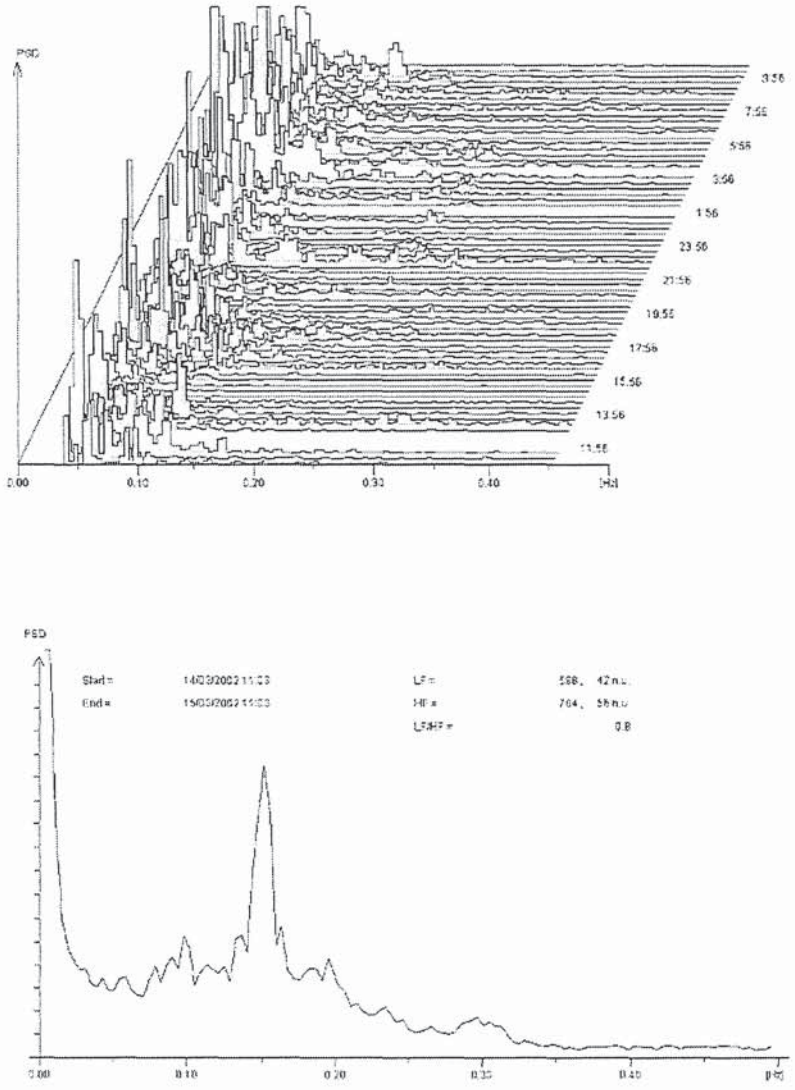


Figure 1.14: Analyzing heart rate variability (HRV) in the frequency domain. (a) The compressed spectral array (CSA), which represents a set of 72 power spectral density (PSD) charts calculated with 20-minute intervals for 24 hours, based on the first 4 minutes of each 20-minute interval. The vertical axis represents PSD and the horizontal axis represents the frequency in hertz (Hz). (b) PSD is calculated using FFT. The vertical axis represents PSD and the horizontal axis represents the frequency. LF: low frequency component; HF: high frequency component (see text).

Technique	Description
Heart rate (HR) and heart rate variability (HRV)	• Resting heart rate (Hathaway *et al.*, 1998); • Routine exercise tolerance test; • Time-domain analysis of HRV – analysis or RR intervals and frequency-domain analysis of HRV (HF and LF parameters).
Provocation tests	• Valsalva maneuver (Sarnoff *et al.*, 1948) ; • Pressor drug infusion (Goldberger, 1999); • Other methods: lower body negative pressure, neck chamber pressure, head-upright tilt (Eckberg, 1980; Jardeh and Prieto, 2003).
Other techniques	• Plasma levels of norepinephrine for the overall level of sympathetic activity (Cryer, 1977); • Microneurography (Hagbarth, 1979); • Pupillography (Shirakawa and Ishikawa, 1992); • Cardiac norepinephrine spillover for the sympathetic firing rate in the heart (Esler, 1993); • Blood pressure variability (Lanfranchi and Somers, 2002).

Table 1.4: Autonomic assessment tests. HF: high frequency; LF: low frequency

1.11.5. Systemic blood pressure

During the contraction (systole) and expulsion of the blood from the left ventricle, the aortic pressure rises from its resting value of about 80 mm Hg (DBP) to about 120 mm Hg (SBP). It is this pressure that is measured with a sphygmomanometer. The given normal BP values for adults are <135/85 mmHg for daytime, <120/75 mmHg for nighttime and <130/80 mmHg for 24 hours (McGrath, 2002). However, these values can vary due to various factors such as (Sunthareswaran, 2002):

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

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

1.11.5.1. Autonomic control of systemic blood pressure

The main integrating system for BP control is situated in the brain, in medullary cardiovascular centre. This centre maintain adequate blood flow to the brain and heart (Silverthorn, 2001). In order to achieve this goal, it requires sensory inputs from the baroreceptors located in the walls of the aortic arch and carotid artery; these baroreceptors monitor the pressure of the blood through the main arteries. After proper integration and analysis of the input, the medullary cardiovascular centre sends appropriate efferent outputs *via* both the sympathetic and parasympathetic divisions of the ANS (Silverthorn, 2001); these stimuli regulate the heart function and thus, the circulation through the body.

The final MBP is a function of the cardiac output and the resistance in the arterioles. As a rule of thumb, MBP approximates:

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

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

Equation 1.7: Calculation of mean blood pressure

1.11.5.2. Circadian variations in systemic blood pressure

Typically, after waking, SBP rises rapidly by 20 to 25 mmHg and DBP by 10 to 15 mmHg. In humans, both SBP and DBP are highest in the late afternoon, beginning to decline in the evening and attaining lowest levels during sleep (Baumgart, 1991; Smolensky and Haus, 2001). This variation in systemic BP is due to circadian variations in physical and mental activity, stress, and also to the change in posture while lying down during sleep. Circadian autonomic and endocrine rhythms directly influence the 24-h profile of systemic BP (Portaluppi and Smolensky, 2001), thereby controlling BP in order to maintain constant blood flow during the organic and functional changes in arteries throughout the body (Tochikubo *et al.,* 1997).

Typically, the physiologic nocturnal dip in BP (representing the fall in blood pressure during night-time expressed as a percentage of the average day-time reading level) is around 10% to 20%, and is present in approximately two thirds of the healthy population (known as dippers). The so-called extreme dippers have a nocturnal fall in BP of more than 20%, which may occur naturally or due to the use of antihypertensive medications. These patients could also exhibit ischaemic phenomena, including cardiac ischaemia (Pierdomenico *et al.,* 1998), silent cerebrovascular damage (Kario *et al.,* 1998), and anterior ischaemic optic neuropathy (AION) (Hayreh *et al.,* 1994). Extreme dipping may also be associated with increased BP variability (Kario *et al.,* 1999).

Non-dippers, have a nocturnal BP fall of less than 10%, and are characterized by increased frequency of myocardial ischaemia, cerebrovascular damage including stroke, haemorrhages, thrombosis and vascular dementia (Shimada and Kario, 1997) possibly because these patients suffer a longer duration of exposure to high BP levels over 24 hours (Verdecchia *et al.,* 1991) .

The three possible types of 24-h BP curve are presented in Figure 1.15.

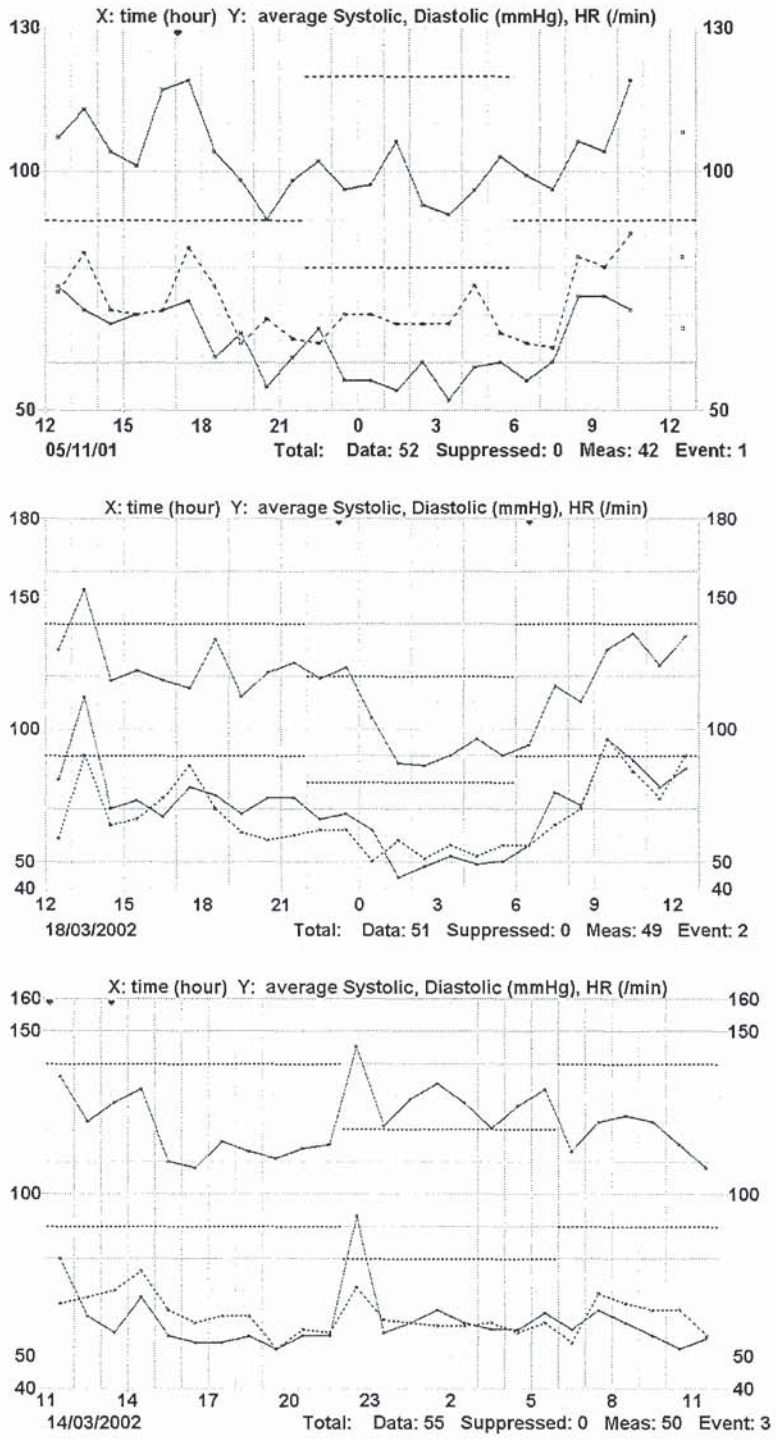


Figure 1.15: 24-h BP assessment. Top: normal dipping; middle: over-dipping; bottom: non-dipping. Upper solid line: systolic BP; lower solid line: diastolic BP.

Although extremely important, the extreme dipper group is often overlooked in the medical literature; the vast majority of studies on diseases of the

cardiovascular system in elderly patients refer only to dippers (BP fall> 10%) and nondippers (BP fall < 10%, Figure 1.16). It is well known that advancing age is associated with attenuated dipping in both men and women (Staessen *et al.,* 1997), and that BP dipping during the night may be diminished in women following the menopause (Sherwood *et al.,* 2001); whether or not this fact contributes to the smaller interest in over-dipping is debatable. Although the group of non-dippers seems to exhibit a large variety of cardio- and cerebrovascular abnormalities, the extreme dippers group should also be addressed in these studies.

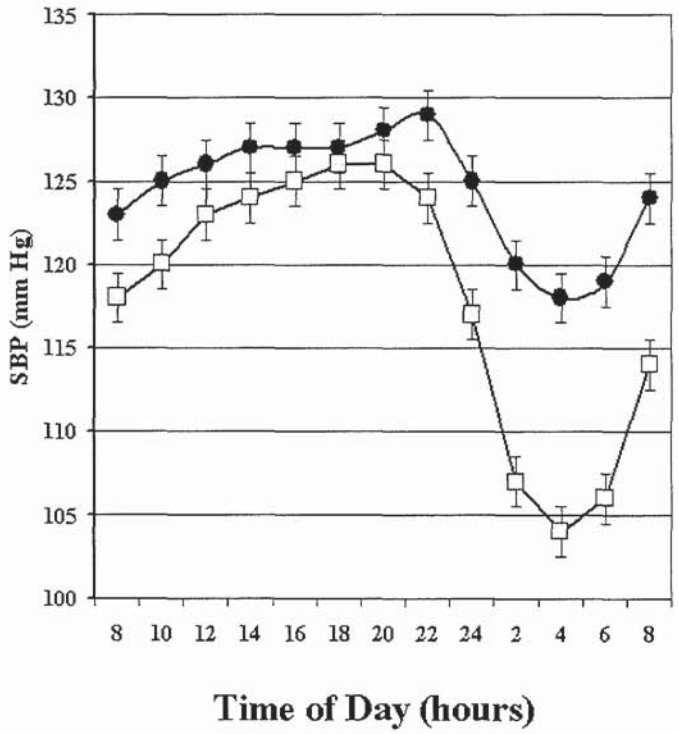


Figure 1.16: Circadian variation in systolic blood pressure (SBP) in dippers (squares) and non-dippers (circles). Periods of sleep were defined according to a diary activity rating. Reproduced with permission from Sherwood et al., 2002 (Sherwood *et al.,* 2002).

BP values seem to be influenced by the rhythmic release of catecholamines (Yamasaki *et al.,* 1998). It has been shown that, under physiological conditions,

the nocturnal BP reduction is caused mainly by reduced sympathetic activity (Sherwood *et al.,* 2002). This theory is supported by observations that plasma levels of catecholamines (Akerstedt and Froberg, 1979; Linsell *et al.,* 1985; Harisson, 1985) and muscular sympathetic nerve activity (Somers *et al.,* 1993) decrease at night. Moreover, there is a decrease in plasma cortisol, resulting in reduced vascular sensitivity to catecholamines (Reis, 1960). The nocturnal reduction in BP is further exacerbated by a lower venous return due to translocation of blood to the peripheral circulation (Cranston, 1964), and the effects of the recumbent posture typically adopted during sleep which suppresses renal sympathetic activity and the renin-angiotensin system, and facilitates sodium and water excretion (Takakuwa *et al.,* 2001).

The origin of the circadian variation observed in BP is also influenced to a large extent by exogenous factors; for example, physical activity is sometimes implicated in shifting subjects with a non-dipper status to a dipper one, or a dipper to a non-dipper one (Mochizuki *et al.,* 1998). BP may also be influenced by other exogenous factors such as stress, emotions, talking and mental activity (Mansoor *et al.,* 2000). Several attempts have been made to eliminate these influences and determine the "true" circadian variation in BP. Mann et al. (Mann *et al.,* 1979) and Van der Meirecker et al. (Van der Meiracker *et al.,* 1988) recorded subjects during 24-h of bed rest and found that the circadian rhythm of BP had a smaller amplitude compared to those reported under normal, ambulatory conditions. Within subjects confined to 24-h bed rest without sleep and with meals distributed evenly across a 24 hour period, Kerkhof et al. (Kerkhof *et al.,* 1998) and Van Dongen et al. (Van Dongen *et al.,* 2001) found no circadian rhythm in BP. These findings could indicate that there is no endogenous factor regulating the circadian variation of BP.

Catecholamine levels and changes in activity are not the only variables that influence nocturnal BP values. It has been demonstrated that, in addition to normal dipping, systemic BP may show a phasic surge during REM sleep (Sei and Morita, 1999). This surge may be similar in part to that thought to be responsible for triggering acute ischaemic events associated with extreme stress (Mittleman *et al.,* 1995). In addition, a non-dipping behaviour may occur

due to an inability to suppress sympathetic activity during sleep (Ziegler, 2003). It is possible that physiological and pathological events occurring during sleep may precipitate acute ischaemic episodes in predisposed patients (Lavery *et al.,* 1997). If and to what extend the eye is affected by such episodes is still to be determined.

1.11.5.3. Ambulatory blood pressure monitoring

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

There are a large variety of devices for measuring 24-hour BP, with or without additional ECG registration capabilities. Their pressure detection relies on the following principles (McGrath, 2002):

- **Auscultation.** This method uses a microphone placed on one arm, over an artery to detect the onset and disappearance of the so-called Korotkoff sounds, which are caused by the vibration in the arteries' wall when the cuff pressure is slowly decreased and the turbulent blood flow is audible;
- **Cuff oscillometry.** This method does not determine the blood pressure instantaneously but it determines it from the curves of the changes in the pressure and its oscillation. When the pressure in the cuff is decreasing slowly, the magnitude of the pressure oscillation in the cuff gradually increases and eventually reaches a peak. Further decrease of the cuff pressure causes the oscillation to decrease. The relationship between the changes of cuff pressure and its oscillation is stored in memory and

used to determine BP.The systolic value is taken when the oscillation increases rapidly, the diastolic value when the oscillation decreases rapidly and the MAP when the oscillation reaches a peak;

- **Volumetric oscillometry.** This method detects volume pulsations under a cuff placed usually on a finger. Systolic and mean pressures are estimated as the cuff pressures at which finger volume oscillations commence and become maximal, respectively, while diastolic pressure is derived.

Only devices validated to international standards should be used for BP measurement (O'Brien *et al.,* 1993). Patients should be monitored on a normal working day; however, the fact that the BP rhythm shows immediate adaptation to shifted phases of activity and sleep could decrease the importance of interpreting 24-hour BP data without monitoring the patient's activity (O'Shea and Murphy, 2000). Appropriate use of traditional or computerized diaries, as well as of actigraphy (electronically monitored activity using a wrist device (Shapiro and Goldstein, 1998) should thus be recommended for 24-hour BP monitoring and data should be analysed according to the true sleep and wake periods for each individual.

1.11.6. CardioTens ambulatory blood pressure and electrocardiogram monitor

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

The final data reading consists on graphical (Figure 1.17) or worksheet-like displays, printouts and statistical analysis for evaluation.

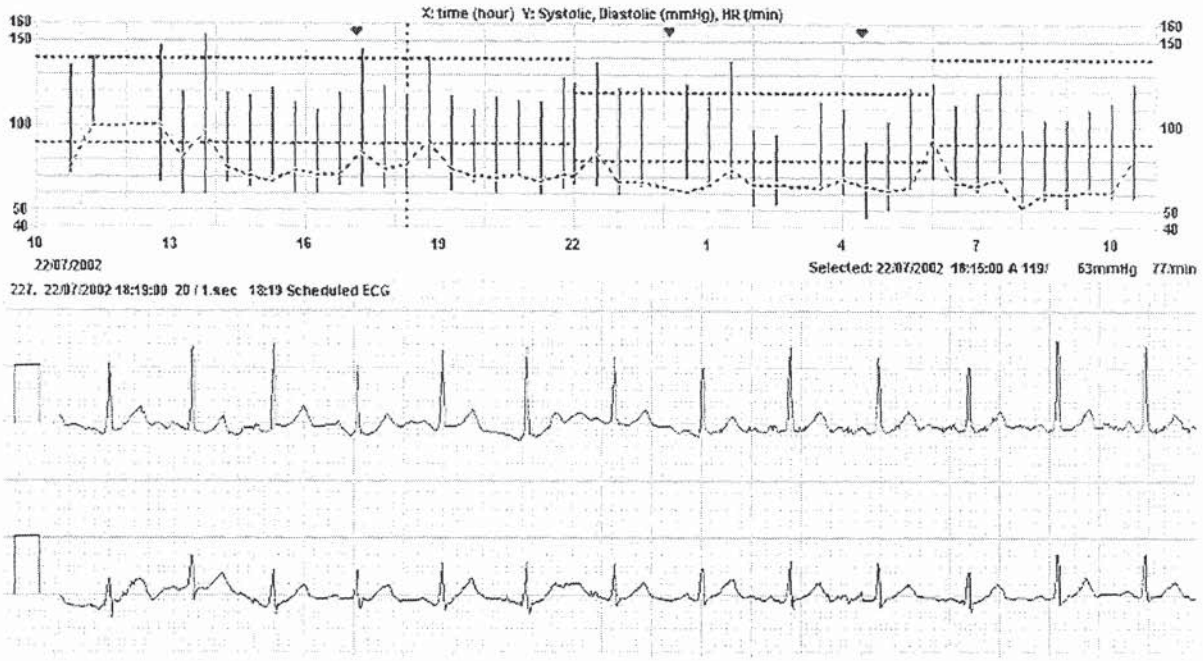


Figure 1.17: 24-hour blood pressure and electrocardiogram recordings using CardioTens-01 (graphic display). Top: 24-h blood pressure readings (vertical lines) and heart rate curve (interrupted line). Bottom: Sample electrocardiogram.

1.12. Biochemical studies

1.12.1. Background

Besides more extensively investigated factors such as increased intraocular pressure (IOP) (Krakau, 1981; Bonomi *et al.,* 2001), reduced ocular blood flow (OBF) (Rojanapongpun *et al.,* 1993; Nicolela *et al.,* 1996*b*; Kaiser *et al.,* 1997; Butt *et al.,* 1997; Findl *et al.,* 2000), ocular vascular dysregulation (Flammer, 1994; O'Brien, 1998; Chung *et al.,* 1999 *a*; Anderson, 1999; Gherghel *et al.,* 1999; Gherghel *et al.,* 2000; Hosking *et al.,* 2004, and systemic BP alterations (Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham *et al.,* 1995), oxidative stress has also been

proposed as a contributing factor in the etiology of GON (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004).

A free radical is defined as any species that has one or more unpaired electrons. Normally, more than 95% of the oxygen consumption by aerobic organisms is the result of enzymatic reduction to H_2O in mitochondria by the terminal oxidase of the respiratory chain. When molecular oxygen is reduced by one electron, the product is a superoxide radical (O_2^-). The addition of a second electron to O_2^- at physiologic pH levels gives rise to hydrogen peroxide (H_2O_2), an oxidizing species that has no unpaired electrons and thus is not a free radical. However, the one electron reduction of H_2O_2 yields H_2O and hydroxyl radical (OH^-), the strongest oxidant produced in biological systems. Together, O_2^-, H_2O_2 and OH^- are known as the ROS and are continuously produced by aerobically growing cells (Castro and Freeman, 2001).

Oxidative stress is defined by the presence of pathological levels of ROS relative to the antioxidant defence. Enzymatic sources of ROS in mammalian cells are: xanthine oxidase, cyclooxygenase, the cytochrome p450, NOS and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Cohen, 1994). Oxidative stress results in vascular endothelial dysfunction (Nedeljovic *et al.,* 2003). This fact is of extreme importance for human circulation. The vascular endothelium plays important roles in blood circulatory homeostasis. In particular, its integrity is crucial for the maintenance of blood flow and antithrombotic capacity in various vascular beds, therefore contributing to BP control, blood flow and vessel patency (Luscher and Barton, 1997). Consequently, endothelial dysfunction may result in both disturbances in blood perfusion and vascular motility throughout the body as well as in individual organs such as the eye. Excessive production of ROS can lead to deoxyribonucleic acid (DNA) mutation, lipid peroxidation, protein damage and ultimately cell death via necrosis or apoptosis (Carmody and Cotter, 2001; Ueda *et al.,* 2002).

In the late 1980s, in the course of defining the mechanisms of endothelial control of blood flow (Castro and Freeman, 2001), another free radical species,

nitric oxide (NO), was described (Wilson, 1988). It has also been shown that excessive production of NO can be cytotoxic. The rapid reaction between NO and O_2^- yields peroxynitrite ($ONOO^-$) an extremely potent oxidizing species (Koppenol *et al.,* 1992). Peroxynitrite, referred to as reactive nitrogen species (RNS), is assumed to react preferentially with CO_2 *in vivo* to produce nitrogen dioxide (NO_2) and trioxocarbonate radicals. It also nitrates tyrosine residues of the proteins, stimulating the production of 3-nitrotyrosine (3-NT), which disrupts the normal function of the proteins. Nitric oxide and secondary species derived from it are known as reactive nitrogen species (RNS) (Castro and Freeman, 2001).

1.12.2. Reactive species in human pathology

In certain situations free radicals can be generated in an exaggerated manner and can injure tissues and organs by interacting with their anatomy, physiology or genetic activity (Valencia *et al.,* 2002). Oxidative stress has been implicated in a large number of human diseases including different autoimmune diseases (Ahsan *et al.,* 2003), alcoholic liver disease (Videla and Guerri, 1990), cancer (Beutler and Gelbart, 1985), infection with human immunodeficiency virus (HIV) (Buhl *et al.,* 1989) as well as in ischaemia-reperfusion injury (Ferdinandy and Schulz, 2003). Oxidative stress also results in vascular endothelial dysfunction (Nedeljovic *et al.,* 2003). It has been demonstrated that patients with poor endothelial function are at increased risk for developing cardiovascular and cerebral ischaemic events (Perticone *et al.,* 2001).

1.12.3. Oxidative stress in ophthalmic diseases in general and glaucoma in particular

As disturbances in systemic blood flow have a direct influence on blood perfusion of various tissues, it can be speculated that any systemic oxidative insult results in both alterations of the circulatory homeostasis and disturbances of the vascular motility throughout the body as well as in individual organs such as the eye.

Oxidative stress has been implicated in the aetiology of certain eye diseases such as cataract (Harding, 1970; Spector, 1995; Lou, 2003), proliferative diabetic retinopathy (PDR), proliferative vitreoratinopathy (PVR) (Cicik *et al.,* 2003), age-related macular degeneration (ARMD) (Nowak *et al.,* 2003; Cai *et al.,* 2003) and glaucoma (Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004). The eye is protected against oxidative stress by several mechanisms involving antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), as well as by low-molecular-weight antioxidants such as glutathione (GSH) and ascorbate (Richer and Rose, 1998).

Oxidative stress has also been proposed as a contributing factor in the aetiology of GON (Ferreira *et al.,* 2004). Observations relating to oxidative stress in glaucoma are represented by studies showing:

- An increased nitrotyrosine levels in ONH of POAG patients (Neufeld, 1999);
- A decreased NOS in the trabecular meshwork (TM) of POAG patients (Nathanson and McKee, 1995);
- An imbalance between ET_1 and NO production in POAG (Sugiyama *et al.,* 1995);
- A decreased concentration of NO_2^- and cGMP in plasma and AH of POAG patients (Galassi *et al.,* 2004);
- Glutahione-S-transferase (GST) is targeted by the serum antibodies detected in some glaucoma patients. High levels of GST in retinal glial cells suggest one native neuroprotective mechanism provided at this level (Polyak *et al.,* 1997). Whether the circulating antibodies against GST in glaucoma are a consequence of tissue stress and damage in the retina or whether they have a pathogenic significance should be further evaluated.

1.12.4. Antioxidative defence

To combat the cytotoxic action of the ROS and RNS, cells are equipped with a large variety of antioxidant defences that include (Castro and Freeman, 2001):

- Enzymes which catalyse the dismutation of $O_2^{\cdot}$ to H_2O_2;
- Hydroperoxide scavenging enzymes such as catalase and glutathione peroxidase which convert H_2O_2 to H_2O;
- Hydrophilic radical scavengers such as ascorbate, urate, and glutathione (GSH);
- Lipophilic radical scavengers such as tocopherols, flavonoids, carotenoids, and ubiquinol;
- Enzymes involved in the reduction of oxidized forms of small molecular antioxidants (GSH reductase); and
- Cellular enzyme systems that maintain a nicotinamide adenine dinucleotide (NADH) and NADPH-dependent reducing environment (i.e., glucose-6-phosphate dehydrogenase).

1.12.4.1. Glutathione

Glutathione (GSH, L-(-glutamyl-L-cysteinylglycine), a tripeptide consisting of glycine, cysteine and glutamic acid, is among the most efficient substance that cells and tissues can use in their defence against oxidative stress (Pompella *et al.,* 2003). It prevents the devastating effects of the ROS either directly as an antioxidant or indirectly, by maintaining other cellular antioxidants in a functional state (Benedich, 1990; Pompella *et al.,* 2003). GSH conjugates with a large variety of products of oxidative stress and carcinogens via reactions facilitated by an enzyme called glutathione S-transferase (GST). In living organisms, glutathione exists in two forms: reduced (GSH) and oxidized (GSSG) (Figure 1.18). An optimal GSH: GSSG ratio is critical for survival of the cells and tight regulation of the system is, therefore, very important (Townsend *et al.,* 2003).

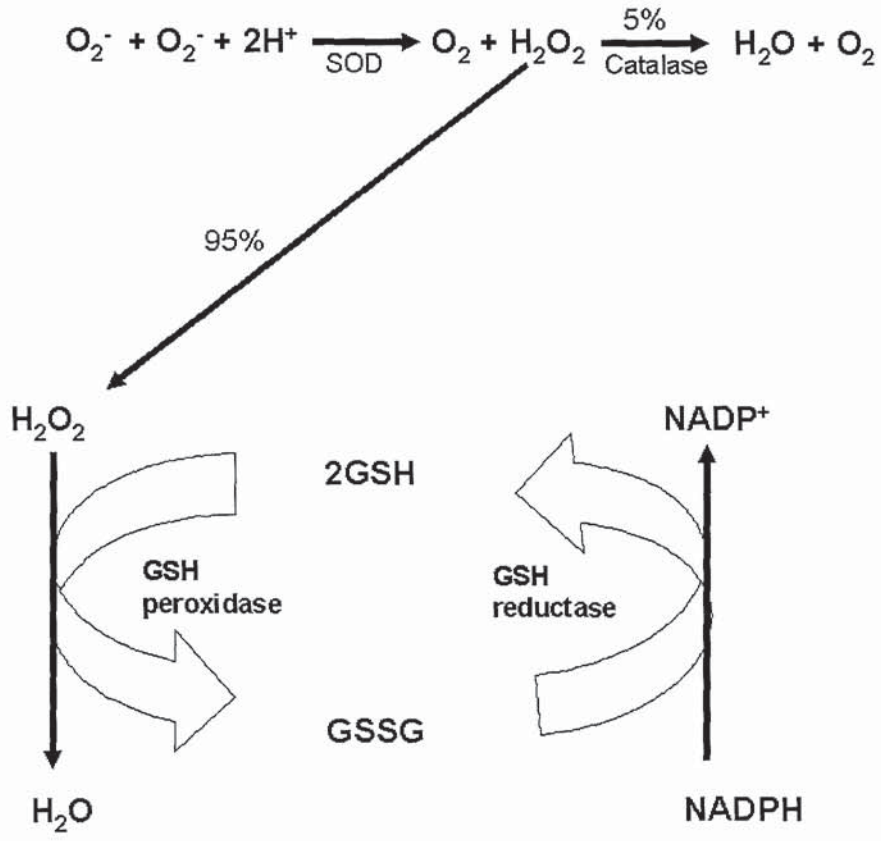


Figure 1.18: GSH intervention in the protection against free radicals (normal redox cycle): O_2^-: superoxide anion; H^+: ; H_2O_2: hydrogen peroxide; O_2: oxygen; H_2O: water; GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; $NADP^+$, NADPH: nicotinamide adenine dinucleotide phosphate

Although both GSH and GSSG occur in tissues, GSH is by far the predominant form (more than 98% GSH). GSSG is rapidly reduced back to GSH by glutathione reductase at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH), thereby forming the redox cycle (Wang and Ballatori, 1998).

GSH is found in high levels in the ocular tissues where it is involved in multiple functions, including serving as an antioxidant and as an electron donor for peroxidases. Fujii et al. (Fujii *et al.,* 2001) has immunohistochemically localized GSH-reductase in the adult rat eye. The reductase was distributed in the corneal and conjunctival epithelium, corneal keratocytes and endothelium, iris

and ciliary body, neural retina, and retinal pigment epithelium. In addition, GSH-reductase was highly expressed in the retinal ganglion cells. They have concluded that, except for the lens epithelium, the presence of glutathione reductase in the ocular tissues suggests its pivotal role in the protection of the tissues against oxidative stress.

Although it has already been shown that GSH activity is disturbed in the TM and AH of glaucoma patients (Izzotti *et al.,* 2003; Ferreira *et al.,* 2004), to date no study demonstrated an association between low plasma GSH level and GON. A low level of circulating GSH could result in a higher rate of oxidative reactions that reduce the bioavailability of NO. A number of studies have shown that among other important functions, NO is also involved in the regulation of systemic hemodynamics (Vallance *et al.,* 1989; Vallance and Chan, 2001). A low NO production could also have important consequences on the equilibrium between the endothelial vasoconstrictory and vasodilatory factors. Assessment of plasma GSH levels in glaucoma patients is, therefore, necessary. A possible low plasma GSH level in glaucoma patients could explain, at least partially, the general vasospastic tendency manifested at both peripheral (Gasser *et al.,* 1999*a*; Pache *et al.,* 2003) and ocular vascular beds (Guthauser *et al.,* 1988; Mahler *et al.,* 1989; Schmetterer and Polak, 2001; Gherghel *et al.,* 2004*a*).

1.13. Summary

Vascular risk factors, both ocular and systemic, play an important role in pathogenesis of glaucoma. Although substantial, progress in investigating these parameters has been slow, possible due to numerous difficulties in choosing the way of applying this information in the process of finding improvements or alternatives of the existent therapeutic regimens. Nevertheless, more evidences are pointing towards the fact that OBF parameters should not be neglected and indeed considered when managing glaucoma patients with multiple risk factors. Moreover, systemic haemodynamic parameters have a great impact on supporting perfusion to the

eye. Therefore, an understanding of both their physiology and various dysregulations is mandatory.

Due to their roles in the aetiology of systemic vascular disturbances and glaucoma, both oxidative stress and antioxidative defence mechanisms should also be studied thoroughly. This review highlights all these important variables as well as the blank areas that remain to be clarified by further research.

2. Research Rationale

2.1. Introduction

Although the role of vascular risk factors in the onset or progression of GON has been known for many decades, a diagnostic and therapeutic reliance on IOP still persists. It is likely that the relatively late advancement in OBF measurement technologies, together with the sometimes inconsistent research results about the implication of ocular vascular risk factors in glaucoma pathogenesis, have played a role.

Glaucoma has been associated with alterations in various ocular vascular beds and the systemic circulation. However, it is still unclear how haemodynamic alterations in vascular beds other than the ONH could interfere with retinal ganglion cells survival. Furthermore, the importance of various disturbances in systemic circulation in the aetiology and clinical course of POAG has also to be clarified. Elucidating how these factors influence the course of glaucomatous disease may open new therapeutic avenues for those glaucoma cases that progress despite therapeutically controlled IOP. Consequently, new research aimed to address these issues is of importance.

An important factor that mediates the onset and progression of various vascular diseases is oxidative stress. Although POAG itself has been associated with both vascular dysregulation manifested at a multitude of vascular beds throughout the body and high oxidative stress at the ocular level, to date no disturbed systemic antioxidative capacity has been proven in this category of patients.

Some glaucoma medications can have different effects on improving ocular perfusion. With regard to latanoprost, the results are variable, possible due to factors such as the type of glaucoma, period of treatment, target vessels for evaluation and technology used for OBF measurement. Nevertheless, in order to demonstrate an OBF improvement effect with possible consequences on visual function, measurement of capillary blood flow should be performed in more treatment follow-up protocols.

The principal purpose of the present work is to investigate the presence and impact of ocular and systemic vascular risk factors in POAG pathogenesis as well as to identify the possible role played by defective systemic anti-oxidative defence mechanism in the occurrence of this disease. The effect of treatment with latanoprost 0.005% on ONH and retinal blood flow is also investigated.

2.2. Research aims

The aims of the studies presented in this thesis are:

- To investigate the presence and impact of ocular and systemic vascular risk factors in POAG;
- To assess the relationship between ocular and systemic vascular risk factors in POAG;
- To assess the level of the systemic anti-oxidative defence in POAG patients; and
- To investigate possible effects of Latanoprost 0.005% on ocular blood flow.

2.3. Outline of investigations

The major areas of investigations included in the present thesis are outlined below:

2.3.1. The role of ocular and systemic haemodynamic disturbances in the aetiology of POAG

2.3.1.1. Systemic and ocular vascular response to temperature provocation in POAG patients

It has been suggested that disturbed autonomic activity may play a role in the pathogenesis of the glaucomatous damage. The status of the ANS can be assessed using a large variety of tests including cold stimulation, which is a well established provocation test used to detect abnormal vascular reactivity in patients with autonomic failure. The purpose of this work was to assess the

systemic vascular reactivity as well as retinal and ONH blood flow changes in response to temperature provocation in POAG patients and to compare them with the haemodynamic response recorded in a normal control group.

2.3.1.2. Relationship between silent myocardial ischaemic episodes and autonomic dysfunction in untreated POAG patients: relationship to abnormal variations in systemic blood pressure and heart rate

It has been demonstrated that chronic autonomic dysfunction could result in vascular deficiencies at the level of individual organs, such as the heart. Moreover, a systemic circulatory imbalance could affect the eye either by leading to or exacerbating glaucoma in susceptible individuals.

HRV analysis represents a method used in cardiovascular research for the assessment of ANS activity. Using a newly developed device capable of triggering BP recordings during episodes of abnormal cardiac activity, this study seeks to determine a possible relationship between the onset of silent cardiac ischaemia and alterations in BP, HR and HRV parameters during the daily normal routine of consecutive newly diagnosed and untreated POAG patients.

2.3.1.3. Ocular blood flow alterations in POAG patients: relationship to autonomic nervous system function.

In some glaucoma patients both ocular and systemic vascular disturbances contribute to the occurrence and progression of the disease (Emre *et al.,* 2004). However, establishing a direct link between the two types of risk factors needs caution. It still unclear whether the OBF alterations associated with glaucoma represent isolated events or occur as a result of more complex systemic haemodynamic disturbances due to autonomic nervous dysfunction.
In this clinical study, a cluster analysis based on OBF parameters was performed in newly diagnosed and previously untreated POAG patients and normal controls and differences with regard to HRV parameters were assessed between clusters.

2.3.2. The anti-oxidative defence status in POAG patients

2.3.2.1. Systemic circulatory glutathione levels in newly diagnosed primary open-angle glaucoma patients

GSH is among the most efficient substance that cells and tissues can use in their defence against oxidative stress (Pompella *et al.,* 2003). Age and disease, however, act to decrease the amount of GSH available in the organism. Moreover, POAG has also been associated with low levels of GSH and impaired GSH activity at both the trabecular meshwork level and in the aqueous humor. A low GSH level could reduce the bioavailability of NO for vasodilation, with consequential disruption of vascular tone. At the ocular level this could result in ocular perfusion pressure (OPP) alterations (Galassi *et al.,* 2004); however, to date no study has investigated whether altered systemic GSH levels occur in patients suffering from POAG. The aim of this work was to determine whether POAG patients have a reduced anti-oxidative defence capacity due to low levels of circulating GSH.

2.3.3. The effect of Latanoprost 0.005% on ocular haemodynamics.

Although the effects of both one-dose and short period treatment with latanoprost 0.005% on ocular haemodynamics have been reported in normals, much less information exists on the effect of this drug on the ocular circulation in glaucoma patients. Most of the studies report that latanoprost 0.005% increases POBF. To date, however, it is not know whether a similar or indeed an opposite effect occurs at the ONH or retinal circulations level. This research examined the long-term effect of latanoprost on ONH and peripapillary retinal blood flow in previously untreated POAG patients.

2.4 Summary

The importance of vascular risk factors, both ocular and systemic, in glaucoma pathogenesis has been extensively researched. However, it is still unclear how haemodynamic disturbances occurring in various ocular and systemic vascular beds could interfere with retinal ganglion cells survival, therefore contributing to

glaucoma onset and progression. Moreover, the link between local and systemic vascular dysregulation in glaucoma pathogenesis is not clear. Elucidating these mechanisms could open new diagnosis and therapeutic avenues especially for those glaucoma cases that progress despite therapeutically controlled IOP. The purpose of the following studies was to investigate the presence and impact of both ocular and systemic vascular dysregulation in POAG pathogenesis and, in particular, to investigate a possible relationship between these two types of risk factors. A possible effect of latanoprost 0.005% on ocular blood flow of patients suffering from POAG was also assessed.

3. Systemic and Ocular Vascular Response to Temperature Provocation in Untreated Primary Open-Angle Glaucoma Patients

The publication of this work is presented in Appendix 3.2.

3.1. Abstract

Purpose: To assess systemic and ocular vascular reactivity in response to warm and cold provocation in primary open-angle glaucoma patients and normal control subjects.

Methods: Twenty-four previously untreated POAG patients and 22 normal control subjects were subjected to a modified CPT involving immersion of the right hand in 40°C warm water followed by 4 °C cold water exposure, while finger and ocular blood flow were assessed by means of peripheral laser Doppler flowmetry and Heidelberg Retina Flowmetry respectively. Finger and body temperature as well as IOP, systemic BP, systemic PP, HR and OPP were also monitored. Differences between the groups at baseline were calculated using Student's t-test for independent variables. Warm- and cold-induced changes were calculated using repeated measurements ANOVA (re-ANOVA) followed by post-hoc analysis using Tukey HSD test.

Results: Glaucoma patients demonstrated an increase in DBP ($p=0.023$), HR ($p=0.010$), and mean OPP ($p=0.039$) during immersion of the tested hand in 40°C water. During cold provocation, glaucoma patients demonstrated a significant decrease in finger ($p=0.0003$) and ocular blood flow (velocity measured at the temporal neuroretinal rim; ($p=0.021$). Normal subjects did not demonstrate any blood flow or finger temperature changes during immersion of the tested hand in 40°C water ($p>0.05$); however, they exhibited an increase in systolic blood pressure ($p=0.034$), pulse pressure ($p=0.0009$), and a decrease in finger blood flow ($p=0.0001$) during cold provocation. In normals the ocular blood flow was unchanged during high and low temperature challenge.

Conclusions: Temperature provocation elicits a different BP and OBF response in primary open-angle glaucoma patients compared to control

subjects. These findings suggest a systemic autonomic failure and ocular vascular dysregulation in POAG patients.

3.2. Introduction

In the search for risk factors involved in the pathogenesis of glaucoma, vascular risk factors are the most extensively studied variables other than increased IOP. In particular, a vascular dysregulation leading to local vasospasm or impaired autoregulation has been advocated as a possible contributing factor in the aetiology of POAG (Guthauser *et al.,* 1988; Harris *et al.,* 1994; Pillunat *et al.,* 1994; Flammer *et al.,* 2001; Hosking *et al.,* 2004).

Among the factors that may influence blood flow physiology in the human body, variables such as BP and HR are regulated by the ANS (Appenzeller and Orbie, 1997). It follows that any autonomic disturbances could result in severe haemodynamic consequences. POAG itself has also been directly linked to ANS dysfunctions. Systemic parasympathetic and sympathetic neuropathies have been reported in patients with both POAG and NTG (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003) and it has already been suggested that disturbed autonomic activity may play a role in the pathogenesis of the glaucomatous damage. Nevertheless, further research is needed to prove a direct relationship between glaucoma and ANS failure.

Beside other autonomic function tests such as the measurement of HRV, Valsalva manoeuvre (Sarnoff *et al.,* 1948), pressor drug infusion (Goldberger, 1999), lower body negative pressure, neck chamber pressure, head-upright tilt (Eckberg, 1980; Jardeh and Prieto, 2003), cold stimulation is also a well established provocation test used in detecting abnormal vascular reactivity in patients with autonomic failure (Stancak *et al.,* 1996). The CPT generally involves immersion of one hand in cold water and is a well-known stress test able to induce a reproducible sympathetic activation in both normals and subjects with arterial hypertension (Victor *et al.,* 1987; Benetos and Safar, 1991). Some of the physiological responses to this test include arteriolar

vasoconstriction, increased BP and plasma catecholamines, and reduction in peripheral blood flow (Lafleche *et al.,* 1998). A well-known variation of the CPT involves immersion of the right upper limb in 40°C warm water followed by a hand-cooling test (Drance *et al.,* 1988; Rojanapongpun and Drance, 1993; Broadway and Drance, 1998).

Although cold provocation has a dramatic effect on the systemic vasculature, little is known about its consequences on ocular blood flow. Rojanapongpun and Drance (Rojanapongpun and Drance, 1993) and Nicolela et al. (Nicolela *et al.,* 2003) failed to identify blood flow changes in the ophthalmic artery and retinal circulation of patients suffering from POAG as a response to cold stimulation. However, responses elicited by temperature provocation in other ocular vascular beds still remain to be proved.

3.3. Hypothesis

In patients suffering from vascular dysregulation manifested by peripheral vasospasm, VF defects have been reported after cold provocation (Guthauser *et al.,* 1988; Mahler *et al.,* 1989); it has been suggested that this effect could be the result of an ocular circulatory insufficiency triggered by temperature stress. Since vascular dysregulation has been demonstrated to play a role in the pathogenesis of glaucomatous neuropathy (Guthauser *et al.,* 1988; Harris *et al.,* 1994; Pillunat *et al.,* 1994; Flammer *et al.,* 2001; Hosking *et al.,* 2004) we can hypothesize that cold stimulation results in an abnormal haemodynamic response in normally autoregulated ocular vascular beds.

3.4. Aims

The aim of this study was to assess the systemic and ocular vascular reactivity in response to temperature provocation in untreated POAG patients and normal control subjects.

3.5. Subjects and methods

3.5.1. Recruitment of untreated primary open-angle patients

Successive, newly diagnosed and previously untreated POAG patients attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust, Birmingham, were recruited for the study.

3.5.1.1. Inclusion criteria

Patients were diagnosed as having POAG based on IOP diurnal curve with at least two measurements higher than 24 mmHg, the existence of glaucomatous cupping of the optic disc on indirect funduscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with a diagnosis of glaucoma using program SITA 24-2 of the Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA). The characteristics of glaucomatous VF loss are included in Table 3.1 (Steele, 2003).

- Paracentral scotoma: relatively steep depression of sensitivity that respects the nerve fibre distribution;
- Arcuate scotoma: results from an elongation of a paracentral scotoma or coalescence of multiple VF defects. It respects the horizontal midline.
- Nasal step: defect associated with a difference in the sensitivity above and below midline in the nasal field. If greater that 5-10°, the nasal step is considered pathological;
- Overall depression: a gradual shrinking of the island of vision. Results in reduced sensitivity measures;
- Extensive VF defects, involving one or both hemifields.

Table 3.1: Types of glaucomatous visual field defects

3.5.1.2. Exclusion criteria

Exclusion criteria are outlined in Table 3.2:

- Patients with narrow iridocorneal angles;
- Evidence of secondary glaucoma;
- Pseudoexfoliation;
- Pigmentary dispersion;
- A history of intraocular surgery;
- Any form of retinal or neuro-ophthalmologic disease that could result in VF defects; and
- A history of chronic systemic disease, especially diabetes mellitus, or occlusive vascular disorders.

Table 3.2: Exclusion criteria for the glaucoma patients' group

3.5.2 Normal subject recruitment

The normal control group was recruited from patients' spouses and other volunteers. The group included only those subjects who had never had any significant systemic or ocular disease, including chronic cardiovascular diseases and glaucoma. The criteria used for the exclusion of POAG in this group are found in Table 3.3.

1. An ophthalmoscopically normal ONH, defined by:
   - The proportion of NRR thickness according to ISNT rule
   - A vertical cup to disc ratio (C/D) of less than 0.7
   - Absence of focal narrowing of the NRR
   - A healthy coloured NRR
   - Absence of disk haemorrhages
   - Absence of slit defects in the NFL on red-free ophthalmoscopy
   - Absence of any other signs indicative of ONH pathology
2. Normal VF as measured by automated VF examination (SITA 24-2 of the Humphrey Field Analyzer)

Table 3.3: Criteria used to define absence of primary open-angle glaucoma in an eye (Hodapp *et al.*, 1993; Jonas *et al.*, 1999).

3.5.3. Other exclusion criteria for both study groups

Further exclusion criteria for both groups were refractive errors higher than +2 and -3 diopters, a medical history of Raynaud's phenomenon, neurological or metabolic diseases, chronic intake of vasoactive drugs and physical or mental incapacity to perform experimental procedures.

3.5.4. Ethical approval

Prior to the study, ethical approval was obtained from the local medical ethics committee (Aston University and Heartlands and Solihull NHS Trust), and written informed consent was received from all subjects. The study was designed and conducted in accordance with the Tenets of Declaration of Helsinki.

3.5.5. Experimental protocol

3.5.5.1. Visual field assessment

VF was assessed by means of Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA) with a size III white stimulus using the full threshold program 24-2. The instrument is described in detail below.

HFA represents a standard device used for the diagnosis and monitoring of glaucoma and other ocular and neurological diseases. It tests the patient's response to a visual stimulus and is able to pick up the earliest changes to the eye's function. The test works by displaying flashes of light around a bowl with a fixed background light level. The machine aims to find the dimmest light that the eye is able to detect at various locations in the visual field.

The luminance of the test being projected on to the interior of the white bowl of the apparatus ranges from 10,000 to 0.1 apostilbs (asb). When converted to logarithms and then to decibels (dB), one log unit below the 10,000 asb or 1000 asb becomes equal to 10 or 10 dB.

A systematic approach is necessary whenever trying to interpret the large amount of information provided by the HFA. An indication of how the patients' results compare to the normal values stored in the apparatus is given by the so-called "reliability indices". These are (Steele, 2003):

- **Fixation losses.** The instrument checks periodically the location of the blind spot and the fixation is determined by projecting a bright stimulus in the blind spot. If the stimulus is seen, we have a false positive response (see below);
- **False negative errors.** Sometimes, a brighter stimulus is presented at a test point in the field that was earlier reported as "being seen". If the patient does not respond to the bright stimulus we have a false negative error;

- **False positive errors.** Occur when the patient is distracted by outside factors or when trying to guess. A high false positive score indicates that the patient is "Trigger Happy".

Statpac II represents a statistical package incorporated into the software of the HFA. The parameters measured with HFA are (Steele, 2003):

- **Mean Deviation (MD):** represents the mean difference in dB between the "normal" expected field of vision and the patient's real field of vision. This index is a measure of overall depression, or significantly deep losses in one part of the field and not in others;
- **Pattern Standard Deviation (PSD):** represents a measurement of the degree of abnormality of the patient's measured field of vision when comparing to the "normal" age-corrected reference VF;
- **Short-Term Fluctuation (SF):** represents the consistency of the patients responses during the field testing;
- **Corrected Pattern Standard Deviation (CPSD):** represents a measurement of the degree of abnormality of the patient's measured field of vision when comparing to the "normal" age-corrected reference VF after being corrected for intra-test variability (as noted by the SF).

3.5.5.2. Intraocular pressure measurement

Because the design of the experiment did not allow the patient to be seated at a slit-lamp, IOP was measured by means of the Tono-Pen XL (Mentor Ophthalmics Inc., Norwell, MA). The Tono-Pen XL is an easy to use, portable, hand-held instrument which provides fast and accurate IOP readings, with the accuracy comparable to the Goldman Tonometer. The device utilizes micro strain gage technology and a 1.5mm transducer tip. The Tono-Pen gently contacts the cornea, and displays the average of four independent readings, along with a statistical coefficient.

The measurements were taken after applying 1 drop of 0.4% benoxinate hydrocloride.

3.5.5.3. Temperature provocation

All subjects were tested in the morning between 8 and 10 am, in a room with a constant temperature, ranging between 21° and 23°C (mean±SD: 21.31±1.15°C). Subjects were instructed to avoid ingesting any stimulants including coffee, tea or cigarette smoke on the morning of the study. In preparation for the investigations, each subject rested in a sitting position for 15-20 minutes in a quiet room in order to achieve sufficient mental and physical calm. The steps of the experiment are outlined in Table 3.4.

Duration	Action	Measurement (see explanations in the text)
10 minutes	Patient resting in seating position	FBF, FT, TT, IOP, BP, HR, OBF.
5 minutes	Immersion of the right hand up to the wrist in 40°C water	FBF, FT, TT, IOP, BP, HR, OBF.
5-10 minutes	Patient recovered with the hand wrapped in a towel to avoid a cooling evaporation effect on hand temperature	FBF, FT, TT, IOP, BP, HR, OBF.
1 minute	Immersion of the right hand in ice water (4°C)	FBF, FT, TT, IOP, BP, HR, OBF.
15 minutes	Patient recovered with the hand wrapped in a towel to avoid a cooling evaporation effect on hand temperature	FBF, FT, TT, IOP, BP, HR, OBF.

Table 3.4: The steps of the temperature provocation experiment. FBF: finger blood flow; FT: finger temperature; TT: tympanic temperature; IOP: intraocular pressure; BP: blood pressure; HR: heart rate; OBF: ocular blood flow.

For each subject, peripheral finger blood flow (FBF) by means of peripheral lased Doppler flowmetry, and finger temperature (FT) in the left hand were measured continuously during the experiment. In addition, IOP, tympanic temperature (TT), BP, HR were recorded and OBF parameters (Volume, Flow, Velocity) in the temporal areas of the neuroretinal rim (NRR) and peripapillary retina were measured using the Heidelberg Retina Flowmeter (HRF, Heidelberg Engineering, GmbH, Heidelberg, Germany) were assessed at the end of each step of the experiment. These techniques are described below.

a. Peripheral blood flow measurement

Skin perfusion was assessed by means of a blood perfusion monitor (Periflux System 5001, Perimed, Sweden) that uses a low-power solid-state laser diode as a coherent source of light. The optical output power at the probe end is less than 2mW and the wavelength is typically 780 nm. The apparatus and the technique have been described in detail in Chapter 1.6.1.3 of the present thesis.

All measurements were performed with an upper frequency limit of 32 kHz, an output circuit time constant of 0.2 second and a gain factor of 3x. For measurements of FBF, the probe was attached with an adhesive tape to the pulp of the second finger of the left hand. The hand of the seated subjects rested comfortably on a table, at heart level. The probe of the perfusion monitor was placed to avoid any undue pressure, and kept in this position in order that the signal level indicator remains green. The FBF was then recorded continuously on a computer (Figure 3.1).

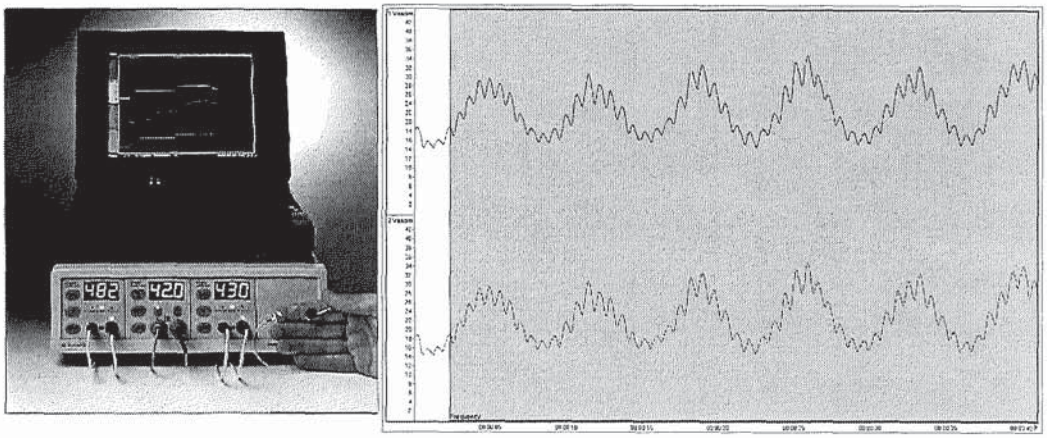


Figure 3.1: The Periflux System 5001 (left) and the graphic display of the peripheral blood flow measurements (right).

The software version used in the present experiment was PeriSoft for Windows 2.0. This software allows us to program different scripts that indicate how much time there is left until the next step is performed and how long the current step has reached. After each measurement a change report is made. When the recording is finished, the software generates a report.

b. Finger temperature measurement

The FT was measured using a copper-constantan sensor (ML309 Thermistor Pod) connected to a PowerLab/4SP unit (A&D Instruments Ltd., Oxford, UK). This type of sensor is isolated from the environment with neoprene, and is suitable for skin temperature measurements in the range of 5° to 45 (C. The sensor was fixed with a thin layer of adhesive tape on the dorsal aspect of the second finger of the left hand near the area of peripheral blood flow measurement. The PowerLab unit was connected to a computer permitting continuous recording of peripheral temperature during the experiment (Figure 3.2). The software used for the present experiment was Chart for Windows, version 4.1.2. The start and finish points for each step of the experiment has been entered manually by the operator (DG).

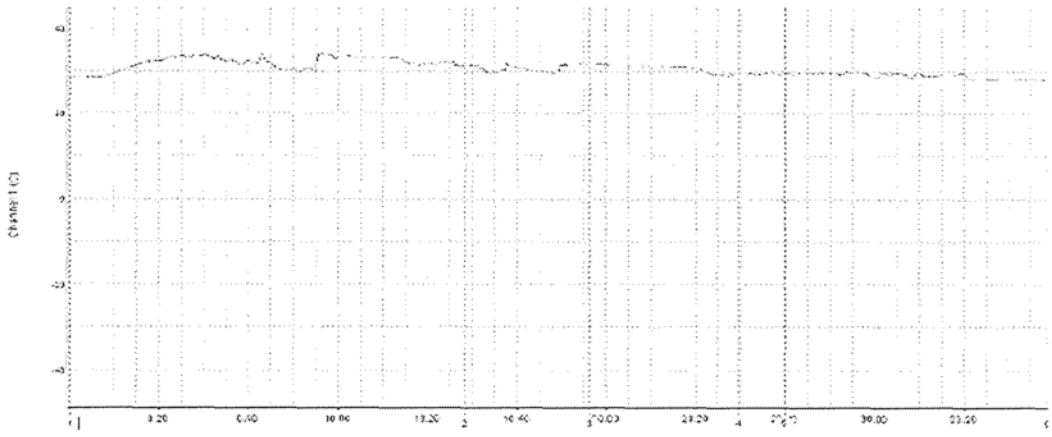


Figure 3.2: Graphic representation of the FT values measured during the experiment

The FBF and FT values were measured continuously during the experiment and average values were calculated for each step of the experiment from the reports generated by the computer.

c. Central temperature measurement

A noncontact infrared ear thermometer (ThermoTek One Second Tympanic Thermometer, P.M.S. Instruments Ltd., UK) was used to measure body temperature. Infrared ear thermometry findings are reproducible and provide a relatively close estimate of pulmonary artery core temperature (Edge & Morgan, 1993; Erickson & Kirklin, 1993). Within 1 second, the ThermoTek One Second noncontact infrared ear thermometer measures tympanic temperature, which represents core body temperature. This device can evaluate temperatures between 20°C and 42.2°C and works by detecting infrared energy emitted from the deep auditory canal and tympanic membrane.

The measurements were performed at the end of each step of the experiment. The thermometer's tip with a protective cap was inserted in the right ear of the patient and the reading was taken from the LCD display of the instrument.

d. Systemic blood pressure and heart rate

Clinical BP and HR values were obtained using a BP automatic monitor (UA-779, A&D Instruments Ltd., Oxford, UK). This device measures BP automatically, using the same principle as a conventional mercury sphygmomanometer, with a cuff and microphone. During the BP measurements, the cuff was placed around the upper right arm approximately at heart level. The SBP and DBP values were measured 3 times (1 minute apart); the average readings for SBP and DBP were then used to calculate the mean BP (MBP) according to the formula given in the Equation 1.7.
Pulse pressure (PP) was calculated according to the Equation 1.1.

The IOP and MBP measurements were used to calculate the mean ocular OPP according to the Equation 1.2.

e. Ocular blood flow measurements

For data accuracy, in glaucoma patients measurements were performed in the eye with the most advanced disease (as determined by ophthalmoscopy and VF analysis); in normals the test eye was randomly selected. Perfusion parameters in the superior temporal regions of the neuroretinal rim and peripapillary retina were measured for each step of the experiment using the HRF system. The HRF principle has previously been described in detail elsewhere (Michelson and Schmauss, 1995; Michelson *et al.,* 1995) and in this thesis (see Chapter 1.9.2).

All HRF recordings were obtained through dilated pupils using one drop of Tropicamide 1% (Alcon, Fort Worth, Texas, USA). To avoid errors resulting

from accommodative changes between single images a fixation point was utilised.

Three images were recorded at the end of each step of the experiment. Measurements were performed first on one location on the temporal NRR (TNRR) using a central alignment technique (Sehi and Flanagan, 2004) and then on one location on the temporal retina (TR) while avoiding the large vessels. Quality inclusion criteria were lack of movements during the recording and good illumination of the image.

To analyse HRF data we needed to define the border between the cup and the rim as well as the disc margin. For this purpose, we used a confocal laser retina Tomograph, a Heidelberg Retina Tomograph (HRT, Heidelberg Engineering, GmbH, Heidelberg, Germany). The method used by this device has been previously described in detail (Burk *et al.,* 1992; Burk *et al.,* 1993; Rohrschneider *et al.,* 1993; Bartz-Schmidt *et al.,* 1994; Iester *et al.,* 1997*a*; Iester *et al.,* 1997*b*; Jonescu-Cuypers *et al.,* 1999). In short, this instrument is a confocal scanning laser (670 nm) ophthalmoscope that obtains topographic images as a series of 32 optical sections perpendicular to the optical axis of the eye at consecutive focal planes. The image consists of 256 by 256 pixels, and at each pixel the retinal height is determined.

In eligible eyes, one topographic image was obtained through a dilated pupil (by using Tropicamide 1%- Alcon, Fort Worth, Texas, USA). This is in contrast to the standard protocol of 3 images recommended usually (Weinreb *et al.,* 1993). However, because of the principal of regression to the mean, limiting the number of acquired topographic images to one image per eye is not expected to alter the results in a screening procedure such as the one described here (Saruhan *et al.,* 1998).

During the imaging procedure, the subjects fixated on a distant target with the fellow (non-test) eye. For the topographic images, the optic disc margin was outlined along the inner margin of the scleral ring. Using a transparency overlaid on the monitor's screen, anatomical landmarks (blood vessels, optic

nerve contour and contour of the cup) have been drawn manually (Jonescu-Cuypers *et al.,* 2001). The transparency obtained from the HRT image was overlaid on the HRF image of the same subject, ensuring the best possible adjustment; a 10 X 10 pixel frame was then drawn on the area of interest. The same transparency was then used for all subsequent images obtained for the same subject during the experiment to ensure that the exact location of the measurement frames was reproduced. Acceptable brightness of the examined area was considered when DC (direct current) values were between 70 and 200 arbitrary units (AU) (Kagemann *et al.,* 1998; Hosking *et al.,* 2001*a*). Blood flow, volume, and velocity (AU) were determined for each image.

3.5.6. Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Differences between the groups at baseline were calculated using Student's t-test for independent variables. Warm- and cold-induced changes were calculated using repeated measurements ANOVA (re-ANOVA) followed by post-hoc analysis using Tukey HSD test. Peripheral blood flow and temperature were corrected for the influence of ambient and central temperatures, while OBF values were corrected for changes in MOPP in an analysis of covariance (ANCOVA). This approach was performed using a multivariate analysis to ensure independence of the change at each step from the other changes in re-ANOVA. Pearson's linear correlation factor was used to study the relationship between changes in peripheral and ocular blood flow parameters. Holm's sequentially rejective method was used to correct for multiple comparisons (Holm, 1979). Statistical analyses were performed in Statistica ® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. Statistical significance was defined as $p<0.05$.

3.6. Results

3.6.1. Sample

Thirty-two glaucoma patients (12 men and 20 women) and 34 control subjects (20 men and 14 women) were subjected to the experiment. However, as a result of this careful image analysis and subsequent rejection of those subjects

who exhibited poor HRF image quality during one or more steps of the experiment, only 24 glaucoma patients (9 men and 15 women) and 22 normal subjects (13 men and 9 women) were included in the final statistical analysis.

3.6.2. Baseline values

There was no significant difference in age between study groups (mean±SD: 68.38±11.92 and 62.59±9.43 years respectively, $p>0.05$). Table 3.5 shows the IOP and VF defects (MD and PSD parameters) as well as systemic haemodynamic parameters (BP, PP, MOPP, HR), and TT for both study groups. Peripheral and ocular blood flow parameters and FT values are summarized in Table 3.6. The IOP was significantly higher in the untreated glaucoma patients compared to normal subjects ($p=0.0005$). The VF damage was also more accentuated in the glaucoma group compared with normal controls ($p=0.016$ and $p=0.0005$ respectively). There were no significant differences between study groups with regard to systemic BP, PP, MOPP, HR and central temperature ($p>0.05$, Table 3.5). Moreover, the study groups were also comparable with regard to FT, FBF and OBF parameters (Flow, Velocity, Volume; $p>0.05$, Table 3.6).

3.6.3. Warm stimulation

3.6.3.1. Glaucoma patients

Warm stimulation resulted in a significant increase in DBP (% mean change ± SD: 6.56±13.40, $p=0.023$), HR, (% mean change ± SD: 5.40±8.06, $p=0.010$) and MOPP (% mean change ± SD: 7.82±19.64, $p=0.039$) compared to baseline values (Figures 3.3 and 3.4). OBF parameters, however, remained unchanged after immersion of the right hand in 41°C warm water ($p>0.05$, after correcting for the influence of MOPP).

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
IOP (mmHg)	23.63±4.89	17.95 ± 3.74	0.00005	0.0005
MD (dB)	-5.03±2.47	-1.05±2.00	0.002	0.016
PSD (dB)	5.74±3.62	1.70±0.42	0.00005	0.0005
SBP (mmHg)	134.46 ±18.90	127.95±21.95	0.286	NS
DBP (mmHg)	76.04 ±11.80	77.73 ±9.83	0.603	NS
MBP (mmHg)	95.51±13.29	94.47 ± 13.01	0.789	NS
PP (mmHg)	58.42±12.61	50.23±15.84	0.058	NS
MOPP (mmHg)	39.63±8.56	44.30±9.92	0.094	NS
HR (beats/min)	66.35±12.06	68.86±9.09	0.435	NS
TT (°C)	35.59 ±0.62	35.65± 0.48	0.707	NS

Table 3.5. Systemic BP, HR, IOP, and central temperature in the study groups at baseline. IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; PP: pulse pressure; MOPP: mean ocular perfusion pressure; HR: heart rate; TT: tympanic temperature; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
FT (°C)	32.26 ±3.35	34.27 ±2.66	0.030	NS
FBF (AU)	218.70±129.42	297.90 ±144.16	0.056	NS
Volume TNRR (AU)	13.44±4.08	12.81±2.69	0.565	NS
Flow TNRR (AU)	236.43±86.97	212.83±52.18	0.317	NS
Velocity TNRR (AU)	0.76 ±0.38	0.76±0.18	0.983	NS
Volume TR (AU)	16.55 ±3.74	16.90 ± 2.66	0.730	NS
Flow TR (AU)	292.58±88.72	317.97±79.52	0.339	NS
Velocity TR (AU)	1.04±0.30	1.14±0.27	0.312	NS

Table 3.6: Peripheral temperature and peripheral and ocular blood flow parameters at baseline. FT: finger temperature; FBF: finger blood flow; TNRR: temporal neuroretinal rim; TR: temporal retina; °C: degrees Celsius; AU: arbitrary units; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

3.6.3.2. Normal subjects

In normal subjects, warm stimulation had no effect on any systemic or ocular measured parameters when compared to baseline values ($p>0.05$).

3.6.3.3. Intergroup differences

The measured systemic, peripheral and ocular parameters after warm stimulation are listed in Tables 3.7 and 3.8. The only statistically significant difference between groups was in the level of IOP ($p=0.0005$). The changes between baseline and warm stimulation are shown in Tables 3.9 and 3.10.

There were no statistically significant differences between the two study groups ($p>0.05$).

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
IOP (mmHg)	23.42±4.99	17.68±3.27	0.00004	0.0003
SBP (mmHg)	135.67 ±24.40	126.77±21.80	0.200	NS
DBP (mmHg)	81.08±16.50	79.68 ±12.69	0.750	NS
MBP (mmHg)	99.28 ± 18.55	95.38 ±15.32	0.444	NS
MOPP (mmHg)	42.77 ±11.22	45.90± 11.27	0.345	NS
PP (mmHg)	54.58 ±12.68	47.09±11.79	0.044	NS
HR (beats/min)	69.29 ±12.53	69.59 ±8.82	0.926	NS
TT (°C)	35.57±0.60	35.67±0.49	0.710	NS

Table 3.7. Systemic BP, HR, IOP, and central temperature in the study groups after warm stimulation. IOP: intraocular pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; PP: pulse pressure; MOPP: mean ocular perfusion pressure; HR: heart rate; TT: tympanic temperature; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
FT (°C)	32.81±3.41	34.48 ±2.59	0.070	NS
FBF (AU)	211.46 ±124.60	263.72±142.78	0.192	NS
Volume TNRR (AU)	13.04 ±4.37	11.90 ±2.98	0.368	NS
Flow TNRR (AU)	226.97±88.74	205.46±42.12	0.367	NS
Velocity TNRR (AU)	0.80±0.30	0.74±0.15	0.480	NS
Volume TR (AU)	17.00±3.68	16.87±3.69	0.912	NS
Flow TR (AU)	294.43±83.60	318.56±67.83	0.330	NS
Velocity TR (AU)	1.04±0.29	1.14±0.23	0.265	NS

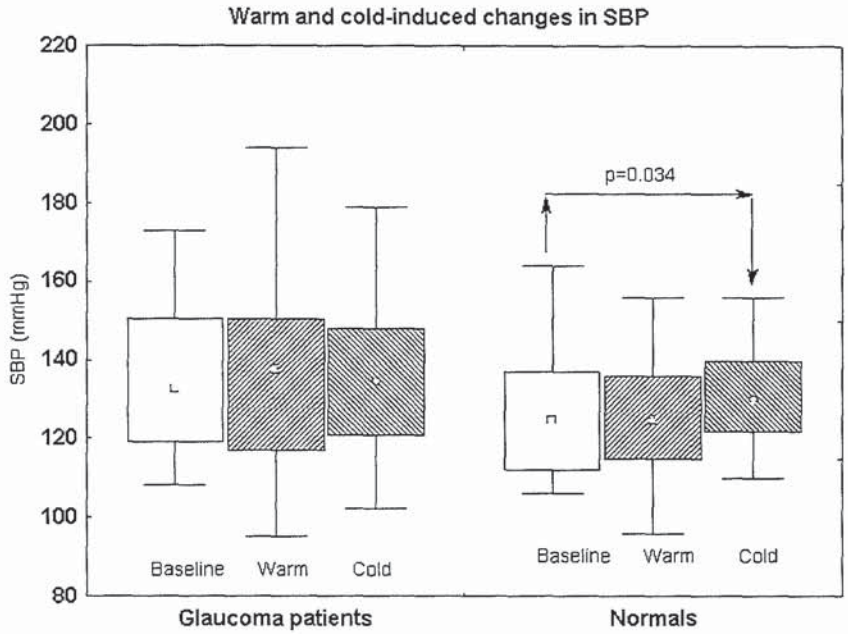
Table 3.8: Peripheral temperature and peripheral and ocular blood flow parameters after warm stimulation. FT: finger temperature; FBF: finger blood flow; TNRR: temporal neuroretinal rim; TR: temporal retina; °C: degrees Celsius; AU: arbitrary units; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Parameter	Warm-induced changes (%)		p-value	Cold-induced changes (%)		p-value	p-value*
	Glaucoma patients (n=24)	Control subjects (n=22)		Glaucoma patients (n=24)	Control subjects(n=22)		
IOP (mmHg)	-2.52±4.02	-1.32±3.56	0.294	1.80±4.57	1.63±3.52	0.886	NS
SBP (mmHg)	0.87±11.36	-0.75±7.13	0.570	0.04±6.28	4.65±2.53	0.015	NS
DBP (mmHg)	6.56±13.40	2.37±7.82	0.208	3.05±5.39	1.39±6.51	0.273	NS
MBP (mmHg)	3.80±11.85	0.81±6.27	0.297	1.61±5.67	2.78±4.32	0.458	NS
PP (mmHg)	-5.93±15.94	-3.34±18.43	0.611	-3.82±16.66	11.58 ±16.58	0.003	0.021
MOPP (mmHg)	6.78±19.72	1.84±10.49	0.302	1.62±5.97	3.34±6.47	0.354	NS
HR (beats/min)	5.40±8.06	1.35±6.92	0.077	0.56±7.13	-1.27±8.78	0.458	NS

Table 3.9: Intergroup differences in warm and cold-induced changes (% of baseline values) for IOP, systemic BP, PP, HR and MOPP. IOP: intraocular pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; PP: perfusion pressure; MOPP: mean ocular perfusion pressure; HR: heart rate. P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Parameter	Warm-induced changes (%)		p-value	Cold-induced changes (%)		p-value
	Glaucoma patients (n=24)	Control subjects (n=22)		Glaucoma patients (n=24)	Control subjects (n=22)	
TT (°C)	0.01±0.01	-0.02±0.01	0.897	0.05±0.04	0.02±0.03	0.833
FT (°C)	1.75±3.28	0.65±1.19	0.144	1.17±4.08	-0.01±2.32	0.236
FBF (AU)	7.21±50.31	-9.44±28.60	0.180	-29.95±44.67	-50.86±26.01	0.062
Volume TNRR (AU)	2.68±17.87	-4.32±16.40	0.243	3.67±29.38	-9.43±12.47	0.485
Flow TNRR (AU)	-0.06±25.49	0.57±27.27	0.944	-12.84±27.10	-6.22±23.61	0.470
Velocity TNRR (AU)	-1.40±24.41	2.04±25.99	0.691	-15.55±22.67	-4.52±15.72	0.135
Volume TR (AU)	0.54±16.20	1.25±8.89	0.874	-10.13±28.31	4.18±19.09	0.103
Flow TR (AU)	-1.02±13.98	3.00±19.18	0.459	-3.45±28.32	-4.75±22.10	0.885
Velocity TR (AU)	-1.96±13.43	2.61±18.44	0.381	-3.72±26.58	-5.06±21.20	0.875

Table 3.10: Intergroup differences in warm and cold-induced changes (% of baseline values) for FT, LDF, and ocular blood flow parameters. TT: tympanic temperature; FT: finger temperature; FBF: finger blood flow; TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units. Values are given in mean ±SD.



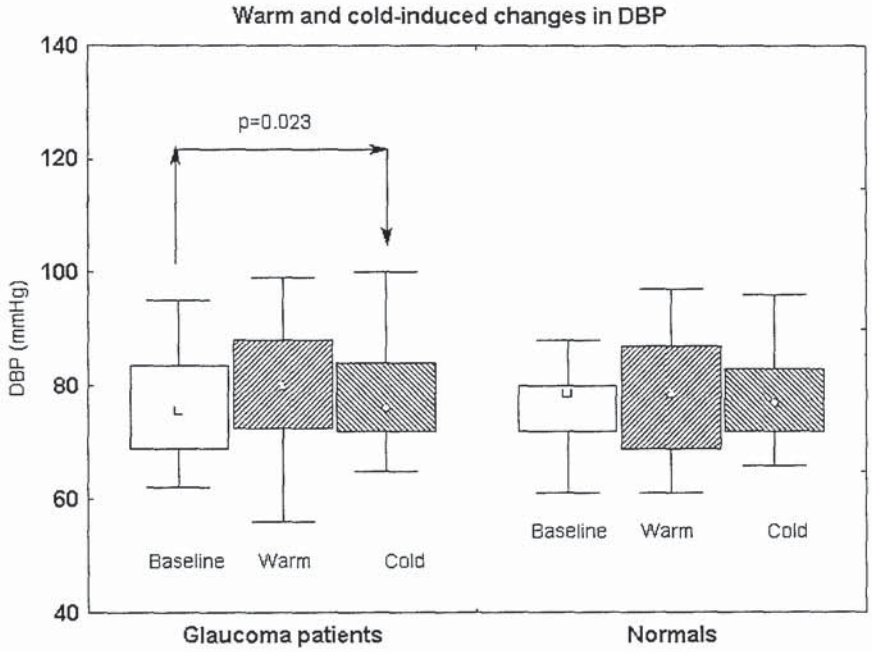


Figure 3.3: Warm and cold induced changes in blood pressure glaucoma patients and normal control subjects. SBP: systolic blood pressure; DBP: diastolic blood pressure.

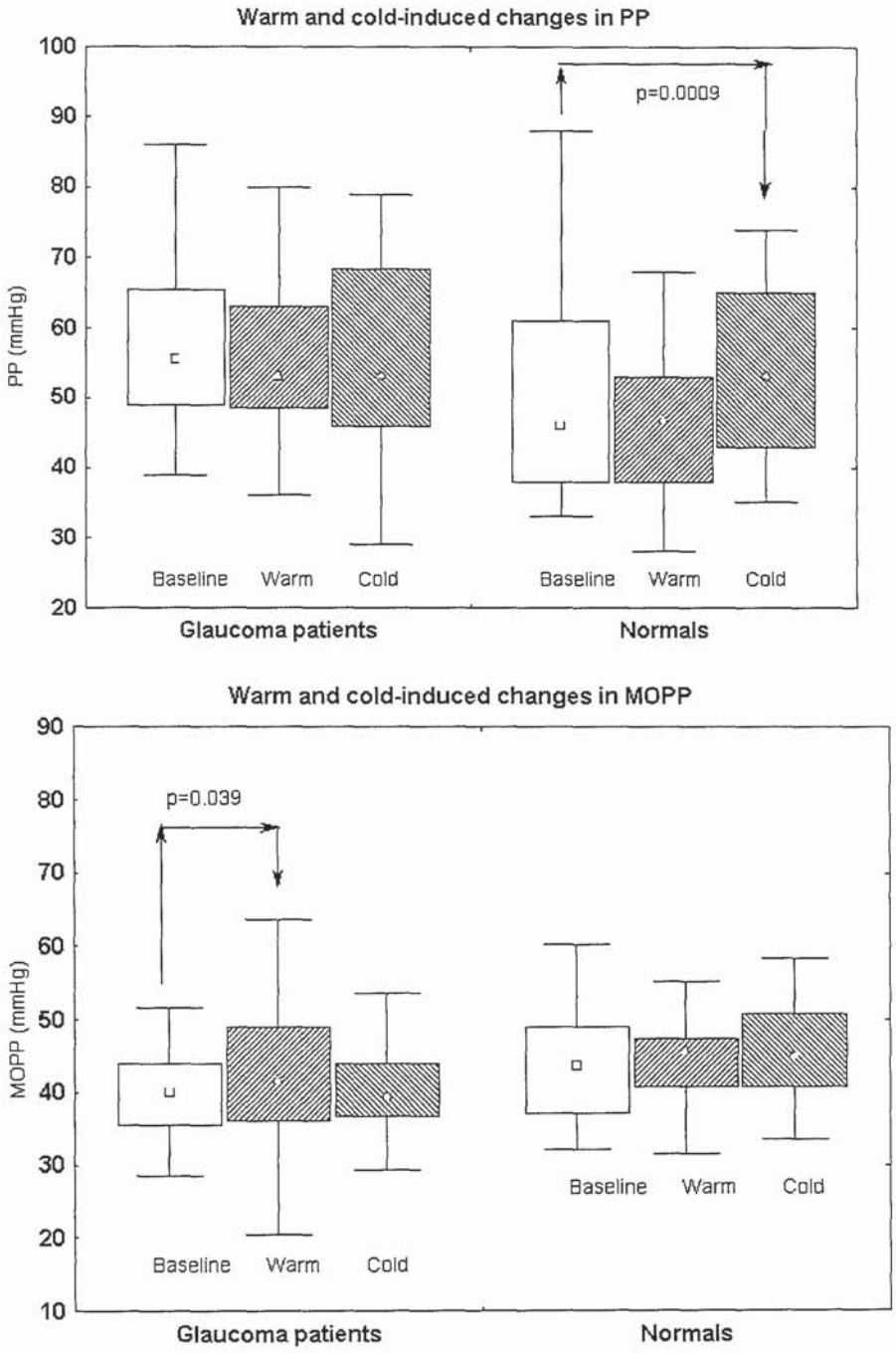


Figure 3.4: Warm and cold induced changes in pulse pressure and ocular perfusion pressure in glaucoma patients and normal control subjects. PP: pulse pressure; MOPP: mean ocular perfusion pressure.

3.6.4. Cold stimulation

3.6.4.1. Glaucoma patients

IOP, systemic BP, PP, MOPP and HR were not significantly modified by cold stimulation; however, after correcting for the influence of room and body temperature, there was a significant decrease in FBF (mean change ± SD: -29.95±44.67, p=0.0003, Figure 3.5) compared to baseline values. In the glaucoma group, cold stimulation determined a significant decrease in the parameter "Velocity" (mean change ± SD: 15.55±22.67, p=0.021, Figure 3.6) measured at the temporal neuroretinal rim level. These changes were significant after correcting for the influence of MOPP on these parameters.

3.6.4.2. Normal subjects

In normal subjects, immersion of the right hand in 4°C cold water for 1 minute resulted in a statistically significant increase in SBP (% mean change ± SD: 4.65±2.53, p=0.034) and PP (% mean change ± SD: 11.58±16.58, p=0.0009). The cold provocation also resulted in a significantly decrease in FBF compared to baseline values (% mean change ± SD: -50.86±26.01, p=0.0001). In normals, cold stimulation did not, however, influence the OBF parameters.

3.5.3.3 Intergroup differences

The measured systemic, peripheral and ocular parameters after immersion of the right hand in 4°C cold water for 1 minute are listed in Tables 3.11 and 3.12. The only statistically significant difference between groups was in the level of IOP (p=0.0003), with higher values in the POAG patients.

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
IOP (mmHg)	23.96± 3.63	18.27±3.72	0.00004	0.0003
SBP (mmHg)	134.21 ±18.55	133.04± 17.26	0.827	NS
DBP (mmHg)	78.33 ±13.02	78.77 ±10.40	0.901	NS
MBP (mmHg)	96.96±13.54	96.86±11.89	0.980	NS
MOPP (mmHg)	40.68±8.58	46.30±9.25	0.038	NS
PP (mmHg)	55.88 ±14.16	54.27±11.62	0.678	NS
HR (beats/min)	66.52 ±13.63	67.54 ±7.63	0.762	NS
TT (°C)	35.53±0.70	35.64±0.40	0.858	NS

Table 3.11. Systemic BP, HR, IOP, and central temperature in the study groups after cold provocation. IOP: intraocular pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; PP: pulse pressure; MOPP: mean ocular perfusion pressure; HR: heart rate; TT: tympanic temperature; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Parameter	Glaucoma patients (n=24)	Control subjects (n=22)	p-value	p-value*
FT (°C)	32.77±3.48	34.25±2.60	0.112	NS
FBF (AU)	131.15 ±96.10	151.44.90 ±119.31	0.527	NS
Volume TNRR (AU)	13.29±4.71	12.05 ±3.35	0.383	NS
Flow TNRR (AU)	209.11±78.09	223.64±57.36	0.533	NS
Velocity TNRR (AU)	0.73 ±0.24	0.81±0.22	0.349	NS
Volume TR (AU)	14.82±4.16	16.76 ± 3.07	0.125	NS
Flow TR (AU)	280.71 ±90.71	300.01±79.60	0.504	NS
Velocity TR (AU)	1.00±0.32	1.08±0.27	0.547	NS

Table 3.12: Peripheral temperature and peripheral and ocular blood flow parameters after warm stimulation. FT: finger temperature; FBF: finger blood flow; TNRR: temporal neuroretinal rim; TR: temporal retina; °C: degrees Celsius; AU: arbitrary units; P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean ±SD.

Intergroup differences in cold induced changes (% of baseline values) are listed in Tables 3.8 and 3.9. These changes were comparable between study groups for all the measured parameters ($p>0.05$) with the exception of PP; normals exhibited higher PP changes from baseline values than glaucoma during cold provocation ($p=0.021$).

The percent change in FBF did not correlate with changes in the measured ocular blood flow parameters for the same stage of the experiment in either of experimental group ($p>0.05$).

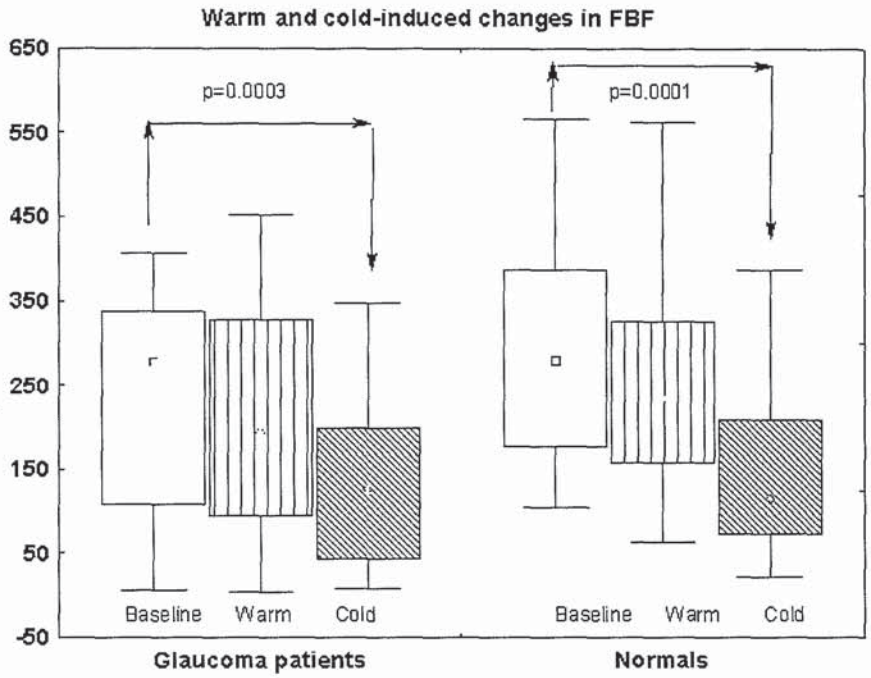


Figure 3.5: Warm and cold-induced changes in finger blood flow (FBF) in glaucoma patients and normal control subjects.

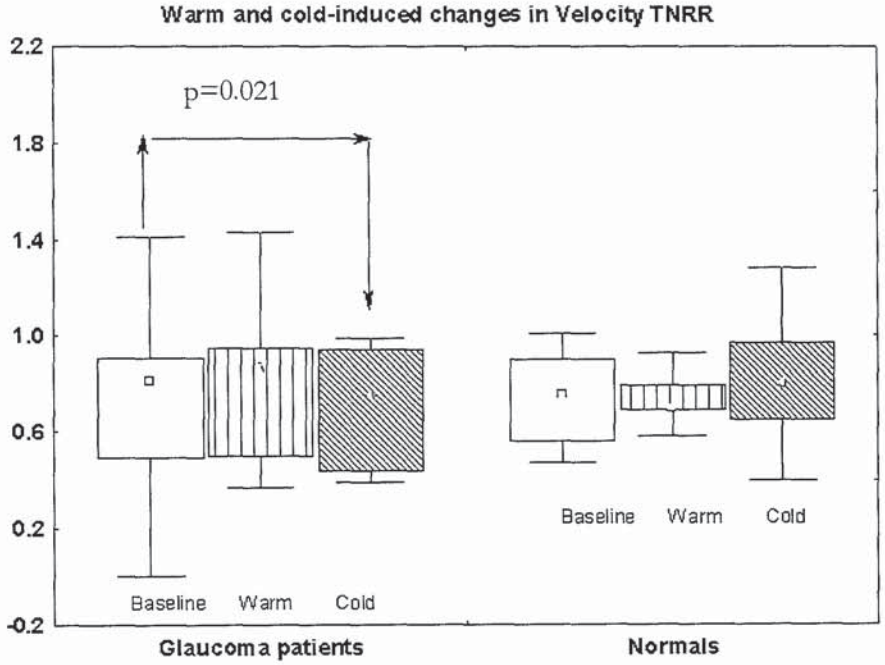


Figure 3.6: Warm and cold-induced changes in HRF parameter "Velocity" measured at the temporal neuroretinal rim level (TNRR) in glaucoma patients and normal control subjects.

3.7. Discussion

3.7.1. Main findings

The present study assessed the response of BP as well as peripheral and ocular blood flow to warm and cold provocation. Our results disclosed a difference in the systemic and ocular vascular response to these stimuli between untreated POAG patients and otherwise healthy age-matched control subjects. While DBP, HR and MOPP increased during immersion of the test hand in 40°C water in POAG patients, in control subjects SBP, and PP increased markedly during cold provocation. Moreover, in glaucoma patients cold stimulation did not affect the systemic BP; however, it resulted in a significant reduction in blood velocity at TNRR level. No OBF changes were observed in normal subjects after cold provocation. Cold provocation resulted in a similar reduction in FBF in both study groups.

3.7.2. Temperature provocation

The CPT is a well-known provocation test able to elicit sympathetic activation. The initial normal response to cold consists of cutaneous vasoconstriction and an increase in systemic BP; a blunt BP response to the CPT may indicate autonomic dysregulation (Velasco *et al.,* 1997; Lafleche *et al.,* 1998).

3.7.2.1. The effect of warm stimulation

In our study, glaucoma patients exhibited high DBP and HR during warm but not cold provocation. It is thought that the vascular responses to warm immersion are the result of stimulation of the preoptico-anterior hypothalamic area in the brain (Lovallo, 1975). Severe whole body heating results in an increased HR without changes in systemic BP (Yamazaki *et al.,* 1997) while mild heating has no influence on either HR or systemic BP (Yamazaki and Sone, 2000). Changing the stimulated skin area further decreases the temperature effect on systemic BP (Yamazaki and Sone, 2000). Therefore, the immersion of one hand in 40°C warm water for 5 minutes was unlikely to provoke any HR or BP changes in our subjects. Indeed, the control group did not exhibit any systemic circulatory changes during warm stimulation. The most

obvious explanation for the high BP and HR during warm provocation in our glaucoma patients could be an anticipatory reaction to the physical stress; this reaction occurred despite the fact that patients were allowed 15-20 minutes in a quiet room in order to achieve a sufficient mental and physical calm before the experiment. In glaucoma patients, the haemodynamic response due to the anticipation of the test may have been stronger than the effect of the warm stimulation itself and the result was an increase in BP and HR. This type of vascular hyperactivity seen in the glaucoma group is similar to that demonstrated in subjects at risk for cardiovascular diseases in whom the anticipatory and recovery vascular responses are better predictors for subsequent circulatory disturbances than the direct stress-induced reactivity (Gregg *et al.,* 1999).

3.7.2.2. The effect of cold stimulation on systemic and ocular blood flow

In our glaucoma patients, immersion of the tested hand in 4°C cold water did not affect the systemic BP. Similar results were reported by Nicolela et al. (Nicolela *et al.,* 2003) in POAG patients and control subjects after 30 minutes body surface cooling; the authors observed that despite the increase in plasma ET_1, glaucoma patients did not exhibit any change in their systemic BP, while to control subjects demonstrated a significant increase in DBP and MOPP with cooling. No explanation was offered for this finding. We suggest that a blunted BP response to cold provocation in POAG patients could signal an abnormal neural pathway resulted from a lack of sympathetic participation. Whether this type of reaction has a role in glaucoma pathogenesis is still to be clarified.

This study demonstrated an abnormal decrease of the blood velocity in the temporal region of the NRR after 1-minute cold provocation in patients suffering from POAG. To our knowledge this is the first observation of an abnormal ocular haemodynamic response to cold provocation in POAG patients. Previous studies failed to report any OBF changes to CPT in either retrobulbar (Rojanapongpun and Drance, 1993) or retinal circulations (Nicolela *et al.,* 2003). Similarly, the study showed no haemodynamic changes in temporal peripapillary retinal circulation; however, cooling significantly decreased the

blood velocity measured at the temporal NRR level. Since the vessels in the retina and in the prelaminar portion of the optic nerve have no neural innervation (Laties, 1967; Ye *et al.,* 1990) it is reasonable to assume that a local neural-mediated vasoconstriction cannot explain our observation. We can not exclude, however, a possible sympathetic influence on the blood flow downstream to the measurement point, at the level of PCAs and CRA; these vessels have a rich supply of autonomic nerves (Ehinger, 1966; Laties, 1967; Ernest, 1979) and could, in theory, react to a powerful vasoconstrictor stress such as CPT. Nevertheless, our glaucoma patients did not demonstrate a systemic sympathetic activation during cold provocation as measured by an increase in systemic BP. Although activation of thermoreceptors in the skin during CPT could result in different activation of the arteries situated closer to than of those situated away from the body surface (e.g., ocular arteries) (Lafleche *et al.,* 1998), it is difficult to presume that in our patients, the sympathetic response to cooling manifested only at the retrobulbar vessels level.

A more probable explanation could be the involvement of endothelial factors. Indeed, in a recently published study Nicolela et al. (Nicolela *et al.,* 2003) demonstrated a significant increase in ET_1 levels after body cooling in POAG patients. Although they failed to demonstrate any haemodynamic changes in the retinal circulation, they speculated that a high ET_1 concentration following CPT could influence the optic nerve blood flow. This conclusion does not come as a surprise; it has already been demonstrated that in humans, systemic injection of ET_1 results in a reduction of both pulsatile and optic nerve blood flow (Schmetterer *et al.,* 1997). Although we did not measure plasma ET_1 levels in our patients, in the light of previous research it is possible that high plasma levels of this extremely potent vasoconstrictor peptide resulted in a decreased blood flow at the NRR level in our glaucoma patients.

In our study, optic nerve blood flow was assessed by means of HRF technology. This is a noninvasive, high-resolution mapping technique estimating capillary blood flow of the ONH and retina with an acceptable reliability (Michelson *et al.,* 1996*b*; Chauhan and Smith, 1997; Bohdanecka *et*

al., 1998) and adequate ability to show blood flow alterations (Lietz *et al.,* 1998). Moreover, in our study we used a central alignment technique described by Sehi and Flanagan (Sehi and Flanagan, 2004), which allows an optimal focal plane for the flowmetry of the NRR and more repeatable measurements of the blood flow at this level.

Using this technique we observed that blood flow in the anterior optic nerve of glaucoma patients was normal under baseline conditions but became disturbed when challenged. This observation suggests that in glaucoma patients the blood flow may not be reduced continuously. If chronically reduced blood flow were able to induce glaucomatous damage, vascular diseases with chronic reduction in blood flow such as arteriosclerosis would have to be related to the prevalence of glaucoma. However, neither risk factors for arteriosclerosis nor arteriosclerotic alterations in the carotid vessels could be demonstrated to be related to the prevalence of glaucomatous optic neuropathy (Morgan and Drance, 1975; Carter *et al.,* 1990; Schulzer *et al.,* 1990; Klein *et al.,* 1993; Gasser *et al.,* 1999*a*). An alternative explanation could be that these patients suffer from ocular vascular dysregulation, which could predispose the ONH to ischaemia and reperfusion damage. Indeed, vascular dysregulation has been advocated as a possible contributing factor in the aetiology of POAG (Chung *et al.,* 1999 *a*; Anderson, 1999; Gherghel *et al.,* 1999; Gherghel *et al.,* 2000; Hosking *et al.,* 2004). The abnormal reduction of blood flow at the NRR in response to a stress factor such as cold could increase the susceptibility of the optic nerve to high IOP and low perfusion pressure in these individuals.

3.7.3. Conclusion

In summary, patients suffering from POAG appear to show an abnormal BP and HR response to warm provocation. In addition, they also demonstrated a blunted BP response and a reduction in ONH blood flow in response to cold provocation. It can be concluded that this reaction is indeed a manifestation of an autonomic dysfunction, which could contribute to the onset or progression of glaucomatous optic neuropathy. The presence of autonomic dysfunction in glaucoma patients will be tested in the Chapter 4 of the present thesis.

4. Relationship between Silent Myocardial Ischaemic Episodes and Autonomic Function in Untreated Primary-Open Angle Glaucoma Patients: Relationship to Abnormal Variations in Systemic Blood Pressure and Heart Rate

4.1. Abstract

Purpose: To investigate the relationship between the onset of silent cardiac ischaemic episodes and alterations in BP, HR and HRV parameters in newly diagnosed and untreated POAG patients.

Methods: Twenty-four glaucoma patients and 23 age-matched control subjects were subjected 24-hour ambulatory BP and ECG monitoring by using a device capable of both triggering BP recordings during episodes of abnormal cardiac activity and computing HRV parameters (Cardiotens-01, Meditech Ltd., Hungary). Systemic BP, HR, number and duration of silent cardiac ischaemic episodes (defined as transient myocardial ischaemia in the absence of angina or other cardiac symptoms) as well as HRV frequency domain analysis parameters (LF, HF and LF/HF) ratio were measured.

Results: In comparison to control subjects, glaucoma patients demonstrated higher LF and LF/HF ratio parameters (LF-24h: $p=0.022$; LF/HF-24h: $p=0.032$; LF-active: $p=0.020$; LF/HF-active: $p=0.029$; LF-passive: $p=0.044$; LF/HF-passive: $p=0.050$). Glaucoma patients free from cardiac ischaemia (10 patients) also demonstrated higher LF and LF/HF during the active period of the measurement ($p=0.010$ and $p=0.021$ respectively). In both glaucoma (14 patients) and control subjects (10 subjects) with electrocardiogram alterations, the longest silent cardiac ischaemic episode was associated with a significant increase in HR ($p=0.007$ and $p=0.010$ respectively) but not with changes in HRV parameters ($p>0.05$). In glaucoma patients, the longest ischaemic episode was also associated with a significant increase in diastolic and mean BP ($p=0.007$ and $p=0.003$ respectively).

Conclusion: Independent of a history of cardiovascular diseases, glaucoma patients exhibit blunted HRV, which may be indicative of abnormal autonomic function.

4.2. Introduction

The substantial number of POAG sufferers that continue to exhibit disease progression despite therapeutically lowered IOP and the common finding of patients with normal IOP that develop glaucoma has prompted the search for other risk factors. It has already been demonstrated that POAG patients suffer from blood flow disturbances in both the ocular (Rojanapongpun *et al.,* 1993; Wolf *et al.,* 1993; Costa *et al.,* 1994; Nicolela *et al.,* 1996*b*; Kaiser *et al.,* 1997; Butt *et al.,* 1997; Findl *et al.,* 2000; Gherghel *et al.,* 2000; Gherghel *et al.,* 2001; Emre *et al.,* 2004; Fuchsjager-Mayrl *et al.,* 2004) and systemic (Susanna and Basseto, 1992; Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham *et al.,* 1995; Stroman *et al.,* 1995; Waldmann *et al.,* 1996; O'Brien and Butt, 1999; Kashiwagi *et al.,* 2000; Kashiwagi *et al.,* 2001; Emre *et al.,* 2004; Fuchsjager-Mayrl *et al.,* 2004) circulations.

Among the factors that may influence blood flow physiology throughout the human body, variables such as BP and HR have a circadian rhythm, dependent on the ANS (Appenzeller and Orbie, 1997). Chronic imbalances of the ANS could lead to adverse cardiovascular events, such as myocardial ischaemia (Moore and Chester, 2001; Cohn *et al.,* 2003). Moreover, a systemic circulatory imbalance could affect the eye either by leading to or exacerbating glaucoma in susceptible individuals. POAG itself has been directly linked to ANS dysfunction. Systemic parasympathetic and sympathetic neuropathies have been reported in patients with both open-angle and NTG (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003) and it has been suggested that disturbed autonomic activity may play a role in the pathogenesis of the glaucomatous damage. Moreover, POAG has also been associated with episodes of silent myocardial ischaemia (Kaiser *et al.,* 1993*a*; Waldmann *et al.,* 1996). Since the ECG changes associated with asymptomatic myocardial ischaemia occurred mainly during the night while the patients were asleep, the authors concluded that the major cause were not atherosclerotic changes, but functional vascular dysregulation. This dysregulation could occur in the context of a more general autonomic dysfunction.

HRV analysis is a widely used method in cardiovascular research providing an indirect tool for the assessment of ANS activity. HRV abnormalities (Kashiwagi *et al.,* 2000) have also been significantly associated with the presence of glaucoma. Although glaucoma patients seen in clinical practice, especially those suffering from normal NTG or progressive glaucomatous neuropathy, frequently suffer from a variety of cardiovascular diseases, such as systemic arterial hypertension and silent myocardial ischaemia (Flammer *et al.,* 2001), studies performed so far carefully selected only those glaucoma patients free from any systemic vascular diseases. This is, however, a rare encounter in clinical practice.

4.3. Hypothesis

POAG has been associated with both ANS dysfunctions (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003) and occurrence of silent myocardial ischaemic episodes (Kaiser *et al.,* 1993*a*; Waldmann *et al.,* 1996). However, to date no link has been made between the two entities in patients suffering from POAG. We hypothesize that in some patients suffering from POAG, the occurrence of silent cardiac ischaemic events is associated with episodes of autonomic dysfunction, manifested as BP, ECG alterations or both.

4.4. Aims

The aim of this study was to examine the relationship between the onset of silent cardiac ischaemia, and alterations in BP, HR and HRV parameters during the normal daily routine of consecutive newly diagnosed and untreated POAG patients.

4.5. Subjects and methods

4.5.1. Recruitment of untreated primary open-angle glaucoma patients

Consecutive, newly diagnosed and previously untreated POAG patients attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust, Birmingham, UK, were included in this prospective study.

4.5.1.1. Inclusion criteria

Patients underwent diurnal IOP phasing and were diagnosed as having POAG if at least two IOP measurements were greater than 24 mmHg, glaucomatous cupping of the optic disc on funduscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with a diagnosis of glaucoma using program 24-2 of the Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA). The characteristics of glaucomatous VF defects have previously been described (see Table 3.1).

4.5.1.2. Exclusion criteria

Exclusion criteria are outlined in Table 4.1.

- Patients with narrow iridocorneal angles;
- Evidence of secondary glaucoma;
- Pseudoexfoliation;
- Pigmentary dispersion;
- A history of intraocular surgery;
- Any form of retinal or neuro-ophthalmologic disease that could result in VF defects; and
- Any significant cardiovascular and metabolic disease except systemic hypertension and cardiac ischaemia;
- Treatment with beta-blockers and digitalis and pacemaker wear.

Table 4.1: Exclusion criteria for the glaucoma patients' group

4.5.2. Control subject recruitment

The control group was comprised of subjects who have never been diagnosed with glaucoma and were recruited from patients' spouses and other volunteers. A history of cardiovascular ischaemic disease and of abnormal systemic BP, as well as a history of chronic anti-anginal and antihypertensive medication (with the exception of beta-blockers) were, however, neither inclusion nor exclusion criteria for the control group.

4.5.3. Ethical approval

Ethical approval was obtained from the local medical ethics committees (Aston University and Heartlands and Solihull NHS Trust), and written informed consent was received from all subjects prior to entry into the study. The study was designed and conducted in accordance with the Tenets of Declaration of Helsinki.

4.5.4. Experimental protocol

4.5.4.1. Visual field assessment

VF was assessed by means of Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA) with a size III white stimulus using the full threshold program 24-2. The instrument has been described in the Chapter 3.5.5.1 of the thesis.

Two VF examinations were performed. The results from the second VF measurement were included in the analysis.

4.5.4.2. Intraocular pressure measurement

IOP was measured using a standard Goldmann tonometry technique after instillation of a topical anaesthetic (Benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd). The technique has been described in the Chapter 3.5.5.2 of the present thesis.

4.5.4.3. Ambulatory blood pressure and electrocardiogram measurements

A computer-operated ambulatory blood pressure and ECG monitor (Cardiotens-01, Meditech Ltd., Hungary) was installed for each subject between 9 am and 11am at the Fast Track Glaucoma Clinic. A detailed description of this device has been offered in Chapter 1.11.6.

Measurements were performed in ambulatory conditions. All subjects maintained their normal activity and were carefully instructed to complete a diary each time their activities changed, and when any chronic medication was taken. The 24-hour BP, and ECG data were later downloaded and analyzed (see Appendix 1). Recordings were analysed using the "Medibase" software program Version 1.42 (Meditech, Budapest, Hungary).

For every subject, the BP unit was programmed to measure BP oscillometrically every 30 minutes over a 24-h period. The monitor was also programmed to execute extra BP recordings triggered by automatically identified episodes of silent cardiac ischaemia (Uen *et al.,* 2003). Average daytime (active period), average night-time (passive period) and average 24-h SBP and DBP were evaluated. Active and passive periods were determined for each patient based on the true wake and sleep times recorded in each individual subject diary. From these readings, the active and passive MBP levels were calculated according to the Equation 1.7.

A measurement outlier rejection method, based on PP determination (calculated according to Equation 1.1) was applied. A PP of less than 10 mmHg when the SBP was below 100 mmHg and of less than 10% of the systolic reading when the SBP was larger than 100 mmHg were considered non-physiologic and rejected (Graham *et al.,* 1995). Furthermore, at least 80% of the programmed recordings were required for a diurnal curve to be considered in the present analysis.

The nocturnal BP dip, was expressed as the percentage change in MBP between the active and passive periods for each subject. According to the reduction in average MBP values from the active to the passive period, subjects were classified as nondippers (<10%), dippers (>10% and <20%) and overdippers (>20%) (Verdecchia *et al.,*1991; Kario *et al.,* 1996). More details are offered in the Chapter 1.11.5.2 of the present thesis.

Circadian changes in BP (SBP, DBP and MBP) were calculated as: *active period – passive period* values.

Details on HR measurement are offered in Chapter 1.11.1. The Cardiotens-01 was programmed to measure HR every 30 minutes over a 24-h period. HR values during episodes of silent cardiac ischaemia were also determined.

Average HR values were calculated for the active, passive and 24-h periods. Circadian changes in HR were calculated as: *active period – passive period* values.

ST-segment depression represents the ECG sign for silent myocardial ischaemia. More details are provided in the Chapter 1.11.3.4.

The ECG unit of the CardioTens-01 monitor performed ECG an registration via two independent channels, producing an strip with two leads (CM5 and CC5) of 30s duration every 5 minutes (Figure 4.1); these leads are recommended by the manufactures as best suitable for ST analysis targeted procedures.

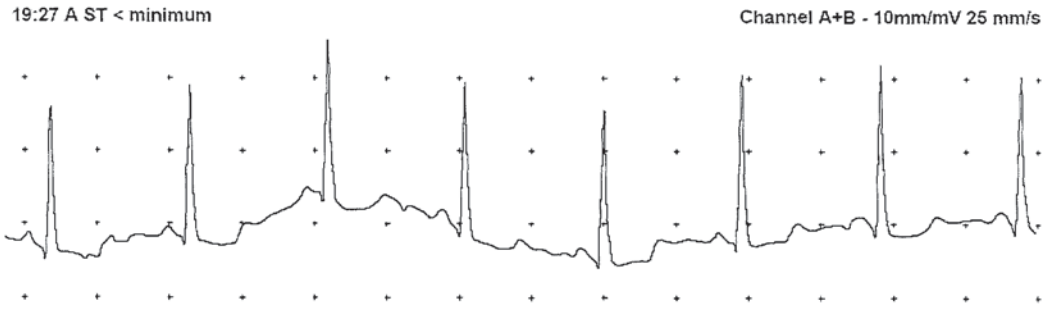


Figure 4.1: ECG strip recorded with CardioTens-01.

Only ECG recordings free of artefacts (missing data, noise, etc.) were included in the analysis. A silent ischaemic response was defined according to the 1-1-1 rule (Uen *et al.,* 2003) (i.e,, as horizontal or downsloping ST-segment depression of $\geq$ 1mm below the isoelectric baseline, persisting for $\geq$ 1 min and situated at 1 min interval from the previous episode).

The total number of ischaemic events (TIE), total ischaemic burden (TIB) and the LIE were automatically reported by the device for each subject.

BP and HR values measured following a trigger due to silent cardiac ischaemic events were also analyzed. These readings were taken approximately 60 seconds after the onset of the ischaemic event, this being the time required by the device to inflate and deflate the cuff. A significant change in BP and HR during the silent cardiac ischaemic events was arbitrarily defined by us as a 10% increase or decrease in BP when compared to 24-h MBP values. For the simplicity of data analysis, only BP and HR values measured during the longest LIE were included in the analysis.

4.5.4.4. Heart rate variability measurement

HRV measurement offers details about ANS activity. This parameter is described in the Chapter 1.11.4 of the present thesis.

HRV values are calculated using the "Medibase" software program Version 1.42 (Meditech, Budapest, Hungary) after the recordings were uploaded intoa a computer. This software allows us to measure both frequency and time domain parameters (see Chapter 1.11.4). For the simplicity of data analysis, only frequency domain parameters (LF, HF and LF/HF ratio) were included in our study. These parameters were calculated for both the active and passive periods of the recording. Circadian changes in LF, HF and LF/HF parameters were also calculated as: *active period – passive period* values. Since transient variations in HRV have been validated as measures of short-term changes in autonomic tone (Freed *et al.,* 1994), HRV parameters were determined for a period of 10 minutes before and after the LIE.

4.5.5. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences between the study groups at baseline were calculated using Student's *t*-test for independent variables. Differences in the frequency of subjects using various systemic drugs were tested by means of chi-square tests. Differences in ECG, BP and HRV parameters measured during different steps of the experiment were analyzed using a repeated measurements analysis of variance (re-ANOVA) followed by post-hoc analysis using Tukey HSD test. The influence of circadian variations in BP and HR on HRV parameters was calculated using a stepwise linear multiple regression analysis. Statistical analyses were performed with Statistica ® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. Statistical significance was defined as $p<0.05$.

4.6. Results

4.6.1. Sample

Twenty-nine glaucoma patients (10 men and 19 women) and 30 control subjects (14 men and 16 women) were subjected to the experiment. Due to either multiple artefacts or electrode loss during the ambulatory recording (mainly during sleep), ECGs could not be interpreted in 6 glaucoma patients and in 7 control subjects, thus reducing the number of subjects included in the

final analysis to 23 glaucoma patients (9 men and 14 women) and 22 control subjects (12 men and 10 women). The sample characteristics of the remaining participants are given in Table 4.2. There were no statistically significant differences in age, body mass index and number of smokers between study groups ($p>0.05$). Moreover, no significant difference was evident between the groups regarding the number of subjects taking antihypertensive and anti-ischaemic medication ($p>0.05$). However, there were significant differences in IOP ($p=0.0002$) and VF defects ($p=0.017$ and $p=0.00003$ respectively) with glaucoma patients showing higher values than the control group.

The duration of night-time sleep was similar among subjects from both groups (6.67±1.27 h and 6.93±1.28h respectively, $p>0.05$).

Parameter	Glaucoma patients (n=23)	Control subjects (n=22)	p-value
Age (years)	62.52±9.22	68.52±11.92	0.067
IOP (mmHg)	24.04±4.47	18.61±3.53	0.0002
MD (dB)	-5.03±2.47	-1.04±1.95	0.017
PSD (dB)	5.74±3.61	1.67±0.42	0.00003
Body Mass Index (kg/m^2)	25.10±4.23	24.82±5.62	0.832
Smokers (number)	3	3	0.955
Positive history of systemic hypertension and/or chronic cardiac ischaemic disease (number)	7	5	0.397
Duration of sleep (hours)	6.67±1.27	6.93±1.28	0.484

Table 4.2: Clinical characteristics of the study groups. IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing. Values are given in mean ± SD.

4.6.2. 24-h blood pressure and heart rate measurements: intergroup comparison

The measured BP and HR values are listed in Table 4.3. There were no statistically significant differences between the study groups ($p>0.05$). Although there were significantly more over-dippers in the glaucoma group (6 patients) than among the control subjects (1 subject), $p=0.037$, the numbers of glaucoma patients and control subjects with normal dipping (8 patients and 10 control subjects) and non-dipping (9 patients and 11 control subjects) in MBP were statistically comparable ($p>0.05$).

4.6.3. ST-segment changes with respect to blood pressure and heart rate fluctuation

Silent cardiac ischaemic events were recorded in 14 glaucoma patients (58.88%, mean age ± SD: 66.14 ±11.16 years) and 10 control subjects (43.48%, mean age ± SD: 63.40 ±9.41 years); the age and number of subjects with silent cardiac ischaemia were similar between groups ($p>0.05$).

a) Comparision between subgroups of patients and control subjects who experienced silent myocardial ischaemic episodes during the test period

The mean TIE, TIB, and LIE as well as BP and HR values measured when triggered by LIE in these subgroups of the study populations are given in Table 4.3. These parameters were not statistically different between subgroups ($p>0.05$). The subgroups were also similar with regard to the circadian distribution of the ischaemic episodes ($p>0.05$, data given below).

Parameter	Glaucoma patients (n=23)	Control subjects (n=22)	p-value
SBP-24h (mmHg)	128.88±12.59	124.25±8.74	0.327
DBP-24h (mmHg)	71.37±8.20	71.80±11.29	0.882
MBP-24h (mmHg)	90.54±8.89	89.28±13.35	0.707
HR-24h (beats/min)	69.00±11.55	68.14±8.03	0.778
D-SBP (mmHg)	136.19±12.79	130.30±19.13	0.220
D-DBP (mmHg)	78.87±11.57	76.74±11.14	0.525
D-MBP (mmHg)	97.97±11.21	94.59±13.35	0.352
D-HR (beats/min)	73.35±12.66	72.59±9.58	0.821
N-SBP (mmHg)	121.58±16.05	118.41±19.38	0.548
N-DBP (mmHg)	63.87±10.19	67.19±12.29	0.322
N-MBP (mmHg)	83.11±11.48	84.26±14.22	0.762
N-HR (beats/min)	64.66±11.26	64.00±7.23	0.820
Circadian SBP (mmHg)	14.61±14.45	11.68±10.57	0.441
Circadian DBP (mmHg)	15.00±14.36	9.22±6.85	0.093
Circadian MBP (mmHg)	14.87±14.09	10.04±7.81	0.163
Circadian HR (beats/min)	8.71±6.36	9.14±5.96	0.814

Table 4.3: Blood pressure and heart rate characteristics of the study groups. SBP: systolic blood pressure; DBP: diastolic BP; MBP: mean BP; D: diurnal (active) value; N: nocturnal (passive) value; circadian: circadian variation=active period-passive period values. Values are given in mean ± SD.

Parameter	Glaucoma patients (n=14)	Control subjects (n=10)	p-value
TIE (number)	5.67 ±5.94	5.42±8.12	0.927
TIB (minutes)	13.64±13.86	9.17±10.74	0.368
LIE (minutes)	5.27± 5.48	3.08±1.86	0.193
SBP (mmHg)	141.07±19.24	132.42±19.24	0.377
DBP (mmHg)	83.60±17.17	69.75±21.58	0.075
MBP (mmHg)	102.76±14.75	90.64±23.21	0.111
HR (beats/min)	85.53±16.66	83.92±20.61	0.818

Table 4.4: ECG and BP parameters measured during LIE. TIE: total ischaemic events; TIB: total ischaemic burden; LIE: longest ischaemic event; SBP: systolic blood pressure; DBP: diastolic BP; MBP: mean BP-values measured during the LIE. Values are given in mean ± SD.

b) Glaucoma patients suffering from silent cardiac ischaemic episodes (14 patients)

A total of 40 ischaemic events were recorded in this subgroup of glaucoma patients. All ischaemic events were asymptomatic. From these ischaemic events, LIE was determined for each patient, and their circadian distribution is illustrated in Figure 4.2. Only 2 of these glaucoma patients (14.29%) demonstrated an important silent ischaemic event (determined by ST-segment depression ≥ 1mm, persisting for ≥ 1 min) during sleep or early hours of the morning; in all the others, the LIE occurred during late morning (3 patients: 21.43%) and during the afternoon or evening (9 patients: 64.28%), while (according to patient's diaries) patients either performed various physical activities, such as walking or house work of various intensity (9 patients, 64.28%) or rested in bed without sleeping (5 patients, 35.72%). The difference between the type of activity performed by patients while LIE occurred was not statistically significant ($p>0.05$).

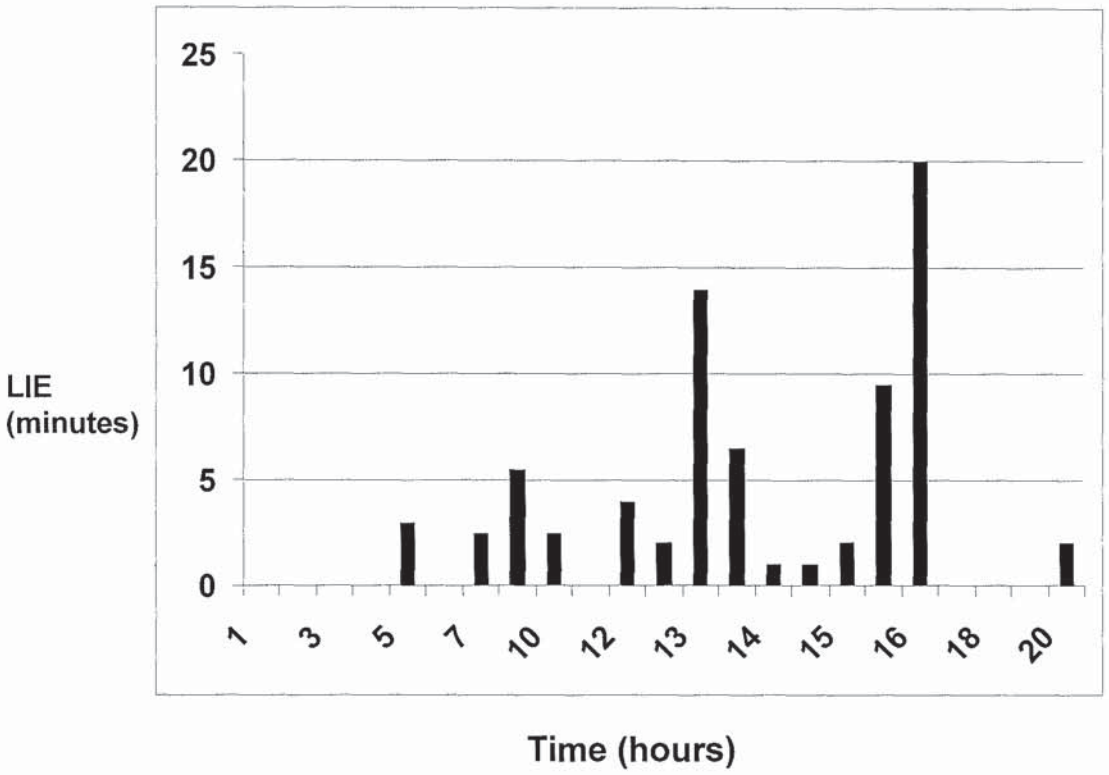


Figure 4.2: The circadian distribution of longest ischaemic event (LIE) for each glaucoma patient (n=14).

In this subgroup of glaucoma patients, the SBP value measured during the LIE was similar to the 24-h value ($p>0.05$); however, DBP and MBP measurements triggered by LIE were statistically significantly higher than the 24-h individual mean value (mean difference ±SD: 14.09 ±13.92 mmHg, $p=0.007$; and 14.02±10.85 mmHg, $p=0.003$ respectively), Figure 4.3 and 4.4. In the 8 glaucoma patients showing a cardiac ischaemic event at any time (57.14%) the LIE occurred during episodes of significant increase in MBP (>10% than 24-h MBP; mean difference ± SD: 20.81 ±9.46 mmHg); however, the remaining 6 patients (42.86%) failed to demonstrate any significant BP changes during the LIE ($p>0.05$).

HR values during the LIE were also significantly higher than 24-h mean HR values (mean difference ±SD: 15.75±11.16 beats/min, $p=0.007$), Figure 4.5. In 10 of these patients (71.43%) the LIE was associated with a significant increase in HR (>10% than 24-h HR; mean difference ± SD: 19.87 ± 10.56beats/min);

however, the remaining 4 patients (42.86%) failed to demonstrate any significant BP changes during the LIE ($p>0.05$). These 4 patients also failed to demonstrate a significant BP change during LIE and were previously diagnosed with systemic hypertension and/or chronic cardiac ischaemic disease.

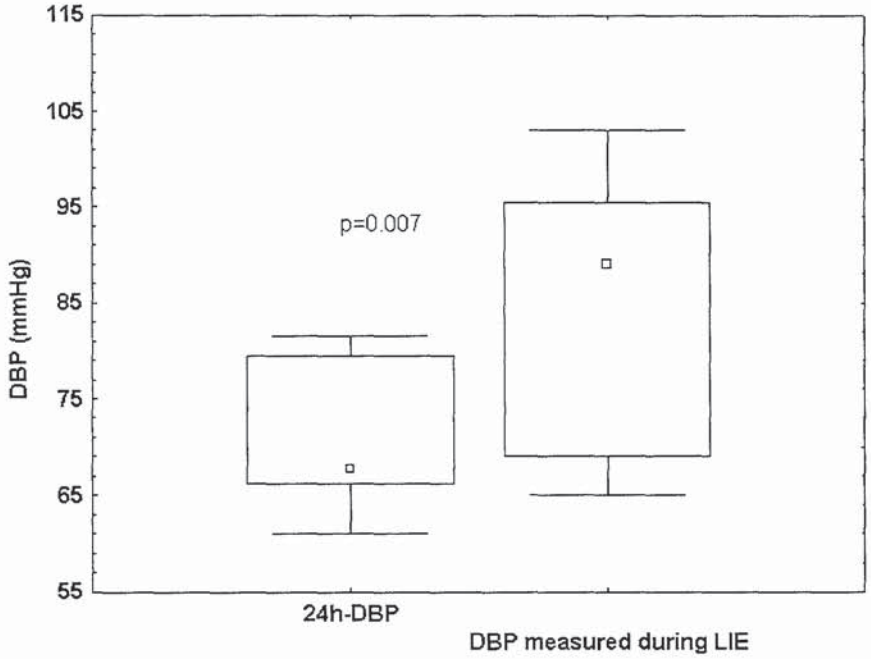


Figure 4.3: Diastolic blood pressure (DBP) in glaucoma patients suffering from silent cardiac ischaemic events. LIE: longest ischaemic event

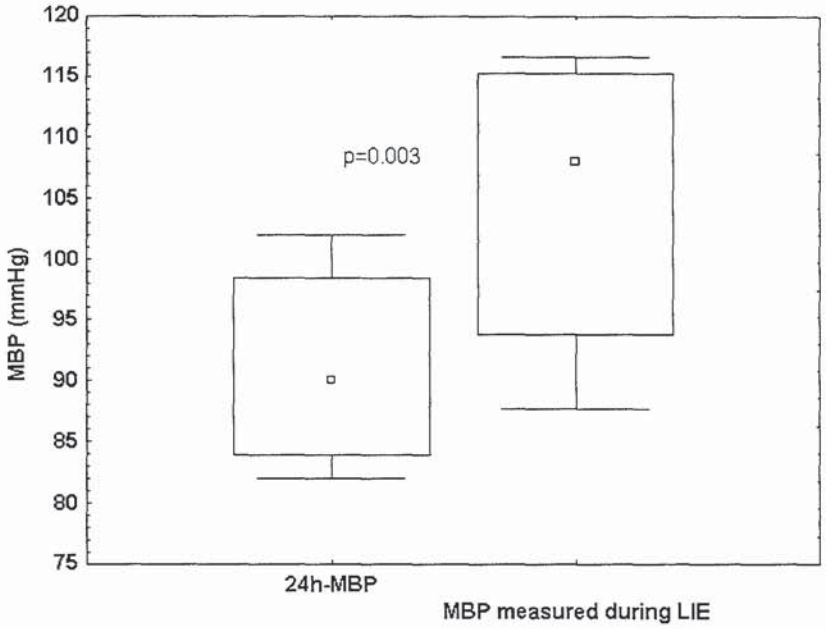


Figure 4.4: Mean blood pressure (MBP) in glaucoma patients suffering from silent cardiac ischaemic events. LIE: longest ischaemic event.

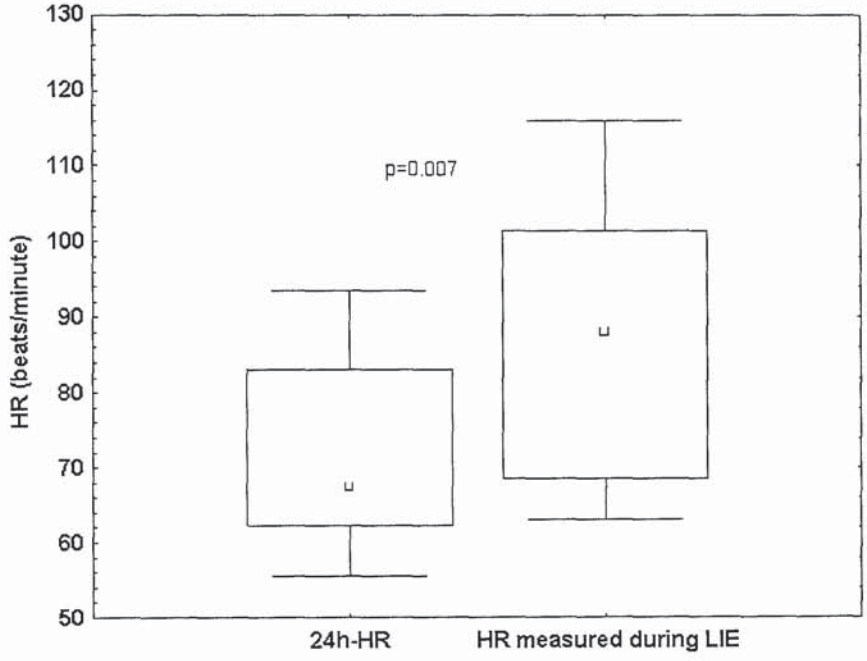


Figure 4.5: Heart rate (HR) in glaucoma patients suffering from silent cardiac ischaemic events. LIE: longest ischaemic event

c) Control subjects suffering from silent cardiac ischaemic episodes (10 subjects)

A total of 25 ischaemic events were recorded in this subgroup of control subjects. Their circadian distribution is illustrated in Figure 4.6. Only one subject (10%) demonstrated an important silent ischaemic event during sleep; the remaining 9 subjects (90%) suffered from LIE during late morning (4 subjects: 40%) or afternoon (5 subjects: 50%) while subjects performed various physical activities (walking, driving or house work: 6 control subjects, 66.67%) or were resting in bed (3 subjects, 33.33%). As for the glaucoma group, the difference between the type of activity performed by control subjects during LIE was not statistically significant ($p>0.05$).

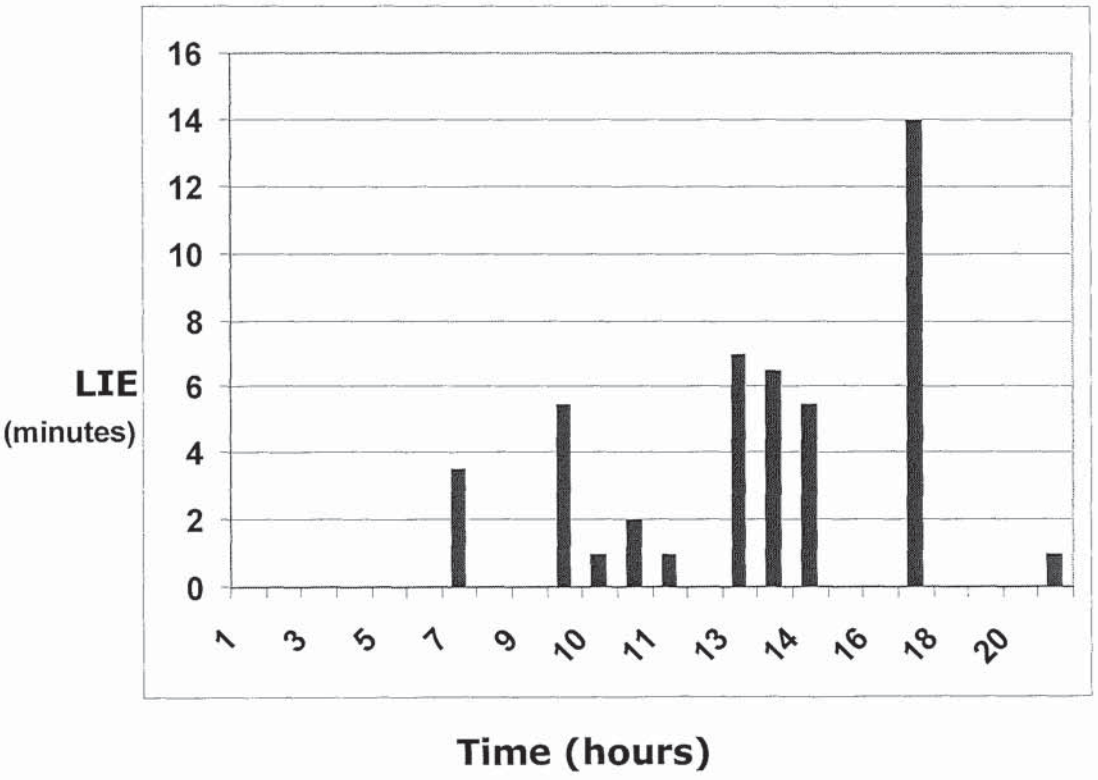


Figure 4.6: circadian distribution of longest ischaemic event (LIE) in control subjects.

SBP, DBP and MBP measurements triggered by the LIE were statistically comparable with the 24-h mean values for these parameters (p>0.05). However, in 4 control subjects showing a cardiac ischaemic event at any time (40%) the LIE occurred during episodes of significant increase in MBP (>10% than 24-h MBP; mean difference ±SD: 28.42 ±15.23 mmHg) and in 2 (20%) during significant MBP drop (>10% than 24-h MBP; mean difference ±SD: -16.67 ±4.24 mmHg). Four subjects (40%) failed to demonstrate any significant BP changes during the LIE (p>0.05).

HR values measured during LIE were significantly higher than the 24-h HR value (mean difference ±SD: 15.34±10.88 beats/min, p=0.010, Figure 4.5). In 9 of the 10 control subjects showing an ischaemic event at any time (90%) the LIE was associated with a significant increase in HR (>10% than 24-h HR; mean difference ±SD: 24.89± 12.14 beats/min). In one patient (10%) the LIE was not associated with significant changes in HR (p>0.05)

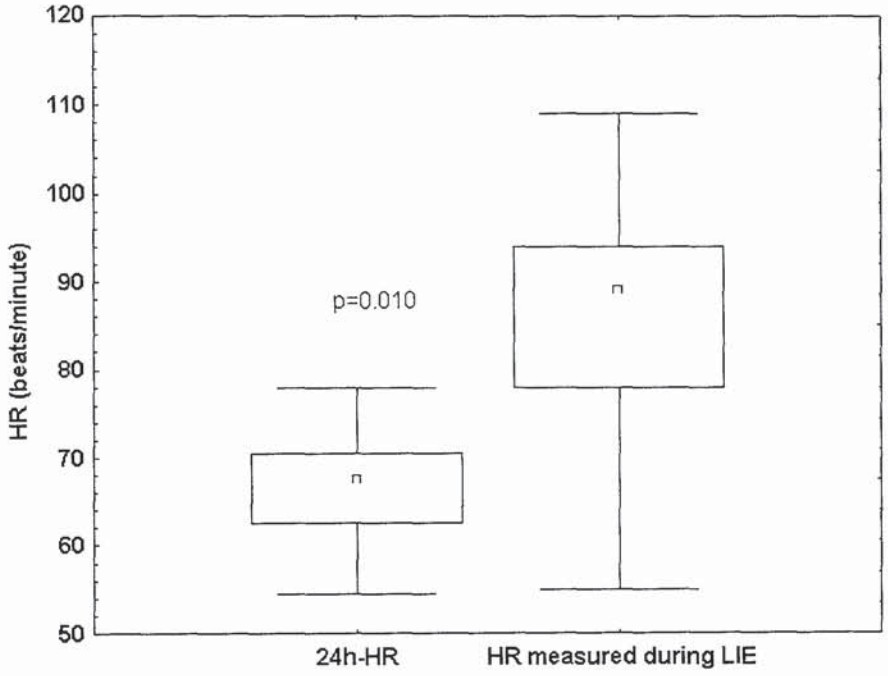


Figure 4.7: Heart rate (HR) measurements in control subjects suffering from silent cardiac ischaemia. LIE: longest ischaemic event

4.6.4. Heart rate variability analysis

a) Intergroup differences (all glaucoma patients and control subjects)

Glaucoma patients included in the experiment exhibited significantly higher LF and LF/HF values than the control group during 24-h interval (p=0.022 and p=0.032 respectively; Table 4.5) as well as during both active and passive periods of the measurement than the control subjects (p=0.020, p=0.029; and p=0.044, p=0.050 reprectively, Table 4.5). The circadian variations of the three measured HRV parameters (circadian variation=active period-passive period values) were, however, similar in both study groups (p>0.05).

Parameter	Glaucoma patients (n=23)	Control subjects (n=22)	p-value
LF-24h (nu)	65.43±12.02	55.52±14.77	0.022
HF-24h (nu)	29.52±11.00	35.95±10.56	0.060
LF/HF-24h	2.70±1.52	1.81±1.03	0.032
LF-day (nu)	67.50±10.92	57.50±14.75	0.020
HF-day (nu)	28.75±10.49	35.05±11.56	0.079
LF/HF-day	2.91±1.50	1.97±1.09	0.029
LF-night (nu)	64.55±13.65	53.95±17.91	0.044
HF- night (nu)	30.50±12.62	37.53±12.01	0.083
LF/HF- night	2.68±1.60	1.77±1.17	0.050
Circadian LF (nu)	2.95±9.27	4.37±15.97	0.738
Circadian HF (nu)	-1.75±8.76	-2.89±10.52	0.714
Circadian LF/HF	0.23±0.87	0.26±0.88	0.921

Table 4.5: HRV parameters measured in the study groups. LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

When comparing subjects suffering from episodes of silent cardiac ischaemia from both study groups (14 glaucoma patients and 10 control subjects) the measured HRV parameters were similar (p>0.05, Table 4.6). However, when

comparing subjects free from any ECG changes (9 patients and 12 control subjects), glaucoma patients demonstrated higher LF and LF/HF ratio values than control subjects ($p=0.010$ and $p=0.021$ respectively, Table 4.7). The HRV measurements recorded during the test period in glaucoma patients and control subjects free of cardiac ischaemic episodes, were similar to those measured in patients and control subjects who suffered from silent myocardial ischaemia ($p>0.05$, Table 4.8).

Parameter	Glaucoma patients (n=14)	Control subjects (n=10)	p-value
LF-24h (nu)	64.08±13.49	60.30±14.10	0.520
HF-24h (nu)	34.00±9.27	33.30±7.06	0.522
LF/HF-24h	2.14±1.10	1.99±0.97	0.845
LF-day (nu)	66.85±13.45	64.14±9.63	0.739
HF-day (nu)	30.85±10.11	28.29±6.75	0.645
LF/HF-day	2.51±1.22	2.41±1.0.78	0.858
LF-night (nu)	58.46±14.15	54.00±20.45	0.572
HF- night (nu)	36.23±11.87	35.00±11.00	0.823
LF/HF- night	1.98±1.28	1.87±1.25	0.850

Table 4.6: HRV parameters measured in the subgroups suffering from silent episodes of myocardial ischaemia. LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

Parameter	Glaucoma patients (n=9)	Control subjects (n=12)	p-value
LF-24h (nu)	69.22±18.41	55.00±15.05	0.066
HF-24h (nu)	29.89±11.02	33.83±11.97	0.449
LF/HF-24h	2.73±1.63	1.90±0.97	0.161
LF-day (nu)	74.25±11.13	55.08±16.36	0.010
HF-day (nu)	25.00±8.72	35.50±12.35	0.052
LF/HF-day	3.42±1.62	1.87±1.14	0.021
LF-night (nu)	59.12±18.48	55.17±15.56	0.611
HF- night (nu)	28.75±11.60	39.67±12.77	0.068
LF/HF- night	2.54±1.70	1.72±1.16	0.213

Table 4.7: HRV parameters measured in the subgroups free from silent episodes of myocardial ischaemia. LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

Parameter	Glaucoma patients		p-value	Control Subjects		p-value
	9 patients without silent cardiac ischaemia	14 subjects with silent cardiac ischaemia		12 patients without silent cardiac ischaemia	10 subjects with silent cardiac ischaemia	
LF-24h (nu)	69.22±18.41	64.08±13.49	0.457	55.00±15.05	60.30±14.10	0.408
HF-24h (nu)	29.89±11.02	34.00±9.27	0.355	33.83±11.97	33.30±7.06	0.903
LF/HF-24h	2.73±1.63	2.14±1.10	0.323	1.90±0.97	1.99±0.97	0.822
LF-day (nu)	74.25±11.13	66.85±13.45	0.208	55.08±16.36	64.14±9.63	0.202
HF-day (nu)	25.00±8.72	30.85±10.11	0.192	35.50±12.35	28.29±6.75	0.175
LF/HF-day	3.42±1.62	2.51±1.22	0.159	1.87±1.14	2.41±1.0.78	0.281
LF-night (nu)	59.12±18.48	58.46±14.15	0.927	55.17±15.56	54.00±20.45	0.890
HF- night (nu)	28.75±11.60	36.23±11.87	0.173	39.67±12.77	35.00±11.00	0.431
LF/HF- night	2.54±1.70	1.98±1.28	0.402	1.72±1.16	1.87±1.25	0.788

Table 4.8: HRV parameters measured in subjects free from any cardiac ischaemic event compared to those suffering from silent myocardial ischaemia episodes. LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

HRV measurements measured 10 minutes before and after the LIE are given in Table 4.9. There were no statistically significant differences between patients and control subjects (p>0.05).

b) Glaucoma patients suffering from silent cardiac ischaemic episodes (14 patients)

The active LF, HF and LF/HF ratio values were statistically comparable to those measured during the passive period of the measurement (p>0.05, Table 4.9).

Parameter	Glaucoma patients (n=14)	Control subjects (n=10)	p-value
LF-before (nu)	62.92±22.46	57.29±17.15	0.476
HF-before (nu)	29.08±18.42	31.64±12.98	0.682
LF/HF-before	3.58±3.15	2.51±2.16	0.316
LF-after (nu)	68.67±17.78	58.57±19.12	0.179
HF-after (nu)	25.42±16.89	29.07±11.86	0.525
LF/HF-after	4.19±3.12	2.81±2.37	0.211

Table 4.9: HRV parameters measured 10 minutes before and after LIE in the patients and subjects suffering from silent myocardial ischaemia episode.LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; data given in normalized units; nu: normalized units. Values are given in mean±SD.

In glaucoma patients suffering from silent cardiac ischaemia, LF, HF and LF/HF ratio values measured 10 min before LIE were not statistically different from the corresponding variable measured after the ischaemic event (p>0.05,Table 4.9). There was also no difference between LF, HF and LF/HF ratio values measured before and after the ischaemic and the 24-h mean value for the corresponding parameter (p>0.05).

To determine if the circadian variations in BP and HR influence circadian variation in the LF, HF and LF/HF parameters, multivariate analysis was performed. In the

analysis, which included 4 variables (circadian variation values for SBP, DBP, MBP, and HR), circadian variations in BP and HR did not correlate with the circadian variation in either of the frequency domain analysis parameters ($p>0.05$).

c) Control subjects suffering from silent cardiac ischaemic episodes (10 subjects)

In the control group, nocturnal LF, HF and LF/HF values were not significantly different from diurnal values ($p>0.05$). Moreover, the HRV parameters measured before LIE were not statistically different from those measured after the ischaemic event ($p>0.05$, Table 4.9). The LF, HF and LF/HF ratio values measured 10 min before the LIE did not differ from the 24-h corresponding value ($p>0.05$, Table 4.9).

As for the glaucoma group, circadian variations in BP and HR in the control group did not correlate with the circadian variation in either of the frequency domain analysis parameters ($p>0.05$).

4.7. Discussion

4.7.1. Main findings

Using a new device (Cardiotens-01, Meditech Ltd., Hungary), capable of triggering BP recordings during episodes of abnormal cardiac activity, the present study assessed the relationship between the occurrence of silent cardiac ischaemia episodes and alterations in BP, HR and HRV parameters in consecutive, newly diagnosed and previously untreated POAG patients during their normal daily routine. Our results disclosed that independent of a history of systemic hypertension or chronic cardiac ischaemic disease, glaucoma patients may exhibit blunted circadian fluctuations of HRV, translated by high sympathetic tone during both day and nigh. In both glaucoma patients and control groups, the LIE was associated with raised systemic BP and HR.

4.7.2. Circadian pattern of blood pressure and heart rate

There were no differences between glaucoma patients and control subjects with regard to the 24-h BP and HR values. However, in the glaucoma group there were significantly more over-dippers than in the control group. Our finding is in accordance with previous studies describing low BP levels and over-dipping in glaucoma patients (Kaiser and Flammer, 1991; Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Graham *et al.,* 1995; Yazici *et al.,* 2003). It has been reported that not only the physiologic nocturnal BP dip may be exaggerated in some glaucoma patients, but that the long-term outcome of the glaucomatous disease might be worse among glaucoma patients with lower nocturnal BP variables, suggesting that the exaggerated nocturnal reduction in BP may be a risk factor in this disease (Graham and Drance, 1999). The present study did not, however, evaluate the preponderance of excessive nocturnal BP decrease in glaucoma patients. It simply demonstrated again that, despite reports which have failed to find a correlation between nocturnal BP dips and glaucoma (Detry *et al.,* 1996; Kashiwagi *et al.,* 2001), over-dipping in systemic BP is frequently encountered in the glaucoma's clinical and research practice.

4.7.3. Silent cardiac ischaemia: circadian rhythm and relationship to fluctuations in blood pressure and heart rate

The total number of silent cardiac ischaemic episodes and their circadian distribution was similar in glaucoma patients and control subjects, with more ischaemic events occurring during late morning and in the afternoon hours than during the night and early hours of the morning. This pattern is similar to that reported in the general elderly population (Rautaharju *et al.,* 1995) and in patients with stable coronary artery disease (CAD) (Fox and Mulcahy, 1990; Goseki *et al.,* 1994) but different from the circadian rhythm of acute coronary events (Patel *et al.,* 1997). In the glaucoma patients that exhibited silent cardiac ischaemic episodes at any time, in 8 patients (57.14%) the LIE was related to high systemic BP values, 10% or more increase in 24-h MBP, and in 10 patients (71.43%) to a significant increase in HR (10% or more than the mean 24-h HR values). In control subjects exhibiting silent cardiac ischaemic episodes, 4 patients (40%) exhibited at least 10% increase in systemic BP during LIE in comparison to the 24-h MBP; in 9

subjects (90%) the LIE was related to at least a 10% increase in HR compared to 24-h HR. These observations are consistent with previous studies showing that the majority of silent cardiac ischaemic episodes are associated with an increase in BP and HR before the onset of ST-segment depression (Quyyumi *et al.,* 1985; Quyyumi *et al.,* 1986; Deedwania and Nelson, 1990; Panza *et al.,* 1992; Rehman *et al.,* 1997). However, in 6 glaucoma patients (42.86%) and 4 control subjects (40%) LIE was not associated with significant changes in systemic BP. Moreover, in 2 control subjects (20%) LIE occurred in association with a significant systemic BP drop (10% or more of the 24-h MBP).

The mechanism behind the occurrence of silent cardiac ischaemic episodes is much debated. Based on the frequent increase in BP and HR values prior to the ischaemic event it may be concluded that ischaemia is due to an increase in myocardial oxygen demand (Deedwania and Nelson, 1990). However, in our study some glaucoma patients and control subjects failed to exhibit BP and HR changes during cardiac ischaemic episodes or changes occurred in the opposite direction. In these cases the ischaemia could have been the result of a reduction in coronary blood flow as a result of a vascular dysregulation (Asmar *et al.,* 1996; Rehman *et al.,* 1997) dependent, at least partially, on variations in the sympathetic tone (Panza *et al.,* 1991). A combination of the two previously mentioned mechanisms (increased oxygen demand and decreased blood flow) seems to be the cause for the silent cardiac ischaemic episodes encountered among subjects included in the present study.

4.7.4. Autonomic nervous system activity

In this study, we demonstrated an overall blunted circadian fluctuation of the HRV in glaucoma patients, interpreted as high sympathetic tone during both day and night (Figure 3.6). Moreover, glaucoma patients free from any cardiac ischaemic episode demonstrated higher sympathetic tone during the day comparing to control subjects with similar ECG findings. When comparing patients and control subjects with ECG modifications consistent with the occurrence of silent myocardial ischaemia the measured HRV parameters were, however, similar. In addition, when comparing patients and control subjects suffering from silent cardiac

ischaemic episodes with those free from any ECG disturbances, the measured HRV parameters were also statistically comparable. These findings are of great interest. It seems that while both subgroups of glaucoma patients (with and without silent cardiac ischaemic events) have a constant high sympathetic tone, in control subjects only those subjects suffering from episodic silent myocardial ischaemia demonstrate such an autonomic disturbance. Therefore, we suggest that in glaucoma, the observed autonomic dysfunction is not related to a concurrent cardiovascular disease but may be associated with the occurrence and possibly the progression of glaucomatous optic neuropathy.

A high sympathetic tone during both day and night has previously been reported in NTG patients (Kashiwagi *et al.,* 2000) and could result in endothelial damage by either platelet activation or by mechanical injury to the vascular wall as a result of high systemic BP and increased blood velocity (Remme, 1998). A constant high sympathetic tone represents not only a sign of blunted HRV (Korpelainen *et al.,* 1997) but also an indicator of increased oxygen demand in various tissues (Remme, 1998). It could also result in a low ischaemic threshold in all organs, including the eye. In glaucoma patients suffering from either blunted circadian fluctuations of the HRV or high diurnal sympathetic tone, the eye could be more susceptible to minor changes in perfusion pressure and ocular diseases with vascular risk factors such as glaucoma could occur with higher frequency.

The mechanism behind a constant high sympathetic tone and an abnormal HRV in glaucoma is still under investigation. Glaucoma has been associated not only with an increased frequency of silent myocardial ischaemia (Waldmann *et al.,* 1996) but also with diffuse cerebral small-vessel ischaemic changes that have been observed in more patients with normal tension glaucoma than in control subjects (Stroman *et al.,* 1995). A clinical association between these two types of systemic circulatory disturbances has never been demonstrated. An abnormal HRV in some glaucoma patients could be the result of such ischaemic lesions in those areas of the brain responsible for the cardiovascular circadian rhythm situated in the anterior hypothalamus (Moore and Silver, 1998; Rensing *et al.,* 2001). Moreover, some cerebral ischaemic lesions may also result in alterations of the serum levels of antidiuretic hormone, catecholamines, and cortisol; these endocrinological

disturbances may have as a direct consequence a disturbed HRV (Korpelainen *et al.,* 1997). Further studies are necessary to test these hypotheses.

4.7.5. Conclusion

In conclusion, our study demonstrates that, independent of a positive history of systemic hypertension and/or chronic cardiac ischaemic disease, newly diagnosed and previously untreated glaucoma patients demonstrate blunted circadian fluctuations in HRV and/or a high diurnal sympathetic tone. As a consequence, these patients may be more susceptible to defective perfusion pressure in target organs such as the heart or eye. The effect of autonomic disturbances on ocular blood flow will be tested in the Chapter 5 of the present thesis.

4.7.6. Possible clinical consequences

More extensive investigations are necessary to determine the risk factors for each individual case of POAG. Clinicians should consider potential autonomic effects of various systemic and ocular therapies in patients suffering from glaucoma. It is well known that any therapy that activates the sympathetic division of ANS will increase the risk of systemic circulatory events and any drugs that increase the vagal tone or decrease the sympathetic hyperactivity may improve cardiovascular outcome (Tulppo *et al.,* 2001; Curtis and O'Keefe, 2002; Malfatto *et al.,* 2003). Since both chronic cardiovascular diseases and their treatment may represent important contributory factors in glaucoma pathogenesis, clinicians should consider carefully any possible danger arising from this strategy. Moreover, IOP-lowering treatment often consists of drugs that either mimic or inhibit the sympathetic and parasympathetic divisions of ANS (Brown *et al.,* 2002). For these reasons, an autonomic assessment, together with 24-h BP measurement could be useful in monitoring the efficacy and possible circulatory side effects of therapies for glaucoma and systemic diseases. Together with existing antiglaucoma drugs that have a possible positive effect in restoring the autonomic balance, such as beta-blockers (due to their effect in cutting a high sympathetic tone) (Nino *et al.,* 2002), "timed" therapeutic strategies should be developed as possible alternative in the treatment for glaucoma.

5. Ocular Flow Alteration in Newly Diagnosed and Untreated Primary Open-Angle Glaucoma Patients; Relationship to Autonomic Nervous System Function.

5.1. Abstract

Purpose: To investigate the relationship between ocular and systemic vascular disturbances in newly diagnosed and previously untreated POAG patients

Methods: In 18 POAG patients (mean age±SD: 67.11±10.89 years) and 17 normal controls (mean age±SD: 63.82±10.05 years), OBF parameters at the NRR and peripapillary retina were obtained form one eye by means of HRF. Based on these haemodynamic parameters, a cluster analysis was performed and differences with regard to ocular and systemic risk factors were assessed between clusters.

Results: Glaucoma patients exhibited higher IOP ($p=0.0144$), and VF damage (MD: $p=0.009$ and PSD: $p=0.008$), as well as higher LF ($p=0.036$) and LF/HF ($p=0.028$) values than normal controls. They also demonstrated lower HF values ($p=0.026$). Based on the OBF measurement results, two clusters have been identified in each study group: cluster 1 (8 glaucoma patients, mean age±SD: 71.63±6.84 years, and 8 normal controls, mean age±SD: 64.50±8.78 years who exhibited higher OBF values); and cluster 2 (10 glaucoma patients, mean age±SD: 63.50±12.46 years, and 9 normal controls, mean age±SD: 63.22±11.55 years who exhibited lower OBF values). No differences in age, gender distribution, IOP, VF damage or BP parameters were observed between the two clusters in either of the study groups ($p>0.05$). Glaucoma patients with higher OBF (cluster 1) demonstrated higher HF value ($p=0.044$) than those with low ocular perfusion (cluster 2). They also demonstrated higher LF ($p=0.012$) and LF/HF ($p=0.031$) parameters than normal controls with similar ocular haemodynamic parameters (cluster 1). Glaucoma patients with lower OBF (cluster 2) exhibited higher 24-h SBP ($p=0.020$), MBP ($p=0.026$), LF/HF ($p=0.020$), and lower HF ($p=0.015$) values than normal controls with similar ocular perfusion characteristics (cluster 2).

Conclusion: In some glaucoma patients, autonomic system disturbances appear to result in both systemic and ocular blood flow alterations.

5.2. Introduction

There is substantial evidence to suggest that both ocular (Rojanapongpun *et al.,* 1993; Wolf *et al.,* 1993; Costa *et al.,* 1994; Nicolela *et al.,* 1996*b*; Kaiser *et al.,* 1997; Butt *et al.,* 1997; Findl *et al.,* 2000; Gherghel *et al.,* 2000; Gherghel *et al.,* 2001; Emre *et al.,* 2004; Fuchsjager-Mayrl *et al.,* 2004) and systemic haemodynamic disturbances (Susanna and Basseto, 1992; Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham *et al.,* 1995; Stroman *et al.,* 1995; Waldmann *et al.,* 1996; O'Brien and Butt, 1999; Kashiwagi *et al.,* 2000; Kashiwagi *et al.,* 2001; Emre *et al.,* 2004; uchsjager-Mayrl *et al.,* 2004) are involved in the pathogenesis of glaucoma. Moreover, in some glaucoma patients both ocular and systemic vascular disturbances seem to act concomitantly in producing disease. Indeed, in a recent study, Emre et al. (Emre *et al.,* 2004) that in some patients suffering from POAG there is an association between low OBF values and occurrence of peripheral vasospasm. However, establishing a direct link between the two types of risk factors needs caution. It still unclear whether the OBF alterations associated with glaucoma represent isolated events or occur as a result of more complex systemic haemodynamic disturbances. More investigation is, therefore, necessary to demonstrate the cause responsible for a haemodynamic insufficiency occurring in multiple vascular beds as well as its importance for the onset and progression of GON.

5.3. Hypothesis

It is possible that in some glaucoma patients, similar vascular disturbances occur concomitantly in various vascular beds, leading ultimately to low blood flow in the affected tissues. These haemodynamic disturbances could be the result of a systemic autonomic dysfunction.

5.4. Aim

In this clinical study, a cluster analysis based on OBF parameters was performed in newly diagnosed and previously untreated POAG patients and normal controls and differences with regard to HRV parameters were assessed between clusters.

5.5. Subjects and methods

5.5.1. Recruitment of untreated primary open-angle glaucoma patients

Consecutive, newly diagnosed and previously untreated POAG patients attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust, Birmingham, UK, were included in this prospective study.

5.5.1.1. Inclusion criteria

Patients underwent diurnal IOP phasing and were diagnosed as having POAG if at least two IOP measurements were greater than 24 mmHg, and they presented with glaucomatous cupping of the optic disc on funduscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with a diagnosis of glaucoma using program 24-2 of the Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA). The characteristics of glaucomatous VF defects have previously been described in the present thesis (see Table 3.1).

5.5.1.2. Exclusion criteria

Exclusion criteria are outlined in Table 5.1.

- Patients with narrow iridocorneal angles;
- Evidence of secondary glaucoma;
- Pseudoexfoliation;
- Pigmentary dispersion;
- A history of intraocular surgery;
- Any form of retinal or neuro-ophthalmologic disease that could result in VF defects; and
- Any cardiovascular and metabolic disease including systemic hypertension and cardiac ischaemia.

Table 5.1: Exclusion criteria for the glaucoma patients' group

5.5.2. Control subject recruitment

The control group was comprised of subjects who have never been diagnosed with glaucoma and were recruited from patients' spouses and other volunteers. A history of cardiovascular ischaemic disease and/or abnormal systemic BP, as well as a history of chronic vasoactive medication such as anti-anginal or antihypertensive drugs, was also exclusion criteria for the control group.

5.5.3. Ethical approval

Ethical approval was obtained from the local medical ethics committees (Aston University and Heartlands and Solihull NHS Trust), and written informed consent was received from all subjects prior to entry into the study. The study was designed and conducted in accordance with the Tenets of Declaration of Helsinki.

5.5.4. Experimental protocol

5.5.4.1. Visual field assessment

VF was assessed by means of Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA) with a size III white stimulus using the full threshold program 24-2. The instrument has been described in the Chapter 3.5.5.1 of the thesis.

Two VF examinations were performed. The results from the second VF measurement were included in the analysis.

5.5.4.2. Intraocular pressure measurement

IOP was measured using a standard Goldmann tonometry technique after instillation of a topical anaesthetic (Benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd). The technique has been described in the Chapter 3.5.5.2 of the present thesis.

5.5.4.3. Ambulatory blood pressure and electrocardiogram measurements

The ambulatory BP and ECG measurement method has been described in the Chapter 4.5.4.1 and a detailed description of device used has been offered in the Chapter 1.11.6 of the present thesis. The 24-hour BP and ECG data obtained from the patients and controls were later downloaded and analyzed using the "Medibase" software program Version 1.42 (Meditech, Budapest, Hungary). At least 80% of the programmed recordings were required for a diurnal curve to be considered in the present analysis.

24-h average values for SBP and DBP were used to calculate 24-h MBP value according to the Equation 1.7. OPP was calculated according to Equation 1.2.

5.5.4.4. Heart rate variability measurement

HRV values are calculated using the "Medibase" software program Version 1.42 (Meditech, Budapest, Hungary) after the recordings was uploaded into a computer. This software allows us to measure both frequency and time domain parameters (see Chapter 1.11.4). For the simplicity of data analysis, only 24-h average values for the frequency domain parameters (LF, HF and LF/HF ratio) were included in the final analysis.

5.5.4.5. Ocular blood flow measurement

For data simplicity, in glaucoma patients measurements were performed in the eye with the most advanced disease (as determined by ophthalmoscopy and VF analysis); in normals the test eye was randomly selected. Perfusion parameters in the superior temporal regions of the NRR and peripapillary retina were measured using the HRF system (HRF, Heidelberg Engineering, GmbH, Heidelberg, Germany). The HRF principle has previously been described in detail elsewhere (Michelson and Schmauss, 1995; Michelson *et al.,* 1995) and a summary is presented in this thesis (see Chapter 1.9.2). The method used to obtain HRF measurements has been described in the Chapter 3.5.5.6 of the thesis. Parameters "flow", "volume" and "velocity" were obtained for the superior temporal regions of both NRR and peripapillary retina.

The BP and HRV monitoring, the IOP curve and the OBF measurements were obtained during the same 24-h interval for each patient. OBF measurements were obtained in the morning between 8 and 10 am and subjects were instructed to avoid ingesting any stimulants including coffee, tea or cigarette smoke on the morning of the study. In preparation for the OBF measurements, each subject rested in a sitting position for 10-15 minutes in a quiet room in order to achieve stable baseline conditions.

5.5.5. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Differences between the study groups at baseline were calculated using Student's *t*-test for independent variables. Based on OBF data, two distinct subgroups for both patients and normal controls groupswere computed by means of cluster analysis (K-means clustering test) using Statistica ® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. The multivariate approach in a cluster analysis allows identifying systematic relations between variables when there are no (or not complete) *a priori* expectations as to the nature of those relations. K-means clustering analysis allows us to examine the means for each cluster and to assess how distinct our *k* clusters are.

For each group, differences between the two clusters in age, VF damage, IOP, 24-h BP values (SBP, DBP and MBP), HR, OPP and HRV parameters averaged for 24-h were evaluated using Student-*t* test for independent variables. Gender distribution was studied using Fisher's exact test. Holm's sequentially rejective method was used to correct for multiple comparisons (Holm, 1979). Differences were considered statistically significant if $p<0.05$.

5.6. Results

5.6.1. Sample

After careful selection based on all inclusion and exclusion criteria, 23 glaucoma patients (10 men and 13 women) and 20 control subjects (8 men and 12 women)

were included in the study. However, as a result of subsequent rejection of those patients and controls who exhibited both poor ECG recording and HRF image quality (See experimental Chapters 3 and 4), only 18 glaucoma patients (7 men and 11 women, mean age±SD: 67.11±10.89 years) and 17 normal subjects (8 men and 9 women, mean age±SD: 63.82±10.05 years) were included in the final statistical analysis.

5.6.2. Intergroup differences at baseline

The baseline clinical characteristics of the study main groups are given in Table 5.2. After correcting for multiple comparisons, there were no statistically significant differences in age, systemic BP, HR, OPP or OBF values ($p>0.05$); however glaucoma patients exhibited higher IOP ($p=0.0144$) and VF damage (MD: $p=0.009$ and PSD: $p=0.008$ respectively) than normal controls. They also demonstrated higher LF and LF/HF ($p=0.036$ and $p=0.028$ respectively) and lower HF values ($p=0.026$) than normal subjects.

Parameter	Glaucoma patients (n=18)	Control subjects (n=17)	p-value	p-value*
IOP (mmHg)	24.56±4.25	18.88±2.79	0.0009	0.0144
MD (dB)	-4.70±2.83	-0.96±1.10	0.0005	0.009
PSD (dB)	5.13±3.59	1.76±0.44	0.0005	0.008
24-h SBP (mmHg)	139.64±11.46	129.06±20.13	0.063	NS
24-h DBP (mmHg)	82.49±10.35	76.29±12.23	0.115	NS
24-h MBP (mmHg)	101.54±9.90	93.88±14.41	0.075	NS
24-h HR (beats/min)	75.75±12.64	73.31±8.85	0.525	NS
OPP (mmHg)	51.84±6.45	50.00±10.24	0.534	NS
Volume TNRR (AU)	13.87±3.91	13.43±3.23	0.718	NS
Flow TNRR (AU)	248.35±89.00	218.56±59.67	0.256	NS
Velocity TNRR (AU)	0.85±0.36	0.77±0.20	0.469	NS
Volume TR (AU)	15.81±3.91	16.50±2.85	0.560	NS
Flow TR (AU)	301.03±89.50	289.28±65.24	0.662	NS
Velocity TR (AU)	1.08±0.31	1.04±0.22	0.657	NS
LF-24h (nu)	69.59±9.91	56.59±13.38	0.003	0.036
HF-24h (nu)	24.94±9.07	36.35±10.36	0.002	0.026
LF/HF-24h	3.20±1.50	1.79±0.92	0.002	0.028

Table 5.2: Intergroup differences at baseline. IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; OPP: ocular perfusion pressure; TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units; LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. P-value*: p-value corrected by means of Holm's sequentially rejective method; NS: non-significant. Values are given in mean±SD.

5.6.3. Cluster analysis: glaucoma patients

Among the 18 glaucoma patients, cluster analysis grouped 8 patients (3 men and 5 women, mean age±SD: 71.63±6.84 years) in a first group with higher blood flow parameters (cluster 1) and 10 patients (4 men and 6 women, mean age±SD: 63.50±12.46 years) in a second group with lower blood flow parameters (cluster 2).

There was no significant difference in age and sex distribution between the two subgroups (p>0.05). OBF parameters of the two groups are displayed in Table 5.3.

Parameter	Cluster 1 (n=8)	Cluster 2 (n=10)	p-value
Volume TNRR (AU)	16.24±3.37	11.97±3.35	0.016
Flow TNRR (AU)	317.39±83.41	193.12±43.87	0.0009
Velocity TNRR (AU)	1.11±0.31	0.63±0.25	0.002
Volume TR (AU)	18.08±3.02	14.00±3.67	0.022
Flow TR (AU)	377.55±52.15	239.81±60.40	0.0001
Velocity TR (AU)	1.34±0.18	0.87±0.21	0.0001

Table 5.3: Cluster analysis results for OBF parameters in glaucoma patients. TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units; LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

There were significant differences in all measured OBF parameters (volume, flow and velocity) measured at both NRR (volume: p=0.016, flow: p=0.0009, and velocity: p=0.002 respectively, Figures 5.1, 5.2 and 5.3) and peripapillary retina levels (p=0.022, p=0.0001, and p=0.0001 respectively, Figures 5.4, 5.5, 5.6).

Figure 5.1: Differences between clusters in OBF parameter Volume measured at the superior temporal neuroretinal rim level.

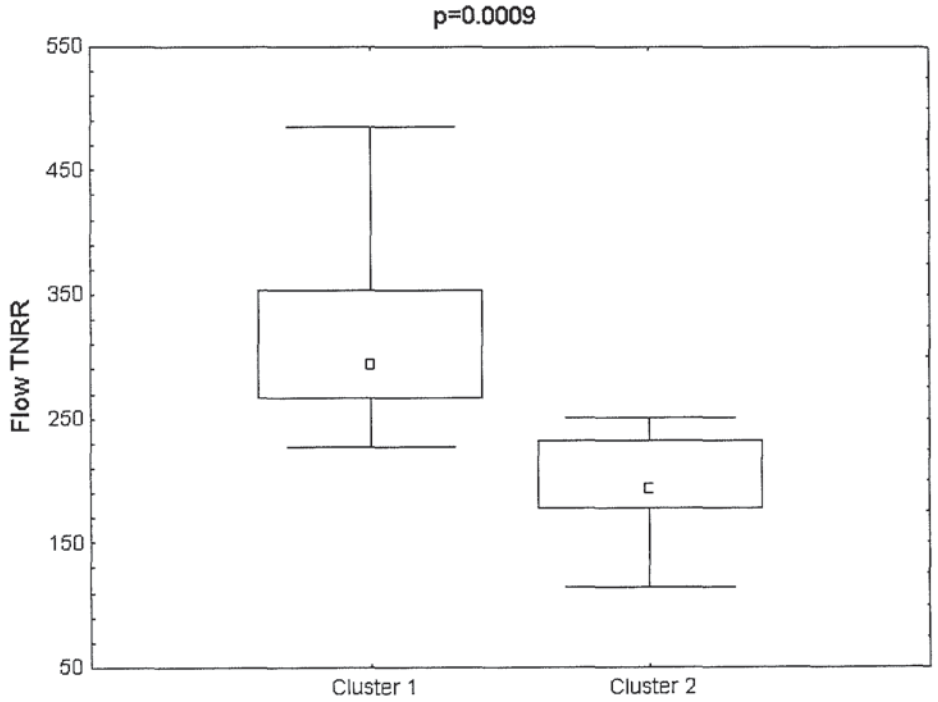


Figure 5.2: Differences between clusters in OBF parameter Flow measured at the superior temporal neuroretinal rim level.

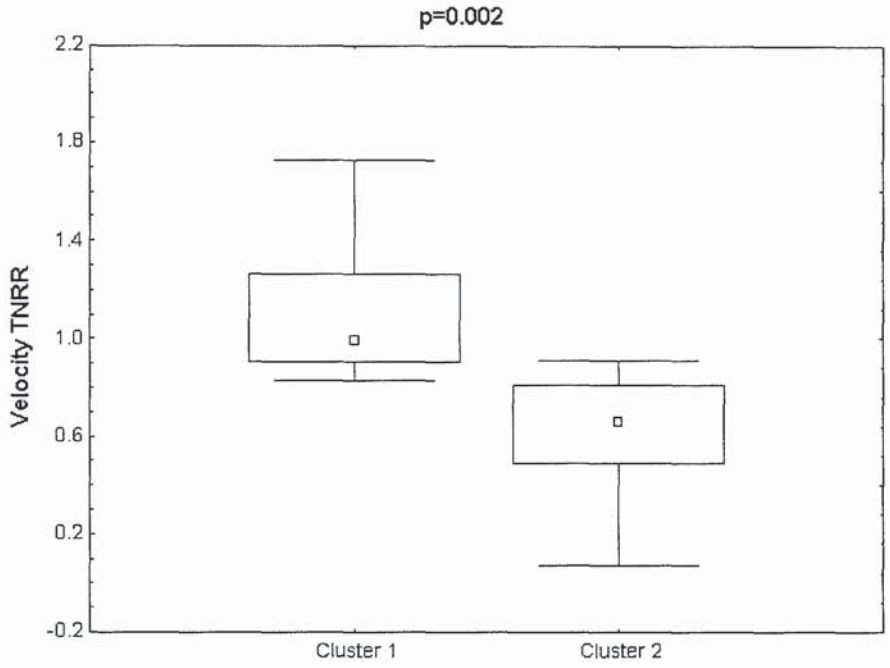


Figure 5.3: Differences between clusters in OBF parameter Velocity measured at the superior temporal neuroretinal rim level.

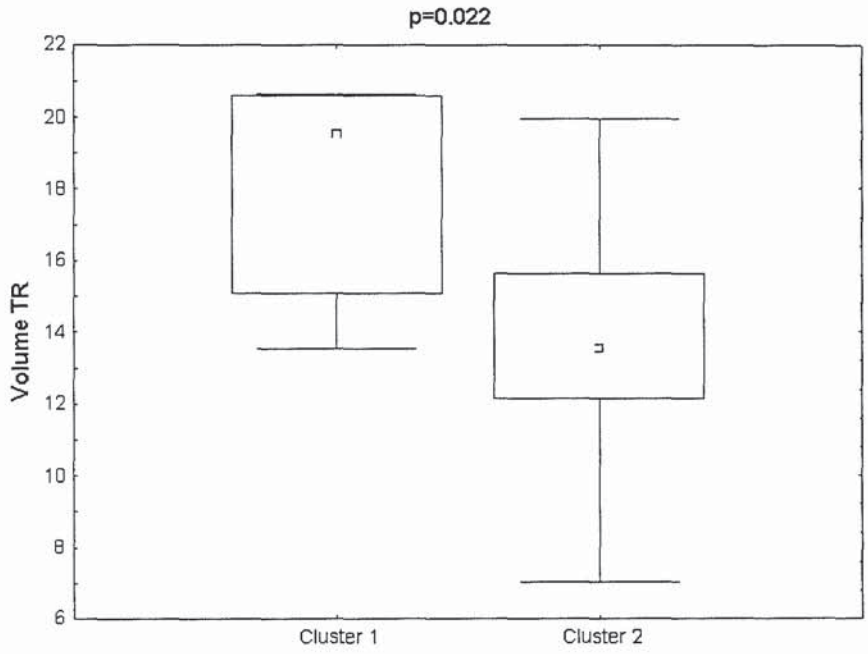


Figure 5.4: Differences between clusters in OBF parameter Volume measured at the superior temporal peripapillary retina.

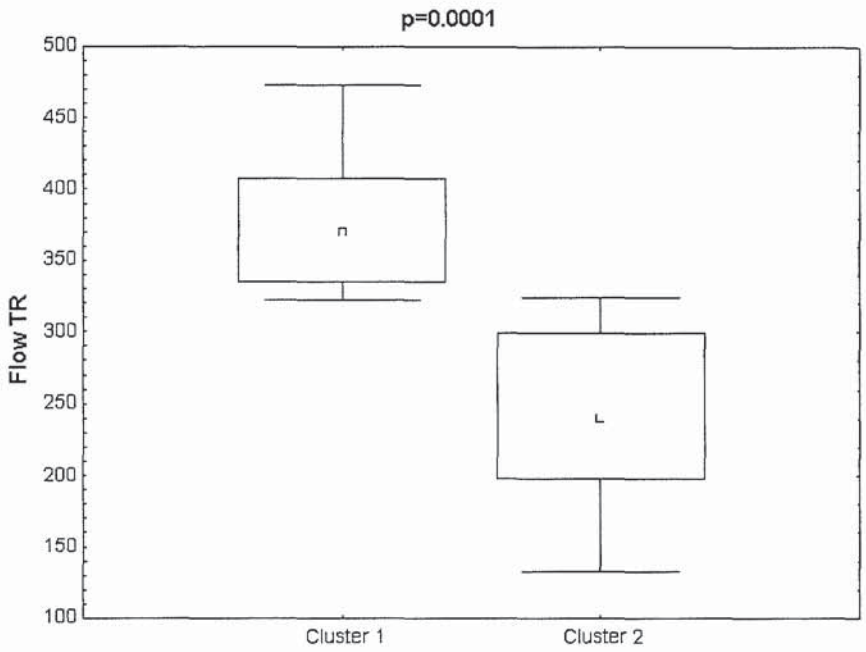


Figure 5.5: Differences between clusters in OBF parameter Flow measured at the superior temporal peripapillary retina.

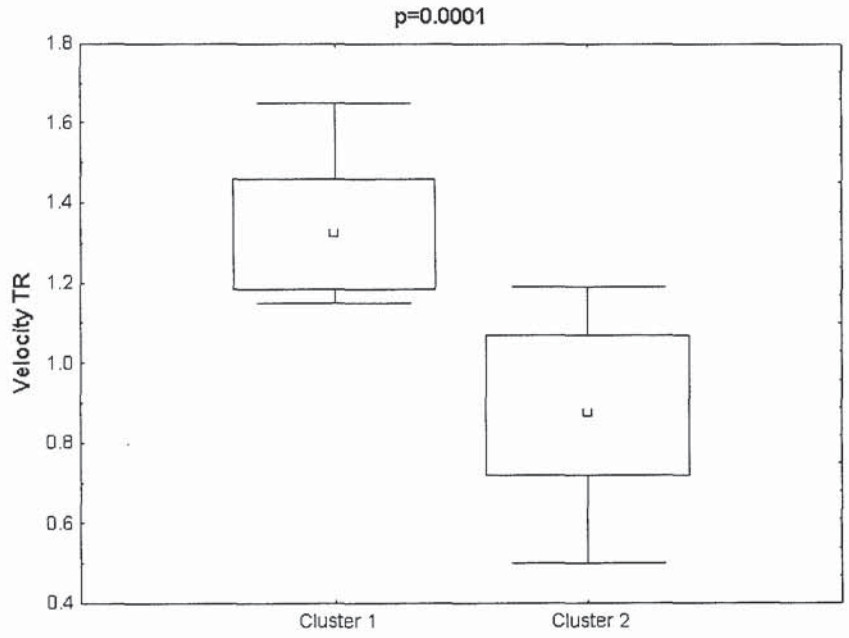


Figure 5.6: Differences between clusters in OBF parameter Velocity measured at the superior temporal peripapillary retina.

IOP, VF damage, 24-h BP, 24-h HR, and OPP values did not show significant differences between the two clusters ($p>0.05$, Table 5.4).

Parameter	Cluster 1 (n=8)	Cluster 2 (n=10)	p-value
IOP (mmHg)	25.00±3.09	24.20±3.61	0.759
MD (dB)	-5.36±4.96	-4.18±3.00	0.726
PSD (dB)	4.50±3.22	5.63±3.96	0.522
24h SBP (mmHg)	141.57±15.91	138.10±6.74	0.540
24h DBP (mmHg)	87.16±13.39	78.75±5.27	0.087
24-h MBP (mmHg)	105.29±13.58	98.53±4.36	0.156
24-h HR (beats/min)	78.74±11.43	73.35±13.63	0.384
OPP (mmHg)	53.53±8.64	50.35±3.53	0.326

Table 5.4: Inter-clusters differences in glaucoma patients . IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; OPP: ocular perfusion pressure. Values are given in mean±SD.

LF and LF/HF parameters were also statistically comparable between the two clusters (67.71±8.69 nu versus 70.90±10.94 nu, $p>0.05$; and 2.44±1.01 versus 3.73±1.61, $p>0.05$). In contrast, HF parameter was significant higher in the glaucoma patients from the first cluster (with high OBF values) than those grouped in the cluster 2 (30.14±8.23 nu versus 21.30±8.08 nu, $p=0.044$, Figure 5.7).

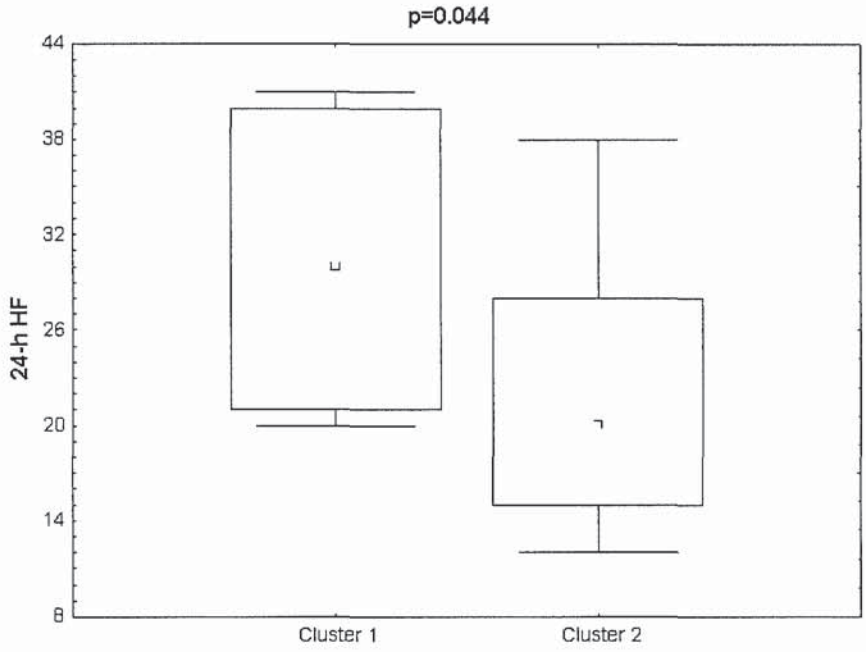


Figure 5.7: Differences between clusters in 24-h HF parameter.

5.6.4. Cluster analysis: normal controls

Among the 17 normal controls, cluster analysis grouped 8 subjects (4 men and 4 women, mean age±SD: 64.50±8.78 years) in a first group with higher blood flow parameters (cluster 1) and 9 subjects (4 men and 5 women, mean age±SD: 63.22±11.55 years) in a second group with lower blood flow parameters (cluster 2). There was no significant difference in age and sex distribution between the two subgroups ($p>0.05$). OBF parameters of the two clusters are displayed in Table 5.5.

Parameter	Cluster 1 (n=8)	Cluster 2 (n=9)	p-value
Volume TNRR (AU)	14.73±3.60	12.27±2.52	0.121
Flow TNRR (AU)	267.99±35.07	174.62±37.80	0.0001
Velocity TNRR (AU)	0.93±0.11	0.63±0.14	0.0001
Volume TR (AU)	17.52±2.45	15.59±2.99	0.171
Flow TR (AU)	329.07±57.79	253.92±50.99	0.012
Velocity TR (AU)	1.18±0.20	0.92±0.18	0.012

Table 5.5: Cluster analysis results for OBF parameters in normal controls. TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units; LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

OBF parameter "volume" measured at both NRR and peripapillary retina locations was not statistically different between the two clusters ($p>0.05$). However, all the other measured OBF parameters measured at both NRR and peripapillary retina levels (flow: $p=0.0001$ and $p=0.012$ respectively, Figures 5.8 and 5.9; and velocity: $p=0.0001$ and $p=0.012$ respectively, Figures 5.10 and 5.11) were significantly different between clusters 1 and 2.

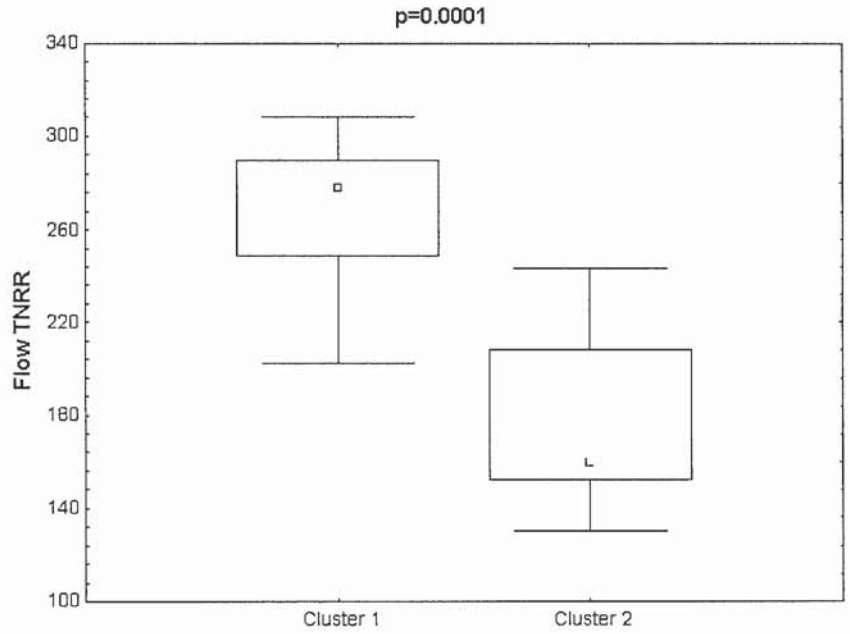


Figure 5.8: Differences between clusters in OBF parameter Flow measured at the superior temporal neuroretinal rim level.

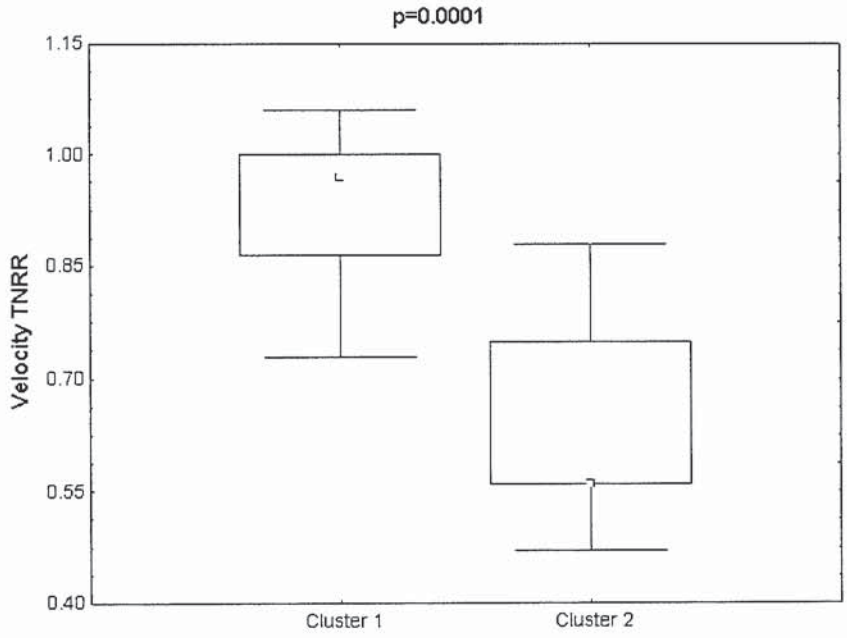


Figure 5.9: Differences between clusters in OBF parameter Velocity measured at the superior temporal neuroretinal rim level.

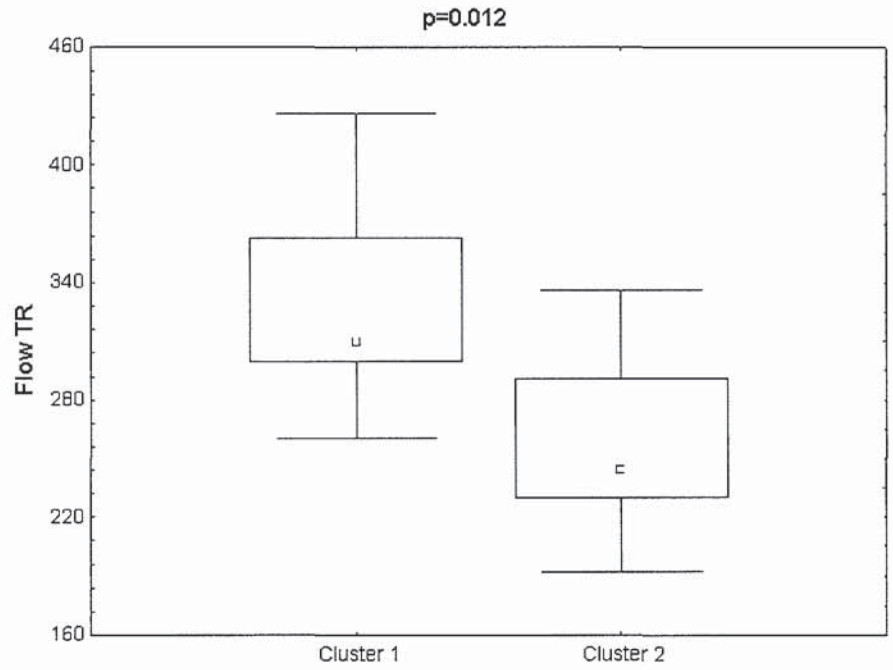


Figure 5.10: Differences between clusters in OBF parameter Flow measured at the superior temporal peripapillary retina.

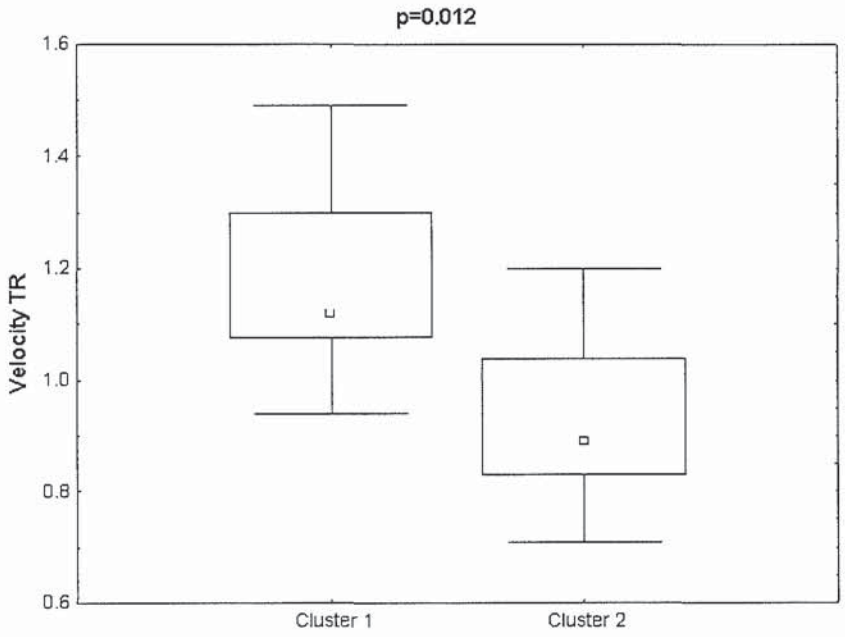


Figure 5.11: Differences between clusters in OBF parameter Velocity measured at the superior temporal peripapillary retina.

IOP, VF damage, 24-h BP, 24-h HR, and OPP values did not show significant differences between the two clusters (p>0.05, Table 5.6).

Parameter	Cluster 1 (n=8)	Cluster 2 (n=9)	p-value
IOP (mmHg)	18.75±1.65	19.00±2.00	0.896
MD (dB)	-1.17±1.38	-0.78±1.00	0.712
PSD (dB)	1.98±0.44	1.57±0.36	0.054
24h SBP (mmHg)	134.13±14.65	124.56±15.17	0.344
24h DBP (mmHg)	80.13±15.06	72.89±8.55	0.235
24-h MBP (mmHg)	98.13±14.99	90.11±10.90	0.266
24-h HR (beats/min)	70.63±9.44	76.00±7.89	0.236
OPP (mmHg)	52.92±12.82	47.41±7.07	0.282

Table 5.6: Inter-clusters differences in normal controls. IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; OPP: ocular perfusion pressure. Values are given in mean±SD.

In the control group, all measured HRV parameters (LF, HF and LF/HF ratio) were statistically comparable between the two clusters (52.13±11.63 nu versus 60.56±14.13 nu, p>0.05; 38.13±6.13 nu versus 34.78±13.26 nu, p>0.05; and 2.44±1.01 versus 3.73±1.61, p>0.05).

5.6.5. Cluster analysis: intergroup differences

5.6.5.1. Glaucoma patients and normal controls characterised by higher ocular blood flow values

There were no statistically significant differences in OBF values between these two groups (p>0.05, Table 5.7).

Parameter	Glaucoma patients (n=8)	Normal controls (n=8)	p-value
Volume TNRR (AU)	16.24±3.37	14.73±3.60	0.397
Flow TNRR (AU)	317.39±83.41	267.99±35.07	0.145
Velocity TNRR (AU)	1.11±0.31	0.93±0.11	0.147
Volume TR (AU)	18.08±3.02	17.52±2.45	0.687
Flow TR (AU)	377.55±52.15	329.07±57.79	0.100
Velocity TR (AU)	1.34±0.18	1.18±0.20	0.101

Table 5.7: OBF parameters in glaucoma patients and normal controls characterised by high OBF values (cluster 1). TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units; LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

However, the glaucoma patients group exhibited higher IOP (p=0.022), and VF damage (MD: p=0.031and PSD: p=0.046) than normal controls (Table 5.8).

Parameter	Glaucoma patients (n=8)	Normal controls (n=8)	p-value
IOP (mmHg)	25.00±3.09	18.75±1.65	0.022
MD (dB)	-5.36±4.96	-1.17±1.38	0.031
PSD (dB)	4.50±3.22	1.98±0.44	0.046
24h SBP (mmHg)	141.57±15.91	134.13±14.65	0.485
24h DBP (mmHg)	87.16±13.39	80.13±15.06	0.340
24-h MBP (mmHg)	105.29±13.58	98.13±14.99	0.384
24-h HR (beats/min)	78.74±11.43	70.63±9.44	0.144
OPP (mmHg)	53.53±8.64	52.92±12.82	0.912

Table 5.8: Intergroup differences in patients and controls characterised by high ocular blood flow (cluster 1). IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; OPP: ocular perfusion pressure. Values are given in mean±SD.

Glaucoma patients characterised by high OBF values also exhibited high LF and LF/HF parameters than normal controls (67.71±8.69 nu versus 52.13±11.63 nu, p=0.012 and 2.44±1.01 versus 1.46±0.51, p=0.031, Figures 5.12 and 5.13). HF values were, however, statistically comparable between the two groups (30.14±8.23 nu versus 38.13±6.13 nu, p>0.05).

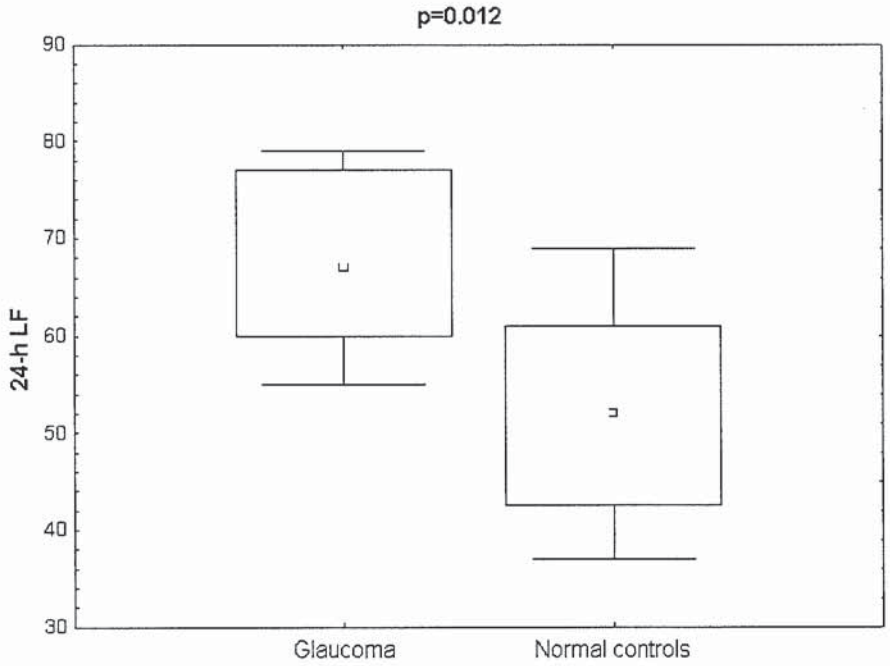


Figure 5.12: Differences in 24-h LF parameter between glaucoma patients and normal controls with high OBF values.

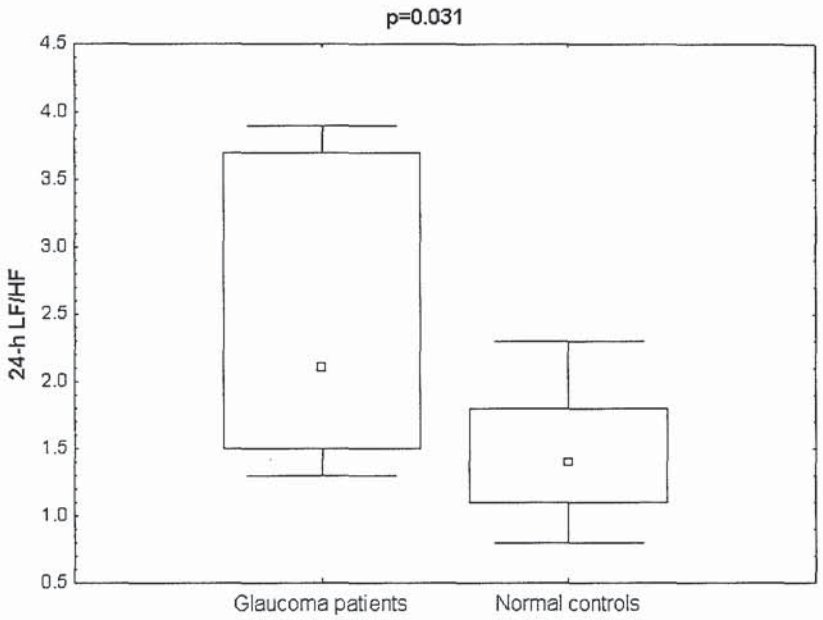


Figure 5.13: Differences in 24-h LF/HF ratio between glaucoma patients and normal controls with high OBF values.

5.6.5.2. Glaucoma patients and normal controls characterised by lower ocular blood flow values

There were no statistically significant differences in OBF values between these two groups ($p>0.05$, Table 5.9). However, the glaucoma patients group exhibited higher 24-h SBP ($p=0.020$), 24-h MBP ($p=0.026$), IOP ($p=0.022$), and VF damage (MD: $p=0.006$ and PSD: $p=0.007$) than normal controls (Table 5.10).

Parameter	Glaucoma patients (n=10)	Normal controls (n=9)	p-value
Volume TNRR (AU)	11.97±3.35	12.27±2.52	0.830
Flow TNRR (AU)	193.12±43.87	174.62±37.80	0.341
Velocity TNRR (AU)	0.63±0.25	0.63±0.14	0.974
Volume TR (AU)	14.00±3.67	15.59±2.99	0.318
Flow TR (AU)	239.81±60.40	253.92±50.99	0.592
Velocity TR (AU)	0.87±0.21	0.92±0.18	0.619

Table 5.9: OBF parameters in glaucoma patients and normal controls characterised by low OBF values (cluster 2). TNRR: temporal neuroretinal rim; TR: temporal retina; AU: arbitrary units; LF: low frequency component of the heart rate variability (HRV); HF: high frequency component of the HRV; nu: normalized units. Values are given in mean±SD.

Parameter	Glaucoma patients (n=10)	Normal controls (n=9)	p-value
IOP (mmHg)	24.20±3.61	19.00±2.00	0.024
MD (dB)	-4.18±3.00	-0.78±1.00	0.006
PSD (dB)	5.63±3.96	1.57±0.36	0.007
24h SBP (mmHg)	138.10±6.74	124.56±15.17	0.020
24h DBP (mmHg)	78.75±5.27	72.89±8.55	0.086
24-h MBP (mmHg)	98.53±4.36	90.11±10.90	0.026
24-h HR (beats/min)	73.35±13.63	76.00±7.89	0.633
OPP (mmHg)	50.35±3.53	47.41±7.07	0.281

Table 5.10: Intergroup differences in patients and controls characterised by low ocular blood flow (cluster 2). IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure; HR: heart rate; OPP: ocular perfusion pressure. Values are given in mean±SD.

Glaucoma patients characterised by low OBF values also exhibited lower HF and higher LF/HF parameters than normal controls (21.30±8.08 nu versus 34.78±13.26

nu, p=0.015 and 3.73±1.61 versus 2.09±1.11, p=0.020, Figures 5.14 and 5.15). LF values were, however, statistically comparable between the two groups (70.90±10.94 nu versus 60.56±14.13 nu, p>0.05).

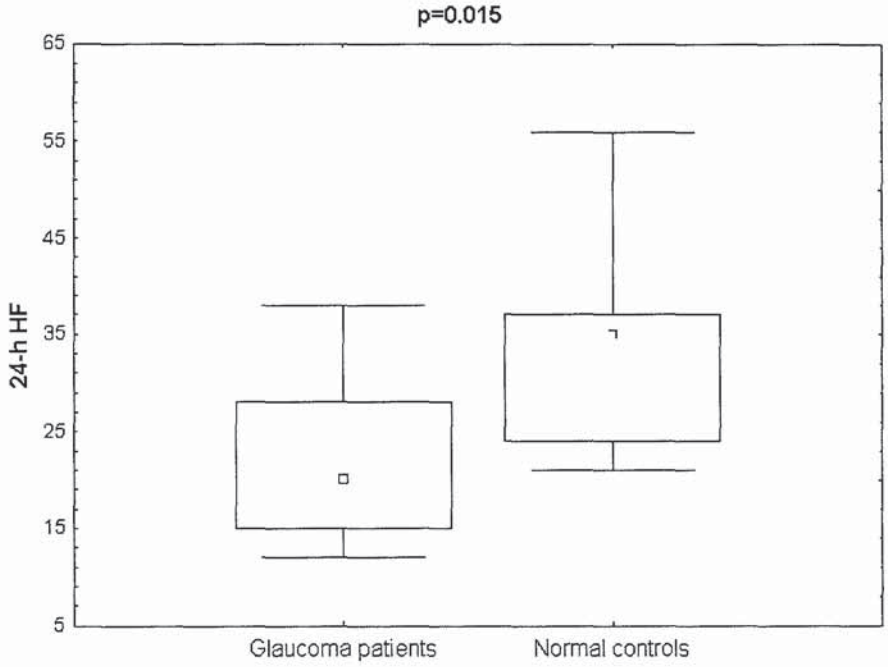


Figure 5.14: Differences in 24-h HF ratio between glaucoma patients and normal controls with low OBF values.

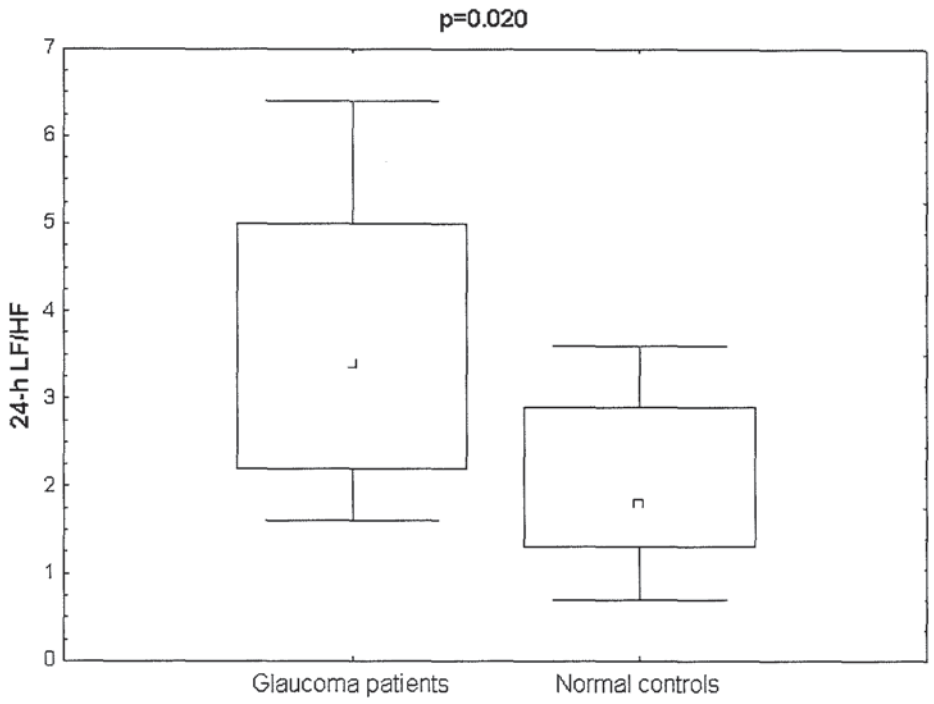


Figure 5.15: Differences in 24-h LF/HF ratio between glaucoma patients and normal controls with low OBF values.

5.7. Discussion

Glaucoma patients exhibited higher IOP and VF damage than normal controls. In addition, POAG patients also exhibited higher sympathetic and lower parasympathetic activity measured over a 24-h period than normal controls. However, OBF parameters were similar between the two groups.

In this study we used a multivariate analysis designed to identify patterns in multivariate data sets. This approach differs from more traditional analysis where groups are defined based on obvious criteria. A cluster analysis allows us to define groups of patients based on more complex characteristics and gives us an indication of what should be investigated (Emre *et al.,* 2004).

Two clusters of patients and controls were computed based on OBF parameters. Two groups of patients and controls were obtained by using this approach: a) patients and normal controls with higher OBF values; and b) patients and normal

controls with lower OBF values. Although the two computed groups differed in the measured OBF parameters, no difference in gender distribution, IOP, VF damage, 24-h BP, 24-h HR or OPP were observed between either the two glaucoma or normal controls clusters.

Besides the OBF parameters used to compute the two clusters, the only statistically significant difference between the two computed glaucoma clusters, was a higher parasympathetic nervous system activity (as measured by HF parameter) over a 24-h period in those patients with higher OBF values (cluster 1) than the patients included in cluster 2. Since glaucoma patients exhibited higher sympathetic nervous system activity (as expressed by LF and LF/HF) than normal controls, an increased parasympathetic tonus in those glaucoma patients with high OBF when compared to those with low blood flow parameters could play a role in maintaining a normal ocular perfusion. This quality may play a crucial role in the event of haemodynamic challenges.

Glaucoma patients exhibiting lower OBF values showed higher systemic BP (SBP and MBP) values, higher sympathetic and lower parasympathetic nervous activity when compared to normal controls with similar ocular perfusion characteristics. In these patients, higher BP values could be the direct result of a disturbed ANS function characterised by continuously high sympathetic tonus. Since the vessels in the retina and in the prelaminar portion of the optic nerve have no neural innervation (Laties, 1967; Ye *et al.,* 1990) only a possible sympathetic influence on the blood flow downstream to the measurement point, at the level of PCAs and CRA (which vessels have a rich supply of autonomic nerves) can offer an explanation for our finding. However, involvement of endothelial factors can not be excluded. ET_1 is an endothelium-derived compound that exerts a variety of hemodynamic and structural alterations in the cardiovascular and pulmonary circuits (see Chapter 1.3.4.2 of the present thesis). The ET_1 system is activated in a large number of cardiovascular diseases, including systemic hypertension (Ram, 2003). High ET_1 levels could also contribute to ischaemia-related cupping of the ONH (Oku *et al.,* 1999). It can be concluded that in these patients, systemic blood flow alterations due to autonomic and/or endothelial factors could, at least partially,

determine the observed OBF disturbances and together contribute to the occurrence of glaucomatous damage.

A high sympathetic tone during both day and night has previously been reported in NTG patients (Kashiwagi *et al.,* 2000) and could result in endothelial damage by either platelet activation or by mechanical injury to the vascular wall as a result of high systemic BP and increased blood velocity (Remme, 1998). A constant high sympathetic tone represents not only a sign of blunted HRV (Korpelainen *et al.,* 1997) but also an indicator of increased oxygen demand in various tissues (Remme, 1998). It could also result in a low ischaemic threshold in all organs, including the eye. In glaucoma patients suffering from a high sympathetic tonus during both day and night, the eye could be more susceptible to minor changes in perfusion pressure and ocular diseases with vascular risk factors such as glaucoma could occur with higher frequency.

No difference in IOP or VF degree of damage was observed between glaucoma patients characterized by high and those by lower OBF parameters. This observation is in concordance with previous studies. Emre et al. (Emre *et al.,* 2004) also reported that glaucoma patients with low and high OBF values measured at different vascular beds demonstrate similar visual function. It can, therefore, be suggested that the OBF alterations observed in some glaucoma patients could be either primary or secondary to systemic haemodynamic disturbances. Concomitant alterations in systemic BP and ANS function observed in glaucoma patients exhibiting low OBF values included in the present study support this hypothesis.

5.7.2. Conclusions

In some glaucoma patients, systemic autonomic system disturbances appear to result in both systemic and local vascular alterations. Although more studies are necessary to confirm this hypothesis, this fact could have important clinical and therapeutic consequences. Beside an autonomic dysfunction other variables could, however, be responsible for multiple vascular dysregulation in POAG patients. This hypothesis will be tested in Chapter 6 of the present thesis.

5.7.3. Possible clinical and therapeutic consequences

The fact that vascular risk factors are, at least partially, responsible for the occurrence and progression of GON had little if any therapeutic impact so far. Given the success of IOP lowering upon glaucoma prognosis, consideration of therapeutic alternatives seems to be inappropriate. Nevertheless, we should consider addressing other risk factors whenever IOP-lowering strategy fails to arrest the progression of the disease. A more clear understanding of the complex relationship between ocular and systemic haemodynamic disturbances could represent the first step in finding alternatives to the current antiglaucoma medication.

6. Systemic Circulatory Glutathione Levels in Newly Diagnosed Primary Open-Angle Glaucoma Patients

The publication of this work is presented in Appendix 3.3.

6.1. Abstract

Purpose: To assess the level of plasma glutathione in untreated primary open-angle glaucoma patients.

Methods: Twenty-one newly diagnosed primary open-angle glaucoma patients (mean age±SD: 72.40± 11.00 years) and 34 control subjects (mean age±SD: 68.45± 10.11 years) were subjected to a blood analysis in order to detect the level of circulating glutathione in both its reduced and oxidized forms. The effects of gender and systemic blood pressure on circulating glutathione levels were also analyzed.

Results: Age had a negative effect on the level of both reduced and total glutathione ($p=0.002$, $r=-0.52$ and $p=0.002$, $r=-0.52$ respectively) in control subjects but not in glaucoma patients. In the control group, men demonstrated higher levels of both reduced and total glutathione than women ($p=0.024$ and $p=0.032$ respectively). After correcting for age and gender influences on blood glutathione levels, glaucoma patients exhibited significantly lower levels of reduced and total glutathione than control subjects ($p=0.010$ and $p=0.006$). No differences between study groups were observed in either oxidized glutathione levels or redox index.

Conclusions: Glaucoma patients exhibit low levels of circulating glutathione, suggesting a general compromise of the antioxidative defence.

6.2. Introduction

POAG represents a chronic, slowly progressive optic neuropathy, characterized by excavation of the ONH and a distinctive pattern of visual field VF defects (Van Buskirk and Cioffi, 1992). The disease is multifactorial in origin and is associated more closely with elevated IOP resulting in the main from reduced drainage of AU. Nevertheless, about one third of glaucoma patients exhibit an apparently normal IOP. Moreover, a substantial number of POAG cases continue to progress despite therapeutically lowered IOP. All these observations have spurred further research to determine the existence of other possible risk factors for glaucoma (Flammer et al., 2002).

Besides more extensively investigated factors such as increased IOP (Krakau, 1981; Bonomi *et al.,* 2001), reduced OBF (Rojanapongpun *et al.,* 1993; Nicolela *et al.,* 1996*b*; Kaiser et al., 1997; Butt *et al.,* 1997; Findl *et al.,* 2000), ocular vascular dysregulation (Flammer, 1994; O'Brien, 1998; Chung *et al.,* 1999 *a*; Anderson, 1999; Gherghel *et al.,* 1999; Gherghel *et al.,* 2000; Hosking *et al.,* 2004), and systemic BP alterations (Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham *et al.,* 1995), oxidative stress has also been proposed as a contributing factor in the aetiology of GON (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004). Oxidative stress represents a harmful state defined by the presence of pathological levels of ROS relative to the antioxidant defence. ROS are molecules more strongly oxidizing than O_2 itself, or molecules containing oxygen that generate free radicals. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$), which is the strongest oxidant produced in biological systems. Another reactive species, the so-called RNS, include nitric oxide and peroxynitrite ($ONOO^-$), a product resulting from the reaction between ROS and NO.

In certain situations free radicals can be generated in an exaggerated manner and can injure tissues and organs by interacting with lipids, proteins or DNA (Valencia *et al.,* 2002). Oxidative stress has been implicated in a large number of human diseases including different autoimmune diseases (Ahsan *et al.,* 2003), alcoholic liver disease (Videla and Guerri, 1990), cancer (Beutler and Gelbart, 1985),

infection with human immunodeficiency virus (HIV) (Buhl et al., 1989) as well as in ischaemia-reperfusion injury (Ferdinandy and Schulz, 2003).

In order to survive, the human body has developed a complex, efficient and highly adaptive antioxidant defence system. This system includes two categories of antioxidants:

- Enzymatic, such as glutathione peroxidase (GPx), glutathione reductase (GSSGR), superoxide dismutase (SOD), catalase (CAT) and peroxiredoxin (Erden and Bor, 1984); and
- Non-enzymatic, such as reduced glutathione (GSH), antioxidant vitamins and low molecular weight compounds such as urate (Portakal and Inal, 1999).

The eye is also protected against oxidative stress by several mechanisms involving antioxidant enzymes such as CAT and SOD, as well as by low-molecular-weight antioxidants such as GSH and ascorbate (Richer and Rose, 1998). Glutathione (GSH, L-glutamyl-L-cysteinylglycine), a tripeptide consisting of glycine, cysteine and glutamic acid, prevents the effects of ROS either directly as an antioxidant or indirectly, by maintaining other cellular antioxidants in a functional state (Benedich, 1990; Pompella *et al.,* 2003). GSH conjugates with a large variety of products of oxidative stress and carcinogens via reactions facilitated by an emzyme called glutathione S-transferase (GST). In living organisms, glutathione exists in two forms: reduced (GSH) and oxidized (GSSG). An optimal GSH:GSSG ratio is critical for survival of the cells and tight regulation of the system is, therefore, very important (Townsend *et al.,* 2003).

Abnormal levels of GSH have already been reported in the lens (Harding, 1970; Spector, 1995; Lou, 2003) and whole blood (Donma *et al.,* 2002) of age-related cataract patients, in the vitreous humor and blood of patients with proliferative diabetic retinopathy (PDR) and proliferative vitreoratinopathy (PVR) (Cicik *et al.,* 2003) and in the blood of patients with age-related macular degeneration (ARMD)

(Nowak *et al.,* 2003; Cai *et al.,* 2003). Altered GSH and GSH activity has also been reported in the trabecular meshwork and aqueous humor of glaucoma patients (Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004). In a recent study, Yang et al. (Yang *et al.,* 2001) demonstrated the presence of serum antibodies against GST in glaucoma patients. The results of these studies are of extreme importance. A deficit in the scavenging capacity of glutathione could reduce the bioavailability of NO for vasodilation, with consequent disruption of vascular tone.

7.3. Hypothesis

Assumptions have been made that oxidative stress plays a role in the aetiology of POAG (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004). GSH is one of the most potent factors acting to protect human body against oxidative stress. A low level of circulating GSH could result in a higher rate of oxidative reactions that reduce the bioavailability of NO. Since NO is involved in the regulation of systemic hemodynamics (Vallance *et al.,* 1989; Vallance and Chan, 2001), a low NO production could have important consequences on the equilibrium between the endothelial vasoconstrictory and vasodilatory factors; in glaucoma this could result in a general vasospastic tendency manifested at both peripheral (Gasser *et al.,* 1999*a*; Pache *et al.,* 2003) and ocular vascular beds (Guthauser *et al.,* 1988; Mahler *et al.,* 1989; Schmetterer and Polak, 2001; Gherghel *et al.,* 2004*a*) in susceptible glaucoma patients.

Although low GSH and GSH activity have been reported in the trabecular meshwork and aqueous humour of glaucoma patients (Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004), to date no study has investigated whether altered systemic GSH levels occur in patients suffering from this disease. If oxidative stress plays a role in the pathogenesis of POAG, this should be translated at the systemic level by low plasma GSH levels.

7.4. Aim

The aim of this study was to establish circulating GSH and GSSG levels in newly diagnosed and previously untreated POAG patients.

7.5. Subjects and methods

7.5.1. Recruitment of untreated primary open-angle glaucoma patients

Consecutive, newly diagnosed and previously untreated POAG patients attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust, Birmingham, UK, were included in this prospective study.

7.5.1.1. Inclusion criteria

Patients underwent diurnal IOP phasing and were diagnosed as having POAG if at least two IOP measurements were greater than 24 mmHg, glaucomatous cupping of the optic disc on funduscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with a diagnosis of glaucoma using program 24-2 of the Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA). The characteristics of glaucomatous VF defects have previously been described (see Table 3.1).

7.5.1.2. Exclusion criteria

Exclusion criteria for glaucoma patients group are outlined in Table 6.1.

- Narrow iridocorneal angles;
- Evidence of secondary glaucoma;
- Pseudoexfoliation;
- Pigmentary dispersion;
- A history of intraocular surgery; and
- Any form of retinal or neuro-ophthalmologic disease that could result in VF defects;

Table 6.1: Exclusion criteria for the glaucoma patients group

6.5.2. Normal subjects recruitment

The control group was recruited from patients' spouses and from other volunteers and it was composed of subjects who had never had neither glaucoma nor other ocular diseases such as cataract, DR and ARMD.

6.5.3. Other exclusion criteria for both study groups

Further exclusion criteria for both study groups were smoking and a history of any chronic systemic disease with presumed low GSH levels including autoimmune diseases (Ahsan *et al.,* 2003), alcoholic liver disease (Videla and Guerri, 1990), cancer (Beutler and Gelbart, 1985), and diabetes mellitus (Beard *et al.,* 2003).

6.5.4. Ethical approval

Ethical approval was obtained from the local medical ethics committees (Aston University and Heartlands and Solihull NHS Trust), and written informed consent was received from all subjects prior to entry into the study. The study was designed and conducted in accordance with the Tenets of Declaration of Helsinki.

6.5.5. Experimental protocol

6.5.5.1. Visual field assessment

VF was assessed by means of Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA) with a size III white stimulus using the full threshold program 24-2. The instrument has been described in the Chapter 3.5.5.1 of the thesis.

Two VF examinations were performed. The results from the second VF measurement were included in the analysis.

6.5.5.2. Intraocular pressure measurement

IOP was measured using a standard Goldmann tonometry technique after instillation of a topical anaesthetic (Benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd). The technique has been described in the Chapter 3.5.5.2 of the present thesis.

6.5.5.3. Systemic blood pressure measurement

BP was measured for each patient and subject in the morning between 8 and 9 am. In preparation for this BP measurement, each subject rested in a sitting position for about 10 minutes in a quiet room in order to achieve sufficient mental and physical calm. The BP value was obtained from the nondominant arm using a BP monitor (UA-779, A&D Instruments Ltd., Oxford, UK). This device measures BP automatically, using the same principle as a conventional mercury sphyngomanometer, with a cuff and a microphone. During the BP measurements, the cuff was placed around the upper arm approximately at heart level. The SBP and diastolic DBP values were measured 3 times (1 minute apart); the average readings for SBP and DBP were then used to calculate the MBP as previously described in Equation 1.7.

6.5.5.4. Blood sampling

In order to avoid diet influence on plasma GSH levels (Jones *et al.,* 1992), subjects were instructed to fast between 9pm and retiring on the evenings before the blood

drawing. On the morning of the test, subjects were requested to have only a light breakfast such as simple toast, while avoiding cooked breakfast, meat, cereal, fresh fruits and fruit juice. Vitamins, minerals or other nutritional supplements were also stopped. In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least two hours before the visit.

All blood samples were collected by a qualified phlebotomist in the morning, between 9am and 10am. Seven ml of blood was collected in EDTA (ethylenediamide tetra-acetic acid) tubes (in order to prevent oxidation) (Anderson, 1996) by venipuncture to the antecubital vein. Thirty µl of blood was then transferred into centrifuge tubes for the initial processing. The GSH was released from the blood cells by protein precipitation and cellular disruption achieved by addition of 33.3 µl of 5-sulphosalicylic acid (SSA), 100 mg/ml within 10 minutes from the blood collection (Anderson and Meister, 1980). Each sample was then diluted with 936.7 µl sodium phosphate buffer (pH 7.5) and the content of each tube was mixed rapidly in a centrifuge, at 13 000 rpm for 5 minutes. 150 µl of supernatant were then collected into clean centrifuge tubes and immediately cooled at –70 degrees Celsius. Samples stored at this temperature are stable for at least 2 months and can be transported on dry ice without deterioration (Jones *et al.*, 1998).

6.5.5. Blood assays

6.5.5.1. GSH assay

Total GSH levels (t-GSH) were assessed by the glutathione reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling procedure (Tietze, 1969; Anderson, 1996). The reagents and equipment used for this assay are listed in Table 6.2.

Reagents:
Sodium phosphate buffer: 125 mM containing 6.3 mM disodium EDTA, pH 7.5
Daily buffer: 3 mg NADPH into 10 ml of sodium phosphate buffer
DTNB solution: 47.56 mg of 5.5 DTNB into 20 ml sodium phosphate buffer
SSA solution: 1 g SSA in 1 ml distilled water
GSH solution: 15.35 mg GSH in 0.5 ml distil water
Enzyme: 41. 6 µl GSR in 5.158 ml sodium phosphate buffer
Equipment:
pipettes: 1000µl, 100µl, 2µl
Adjustable multi-channel pipettes: 5-50µl, 50-250µl
Flat-bottomed 96 well assay plate
96-well plate reader with kinetics

Table 6.2: Reagents and equipment used for the GSH assay. EDTA: ethylenediamide tetra-acetic acid; NADPH: nicotinamide adenine dinucleotide phosphate; DTNB: dithiobis-2-nitrobenzoic acid; SSA: 5-sulphosalicylic acid; GSH: reduced glutathione; GSR: glutathione reductase.

A standard curve from 0 to 2 nmol in 0.5 nmol increments using GSH solution (0.2-0.8µl) was prepared. The standards contained the same final concentrations of SSA as for the samples (1%). To each well of a 96 well plate, 150 µl of daily buffer, 50 µl of DTNB solution and 25 µl of standards or samples were added in quadruplicate and the plate was incubated at 37 °C for 3 minutes. Finally, 25 µl of GSR was added to the previous mixture and the plate was read at 410nm using a 96-well plate reader with kinetics (Punchard *et al.,* 1994). A standard curve was then generated using a linear regression program (Microsoft Excel® - Figure 6.1).

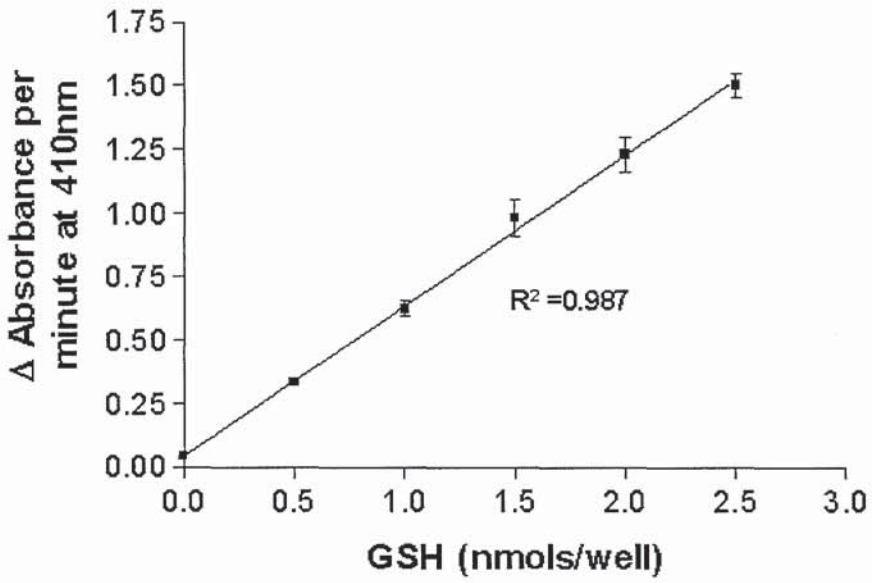


Figure 6.1: The standard curve for the GSH assay

6.5.5.2. GSSG assay

The GSSG levels were assessed using a DTNB-GSSG reductase recycling assay (Anderson, 1996). The reagents used in this assay were those already described in Table 6.1 and, in addition, triethanolamine (TEA) and 2-vinyl pyridine (2-VP). TEA prevents a local high local pH and oxidation while 2-VP is used to stabilize GSH.

A GSSG standard curve from 0 to 0.25 nmol, in 0.025 nmol increments was prepared. 100 µl of standards and samples were transferred into separate centrifuge tubes and 2 µl of 2-VP was added to each tube. TEA was then used to adjust the pH of the standards/samples to 7.5.

To each well of the 96 well plate, 150 µl of daily buffer, 50 µl of DTNB solution, and 25 µl of standards or samples were added in quadruplicate and the plate was incubated at 37°C for 3 minutes. Finally, 25 µl of GSR were added to the previous mixture and the plate was read at 410nm using a 96-well plate reader with kinetics. A standard curve was then generated using a linear regression program (Microsoft Excel® - Figure 6.2).

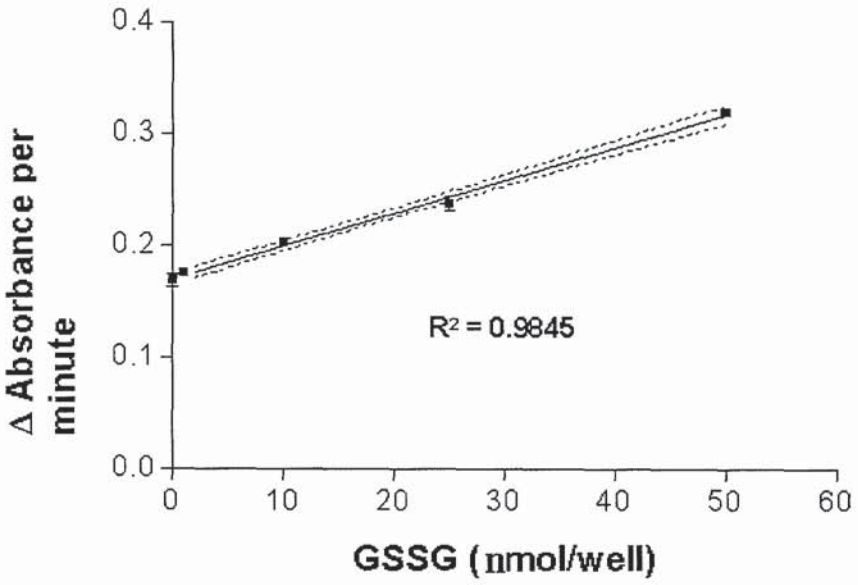


Figure 6.2: The standard curve for GSSG assay

Finally, the GSH levels and the redox index were calculated according to the Equations 6.1 and 6.2.

Plasma GSH = t-GSH – GSSG

- T-GSH: total reduced glutathione
- GSSG: oxidized glutathione

Equation 6.1: Calculation of plasma GSH levels

Redox index = GSH/GSSG

- GSH: reduced glutathione
- GSSG: oxidized glutathione

Equation 6.2: Calculation of redox index

6.5.6. Statistical analysis

The statistical analysis was performed using Statistica ® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. Data are expressed as mean ± standard deviation (SD). Differences between groups at baseline for age and systemic BP parameters were calculated using Student's *t*-test for independent variables. A multivariate analysis was performed to test the influence age, gender and systemic BP on blood glutathione levels. Differences between the two study groups in blood glutathione levels were computed in a covariance analysis model (ANCOVA). P-values of less than 0.05 were considered statistically significant.

6.6. Results

6.6.1. Sample

Twenty-one POAG patients (7 men and 14 women, mean age±SD: 72.40 ± 11.00 years) and 34 control subjects (15 men and 19 women, mean age±SD: 68.45 ± 10.11) years were included in the study. The characteristics of the study groups are given in Table 6.3. There was no significant difference in age and systemic BP between glaucoma patients and control subjects ($p>0.05$); however, there were significant differences in IOP ($p=0.0001$) and the measured VF parameters (MD: $p=0.010$ and PSD: $p=0.0001$ respectively).

Parameter	Glaucoma patients (n=21)	Control subjects (n=34)	p-values
Age (years)	72.40±11.00	68.45±10.11	>0.05
IOP (mmHg)	24.04 ± 4.47	18.68±3.59	0.0001
MD (dB)	-5.03± 3.44	-1.05±0.99	0.010
PSD (dB)	5.74 ± 2.62	1.70±0.42	0.0001
SBP (mmHg)	142.72±20.95	142.24 ± 23.66	>0.05
DBP (mmHg)	79.99± 12.34	82.76±12.36	>0.05
MBP (mmHg)	100.90±13.58	102.58 ± 15.24	>0.05

Table 6.3: Characteristics of the study groups. IOP: intraocular pressure; MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure. Values are given in mean ± SD.

6.6.2 The effect of age and systemic blood pressure

In the control group age had a negative effect on the level of both GSH (p=0.002, r= -0.52, Figure 6.3) and t-GSH (p=0.002, r= -0.52, Figure 6.4) but not on the level of GSSG and redox index (p>0.05). In glaucoma patients, however, blood glutathione levels were not significantly influenced by age. There was no significant association between blood glutathione levels and systemic BP values in either of the study groups (p>0.05).

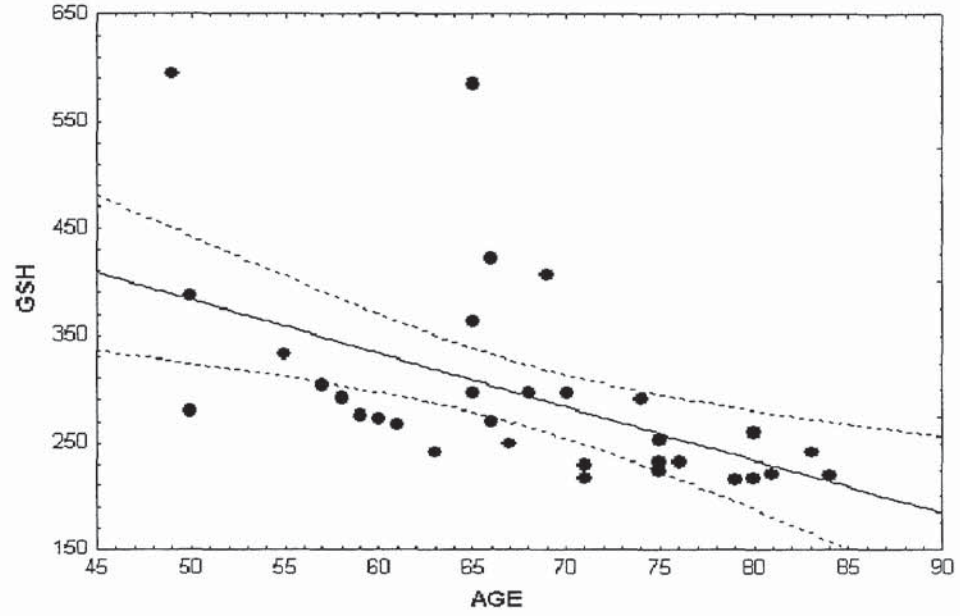


Figure 6.3: Scatter plot showing the effect of age on GSH levels in normal subjects

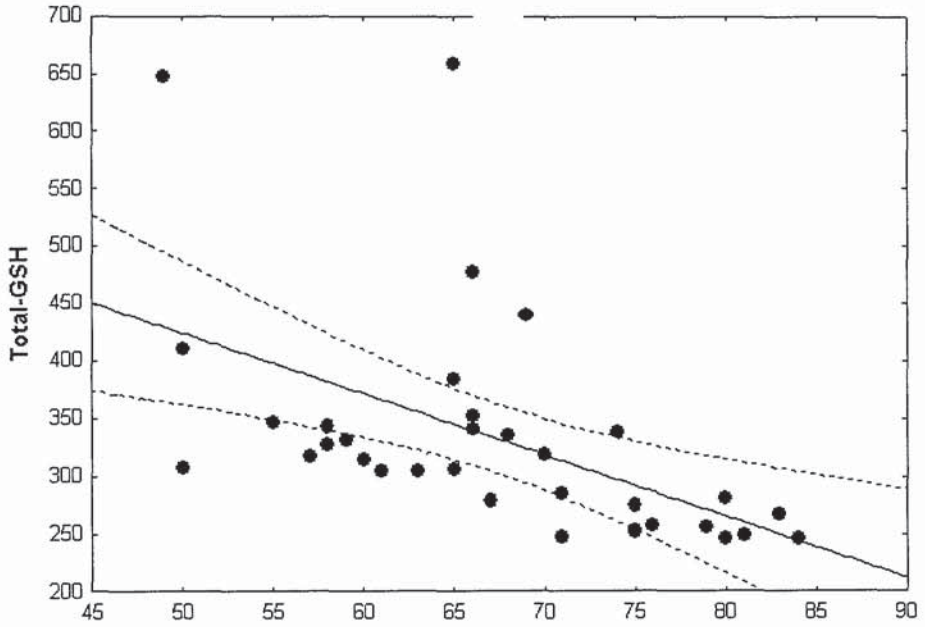


Figure 6.4: Scatter plot showing the effect of age on total-GSH levels in normal subjects

6.6.3 The effect of gender

There were no differences in the number of men and women included in the study groups ($p>0.05$, Chi-square test).

The relationship between gender and blood glutathione levels is illustrated in Table 6.4. In the control group, men demonstrated higher levels of both GSH ($p=0.024$, Figure 6.5) and t-GSH ($p=0.032$, Figure 6.6). GSSG and redox index levels were, however, similar in both men and women ($p>0.05$). In glaucoma patients group, however, men and women had similar levels of blood glutathione ($p>0.05$).

	Glaucoma patients (n=21)			**Control subjects (n=34)**		
Parameter	**Men (7 subjects)**	**Women (14 subjects)**	**p-value**	**Men (15 subjects)**	**Women (19 subjects)**	**p-value**
GSH (nmol)	225.42±111.5	224.85±87.36	>0.05	338.47±120.32	265.62±50.68	0.024
GSSG (nmol)	34.13±8.94	25.22±20.80	>0.05	36.10±16.98	34.94±18.09	>0.05
Redox index	10.29±7.13	9.13±6.27	>0.05	10.90±5.24	10.24±7.72	>0.05
t-GSH (nmol)	249.93±96.45	258.98±80.44	>0.05	372.35±126.16	300.55±54.85	0.032

Table 6.4: Gender influence on blood glutathione levels. GSH: reduced glutathione; GSSG: oxidized glutathione; t-GSH: total glutathione. Values are given in mean ± SD.

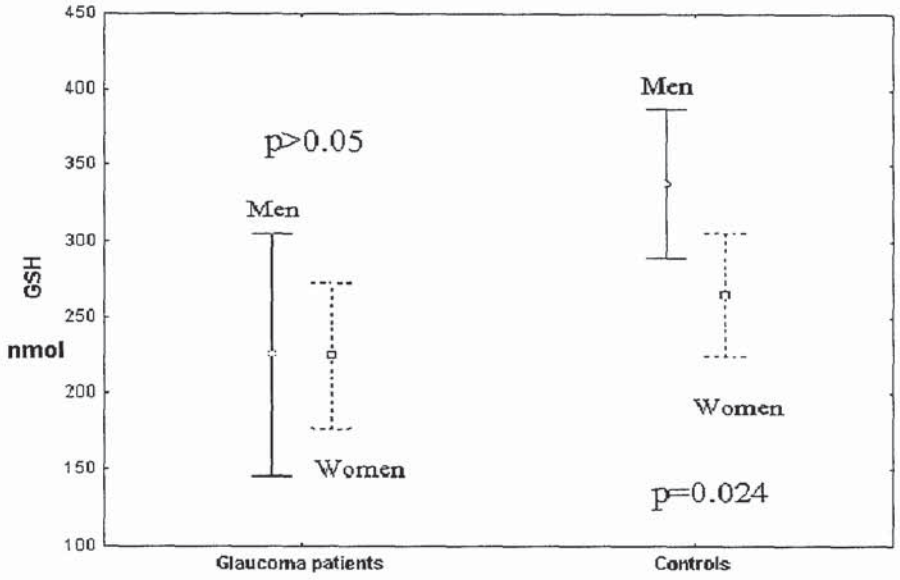


Figure 6.5: In control subjects, men exhibited higher GSH levels than age-matched women. This effect was not evident in glaucoma patients

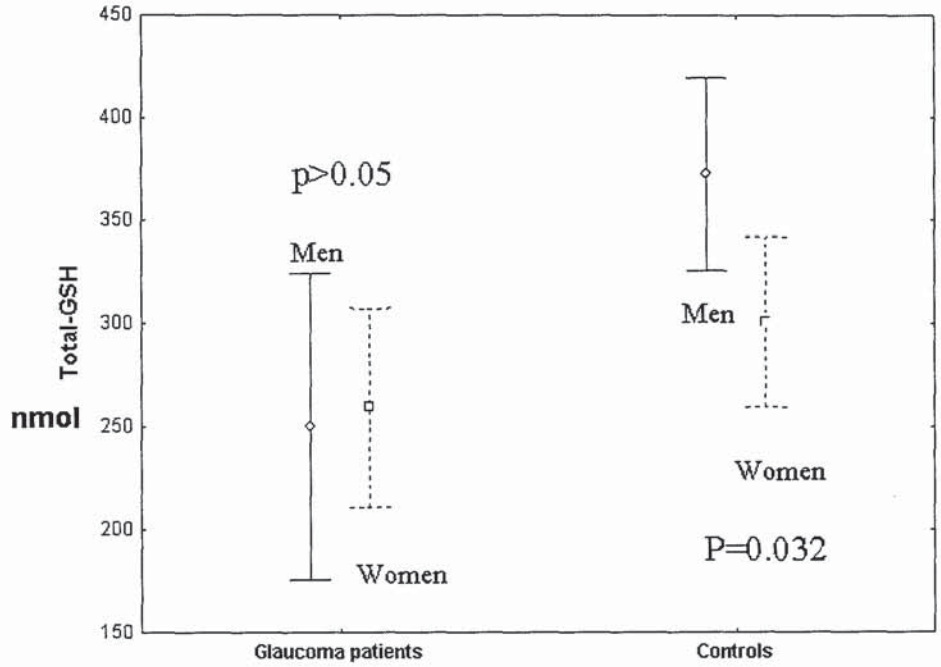


Figure 6.6: In control subjects, men exhibited higher total-GSH levels than age-matched women. This effect was not evident in glaucoma patients

7.6.4. Intergroup differences in blood glutathione

Table 6.5 shows blood GSH and GSSG levels in both study groups. After correcting for age and gender influence on blood glutathione levels, glaucoma

patients exhibited significantly lower levels of GSH and t-GSH than control subjects (p=0.010 and p=0.006 respectively, Figures 6.7 and 6.8). There were no significant differences between study groups in GSSG levels and redox index (p>0.05).

Parameter	Glaucoma patients (n=21)	Control subjects (n=34)	p-values
GSH (nmol)	225.27 ± 83.03	332.23 ± 98.50	0.010
GSSG (nmol)	31.79 ± 18.61	35.34 ± 17.64	>0.05
Redox index (GSH/GSSG)	9.44 ± 6.34	10.52 ± 6.69	>0.05
t-GSH (nmol)	256.28 ± 83.03	333.04 ± 99.92	0.006

Table 6.5: Blood glutathione levels in the study groups. GSH: reduced glutathione; GSSG: oxidized glutathione; t-GSH: total glutathione. Values are given in mean ± SD.

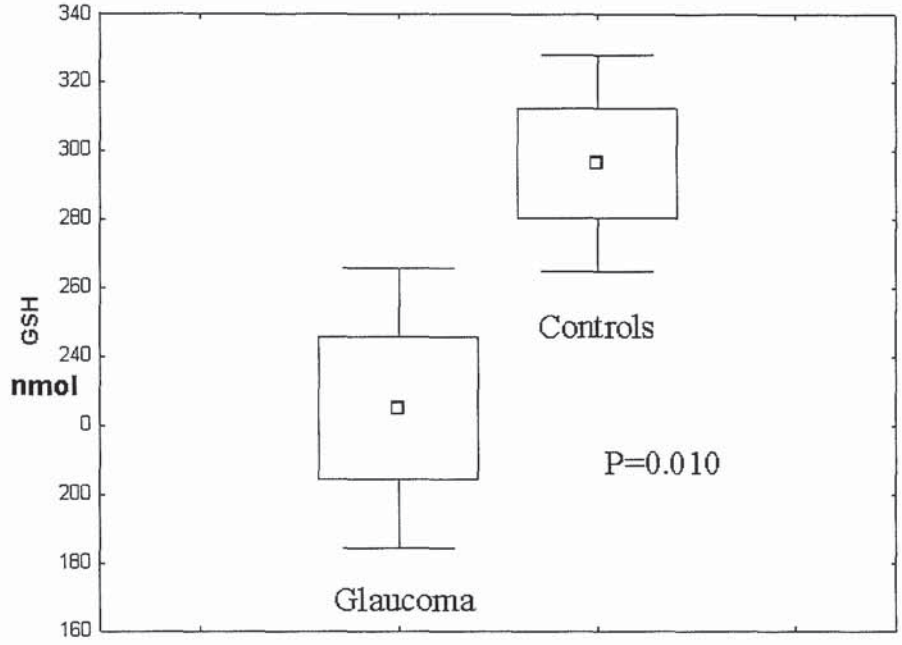


Figure 6.7: Glaucoma patients exhibited lower GSH levels than age-matched control subjects

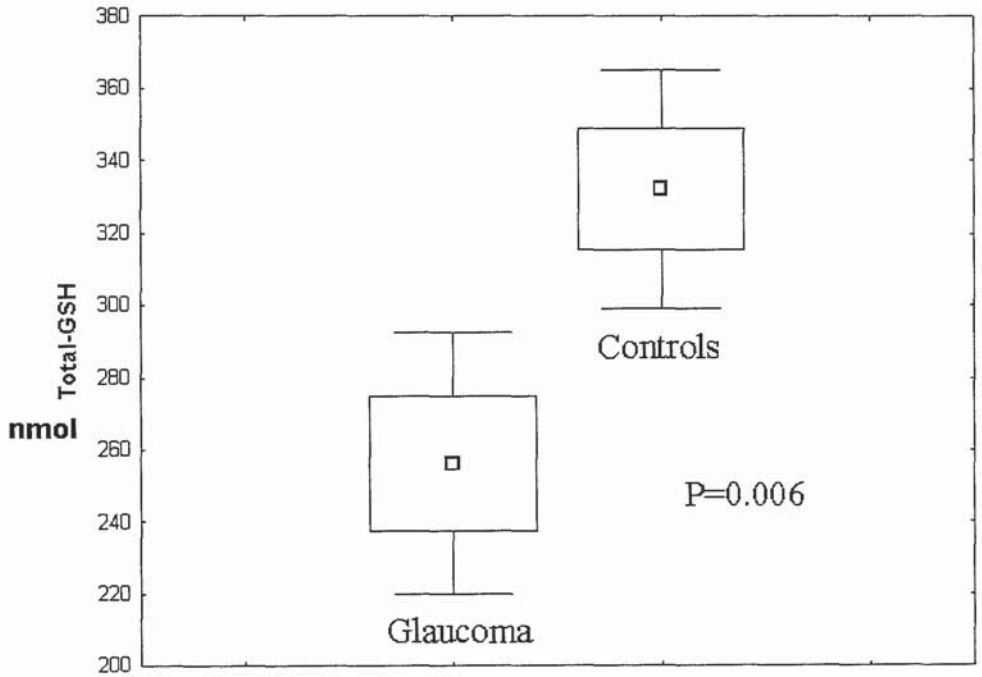


Figure 6.8: Glaucoma patients exhibited lower total-GSH levels than age-matched control subjects

6.7. Discussions

6.7.1. Main findings

The present study assessed the circulating GSH and GSSG levels in newly diagnosed and previously untreated POAG patients. Our results disclosed that independent of age and gender, glaucoma patients demonstrated lower GSH and t-GSH levels than age-matched control subjects. GSSG levels and redox index were, however, similar in both study groups.

6.7.2. Age effect on circulating glutathione levels

GSH is among the most efficient substance that cells and tissues can use in their defence against oxidative stress (Pompella *et al.,* 2003). Age and disease, however, act to decrease the amount of GSH available in the organism. It has been demonstrated that at least half of apparently healthy elderly individuals show low blood GSH levels (Matsubara and Machado, 1991; Lang *et al.,* 1992; Erden-Inal *et al.,* 2002). Indeed, our results show a negative correlation between age and both GSH and t-GSH levels in control subjects. This effect was, however, lost in the glaucoma patients group. In the general population, age is strongly associated with increased oxidative stress and reduced antioxidant status (Knight, 2000). The loss of this correlation between age and GSH loss in POAG patients demonstrated in the present study could indicate that a more important, independent factor than age influencing GSH levels in this cohort.

6.7.3 Gender effect on circulating glutathione levels

It has been shown that blood GSH levels are influenced by gender, with men demonstrating higher values than women (Flagg *et al.,* 1993). Our results also show that in control subjects, men have higher levels of GSH and t-GSH than women. Nevertheless, in glaucoma patients both genders demonstrated similar blood glutathione levels. This again suggests an overriding modulation of GSH in POAG which exceeds that afforded by either gender or age.

6.7.4. Circulating glutathione levels in primary open-angle glaucoma patients

Circulating GSH can be depleted either by subjecting cells to oxidant stress, or by inhibition of synthesis. In glaucoma patients, due to a high level of oxidative stress (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004), GSH could be overused in reactions that result in GSSG production (Anderso and Meister, 1980). Nevertheless, our study groups demonstrated similar GSSG levels. One explanation could be that the GSSG resulted from the reaction between GSH and ROS is rapidly reduced back to GSH, thus completing the normal redox cycle (Wang and Ballatori, 1998). In normal conditions, glutathione reductase is quite effective in maintaining GSH in its reduced state (Wang and Ballatori, 1998). Our glaucoma patients, however, demonstrated a significantly lower GSH level than control subjects indicating that this mechanism played a role in our model and implying that the protection against ROS afforded by GSH could be reduced in patients suffering from this disease, possibly due to a defective redox cycle.

Another possible reason for low GSH levels in our glaucoma patients could be defective intracellular synthesis. The liver is the major site for GSH synthesis (Deleve and Kaplowitz, 1991). The precursors necessary for this synthesis are L-glutamate, L-cysteine and L-glycine and the first step of the reaction, catalyzed by γ-glutamylcysteine syntethase (GSC), is controlled by a negative feedback from its end product, GSH (Richman and Meister, 1975). In the present study, however, the level of GSH in glaucoma patients was found to be low, therefore suggesting that the limiting factors in this case could be the availability of GSH's precursors (Wang and Ballatori, 1998). Although both glutamate and glycine are important, it seems that the major determinant of the rate of GSH synthesis is the availability of the amino acid cysteine (Cys). Cysteine results from the metabolism of homocysteine (Hcy) in the presence of cofactors such as Vitamins B6, B12 and folate. Any interruption in the Hcy-Cys pathway could result not only in the accumulation of the former but also in less available quantities from the second amino acid. In high concentrations Hcy has been implicated in the aetiology of various cardio- and cerebrovascular diseases (McCully, 1969; (Clarke *et al.,* 1991; Selhub *et al.,* 1995). Because vascular risk factors are implicated in the aetiology

of POAG (Rojanapongpun *et al.,* 1993; Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham *et al.,* 1995; Nicolela *et al.,* 1996*b*; Kaiser *et al.,* 1997; Butt *et al.,* 1997; Findl *et al.,* 2000; Chung *et al.,* 1999*a*; Anderson, 1999; Gherghel *et al.,* 1999; Gherghel *et al.,* 2000; Hosking *et al.,* 2004), one could hypothesize that abnormal levels of circulating Hcy are to be found in patients suffering from this disease. The results of recent research published on this subject have, however, been contradictory. Bleich et al. (Bleich *et al.,* 2002) reported higher plasma Hcy levels in patients suffering from POAG than in control subjects while, in a more recent study, Wang et al. (Wang *et al.,* 2004) did not detect any significant difference in plasma Hcy between POAG patients and control subjects. The present study did not aim to quantify the plasma Hcy in our patients and control subjects. We suggest that the abnormal low level of GSH found in glaucoma patients could be at least partially explained by a lower availability of Cys due to an abnormal Hcy-Cys metabolic pathway.

A low level of circulating GSH may also result in a higher rate of oxidative reactions that reduce the bioavailability of NO. A number of studies have shown that NO is also involved in the regulation of systemic haemodynamics (Vallance *et al.,* 1989; Vallance and Chan, 2001), a disturbed NO homeostasis contributing to alterations in systemic vascular diseases such as hypertension or diabetes, where basal formation of NO is impaired (Calver *et al.,* 1992; Chan *et al.,* 2000). It has also been hypothesized that a low NO production has important consequences on the equilibrium between the endothelial vasoconstrictory and vasodilatory factors at the ocular level that could also result in a decreased OBF in susceptible glaucoma patients (Schmetterer and Polak, 2001). In addition to the haemodynamic role, NO has also been shown to induce relaxation of the trabecular meshwork and the ciliary muscle resulting in a decrease in IOP (Wiederholt *et al.,* 1994); any disturbance in the NO balance could, therefore, act both at the ocular and systemic levels (Galassi *et al.,* 2004) and have dramatic consequences on the progression of glaucoma. It can been suggested that in some glaucoma patients, not only the high level of IOP but also the occurrence of vasospasm (Pache *et al.,* 2003) and thrombosis (O'Brien *et al.,* 1997) can be the result of reduced NO bioavailability due to low levels of circulating GSH.

6.7.5. Possible clinical implications

The results of this study could have clinical consequences. Since GSH supplements are well absorbed and results in a substantial increase in plasma GSH levels (Hagen *et al.,* 1990), any GSH deficiency could be corrected by administration of GSH or GSH-precursors supplements. Indeed, the beneficial effect of antioxidative supplementation is well known in cardiovascular medicine where dietary GSH has been proven to increase myocardial resistance to ischaemia-reperfusion injuries (Ramires and Ji, 2001). Since ischaemia plays such an important role in glaucoma pathogenesis, it could be hypothesized that GSH supplementation could have beneficial effects in those glaucoma patients with low antioxidative defence and vascular risk factors. This hypothesis should, however, be tested by further research.

6.7.6. Conclusion

In conclusion, this study demonstrated for the first time that untreated POAG patients exhibit low levels of circulating GSH, suggesting a general compromise to antioxidative defence. Further investigations are, however, necessary to identify the cause for this biochemical deficiency.

7. The Effect of Treatment with Latanoprost 0.005% on Visual and Circulatory Parameters of Newly Diagnosed and Previously Untreated Primary Open-Angle Glaucoma Patients

7.1. Abstract

Purpose: To evaluate the effect of latanoprost 0.005% on the visual field (VF) as well as on the ONH and retinal circulation in newly diagnosed and previously untreated POAG patients.

Methods: Twenty-two newly diagnosed and previously untreated POAG patients (mean age±SD: 68.38±11.92 years) were included in this longitudinal open-label study. Patients were treated with latanoprost 0.005% once a day. VF, IOP, systemic BP, OPP, POBF and OBF measured at the ONH and retina levels were evaluated during a 6-month follow-up period.

Results: Treatment with latanoprost 0.005% resulted in significant decrease in IOP ($p<0.0001$) and increase in OPP ($p<0.0001$). After correcting for changes in OPP, the blood velocity measured at the ONH level was significantly higher after 6 month of treatment than at the baseline ($p=0.0310$). In addition, blood volume and flow measured at the peripapillary retina level were also improved after 3 and 6 months of treatment ($p=0.0170$; $p=0.0260$, and $p=0.0170$; $p=0.0240$ respectively). There was no significant change in either VF or POBF parameters.

Conclusion: Latanoprost 0.005% significantly reduced IOP and increase OPP and OBF measured at the ONH and peripapillary retina levels in previous untreated patients with POAG. Although no improvement has been registered in the VF, its progression was also stopped. These effects could be beneficial for glaucoma patients suffering from ocular vascular dysregulation.

7.2. Introduction

Since various ocular (Rojanapongpun *et al.,* 1993; Wolf *et al.,* 1993; Costa *et al.,* 1994; Nicolela *et al.,* 1996*b*; Kaiser *et al.,* 1997; Butt *et al.,* 1997; Findl *et al.,* 2000; Gherghel *et al.,* 2000; Gherghel *et al.,* 2001; Emre *et al.,* 2004; Fuchsjager-Mayrl *et al.,* 2004) and systemic (Susanna and Basseto, 1992; Kaiser *et al.,* 1993*b*; Hayreh *et al.,* 1994; Bechetoille and Bresson-Dumont, 1994; Tielsch *et al.,* 1995; Graham et al., 1995; Stroman *et al.,* 1995; Waldmann *et al.,* 1996; O'Brien and Butt, 1999; Kashiwagi *et al.,* 2000; Kashiwagi *et al.,* 2001; Emre *et al.,* 2004; Fuchsjager-Mayrl *et al.,* 2004) vascular risk factors have been linked to the occurrence and progression of GON it would follow that they should be addressed by specific therapy. Despite this knowledge, however, the principal treatment objectives remain reduction of IOP using the large therapeutic arsenal available on the market. Although designed to improve AH drainage via trabecular meshwork or uveoscleral routes, some of these drugs have additional therapeutical effects that could be exploited in order to improve the prognosis in patients with additional risk factors such as vascular dysregulation.

Previous studies have indicated that some antiglaucoma medication, such as beta-blockers (Harris *et al.,* 1995; Yoshida *et al.,* 1998; Sponsel *et al.,* 1999; Bergstrand *et al.,* 2001), alpha-agonists (Vetrugno *et al.,* 2001), and carbonic anhidrase inhibitors (CAI) (Martinez *et al.,* 1999; Harris *et al.,* 1999; Bernd *et al.,* 2001; Galassi *et al.,* 2002) could improve ocular circulation in glaucoma. Prostaglandin analogues, such as latanoprost (a newly developed prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$)-related FP receptor agonist substance (Resul *et al.,* 1997), have also been investigated for their effect on OBF. However, the results reported by the few studies performed so far are contradictory. Although acute administration of latanoprost increases ONH blood flow in normal eyes (Ishii *et al.,* 2001; Tamaki *et al.,* 2001), in glaucoma patients, the only consistent effect demonstrated by administration of this drug was an increase in POBF (Vetrugno *et al.,* 1998; McKibbin and Menage, 1999; Geyer *et al.,* 2001; Georgopoulos *et al.,* 2002). To date, no research has demonstrated an effect on ONH or retinal circulation after administration of latanoptost 0.005% in POAG patients.

7.3. Aim

This longitudinal prospective study investigates the effect of treatment with latanoprost 0.005% on the VF and ocular circulation in newly diagnosed and previously untreated POAG patients.

7.4. Subjects and Method

7.4.1. Recruitment of untreated primary open-angle glaucoma patients

Successive newly diagnosed and previously untreated POAG patients attending the Fast Track Glaucoma Clinic at the Heartlands and Solihull NHS Trust, Birmingham, were recruited for the study.

7.4.1.1. Inclusion criteria

Patients underwent diurnal IOP phasing and were diagnosed as having POAG if at least two IOP measurements were greater than 24 mmHg, and presented with glaucomatous cupping of the optic disc on funduscopic examination, normal open anterior chamber angles by gonioscopy, and repeatable VF defects consistent with a diagnosis of glaucoma using program 24-2 of the Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA). The characteristics of glaucomatous VF defects have previously been described in the present thesis (see Table 3.1).

7.4.1.2. Exclusion criteria

Exclusion criteria are outlined in Table 7.1.

- Narrow iridocorneal angles;
- Evidence of secondary glaucoma;
- Pseudoexfoliation;
- Pigmentary dispersion;
- A history of intraocular surgery;
- Any form of retinal or neuro-ophthalmologic disease that could result in VF defects;
- Refractive errors higher than +2 and -3 diopters;
- A history of chronic systemic disease, including diabetes mellitus, or occlusive vascular disorders; and
- Chronic intake of vasoactive drugs.

Table 7.1: Exclusion criteria for the glaucoma patients' group

7.4.2. Ethical approval

Prior to the study, ethical approval was obtained from the local medical ethics committees (Aston University and Heartlands and Solihull NHS Trust), and written informed consent was received from all subjects. The study was designed and conducted in accordance with the Tenets of Declaration of Helsinki.

7.4.3. Treatment method and follow-up

After confirmation of the diagnosis, patients received a prescription for latanoprost 0.005% ophthalmic solution (Xalatan; Pharmacia, Uppsala, Sweden) and were fully instructed in how to self-administer the drug. Patients were evaluated at 1, 3 and 6 months interval after initiation of therapy.

7.4.4. Experimental protocol

At each visit patients underwent the following assessments: VF, IOP, systemic BP and HR. In addition, POBF and OBF at the NRR and peripapillary retina levels

were also determined. Although the drops have been administrated in both eyes, only one eye per patients has been included in the final analysis. Based on the ophthalmoscopy and VF results, this was chosen as the eye with the most advanced disease at the time of diagnosis. The investigations performed in this study are described in order below.

7.4.4.1. Visual field assessment

VF was assessed by means of Humphrey Field Analyzer (HFA: Zeiss-Humphrey, San Leandro, CA) with a size III white stimulus using the full threshold program 24-2. The instrument has been described in the Chapter 3.5.5.1 of the thesis.

Two VF examinations were performed at baseline. The results from the second VF measurement were included in the analysis. Follow-up VF measurements were performed at 1, 3 and 6 month of treatment with latanoprost 0.005%.

7.4.4.2. Intraocular pressure measurement

IOP was measured using a standard Goldmann tonometry technique after instillation of a topical anaesthetic (Benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd). The technique has been described in the Chapter 3.5.5.2 of the present thesis.

7.4.4.3. Systemic blood pressure and heart rate measurement

In preparation for this measurement, each subject rested in a sitting position for 5-10 minutes in the consulting room in order to achieve a sufficient mental and physical calm. The device and technique used for systemic BP measurements were previously described in the Chapter 3.5.5.4 of the present thesis.

The SBP and diastolic DBP values were measured 3 times (1 minute apart); the average readings for SBP and DBP were then used to calculate the MBP according to Equation 1.7. OPP was calculated according to Equation 1.2.

7.4.4.4. Ocular blood flow measurements

POBF parameters were determined immediately after IOP measurement, be means of an OBF analyser (OBFA – Paradigm Medical Industries Inc., Utah, USA). This instrument has been described in detail in the Chapter 1.9.1 of the present thesis.

Three consecutive series of POBF measurements were recorded by a single experienced operator (DG) for each patient. Measurements were performed with the patients in the sitting position and the pneumotonometer mounted on slit lamp microscope, after instillation of a topical anaesthetic (Benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd). The mean OBFA measurements (PA, PV, PR and POBF) were used for the analysis.

ONH and retinal BF were measured by a method already described elsewhere in this thesis (see Chapter 1.9.2). Perfusion parameters (flow, volume and velocity) at the superior temporal regions of the NRR and peripapillary retina were determined at each visit.

7.4.5. Statistical analysis

The influence of OPP on OBF parameters was calculated using a stepwise linear multiple regression analysis. The pre- and post-treatment differences in VF, IOP, BP, HR, and OPP were assessed using repeated measures analysis of variance (re-ANOVA). Similar differences in OBF were assessed by using repeated measures analysis of co-variance (re-ANCOVA), with OPP as changing covariant. Differences between visits were assessed by post-hoc analysis using the Turkey HSD test. The statistical analysis was performed using Statistica ® (version 6.0, StatSoft Inc., Tulsa, OK, USA) for Windows. A p-value of less than 0.05 was considered statistically significant.

7.5. Results

7.5.1. Sample

Thirty-two glaucoma patients (12 men and 20 women) were included in the study. Careful image analysis and subsequent rejection of those subjects who exhibited poor HRF image quality during the follow-up period resulted in exclusion of 10 glaucoma patients (4 men and 6 women). Finally, 22 glaucoma patients (9 men and 15 women, mean age±SD: 68.38±11.92 years) were included in the final statistical analysis.

7.5.2. The effect of latanoprost 0.005% on the visual field, intraocular pressure, systemic blood pressure, heart rate and ocular perfusion pressure

These results are presented in Table 7.2. Although the VF defect was stable during the 6 months observation period ($p>0.05$), treatment with latanoprost 0.005% resulted in a significant decrease in IOP ($p<0.0001$). This effect was evident during all three visits ($p<0.0001$, $p<0.0001$, and $p<0.0001$ respectively, Figure 7.1).

Latanoprost 0.005% did not have any significant effect on systemic BP parameters (SBP, DBP, MBP) or HR ($p>0.05$). However, OPP increased significantly after 1 month ($p=0.0017$), 3 month ($p=0.0014$) and 6 month ($p=0.0001$) of therapy (Figure 7.2).

Parameter	Baseline	1 month	3 month	6 month	p-value
MD (dB)	-4.00± 3.02	-5.03± 4.73	-5.01± 4.75	-5.15± 4.82	>0.05
PSD (dB)	6.09± 4.22	5.01± 3.88	5.05± 4.32	5.20± 4.28	>0.05
IOP (mmHg)	26.36± 3.32	18.93± 2.87	17.86± 2.07	15.64± 2.37	<0.0001
SBP (mmHg)	134.75± 15.66	135.25± 15.42	132.08± 10.63	139.33±12.76	>0.05
DBP (mmHg)	79.33± 11.47	81.00± 8.99	80.17± 9.27	79.67± 12.68	>0.05
MBP (mmHg)	97.81± 12.34	99.08± 10.04	97.47± 8.53	99.56±10.89	>0.05
OPP (mmHg)	39.04± 7.93	46.89± 5.38	47.03± 5.02	50.35± 7.45	<0.0001
HR (beats/min)	64.73± 12.25	64.33± 12.30	67.67± 11.65	65.54± 14.45	>0.05

Table 7.2: The effect of Latanoprost 0.005% on VF, IOP and POBF parameters. MD: mean defect in automated visual field testing; PSD: pattern standard deviation in automated visual field testing; IOP: intraocular pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; MBP: mean blood pressure. Values are given in mean ± SD.

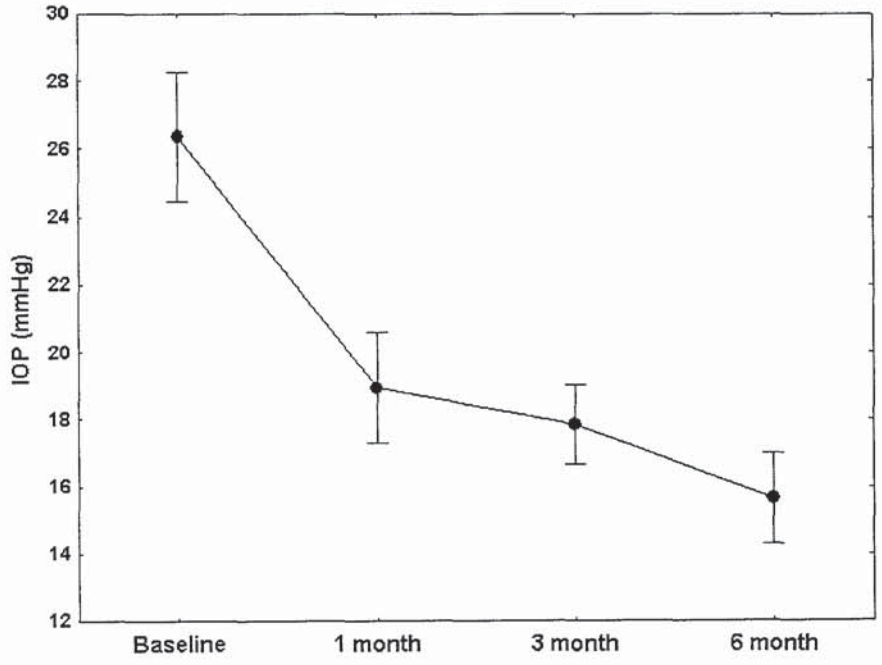


Figure 7.1: Treatment with latanoprost 0.005% results in IOP decrease. The baseline values differ from each of the 1, 3, and 6 month at p<0.0001.

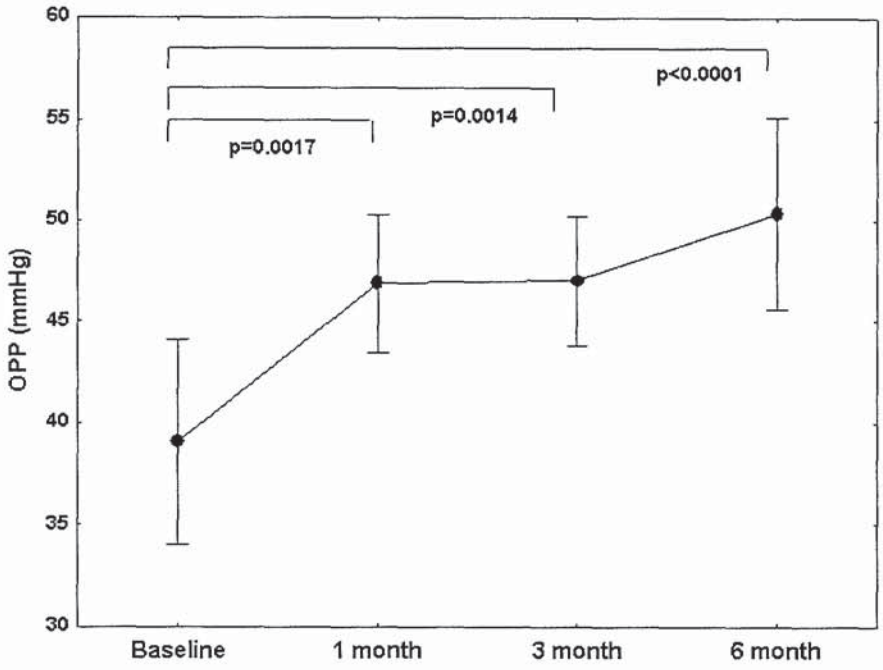


Figure 7.2: OPP increases after treatment with latanoprost 0.005%.

7.5.3. Change in pulsatile ocular blood flow parameters

The effects of 6-month treatment period with latanoprost 0.005% on POBF parameters are listed in the Table 6.3. After correcting for changes in OPP, treatment with latanoprost 0.005% did not have any significant effect on the measured POBF parameters ($p>0.05$).

6.5.4. Change in optic nerve head and peripapillary blood flow

These results are presented in the Table 6.4. Although the overall effect did not show any significant difference, after correcting for changes in OPP the parameter "Velocity" measured at the NRR level was significantly higher after 6 month than at the baseline ($p=0.0310$, Figure 6.3). At the temporal peripapillary retina level, parameter "Volume" was higher after 3 months ($p=0.0330$, Figure 6.4), parameter "Flow" after 3 and 6 months ($p=0.0170$ and $p=0.0260$ respectively, Figure 6.5), and parameter "Velocity" after 3 and 6 months ($p=0.0170$ and $p=0.0240$ respectively,

Figure 6.6) of treatment with latanoprost 0.005% than the baseline value. These values were corrected for changes in OPP.

Parameter	Baseline	1 month	3 month	6 month	p-value
PA (mmHg)	4.45 ±2.10	4.15± 1.75	3.77 ±1.44	3.55 ±1.76	>0.05
PV (µl)	6.79 ±3.34	8.44± 4.22	7.80± 3.35	7.75± 4.19	>0.05
PR (beats/min)	62.36 ±15.06	64.82± 12.96	67.55 ±12.98	64.45± 14.88	>0.05
POBF (µl/sec)	15.10± 8.39	16.45± 8.31	15.88 ±5.93	14.65± 4.76	>0.05

Table 7.3: The change in POBF parameters. IOP-POBF: intraocular pressure measured with the Ocular Blood Flow Analyser (OBFA); PA: pulse amplitude; PV: pulse volume; PR: pulse rate; POBF: pulsatile ocular blood flow. Values are given in mean ± SD.

Parameter	Baseline	1 month	3 month	6 month	p-value
Volume NRR (AU)	13.73±1.81	13.74± 2.33	15.38 ±2.67	14.50± 2.52	>0.05
Flow NRR (AU)	250.87± 73.09	250.51± 89.14	263.96± 76.68	271.15± 46.25	>0.05
Velocity NRR (AU)	0.77± 0.18	0.82 ±0.30	0.94± 0.26	0.99 ±0.15•	>0.05
Volume TR (AU)	15.28 ±4.20	16.50± 4.38	17.77 ±2.82•	16.68± 1.80	>0.05
Flow TR (AU)	267.56± 87.05	301.67 ±93.73	341.38± 72.92•	335.44±73.47•	>0.05
Velocity TR (AU)	0.97± 0.31	1.09 ±0.33	1.26 ±0.26•	1.21± 0.26•	>0.05

Table 7.4: The change in optic nerve head and peripapillary ocular blood flow (after correcting for OPP in a re-ANCOVA analysis). These parameters are measured in arbitrary units (AU). NRR: neuroretinal rim; TR: temporal peripapillary retina. Values are given in mean ± SD. • Statistically significant differences from baseline values

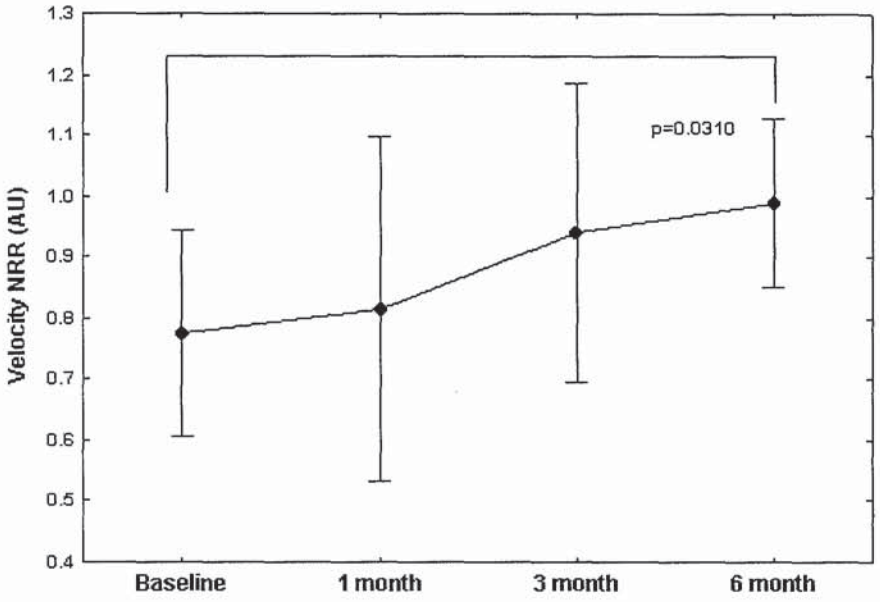


Figure 7.3: Blood velocity measured at the NRR was higher at 6 months than the baseline value

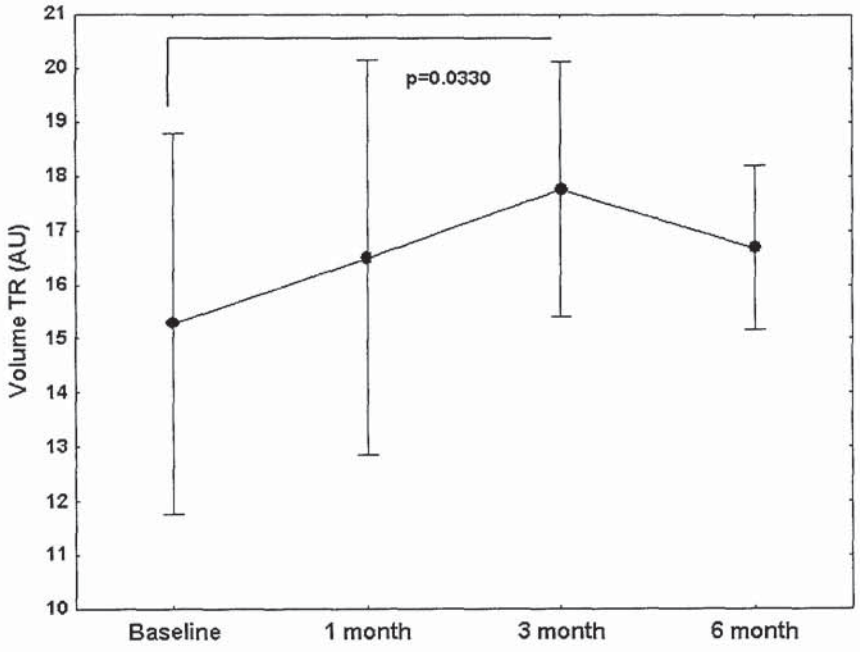


Figure 7.4: Blood volume measured at the peripapillary TR was higher at 3 months than the baseline value

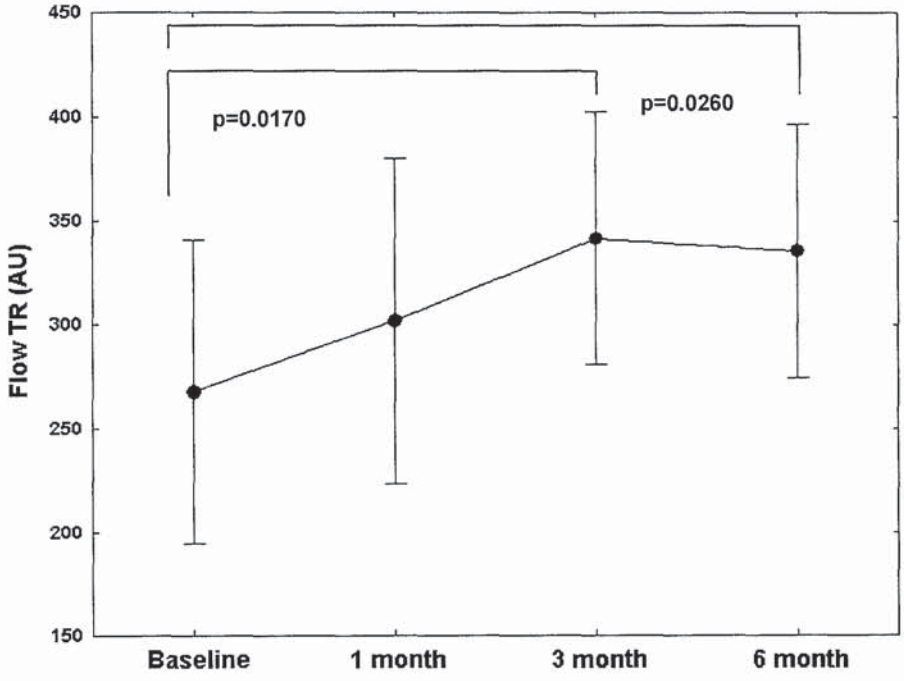


Figure 7.5: Blood flow measured at the peripapillary TR was higher at 3 and 6 month than the baseline value.

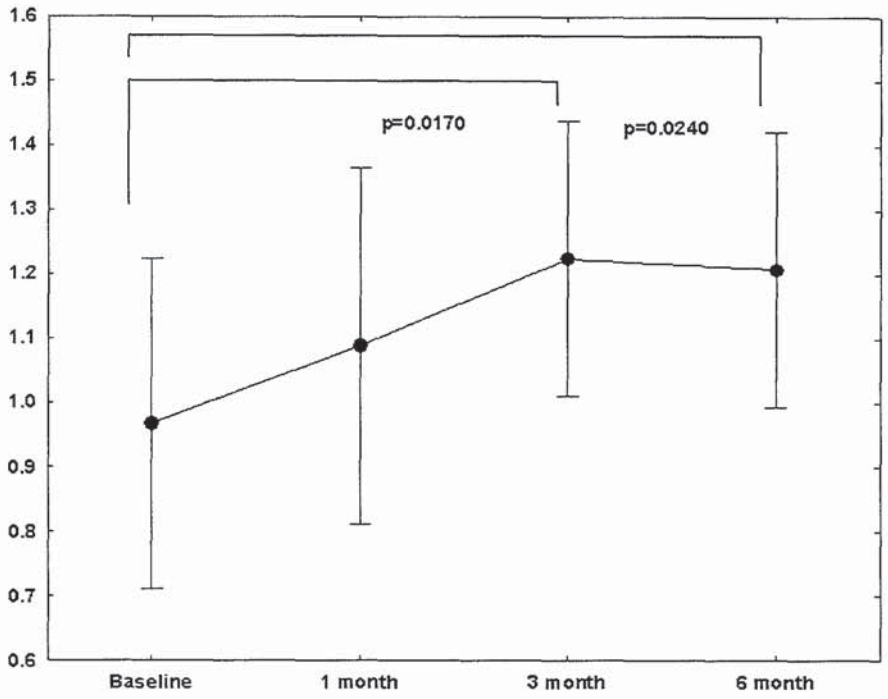


Figure 7.6: Blood velocity measured at the peripapillary TR was higher at 3 and 6 month than the baseline value.

7.6. Discussion

7.6.1. Main findings

The present study assessed the effects of a 6-month period of treatment with latanoprost 0.005% on the VF and OBF of newly diagnosed and previously untreated POAG patients. Our results demonstrated that the most rapid and persistent therapeutic effect obtained after administration of latanoprost 0.005% was represented by both a decrease in IOP and an increase in OPP; these effects were evident after 1 month of therapy and persisted during the entire follow-up period. Moreover, after correcting for the changes in OPP, patients also demonstrated an improvement in the OBF parameters measured at the ONH and peripapillary retina levels; this result was evident only after 3 to 6 months of treatment. No significant differences were, however, demonstrated in either VF or POBF parameters between the follow-up visits.

7.6.2. Effect on the visual field

Treatment with latanoprost 0.005% did not caused significant changes in VF during the follow-up period. This finding is in agreement with previous studies (Erkin *et al.,* 2004). However, successfully arresting the progression of the VF defect for the 6-month follow-up period could be regarded as a positive effect. The Advanced Glaucoma Intervention Study (AGIS) (The AGIS investigators, 2000) found that both the degree and the consistency of IOP-lowering by antiglaucoma medication are directly correlated with the preservation of VF. Indeed, our study has proven that treatment with latanoprost 0.005% resulted in a rapid and consistent IOP-lowering effect. Nevertheless, many other factors, such as OBF play an important role in preserving visual function (Halpren *et al.,* 2002). Improvements in ONH and retinal circulations could have also played a role in preserving the VF in POAG patients included in the present study.

7.6.3. Effect on intraocular pressure and ocular perfusion pressure

Latanoprost's IOP-lowering and OPP-increasing effects have already been reported in previous studies (Alm *et al.,* 1993; Camras and The United States Latanoprost Study Group, 1996; Drance *et al.,* 1998). Our results also showed rapid and persistent decrease in IOP and increase in OPP, therefore reinforcing the beneficial effect of latanoprost for patients suffering from POAG. Because blood flow to an organ depends directly on the perfusion pressure, an increase in OPP could have a beneficial effect on blood flow to the ONH; this effect, however, is regulated by changes in local vascular resistance (Liu *et al.,* 2002*a*). However, the clinical significance of OPP improvement in glaucoma still needs more clarification.

7.6.4. Effect on ocular blood flow

7.6.4.1. Effect on pulsatile ocular blood flow parameters

POBF analysis is a widely used method for the non-invasive assessment of choroidal blood flow (ChBF). Abnormal ChBF has been postulated to play a role in the aetiology of POAG (Duijm *et al.,* 1997*a*; Duijm *et al.,* 1997*b*; Grunwald *et al.,*

1998; Gugleta *et al.,* 2003). Since it has been shown that a decrease in IOP results in choroidal blood flow improvement (Findl *et al.,* 1997), treatment with latanoprost should have beneficial effects on ChBF. Indeed, several authors reported that treatment with latanoprost 0.005% increases the POBF parameters (Vetrugno *et al.,* 1998; McKibbin and Menage, 1999; Georgopoulos *et al.,* 2002; Liu *et al.,* 2002*a*). After initially reporting an improvement Liu et al. (Liu *et al.,* 2002*a*) found, however, that when adjusting for changes in IOP the latanoprost effect on POBF was lost. Our results also show that after correcting for changes on OPP, treatment with latanoprost 0.05% did not results in any change in the measured POBF parameters. This fact could indicate that treatment with latanoprost 0.005% does not have a direct vasomotor effect on POBF as measured with the OBFA.

7.6.4.2. Effect on optic nerve head and retinal circulation

The present study demonstrates for the first time an improvement in both ONH and retinal circulation after chronic therapy with latanoprost 0.005% in POAG patients. Previous studies also reported a vasodilator effect produced by latanoprost at the ONH circulation level. Tamaki et al. (Tamaki *et al.,* 2001) demonstrated an increase in the ONH tissue blood velocity (as measured by laser speckle method) after one instillation of latanoprost 0.005% in normal human eyes. This result has been confirmed by Ishii et al. (Ishii *et al.,* 2001); the authors measured ONH tissue perfusion after a 7-day instillation regimen using the same OBF measurement technique. The conclusion from both studies was that the observed effect was independent of IOP-lowering properties of latanoprost and that the mechanism responsible for this improvement is still unknown.

Although small quantities of a topically administrated drug can reach the posterior parts of the eye and influence the ONH circulation, at least in rabbits (Sugiyama *et al.,* 1992) , this fact has never been demonstrated for latanoprost. However, recent studies have demonstrated an improvement in the retrobulbar blood velocities measured in POAG patients after 3-month period treatment with latanoprost 0.005% (Inan *et al.,* 2003; Erkin *et al.,* 2004); therefore, we could hypothesize that latanoprost could penetrate to deep ocular structures in effective concentrations and results in measurable pharmacologic effects.

It is well known that PGs play an important role in regulation of the local blood flow throughout the human body (Kaley *et al.,* 1985) . However, administration of $PGF_{2\alpha}$ results in both vasoconstriction and vasodilation (Astin *et al.,* 1994); the later effect is probably due to the release of NO. Latanoprost represents, however, a $PGF_{2\alpha}$-analogue and therefore, its vascular effects might be different from those induced by $PGF_{2\alpha}$. Indeed, studies performed in animals showed a vasodilator effect in the retina and choroid after administration of latanoprost (Stjernschantz *et al.,* 2000). The results reported by the present study could also be explained by a potential vasodilator effect exerted by latanoprost on the ONH and retinal circulation where the blood flow improved independent of changes in OPP. However, more research is needed to confirm the mechanism behind improvements in ocular circulation after treatment with latanoprost 0.005%.

7.6.5. Conclusion

Topical latanoprost significantly reduced IOP and increase OPP and OBF measured at the ONH and retina levels in patients with POAG. The VF defect progression was also stopped. These effects could be beneficial for glaucoma patients suffering from ocular vascular dysregulation.

8. Summary and Conclusions

8.1. Summary

The importance of vascular risk factors, both ocular and systemic, in glaucoma pathogenesis has been researched for decades and relevant scientific literature has been reviewed in Chapter 1 of this thesis. However, it is still unclear how haemodynamic disturbances occurring in various ocular and systemic vascular beds could interfere, either separately or in association, with retinal ganglion cell survival, therefore contributing to glaucoma onset and progression. Elucidating these mechanisms could open new diagnosis and therapeutic avenues especially for those glaucoma cases that progress despite therapeutically controlled IOP. This thesis has been concerned with investigating the presence and impact of ocular and systemic vascular risk factors in POAG pathogenesis. Moreover, the possible role played by the systemic anti-oxidative defence mechanism in the occurrence of GON has also been investigated.

The patients recruited for the purpose of the present thesis were subsequently treated with latanoprost 0.005%. Since a very small number of research concentrate on the effect of the current antiglaucoma medication on OBF, we used this opportunity to investigate the possible effect of this drug on various OBF parameters.

In summary, the findings of this work were:

8.1.2. Systemic and ocular vascular response to temperature provocation in newly diagnosed and previously untreated primary open-angle glaucoma patients

Publication of this research is shown in Appendix 3.2.

The idea for this study came from the observation that cold provocation can result in transitory VF defects in patients suffering from peripheral vasospasm (Guthauser

et al., 1988; Mahler *et al.,* 1989). Since vasospasm has been demonstrated to play a role in the pathogenesis of glaucomatous neuropathy (Guthauser *et al.,* 1988; Harris *et al.,* 1994; Pillunat *et al.,* 1994; Flammer *et al.,* 2001; Hosking *et al.,* 2004) it can be hypothesized that cold stimulation results in an abnormal haemodynamic response in normally autoregulated ocular vascular beds.

Systemic parasympathetic and sympathetic neuropathies have been reported in patients with both POAG and NTG (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003) and cold stimulation is a well established provocation test used in detecting abnormal vascular reactivity in patients with autonomic failure (Stancak *et al.,* 1996). Although POAG has been labelled as autonomic disease, research performed so far failed to demonstrate any retrobulbar or retinal OBF alteration induced by cold provocation in patients suffering from this disease (Rojanapongpun and Drance, 1993; Nicolela *et al.,* 2003). However, the effect on ONH blood flow was not studied. The aim of this study was to assess the systemic and ocular vascular reactivity in response to temperature provocation (warm followed by cold) in untreated POAG patients and normal control subjects.

The systemic circulatory changes to warm provocation observed in glaucoma study group has been explained as being the result of an anticipatory reaction to the physical stress; the haemodynamic response due to the anticipation of the test may have been stronger than the effect of the warm stimulation itself and the result was an increase in BP and HR. This type of vascular hyperactivity seen in the glaucoma group is similar to that demonstrated in subjects at risk for cardiovascular diseases in whom the anticipatory and recovery vascular responses are better predictors for subsequent circulatory disturbances than the direct stress-induced reactivity (Gregg *et al.,* 1999).

The blunted BP reaction to cold provocation observed in the glaucoma patients group was similar to the response reported by Nicolela et al. (Nicolela *et al.,* 2003) in POAG patients and after 30 minutes body surface cooling. This particular vascular response could signal an abnormal neural pathway resulted from a lack of sympathetic participation. The concomitant decrease on OBF parameters

measured at the NRR level was possible due to the involvement of endothelial factors as previously reported by Nicolela et al. (Nicolela *et al.,* 2003). In the light of previous research (Schmetterer *et al.,* 1997) it is possible that high plasma levels of ET_1 resulted in a decreased blood flow at the NRR level in our glaucoma patients. The abnormal reduction of blood flow at the NRR in response to a stress factor such as cold could increase the susceptibility of the optic nerve to high IOP and low perfusion pressure in these individuals.

In conclusion, the observed ocular and systemic haemodynamic response of glaucoma patients to temperature provocation could be a manifestation of a systemic autonomic dysfunction and might result in the onset or progression of glaucomatous optic neuropathy. To further test the presence of systemic autonomic dysfunction in glaucoma patients has been tested in the Chapter 4 of the present thesis. A summary of this work is presented below (Chapter 8.1.2).

8.1.3. Silent cardiac ischaemia and autonomic function in newly diagnosed and previously untreated primary open-angle glaucoma patients

POAG has been associated with both ANS dysfunctions (Clark and Mapstone, 1986; Kumar and Ahuja, 1999; Brown *et al.,* 2002; Riccadonna *et al.,* 2003) and silent myocardial ischaemia (Kaiser *et al.,* 1993*a*; Waldmann *et al.,* 1996). Although chronic imbalances of the ANS could lead to adverse cardiovascular events including myocardial ischaemia (Moore and Chester, 2001; Cohn *et al.,* 2003), to date no link has been made between the occurrence of the two entities in patients suffering from POAG. The hypothesis behind this study was that in some patients suffering from POAG, the occurrence of silent cardiac ischaemic events is associated with episodes of autonomic dysfunction, manifested as BP, ECG alterations or both.

It has been established that autonomic dysfunction could affect the eye either by leading to or exacerbating glaucoma in susceptible individuals. Although glaucoma patients seen in clinical practice, especially those suffering from NTG or progressive glaucoma, frequently suffer from a variety of cardiovascular diseases, such as systemic arterial hypertension and silent myocardial ischaemia (Flammer

et al., 2001), studies performed so far carefully selected only those glaucoma patients free from any systemic vascular diseases. This is, however, a rare encounter in clinical practice. The aim of this study was to examine the relationship between the onset of silent cardiac ischaemia, and alterations in systemic BP and autonomic function (as determined by measuring HRV parameters) during the normal daily routine of consecutive newly diagnosed and untreated POAG patients with/without a positive history for cardiovascular diseases.

Independent of a positive history for cardiovascular diseases, glaucoma patients exhibited a blunted HRV translated by high sympathetic tone during both day and night. This represents an indicator of increased oxygen demand in various tissues (Remme, 1998). It could also result in a low ischaemic threshold in all organs, including the eye. Therefore, the observed autonomic dysfunction may not related to a concurrent cardiovascular disease but be associated with the occurrence and possibly the progression of glaucomatous optic neuropathy. For these reasons, an autonomic assessment, together with 24-h BP measurement could be useful in monitoring both the progression of the diseases and the efficacy of various antiglaucoma medications with effect on the ANS, such as beta-blockers.

The effect of autonomic disturbances on ocular blood flow has been tested in the Chapter 5 of the present thesis; a summary of this work is presented below (Chapter 8.1.4).

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8.1.4. Relationship between ocular and systemic vascular risk factors in primary open-angle glaucoma patients

The idea behind this work was inspired by the observation that in some patients suffering from POAG there is an association between low blood flow values measured in various ocular vascular beds and occurrence of peripheral vasospasm (Emre *et al.,* 2004). It can be hypothesized that in these patients both ocular and systemic vascular disturbances act concomitantly in producing disease.

In this clinical study, a cluster analysis based on OBF parameters was performed in newly diagnosed and previously untreated POAG patients and normal controls

and differences with regard to ANS function (as determined by HRV measurement) were assessed between clusters. This approach differs from more traditional analysis where groups are defined based on obvious criteria and has been chosen in order to define groups of patients based on different OBF characteristics.

Similar to previous studies (Kashiwagi *et al.,* 2000), a high sympathetic tone during both day and night has been observed in the glaucoma patients included in this study. In glaucoma patients suffering from a high sympathetic tonus during both day and night, the eye could be more susceptible to minor changes in perfusion pressure and ocular diseases with vascular risk factors such as glaucoma could occur with higher frequency. Moreover, in glaucoma patients with lower OBF in addition to the high sympathetic tone, a higher systemic BP level has also been observed. If both low OBF and high BP represent a direct consequence of the increased sympathetic activity is yet to be established. However, involvement of endothelial factors such as ET_1 can not be excluded. It can be concluded that in these patients, systemic blood flow alterations due to autonomic and/or endothelial factors could, at least partially, determine the observed OBF disturbances and together contribute to the occurrence of glaucomatous damage.

Although we have been demonstrated that autonomic dysfunction plays an important role in both systemic and ocular vascular disturbances in POAG, other variables could, however, be responsible for the multiple vascular dysregulation associated with this disease. This hypothesis has been tested in Chapter 6 of the present thesis and a summary of this research is presented below (Chapter 8.1.5).

This research suffered from some limitations. The patients and controls included in this study were selected from those included in the Chapter 3 and Chapter 4 of the present thesis. Therefore, exclusions based on both HRF and ECG data quality were applied. Moreover, patients and controls taking chronic medication (which were included in Chapter 4) were also excluded. This fact resulted in a smaller sample size than in the previous two studies. Although the present research draws important conclusions that sustain those of the Chapters 3 and 4, the special statistical approach used in this paper could benefit from analysing results obtained

from a larger number of glaucoma patients and controls. This issue will be addressed in future research.

8.1.5. Systemic anti-oxidative defence in patients suffering from primary open-angle glaucoma

Publication of this work is shown in Appendix 3.3.

Oxidative stress has been proposed as a contributing factor in the aetiology of GON (Alvarado *et al.,* 1984; Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004) and GSH is one of the most potent factors acting to protect human body against oxidative stress. A low level of circulating GSH could result in a higher rate of oxidative reactions with potential harmful effect in various tissues and organs including the eye.

Although low GSH and GSH activity have been reported in the trabecular meshwork and aqueous humour of glaucoma patients (Nguyen *et al.,* 1985; Levin *et al.,* 1996; Izzotti *et al.,* 2003; Ferreira *et al.,* 2004), to date no study has investigated whether altered systemic GSH levels occur in patients suffering from this disease. If oxidative stress plays a role in the pathogenesis of POAG, this should be translated at the systemic level by low plasma GSH levels. Therefore, the aim of this study was to establish circulating GSH levels (as a measure of systemic anti-oxidative defence capacity) in newly diagnosed and previously untreated POAG patients. Our results disclosed that independent of age and gender, glaucoma patients demonstrated lower GSH levels than age-matched control subjects implying that the protection against ROS afforded by GSH could be reduced in patients suffering from this disease, possibly due to a defective redox cycle. A defective GSH intracellular synthesis can also be implicated. This avenue should be explored in further research.

A low level of circulating GSH may also result in a higher rate of oxidative reactions that reduce the bioavailability of NO. Possible implications of this statement are explored including the possibility that a low NO production has important

consequences on the equilibrium between the endothelial vasoconstrictory and vasodilatory factors at the ocular level that could also result in a decreased OBF in susceptible glaucoma patients (Schmetterer and Polak, 2001). It can been suggested that in some glaucoma patients, not only the high level of IOP but also the occurrence of vasospasm (Pache *et al.,* 2003) and thrombosis (O'Brien *et al.,* 1997) can be the result of reduced NO bioavailability due to low levels of circulating GSH.

8.1.6. The effect of treatment with latanoprost 0.005% on ocular circulation in newly diagnosed and previously untreated primary open-angle glaucoma patients

The glaucoma patients included in the present work have been subsequently treated with latanoprost 0.005%. Only few recent studies reported effects of current antiglaucoma medication on OBF; therefore, we used this opportunity to test the effect of this drug on OBF in newly diagnosed and previously untreated POAG patients.

Although the effects of both one-dose and short period treatment with latanoprost 0.005% on ocular haemodynamics have been reported in normals, much less information exists on the effect of this drug on the ocular circulation in glaucoma patients. This longitudinal prospective study investigates the effect of treatment with latanoprost 0.005% on the visual function and ocular circulation in newly diagnosed and previously untreated POAG patients. The effect of latanoprost 0.005% on VF, IOP and OPP was similar with previous observations (Alm *et al.,* 1993; Camras and The United States Latanoprost Study Group, 1996; Drance *et al.,* 1998; Erkin *et al.,* 2004). This study also showed that after correcting for changes on OPP, treatment with latanoprost 0.05% did not results in any change in the measured POBF parameters. This fact could indicate that treatment with latanoprost 0.005% does not have a direct vasomotor effect on POBF as measured with the OBFA.

The present study demonstrates for the first time an improvement in both ONH and retinal circulation after chronic therapy with latanoprost 0.005% in POAG patients.

The results reported in this study could be explained by a potential vasodilator effect exerted by latanoprost on the ONH and retinal circulation where the blood flow improved independent of changes in OPP. This effect, together with a significant decrease in IOP and increase in OPP could be beneficial for glaucoma patients suffering from ocular vascular dysregulation.

The present study involved measuring visual and haemodynamic parameters for a period of 6-month of treatment with latanoprost 0.005%. However, the examiner was not masked to what type of medication was prescribed to the patients included in the study. Before we conclude that treatment with latanoprost 0.005% improves OBF in glaucoma patients, it would be important to overcome this limitation. We need more double-masked, randomized, prospective studies to prove that indeed, the current available antiglaucoma medication have an influence on OBF measured at different levels.

8.2. Conclusions

The aims of this work were:

1. **To investigate the presence and impact of ocular and systemic vascular risk factors in POAG.**

   The findings of this work were:

   - Glaucoma patients exhibit an anticipatory reaction to the physical stress, similar to subjects at risk for cardiovascular diseases.
   - Patients suffering from POAG showed a blunted BP response and a reduction in ONH blood flow in response to cold provocation.
   - Nocturnal over-dip in systemic BP is a frequent encounter among POAG patients.
   - In both glaucoma patients and controls, silent myocardial ischaemic episodes occurred during peaks in systemic BP and HR.
   - Independent of a positive history for cardiovascular diseases, patients suffering from POAG demonstrate a blunt circadian rhythm of the ANS.

2. **To assess the relationship between vascular and systemic vascular risk factors in GON.**

The findings of this work were:

- POAG patients characterised by both high and low OBF values demonstrate a high sympathetic tonus over a 24-h period.
- POAG patients with low OBF demonstrate both 24-h systemic BP and HRV abnormalities.
- OBF alterations observed in some glaucoma patients could be either primary or secondary to systemic haemodynamic disturbances and not a consequence of ONH damage.

3. **To assess the level of systemic anti-oxidative defence in POAG patients.**

The findings of this work were:

- Age and gender had no effect on either GSH or GSSG levels in glaucoma patients.
- Patients suffering from POAG demonstrated significantly lower GSH and t-GSH levels than normal controls.

4. **To investigate the effect of treatment with latanoprost 0.005% on visual function and OBF.**

The findings of this work were:

- VF damage progression has been stopped for 6 months following treatment with latanoprost 0.005%.
- Treatment with latanoprost 0.005% resulted in a significant decrease in IOP and increase in OPP.
- After correcting for changes in OPP, POBF values were not influenced by treatment with latanoprost 0.005%.
- Treatment with latanoprost 0.005% resulted in a significant increase in the OBF parameters measured at the ONH and peripapillary retina levels.

8.3. Proposed practical model for clinical conduit when dealing with new and definite primary open-angle glaucoma or normal-tension glaucoma patients

8.3.1. Background

Various vascular systemic factors are indeed involved in the pathogenesis of POAG. Nevertheless, the principal treatment objectives remain reduction of IOP to a level that the clinician believes will improve the outcome of the disease. Although beneficial in most cases, this approach is sometimes insufficient and in some patients the disease progresses despite our best efforts. Therefore, additional risk factors for each individual case should be identified and treated.

8.3.2. Clinical protocol for primary open-angle glaucoma management

In Figure 8.1 is presented a practical clinical protocol for newly diagnosed glaucoma cases. As one expects, a more complex workup is necessary when the glaucoma suspect exhibit a normal diurnal IOP curve. However, in many patients suffering from POAG peaks in IOP occur outside the normal office hours (Loewenthal, 1977; Asrani *et al.,* 2000; Hughes *et al.,* 2003). Therefore, in these cases 24-h IOP and BP measurement should be performed whenever possible. Such change in clinical routine could have important consequences. Presently the NTG diagnosis is based on diurnal IOP curve showing values of 21 mmHg or less (Levene, 1980). Nevertheless, previous studies reported that IOP could reach its highest level during the night in some glaucoma patients. Therefore, we suggest that 24-h IOP measurement and not the diurnal curve should be considered in the definition and diagnosis of NTG; this kind of assessment can be done with the subject in either sitting or supine position, as the 24-h rhythm of sitting IOP is similar to that of supine IOP (Liu *et al.,* 2003).

Patients should also undergo a thoroughly ANS assessment (Table 1.4), preferably during ambulatory conditions. In addition clinicians should consider potential autonomic effects of various systemic and ocular therapies especially in NTG and progressive POAG cases. It is well known that any therapy that activates the sympathetic division of the ANS will increase the risk of systemic circulatory events, and any drug that increases the vagal tone may improve cardiovascular

outcome (Tulppo *et al.,* 2001; Curtis and O'Keefe, 2002). Since both chronic cardiovascular diseases and their treatment may represent important contributory factors in glaucoma pathogenesis, clinicians should consider carefully any possible danger from this direction. Moreover, IOP-lowering treatment often consists of drugs that either mimic or inhibit the sympathetic and parasympathetic divisions of the ANS (Brown *et al.,* 2002). For these reasons, an autonomic assessment, together with 24-h BP measurement could be useful in monitoring the efficacy and possible circulatory side effects of therapies for glaucoma and systemic diseases.

We still lack a complete understanding of the very complex glaucoma pathogenesis. It is more and more clear that a large variety of risk factors should be considered whenever we face a new glaucoma case. We suggest that a different practical approach could help in improving the diagnosis and outcome in some glaucoma patients with risk factors other than IOP.
Publication of this work is shown in the Appendix 3.1.

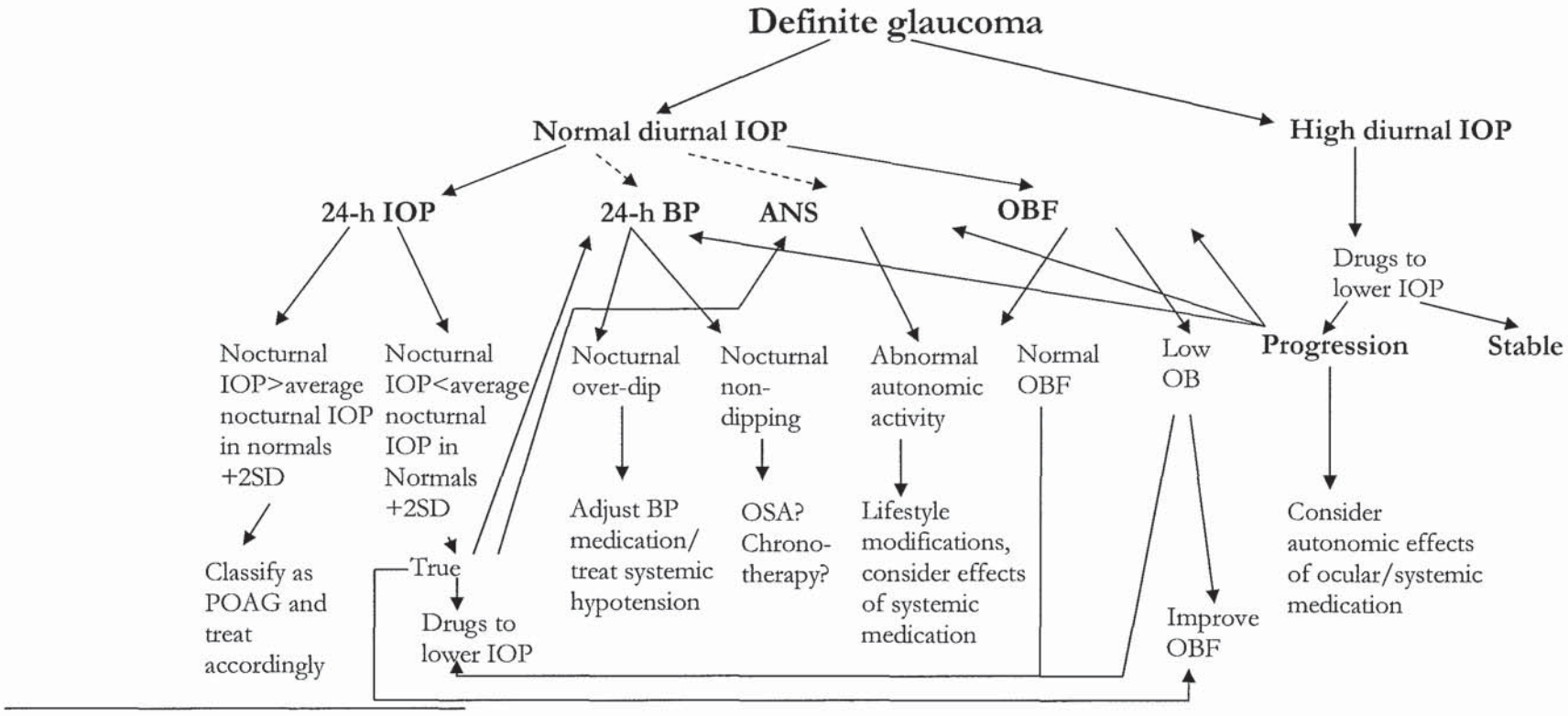

Figure 8.1: Proposed model for clinical conduit when dealing with definite primary open-angle glaucoma (POAG) or normal-tension glaucoma (NTG) patients. IOP: intraocular pressure; BP: blood pressure; ANS: autonomic nervous system; OBF: ocular blood flow; OSA: obstructive sleep apnoea; SD: standard deviation

8.4. Future areas of research arising from this work

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

8.4.1. Magnetic resonance imaging and hormonal studies in primary open-angle glaucoma patients suffering from abnormal autonomic function

The mechanism behind a constant high sympathetic tone and an abnormal HRV in glaucoma is still under investigation. Glaucoma has been associated not only with an increased frequency of silent myocardial ischaemia (Waldmann *et al.,* 1996) but also with diffuse cerebral small-vessel ischaemic changes that have been observed in more patients with normal tension glaucoma than in control subjects (Stroman *et al.,* 1995). However, a clinical association between these two types of systemic circulatory disturbances has never been demonstrated in patients suffering from POAG. An abnormal HRV in some glaucoma patients could be the result of such ischaemic lesions in those areas of the brain responsible for the cardiovascular circadian rhythm situated in the anterior hypothalamus (Moore and Silver, 1998; Rensing *et al.,* 2001). Moreover, some cerebral ischaemic lesions may also result in alterations of the serum levels of antidiuretic hormone, catecholamines, and cortisol; these endocrinological disturbances may have as a direct consequence a disturbed HRV (Korpelainen *et al.,* 1997). Future research to address these hypotheses is necessary.

8.4.2. Peripheral and ocular vasospasm in primary open-angle glaucoma patients with compromised anti-oxidative defence

A low level of circulating GSH results in a higher rate of oxidative reactions that reduce the bioavailability of NO. A number of studies have shown that among other important functions, NO is also involved in the regulation of systemic hemodynamics (Vallance *et al.,* 1989; Vallance and Chan, 2001). A low NO production could also have important consequences on the equilibrium between the endothelial vasoconstrictory and vasodilatory factors; in glaucoma this could result in a general vasospastic tendency manifested at both peripheral (Gasser *et*

al., 1999*a*; Pache *et al.,* 2003) and ocular vascular beds (Guthauser *et al.,* 1988; Mahler *et al.,* 1989; Schmetterer and Polak; Gherghel *et al.,* 2004*a*) in susceptible glaucoma patients. A possible study could explore the presence of peripheral and/or ocular vasospasm in POAG patients with low systemic anti-oxidative capacity, as demonstrated by low plasma GSH levels.

8.4.3. Circadian variation in redox status and endothelial function in primary open-angle glaucoma patients

It has been demonstrated that both oxidative stress (Hardeland *et al.,* 2003) and cardiovascular parameters such as the vascular tone and cardiac output (Veerman *et al.,* 1995) have a circadian rhythm. Endothelium-dependent vasodilation also varies during 24-hour sleep-wake cycle (Walters *et al.,* 2003). A peripheral (Henry *et al.,* 1999) as well as systemic (Buckley *et al.,* 2002) vascular endothelial dysfunction, together with other systemic circulatory disturbances have already been associated with the occurrence and progression of POAG and NTG. Moreover, it has been suggested that a disturbed autonomic activity may play a role in the pathogenesis of the glaucomatous damage (Gherghel *et al.,* 2004*a*; Gherghel *et al.,* 2004*b*). Therefore, a study of the circadian rhythms of oxidative stress markers and endothelial function seems to be important for a better understanding of the pathogenesis of POAG and possible for developing new therapeutic strategies timed to address the diurnal or nocturnal peaks of various risk factors for this disease.

Appendices

Appendix 1: Cardio-Tens report

Appendix 2: Methods of literature search used in Chapter 1

MEDLINE database was systematically searched for articles published until 2004: The search terms used were: ocular blood flow, autonomic nervous system, systemic blood flow and glaucoma, ocular blood flow and antiglaucoma medication, oxidative stress and glaucoma. Additional sources included published books cited in other articles or relevant to the review. Criteria for inclusion or exclusion of articles were originality, importance and new findings regarding ocular and systemic haemodynamics and glaucoma.

Appendix 3: Publication arising from this work

3.1

D. Gherghel, S.L. Hosking, S. Orgul. **Autonomic Nervous System, Circadian Rhythms, and Primary Open-Angle Glaucoma.** Surv Ophthalmol 2004: 49: 491-508

3.2.

D. Gherghel, S.L. Hosking, I.A. Cunliffe. **Abnormal systemic and ocular vascular response to temperature provocation in primary open-angle glaucoma patients: a case for autonomic failure?** IOVS 2004; 45:3546-3554

3.3.

D. Gherghel, H. Griffiths, E.J. Hilton, I.A. Cunliffe, S.L. Hosking. **Systemic reduction in glutathione levels occurs in patients suffering from primary open-angle glaucoma.** IOVS 2004, in press

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