Non-invasive markers of autonomic regulation in response to normal daily activities in health and cardiovascular disease

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Abstract

With the recognition of autonomic nervous dysregulation in the development and progression of cardiovascular diseases, such as hypertension, obesity, sleep apnoea, and heart failure, non-invasive markers of autonomic function have been widely used to predict cardiovascular risks.

Short term recordings (5-10minutes) of ECG and beat-to-beat finger blood pressure are easily accessible, and carry rich information on how the sympathetic and parasympathetic nerve systems modulate the heart and blood vessels. Power spectral analysis of heart rate and blood pressure variability is a well-established tool in evaluating the sympathetic and vagal modulation.

The present study investigated the autonomic responses to normal daily activities using a novel experimental protocol to mimic a common behaviour in western societies – assuming upright posture after eating. The study revealed an enhanced sympathetic activation to orthostatic stress in the postprandial state (within two hours after meal ingestion) with associated pressor and tachycardiac responses in healthy young men. In older subjects, we find a higher basal sympathetic tone, and an attenuated autonomic response to orthostatic stress and meal ingestion; interestingly, meal ingestion induced distinct sympathetic and vagal responses between older men and women, suggesting an additional factor that may increase the risk of cardiovascular diseases in postmenopausal women.

Vascular dysregulation may underlie the pathogenesis of open angle glaucoma, regardless of an increase in intraocular pressure (i.e. high tension glaucoma and normal tension glaucoma). The main study, using the above experimental protocol and approaches, demonstrates for the first time a systemic autonomic dysfunction in both high tension glaucoma and normal tension glaucoma patients, and that the two forms of glaucoma manifest distinct features in autonomic responses. The study provides new evidence that open angle glaucoma may be a systemic cardiovascular autonomic disorder, and glaucomatous visual defects may be an early clinical manifestation of the continuum of multiple target organ damage later in life.

Statement of Candidate

I certify that the work in this thesis entitled “Non-invasive markers of autonomic regulation in response to normal daily activities in health and cardiovascular disease” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I certify that all the experimental studies in the thesis were solely conducted by me, including experimental design, conducting experiments, and data analysis. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by Macquarie University Human Research Ethics Committee; Ethics Reference Numbers: 5201001044 (starting date of approval: 27th Oct, 2010), and 5201100552 (starting date of approval: 31st Aug, 2011).

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Abbreviations

MAP: mean arterial pressure

SBP: systolic blood pressure

DBP: diastolic blood pressure

HR: heart rate

SV: stroke volume

CO: cardiac output

TPR: total peripheral resistance

HRV: heart rate variability

BPV: blood pressure variability

RR: RR interval

LF: low frequency

HF: high frequency

HRV LF power nu: RR interval LF power (RR variance LF component) in normalised unit

HRV HF power nu: RR interval HF power (RR variance HF component) in normalised unit

HRV LF/HF ratio: RR interval LF/HF power (component) ratio

SBP LF power: Systolic blood pressure low frequency power

DBP LF power: Diastolic blood pressure low frequency power

BRS: baroreflex sensitivity

sBRS: sequence method for baroreflex sensitivity estimation

FFT: Fast Fourier Transform

IOP: intraocular pressure

RGC: retinal ganglion cell

POAG: primary open angle glaucoma

NTG: normal tension glaucoma

HTG: high tension glaucoma

BSL: blood sugar level

Contents

Chapter One: Literature review

1. General introduction

Short-term blood pressure fluctuation is mainly regulated by the baroreflex-mediated autonomic nervous system (Cowley Jr *et al.*, 1973; Wieling *et al.*, 2013). The modulation of efferent sympathetic nerve activity and parasympathetic activity may be reflected in the spectral power of blood pressure and heart rate variabilities (Akselrod *et al.*, 1981; Pagani *et al.*, 1986; Pomeranz *et al.*, 1985). The low-frequency spectral power of blood pressure fluctuations may directly reflect sympathetic control to the peripheral vasculature. The low-frequency component of heart rate variability may represent both sympathetic and parasympathetic responses, and high-frequency component of heart rate variability is mostly a marker of cardiac vagal control (Malik, 1996). The ratio of low- and high- frequency components of heart rate variability is commonly recognised as an index of sympathovagal balance, indicating the reciprocal modulation of efferent sympathetic and parasympathetic nerve activities (Malliani *et al.*, 1991). (See Chapter One, Section 4.1 and 4.2 for a detailed discussion.)

Orthostatic stress is a well-established laboratory stimulus in investigating the baroreflex-mediated autonomic function (Montano *et al.*, 1994; Pagani *et al.*, 1986). Postprandial splanchnic hyperaemia also stimulates the baroreflex, and provides a unique approach to examine baroreflex function (Cozzolino *et al.*, 2010; Lipsitz *et al.*, 1993). In addition, carbohydrate ingestion is associated with sympathetic activation (Berne *et al.*, 1989; Young *et al.*, 2010a), particularly in the later (2-3 hour) phase of the postprandial state (Anderson *et al.*, 1991). (See Chapter One, Section 4.3 to 4.7 for a detailed discussion.)

Primary open angle glaucoma is a leading cause of blindness in western societies. Its pathophysiology remains unclear. The signature event of all forms of open angle glaucoma is retinal ganglion cell loss, which is associated with clinical visual field defects (Weinreb *et al.*, 2004). Apart from elevated intraocular pressure, convincing evidence indicates vascular risk factors contribute significantly to the pathogenesis of

glaucoma (Flammer *et al.*, 2007). (See Chapter One, Section 5.2.4 for a detailed discussion)

The instability of ocular blood flow, and not simply a reduction of ocular blood flow, is the main cause of impairment of ocular autoregulation; and may lead to glaucomatous optic neuropathy (Flammer *et al.*, 2002). Retinal circulation proximal to the lamina cribrosa and choroidal circulation are richly innervated by the autonomic nervous system (Pournaras *et al.*, 2008) (See Chapter One, Section 5.2.2 and 5.2.3 for a detailed discussion). It is hypothesised that long-standing systemic autonomic dysfunction may directly or indirectly disturb the ocular autoregulation (Gherghel *et al.*, 2004a) (See Chapter One, Section 5.3.1 for a detailed discussion).

In both high-tension and normal-tension glaucoma, circadian blood pressure monitoring revealed nocturnal blood pressure 'over-dip', which is associated with visual field progression, irrespective of good control of intraocular pressure (Graham *et al.*, 1999; Graham *et al.*, 1995); in response to baroreceptor stimulation, there is impaired baroreflex-mediated autonomic function (Brown *et al.*, 2002) (See Chapter One, Section 5.3.2 for a detailed discussion). Whether or not systemic autonomic dysfunction may underlie the pathogenesis of glaucoma remains to be established. Moreover, it is unknown if there are distinct features of autonomic dysfunction in the two forms of glaucoma.

2. An overview of arterial baroreflex

The arterial baroreflex of the autonomic nervous system contributes importantly to short term cardiovascular hemodynamic perturbations. Beat-to-beat blood pressure fluctuations are instantaneously controlled by the baroreflex negative feedback.

The arterial baroreflex consists of afferent and efferent nerve fibres, and integrative components within the brainstem and spinal cord. During resting and normal physiological conditions, the moment to moment blood pressure fluctuations are sensed by afferent sensory nerve endings in the blood vessel walls of the aortic arch and carotid sinus (McNeill *et al.*, 2010; Wieling *et al.*, 2013). This information is conveyed to the central nervous system within the brainstem (Fan *et al.*, 1998; Paton, 1998) via axons of different sizes (see Chapter One, Section 2.1 below), and then

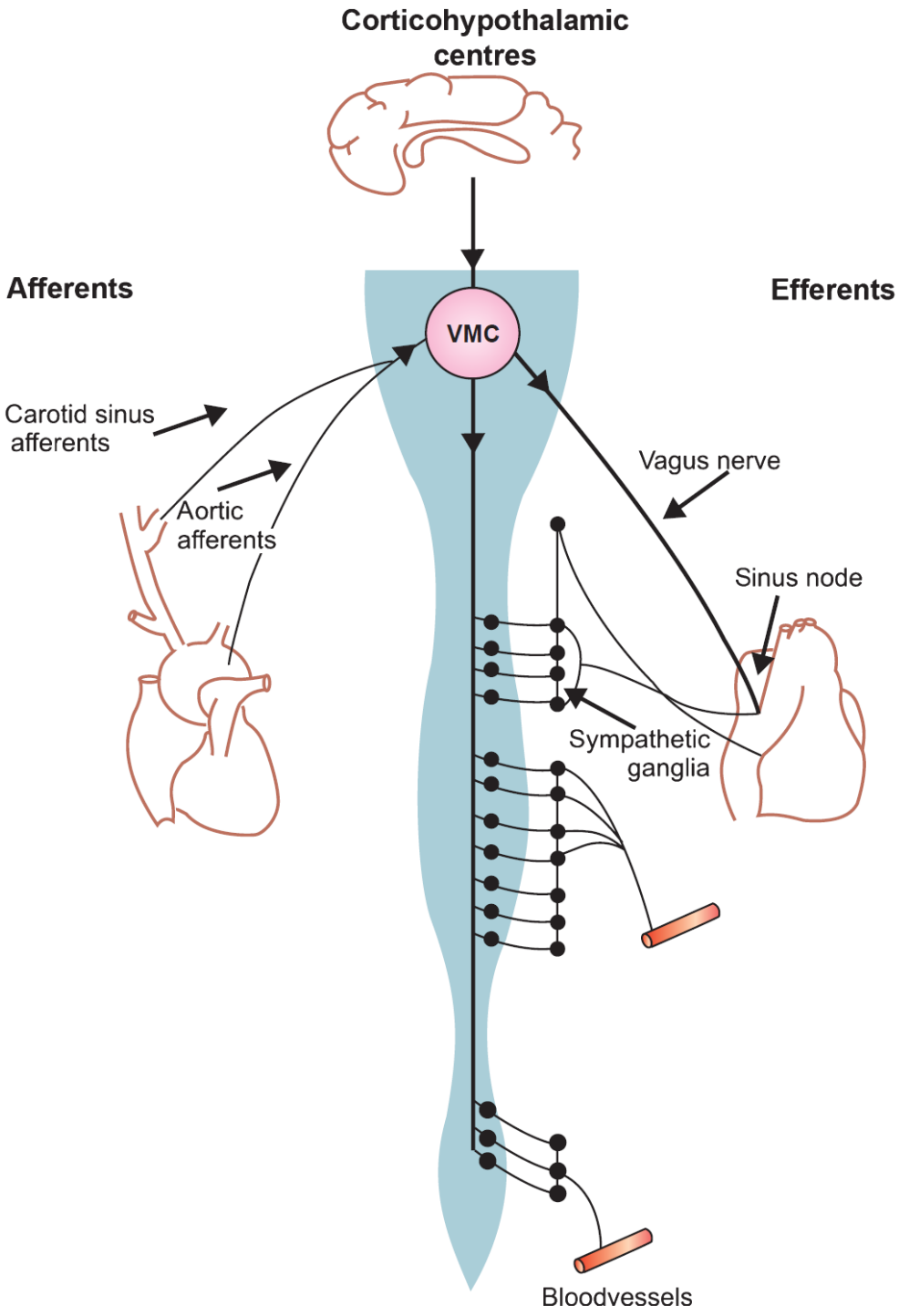
the processed information is delivered via the efferent nerve fibres to the effectors, such as the heart, blood vessels. The cardiovascular efferent pathways comprise two branches: sympathetic and parasympathetic (vagal). The sympathetic nervous system innervates both the heart and blood vessels, whereas the parasympathetic nerves only innervate the heart (Wieling *et al.*, 2013) (Figure1.). In most circumstances, these two nerve branches reciprocally adjust heart rate and contractility, and resistance artery diameter (sympathetic), in order to maintain optimal arterial blood pressure (Malliani *et al.*, 1991).

2.1 Baroreceptors and afferent discharge

Baroreceptors are afferent nerve endings that surround the adventitia of vessel walls of the carotid sinus and aortic arch. Baroreceptors sense arterial pressure by detecting the extent of stretch of vessel walls. With each arterial pressure pulse, the highest rate of firing occurs when pressure is rapidly rising, demonstrating that the arterial baroreceptors are sensitive to the rate of stretch. When arterial pressure is increased, baroreceptor discharge increases; when arterial pressure is decreased, baroreceptor discharge decreases. Importantly, even with the same mean arterial pressure, an increase in pulse pressure (pulse pressure = systolic pressure – diastolic pressure) will increase the baroreceptor discharge (Angell James *et al.*, 1971; James *et al.*, 1970).

Two types of baroreceptors are identified on the basis of their stimulus-response characteristics: 1) type 1 receptors, with larger myelinated fibres (A fibres), are highly sensitive to sudden onset of dynamic changes in arterial pressure above a threshold; 2) type 2 receptors, with predominantly smaller myelinated A fibres and unmyelinated C-fibres, serve to provide input information on tonic or baseline levels of arterial pressure. In dog models, selectively and sequentially anodal blocking of larger A fibres, smaller A fibres and then C-fibres significantly diminishes baroreflex sensitivity without changes in baseline blood pressure; conversely, selectively and sequentially anaesthetic blocking of C- fibres and larger A fibres results in significant elevation in baseline blood pressure with minimal baroreflex downregulation . The coordination of the two types of baroreceptor fibres ensures the most sensitive part of the relationship between mean arterial pressure changes and baroreceptor-

Figure 1. Efferent vagus nerve regulates the sinus node of the heart; efferent sympathetic nerve controls both the heart and blood vessels.
Adapted from Wieling, W., C. T. P. Krediet, et al. J Intern Med. 2013; 273(4): 345-358.



mediated afferent discharges, is around the set point of blood pressure, independent of baseline blood pressure resetting. This is of physiological importance in maintaining circulatory homeostasis (Seagard *et al.*, 1993; Seagard *et al.*, 1990).

2.2 Efferent components of the arterial baroreflex

Arterial baroreceptors send afferent signals into the central nervous system (CNS), mainly within the brainstem (medulla) nuclei, such as solitary tract neurons (Paton, 1998; Pilowsky *et al.*, 2002), which trigger a reflex adjustment to buffer or oppose the changes in blood pressure. The processed information descends from the CNS to the effectors via two branches of efferent autonomic nerves: sympathetic and vagus nerves (Pilowsky *et al.*, 2002). The Efferent sympathetic nervous system regulates cardiac ventricular muscle and vascular smooth muscle, and cardiac sinus node and atrioventricular node. The Efferent parasympathetic nervous system predominantly controls heart beat period (Malpas, 2002; Pilowsky *et al.*, 2002).

At baseline, there is activity in sympathetic nerves providing neurogenic sympathetic tone to the vasculature, and therefore both resistance and capacitance vessels are partially constricted. This is in the normal resting condition (Sundlof *et al.*, 1978; Sundlof *et al.*, 1977). If sympathetic nerve activity increases, the vessels constrict further and arterial pressure will increase. If sympathetic nerve activity decreases, the vessels will dilate and arterial pressure will decrease (Charkoudian *et al.*, 2006; Fu *et al.*, 2004b). With regard to heart rate, the sinoatrial and atrioventricular nodes receive sympathetic and parasympathetic (vagal) innervation, and both efferent outflows are active at rest. Baseline heart rate is determined by the balance between these two opposing influences. In conscious humans, basal parasympathetic nerve activity to the heart is the major determinant of baseline heart rate, whereas basal sympathetic nerve activity has small and negligible effects (Eckberg *et al.*, 1985). Stroke volume is influenced by venous return and cardiac contractility (Fu *et al.*, 2010; Fu *et al.*, 2012; Fu *et al.*, 2005). Increases or decreases in sympathetic nerve activity to the capacitance vessels and the ventricles therefore will affect stroke volume (Charkoudian *et al.*, 2005; Hart *et al.*, 2009a).

3. Arterial baroreflex mediated blood pressure and heart rate fluctuations

3.1 Experimental observation of blood pressure oscillations (Mayer wave)

Under the neural regulation of arterial baroreflex, blood pressure fluctuates periodically. The phenomenon of blood pressure oscillations was recorded by Sigmund Mayer in 1876. In his experiment, pronounced oscillations in blood pressure (amplitude between 15 to 40 mmHg) were observed in rabbits under an anesthetised condition with spontaneous breathing. The frequency of the blood pressure oscillations was about 0.05Hz (approximately 20 seconds per period of oscillation). In 1951, Arthur Guyton et al (Guyton *et al.*, 1951) observed that in dogs under sodium pentobarbital anaesthesia and mechanical ventilation, with haemorrhage of 25% blood volume, there was a fall in mean blood pressure and pronounced blood pressure oscillations (vasomotor waves). In another group of dogs, following sequential stripping of the baroreceptors, in the carotid sinus, or after spinal anaesthesia, blood pressure oscillations were abolished. The average period of spontaneous and sustained vasomotor waves in dogs was found to be 25.2 seconds. Guyton concluded the “vasomotor waves” may be autonomic in origin (Guyton *et al.*, 1951). In 1962, Killip et al found evidence that the Mayer slow waves of blood pressure oscillations in anaesthetised dogs are accompanied by spontaneous *rhythmic* regional vascular resistance and blood flow changes (Killip, 1962). Some recent studies observed that, in haemorrhagic conscious rabbits, the sympathetically mediated blood pressure oscillations (transfer function analysis) was at a frequency of approximately 0.3 Hz during the initial 10minutes compensation (Malpas, 2002; Malpas *et al.*, 2000). This frequency seems much faster than the Mayer wave blood pressure oscillations found in the previous studies, and may be explained by different species and experimental animals in different conscious states.

3.2 Experimental observation of blood pressure fluctuations in humans

The *rhythmic* blood pressure and RR interval oscillations were first identified in 1733 by Stephen Hales. As known, the mechanical cycle of inspiration and expiration is associated with fluctuations in intra-thoracic pressure and venous return (stroke volume), and thus the rise and fall in blood pressure. In 1952, Dornhurst et al

Figure 2. In a resting seated adult subject, the relative arterial blood pressure fluctuates. The upper panels show, with the rise and fall in thorax volume at 15 seconds per cycle, there are associated decreases and increases in blood pressure. Notably, there are also two dominant low frequency components (also 15 seconds per cycle) in the blood pressure waveforms. The lower panels show the low frequency components of blood pressure become *invisible* while respiratory frequency increase to 5 seconds per cycle.
Adapted from Hyndman, B. W., R. I. Kitney, et al. Nature. 1971; 233(5318): 339-341.

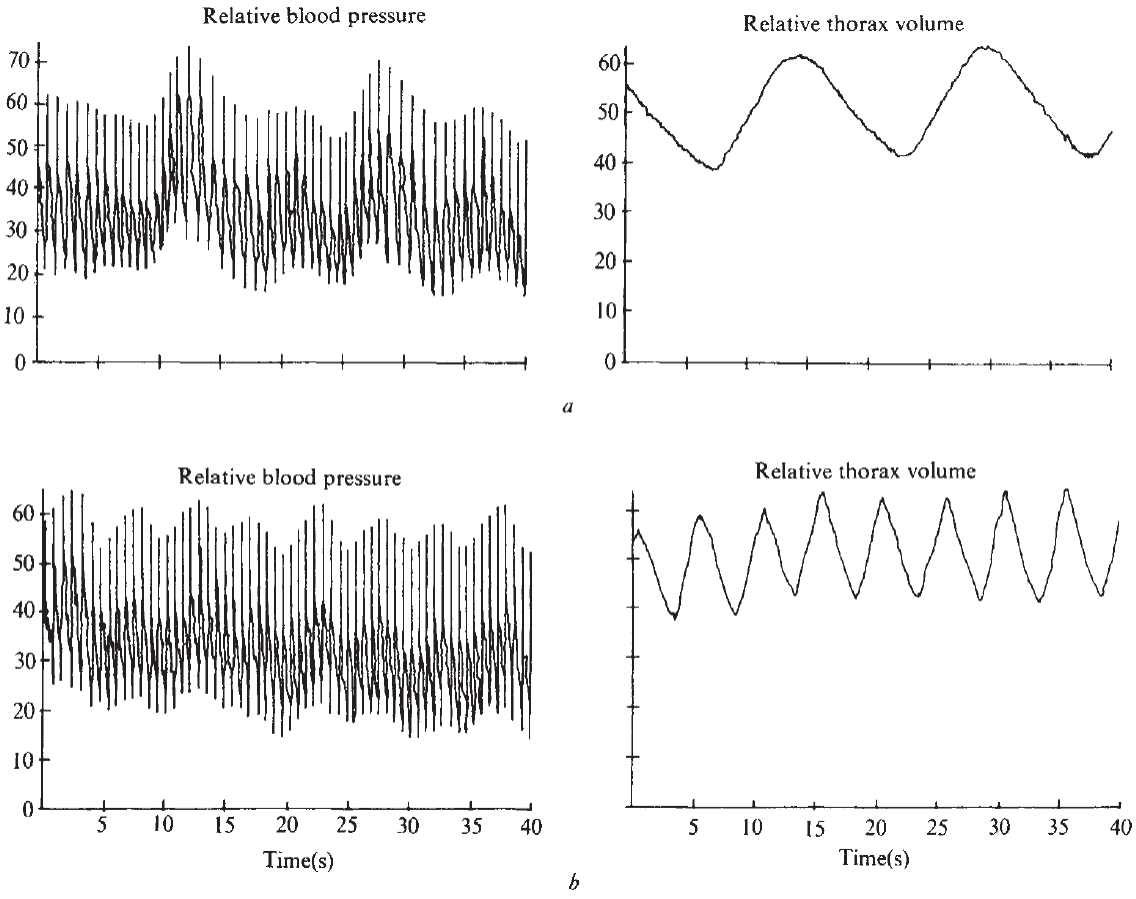
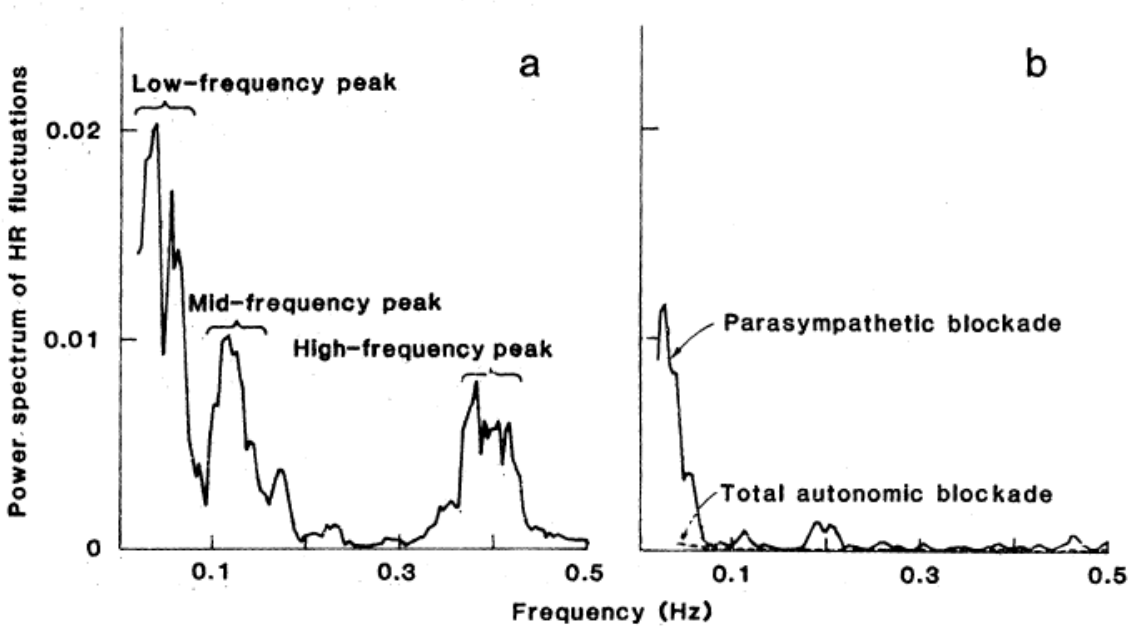


Figure3. Power spectrum analysis decompose beat-to-beat heart rate fluctuations into components at low-, mid-, and high frequency domains. a) a histogram shows the three peaks (spectral powers) of heart rate fluctuations; b) shows parasympathetic blockade minimise both mid- and high- frequency spectral powers. Adapted from Akselrod, S., D. Gordon, et al. Science. 1981; **213**(4504): 220-222.



(Dornhorst *et al.*, 1952) reported that, in conscious humans, in the supine resting state, the most prominent swings in blood pressure (amplitude of the oscillation) is accompanied with cyclical respiration, irrespective of different breathing modes (thoracic or abdominal); a slower respiration rate and erect posture exaggerate the swings in blood pressure (pulse pressure). The researchers also observed that slower wave (6 cycles per minute) blood pressure fluctuations occurred intermittently throughout the rhythmic blood pressure swings described above (Dornhorst *et al.*, 1952). In 1971, Hyndman et al (Hyndman *et al.*, 1971) reported that slow wave blood pressure oscillations can be more readily observed during slower, rather than faster respiration cycles (Figure 2.). They believed that the nonlinear lower frequency (LF) components of blood pressure oscillation is intrinsic in the baroreflex for regulating blood pressure, and is subject to entrainment by the respiratory rhythm. Furthermore, Hyndman et al proposed that, apart from respiratory activity at rest, other physiological challenges may also evoke this baroreflex mediated slow wave blood pressure oscillation. They observed that, when subjects' one hand was immersed into hot (40 °C) and cold (18 °C) water alternatively for 10-20 seconds, without changing core temperature (37 °C), the digital blood flow oscillation was quite different from spontaneous resting oscillation, and the effect was of similar magnitude but longer period stimulus. Therefore, the researchers suggested that the regulation of cutaneous circulation by the thermoregulatory system supports the notion that LF blood pressure oscillation occurs due to changes of peripheral vascular resistance (Hyndman *et al.*, 1971).

3.3 Blood pressure and heart rate fluctuations may reflect efferent autonomic outflow (modulation)

The blood pressure and heart rate may be most available haemodynamic parameters. Blood pressure and heart rate fluctuations were first identified nearly 300 years ago. Until the1970s', following progress in the physiology of cardiovascular regulation and computer technology, researchers recognised that the beat-to-beat fluctuations (variability) reflects cardiovascular neural control (Akselrod *et al.*, 1981).

The beat-to-beat arterial pressure and heart rate tracings can be recorded with computers, and sophisticated mathematical algorithms can be used to subdivide the blood pressure and heart rate fluctuations into different frequency domains (bands). Components (i.e. peaks, distributions, densities, variances) in different frequencies may be an index to characterise the information on rhythmic autonomic neural regulation to the cardiovascular effectors.

In a pioneering physiological study, Chess G F et al (Chess *et al.*, 1975) investigated variations of heart rate using power spectral analysis to understand the efferent neural control to the heart rhythm (pace-making tissue, cardiac sinus node) in 13 decerebrated cats. There were three well-defined peaks (components) in autospectrum in intact control propranolol injection (vagal only) cats, i.e. low (1.5-2.5 cycles/min), medium (6-10 cycles/min) and high frequency (respiratory frequency) domains. Respiratory sinus arrhythmia consistently occurred at the high frequency (HF), and the value of coherence function obtained from heart beat period and respiratory data was consistently greater than 0.8 at the HF. In contrast, after recovery, atropine injection (sympathetic only) significantly reduced the peaks in HF of heart rate variability. Since the low and medium frequency components in heart rate variability were largest for vagal only intact cats (propranolol injection), and were significantly reduced after vagotomy in unconscious cats, the authors claimed that these low and medium components are an intrinsic property of the closed loop vagal control of the heart rate, and that sympathetic activity plays no role in the genesis of heart rate variability (Chess *et al.*, 1975).

However, the Chess et al. study was conducted in anaesthetised unconscious (decerebrated) cats after a significant neurosurgical operation. In 1981, Akselrod S et al. reported their study in 7 trained, unanaesthetised conscious dogs using power spectrum analysis of heart rate fluctuations as an index of cardiovascular regulation. After baseline recordings, each animal underwent multiple pharmacologic interventions. Three peaks (components) of heart rate power spectrum were shown (Figure 3.). Intravenous infusion of glycopyrrolate (parasympathetic blockade) abolished mid- and high- frequency peaks, while the LF peak was reduced. Sympathetic blockade (Propranolol) tends to reduce LF peak’s amplitude, but the

effect was not consistent in each dog. To further investigate the effects of autonomic control on the LF peak, manoeuvres were used to increase efferent tonic control to the heart in the resting dogs. Under the condition of parasympathetic blockade (sympathetic only), vasodilator sodium nitroprusside was infused to reflexly enhance the sympathetic activity, and LF peaks were augmented; under the condition of sympathetic blockade (vagal only), vasoconstrictor methoxamine was infused to reflexly increase parasympathetic activity, LF peaks were also augmented. The authors therefore for the first time concluded that efferent sympathetic and parasympathetic nervous activity make frequency-specific contributions to the heart rate power spectrum (Akselrod *et al.*, 1981).

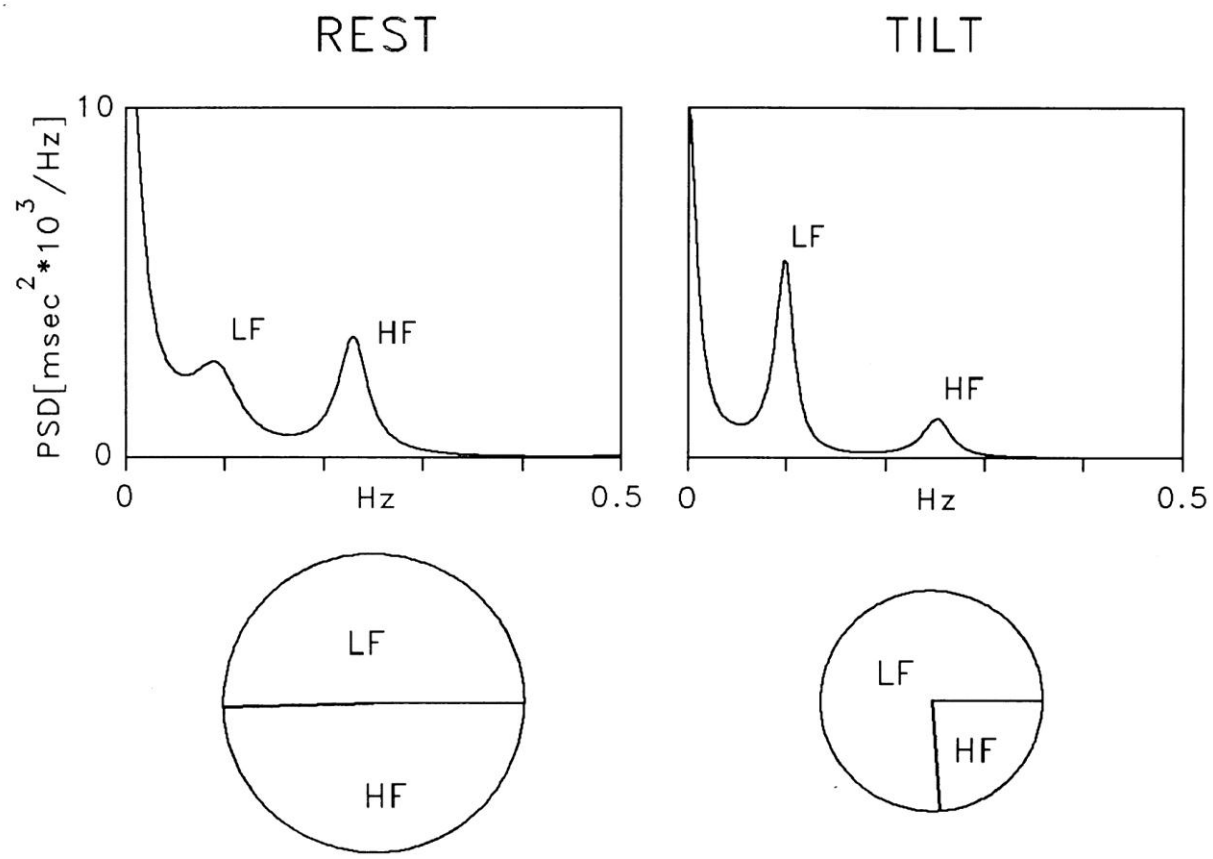
In 1985, Pomeranz B et al, from the same research group of the Akselrod's study at the Harvard University, reported a human experiment in the assessment of autonomic function by heart rate spectral analysis (Pomeranz *et al.*, 1985). In comparison with the pharmacologic intervention used in conscious dogs to increase sympathetic efferent control to the heart to elicit the LF (<0.12 Hz) peaks of heart rate fluctuations (Akselrod *et al.*, 1981), using a postural challenge (from supine to erect posture) is a simple and non-invasive approach in humans. All subjects were under metronome guided breathing. The researchers found that, from supine to standing posture, each subject developed slow wave (about 10 seconds) heart rate oscillations causing a 10 fold increase in the components within the LF band. In response to autonomic blockade, atropine abolished the HF components in both supine and upright positions. In the supine position, atropine (but not propranolol) also reduced LF components by 84%. Importantly, in contrast, in the standing position, atropine only suppressed 72% of LF components, and propranolol contributed to an additional decrease to -89% compared to baseline. Propranolol alone reduced the LF components by 73% in the standing position. These findings indicate the presence of a strong sympathetic influence on LF heart rate fluctuations in standing posture. This is in keeping with the conclusion of Akselrod' study, when baroreceptors are highly engaged (pharmacologic intervention) in conscious animals, power spectrum analysis may be a powerful tool in assessing the efferent *sympathetic* modulation to the heart (Akselrod *et al.*, 1981).

It is worthwhile to address the significant contribution of the Milan research group on the quantitative evaluation of heart rate and blood pressure fluctuations provided by spectral domain analysis. They highlighted the role of quantitative information of blood pressure and heart rate variability that reflects the interaction between efferent sympathetic and parasympathetic regulatory activities (outflows) in most of physiological conditions (Malliani *et al.*, 1991; Pagani *et al.*, 1986).

In order to understand the role of efferent sympathetic and parasympathetic activity on heart rate and blood pressure fluctuations in different frequency domains, Pagani et al (Pagani *et al.*, 1986) performed an experimental study in 57 humans, and achieved more direct findings from 12 conscious dogs. At rest, two clearly separated frequency domain components were seen in the histogram at 0.1 Hz and 0.25 Hz in humans (the study used an autoregressive modelling algorithm but not fast Fourier Transform). During tilting test in humans (90° upright position), the LF component and the ratio of LF and HF components (LF/HF ratio) became largely predominant (Figure 4.). Acute sympathetic receptor blockade (propranolol) significantly reduced LF components and the LF/HF ratio during 90° tilting position, this is consistent with earlier results (Pomeranz *et al.*, 1985). Furthermore, 3 weeks of chronic propranolol administration in healthy subjects demonstrated significantly smaller LF components and LF/HF ratio, and a greater HF component of heart rate fluctuations in the supine resting condition. During tilting, the increase in LF components and LF/HF ratio were markedly attenuated. These findings highlighted the role of efferent sympathetic activity to the LF component of heart rate fluctuations. Another major contribution of the study is that, similar to heart rate fluctuations, blood pressure fluctuations also showed low- and high- components, and there are simultaneous component changes in the heart rate and blood pressure variability from resting to during tilting. More direct evidence for efferent cardiac sympathetic discharge to the cardiac-interval variation was based on the dogs with bilateral stellectomy (excision of stellate ganglion and branches). Before stellectomy, the dogs had an increase in LF component of heart rate variability reflexly mediated by IV nitro-glycerine, whereas after 15 days of postoperative recovery, the conscious dogs showed no response in

Figure 4. The changes of power spectral densities (spectral powers) from rest to tilt. In a young adult subject, there are low frequency (LF) and high frequency (HF) spectral powers at rest shown in a histogram; tilting-up increases the LF power and reduces the HF power; pie charts show from rest to tilt, the ratio of LF/HF increases.

Adapted from Pagani, M., F. Lombardi, et al. Circ Res. 1986; **59**(2): 178-193.



LF heart rate fluctuations, but the response in blood pressure fluctuations was still present.

It is worth noting that in humans, metronome guided breathing exaggerated the HF components of heart rate variability compared to spontaneous breathing (Pomeranz *et al.*, 1985), while in spontaneous breathing, the LF components were more prominent. This suggests respiration (frequency and tidal volume) may influence the contribution of autonomic regulatory activity to the heart rate fluctuations through cyclical variation in intra-thoracic pressure and venous return induced baroreflex engagement, or respiration mediated phasic central neuron firing (changes in PaO_2 or $PaCO_2$) (Katona *et al.*, 1970).

In summary, 1) in humans, although the LF (0.1 Hz) component of heart rate fluctuations may be mediated by both efferent sympathetic and vagal activity, as well as compensatory peripheral vascular resistance, it should not be considered as a simple reflection of neural and peripheral compensatory effects, but rather a useful marker of sympathetic activation; 2) power spectral analysis can also reflect the *reciprocal* changes of instantaneous autonomic neural regulation in most physiological conditions. For example sympathetic activation and vagal inhibition during tilting and sympathetic inhibition and vagal enhancement during metronome guided breathing or normal spontaneous breathing (Pagani *et al.*, 1986). In particular, the LF/HF ratio of RR-interval fluctuations is regarded as a useful marker for reciprocal balance shift between sympathetic and parasympathetic activity (Malliani *et al.*, 1991).

It is believed that, in humans, LF fluctuations in blood pressure and heart rate (RR interval) appear across a broad frequency range, as low as 0.04 Hz and up to 0.15 Hz, although generally close to 0.1 Hz (10 seconds cycle). Apart from the LF fluctuations, there exists a clear HF component (0.15 - 0.4 Hz) in blood pressure and heart rate fluctuations (Akselrod *et al.*, 1981; Hyndman *et al.*, 1971; Malik, 1996; Malliani *et al.*, 1991; Pagani *et al.*, 1986).

3.4 Respiration mediated HF blood pressure and heart rate fluctuations in resting condition

It is widely accepted that the respiratory frequency (HF) component in RR interval fluctuations are primarily mediated by cardiac vagal outflows in both humans and experimental animals (Akselrod *et al.*, 1981; Chess *et al.*, 1975; Malik, 1996; Pagani *et al.*, 1986; Pomeranz *et al.*, 1985). The relatively sluggish sympathetic outflows may not be possible to contribute to the HF fluctuations in heart rate and blood pressure (Malliani *et al.*, 1991).

Elghozi et al (Japundzic *et al.*, 1990) demonstrated that, in conscious rats, atropine inhibited LF and HF heart rate oscillations, atenolol also diminished the LF heart rate oscillations. Interestingly, atropine did not change the SBP fluctuations in both LF and HF bands; in contrast, α-sympathetic blockade (prazosin) increased the HF (respiratory frequency) spectral power and reduced the LF spectral power, indicating that SBP fluctuations may not be predominantly influenced by HR oscillations (unchanged SBP variability to atropine), but is likely to be regulated by the peripheral resistance (α-sympathetic blockade), and thus venous return. Furthermore, the increased SBP fluctuations in respiratory frequency band may indicate a counteracting effect of cardiac adaptation to the change in peripheral resistance. The balance between cardiac output and sympathetic-mediated peripheral vascular resistance has been recently demonstrated in healthy young men (Charkoudian *et al.*, 2005).

At rest, with the inspiration and expiration cycle, there is variation in intra-thoracic pressure and central venous return to the heart (preload). This mechanical respiratory activity will lead to cyclical variation in stroke volume, and thus swings in blood pressure (Dornhorst *et al.*, 1952; Hyndman *et al.*, 1971). The baroreceptors instantaneously sense the fluctuations in blood pressure, which will be reflexly buffered by efferent sympathetic and vagal nerves. Since cardiac sinus node and artrioventricular node are effectors of autonomic nervous control, the cyclical respiratory activity may indirectly alter the heart rate variations through the baroreflex(DeBoer *et al.*, 1987). These baroreflex mediated RR-interval fluctuations

are the determinants of HF spectral power, and modulated by the fast firing of efferent vagus nerve (Eckberg, 1983; Katona *et al.*, 1975; Katona *et al.*, 1970).

Montano et al. demonstrated that in subjects with spontaneous respiration, during both lying and tilting positions at rest, there is high coherence of HF component oscillations between respiration and RR interval (Montano *et al.*, 1994). Bernardi L et al. reported one representative subject's result that there is only a single component in low frequency heart rate oscillation during slow free breathing (but did not record the respiratory rate) (Bernardi *et al.*, 1994). In contrast, controlled breathing (15 breaths per minute) showed low- and high- frequency components of heart rate oscillations. Group results also showed that the RR interval, RR interval variability (SD) and baroreflex sensitivity between spontaneous breathing and controlled breathing did not differ; whereas controlled breathing induced significant decreases in LF nu and ratio of LF/HF, with significant increase in HF nu (Radaelli *et al.*, 1994). Since uncontrolled *slow* breathing may interfere with the calculation of sympathetic outflow while controlled breathing allows discrimination of the two autonomic frequency components, the researchers preferred to choose controlled breathing during the experiment. Pagani et al find the same frequency component values in controlled breathing rates of 20 breaths per minute, and researchers did observe more than just a single low frequency component during spontaneous breathing; thus the researchers recommended spontaneous breathing is more consistent with the normal physiological condition during human experiments (Pagani *et al.*, 1986). Eckberg et al. further studied the influences of breathing rate and pattern on the power spectral analysis of cardiovascular variability, and showed that breathing rate >15 cycles/min reduced both LF and HF spectral power in RR interval fluctuations, and respiratory tidal volume of 1500ml significantly increased HF power over that of 1000ml. The researchers argued that respiration should be controlled when interpreting RR interval power (Brown *et al.*, 1993). Taken together, in practice, either controlled breathing or monitored spontaneous breathing, with the respiratory rate of 12-15 breaths per minute and normal tidal volume, is recommended (Malik, 1996).

3.5 Arterial baroreflex mediated LF oscillations in blood pressure and heart rate

It is now known that the baroreflex mechanism plays a crucial role in buffering short term blood pressure fluctuations. A change in blood pressure is sensed by arterial baroreceptors, and the central nervous system modulates the effectors accordingly, i.e. the cardiac interval, rhythm and contractility by both the fast vagal action and the slower sympathetic action; and the sympathetic mediated peripheral vascular resistance, in an attempt to buffer the initial change in blood pressure.

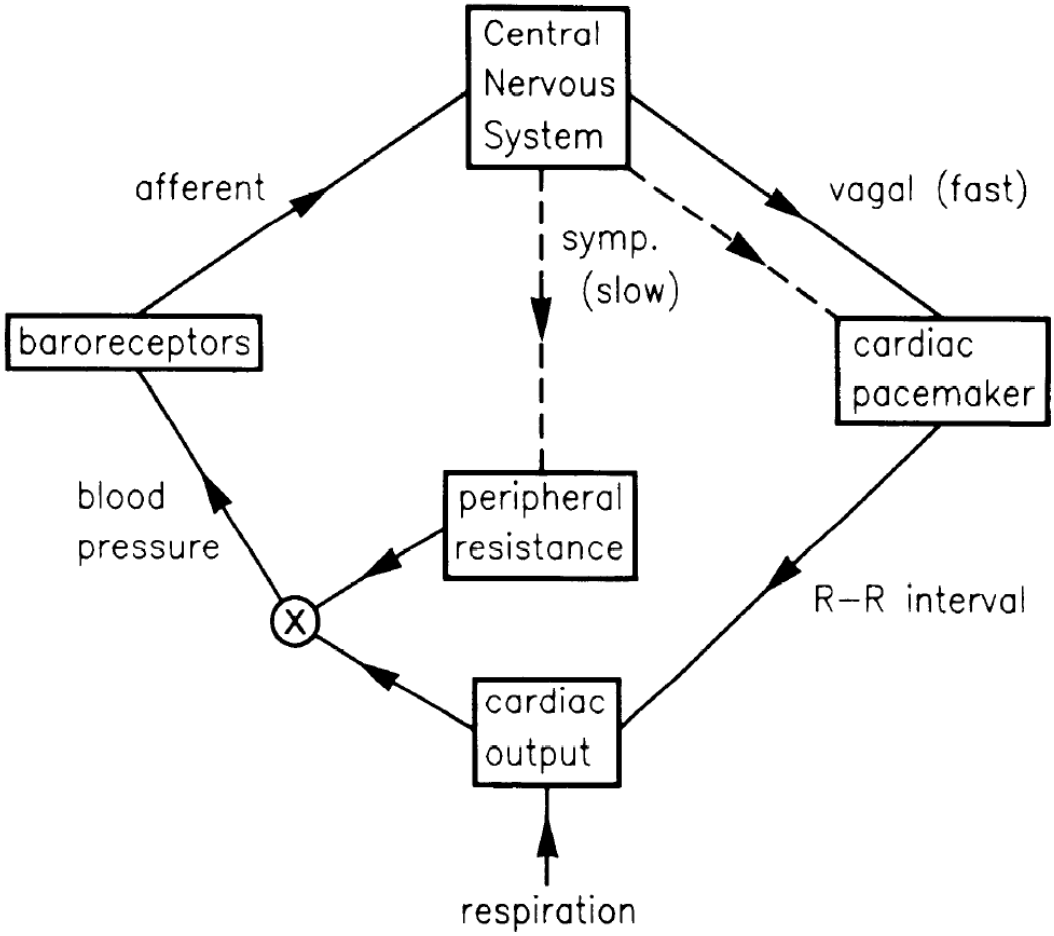
At rest, mechanical respiratory activity mediated blood pressure variations induce cardiac autonomic response via the baroreflex (sensed by baroreceptors).The cardiac interval variations (respiratory sinus arrhythmia) may in turn reflexly buffer blood pressure fluctuations (DeBoer *et al.*, 1987) (Figure 5.). De Boer et al established a mathematical model to interpret the respiration mediated LF oscillations of heart rate at resting condition in humans (DeBoer *et al.*, 1987).

There exists a series of time delays (afferent conduction - CNS interaction - efferent conduction – the response of effectors) between the initial blood pressure change and the baroreflex damped oscillation in blood pressure (Cevese *et al.*, 2001). Bernardi L et al. investigated their hypothesis in humans that, during a 20 second non-respiration period (breath-holding), a 0.6 second applied neck suction may cause a LF fluctuation in RR interval generated by the baroreflex, but is followed by a delayed efferent sympathetic outflow to the vasculature. With the artificial apnoea starting from the end of expiration, followed by the brief neck suction, researchers observed an initial bradycardia and hypotension, followed by arteriole vasoconstriction presumably as a secondary compensation to the initial hypotension. Subsequently a damped LF oscillation in RR interval at 0.1 Hz was detected. This observation demonstrates that, in humans, LF RR-interval fluctuations can be produced from the baroreceptor sensed blood pressure fluctuations (Bernardi *et al.*, 1994).

Saul et al reported that, in humans with resting supine conditions, respiratory sinus arrhythmia is predominantly regulated by the efferent vagus nerve; atropine abolishes these heart rate variations at higher frequencies (>0.1 Hz) (Saul *et al.*,

Figure 5. Schematic diagram of cardiovascular system. Blood pressure affects, through both baroreceptor and central nervous system, cardiac pace-making tissue (sinus node) and peripheral vascular resistance via vagus and sympathetic nerves. Cardiac output is determined by the heart rate (RR-interval lengths). Mathematical stimulation demonstrates that respiration first affects blood pressure may be through mechanical effects (thorax volume and central venous return).

Adapted from DeBoer, R. W., J. M. Karemaker, et al. Am J Physiol - Heart Circ Physiol. 1987; 253 H680-H689.



1991). Their mathematical model (transfer function analysis) also revealed that the respiratory sinus arrhythmia itself may in turn contribute significantly to blood pressure fluctuations. Other physiological experimental studies also demonstrate that the heart rate oscillation may in turn buffer blood pressure oscillation. Toska, K. et al examined the hypothesis that elimination of RR-interval fluctuations by cholinergic blockade increased blood pressure fluctuations in 10 healthy subjects in the supine state. They found that 0.035mg/kg atropine abolished RR interval fluctuations, whereas mechanical respiration-induced variations in stroke volume persisted as before drug administration, and thus cardiac output and mean arterial pressure fluctuations increased (Toska *et al.*, 1993). In their study, the arterial pressures in all subjects increased after cholinergic blockade. In order to avoid the atropine induced hypertensive effect and associated changes in arterial pressure variability, Taylor A J and Eckberg D L (Taylor *et al.*, 1996) prepared a fixed-rate cardiac pacing method (trans-esophageal electrical stimuli) to override normal sinus rhythm, and observed the effects on blood pressure fluctuations in either supine or 40 degree tilting position. Elimination of RR-interval fluctuations did not change the absolute level of blood pressure in both postures, but significantly reduced high (respiratory) frequency blood pressure fluctuations in the supine position, and increased both low- and respiratory- frequency blood pressure fluctuations in tilting position. With the different effects between resting supine and orthostatic states, the authors concluded that RR interval fluctuations are more likely to subserve contributions on buffering the blood pressure fluctuations when mechanical influences are greater with baroreceptors highly activated. Thus, the above studies suggest that respiration mediated RR interval fluctuations may play a role in buffering both the low- and high- frequency oscillations in blood pressure.

However, when baroreceptors are unloaded artificially by using oscillatory lower body negative pressure (LBNP), a highly variable relation between LF oscillations in blood pressure and cardiac interval is revealed (Hamner *et al.*, 2001). 18 healthy young male subjects underwent 10 seconds cycles of oscillatory LBNP at -10mmHg and -30mmHg levels, both LF oscillations in both blood pressure and RR interval were increased. 17 subjects showed significant cross spectral coherence between

these two oscillations during supine resting condition (baseline); in contrast, only 7 subjects maintained significant coherence during oscillatory LBNP. This study implies, in resting supine conditions, RR interval variability is almost entirely accounted for baroreflex feedback in buffering blood pressure fluctuations (Cevese *et al.*, 2001); while during LBNP or isometric exercise, central command is involved, other factors other than simply baroreflex mediated autonomic neural control, e.g. humoral and/or metaboreflex, may participate in generating cardiovascular oscillations and influence buffering effects between these cardiovascular variabilities (RR interval and blood pressure fluctuations) (Cohen *et al.*, 2002; Hamner *et al.*, 2001).

In order to examine the role of baroreflex feedback in buffering blood pressure oscillation, sinoaortic baroreceptor denervation (SAD) was established as a major approach in animal experiments. Guyton and colleagues examined sinoaortic baroreceptor denervation (SAD) dogs in early 1970's (Cowley Jr *et al.*, 1973). The 24 hour blood pressure tracings were recorded from 15 SAD and 12 control dogs. SAD and control dogs had similar systolic/diastolic blood pressure, while mean arterial pressure was 112.7 mmHg in SAD dogs and 101.6 mmHg in normal dogs indicating a modest increase in arterial pressure in SAD dogs. Notably, SAD dogs exhibited markedly unstable, wide blood pressure excursion during resting conditions; in the 24 hour frequency distribution curve for mean arterial pressure, normal dogs showed a narrowly distributed curve, but SAD dogs showed significantly twice as great a distribution. Similar analysis indicated that heart rate is less influenced by baroreceptor deafferentation than is blood pressure. The authors concluded that the main function of baroreflex is not to set the absolute (chronic) level of blood pressure, but to minimise the variation of blood pressure in response to daily challenges. In SAD rats, there is same mean arterial pressure 119 mmHg during continuous recording of arterial pressure whereas increased liability of mean arterial pressure, compared with intact rats (Norman Jr *et al.*, 1981). Similar results were also observed in SAD rabbits (Saito *et al.*, 1986). Recently, researchers argue SAD may not be optimal model to investigate the role of baroreceptor induced long-term blood pressure control (Schreihofer *et al.*, 1992). Thrasher et al (Thrasher, 2002)

developed a carotid baroreceptor unloading dog model, i.e. unilateral ligation to the carotid artery proximal to the sinus, and observed a sustained and significant hypertensive effect. This long term elevation of mean arterial pressure is normalised after the removal of ligature and restoration of carotid flow. Thus, the study suggests chronic baroreceptor unloading may lead to neurogenic hypertension (Thrasher, 2005; Thrasher, 2002).

Cerutti et al. examined the SAD effect on the LF fluctuations in mean arterial pressure and heart rate in conscious rats using power spectral analysis (Cerutti *et al.*, 1994). They found a significant decreased LF power in the SAD rats; a further reduction in LF power achieved by ganglionic blockade (chlorisondamine) was only found in control rats, but not in SAD rats. Transfer function analysis of oscillations in blood pressure and heart rate revealed that coherence was high in both LF and HF bands in control rats; in contrast, in rats without intact baroreflex function, the coherence in LF band was abolished. A similar study also found that, compared to sham rats, the arterial pressure liability in SAD rats is not just an exaggeration of blood pressure fluctuation, as the LF (0.3-0.5 Hz) blood pressure fluctuation only presented in sham rats, but not in SAD rats, suggesting an attenuation of sympathetic mediated blood pressure fluctuations in SAD model (Jacob *et al.*, 1995).

Di Rienzo et al. investigated 9 SAD cats. After 7-10 days of postoperative recovery, the unanaesthetised cats had markedly reduced pulse interval variance in all frequency bands, but accompanied with a significant increase in systolic blood pressure variance, i.e. increase in LF power (0.025-0.07 Hz), a decrease in MF power (0.07-0.14 Hz), and no change in HF power (0.14-0.60 Hz). Although there is reduction in RR interval variability in SAD cats, the increase, but not decrease, in LF power of blood pressure variability is inconsistent with the above experimental results in SAD rats. The authors suggested that removal of baroreflex buffering may cause a complex rearrangement of the blood pressure variability pattern (Di Rienzo *et al.*, 1991)., In conclusion, they propose that other compensatory cardiovascular control, e.g. central neuronal interaction (baroreflex resetting) and /or humoral influence may contribute to the increase in LF power of blood pressure fluctuations. In fact, in the Cerutti et al study, following the complete ganglion blockade in SAD

rats, residual variability is still present in both RR interval and blood pressure, indicating the baroreflex may not be the only determinant of the LF rhythm (Cerutti *et al.*, 1994).

It is of concern that the residual variability in the LF band may be of central sympathetic neural origin, which renders the previous observations not entirely conclusive. In consideration of the limitations of SAD model, for example, incomplete denervation, partial restoration of the baroreceptors and other reflexes may account for the residual variability in Mayer wave band. Bertram D et al. (Bertram *et al.*, 1998) investigated the frequency response of mean arterial pressure to aortic depressor nerve stimulation in anaesthetised and ventilated rats. The aortic nerve was chosen because it contains solely baroreceptor nerve fibres in rat, mouse and rabbit. Since RR interval oscillation tends to oppose the blood pressure oscillation (Cerutti *et al.*, 1994; Di Rienzo *et al.*, 1991), autonomic blockade was performed in all experiments. The researchers found that rhythmic aortic depressor nerve stimulation induced regular oscillations in mean arterial pressure at 0.4 Hz (slow Mayer wave) in rats, and the oscillations were attenuated by $\alpha_2$adrenoceptor blockade and abolished by the sympathetic ganglion blockade (chlorisondamine). In addition, the relationship between the electrical baroreceptor stimulation and LF oscillation in mean arterial pressure was characterised by a strong coherence and phase shift at LF band. The phase shift is mainly due to the presence of a fixed time delay (0.8 second) between aortic depressor nerve stimuli and oscillation in mean arterial pressure. Thus, the authors concluded that the electrical baroreceptor stimulation evoked a LF resonance oscillation in arterial pressure occurring within the baroreflex loop. This study therefore provides more direct evidence that initial blood pressure variations may lead to secondary LF blood pressure oscillations via baroreceptor reflex.

3.6 Central origin in LF oscillations in blood pressure and heart rate

Fernandez de Molina and Perl E R were the first to observe, in spinal cats, rhythmic increases in sympathetic discharge simultaneous to slow arterial pressure oscillations (Fernandez de Molina *et al.*, 1965). In 1974, Preiss and Polosa further examined the relationship between sympathetic central neuronal activity and Mayer waves of blood pressure oscillations in 125 anaesthetised or decerebrated cats. In

their study, Mayer wave was defined as: 1 - 7 cycles/min (average 2.5 cycles/min), blood pressure oscillation amplitude 7-80 mmHg (average 27 mmHg). The most frequent slow waves were those with 2 cycles/min (30 seconds per cycle), followed by 1- 3.5 cycles/min. 13 cats developed Mayer waves spontaneously without any experimental induction. Overall, 78 cats exhibited Mayer waves (62%), most of which were evoked by carotid artery bleeding or common carotid artery occlusion. The most common finding is that during Mayer waves, the firing pattern of sympathetic preganglionic neuronal discharge was characterised by a cyclical alteration of increased and decreased activities (sometimes silence). This firing pattern is obviously different from that during control conditions with spontaneous irregular discharges. In cats with respiration modulation, the firing pattern of sympathetic preganglionic neuronal activity did not alter. In order to remove the influence of baroreflex feedback, baroreceptor deafferentation, a systemic blood pressure stabiliser device, or α_2-adrenoceptor blockade were applied in different experiments under haemorrhage conditions. Irrespective of the disappearance of Mayer waves, the sympathetic preganglionic neuron activity persisted (Preiss *et al.*, 1974). Preiss and Polosa' s (Preiss *et al.*, 1974) study strongly indicates that, under critical conditions, it is the "central oscillator" that generates rhythmic sympathetic neuronal discharge, and contributes to the "slower" Mayer wave of blood pressure oscillations (average 25 seconds per cycle, but not 10 seconds).

Montano et al. recently found that, in decerebrated and ventilated cats, both thoracic sympathetic preganglionic neuronal activity and RR interval fluctuation appeared clearly separated as low- and high- frequency components. The LF (0.1 Hz) RR interval variability is positively correlated to the LF variability of sympathetic preganglionic neuronal discharge. HF oscillation of RR interval and systolic blood pressure is consistent with the higher frequency peak of the ventilation rate (Montano *et al.*, 1992). To further understand whether the LF cardiovascular variability may be of central origin, Montano et al. examined the relationship between cardiovascular neuron impulse activity and systolic blood pressure variability in unanaesthetised, decerebrated cats. They found that cardiovascular neuron impulse activity also exhibited low- and high- frequency oscillations, the same as systolic

blood pressure oscillations. Interestingly, LF (0.12 Hz) arterial pressure variability is positively correlated to LF component of neuron impulse activity of rostral ventrolateral medulla (RVLM); high (respiratory) frequency arterial pressure variability is positively correlated to the HF component of neuron impulse activity of caudal ventrolateral medulla (CVLM) (Montano *et al.*, 1996). The studies of Montano et al. indicate that not only under critical conditions, but also under natural conditions in decerebrated cats, cardiovascular neurons may discharge impulses at low frequency (around 0.1 Hz).

Grasso et al. prepared carotid sinus buffered and vagotomised dogs to detect central sympathetic oscillatory regulation to the vascularly isolated femoral artery. In their study the carotid artery was replaced by perfusion pressure to avoid baroreceptor afferent activation, and additionally cervical vagus nerves were bilaterally sectioned to remove the aortic depressor nerve inflow. The Left femoral artery was tied off and perfused, so that the artery is mechanically uncoupled from the systemic circulation. The left femoral perfusion pressure and iliac blood flow were measured for power spectral analysis. The findings were that the vascularly isolated artery exhibited a similar LF oscillation (0.05 Hz) to the contralateral artery (intact with connection to systemic circulation). Thus, the study suggested the LF oscillations of blood flow (i.e. vascular resistance) in the testing femoral artery may be produced by central sympathetic rhythm generators (Grasso *et al.*, 1995). The authors noted that, although the study cannot exclude the rhythm of autoregulation in the peripheral vasculature or local factors, central origin of sympathetic oscillation (baroreflex-independent) may be the primary source of slow oscillation of circulation.

Legramante J M et al. investigated the feed forward heart period (PI) response to beat-to-beat systolic blood pressure (SBP) changes in anaesthetised ventilated rabbits under minimal influence of peripheral baroreflex buffering by using autonomic blockade. Feed forward non-baroreflex sequence (baroreflex-independent) was defined as more than three consecutive PI changes in the same direction of SBP changes, i.e. hypertensive/tachycardiac or hypotensive/bradycardiac, indicative of an index of central neural regulation to the circulatory system. After complete autonomic blockade (guanethidine+propranolol+atropine), the number of non-baroreflex

sequences dramatically and significantly reduced; selective sympathetic blockade or parasympathetic blockade also significantly reduced the number of non-baroreflex sequences. This result suggests the occurrence of spontaneous non-baroreflex sequences are primarily modulated by the autonomic nervous system, and both branches of the autonomic nervous system take part in this positive feedback mechanism of short-term cardiovascular neural regulation (Legramante *et al.*, 1999).

The rostral ventrolateral medulla (RVLM) is critically important in the generation of sympathetic activity (Guyenet, 2006). A recent experiment in rats investigated the discharge of RVLM neurons that may contribute to the LF sympathetic rhythms. In 7 anaesthetised ventilated rats 51 RVLM neurons were recorded, and 41% showed significant correlation with sympathetic nerve LF rhythm, neurons with sympathoexcitatory properties were major contributors. In contrast, in 4 sinoaortic denervation (SAD) rats, 36 RVLM neurons were recorded, with 36% still correlated with the LF sympathetic nerve rhythm, and >40% were still sympathoexcitatory. The study suggested that, in rats under physiological conditions, RVLM neurons contribute to the LF sympathetic rhythm, and baroreflex plays a role in inducing a participation of more neurons to the genesis of LF sympathetic rhythm (Tseng *et al.*, 2009).

Elghozi et al (Elghozi *et al.*, 1990) investigated the role of central angiotensin in regulating the sympathetic tone while baroreflex is supressed. A very low dose of angiotensin II intracisternally infused in conscious rabbits produced prominent hypertensive and bradycardiac responses, which were inhibited by an angiotensin II antagonist. When the rabbits were sinoaortic denervated, the angiotensin II induced pressor effects became dramatically sensitive. Furthermore, intraspinal 6-hydroxydopamine (6-OHDA) injections given one month earlier did not alter dose-response curves in baroreceptor-intact rabbits. However, the SAD-induced increase in sensitivity to angiotensin II was absent in rabbits with the depletion of spinal noradrenergic (NA) pathways. This study elicits both NA and Non-NA action pathways of angiotensin and in particular highlights the important central role of angiotensin in sympathetic modulation when the baroreflex is supressed (baroreflex-independent).The baroreflex-independent central role of angiotensin II in

cardiovascular sympathetic modulation has been demonstrated in other recent studies (Palma-Rigo *et al.*, 2012; Palma-Rigo *et al.*, 2011).

In humans, the genesis of the LF RR-interval oscillation was studied in spinal cord injury patients with disconnection of high cervical centre (absence of sympathetic outflows to the heart and blood vessels). It was observed that, during the resting supine state, there are LF and HF components of RR interval variability in healthy controls, but the LF component was absent in acute quadriplegic patients (Inoue *et al.*, 1991; Inoue *et al.*, 1990). Guttezzi et al investigated further evidence on the relationship between LF cardiovascular oscillation and rhythmic sympathetic regulation. In 15 neurologically complete quadriplegic patients, LF components of RR interval and blood pressure fluctuations were undetectable or reduced at rest, compared to that of control subjects. After 6 months, in five patients who repeated the experiments, there were marked increases in LF components of both RR interval and blood pressure oscillations. The authors postulated that acute disconnection of sympathetic outflow from the high cervical centre can cause the disappearance of LF cardiovascular oscillation; and the chronic development of LF RR interval variability with concurrent increase in blood pressure variability in quadriplegic patients cannot be solely explained by the baroreflex-cardiovagal motor neuronal regulation, rather the presence of an LF component of cardiovascular variability is likely to be modulated by the sympathetic afferent inputs e.g. somatic or muscle (sympathosympathetic reflex theory) (Guzzetti *et al.*, 1994). However, Koh et al found a similar level of low- and high- frequencies RR interval spectral power between 8 quadriplegic patients and 10 healthy subjects. Strikingly, a vagolytic dose of atropine nearly abolished both the low- and high- frequency of RR interval variability in the two study groups, suggesting a vagal contribution to the LF components of heart rate variability. The authors therefore argued that, since there is a significant presence of LF spectral power in RR interval and arterial pressure in quadriplegic patients, the human Mayer wave may result from mechanisms that do not involve stimulation of spinal sympathetic motoneurones by brainstem neurones; these rhythms reflect in an important way rhythmic firing of vagal cardiac motoneurones (Koh *et al.*, 1994). Notably, in the Guzzetti et al study, researchers

found that, in quadriplegic patients with detectable LF components of heart rate variability, orthostatic stress induced a paradoxical autonomic response, i.e. decreases in LF power and increases in HF power. This seems in keeping with the findings in Koh et al study, that vasoactive drugs (nitroprusside or phenylephrine) mediated blood pressure changes induce significant rise or fall in plasma norepinephrine concentration in healthy control subjects, whereas the plasma norepinephrine level remained unchanged in quadriplegic patients. These findings suggest that, under short term baroreceptor challenge, it is the impairment of sympathetic motor outflow that may underlie the paradoxical responses of heart rate oscillations and blunted plasma norepinephrine response in quadriplegic patients. Although vagal motor neurones may contribute to the LF heart rate oscillations, one cannot exclude the important contribution of sympathetic motor neurones at low frequency domain (Akselrod *et al.*, 1981; Pagani *et al.*, 1986; Pomeranz *et al.*, 1985).

At rest, respiration cyclically changes cardiac venous return and the stroke volume. The variations of stroke volume are directly associated with blood pressure fluctuations. As described above, blood pressure fluctuations interact with RR interval fluctuations via baroreflex loop feedback, termed as secondary resonance (DeBoer *et al.*, 1987; Sleight *et al.*, 1995). In order to demonstrate the presence of central origin in RR interval fluctuations, Cooley et al (Cooley *et al.*, 1998) found a unique experimental model that is minimally influenced by circulatory baroreflex feedback,. In severe heart failure patients during implantation of a left ventricular assist device (LVAD), the cardiac output and blood pressure depend on computerised programming mode, thus removing the respiration induced variations in blood pressure and secondary resonance of LF RR interval oscillations via baroreflex(Cooley *et al.*, 1998). Patients with severe heart failure exhibit low or absence of LF heart rate oscillations (Van De Borne *et al.*, 1997); whereas in patients after one month of LVAD implantation, there exists restoration of a clear and predominant LF power of heart rate oscillation in the naïve heart, with no change of oscillations in blood pressure. Since the presence of predominant LF RR interval oscillation is independent of any blood pressure fluctuations, it is therefore unlikely to be explained by the baroreflex mechanism (Taylor *et al.*, 1996). The researchers

concluded that this restoration of LF components represent a fundamental property of central autonomic outflow (Cooley *et al.*, 1998).

Elghozi J L and colleagues also found that in post heart transplant children, those who were *early* after the operation had little or no LF spectrum power of heart rate variability during resting supine and passive tilting; whereas in a cohort of children who had received heart transplantation relatively long time ago (>44 months), a certain level of LF spectrum power was seen in the supine state, and a significant increase in LF spectrum power was achieved during passive tilting test. Although this is not comparable to that of normal control children, the study indicates a cardiac reinnervation with sympathetic outflow related to the LF cardiovascular variability (Constant *et al.*, 1995). The restoration of sympathetic activity in *late* rather than *early* heart transplantation recipients is also observed using cardiac NE spillover method(Kaye *et al.*, 1993).

4. Baroreceptor unloading induced blood pressure and heart rate oscillations

4.1 Cardiovascular physiology during orthostatic stress

Gravitation induced venous pooling occurs almost immediately when changing from supine to upright posture, and the bulk of blood is transferred to regions below the diaphragm. The increase in hydrostatic pressure in lower limbs is associated with an increase in trans-capillary filtration to the interstitial space, which leads to a decline in plasma volume (Lundvall *et al.*, 1996). The process of both venous pooling and plasma volume loss reaches peak within the first 10 minutes of upright posture. As a result, 1-1.5 litre of blood is transferred to the pelvic region and lower limbs, and 10-15% of plasma volume is accumulated to the interstitial space (El-Sayed *et al.*, 1995; Smit *et al.*, 1999). The direct hemodynamic effect is a reduction in central venous return. According to the Frank-Starling Law of the heart, an increase or decrease in stroke volume occurs in response to the increase or decrease in cardiac ventricular filling volume (end diastolic volume). A fall in stoke volume and cardiac output was observed soon after standing or head-up tilt in normal individuals (Chaudhuri *et al.*, 1992; Wang *et al.*, 1960).

In healthy subjects, following mild lower body negative pressure (-10 mmHg and -15 mmHg), carotid artery diameters were significantly decreased (from baseline 0.66 cm to 0.62 cm and 0.64 cm), associated with increases in forearm vascular resistance and heart rate with stable blood pressure; similar responses were also observed during 30° and 60° head up tilt. An ultrasound geometry study provided direct evidence that orthostatic stress may not only stimulate cardiopulmonary receptors (low pressure baroreceptors), but also cause arterial wall deviation in the carotid sinus area (Lacolley *et al.*, 1992).

When assuming upright posture, the initial short-term adjustments of orthostatic stress are mediated exclusively by neural pathways of the autonomic nervous system. Baroreceptors and cardiopulmonary receptors are rapidly activated in response to a fall in stroke volume and cardiac output, and arterial pressure is maintained stable predominantly by increases in peripheral vascular resistance (total peripheral resistance) and heart rate (Johnson *et al.*, 1974). The peripheral vascular resistance is reflexly mediated by efferent sympathetic nerve activity to the musculoskeletal and splanchnic vascular beds (Abboud *et al.*, 1979; Burke *et al.*, 1977; Chaudhuri *et al.*, 1992; Johnson *et al.*, 1974). The sympathetic mediated vasoconstriction contributes importantly to stabilise arterial pressure in an appropriate proportion with the balanced sympathetic and parasympathetic outflows to the heart in normal individuals (Burke *et al.*, 1977; Fu *et al.*, 2012; Fu *et al.*, 2004b).

Sympathetic microneurography refers to the technique to measure the postganglionic sympathetic neural activity, usually to the muscle and skin. Muscle sympathetic nerve activity (MSNA) is a more direct index in evaluating the central sympathetic outflows to the peripheral vascular beds in humans (Burke *et al.*, 1977; Sundlof *et al.*, 1977). It is observed that sympathetic impulses are normally recorded as groups in pulse-synchronous (heart beat) character with a short pause between burst groups. During a resting supine position, the bursts occurred irregularly without distinct grouping; whereas after assuming upright posture, there are fairly long sequences of bursts separated by silent periods (Burke *et al.*, 1977). To quantify the sympathetic impulse activity, burst incidence is commonly used, i.e. the number of

bursts relative to the number of heart beats (bursts / 100 heart beats). Researchers found that there is wide interpersonal variability between subjects (Burke *et al.*, 1977; Sundlof *et al.*, 1977), but from lying to sitting up and from sitting to standing up, in most cases, sympathetic nerve activity doubled in parallel with an increase in heart rate; also interpersonal variability was reduced (Burke *et al.*, 1977). With 2 subjects where their burst incidence was much higher than in the other 6 subjects in the resting supine state, assuming upright induced paradoxical decrease in burst incidence. This study provides direct evidence that posture change is associated with a marked increase in efferent sympathetic nerve activity, suggesting sympathetic mediated vasoconstriction and a balance shift between sympathetic and parasympathetic outflows to the heart are important compensatory mechanisms during posture change. In parallel with MSNA level findings during posture changes, studies also showed the plasma norepinephrine level were increased after 5 -10 minutes standing up from supine position in normal subjects. Jacob et al investigated the neurohumoral effects and plasma volume changes in 10 healthy subjects after standing (60 minutes). The peak plasma volume fell by 13% (375 ml) and occurred at 14 minutes after standing. There are concomitant increases in plasma norepinephrine/epinephrine at this time point and maintained during the whole 60 minutes standing time. Norepinephrine spillover increased by 80% and clearance decreased by 20% at 30 minutes after standing. Plasma renin, aldosterone and vasopressin, were progressively increased along the time of standing. This study suggests that a fall in plasma volume with associated neurohumoral effects may start at the initial phase of upright posture (Jacob *et al.*, 1998). Compared with patients with diagnosed idiopathic orthostatic hypotension, there were lower level of plasma norepinephrine and dopamine-β-hydroxylase, and failure to increase norepinephrine levels after short-term standing (Ziegler *et al.*, 1977).

4.2 Orthostatic stress induced blood pressure and heart rate oscillations

The power spectral analysis of cardiovascular oscillations provides a non-invasive approach to reflect, in most physiological conditions, the reciprocal responses of efferent sympathetic and parasympathetic control to the cardiac sinus node. In humans, orthostatic stress may be the most “reliable” laboratory stimulus to examine

the reciprocal sympathovagal response based on the evidence of experimental studies (Malliani *et al.*, 1991; Pagani *et al.*, 1986; Pomeranz *et al.*, 1985).

Montano et al investigated the graded tilting effects at 15°, 30°, 45°, 60° and 90° on the correlated sympathovagal balance shift in 22 healthy adults. After resting supine, all subjects were tested randomly for 10 minutes of each tilting angle, with 10min resting supine interval between next tilting angle test. Because of the concern that very low frequency (VLF) components (<0.04 Hz) may overlap and influence the low- and high- frequency components (Akselrod *et al.*, 1981), normalised unit (nu) LF and HF power is introduced and expressed as:

LF nu = LF / (total – VLF), Eqn 1

HF nu = HF / (total – VLF). Eqn 2

The study showed that, following the increase in tilting angle from 30° to 90°, there is a significant reduction in RR interval (increase in heart rate), accompanied with graded increases in LF nu, reductions in HF nu and increases in LF/HF ratio. Linear regression of tilting angle to RR interval LF nu, HF nu and ratio of LF/HF showed high correlations (r = 0.78, - 0.70, 0.68, P<0.001). Except for significant reductions in absolute values of HF power (ms^2) to tilting angle increases (r = -0.41), the absolute values of total variance, LF and VLF powers (ms^2) did not alter. The study concluded that use of a “normalised unit” has the greatest consistency in results with tilting angle changes, suggesting LF nu and HF nu may be better marker in reflecting the sympathovagal balance shift to orthostatic stress, i.e. sympathetic excitation and vagal withdrawal (Montano *et al.*, 1994).

Mukai and Hanayo investigated the changes of heart rate and blood pressure variability to graded tilting in 12 young subjects with controlled frequency rate at 0.25 Hz (15 breaths/minute). RR interval and blood pressure spectral power were expressed as absolute units (ms^2 and $mmHg^2$). During the tilting protocol the angle increased every 4 minutes, RR interval HF spectral power reduced progressively and ratio of LF/HF increased progressively, whereas there was no such progressive change in RR interval LF spectral power (Mukai *et al.*, 1995). Other researchers also reported the similar results in a graded tilting test in healthy subjects (Cooke *et al.*,

1999). The researchers also found that systolic and diastolic blood pressure LF frequency spectral powers increased progressively with tilting angle increase (Cooke *et al.*, 1999; Mukai *et al.*, 1995), suggesting LF blood pressure oscillation is a faithful marker of sympathetic mediated arteriolar responses to orthostatic stress (Pagani *et al.*, 1986). Taken together, in response to orthostatic stress in healthy subjects, reduction in RR interval HF spectral power, increase in ratio of LF/HF, and increase in blood pressure spectral power may be reliable index of sympathovagal balance shift. The changes of RR interval LF spectral power in absolute unit may be of limited value, and normalised unit is recommended to better reflect sympathetic modulation (Montano *et al.*, 1994; Pagani *et al.*, 1986).

To further demonstrate the normalised unit of LF cardiovascular variability may better reflect sympathetic modulation, Pagani et al (Pagani *et al.*, 1997) examined the relationship between LF oscillations of heart rate and blood pressure and LF MSNA variability within physiological blood pressure changes in 8 normal subjects under free spontaneous breathing. LF MSNA variability (arbitrary unit squared) is calculated by power spectral analysis of a neurogram derived from the integration of continuous MSNA signal (time × amplitude); each integral window is defined as the MSNA signal between two consecutive diastolic blood pressures. The amplitude, but not burst, of MSNA is positively correlated to the LF MSNA variability and its normalised unit. A graded infusion of nitroprusside induced progressive decreases in blood pressure and cardiac interval; similarly, a graded infusion of phenylephrine induced increases in blood pressure and cardiac interval. The systolic blood pressure changes were inversely correlated to both burst frequency and amplitude of MSNA; in particular, the increase and decrease in systolic blood pressure were greatly correlated to the LF MSNA in normalised unit. Finally, LF RR interval spectral power in normalised unit, ratio of LF/HF, and LF systolic blood pressure spectral power ($mmHg^2$) were all tightly positively correlated to amplitude of MSNA and LF MSNA oscillation in normalised units. The strong link between the oscillatory components of spinal sympathetic motoneurone activity and cardiovascular parameters confirms the physiological potential that LF RR variability in normalised units is a useful marker in interpreting sympathetic efferent control. The frequency

components of heart rate and blood pressure variability were also demonstrated to characterise the oscillatory pattern of sympathetic neural discharge during orthostatic stress in healthy subjects (Furlan *et al.*, 2000). From supine resting to a slowly increasing tilting angle of 75° tilt, then maintained for 30minutes, central venous pressure reduced from 4.4 to – 4.7 mmHg, and heart rate increased, accompanied with increases in MSNA as bursts/min and burst incidence (bursts/100 heart beats), and plasma norepinephrine and epinephrine. Power spectral analysis showed significant increases in LF components and decreases in HF components of MSNA and RR interval in normalised units, as well as increased ratio of LF/HF in MSNA and RR interval variability. This study confirmed the consistency of LF RR interval variability in normalised units in characterising the sympathetic outflow, and suggesting it is a reliable marker indicative of sympathetic regulation during orthostatic stress.

Mounting experimental evidence demonstrated that LF oscillation of blood pressure in absolute units ($mmHg^2$) is consistently increased during upright tilting (total blood pressure variance does not decrease during sympathetic excitation). There is no doubt LF component of blood pressure variability may be a reliable marker of sympathetic vasomotor modulation (Cooke *et al.*, 1999; Furlan *et al.*, 2000; Pagani *et al.*, 1986). HF blood pressure variation is influenced by not only cardiovagal effects to the vasculature via baroreflex, but also mechanical respiration mediated direct cardiac output effect on the vasculature, so HF component of blood pressure variability cannot faithfully reflect vagal outflow (DeBoer *et al.*, 1987).

It is widely accepted that LF/HF ratio reflects sympathovagal balance shift as a marker of autonomic function in humans. The autonomic nervous system plays a crucial role in short term circulatory haemostasis. In the resting quiet state, the circulation is stabilised mainly by the baroreflex buffering. In response to daily stressors, greater central neuron interaction is engaged. The autonomic neural regulation of cardiovascular function is mainly determined, in its efferent side, by sympathetic and vagal mechanisms.

In most physiological conditions, the organisation of efferent regulatory activity is characterised as reciprocal, for example sympathetic excitation and vagal inhibition

or vice versa. Although a co-activation of the two branches may occur in a few circumstances (Paton *et al.*, 2005), sympathovagal balance shift is a synergic mechanism that contributes to the performance of the cardiovascular effectors (Montano *et al.*, 1994; Pagani *et al.*, 1986).

Blood pressure and heart rate are the most accessible parameters. To explore the frequency domain of blood pressure and heart rate oscillations can reflect the function of central neural regulation from resting to the excitatory state. When changing from a resting basal state to a certain physiological condition / behaviour, sympathovagal balance shift provides a quantitative window to evaluate an individual's cardiovascular regulatory function.

4.3 Cardiovascular response to meal ingestion - splanchnic hyperaemia

The stomach and intestines are highly vascularised organs, and receive a substantial proportion of blood distribution of cardiac output at rest (Parks *et al.*, 1985). The coeliac artery (CA) and superior mesenteric artery (SMA) are major arteries that directly branch from the abdominal aorta, and play an important role in the blood supply to splanchnic organs, such as the stomach and small intestine. Following meal ingestion, the splanchnic artery blood flow significantly increases and peaks within an hour (Someya *et al.*, 2008). It is demonstrated that, in the postprandial state, the increase in splanchnic blood distribution, i.e. splanchnic hyperaemia is consistently associated with sustained increases in cardiac output, stroke volume and heart rate in healthy subjects (Sidery *et al.*, 1994; Waaler *et al.*, 1991).

Since the 1980's, sensitive Duplex Doppler ultrasound became extensively used, and splanchnic blood flow can be measured in a non-invasive approach. For example, the SMA blood flow can be calculated by parameters of blood velocity and artery diameter. Sidery et al (Sidery *et al.*, 1991) tested meal composition effect on the SMA blood flow in young healthy subjects and found that, after a meal with the same energy content of 2.5 MJ (≈400kcal), a high carbohydrate meal induced a rapid rise in SMA blood flow and a peak by 87% at 15 minute; whereas after a high fat meal, SMA blood flow increased and reached a peak of 122% at 45 minutes.

Central circulation markers, i.e. heart rate and cardiac output exhibited gradual increases over time after meals, but meal composition did not induce different peak time of heart rate and cardiac output. Qamar et al., (Qamar *et al.*, 1988) also reported after a 400kcal meal (a similar meal size of the above Sidery et al. study), that although the maximal response of SMA blood flow did not differ between meal compositions, the time that SMA blood flow reaches maximal magnitude was significantly different. The peak time of carbohydrate meal was at 15 minutes, high fat was at 30 minutes and high protein meal at 45 minutes.

Previous observations found that postprandial splanchnic hyperaemia is mainly due to the increase in splanchnic artery blood velocity, and there is no change in artery diameter (Sieber *et al.*, 1991). A recent study also demonstrated that in young healthy subjects after a mixed meal, the SMA blood flow doubled at 40 minutes (Someya *et al.*, 2008). This doubling, accompanied a marked increase in mean blood velocity of SMA at the same time postprandially, whereas there was no change in SMA diameters (Someya *et al.*, 2008).

Meal size may be a determinant of the magnitude of splanchnic blood flow. Sidery et al (Sidery *et al.*, 1994) reported a linear correlation between the meal size (1 MJ, 2 MJ, and 3 MJ) and the changes in SMA blood flow. The 3 MJ meal induced significantly greater SMA blood flow than the 1 MJ meal. According to a review of 11 previous studies of postprandial response in SMA blood flow, it is further summarised that a significant correlation exists between energy content in calories and maximal response (magnitude) in SMA blood flow (% of baseline) (Someya *et al.*, 2008).

Intra-duodenal meal perfusion induced a similar increase in the magnitude of SMA blood flow compared to oral liquid food intake with the same meal size and composition, suggesting small intestinal nutrient exposure (intestinal phase) may play a major role in splanchnic hyperaemia (Sieber *et al.*, 1992). A recent study showed that stimulation of brain higher centres via perception (thinking about food, smell, sight of food, i.e. the cerebral phase) may be responsible for the initial increase in CA and SMA blood flow (within 10 minutes). It was found that the marked postprandial increase in SMA blood flow and peak at 40 minutes may be mainly due

to gastric emptying and chyme reaching the small intestine (Someya *et al.*, 2008) and neurohumoral afferent stimulation (Sieber *et al.*, 1991). Furthermore, Gentilcore et al (Gentilcore *et al.*, 2008a) investigated the intra-duodenal nutrient infusion induced changes in SMA blood flow and systemic circulatory response (blood pressure) in healthy older subjects, and found that, compared to intra-duodenal saline infusion, direct small intestinal exposure of glucose, protein and fat all induced a rise in SMA blood flow and fall in systolic blood pressure. The intra-duodenal glucose infusion induced hypotensive effect occurred at 18 minutes, and was significantly earlier than that of protein (33 minutes) and fat (46 minutes), suggesting a carbohydrate rich meal may induce a more rapid haemodynamic challenge to the arterial baroreflex of the autonomic nervous system.

4.4 Baroreflex activation in buffering postprandial blood pressure

Meal ingestion induces splanchnic hyperaemia and is invariably associated with a reduction in total peripheral resistance (Fagan *et al.*, 1986; Waaler *et al.*, 1991). Cardiac output and heart rate are found to be increased after a meal, and therefore, postprandial blood pressure maintained as stable in healthy subjects (Kelbaek *et al.*, 1987; Kelbaek *et al.*, 1989; Sidery *et al.*, 1994; Waaler *et al.*, 1991). The Arterial baroreflex may be the most important mechanism in buffering postprandial haemodynamic changes and stabilising blood pressure (Kelbaek *et al.*, 1987). Failure of this compensatory mechanism may lead to postprandial hypotension (Jansen *et al.*, 1995).

Kelbaek et al (Kelbaek *et al.*, 1987) studied the postprandial hemodynamic changes in six healthy subjects at rest and during exercise with or without autonomic blockade of the heart. Radionuclide cardiography was used to measure left ventricular ejection fraction (LVEF) and end-diastolic volume (EDV), and calculate stroke volume (stroke volume=EDV×LVEF). The researchers found that, after a 6300kJ mixed meal, during the resting condition, cardiac output was enhanced by 61% due primarily to a rise in stroke volume and end-diastolic dilation; treatment with combined metoprolol and atropine blocked the increase in stroke volume but did not affect the heart rate. During exercise, postprandial cardiac output increased 23% with accompanying increases in stroke volume and heart rate; both the increase in

stroke volume and heart rate were inhibited by autonomic blockade, except for the end diastolic dilation. This study suggests postprandial haemodynamic changes are largely under the influence of the autonomic nervous system.

In healthy subjects, increased plasma norepinephrine concentration, but not epinephrine, after food intake was consistently observed in several studies (Berne *et al.*, 1989; Kelbaek *et al.*, 1987; Lipsitz *et al.*, 1993; Mathias *et al.*, 1989; Paolisso *et al.*, 1997; Vaz *et al.*, 1995b), suggesting food intake may not involve an sympatho-adrenal activation at least early postprandially, and that other regional sympathetic postganglionic signalling may be important in counteracting postprandial haemodynamic changes. In older people the postprandial increase in norepinephrine was found to be lower than young healthy subjects, with an associated postprandial fall in blood pressure (Lipsitz *et al.*, 1986; Lipsitz *et al.*, 1993), suggesting postprandial hypotension may be due to a defective sympathetic nervous system activation after a meal.

Isotope dilution methodology provides information about regional sympathetic nerve activation. Esler and coworkers used isotope dilution methodology to measure food intake induced norepinephrine spillover from sympathetic-sensitive organs into the bloodstream, i.e. the rate of appearance of norepinephrine into the plasma from individual organs (Kingwell *et al.*, 1994). In 9 healthy lean subjects, after a large mixed liquid meal (2.64-3.51MJ), whole body norepinephrine spillover rose rapidly from a baseline 3.5 nmol/min to 5.5 nmol/min of peak level at 30 minutes, and returned to baseline level at 90 minutes; renal norepinephrine spillover rate more than doubled from 0.440 nmol/min to 0.937 nmol/min, whereas the hepatomesenteric and cardiac norepinephrine spillover rate only marginally increased or remained unaltered between 60-90 minutes after the meal. In contrast, water ingestion did not change any of the above parameters in two subjects who repeated the protocol. In addition, skeletomuscular sympathetic nerve activity also peaked at 60 minutes after the meal (Cox *et al.*, 1995). This is consistent with the other studies in this research group (Vaz *et al.*, 1995a; Vaz *et al.*, 1995b). Vaz et al also found in young healthy subjects, the whole body norepinephrine clearance rate increased early postprandially and this may even underestimate the measurement of

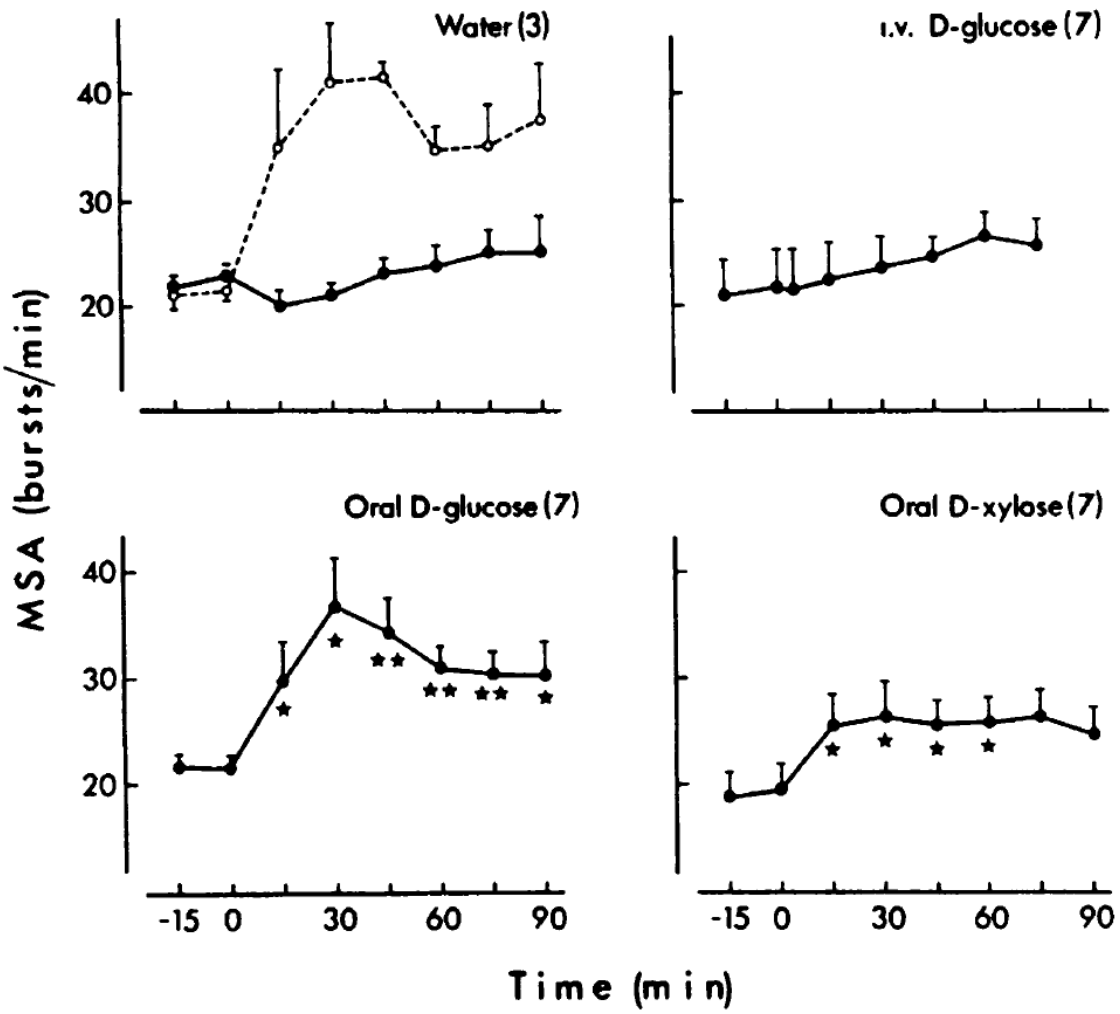
increases in arterial norepinephrine concentration and whole body plasma norepinephrine level (Vaz *et al.*, 1995a). Furthermore, since forearm and kidney norepinephrine spillover markedly increased, the researchers indicated that skeletal muscle and kidney, but not the heart, are two major regional sites of postprandial sympathetic nervous activation (Cox *et al.*, 1995; Vaz *et al.*, 1995a).

It is reported that after meal ingestion, both normal subjects and patients with primary autonomic failure showed superior mesenteric blood flow increase and vascular resistance decrease. However, a reduction in forearm blood flow and a rise in forearm vascular resistance only occurred in normal subjects, but not in autonomic failure patients. This was accompanied with a greater postprandial increase in heart rate and stroke volume in normal subjects than in patients and may explain the postprandial fall in mean arterial pressure observed in patients. This study supports the idea that a baroreflex mediated vasoconstrictor effect in the forearm may be important in preventing a fall in blood pressure (Kooner *et al.*, 1989).

Berne et al (Berne *et al.*, 1989) first demonstrated that oral carbohydrate ingestion was followed by early and sustained increase in MSNA. In healthy non-obese subjects, oral ingestion of 100g of D-glucose in 300 ml water and 75.8g of D-xylose in 300ml water with similar osmolality were followed by a rapid increase in MSNA (bursts/min) and peaked at 30 minutes (Figure 6.). The amplitude of MSNA also increased after both of the carbohydrate ingestion. The increased number of bursts and amplitude (strength) of MSNA were accompanied with plasma noradrenaline increase. In contrast, 300ml water ingestion and intravenous D-glucose did not induce any changes in MSNA and plasma noradrenaline, except a minor increased plasma noradrenaline after water drinking. Since meal ingestion is associated with postprandial splanchnic hyperaemia, and arterial baroreflex may play an essential role in redistributing blood supply and maintaining short-term blood pressure, the researchers suggested that the carbohydrate ingestion associated increase in sympathetic efferent traffic may be attributable to arterial baroreflex regulation (Berne *et al.*, 1989). Fagius and Berne (Fagius *et al.*, 1994) further studied the features of MSNA following oral ingestion of different types of meals. 39 young healthy subjects were randomly allocated into different experimental meal ingestion

Figure 6. The left upper diagram shows in three representative subjects, oral D-glucose (dotted line) causes significant increase in MSA in comparison to water ingestion. In experiments of seven subjects, oral D-glucose (left lower diagram) induces a significantly greater increase in MSA than i.v. D-glucose (right upper) and oral D-xylose (right lower). MSA: Muscle sympathetic nerve activity.

Adapted from Berne, C., J. Fagius, et al. J Clin Invest. 1989; **84**(5): 1403-1409.



groups, i.e. 100g of glucose in 300ml water (n=8), 75.8g of fat in 250ml water (n=8), 100g of lean meat (40g of protein) in 250ml water, and 300ml water only (n=7), and a mixed meal of 1750 kJ energy (n=8). MSNA (bursts/min) significantly increased following all meals (except for water alone), and were consistently sustained at a high level until 90 minutes after meal ingestion. Blood pressure and heart rate increased and remained stable in all meal ingestion groups except water ingestion. It was observed that MSNA increase followed all types of meals, in the absence or minimal increase in serum insulin level after fat or protein meals. This study also indicates that, in healthy young subjects, there is baroreflex-induced postprandial enhancement in sympathetic outflow to the peripheral vasculature, which may be important in regulating the postprandial blood redistribution and maintaining blood pressure (Fagius *et al.*, 1994).

It is well documented that humans who have a higher basal MSNA level are likely to exhibit an attenuated response in MSNA to postural stress resulting in orthostatic intolerance (Burke *et al.*, 1977; Sundlof *et al.*, 1977). The phenomenon of utilisation of MSNA reserve in the resting condition renders a less efficient activation of sympathetic nervous system, so called “ceiling effect” (Fu *et al.*, 2004b). Fagius et al (Fagius *et al.*, 1996) investigated carbohydrate meal ingestion effects on MSNA and blood pressure in young healthy, older subjects and older subjects with insulin resistance. Carbohydrate ingestion induced a profound increase in MSNA and stable blood pressure in healthy young subjects. In contrast, older subjects exhibited an attenuated postprandial response in MSNA because of relatively higher basal MSNA; in those older subjects with insulin resistance, basal MSNA was highest and postprandial MSNA response was weakest. Notably, in older subjects with or without insulin resistance, the blunted postprandial MSNA was accompanied with a significant fall in blood pressure. The researchers thus indicate that it is due to the restricted postprandial MSNA response after carbohydrate ingestion that causes this postprandial hypotension (Fagius *et al.*, 1996).

4.5 Gastrovascular reflex mediated increase in sympathetic efferent nerve activity

Gastric distension may induce a gastric vascular reflex mediated increase in MSNA. Rossi et al (Rossi *et al.*, 1998) first used a insertion of gastric balloon linked to a barostat device that stimulated the mechanoreceptors of the stomach wall in young healthy subjects and demonstrated concomitant and nearly proportional increases in the MSNA and blood pressure in response to graded increase in gastric distension. Similarly to the aged heart and carotid sinus, the mechanoreceptor in the stomach wall may also become less sensitive to stomach stretching following normal ageing. It is demonstrated that, with higher baseline MSNA compared to young healthy subjects, a gastric balloon induced an attenuated increase in MSNA in elderly subjects (van Orshoven *et al.*, 2004). Researchers indicate the gastrovascular reflex may be a fast-acting neural pathway inducing peripheral vasoconstriction and compensates the splanchnic blood pooling (Rossi *et al.*, 1998; van Orshoven *et al.*, 2004). This gastrovascular reflex is claimed to be also important in maintaining blood pressure because it is reported that intraduodenal glucose infusion, i.e. bypassing oral glucose ingestion, evoked similar MSNA responses in young and older subjects (Van Orshoven *et al.*, 2008).

Jordan et al provided the first observation that, in patients with autonomic failure or in healthy older subjects of similar age, within the first 30 minutes after drinking 480ml tap water systolic blood pressure acutely increased 43mmHg or 11mmHg respectively; the pressor response was associated with an increase in plasma norepinephrine level in control subjects, but not in autonomic failure patients (Jordan *et al.*, 1999). A further study in the same research group found that after 480ml tap water-drinking there was a pressor effect in older subjects, and profoundly increased blood pressure in autonomic failure patients; moreover, in autonomic failure patients, the 480ml water induced a greater rise in blood pressure than that of 240ml water. The increases in plasma norepinephrine level were found in both young and older healthy subjects, but not in autonomic failure patients. Plasma volume, renin activity and plasma vasopressin concentration did not differ before and after drinking water in all study groups. Although the mechanism of the pressor effect after drinking water

remains unclear, this study suggests drinking water may be associated with sympathetic activation in normal older subjects; one cannot exclude that drinking a half litre of water may stretch mechanical receptors of gastric wall, and is associated with the activation of gastro-vascular reflex in normal older subjects. The greater blood pressure excursion in autonomic failure patients may reflect an impaired or absent cardiovascular autonomic control (Jordan *et al.*, 2000). The therapeutic potential of drinking water has also been studied (Ando *et al.*, 2009). After 35 minutes drinking 480ml tap water, the pressor effect of water drinking successfully prevented the fall in systolic and diastolic blood pressure in the first minute of standing up; also drinking water immediately prior to a meal significantly attenuated postprandial fall in blood pressure in patients with autonomic failure (either orthostatic hypotension or postprandial hypotension) (Shannon *et al.*, 2002). Thus, studies indicate that drinking water may be “protective” for people with impaired cardiovascular autonomic control.

Gentilcore et al. (Jones *et al.*, 2005), studied gastric distension may attenuate postprandial fall in blood pressure in healthy older subjects. In a study, eight healthy older subjects were given glucose solutions of different concentration and meal volume i.e. 25g-200ml (12.5%), 25g-600ml (4%), 75g-200ml (37.5%) and 75g-600ml (12.5%). Regardless of glucose concentration, the high volume solutions (600ml) induced an increase in blood pressure, and systolic blood pressure is positively correlated to the stomach volume. At the same concentration (12.5%), 600ml solution induced a lower fall in systolic blood pressure than that of 200ml; whereas at the same volume, glucose concentration did provoke different level of changes in blood pressure. This study suggests that the higher volume of glucose ingestion may attenuate the initial postprandial fall in BP healthy elderly subjects, whereas the different glucose concentration of 4% and 37.5% may not affect blood pressure differently. In a recent study, Gentilcore et al (Gentilcore *et al.*, 2008b) also found that systolic and diastolic blood pressure was significantly lower during intra-duodenal infusion of glucose solution (50g glucose in 300ml saline) than that of intra-duodenal infusion of the same glucose solution together with a preceding 500ml water intra-gastric infusion. This study directly confirmed the “protective” role of

gastric distension by drinking water in preventing a fall in blood pressure in healthy older subjects.

The pressor effect of gastric distension in healthy older subjects was further studied by this research group. In eight healthy older subjects, 75g glucose in 300ml saline was orally ingested and gastric emptying rate was calculated for each individual subject in day one. At day two, the same solution was given intraduodenally at a similar gastric emptying rate was detected for each subject. In constrast, following oral ingestion of 300ml glucose solution SMA blood flow, systolic blood pressure was greater and heart rate was slower at day one than after intraduodenal ingestion on day two. The study demonstrates that, at a similar rate of small intestinal exposure to glucose, gastric distension mediated increase in sympathetic outflow to muscle vasculature may be associated with a postprandial “protective” pressor effect in healthy elderly (Gentilcore *et al.*, 2009).

4.6 Sympathetic response after carbohydrate ingestion and central action of insulin

Food intake is controlled by the central nervous system (Schwartz *et al.*, 2000). Multiple gastrointestinal hormones are released following food intake and act as central neurotransmitters. Their role of digestive hormones in cardiovascular regulation has been reviewed in recent years (Sartor *et al.*, 2008; Smith *et al.*, 2012). Of these, the most studied may be central neural action of insulin, i.e. plasma insulin transports across the blood-brain barrier and centrally excites the sympathetic nervous system (Muniyappa *et al.*, 2007; Scherrer *et al.*, 1997).

After carbohydrate ingestion, glucose exposure in the small intestine is associated with immediate insulin secretion and production from the pancreas islet cells. Plasma insulin acts as a potent peripheral vasodilatory hormone to facilitate the process of glucose uptake (Baron, 1994; Baron *et al.*, 1995). Impairment of insulin mediated vasodilation has been found in subjects with insulin resistance (Laakso *et al.*, 1990). The pancreas is the only source of plasma insulin (Banks, 2004). Insulin may be transported across the blood-brain barrier (Banks, 2004) or directly access the neurons within the arcuate nucleus via highly permeable capillaries (Ciofi, 2011). In

self-counteracting this vasodilatory effect (Brooks, 2010), it is demonstrated that brain insulin may increase sympathetic efferent nerve activity to the peripheral vasculature (Anderson *et al.*, 1991; Bardgett *et al.*, 2010; Berne *et al.*, 1989; Muntzel *et al.*, 1994; Spraul *et al.*, 1994; Straznicky *et al.*, 2009; Ward *et al.*, 2011) and enhance arterial sympathetic baroreflex gain in animals (Cassaglia *et al.*, 2011; Pricher *et al.*, 2008) and humans (Young *et al.*, 2010a; Young *et al.*, 2010b).

In 1977, Young and Landsberg reported that, in Sprague-Dawley rats, the norepinephrine turnover rate in the heart (calculated by the half-time disappearance of ^{3}H norepinephrine) increased significantly over 48 hours fasting, and the norepinephrine turnover rate was higher than that of normal control fed rats. After 48 hours fasting, refeeding reduced the turnover rate. In addition, the effect of inhibition of norepinephrine turnover in the heart by using rate-limiting enzyme (α-methylparatyrosine) was present in fed and refed rats but absent in fasting rats. The two independent methods demonstrated that fasting is associated with a supressed sympathetic nervous system and refeeding may reverse this effect (Young *et al.*, 1977b). With the same method, the researchers also reported that voluntary overfeeding with sucrose in the rats for 3 days is associated with increased sympathetic activity in the heart (Young *et al.*, 1977a). Furthermore, the researchers found that gold thioglucose, a chemical compound that destroys the ventromedial hypothalamus, abolished the effect of increased ^{3}H norepinephrine turnover rate in the heart in fasting rats. Therefore, the researchers provide evidence that dietary regulation may be centrally controlled, and indicate that ventromedial hypothalamus may be responsible for suppressive effects of the sympathetic nervous system in fasting state (Young *et al.*, 1980).

In 1981, Young and Landsberg investigated the role of insulin mediated sympathoexcitatory response in humans (Rowe *et al.*, 1981). The researchers designed hyperinsulinaemic-euglycaemic clamp studies using different insulin infusion rates of 2mU/kg/min and 5mU/kg/min, and a hyperglycaemia clamp study (20% glucose infusion and plasma glucose level maintained at 125mg/dl). The euglycaemic and hyperglycaemic studies were conducted in each of 12 healthy young and non-obese subjects on different days. 5 subjects were additionally

clamped with saline and served as control. Plasma norepinephrine level was regarded as marker of sympathetic postganglionic response in this study. Not surprisingly, the high insulin infusion (5mU/kg/min) euglycaemic clamp achieved maximal and steady rise in insulin level over the 2 hours of testing period, followed by the low insulin infusion (2mU/kg/min) euglycaemic clamp and hyperglycaemic clamp. Saline infusion did not change plasma insulin level. Plasma norepinephrine concentration was markedly increased and exhibited a gradual rise from 30 minutes until 120 minutes in both of the euglyaemic clamp studies; the higher insulin infusion group achieved a consistently higher norepinephrine level at all the time points. With minimal or no increase in either the hyperglycaemic clamp study or saline control group, no change in plasma norepinephrine concentration was observed. This study highlights that it is the hyperinsulinaemia, but not high blood glucose (the increase in insulin level was not sufficiently high in the hyperglycaemic clamp group, i.e. from baseline 5.8 μU/mL to 40 μU/mL), that may directly contribute to sympathetic postganglionic activity (Rowe *et al.*, 1981).

This hypothesis was confirmed by the following studies in determining the link of postprandial hyperinsulinaemia and MSNA in healthy humans (Berne *et al.*, 1989). After 100g oral D-glucose ingestion dissolved in 300ml water, serum insulin level maintained at a high level of approximate 40mU/L throughout the testing period, whereas oral D-xylose ingestion only elicited a significantly slight increase in serum insulin level (approximate 10mU/L). Accordingly, MSNA and plasma norepinephrine were markedly increased after D-glucose ingestion; in contrast, after D-xylose ingestion, the increases in MSNA and plasma norepinephrine were less intense. Interestingly, there is a positive correlation between postprandial MSNA and serum insulin level only found after D-glucose ingestion. Blood pressure and heart rate remained stable and postprandial blood glucose level was higher than baseline in both groups. Thus, the researchers demonstrate the role of insulin underlying the mechanism of postprandial MSNA increase, and emphasise that the source of sympathetic response after these two types of carbohydrate ingestion may be quite different (Berne *et al.*, 1989). In a following study, Fagius and Berne further confirmed that glucose meal induced higher insulin level and greater MSNA increase

than mixed meal, protein meal and fat meal in healthy young subjects (Fagius *et al.*, 1994).

To elucidate the role of hyperinsulinaemia per se, rather than insulin induced carbohydrate oxidation as a cause of sympathetic neural activation, Vollenweider et al (Vollenweider *et al.*, 1993) investigated 6 lean healthy subjects with average age of 28 years. Subjects underwent three experimental protocols. Hyperinsulinaemic-euglycaemic clamp was applied in the first protocol, i.e. subjects underwent insulin infusion at a rate of 6pmol/kg/min with 20% glucose infusion at an infusion rate according to the blood sugar levels measured every 5min, so as to maintain euglycaemia. The second protocol was a 20% glucose infusion at a rate equivalent to the clamp, without exogenous insulin infusion. Fructose infusion with a rate similar to glucose infusion was given as a third protocol. MSNA and calf blood flow/vascular resistance were measured and carbohydrate oxidation was calculated. In the clamp group, with marked increase in plasma insulin from baseline 42 pmol/L to 402 pmol/L and 376 pmol/L at 60 and 120min time points, MSNA (bursts/min) significantly increased at 60min and doubled at 120min. Compared to baseline, there was a physiological increase in insulin level during glucose infusion (140 pmol/L and 282 pmol/L), which is associated with modest and significant increase in MSNA at 120min. Fructose only elicited a slight and significant increase in insulin level without MSNA increase compared to baseline. Muscle sympathetic neural response in the clamp group was significantly greater than those in both glucose and fructose infusion groups; similarly, insulin mediated peripheral vasodilatory effect was observed only in the clamp group. The skeletomuscular vasodilation and sympathetic vasoconstrictor effect produced by hyperinsulinaemic-euglycaemic clamp are entirely in accordance with the previous studies (Anderson *et al.*, 1991). Notably, in this study, while carbohydrate oxidation was higher than baseline levels, it was comparable amongst all three experimental protocols. In addition, blood pressure remained stable, heart rate increased to nutrient intake, and no hypoglycaemia was recorded in the study. Glucose infusion elicited a high blood sugar level compared to baseline, whereas the clamp and fructose infusion protocols maintained normal blood sugar level. Based on these convincing observations, the

researchers conclude that hyperinsulinaemia may be directly associated with the increase in sympathetic efferent outflow (Vollenweider *et al.*, 1993).

More recently, the specific sites of the central effects of insulin have been identified by Brooks and coworkers(Cassaglia *et al.*, 2011; Pricher *et al.*, 2008). A Hyperinsulinaemic-euglycaemic clamp was used to increase lumbar sympathetic nerve activity (LSNA) and arterial baroreflex gain of LSNA in experimental Sprague-Dawley rats. Bilateral microinjection of muscimol to either the paraventricular nucleus of the hypothalamus (PVN) or the arcuate nucleus (ArcN) inhibited this sympathetic response. Importantly, microinjection of insulin into the PVN did not elicit an increase in LSNA, but microinjection insulin into the ArcN increased LSNA and the arterial baroreflex gain of LSNA. Histology study confirmed the electrophysiology study. This study demonstrated that ArcN within hypothalamus is the site insulin acts on to initiate the central sympathetic neural excitation (Cassaglia *et al.*, 2011).

Since the above animal studies show that insulin augments not only sympathetic nerve activity, but also resets arterial sympathetic baroreflex gain, Young et al (Young *et al.*, 2010a), for the first time, demonstrated that insulin enhances the gain of arterial baroreflex control of MSNA in humans. In this study, the weighted linear regression analysis between MSNA and diastolic blood pressure was used to determine the baroreflex gain. A liquid mixed meal (57% carbohydrate) was used in 12 healthy young male subjects, and hyperinsulinaemic-euglycaemic clamp was achieved with the comparable level of postprandial plasma insulin level in 8 matched subjects. A subset of 4 subjects served as time control. With similarly increased insulin levels in both mixed meal and the clamp groups, but not in time control group, the arterial baroreflex gain of MSNA (both MSNA bursts incidence gain and total MSNA gain) significantly enhanced compared to baseline, and the mixed meal group and clamp group showed a comparable level of the baroreflex gain from 30min until 120min (Young *et al.*, 2010a). Thus, this study may establish a hypothesis that physiological increases of plasma insulin in the postprandial state may enhance the sensitivity of arterial baroreflex control of sympathetic nerve activity in healthy subjects, suggesting a central baroreflex resetting (Guyenet, 2006).

In summary, the above studies suggest that in humans, after carbohydrate ingestion, the direct central neural action of insulin may contribute to postprandial circulatory haemostasis, in harmony with a baroreflex-mediated autonomic response (Guyenet, 2006; Scherrer *et al.*, 1997).

4.7 Changes in blood pressure and heart rate fluctuations to meal ingestion

Postprandial hypotension was first identified in patients with autonomic failure, an average reduction in systolic blood pressure of 49±6 mmHg was found after a meal (Robertson *et al.*, 1981; Seyer-Hansen, 1977). The meal ingestion induced depressor response becomes widely recognised from 1980's when the condition was reported in a group of nursing home elderly persons with a decline of 24-25mmHg in systolic blood pressure after a meal (Lipsitz *et al.*, 1983).

Postprandial hypotension is frequently absent of clinical symptoms, such as presyncope or syncope. It is thought that the cerebral symptoms may occur when the reduction in mean arterial pressure exceeds the adaptive cerebral autoregulation. Currently, postprandial hypotension is defined as a decrease in systolic blood pressure 20mmHg or more within the start of a meal, but the more appropriate value of definition for postprandial hypotension remains unclear (Jansen *et al.*, 1995).

In elderly persons, postprandial hypotension may cause subclinically compromised cerebral perfusion and lead to insidious detrimental consequences. In 10 institutionalised elderly subjects, blood pressure declined between 30 and 55 minutes after a 400kcal mixed meal, and was accompanied with an increase in cerebral arteriolar resistance detected by transcranial Ultrasound Doppler, suggesting a reduction in cerebral perfusion (Krajewski *et al.*, 1993). It is also reported that a 78 years old man developed repetitive transient ischaemic attacks after almost every meal, with marked fluctuation of systolic blood pressure from 110 mmHg to 200 mmHg. Angiography revealed an occlusion of his left carotid artery and stenosis of his right middle cerebral artery. This observation suggests postprandial hypotension may be a risk factor in triggering cerebral ischaemia(Kamata *et al.*, 1994). It is also demonstrated that postprandial

hypotension is associated with asymptomatic cerebrovascular damage in essential hypertensive patients (Kohara *et al.*, 1999).

Many factors may predispose to the onset of postprandial hypotension. Of these, the impaired sympathetic-mediated vasoconstrictor effect has been demonstrated as an important detrimental factor. As a distinct entity that differs from orthostatic hypotension, the pathophysiology of postprandial hypotension remains to be elucidated (Jansen *et al.*, 1995).

Compared with conventional blood pressure and heart rate measurement, continuous record blood pressure and heart rate is a non-invasive and applicable approach and provides rich information on beat-to-beat blood pressure and RR interval fluctuations (Malik, 1996). Power spectral analysis of blood pressure and heart rate reflects cardiovascular autonomic modulation and has shed light on understanding the pathophysiology of postprandial hypotension (Jansen *et al.*, 1995).

In 1990, Hayano et al carried out a study to examine the autonomic response to diurnal changes in healthy young subjects, and all the study subjects remained in a laboratory in a physiological steady state over 16 hours throughout the experiment. The cardiac interval fluctuations were calculated using an autoregressive model (but not Fast Fourier Transform). The researchers reported that after each standard meal, there exists a reduction in HF component of RR interval fluctuations at 30min and an increase in LF component of RR interval fluctuations at 90 min. This implies that meal ingestion may be associated with significant cardiac sympathetic activation and parasympathetic withdrawal in daily life (Hayano *et al.*, 1990).

Lipsitz and coworkers pioneered to examine meal ingestion induced changes in cardiovascular parameters (Lipsitz *et al.*, 1983) and variabilities in normal ageing (Ryan *et al.*, 1992). Ryan et al reproduced the fall in blood pressure in institutionalised elderly subjects after an ordinary breakfast. Increases in LF component of RR interval oscillations (arbitrary units) and LF/HF ratio to the meal occurred only in control young subjects; compared with young subjects, elderly subjects manifested attenuated RR interval LF oscillations, suggesting a defective baroreflex mediated compensation in the elderly, which may underlie the onset of postprandial hypotension (Ryan *et al.*, 1992). In a study of postprandial

haemodynamic and autonomic responses in healthy young and older subjects as well as dysautonomia patients with postprandial hypotension, in response to a 400kcal liquid mixed meal, splanchnic blood pooling increased, heart rate increased, while total peripheral resistance and blood pressure remained stable (Lipsitz *et al.*, 1993).

In healthy young subjects, LF RR interval spectral power (arbitrary units) was modestly but significantly increased, suggesting a cardiac sympathoexcitation in buffering blood pressure. On the other hand, in healthy elderly, forearm vascular resistance and cardiac index (ml/min/m^2) were increased with associated increases in plasma norepinephrine, suggesting a sympathetic vasoconstrictor effect in the postprandial state. In contrast, dysautonomia patients manifested a large decline in blood pressure after the meal corresponding with a fall in systemic vascular resistance and lack of increase in plasma norepinephrine level. Forearm vascular resistance and cardiac interval LF spectral power did not increase, suggesting a failure of cardiac and vascular sympathetic activation (Lipsitz *et al.*, 1993). This study provides convincing evidence on the role of autonomic nervous system in maintaining circulatory haemostasis in the postprandial state.

We reviewed all relevant original articles in examining meal ingestion induced blood pressure and heart rate fluctuations and summarised in Table 1.

Key words used for searching all the published and relevant studies about meal ingestion induced cardiovascular variability are: ((meal ingestion) or postprandial) and (heart rate variability)

According to the studies reviewed, we suggest that:

Following a meal, an increase in LF component of RR interval fluctuation (ms^2) (Hayano *et al.*, 1990; Tentolouris *et al.*, 2003) and a decrease in HF component of RR interval fluctuation (ms^2) (Hayano *et al.*, 1990; Lu *et al.*, 1999; Oberman *et al.*, 2000; Sauder *et al.*, 2012; Tentolouris *et al.*, 2003) were observed in healthy young subjects. Lipsitz and coworkers calculated percentage changes of LF and HF components of RR interval fluctuations, i.e. normalised units (expressed as arbitrary units in their studies) and showed that RR interval LF fluctuations in arbitrary units

Table 1. Meal ingestion induced changes in blood pressure and heart rate variability

	Subjects Profile	Meal			Method	Responses to meal ingestion						Group comparison for meal response
		calories	pattern	composition		LF_{RR} (mms^2)	LF_{RR} nu	HF_{RR} (mms^2)	HF_{RR} nu	Ratio of LF/HF	SBP LF power ($mmHg^2$)	
Hayano etal, 1990	8 healthy young males, 23-25yrs	680 kcal	Semi-liquid	Carbohydrate 110g, Fat 16g, Protein 25g	Autoregressive model	↑ at 90min		↓ at 30min				Nil
Ryan etal, 1992	7 young (24yrs) and 13 institutionalised elderly (89yrs)		Semi-liquid	112g of milk, 1-2 slices of toast with margarine, 140-196g of orange juice, 1 cup of decaffeinated coffee	FFT		↑ at 30-50min in young subjects		↔ in either young or elderly subjects	↑ in young subjects		The responses of LF_{RR} nu and LF/HF ratio attenuated in elderly than young subjects
Lipsitz etal, 1993	11 young healthy (26yrs), 9 healthy elderly (80yrs), 10 dysautonomic patients (65yrs)	400 kcal	liquid	40% carbohydrate, fat 45%, 12% protein	FFT		↑ at 80min in young subjects		↔ in all groups			The responses of LF_{RR} nu, HF_{RR} nu attenuated in elderly and dysautonomia patients
Vaz etal, 1995	Young healthy subjects	3.15MJ (752 kcal)	liquid	53% carbohydrate, 32% fat and 15% protein		↔		↔		↔	↑	Nil
Paolisso etal 1997	17 healthy normotensive subjects (31yrs)	300 kcal	liquid	OGTT, 75g glucose	FFT					↑ at 60min		Nil
Imai etal, 1998	20 elderly subjects (70yrs), divided into with or without postprandial hypotension groups	400 Kcal	400 ml liquid	66.7 g of carbohydrate, 8.9 g of fat, and 13.3 g of protein	FFT			↔ in both groups		↔ in both groups	↑ in those who without postprandial	Sympathetic vasomotor (SBP LF power) response

											hypotension	attenuated in postprandial hypotension subjects
Lu etal, 1999	9 healthy volunteers (31yrs)	500 kcal	turkey sandwich	50.1% carbohydrate, 32.4% fat, 17.5% protein	FFT		↔		↓ at 30min and 60min	↑ at 30min and 60min		Nil
Oberman etal, 2000	89 eligible subjects entered the study: young (n=30, 20-39yrs), middle aged (n=27, 45-59yrs), elderly (n=32 , 60+yrs)	425 kcal	Semi-liquid	Carbohydrate 78.1g, Fat 2.8g, Protein 20.7g	FFT			↓ at 30min and 60min in all three groups			↔ in all three groups at 30 and 60min	No difference between young and elderly subjects
Dionne etal, 2002	14 young healthy subjects (21-22yrs); two sessions: breathing control (n=11) or spontaneous breathing (n=14)	700 kcal	Semi-liquid	55% carbohydrate, 30% fat, 15% protein	FFT	↔ in both testing sessions		↔ in both testing sessions		↔ in both testing sessions		No difference in controlled or spontaneous breathing
Kawaguchi etal, 2002	20 young healthy subjects (25yrs) and 20 healthy elderly subjects (78yrs)	700 kcal	solid	Carbohydrate 120g, Fat 23g, Protein 30g	FFT	↔ in either young or elderly subjects		↔ in either young or elderly subjects		↑ at 30, 60 and 90min in elderly subjects		Elderly exhibited sympathetic hyperresponsiveness to meal, suggesting noradrenaline down-regulation
Tentolouris etal, 2003	15 lean (35yrs) and 15 obese (37yrs) healthy women	546 kcal carbohydrate meal; or 532 kcal fat meal	Semi-liquid	Carbohydrate rich meal: Carbohydrate 130g, Fat 0.26g, Protein 6.1g Fat rich meal: carbohydrate 5g, Fat 52g, Protein	FFT	↑ in lean women after carbohydrate rich meal through		↓ in lean women after carbohydrate rich meal throughout 3 hours		↑ in lean women after carbohydrate rich meal through		Greater response of $InLF_{RR}$, $InHF_{RR}$ and LF/HF ratio in lean women than obese

				11g		out 3 hours				out 3 hours		women after carbohydrate rich meal
Masuda etal, 2003	10 healthy control (22-57yrs) and 16 elderly (67-92yrs) subjects; the elderly subjects were divided into with or without postprandial hypotension (PPH)	600 kcal	Lunch	Not specified	Maxium Entrop spectral analysis		↑ in elder subjects early after eating					Within 60min after eating, LF_{RR} nu greater in elderly without PPH than younger control and elderly with PPH.
Ambarish etal, 2005	15 healthy subjects (29yrs)		Lunch meal		FFT		↔ for 2 hours		↔ for 2 hours	↔ for 2 hours		Nil
Millis etal, 2009	6 young healthy African-American subjects (18-20yrs)	900 kcal	liquid	High carbohydrate drink: pure fruit juice; High fat drink: 23% carbohydrate, 67% fat, 10% protein						↑ in both carbohydrate drink and high fat drink		Nil
Cozzolino etal, 2010	15 healthy controls (25.5yrs) and 15 type 1 diabetic patients (24.5yrs)	500 kcal	Semi-liquid	50% carbohydrate, 20% fat, 30% protein	Autoregressive model	↑ in control subjects at 20min after meal	↑ in control subjects at 20min after meal	↓ in control subjects at 20min after meal	↓ in control subjects at 20min after meal	↑ in control subjects at 20min after meal		The responses of LF_{RR} nu, HF_{RR} nu and LF/HF ratio were attenuated in diabetic patients; exogenous insulin did not reverse it.

Sauder etal, 2012	20 healthy young subjects (20-31yrs)	826-855 kcal	Semi-liquid	Two types of high fat meals and a low fat control (carbohydrate rich) meal	Autoregressive model				↓ in all types of meals			No difference in three types of meals
Purtell etal, 2013	10 Prader-Willi syndrome (PWS) patients, 11 matched healthy obese subjects, 9 healthy lean subjects	60 kcal	Semi-liquid	50% carbohydrate, 35% fat, 15% protein	FFT	↔ in all three groups		↔ in all three groups		↔ in all three groups		Attenuated LF_{RR} response in PWS patients compared to the matched healthy obese subjects.

were increased in response to a mixed meal in healthy young control subjects (Lipsitz *et al.*, 1993; Ryan *et al.*, 1992). Cozzolino et al, using an autoregressive model (a more complicated mathematical approach, discussed in the Method Chapter) demonstrated the increases in LF peak of RR interval oscillations and decreases in HF of RR interval oscillations in both absolute and normalised units after a mixed meal in healthy control subjects of their study (Cozzolino *et al.*, 2010).

Conversely, unchanged RR interval fluctuations after a meal were also observed in healthy subjects by several lines of studies (Ambarish *et al.*, 2005; Dionne *et al.*, 2002; Kawaguchi *et al.*, 2002; Purtell *et al.*, 2013; Vaz *et al.*, 1995b). The unchanged RR interval fluctuations may be in keeping with the finding that norepinephrine spillover from the heart was unaltered within 60min after a large meal, although renal and whole body norepinephrine spillover levels were increased (Cox *et al.*, 1995). Researchers argue that LF component of RR interval fluctuations may not be a direct cardiac sympathetic index, but a measure of modulation of cardiac autonomic outflows by baroreflex (Goldstein *et al.*, 2011).

The LF/HF ratio of RR interval fluctuations may be a more reliable marker representing sympathovagal balance (Malliani *et al.*, 1991). In most physiological circumstances, LF/HF ratio may reflect a reciprocal sympathetic activation and vagal inhibition (Montano *et al.*, 1994; Pagani *et al.*, 1986; Pagani *et al.*, 1997). In healthy subjects, when changes in RR interval fluctuation spectral power observed in the postprandial state, LF/HF ratio was found consistently increased in several lines of studies, suggesting meal ingestion is likely to be associated with cardiac sympathovagal balance shifting towards sympathetic dominance (Cozzolino *et al.*, 2010; Lu *et al.*, 1999; Millis *et al.*, 2009; Paolisso *et al.*, 1997; Ryan *et al.*, 1992; Tentolouris *et al.*, 2003).

After meal ingestion, in response to the splanchnic blood pooling, forearm vascular resistance is augmented in healthy adults (Kooner *et al.*, 1989) or elderly person (Lipsitz *et al.*, 1993), with associated increases in plasma norepinephrine level (Lipsitz *et al.*, 1993). In healthy young subjects, carbohydrate ingestion increased MSNA (Berne *et al.*, 1989) and enhanced the gain of arterial baroreflex control of

MSNA (Young *et al.*, 2010a). The above studies suggest meal ingestion is likely to be associated with an increase in sympathetic outflow to the peripheral vasculature.

Surprisingly, compared to heart rate variability, the spectral power of blood pressure oscillation as a non-invasive and more direct index of sympathetic outflow to the vasculature (Pagani *et al.*, 1986; Pagani *et al.*, 1997) has been less appreciated in meal ingestion studies. To date, there are only three studies documenting the blood pressure oscillations in response to meal ingestion. Vaz et al observed an increase in blood pressure fluctuation at 0.1 Hz in healthy adult subjects, in accordance with increases in plasma whole body and forearm noradrenaline spillover at 60min postprandially(Vaz *et al.*, 1995b). Imai et al found a similar increase in LF spectra power of blood pressure oscillations after a meal (60min time point) in elderly subjects without postprandial hypotension; whereas in elderly subjects with postprandial hypotension, the LF spectra power of blood pressure fluctuations did not increase and was significantly lower than their counterpart subjects, suggesting attenuated sympathetic outflows to the peripheral vasculature (Imai *et al.*, 1998). However, it is reported that early after a meal (within an hour), the LF component of blood pressure oscillations remains unchanged in young, middle-aged and elderly persons (Oberman *et al.*, 2000).

There are inconsistent findings of autonomic responses to meal ingestion in normal aging. Lipsitz et al demonstrated a modest but significant increase in LF RR interval oscillations in healthy young subjects, but this finding is absent in healthy elderly subjects (Lipsitz *et al.*, 1993). However, the same research group later reported that, after the mixed meal, HF component of RR interval oscillations reduced in healthy young, middle-aged and elderly persons, but there was no difference for cardiovascular oscillation responses to meal ingestion in healthy subjects (Oberman *et al.*, 2000). Kawaguchi et al showed that LF and HF components of RR interval oscillations were unchanged to meal ingestion in both healthy young and elderly subjects. However, in elderly subjects, the ratio of LF/HF increased at 30, 60 and 90min in the postprandial state, suggesting a sympathetic hyper-responsiveness to meal ingestion. With the findings of higher baseline and blunted postprandial plasma norepinephrine levels in elderly subjects, although inconsistent with other studies

(Lipsitz *et al.*, 1993), the researchers implied that the paradoxical sympathetic efferent overactivity may be due to normal aging mediated norepinephrine down-regulation (Kawaguchi *et al.*, 2002).

Undoubtedly, in elderly persons with postprandial hypotension, there are attenuated response to meal ingestion in LF RR interval fluctuations and LF/HF ratio compared to healthy young subjects (Ryan *et al.*, 1992) and age-matched healthy elderly subjects (Masuda *et al.*, 2003). In addition, LF blood pressure fluctuations also blunted in elderly subjects with postprandial hypotension than their counterpart subjects (Imai *et al.*, 1998).

After a carbohydrate rich meal, LF RR interval fluctuations increased, HF RR interval fluctuations decreased, with accompanied LF/HF ratio increased in lean women. The cardiac autonomic responses to meal ingestion were absent in obese women (Tentolouris *et al.*, 2003). A reduced LF RR interval fluctuation was recently found in patients with a genetic type of obesity, i.e. Prader-Willi Syndrome, compared with healthy obese matched subjects, supporting the idea that Prader-Willi Syndrome may be associated with cardiovascular risk (Purtell *et al.*, 2013). It has been recently demonstrated that Type 1 diabetic patients exhibited an attenuated LF and HF cardiac interval fluctuations to meal ingestion compared with age-matched healthy control subjects; exogenous insulin infusion did not reverse the failed response, suggesting that type 1 diabetic patients may have impaired baroreflex-mediated autonomic response to haemodynamic challenge, independent of the role of insulin (Cozzolino *et al.*, 2010).

Meal ingestion may be a unique laboratory haemodynamic stimulation, which is relatively subtle to orthostatic stress, and may elicit distinguishing autonomic features between study groups (Cozzolino *et al.*, 2010; Purtell *et al.*, 2013). Meal ingestion mediated blood pressure and heart rate fluctuations remain to be studied in hypertension (Lucini *et al.*, 2002a), type 2 diabetes (Straznicky *et al.*, 2012) and other common cardiovascular diseases (or subclinical-cardiovascular conditions) (Brown *et al.*, 2002) that may be associated with autonomic dysfunction.

5. Cardiovascular autonomic dysfunction in the pathophysiology of glaucoma

5.1 Intraocular pressure and associated pathophysiology of glaucoma

Glaucoma is a leading cause of blindness in western society (Quigley, 1996). Primary open angle glaucoma (POAG) may be the most common form of glaucoma, and is a chronic neurodegenerative optic neuropathy (Quigley, 1999). In glaucoma, the neuroretinal rim of the optic nerve become progressively thinner, thereby enlarging the optic nerve cup. During clinical ophthalmoscopy screening examination, the cup-to-disc ratio increases and cup-to-disc asymmetry may appear (Hollands *et al.*, 2013; Weinreb *et al.*, 2004).

5.1.1 Intraocular pressure elevation in the pathophysiology of glaucoma

The elevation of intraocular pressure (IOP) remains an important risk factor leading to progressive and irreversible optic neuropathy (Ernest *et al.*, 2013; Hollands *et al.*, 2013; Sommer *et al.*, 1991). The term POAG refers to the histological feature that the iridocorneal angle is open and no other apparent cause can be found for an increased IOP. Aqueous humor is secreted by the cillary body into the posterior chamber, passes posterior to the iris and through the pupil into the anterior chamber, then exits through the trabecular meshwork into Schlemm's canal or uveoscleral angle, and travels from there into the episcleral venous system. As the cornea and lens in the anterior chamber has no blood supply, aqueous humor is physiologically important for the metabolic demands of the lens and cornea. With imbalanced secretion and drainage of aqueous humor, IOP rises (Figure7.) (Kwon *et al.*, 2009; Weinreb *et al.*, 2004). It is believed that the elevation of IOP may be mainly attributable to diminished aqueous outflow rather than the increased production of aqueous humor(Toris *et al.*, 1993; Wang *et al.*, 2008).

Elevated IOP has direct effects on retinal ganglion cells (RGC). It is reported that acute IOP elevation can substantially inhibit the axonal retrograde transport of brain-derived neurotrophic factor (BDNF) and cause neuron loss in a rat model (Quigley *et al.*, 2000). In response to moderate IOP elevation, reactive oxygen species (ROS), such as superoxide significantly increased and accumulated in the retinal tissue,

indicating IOP-related ischaemia and oxidative stress may contribute to the RGC death (Ko *et al.*, 2005; Peng *et al.*, 2009). Glutamate excitotoxicity was demonstrated to directly kill RGC in a rat model (Vorwerk *et al.*, 1996), and the concentration of glutamate was more than doubled in the vitreous humor of eyes in patients with open angle glaucoma and monkeys' glaucomatous eyes induced by laser photocoagulation (Dreyer *et al.*, 1996). DBA/2J mice with deficiency in the pro-apoptotic molecule BCL-2 associated X protein (BAX deficiency) may be a valuable experimental model since they develop variable onset of IOP elevation and progressing axonal damage and RGC loss with age. It is demonstrated that the RGC soma in DBA/2J mice were susceptible to N-methyl-D-aspartate (NMDA) mediated excitotoxicity, but RGC axonal degeneration is not affected by this biochemical pathway, suggesting the neurodegenerative pathway between the RGC's body and axon may be molecularly different, and RGC loss is closely related to glutamate excitotoxicity(Libby *et al.*, 2005).

Experimental evidence shows that increased IOP causes activation of glial cells in the optic nerve head (ONH). In BAX-deficient DBA/2J mice, activations of complement cascade and endothelin system were found in the early stage of glaucoma (Howell *et al.*, 2011). More recently, transendothelial migration of leukocytes into the neural tissue of eyes was identified as the first signalling pathway of early inflammation that leads to glaucomatous neural damage (Howell *et al.*, 2012). It was previously documented that raised IOP induces Tumour necrosis factor- α (TNF-α) upregulation(Nakazawa *et al.*, 2006; Tezel *et al.*, 2000) and overexpression of elastin in astrocytes of the lamina cribrosa(Pena *et al.*, 2001). The extracellar matrix remodelling may in turn increase mechanical stress onto the RGC axons. Chronic elevation of IOP induced retrobulbar structural changes were observed in experimental rhesus monkeys; the mechanical strain to the retrobulbar tissue causes lamina cribrosa deformation in glaucomatous eyes, i.e. posteriorly displaced lamina cribrosa with associated lamina thickness and elongated scleral canal diameter (Bellezza *et al.*, 2003). The thickening of prelaminar tissure while bowing posteriorly may account for the clinical cupping in early glaucomatous eyes (Yang *et al.*, 2007). While RGC axons and lamina cribrosa undergo chronic aversive stress, the

Figure 7. Imbalance of aqueous Humor causes an increase in intraocular pressure, i.e. over-secretion from ciliary body and/or reduction of drainage (blockage of Schlemm's canal).

Adapted from Kwon, Y. H., J. H. Fingprt, et al. N Engl J Med. 2009; **360**(11): 1113-1124.

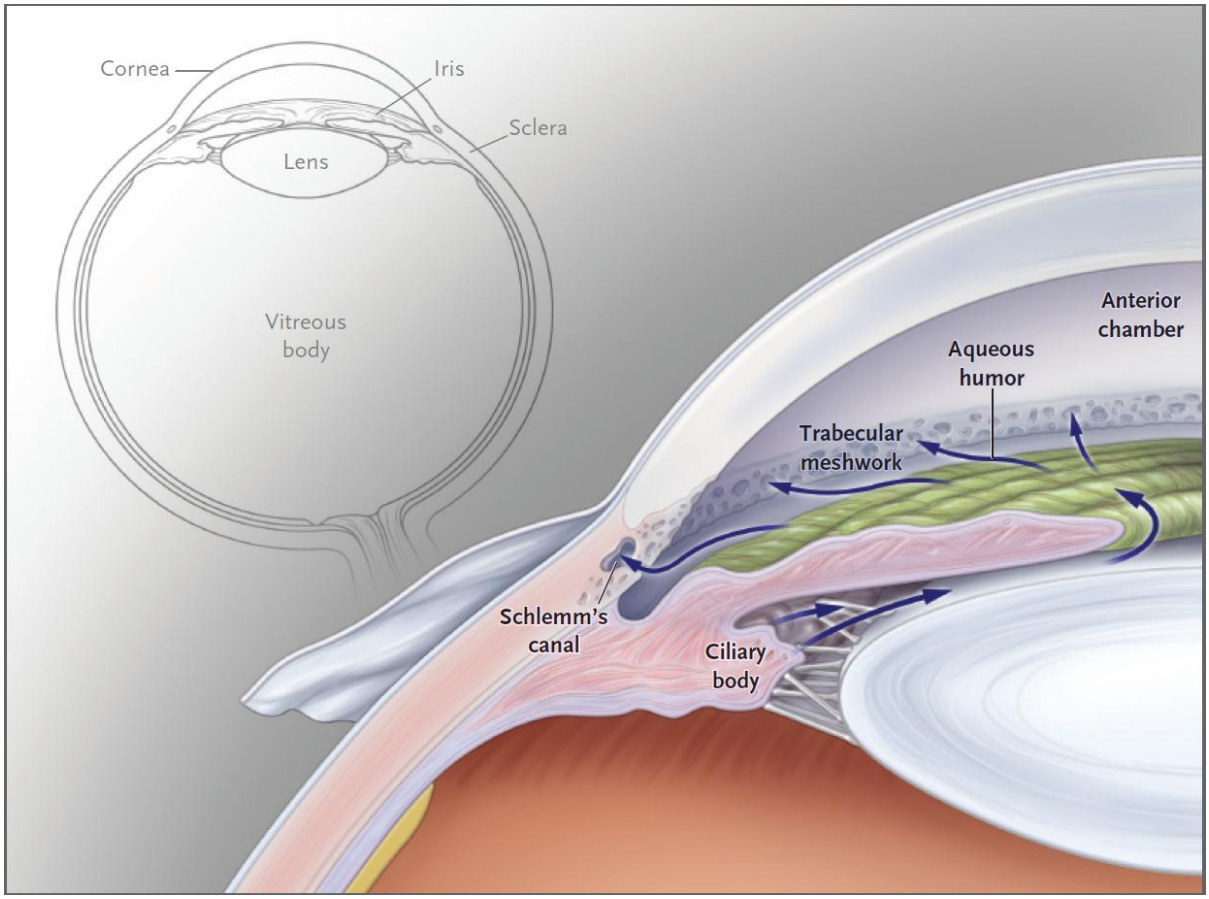
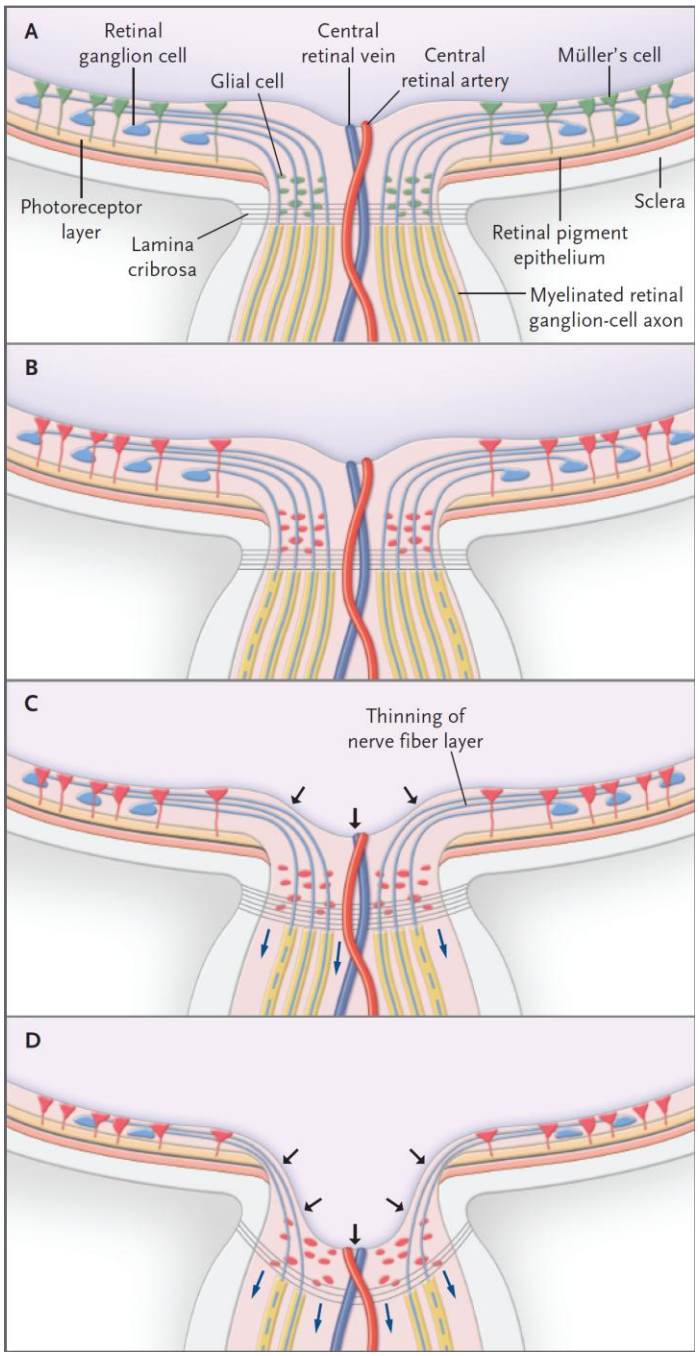


Figure 8. Increased intraocular pressure causes loss of retinal ganglion cell, and subsequently clinically detectable glaucomatous signs. Panel A, normal optic nerve head and retina; Panel B, increasingly elevated intraocular pressure puts stress on retinal ganglion cells and glial cells become reactive (red dots); Panel C, damaged retinal ganglion cells result in thinning of the nerve fibre layer, and black arrows show the posterior bowing of the optic nerve head; Panel D, In advanced stage (visual defects and clinical detectable signs), nerve fibre layers become thinner, and optic nerve head bowed further posteriorly.

Adapted from Kwon, Y. H., J. H. Fingprt, et al. N Engl J Med. 2009; **360**(11): 1113-1124.



demyelinated axonal segment at the lamina cribrosa in the ONH may be the initial site of glaucomatous damage that precedes the RGC death (Howell *et al.*, 2007; Quigley, 1999). With the axonal degeneration of optic nerve fibre, subsequent RGC loss may ensue (Almasieh *et al.*, 2012; Calkins, 2012; Kwon *et al.*, 2009). In advanced stage of glaucoma, the prelaminar tissue is substantially shrunk, and the laminar cribrosa becomes thinner and bowed more posteriorly, resulting in pronounced clinical cupping of the ONH (Kwon *et al.*, 2009). (Figure 8.)

5.1.2 Glaucoma occurs with or without IOP elevation

According to the conventional IOP measurement, a cut-off value of 21 or 22 mmHg is commonly used, and POAG is classified into two types, i.e. high tension glaucoma (HTG) and normal tension glaucoma (NTG).(NB: In practice, POAG is also commonly used to refer to HTG.) However, in considering the mechanistic basis of neurodegeneration in POAG, the arbitrary separation of the two types may not be appropriate (Pache *et al.*, 2006; Sommer, 2011).

Epidemiological studies indicate that POAG can occur with or without elevation of IOP. The Baltimore Eye Survey reports that more than half (59%) the subjects with open angle glaucoma had an IOP lower than 22 mmHg (Sommer *et al.*, 1991). In the Beaver Dam Eye Study (n=4926, age ≥ 40 years), overall prevalence was 2.1% and 33/104 cases of open angle glaucoma patients had IOP less than 22 mmHg (Klein *et al.*, 1992). In a single-centre prospective cohort study (The Rotterdam Study, n=3062, age ≥ 55 years), overall prevalence of open angle glaucoma was 1.1%, and 38.9% patients had IOP less than 21 mmHg (Dielemans *et al.*, 1994). A survey in Australian metropolitan older adults (n=3721, participating rate 83%) reported that 56 persons were identified with open angle glaucoma; of these, 21 persons (39%) had IOP less than 21 mmHg (Wensor *et al.*, 1998). A large scale rural Italian population based (age ≥ 40 years) epidemiology study (The Egna-Neumarkt study) showed that overall prevalence of ocular hypertension, HTG and NTG was 2.1%, 1.4%, and 0.6%, suggesting ocular hypertension itself may not necessarily develop glaucomatous eyes, and the ratio of NTG and HTG prevalence is considerable (0.6% : 1.4% ≈ 42%) (Bonomi *et al.*, 1998). Furthermore, Asian individuals with glaucoma may have lower mean IOP of 15.3 mmHg (Iwase *et al.*, 2004) than European white individuals of 24.2

mmHg (Sommer *et al.*, 1991). A recent Japanese study (n=3021, age ≥ 40 years) showed that overall prevalence of open angle glaucoma is 3.9%; not surprisingly, amongst this, 92% of those diagnosed with glaucoma had IOP levels of 21 mmHg or less, suggesting IOP measurement as a screening examination particularly in an Asian population may require a different diagnostic criterion (Iwase *et al.*, 2004).

In addition, the progression of glaucomatous visual field damage is only weakly correlated with mean IOP measurement (Chauhan *et al.*, 1990; Chauhan *et al.*, 1992; Martínez-Belló *et al.*, 2000). Lowering IOP does not prevent long-term progression of glaucoma in a large proportion of patients (Graham *et al.*, 1999; Leske *et al.*, 2007; Stewart *et al.*, 2000).

5.2 Ocular ischaemia contributing to the pathophysiology of glaucomatous optic neuropathy

Factors independent or in addition to IOP that underlie the pathogenesis of both HTG and NTG have been recognised based on experimental studies (Pache *et al.*, 2006; Weinreb *et al.*, 2004) (Figure 9.). Of these, vascular factors may be a more important one leading to glaucomatous optic neuropathy (Flammer *et al.*, 2007; Flammer *et al.*, 1998; Flammer *et al.*, 2002; Leske *et al.*, 2007).

5.2.1 Anatomy and physiology of ocular blood flow

As mentioned above, cornea, lens and vitreous humor are avascular structures and their metabolic needs are supplied by the aqueous humor. The retina has the highest metabolic demands of any tissue in the body (Buttery *et al.*, 1991). The delivery of nutrients, oxygen and metabolic substrate to the retina in humans is achieved via an abundant blood supply from retinal and the choroidal vascular system. Although retinal and choroidal arteries are both derived originally from the ophthalmic artery, a branch of the internal carotid artery, the two separate vascular systems differ morphologically and functionally (Kur *et al.*, 2012).

The central retinal artery (CRA) is usually the first branch of ophthalmic artery and a main branches of the internal carotid artery (Hayreh, 2006). The central retinal artery, in the strict sense, retinal arteriole, passes through the lamina cribrosa and branches into four main arterioles (i.e. superior- and inferior- temporal and nasal arterioles),

Figure 9. Factors contributing to pathophysiology of glaucomatous neurodegeneration

Adapted from Weinreb, R. N. and P. Tee Khaw. Lancet. 2004; **363**(9422): 1711-1720.

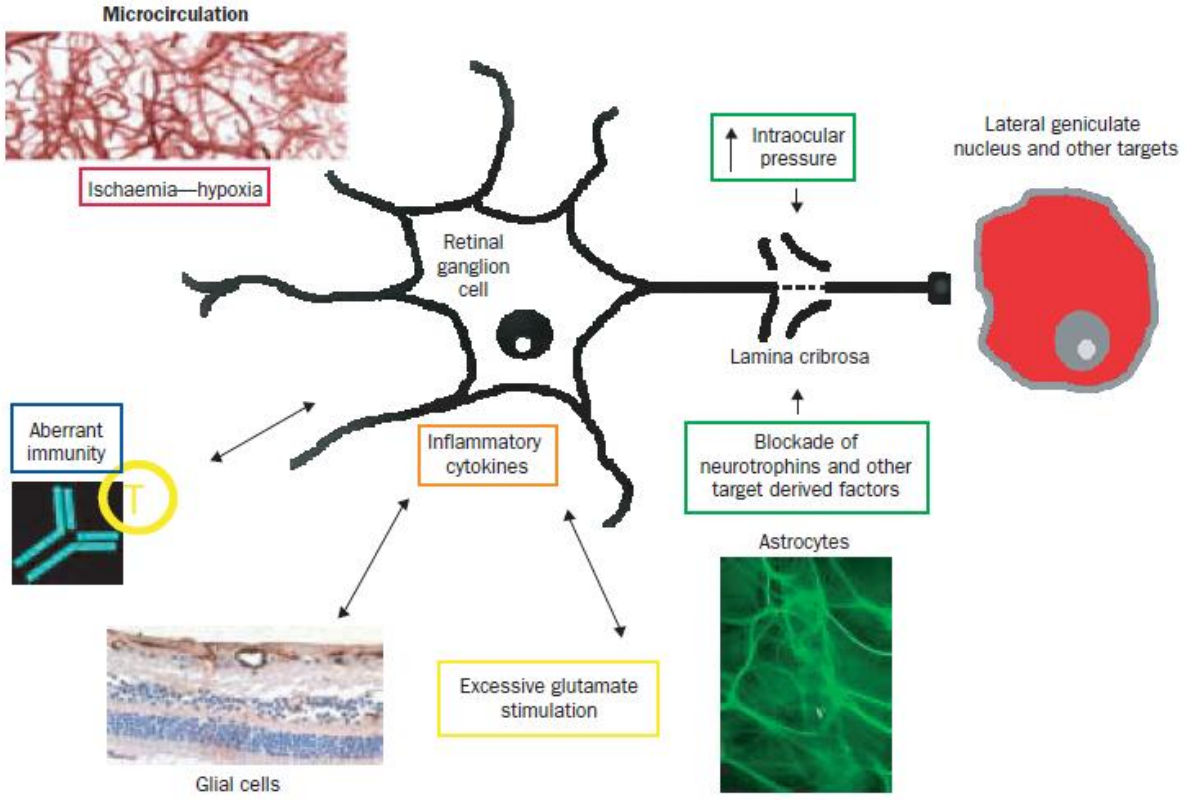
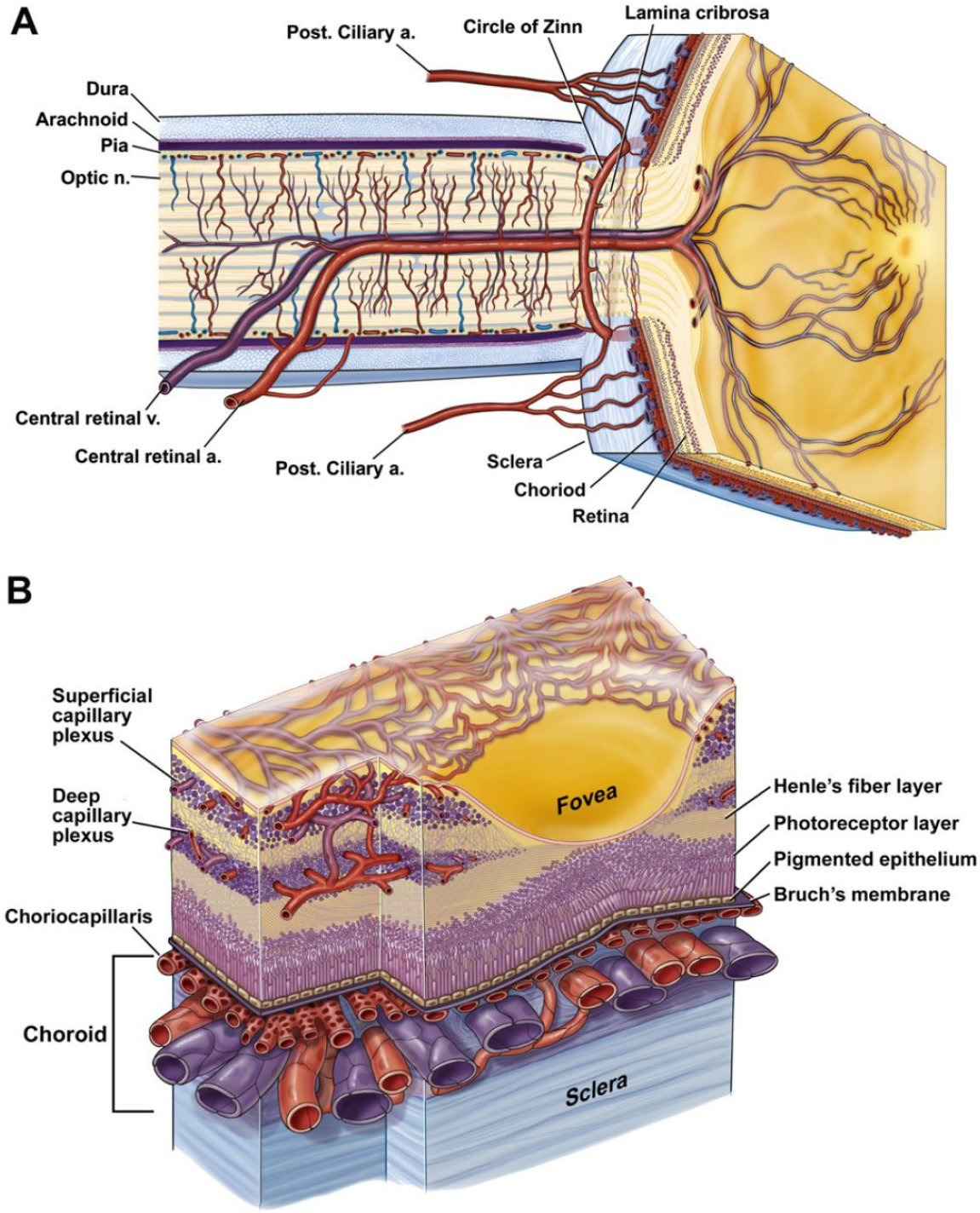


Figure 10. Anatomy of ocular circulation. A) cut-away drawing of superior-inferior axis of human eye through the optic nerve, showing the vascular supply to the retina and choroid; B) drawing showing vasculature of the retina and choroid.

Adapted from Kur, J., E. A. Newman, et al. Prog Retin Eye Res. 2012; **31**(5): 377-406.



supplies the inner two-third of the retina. The retinal artery and retinal arteriole proximal to the lamina cribrosa are sympathetically regulated (See Section 5.2.2 below for a detailed information).The choroidal plexus, derived primarily from the long and short posterior cilliary arteries, supplies the photoreceptors in the outer one-third of the retina. In the retinas of primates and humans, the fovea in the centre of the retina is composed of highest density of retinal ganglion cone cells and facilitates high acuity and colour vision. The four main arterial branches of the retinal circulation deviate around the fovea region to facilitate uninterrupted vision; instead, the thinness of the fovea allows adequate oxygenation via the posterior choroidal circulation (Engerman, 1976). The choroidal circulation may also play a main role for the blood supply of prelamina, lamina cribrosa (Circle of Zinn) and retrolamina regions. Both retinal and choroidal arteries give rise to a plexus of capillaries, and the venous system has a similar arrangement with the arteries(Figure10.).

The retinal circulation is characterised by low level of flow (Alm *et al.*, 1973) and high level of oxygen extraction (Hickam *et al.*, 1963). In contrast, flow in the choroidal circulation is very high and oxygen extraction is relatively low. This explains that besides supplying nutrients, the choroidal circulation may be more important in thermoregulation, volume buffer and aqueous humor production (Nickla *et al.*, 2010).

In humans, the walls of retinal arterioles contains 5-7 layers of smooth muscle cells in the optic disc region; after several branching, the number of layers diminishes to just 1-2 in the retinal periphery. Smooth muscle layers are originated circularly and longitudinally, and surrounded by basal lamina. Endothelial cells are orientated along the longitudinal axis of the vessel (Hogan *et al.*, 1963). Pre-capillary arterioles featured by innervated pre-capillary sphincters are the segments crucial for generating vascular tone. Contrary to other vascular network, human retina lacks pre-capillary sphincters (Henkind *et al.*, 1968), therefore, the retinal capillaries may be continuously perfused. The walls of retinal capillaries and post-capillaries are composed of three distinct elements: endothelial cells, intramural pericytes and a basement lamina. Compared to choroidal capillaries, the endothelium of retinal capillaries and postcapillaries are covered with high-density pericytes with a ratio almost 1:1 of endothelial cells. Indeed, the pericytes represent unique and dynamic

myogenic regulation of retinal microvasculature through the release of vasoactive substrate (Chan-Ling *et al.*, 2011b; Frank *et al.*, 1990; Haefliger *et al.*, 1994; Shepro *et al.*, 1993). Furthermore, choroidal capillaries are fenestrated, although not as frequently as in capillaries of other tissues (Chan-Ling *et al.*, 2011a). In contrast, in retinal capillaries, continuous layered endothelial cells and tight junctional complexes along the opposing surfaces of adjacent cells may serve as structural barrier in maintaining optimal homeostasis for retinal neurons (Pournaras *et al.*, 2008).

5.2.2 Autonomic innervation to retinal and choroidal circulation

The eye has a rich autonomic innervation. Histologically, nerve endings in or on the walls of the retinal vasculature are absent in human, monkey and rat by electron microscopy (Hogan *et al.*, 1963). The autonomic innervation to the eye of monkey, cat and rabbit was then further studied using a sensitive histofluorometric method for adrenergic nerves and the acetylcholinesterase technique for cholinergic nerves. Researchers found a considerable variation of intraocular adrenergic innervation on the retina, cornea and ciliary body between monkey and the other two species, but all species showed adrenergic innervation in choroidal arterioles and capillaries (Laties *et al.*, 1966). A subsequent study using the Falck and Hillarp fluorescent method examined adrenergic innervation in different species of New World monkeys and reported that in primates, adequate adrenergic innervation along the course of central retinal artery and adjacent tissue present in the retrolamina region, but significantly diminishes within the lamina cribrosa and disappears in the intraocular portion of the retinal artery. In comparison, choroidal artery and capillaries are heavily innervated with adrenergics both extraocularly and intraocularly(Laties, 1967). Based on investigative findings from scanning/transmission electron microscopy and the Falck and Hillarp fluorescent method, Furukawa et al found in rabbits (Japanese White), that there exists heavy sympathetic innervation on the retinal arterioles and capillaries, and the nerve fibres disappear 7 days after sympathoganglionectomy(Furukawa, 1987). It is now accepted that the controversial finding may be due to the species difference in considering the retina is such a highly-differentiated organ (Bill *et al.*, 1990; Laties *et al.*, 1966; Ye *et al.*, 1990). In keeping with findings of animal (monkeys and rats) (Bergua *et al.*, 2003; Ye *et al.*,

1990) and human investigations (Denis *et al.*, 1989), recent immunohistochemistry studies further show that in humans there are tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) positive nerve fibres within the vessel wall of the central retinal artery proximal to the lamina cribrosa, supporting the concept that sympathetic innervation terminates as the blood vessel enters the globe and do not follow the branches of the central retinal artery inside the eye (Bergua *et al.*, 2013). Taken together, it is believed that in primates and humans, sympathetic nerves originate from the uppermost superior cervical sympathetic ganglion innervate the choroidal vascular beds as well as the central retinal artery up to the lamina cribrosa, but not further into the retina (Bill *et al.*, 1990; Delaey *et al.*, 2000).

It seems that the role of sympathetic innervation on the ocular haemodynamic is to avoid overperfusion (Robinson *et al.*, 1986). For example, while there is an acute increase in systemic blood pressure due to “fight or flight”, activation of sympathetic nerve fibres to the eyes may give a normal blood flow in the choroid despite a rise in blood pressure; vasoconstriction of extraocular CRA may buffer the stress to intraocular vascular beds in the retina and assist the intrinsic myogenic autoregulation in preventing acute hyperperfusion to the retina and maintaining homeostasis (Bill *et al.*, 1990; Schmidl *et al.*, 2011). During isometric exercise e.g. squatting, an increase in ocular perfusion pressure up to 67% induces an increase in choroidal vascular resistance that limits the increase in choroidal blood flow to approximately 12%. This regulatory process fails when ocular perfusion pressure is further increased (Riva *et al.*, 1997a). On the contrary, acute decrease in ocular perfusion pressure induced by scleral suction (artificial increase in IOP) is associated with phenomenon relative to the involvement of combined neural, passive haemodynamic and autoregulatory mechanisms (Riva *et al.*, 1997b).

Parasympathetic nerves reach the choroidal vasculature through the facial nerve (pterygopalatine ganglion) (Cuthbertson *et al.*, 1997; Ruskell, 1971; Ruskell, 1970), as well as the oculomotor nerve and the upper two divisions of trigeminal nerve (Yasuhara *et al.*, 2004). Vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT) were colocalised in nerve fibres suppling the central retinal artery proximal to lamina cribrosa in humans (Bergua *et al.*, 2013). The muscarinic

binding sites were also found retinal vessels (Ferrari-Dileo *et al.*, 1989). However, as the authors mentioned these cholinergic receptors may not play any physiological role at all because blood-retina-barrier tightly separates the plasma substrate from surrounding environment (Pournaras *et al.*, 2008). On the other hand, lack of anatomical autonomic innervation within retina may abolish the possibility of the presence of acetycholine(Laties, 1967). The precise role of parasympathetic innervation to the orbital vessels and the interaction of sympathetic and parasympathetic neural control are still not clear (Bill *et al.*, 1990; Schmidl *et al.*, 2011).

5.2.3 Intra-retinal vascular autoregulation

Blood-retinal barrier (BRB) refers to the cellular barriers that are composed of highly dynamic and specific structures to maintain optimal ocular homeostasis. Barrier tissues, in particular epithelia and vascular endothelium are capable of responding to physiological requirements and to ever-changing extrinsic conditions; extracellular matrix and glycocalyx sense such external stimuli and modulate the dynamic responsiveness of the barrier cells.

Intra-retinal vascular endothelium is considered as the main component of the BRB due to its elaborate network of tight junctions, the paucity of pinocyticcaveolae and absence of fenestrations. In many respects, the endothelium of the BRB closely resembles the one of the blood-brain barrier (Patton *et al.*, 2005). Compared to brain-derived endothelial cells, retinal endothelial cells release high levels of superoxide, have less glutathione peroxidase activity, and lower levels of superoxide dismutase and junctional protein, suggesting retinal endothelial cells are more vulnerable to oxidative stress (Grammas *et al.*, 2003). The retinal vascular tone and blood flow is by and large autoregulated by intrinsic myogenic and metabolic mechanisms. The highly dynamic endothelial cells release vasoactive substrate, e.g. NO, endothelin and metabolites of arachidonic acid, and therefore these hormonal factors play a crucial role in vascular autoregulation (Kaur *et al.*, 2008).

As mentioned above, the unique fine structure of the intra-retinal capillaries and post-capillaries is a high-density of pericytes(Patton *et al.*, 2005). Pericytes outer-

layered the endothelial cells work in harmony with retinal vessels with smooth muscle cells, and represent the myogenic mechanism for vascular autoregulation(Alm *et al.*, 1973; Peppiatt *et al.*, 2006; Russell, 1973). Furthermore, it is found that pericytes may serve metabolic mechanisms by expressing varieties of receptors for vasoactive substances, which enable them to respond to endothelin-1 for contraction (Chakravarthy *et al.*, 1992) and NO for relaxation (Schönfelder *et al.*, 1998).

Both epithelium and vascular endothelium synthesise a specialised extracellular matrix scaffold, the basal lamina, which separate the endothelial layer from the surrounding pericytes in capillaries and postcapillary venules and from smooth muscle cells present in arteries and arterioles (Hughes *et al.*, 2004). Common features of basal laminas are elasticity and tensile strength.

The glycocalyx is a polyanionic, negatively charged multi-molecule membrane ensheathed onto the vascular bed and acts as an electrostatic coating. A major function of the vascular endothelial glycocalyx is to sense mechanical forces of blood flow, to attenuate the effect of direct fluid shear stress onto the endothelium and preserve the endothelial function (Gouverneur *et al.*, 2006b; Weinbaum *et al.*, 2003). Fluid shear stress stimulates incorporation of hyaluronan into endothelial glycocalyx, which may contribute to its vasculoprotective effects against proinflammatory and pro-atherosclerotic stimuli (Gouverneur *et al.*, 2006a). Constitutes of glycocalyx , such as hyaluronic acid (Mochizuki *et al.*, 2003), albumin (Jacob *et al.*, 2007), and heparansulfates(Florian *et al.*, 2003) are intimately involved in regulating NO release by serving as an important wall-shear-stress mechano-sensor (Kumagai *et al.*, 2009). The glycocalyx contains enzymes, such as extracellular superoxide dismutase (SOD) (Li *et al.*, 1998) that protect from excessive reactive oxygen species (ROS) production. Impairment of glycocalyx may induce oxidative stress-induced cardiovascular diseases (Fukai *et al.*, 2002; Lu *et al.*, 2008; van Deel *et al.*, 2008).

In summary, long-standing intra-vascular wall-shear-stress may interfere with retinal blood flow regulation and contribute to the toll of retinal vascular barrier vulnerability, and therefore lead to retinal vascular-barrier dysfunction and eventually, to the impairment of capillary perfusion.

5.2.4 Evidence of endothelial dysfunction associated ocular ischaemia/hypoxia in the pathogenesis of glaucomatous optic neuropathy

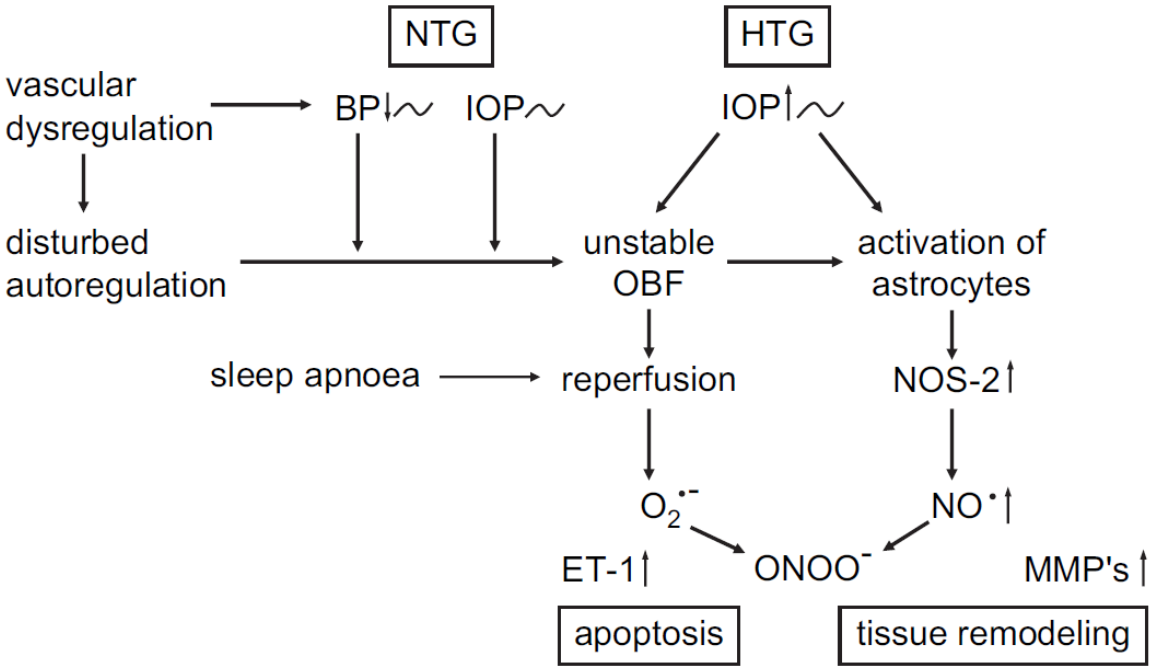
According to the nature of blood supply at the ONH and retina via retinal and choroidal circulation, the eye may be a vulnerable target organ for ischaemic damage (Flammer *et al.*, 2013; Grieshaber *et al.*, 2007a; Grieshaber *et al.*, 2007c). Direct evidence of neuronal tissue hypoxia at the retina and ONH has been found in glaucoma patients (Tezel *et al.*, 2004). One can postulate is that insults from systemic blood flow regulation may chronically disrupt the retinal neuron and axonal homeostasis (Anderson *et al.*, 1998; Flammer *et al.*, 2007; Flammer *et al.*, 2002; Hayreh *et al.*, 1994; Leske *et al.*, 2007; Tielsch *et al.*, 1995). A genetically-predisposed eye exposed to an environment with repetitive aversive stress is likely to develop glaucomatous optic neuropathy (Duggal *et al.*, 2007).

A concomitant upregulation and/or an imbalance of ET-1 and NO is a characteristic of endothelial dysfunction. Endothelial cells line not only ocular vasculature but also trabecular meshwork and Schlemm's canal that are crucial for aqueous humor outflow. Elevated IOP may cause decreased ocular blood flow and microangiopathy (Portmann *et al.*, 2011). Endothelial dysfunction and IOP elevation is linked (Howell *et al.*, 2011; Howell *et al.*, 2012). The following review focuses on the primary role of endothelial dysfunction mediated ocular ischaemia/hypoxia in the pathogenesis of glaucomatous optic neuropathy (Figure 11.).

Endothelin-1 (ET-1) is one of the most potent vasoconstrictor peptides. Higher plasma ET-1 concentration is detected in patients with normal tension glaucoma (Kaiser *et al.*, 1995; Sugiyama *et al.*, 1995) and the elevated plasma ET-1 level may be correlated with the severity of glaucomatous damage (Sugiyama *et al.*, 1995).It is demonstrated that intravenous administration of Endothelin-1 reduces pulsatile blood flow in the choroid and the optic disc at doses which do not affect systemic hemodynamics or flow velocity in the ophthalmic artery, suggesting both ocular circulation at the optic disc and choroidal blood flow are particularly sensitive to changes in local ET-1 concentration (Schmetterer *et al.*, 1997b). Higher ET-1 is associated with lower ocular blood flow (Galassi *et al.*, 2011), inhibition of ET-1 may reverse the ocular hypoperfusion in patients with normal tension glaucoma (Resch *et*

Figure 11. A diagram showing the proposed mechanism that unstable ocular blood flow (OBF) underlies both normal tension glaucoma (NTG) and primary open angle glaucoma (also called high tension glaucoma, HTG). The instability of OBF may be associated with reperfusion injury, and endothelial dysfunction in the retina.

Adapted from Flammer, J. and M. Mozaffarieh. Surv Ophthalmol. 2007; **52**(6 SUPPL.): S162-S173.



al., 2009). Although HTG patients may not invariably demonstrate increased plasma ET-I level (Holló *et al.*, 1998), a significantly higher ET-1 level in the aqueous humor was found in both NTG and HTG patients compared to a control group (Tezel *et al.*, 1997). As known, the progression of glaucomatous damage may be associated with decreased blood supply to the arterioles at the optic nerve head independent of IOP (Schumann *et al.*, 2000; Zeitz *et al.*, 2006), a recent study shows that plasma ET-1 concentration was higher in normal or normalised IOP open angle glaucoma patients with progressive glaucomatous visual field damage than those with stable visual field (Emre *et al.*, 2005).

Clinical evidence suggests that high levels of circulating ET-1 in open angle glaucoma patients may be evoked by vasospastic stimuli during daily life, such as cold provocation (Henry *et al.*, 2006; Nicolela *et al.*, 2003) and abnormal ET-1 response to postural stress (Kaiser *et al.*, 1995). Indeed, ET-1 mediated reduction in ocular blood flow matches that seen in nail-bed capillaries (Emre *et al.*, 2004; Gasser *et al.*, 1991), suggesting that impaired ocular perfusion may be a sign of generalised systemic vascular dysregulation in glaucoma patients (Gherghel *et al.*, 2004a).

The independent role of ET-1 in the pathogenesis of glaucomatous optic neuropathy is demonstrated in ischaemic animal models. Intravenous and intravitreous ET-1 injection to the rabbit eyes caused reduction in ocular blood flow at the ONH independent of IOP (Sugiyama *et al.*, 1995). Two weeks of intraocular ET-1 injection to the surrounding tissue of the optic nerve in one eye via mini-pump, rabbits developed persistent reduction of ocular blood flow (38% lower than contralateral eye) without IOP elevation, the disturbed blood supply was associated with resultant glaucomatous optic disc cupping (Orgül *et al.*, 1996); similar results were demonstrated in the optic nerve of primate (Cioffi *et al.*, 1999). Consistent with the above, besides increased optic cup/disc ratio, histological examination revealed the ET-1 induced ischaemic lesion is relative to axonal loss and demyelination affecting the prelaminar portion of the optic nerve (Oku *et al.*, 1999). A large sample sized rats study showed chronic local ET-1 injection induced RGC soma and axon losses are time-dependent, but not dose-dependent (Chauhan *et al.*, 2004).

In physiological conditions, nitric oxide (NO) production in the endothelial cells triggered by an agonist e.g. acetycholine may induce local vasodilation (Haefliger *et al.*, 1999; Schmetterer *et al.*, 2001), NO synthase inhibition reduces ocular perfusion (Schmetterer *et al.*, 1997a). NO is highly diffusible and unstable small molecule with half-life only a few seconds, therefore NO is not feasible to be directly identified in vivo. NO pathway is valuable in interpreting the role of NO in blood flow regulation (Schmetterer *et al.*, 2001). Endothelial nitric oxide synthase-3 (NOS-3) is responsible for the production NO. The upregulation of NOS-3 in vascular endothelia was found in the donor eyes of patients with open angle glaucoma (Neufeld *et al.*, 1997), suggesting a basal NO excessive production in response to the ischaemia/hypoxia (Arnet *et al.*, 1996).

The two major pathogenetic components of glaucoma are damage to the axons and the activation of the astrocytes (Flammer *et al.*, 2007). Astrocytes are activated by not only mechanical stress, but also endothelin upregulation, such as reperfusion injury (Prasanna *et al.*, 2002; Prasanna *et al.*, 2011). In pathophysiological conditions, the neurotoxic NO synthase-2 (NOS-2) is produced by macrophage tissue in response to the above inflammatory stimuli, and NO itself is not damaging. However, if NO reaches the axons at the ONH where there is high concentration of superoxide radicals, it leads to the formation of the neurotoxic peroxynitirite ($ONOO^-$) (Haefliger *et al.*, 1999; Neufeld *et al.*, 1997). Inhibition of NOS-2 may prevent the progression of glaucomatous optic neuropathy (Neufeld *et al.*, 1999). The presence of NOS-2 in astrocytes of optic lamina cribrosa in glaucoma patients, provides evidence that excessive exposure to NO in the ONH may be neurodestructive and relative to RGC axonal damage (Neufeld *et al.*, 1997).

According to the basal upregulation of NOS in glaucoma patients, endothelial-dependent vasodilatation reserve may be attenuated, resembling the so called “ceiling effect” found in sympathetic-mediated neurovascular response (Burke *et al.*, 1977; Fu *et al.*, 2004b). NOS inhibition failed to reduce the ocular blood flow in the ONH and fundus pulsation amplitude in glaucoma patients, indicating an increased basal NOS activity may be a characteristic of attenuation of NO bioavailability for ocular vasodilaton in glaucomatous ocular tissue (Polak *et al.*, 2007). It is also

shown that intra-arterial infusion of acetylcholine, a NO agonist elicited a blunted response of forearm blood flow in NTG patients, whereas the response of endothelial-independent vasodilator (sodium nitroprusside) did not differ between NTG and matched-control subjects (Henry *et al.*, 1999; Su *et al.*, 2006). This study strongly supports the idea that systemic endothelial dysfunction rather than only a local phenomenon with ocular vascular dysfunction may exist in glaucoma patients (Flammer *et al.*, 2002; Flammer *et al.*, 2001; Mroczkowska *et al.*, 2013; Mroczkowska *et al.*, 2012).

5.2.5 Glutamate excitotoxicity, inflammatory cytokines and aberrant immunity

Other factors may also play an individual or collective role in the ischaemia-related pathogenesis of RGC loss, independent of IOP elevation (Flammer *et al.*, 2007; Pache *et al.*, 2006; Weinreb *et al.*, 2004).

Glutamate excitotoxicity, in particular NMDA subtypes, is known to kill the RGCs; impaired glutamate transporters are proposed to be responsible for a decrease in glutamate clearance by glial cells (Lipton, 2003; Vorwerk *et al.*, 1999). Specifically, glutamate/aspartate transporter (GLAST) expressed in the Muller glial cells may play an important role in both balancing extracellular of gluatmatergic stimulation below neurotoxic level and transporting glutamate into Muller cells for the intracellular synthesis of glutathione (an antioxidant); whereas the main role of excitatory amino acid carrier-1 (EAAC-1) expressed in neurons is to transport cysteine into RGC as a precursor for glutathione neuronal synthesis (Harada *et al.*, 1998). It is demonstrated that mice with knocked-out one of each 3 glutamate transporter, in particular GLAST deficient and EAAC-1 deficient, develop spontaneous RGC loss and typical glaucomatous optic nerve damage without elevation of IOP. In the GLAST deficient mice, glutathione in Muller glia was reduced, and EAAC-1 deficient mice were more vulnerable to oxidative stress. This study implies the role of intact glutamate transporter for the survival of RGC and the synthesis of glutathione in normal tension glaucoma animal model (Harada *et al.*, 2007). More recently, a study shows that blood glutathione level was similarly reduced in patients with high tension glaucoma or normal tension glaucoma, supporting a role of comprised glutamate transporter in glaucoma patients, independent of elevated IOP (Gherghel *et al.*, 2013). Apoptosis

signal-regulating kinase 1 (ASK1) has an important role in stress-induced RGC apoptosis. In the above GLAST deficiency-induced NTG mice model, ASK1 deficiency showed protective effects for the survival of RGC, and supressed TNF-α overactivation (Harada *et al.*, 2010).

Overexpression of TNF-α in retinal glial cells and TNF-α receptor-1 in the RGCs were found from retina of donors with open angle glaucoma compared to age-matched controls (Tezel *et al.*, 2001). The role of TNF-α upregulation in the pathogenesis of RGC loss was also demonstrated in animal experimental eyes induced by oxidative stress (Tezel *et al.*, 2000). The overexpression of TNF-α, TNF-α receptor-1, and matrix metalloproteinase (MMP) were also found in the ONH in post-mortem eyes from both NTG and HTG patients; more importantly, these markers expressed in NTG are greater than in HTG, suggesting TNF-α signalling activation may underlie the progression of normal tension glaucoma (Yan *et al.*, 2000). NTG patients may be more sensitive to IOP (Yan *et al.*, 2000) or IOP-independent oxidative stress (Tezel, 2006).

A failure to properly control immune responses, i.e. aberrant immunity induced by oxidative stress is likely to convert the initial protective immune response to an autonoimmune neurodegenerative process, and lead to glaucomatous loss of RGC (Wax *et al.*, 2008). Abnormal T-cell subtypes (Yang *et al.*, 2001) and increased autoimmune antibodies (Wax *et al.*, 1994; Wax *et al.*, 1998a; Wax *et al.*, 1998b) have been reported in glaucoma patients, irrespective of IOP level. Recent in vitro experiment showed that retinal glial cells exposed to ROS generating compounds induce more potent T-cell proliferation TNF-α secretion, suggesting in the presence of ROS stimulated antigen-presenting ability of the glial cells in the glaucomatous retina and ONH can elicit an activated immune response (Tezel *et al.*, 2007).

5.3 Cardiovascular autonomic dysfunction in the pathogenesis of glaucoma

Both intraocular pressure and blood pressure fluctuate, their balance (preload and afterload to the ocular microcirculation) determines the ocular perfusion. Ocular blood flow becomes insufficient and/or unstable if there is an imbalance of the capacity of normal ocular autoregulation.

The retinal ischaemia derived from the reduction of ocular blood flow is commonly seen as hypertensive retinopathy and diabetic retinopathy, and is not necessarily a characteristic of glaucomatous optic neuropathy (Flammer *et al.*, 2013). It is hypothesised the link between glaucoma and ocular blood flow is not so much the reduction of retinal blood flow per se, but rather the instability of ocular blood flow (Flammer *et al.*, 2007). The instability of ocular blood flow is likely due to systemic and peripheral vascular dysregulation resulting in repetitive mild reperfusion injury (Zheng *et al.*, 2007), which may be crucial in the pathogenesis of glaucoma (Flammer *et al.*, 2007). Abnormal blood pressure fluctuation may be a more damaging factor than a stable arterial hypotension in both NTG and HTG patients that compromises ocular blood flow (Graham *et al.*, 1999; Graham *et al.*, 1995; Mroczkowska *et al.*, 2013). It is demonstrated that abnormal blood pressure fluctuation and peripheral vasospasm may be two distinct risk factors (Orgul *et al.*, 1995; Pache *et al.*, 2003). Patients suffering from clinical or subclinical systemic autonomic dysfunction are likely combined with primary vascular dysregulation (PVD), if not all, may be at critical risk in the development and progression of glaucomatous optic neuropathy (Grieshaber *et al.*, 2007b; Pache *et al.*, 2006).

5.3.1 Clinical evidence of abnormal blood pressure fluctuation in glaucoma – implication of systemic autonomic function

The primary role of baroreflex-mediated autonomic nervous control is to minimise blood pressure fluctuations in response to daily stressors (Cowley Jr *et al.*, 1973). Autonomic dysfunction or failure may frequently lead to exaggerated blood pressure excursions, such as orthostatic hypotension (Robertson *et al.*, 1993; Singleton *et al.*, 2003) and orthostatic hypertension (Robertson, 2011; Yatsuya *et al.*, 2011). Blood pressure instability and inappropriate blood pressure fluctuation are closely relevant to the prevalence and progression of cardiovascular diseases (Rothwell, 2010).

Following the recognition of arterial hypotension in glaucoma (Kaiser *et al.*, 1991), circadian blood pressure fluctuation using 24 hours ambulatory blood pressure monitoring has been most studied so far in glaucoma patients and provides important clinical clue for autonomic nervous regulation in glaucoma.

In 1991, Kaiser et al reported that in four patients with rapid visual field defects and excavation of ONH despite normal or well-treated IOP, both systolic and diastolic blood pressure significantly dropped during sleep. The researchers then recognised that nocturnal hypotension may be significant risk factor and further 24 hours blood pressure monitoring is required for such patients (Kaiser *et al.*, 1991). In a following study, these researchers found that 38 HTG patients with progressive visual field defects with uncontrolled IOP despite maximal treatment had very similar blood pressure levels to that of 32 controls during both day and night, with physiological dip at night. However, 40 HTG patients with progression despite well-controlled IOP and also 39 patients with normal-tension glaucoma had markedly lower systolic blood pressure during both day and night (Kaiser *et al.*, 1993). This study suggests the impaired blood pressure regulation may be a significant risk factor at least in some HTG and NTG independent of IOP elevation.

Hayreh et al performed 24 hours blood pressure monitoring and calculated hourly-averaged data in patients with anterior ischemic optic neuropathy (AION), NTG and HTG, and found a significantly lower night-time mean diastolic blood pressure and a significantly greater percentage decrease in nocturnal mean diastolic blood pressure in NTG than in AION, indicating that abnormal nocturnal hypotension may be related to the reduction in blood flow at the ONH below a critical level, leading to glaucomatous optic neuropathy (Hayreh *et al.*, 1994). The following studies are in keeping with this finding that nocturnal blood pressure fall is more pronounced in NTG patients than controls (Meyer *et al.*, 1996), suggesting the instability of diurnal and nocturnal blood pressure may play a more important role in the pathogenesis of NTG than the absolute blood pressure value (Kario *et al.*, 1997).

The abnormal diurnal-and-nocturnal blood pressure swing is not restricted to NTG. Graham et al (Graham *et al.*, 1995) performed 24 hours ambulatory blood pressure monitoring in 38 NTG, 46 HTG patients with well controlled IOP and 11 control subjects and found that diurnal and nocturnal blood pressure parameters in NTG and HTG patients did not differ and was comparable to control subjects; moreover, in 52 patients whose visual fields were assessed for more than 2 years, the authors noted that nocturnal systolic, diastolic and mean blood pressure were significantly lower in

37 patients with progressive visual field defects than in 15 patients with stable measurement, in particular, the nocturnal dip of systolic blood pressure was significantly greater in progression patients. This study provides evidence that both NTG and HTG patients with progressive visual field defects may share the pathogenesis of exaggerated nocturnal hypotension. In order to determine long term outcome of systemic blood pressure parameters, Graham et al re-evaluated the visual fields of the original 84 patients after mean 5.1 years. Consistent findings were observed, i.e. significantly lower nocturnal blood pressure variables, with the dips of the systolic, diastolic, and mean arterial pressure significantly larger in patients who had visual field progression as well as greater history of disc haemorrhage, despite good control of IOP (Graham *et al.*, 1999). The above observations well established the pathogenetic link between greater nocturnal hypotension and glaucomatous damage progression; in other words the abnormal circadian blood pressure fluctuation may progress glaucomatous optic neuropathy in both NTG and HTG (Gherghel *et al.*, 2004b). The exaggerated blood pressure variations may cause vulnerable target organ damage, such as ischaemic coronary heart disease (Pierdomenico *et al.*, 1998), cerebrovascular lesion (Kario *et al.*, 1997), and anterior ischaemic optic neuropathy (Hayreh *et al.*, 1994).

Gherghel et al (Gherghel *et al.*, 2001) investigated the relationship between circadian blood pressure rhythm and retrobulbar haemodynamic blood flow. Glaucoma patients were divided into three groups according to the degree of nocturnal dip, i.e. patients with a nocturnal decrease in mean systemic blood pressure (MBP) below 20% of the average daytime MBP (over-dippers), patients with a decrease between 10% to 20% (in dippers), and patients with a decrease of less than 10% (in nondippers). The researchers found a significantly lower end-diastolic velocity and a significantly higher resistivity index in the central artery of over-dipping glaucoma patients compared with nondippers or dippers. In addition, 24-hour mean ocular perfusion pressure was calculated:

MOPP, 2/3 × mean arterial pressure [MAP] - intraocular pressure [IOP]

Marked circadian MOPP fluctuation was noted in the overdipper group (Choi *et al.*, 2006) and is correlated with progressive visual field damage in NTG patients (Sung *et al.*, 2009).

Detry et al investigated 36 primary open angle glaucoma (HTG) patients despite well-controlled IOP and classified them into visual field progression and stability groups. The researchers found the overall mean daytime, night-time, and nocturnal dips fell within the normal range of the reference population. In particular, a significantly smaller systolic and diastolic BP dip in the progressive group and a broader distribution of the lower values both for systolic and diastolic BP in the progressive group were noted (Detry *et al.*, 1996). Similarly in NTG patients, a Japanese study showed that the blood pressure dip in NTG patients with progressive visual field defects was significantly smaller than in patients with NTG with stable visual field defects. Nocturnal blood pressure fluctuation in the 'progressive' patients was significantly greater than in the 'stable' patients (Kashiwagi *et al.*, 2001). These observations suggest the relative absence of a nocturnal BP dip and greater blood pressure swing may be interpreted as underlying mechanism that disturbs the microcirculation at the ONH leading to the visual field progression in both HTG and HTG. Indeed, non-dipper subjects were also found in essential hypertensive patients characterized with a decreased physiological circadian fluctuation on autonomic functions compared with dipper subjects (Kohara *et al.*, 1995). It has been demonstrated that the non-dipping phenomenon is closely related to a high incidence of cardiovascular diseases, a poor long-term survival and profound autonomic dysfunction (Liu *et al.*, 2003).

5.3.2 Direct identification of systemic autonomic dysfunction in glaucoma

Systemic autonomic dysfunction has been recognised in glaucoma for almost 30 years. Clark and Mapstone examined circulatory autonomic responses to various laboratory stimuli in 89 patients with primary closure angle glaucoma, 99 patients with primary open angle glaucoma and 76 control subjects. Apart from the findings of patients with closure angle glaucoma, patients with POAG exhibited significantly lower heart rate variation to deep breathing and immediate active standing testings than control subjects. Since the heart rate variation to the above stimuli relies on

intact parasympathetic efferent activity, the authors indicated an impaired parasympathetic function in POAG (Clark *et al.*, 1985). In a following study the researchers used the above standardised techniques and observed that parasympathetic neuropathy was present in 37.3% of 67 open-angle glaucoma patients compared with only 2.6% of 76 control subjects (Clark *et al.*, 1986). Kumar and Ahuja investigated the cardiovascular reflex responses to standardised laboratory stimuli in 50 patients with POAG and 50 control subjects, and found attenuated sympathetic responses in 36 out of 50 patients (73%) and decreased parasympathetic activity in 43 of 50 patients (86%), suggesting POAG is associated with impaired autonomic nervous system (Kumar *et al.*, 1999).

The baroreflex-mediated autonomic function was further studied in glaucoma patients. In 1984, Demailly et al (Demailly *et al.*, 1984) reported the mean difference of systolic blood pressure from lying to standing position was significantly greater in the NTG patients (6.9 mm Hg fall in systolic blood pressure) than in the HTG patients (1.2 mm HG) and the control group (1.5 mm Hg). This suggests that postural hypotension could play a role in the pathogenesis of NTG. Indeed, orthostatic stress is well-established laboratory stimuli to elicit baroreflex-mediated autonomic response (Montano *et al.*, 1994) and commonly used in clinical setting in examining patients with orthostatic intolerance (Robertson *et al.*, 1993). Riccadonna et al investigated autonomic response to passive head-up tilting in 17 NTG patients, 13 HTG patients and 17 control subjects. With power spectral analysis of heart rate variability calculated, researchers revealed that in response to orthostatic stress, the reduction of high frequency components of heart rate variability was attenuated in NTG patients, in particular those with progressive visual field damage (Riccadonna *et al.*, 2003). This study suggests that autonomic dysfunction of cardiovascular response may be a contributing pathogenetic factor in NTG, inducing a chronic ischemia of the optic nerve. In order to examine the baroreflex-mediated autonomic function in glaucoma, a German group performed sinusoidal neck suction (to artificially activate baroreceptors at 0.1 Hz and 0.2 Hz and examine sympathetic and parasympathetic responses respectively) in 14 HTG, 15 NTG and 17 controls. The responses of RR-interval low- and high- frequency oscillations were attenuated in

both HTG and NTG patients compared to controls; low frequency power of diastolic blood pressure increased significantly in controls but not in the two types of glaucoma patients. This study indicates the impaired cardiovascular autonomic responses to baroreflex simulation exist in both HTG and NTG (Brown *et al.*, 2002).

Gherghel et al (Gherghel *et al.*, 2004a) examined systemic autonomic responses and ocular vascular reactivity to temperature provocation in 24 POAG patients and 22 normal control subjects. In response to immersion of the right hand in 40°C warm water, patients exhibited an increase in diastolic blood pressure, heart rate and mean ocular perfusion pressure; in the following cold provocation test, patients manifested significant reduction in both ocular blood flow at the neuroretinal rim area and finger nailbeds capillary blood flow as expected. In contrast, in control subjects, blood pressure remained unchanged during warm provocation and there was a cold pressor response accompanied with a decrease in finger blood flow but not a reduction in ocular blood flow. Therefore, the study demonstrated that, while control subjects have normal blood pressure response to warm and cold provocation with ocular autoregulation, the paradoxical blood pressure responses in POAG patients suggest a systemic autonomic failure, which underlies the disturbed ocular blood flow as detected (Gherghel *et al.*, 2004a).

Heart rate variability has been used in examining circadian rhythm related autonomic dysfunction in glaucoma. Riccadonna et al found that a lower diurnal heart rate variability and nocturnal blood pressure variability in NTG patients compared with HTG patients and control subjects based on 24 hour blood pressure monitoring data (Riccadonna *et al.*, 2003). A Korean study examined the short term heart rate variability, i.e. 5 minutes supine ECG recording after 30 minutes resting during daytime in 77 newly diagnosed and untreated NTG patients compared with 30 healthy controls, and found that a significant reduction in heart rate variability in NTG patients (Na *et al.*, 2010). Importantly, a following report from this research group revealed that visual field defects were more frequent and deepest in NTG patients with lower heart rate variability and abnormal nailbeds capillary response, and central visual field defect is characteristic in this type of NTG patients (Park *et al.*,

2012). Indeed, reduction in heart rate variability relative to cardiovascular risks is well demonstrated in the Framingham Heart Study (Tsuji *et al.*, 1996).

Power spectral analysis of heart rate variability can determine the modulation of sympathetic and parasympathetic nervous system activity affecting the heart using indexes of low- and high- frequency spectral power. In particular, LF/HF ratio may be a reliable index of the sympathovagal balance (Malliani *et al.*, 1991). Kashiwagi et al recorded 24 hours blood pressure data in 32 NTG patients and 32 normal control subjects and found a significantly greater low-frequency spectral power of patients with NTG during the spans of an active day and a resting night than those of normal subjects, and this difference was particularly evident during the night resting span and those with progressive visual field defects, suggesting that a disturbance of the circadian rhythm of the autonomic nervous system may exist in NTG patients (Kashiwagi *et al.*, 2000). Na et al (Na *et al.*, 2010) showed that during daytime, short term resting LF spectral power is lower but LF/HF ratio was higher in NTG patients than in healthy controls, suggesting abnormal autonomic regulation underlie the pathogenesis of NTG. More recently, a study compared the 24 hour blood pressure profile in 54 NTG patients with 43 control subjects and found a significantly higher LF, LF/HF and lower HF values in NTG patients than in control subjects for the 24-h, daytime and night-time periods. In particular, the researchers confirmed that 'Dippers', 'non-dippers' and 'overdippers' with NTG showed significantly higher LF/HF ratio as compared to the same subgroups of control subjects (Wierzbowska *et al.*, 2012). Therefore, these observations demonstrated that sympathovagal balance of autonomic nervous system in patients with NTG shifted towards sympathetic predominance (Na *et al.*, 2010; Wierzbowska *et al.*, 2012). Sympathetic overdrive is prominent cardiovascular risks leading to detrimental consequences in glaucoma.

Gherghel et al investigated the 24 hours circadian rhythm associated autonomic regulation in a group of consecutive, newly-diagnosed and untreated HTG patients compared with matched control subjects. Overall glaucoma patients showed higher LF spectral power and LF/HF ratio than normal control subjects for both the diurnal (active) and nocturnal (passive) period. In subgroup data, glaucoma patients with normal ECG (representing free from cardiac ischaemia in this study) showed higher

LF and LF/HF values during the active period than normal control subjects; whereas glaucoma patients suffering from silent cardiac ischaemia exhibited similar HRV parameters to the counterpart subjects in control group. This study demonstrates HTG patients manifest autonomic dysfunction, independent of silent cardiac ischaemia(Gherghel *et al.*, 2007). Recently, Gherghel and coworkers (Mroczkowska *et al.*, 2013) investigated multiple lines of macro- and micro- vascular function in 19 HTG patients, 19 NTG patients and 20 control subjects. Compared with controls, patients with HTG and those with NTG exhibited similarly increased nocturnal systemic blood pressure variability, peripheral arterial stiffness, carotid intima-media thickness, and reduced ocular perfusion pressure. Also, both glaucoma groups exhibited abnormal retinal vascular reactivity to flicker light stimuli. The researchers demonstrated multiple comparable alterations in systemic and ocular circulation in the early stage of their disease process, therefore concluded that the importance of considering vascular risk factors in both NTG and HTG (Mroczkowska *et al.*, 2013).

5.3.3 Vascular dysregulation and retinal blood flow instability

Vascular dysregulation is a condition in which blood flow is not properly distributed to meet the demands of different tissues leading to hyper-perfusion or hypo-perfusion (Flammer *et al.*, 2001). Primary vascular dysregulation (PVD) and a secondary vascular dysregulation should be differentiated. The latter is usually a consequence of an autoimmune disease, such as rheumatoid arthritis, multiple sclerosis, leading to a marked increase in endothelin, which mainly reduces ocular blood flow, without significant impact on ocular autoregulation (Gherghel *et al.*, 1999). In contrast, PVD has an inherited component, and most subjects are healthy. However, they do have a higher chance to develop certain diseases attributable to the unstable perfusion to vulnerable tissues. Notably, PVD is the main cause for the disturbed autoregulation of ocular blood flow. The unstable ocular blood flow often presents decades before glaucomatous optic neuropathy develops, although endothelin levels may not be particularly high (Flammer *et al.*, 2002; Flammer *et al.*, 2001).

There is no ‘gold standard’ investigation to diagnose PVD. Cold hands, due to vasospasm, can be a common presentation of the subjects due to inappropriate constriction or insufficient dilation of the smooth muscles of the arterioles, leading to

transient hypoperfusion of the terminal tissue. Cold hands is a reported feature in a subgroup of PVD patients with visual field defects. Gasser and Flammer compared the capillary blood flow velocity in the finger nailbeds of 30 patients with NTG, 30 patients with HTG and 30 control subjects using nailbedcapillaroscopy and found a significant reduction of blood flow velocity in NTG patients compared with control group, which was more pronounced after cold provocation (Gasser *et al.*, 1991). Guthauser et al provided an evidence that significant reduction in blood flow of finger nailbed capillary provoked by local cold water immersion is related to a concurrence of transient deterioration of visual field defects in NTG patients (Guthauser *et al.*, 1988).

Vasospasm is not restricted to patients with NTG but may also be a feature of HTG (Flammer *et al.*, 2001). Drance and coworkers(Schulzer *et al.*, 1990) reported that in 26 patients with NTG and 34 patients with HTG, there were overall 15 patients exhibited vasospastic finger blood flow values, which are highly positively correlated with visual field severity and the highest intraocular pressure (Schulzer *et al.*, 1990). In an epidemiological study, Broadway and Drance screened glaucoma patients with optic disc appearances potentially representative of four classic primary open angle glaucoma subgroups, i.e. focal ischaemic, myopic glaucomatous, senile sclerotic, and those with generalised cup enlargement; the researchers revealed that patients with focal ischaemic discs contained more women and had a higher prevalence of vasospasm and cold extremities, irrespective of IOP (Broadway *et al.*, 1998).

Endothelial dysfunction, i.e. the imbalance of vasoconstrictor and vasodilator vasoactive substances may underlie the mechanism of vascular dysregulation. ET-1 as a potent vasoconstrictor may have a role in the pathogenesis of such vascular dysregulation (Haefliger *et al.*, 1999; Nicolela, 2008). Raised ET-1 plasma levels is found evident in NTG patients (Kaiser *et al.*, 1995). Patients with both NTG and HTG had a significant increased plasma ET-1 in response to provocative cooling testing. As noted, patients with glaucoma who had evidence of acral vasospasm were more likely to show deterioration in visual fields after cooling than patients without acral vasospasm. These observations suggest at least in some patients the abnormal

increase in plasma ET-1 in response to vasospastic stimuli may be involved in the pathogenesis of glaucomatous damage (Nicolela *et al.*, 2003).

5.3.4 Interaction of endothelial function and autonomic nervous system

Endothelial dysfunction is recognised as an early sign of cardiovascular atherosclerotic diseases, such as hypertension, diabetes, coronary artery disease (Flammer *et al.*, 2012). The endothelial function may predict the disease progression and cardiovascular event rates. In a prospective study, patients suffering from cardiovascular events had significantly increased vasoconstrictor responses to acetylcholine infusion (NO-dependent) and cold pressor testing (Schächinger *et al.*, 2000). Accumulating evidence demonstrates the imbalance of ET-1/NO system in glaucoma patients, suggesting the existence of systemic endothelial dysfunction in the pathogenesis of glaucoma (Galassi *et al.*, 2011; Henry *et al.*, 1999; Kaiser *et al.*, 1995; Polak *et al.*, 2007; Sugiyama *et al.*, 1995).

The interaction of endothelial function and sympathetic nervous system is well documented. Over-expression of endothelial NO synthase (NOS-3) in the rostral ventrolateral medulla causes hypotension and bradycardia (Kishi *et al.*, 2001), and endogenous overactivity of NOS-3 in the nucleus tractussolitarii contributes to hypertension (Waki *et al.*, 2006). This suggests a central role for NO activation in regulating the set-point of the sympathetic nervous system. On the other hand, sympathetic activation causes transient endothelial dysfunction detected by the brachial flow-mediated dilation after brief episodes of mental stress (Dietz *et al.*, 1994; Ghiadoni *et al.*, 2000). In addition, acute sympathetic activation induced by unloading baroreceptor significantly attenuates flow-mediated dilation response in the brachial artery, and this effect is completely abolished by Local α-adrenergic blockade (intra-arterial infusion of phentolamine), but not nitroglycerin. This suggests shear-stress induced NO release in the macrovasculature is mainly impaired by an alpha-adrenergic mechanism (Hijmering *et al.*, 2002). The sympathetic effect on the NO bioavailability also observed in coronary artery (microvasculature) via intracoronary Doppler catheter. It is reported that the coronary microcirculation of patients with coronary heart disease failed to dilate during sympathetically mediated

mental stress, suggesting a reduction of NO bioavailability (Dakak *et al.*, 1995; Yeung *et al.*, 1991).

Retinal microvasculature shares common features with the cerebral artery (Patton *et al.*, 2005) and the coronary artery of the heart (Flammer *et al.*, 2013). Many cardiovascular diseases may show distinct retinopathies with signs of disease progression, such as hypertension and diabetes (Flammer *et al.*, 2013). Lately, giving the advancement of non-invasive retinal vessel investigation tools, the easily accessible retinal microvasculature may provide a unique and non-invasive window to evaluate endothelial dysfunction, which may define the development and progression of cardiovascular diseases (Koç *et al.*, 2013; Liew *et al.*, 2008) and predict cardiovascular risk (McGeechan *et al.*, 2009; McGeechan *et al.*, 2008). In patients with coronary artery disease (Heitmar *et al.*, 2011) and individuals with impaired glucose tolerance (Patel *et al.*, 2012), retinal and finger nailbed microvascular dysfunction and endothelial dysfunction detected by flow-mediated dilation and carotid-artery intimal media thickness were concomitantly present.

In glaucoma, a new technique for examining the dynamic retinal vessel function is accumulating confirmatory evidence relative to both ocular and systemic vascular dysfunction. In normal tension glaucoma patients, impaired nailbed capillary response and retrobulbarhaemodynamics are accompanied with higher plasma ET-1 concentration (Galassi *et al.*, 2011). The coexistence of macrovascular abnormality, e.g. increased carotid artery initimal media and pulse wave velocity (index of arterial stiffness) and microvascular abnormality, e.g. dynamic retinal vascular response to flickering light, emphasises the need to investigate systemic rather than only local vascular dysregulation, which may underlie the development and progression of NTG (Mroczkowska *et al.*, 2012). More recently, based on the above findings, Gherghel and coworkers further demonstrated that both NTG and HTG patients exhibited similar ocular and systemic vascular function loss (Mroczkowska *et al.*, 2013).

5.3.5 Choroidal blood flow regulation instability

Choroidal blood flow is directly innervated by sympathetic nerve system (Nickla *et al.*, 2010). Based on choroidal circulation and the neuroretinal rim of the optic nerve glaucoma patients were divided into higher ocular blood flow and lower blood flow groups, 80% patients with lower ocular blood flow values showed a vasospastic response in nailbedcapillaroscopy, whereas only 46% counterpart patients manifested vasospasm, suggesting impaired innervation of choroidal circulation may play a part in the mechanisn of vascular dysregulation (Emre *et al.*, 2004).

Choroidal blood flow autoregulation may be important in adapting ocular perfusion pressure changes and exercise-induced changes in blood pressure (Polska *et al.*, 2007). During isometric exercise, choroidal blood flow is regulated better than blood flow at the ONH (Schmidl *et al.*, 2012). During hand-grip exercise, normal healthy subjects with positive cold hands history showed reduced choroidal blood flow than normal subjects without cold hands history, suggesting sympathetically-mediated inappropriate vasoconstriction or insufficient dilation (Gugleta *et al.*, 2003). In another study, in the resting state, both glaucoma patients, and subjects with pure ocular hypertension, showed lower choroidal blood flow than normal subjects; however, during isometric exercise, with comparable blood pressure levels across groups, glaucoma patients but not subjects with pure ocular hypertension, showed significant greater choroidal blood flow than normal subjects, suggesting less autonomic regulatory capacity of choroidal blood flow to exercise-induced blood pressure elevation may underlie the pathogenesis of glaucomatous damage (Portmann *et al.*, 2011).

6. Thesis outline and study aims

I therefore propose that the measurement of autonomic function may be a useful tool in health and disease.

Thus, the aims of this project were to address the following three hypotheses:

1. In healthy young men, there is a cardiac sympathetic activation and vagal inhibition in response to quiet standing (orthostatic stress), and an increased vascular sympathetic outflow to carbohydrate rich meal ingestion; autonomic

regulation and cardiac adaptation are associated with the haemodynamic changes to the interplay of quiet standing and meal ingestion, i.e. orthostatic stress in the postprandial state (See Chapter Three: Result One).

2. There are ageing effects on basal sympathetic nerve activity and autonomic regulation in response to quiet standing and meal ingestion between young men and older men; and there are distinct features of autonomic regulation to the above daily stressors between older men and older women, in particular in response to meal ingestion (See Chapter Four: Result Two).
3. Open angle glaucoma patients manifest systemic autonomic dysfunction; and there are distinct features of autonomic modulation to meal ingestion and orthostatic stress in the two forms of open angle glaucoma, i.e. high tension glaucoma and normal tension glaucoma (See Chapter Five: Result Three).

Chapter Two: Methodology

The following section describes methods commonly used during studies. Please refer to each Chapter for specific methods used for individual studies.

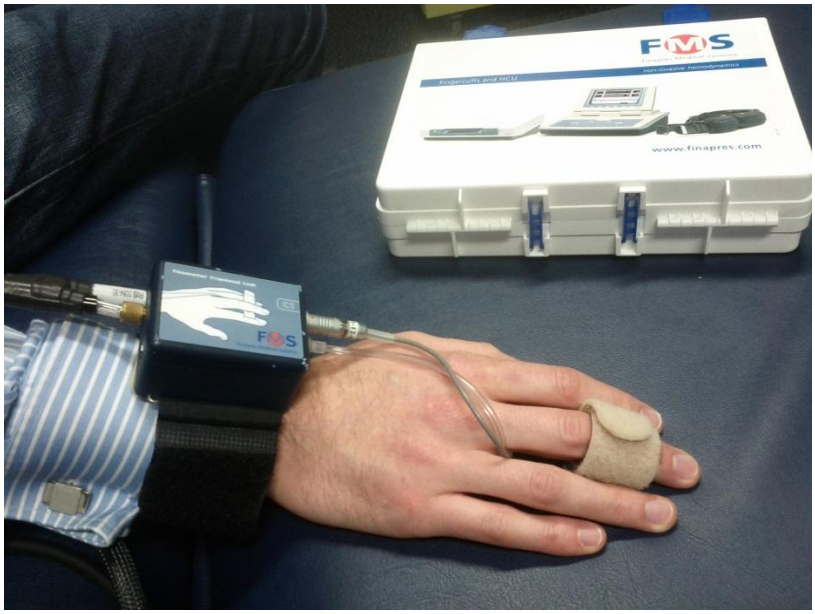
1. Ethical Approval

All experimental procedures and protocols were approved by the Human Ethics Committee of Macquarie University, Sydney, NSW, Australia.

2. Study participants

All young volunteer subjects (N=14) were recruited from a population of postgraduate students in our Department (Australian School of Advanced Medicine, Macquarie University). Subjects received email information of our study advisement, and voluntarily make their initial contact with the investigator (Lei Cao) of the study. Study information documents were provided for the potential volunteer participants to read, including a sheet of participant Information, a sheet of instruction for the experiment day and information consent form. The investigator was responsible for answering all relevant questions from each potential participants and following up their wishes and interests for a consideration of participation without coercion.

Newly or previously diagnosed glaucoma patients (N=36), aged from 45-80 years old, were randomly recruited from Macquarie Ophthalmology clinic. These patients were asked for a permission to be contacted by the study investigator during specialist consultation (Professor Stuart Graham) at Macquarie Ophthalmology clinic. In addition, a proportion of diagnosed glaucoma patients (N=20), and all volunteer subjects without glaucoma (served as control, N=52) were randomly recruited from local communities (see Chapter 5. Figure 1.Recruitment Flowchart). All the participants sourced from local communities were attracted by the study advisements in local newspapers, and expressed their interest for participation to the investigator via telephone or email.



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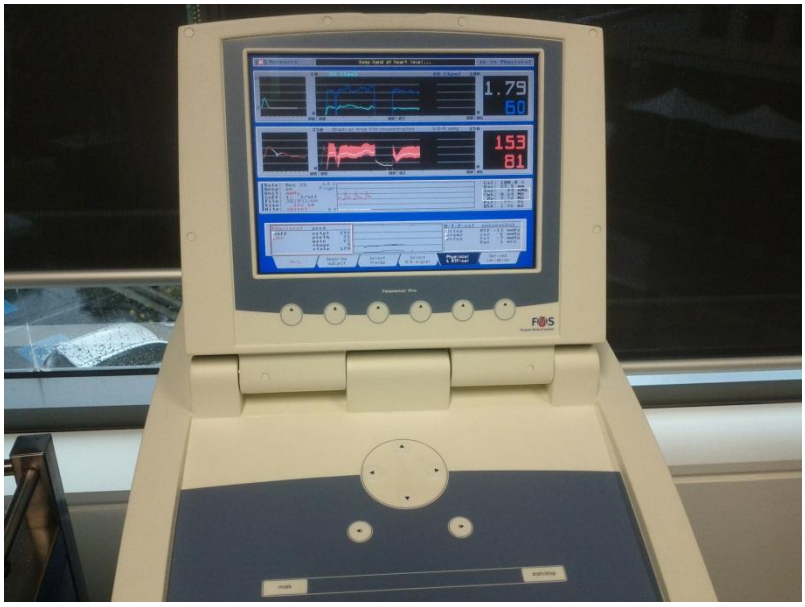

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Figure 1 A. The finger cuff is wrapped around on the middle finger of a representative subject, being attached to the frontend unit placed on the wrist, and connected to the cable of the Finometer main part.

Figure 1B. The main part of the Finometer with built-in acquisition system, being able to display beat-to-beat finger blood pressure waveform, and hemodynamic parameters, such as, blood pressure, heart rate, and estimated cardiac output etc.

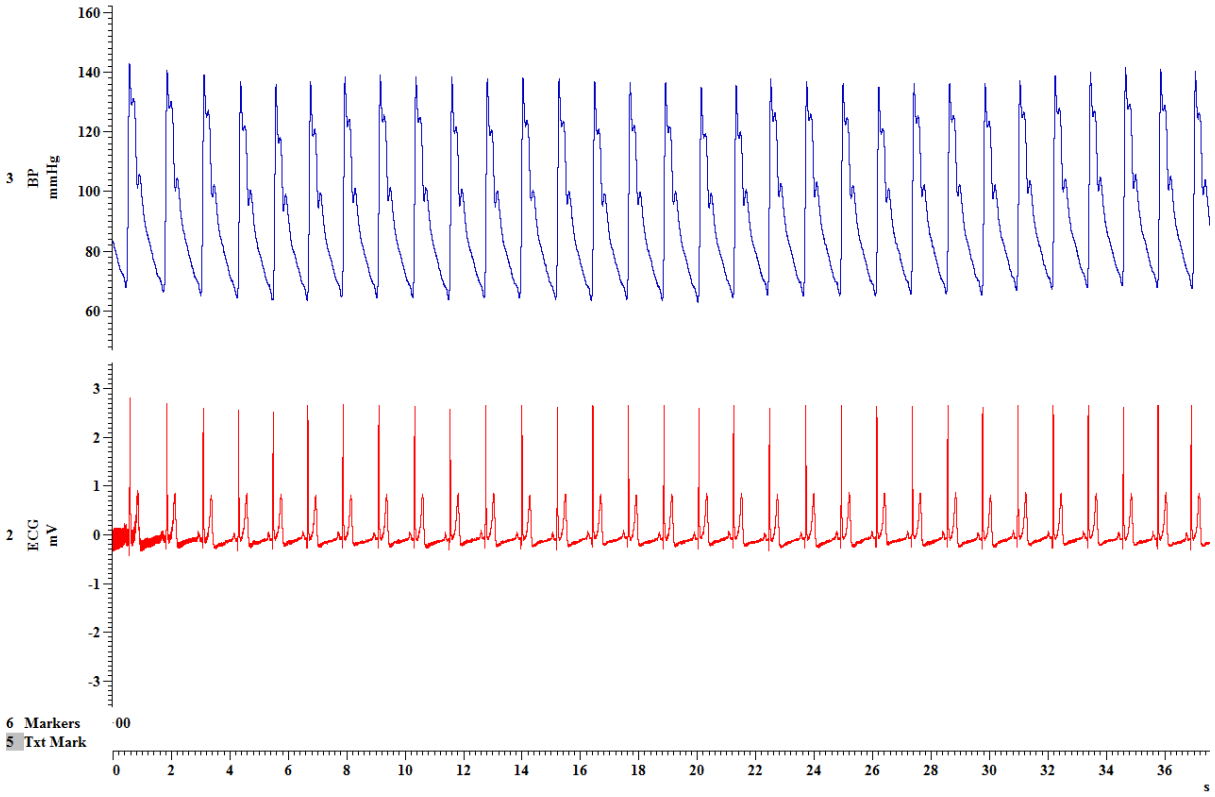


Figure 2. The diagram showing the LabChart records and displays the beat-to-beat blood pressure waveform (top, blue) and ECG (bottom, red) in two different channels.

The investigator explained the purpose and procedures of the study, and sent a sheet of participant Information, a sheet of instruction on the experiment day and information consent form to each potential volunteer participant by email or post mail. The investigator was responsible for answering all relevant questions from each potential participant and following up their wishes and interests for a consideration of participation without coercion.

All subjects were recruited according to the study selection criteria, i.e. no significant history of cardiovascular diseases, no severe heart failure, no heart attack or recent bypass surgery, no severe cardiac arrhythmia, no complicated diabetes, not on vasoactive medication. All subjects recruited from local communities were confirmed with, or without glaucoma by a medical record check, a retinal nerve fibre layer (RNFL) scan (Spectralis OCT, Heidelberg Eng, Germany) after an experiment, and a specialist clinical investigation by Professor Stuart Graham in suspected subjects (See detailed information in Chapter Five).

Prior to the experiment, all subjects were instructed not to drink water 1.5 hours prior to experiments, and to abstain from caffeinated beverages and food for 12 h, alcohol for 24 h, and moderate or strenuous physical activity for 48 h prior to the experimental sessions. On the experiment day, the investigator welcomed the subjects and offered a detailed verbal and written explanation of the intended experimental protocol and measurements, and each subject provided written information consent. For all glaucoma patients and volunteer control subjects, a screening blood test in fasting state was arranged in the pathology officer of Macquarie University Clinic at about 8:45 am on the experiment day. The blood test incorporates renal function, liver function, lipid profile and plasma glucose and insulin. After blood collection, participants were escorted to a cardiovascular laboratory at the Australian School of Advanced Medicine, Macquarie University at about 9am. The cardiovascular lab's temperature is centrally air-conditioned and maintained at 23 degree of Celsius throughout the experiment. The laboratory environment and equipment were introduced to the each subject. A medical history was taken from each subject, paying particular attention to eye and cardiovascular disease, and medication use. Anthropometric measurements comprised body weight, BMI, waist

circumference, and waist/hip ratio. Patient comfort and calmness were observed and appreciated by the investigator throughout the experiment. Subject's privacy was protected and confidentiality is maintained at all time.

3. ECG recording

Subjects were instructed to supine position in a laboratory bed. Three ECG electrodes were attached to the skin; two electrodes were gently placed on the left and right upper chest just under the clavicle (near the shoulder tip but deviating from the high movement muscles to minimise artefacts), and the other one was placed 3-4cm laterally and inferiorly to the heart apex. The ECG wires are then clipped and connected to the three electrodes. The bio-electrical activity of the heart was detected and input into a biological signal amplifier – Bio Amps. The Bio Amps is connected a data acquisition device - Powerlab 8/30, where the biological ECG signals were converted into digital data. A sampling rate of ECG 1000Hz was chosen to ensure the accuracy of HRV analysis. The converted digital data are then transferred to and stored by LabChart 7.2 software in a computer for off line analysis. All the above devices (Bio Amps, Powerlab 8/30, and LabChart 7.2.) are sources from ADInstruments, Bella Vista, Sydney, NSW, Australia.

4. Finger arterial pressure recording

Continuous blood pressure waveform can be non-invasively recorded from a finger cuff via volume-clamp technique, first reported by Ian Penaz in early 1970s. The technique is improved by Wesseling and colleagues, resulting in a device commercialised as the 'Finapres'. An infrared photo-plethysmograph device embedded in a small finger cuff to measure the finger arterial blood volume. The blood volume signal is clamped through a source of compressed air that is connected with the finger cuff. The volume-clamp periodically adjusts to maintain the finger arteries at a set-point where the intramural pressure is zero, therefore, the finger cuff pressure induced by the compressed air pump always reflects intra-arterial pressure (Wesseling *et al.*, 1985).

The finger cuffs are available for three sizes: small, medium and large. The suitable finger cuff size was carefully chosen by the investigator and wrapped around a middle finger of subject. The finger cuff is attached to the frontend unit (air pump compressor) that is placed and gently fixed on the dorsal aspect of the subject's wrist (Figure 1. A.). The frontend unit is then connected the main unit called Finometer Pro via a cable (2.75 meter in length). The Finometer Pro has its own built-in acquisition system, and sampling rate is fixed at 200Hz. All the devices are sourced from Finapres Medical Systems (Ohmeda, Amsterdam, Netherland) (Figure 1.B.). The digital signal of arterial pressure waveform is able to be transferred to the Powerlab and spontaneously shown in the monitor of the computer and stored by Labchart 7.2. (Figure 2.). Once connected, a trial demonstration for the blood pressure recording was then conducted by the investigator to ensure the operation of the Finometer and minimise any concerns from the patient.

To minimise hydrostatic errors, the subjects were instructed to keep the testing finger at the heart level throughout the study, for example, placing hands along the body during supine position, and in particular bending the elbow and keeping hands to the heart level during quiet standing. To keep the hand at the heart level is the priority and the best, otherwise correct systolic and diastolic pressures are not guaranteed due to the change in pressure waveform, even if a height correction system is used (Imholz *et al.*, 1993).

The local temperature of subjects' hands is an important factor in influencing the establishment and detection of signals of finger blood flow while using the Finometer. Besides the ambient temperature is air-conditioned and maintained at 23 degree of Celsius, each subject is regularly asked for feeling warm; otherwise a blanket was provided to cover the whole body. Despite feeling warm, in some patients and subjects, cold hands were noted. Peripheral arteriolar vasoconstriction is likely to affect the quality of finger blood pressure measurement (Wesseling *et al.*, 1985). Measures were taken during experiments, such as rubbing hands, covering with blanket and local heat packs to improve the peripheral blood flow and achieve satisfactory signals.

5. Power spectral analysis of HRV and BPV

5.1 Introduction

"Power spectral density (PSD) analysis provides the basic information of how power (i.e. variance) distributes as a function of frequency. Independent of the method employed, only an estimate of the true PSD of the signals can be obtained by proper mathematical algorithms"(Malik, 1996).

Methods for the calculation of PSD may be generally classified as non-parametric and parametric. In most instances, both methods provide comparable results. The advantages of the non-parametric methods are: (a) the simplicity of the algorithm employed (Fast Fourier Transform in most of the cases) and (b) the high processing speed, whilst the advantages of parametric methods are: (a) smoother spectral components which can be distinguished independently of preselected frequency bands, (b) easy post-processing of the spectrum with an automatic calculation of low and high frequency power components and easy identification of the central frequency of each component, and (c) an accurate estimation of PSD even on a small number of samples on which the signal is supposed to maintain stationarity. The basic disadvantage of parametric methods is the need to verify the suitability of the chosen model and its complexity (Malik, 1996).

5.2 Duration and accuracy of ECG and beat-to-beat arterial pressure recordings

In order to standardise different studies investigating short-term HRV and BPV, a five-minute of continuous recordings of ECG and arterial pressure waveform signals was used in all studies, which contains at least 256 cardiac cycles (≥60 cycles/min * 5min). A 5 minute ECG and arterial pressure recording was obtained in the supine posture and during standing, in fasting state and in postprandial state at each time point (30, 60, 90, 120 minutes). According to the HRV guideline in clinical use, the 5 min recording can be considered to be shorter as a stationary blood pressure signals is difficult to maintain (Malik, 1996). To our experience, a five-minute short-term recording of ECG and arterial pressure were easily accessible. In a few cases, a three or four minutes of recording was considered in patients who developed frequent ectopic beats in ECG tracings (for example, subjects with pre-existing heart

disease), or finger arterial pressure waveform was not maintained stable (for example, normal tension glaucoma patients with cold hands), in particular during orthostatic stress or early after eating in which there is relatively significant haemodynamic and autonomic challenge with associated peripheral vasoconstriction.

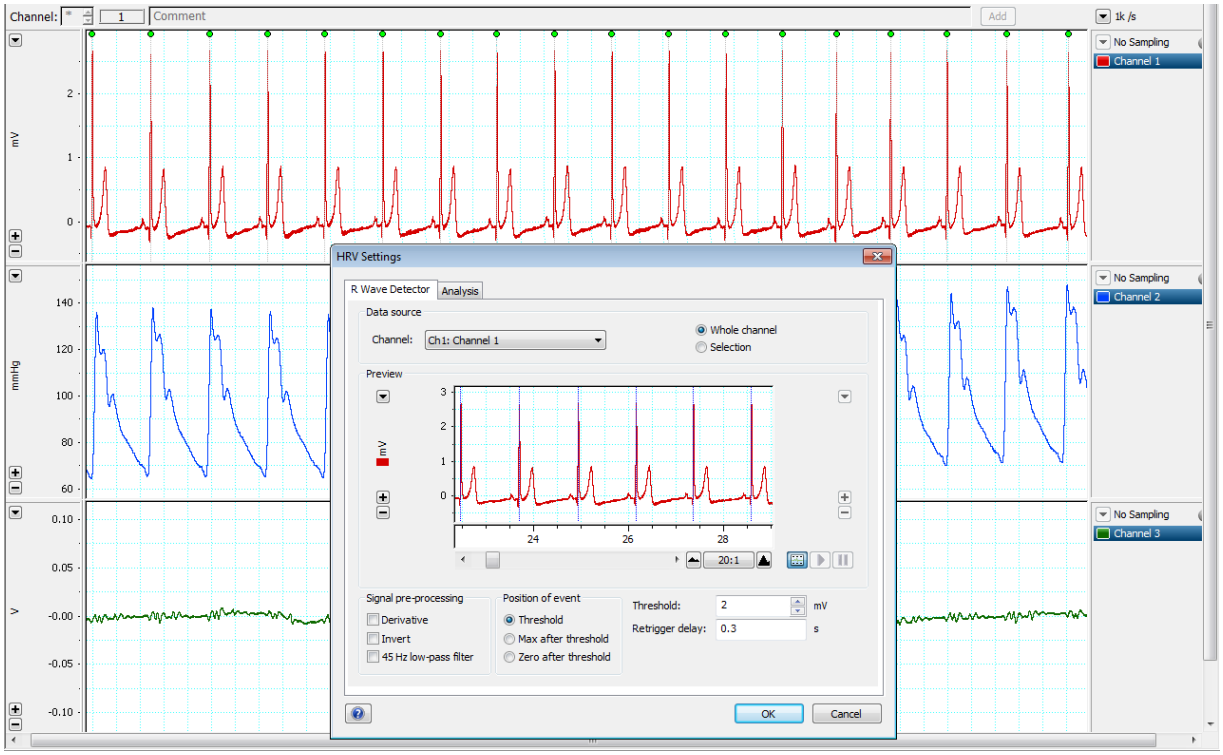
One of the advantages of short-term recording is free of interference. Ectopic beats, arrhythmic events, missing data and noise effects was easily cleared using LabChart 7.2 software tool. Visual inspection and manual correction of the ECG and arterial pressure tracings are used in obtaining accurate ECG arterial pressure waveforms for power spectral analysis; this may introduce significant selection bias, in particular under greater physiological challenge (not “stationary”) or in a diseased heart that has frequent ectopic or arrhythmic events. Medical history and baseline recordings of ECG provide information to minimise this concern.

5.3 Spectral analysis of HRV and BPV using Fast Fourier Transform (FFT)

The location of electrodes and the appropriate ECG wires connection as mention above determines a limb lead II shown in the ECG waveform; there is a normal vector of QRS complex. It can be critical for choosing the fiducial point of the ECG QRS. A well-tested algorithm was used in order to locate a stable and noise-independent reference point. Visual inspection was also used to recheck the fiducial point for each tracing although this may be time consuming.

LabChart 7.2 has a built in HRV module that provides a R wave detector. A threshold value was chosen according to the R wave magnitude of an individual ECG tracing, and a retrigger delay of 0.3 second was used in almost all cases (Figure 3 A.). In HRV analysis module, a default setting was used in all analysis: FFT bin size at 1024Hz, frequency domain defined as very low <0.04Hz, low frequency between 0.04-0.15Hz, and high frequency between 0.15-0.4Hz), and Hanning window was chosen here (Figure 3 B.).

Power spectral analysis of HRV can also be calculated using Spikes2 software (Cambridge Electronic Design Limited, Cambridge, England). Each beat-to-beat R wave peak is found and the value of peak to peak interval (RR interval) can be plotted in a time series in x-axis, so called tachogram (Figure 4.). The tachogram is



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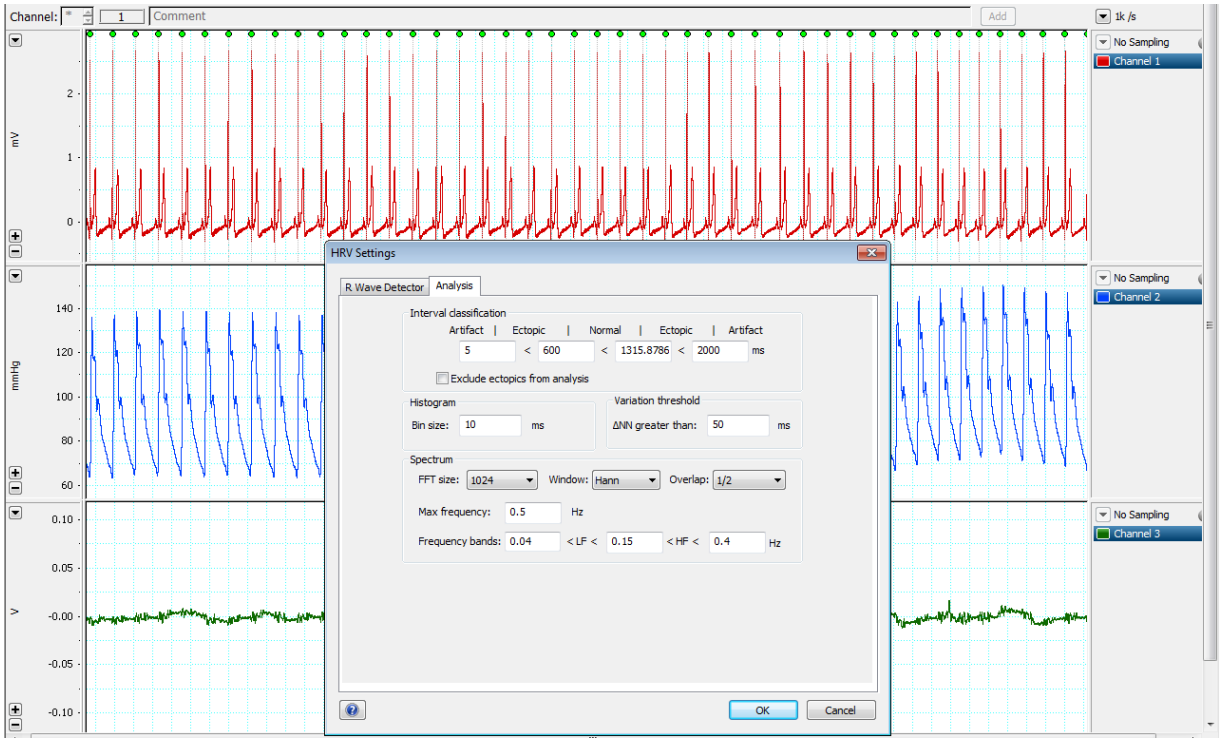

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Figure 3 A, B. The diagrams demonstrate the LabChart 7.2. (HRV module) provides function of step-by-step calculation of heart rate variability using the “HRV settings” dialogue box. A) Detection of the peak of QRS complex. B) Parameters setup for power spectral analysis of heart rate variability.

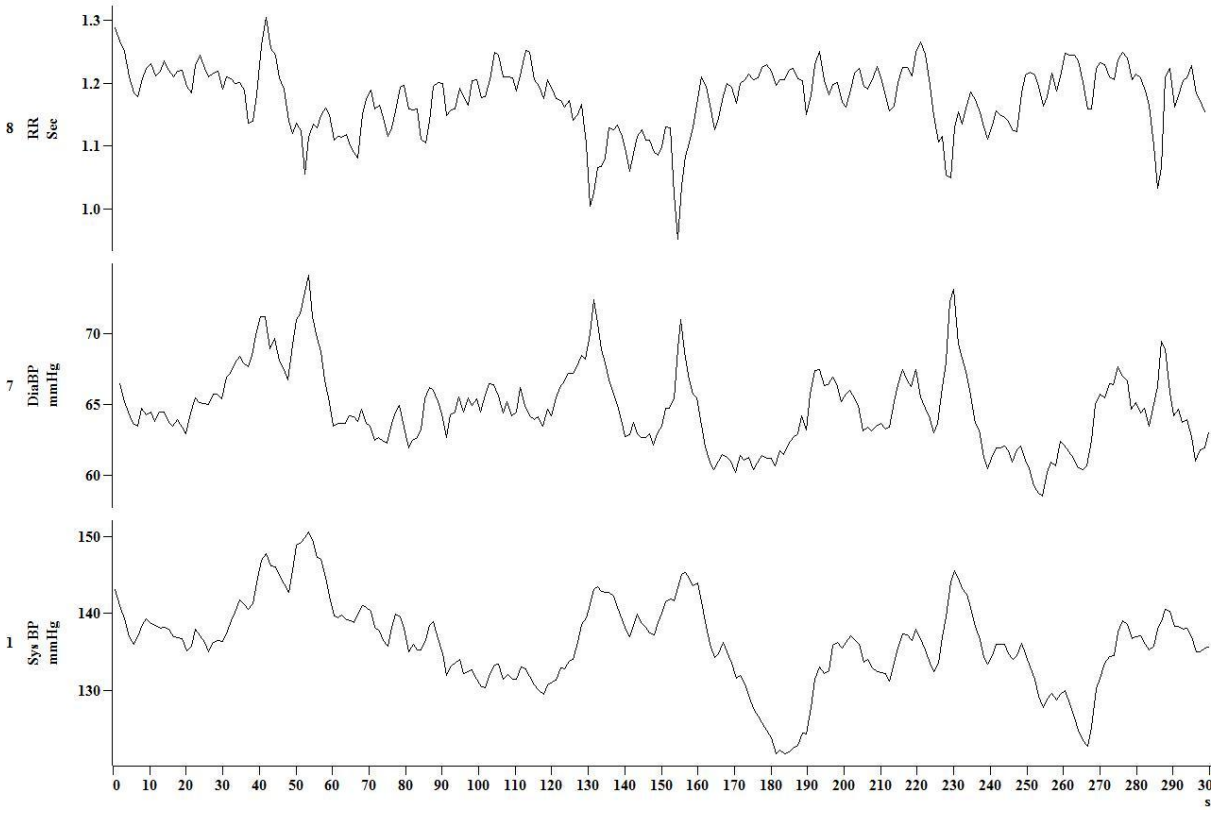

Figure 4. Power spectral analysis of HRV can also be calculated using Spikes2 software. The top channel shows a tachogram in a representative subject, i.e. beat-to-beat variations (fluctuations) of RR intervals in a period of 300 seconds. The middle and bottom channels depict beat-to-beat amplitude changes (fluctuations) of diastolic and systolic blood pressure in the same period of time.

decomposed into power spectral density in different frequency domains (i.e. low frequency and high frequency). Fast Fourier Transform (Hanning window; 512 block size) was applied to the R-R interval. The low-frequency refers to 0.04 - 0.15 Hz and high-frequency refers to 0.15 - 0.40 Hz. Signal powers of each frequency band were calculated as integrals under the respective power spectral density functions and expressed in absolute unit ($msec^2$) (Figure 5.). The method of power spectral analysis of blood pressure variability (BPV) is the same as HRV as described above; simply, the peak and/or trough of beat-to-beat arterial waveform are found, and the fluctuations of systolic and diastolic blood pressure are shown as in a time series respectively (Figure 4.); the fluctuations of blood pressure amplitudeare then decomposed using Fast Fourier Transform algorithm (Hanning window; 512 bin size) to obtain power spectral estimates of systolic/diastolic BPV (Figure 6.). The absolute value of low frequency (0.04 - 0.15 Hz) systolic/diastolic blood pressure spectral powers (mmHg2) are markers of sympathetic vasomotor modulation (Pagani *et al.*, 1986).

6. LF component of cardiovascular variability in comparison with microneurography and noradrenaline spillover methods

6.1 LF and HF components of HRV

With the recognition of a significant relationship of autonomic nervous system and cardiovascular mortality, including hypertension (Guzzetti *et al.*, 1988; Lucini *et al.*, 2002a), ischaemic heart disease (Tsuji *et al.*, 1996), obstructive sleep apnoea (Trimer *et al.*, 2013), and sudden death (Lown *et al.*, 1976), there is a growing need to find simple quantitative markers of autonomic regulation.

The two parameters - Heart rate variability (HRV) and blood pressure variability (BPV) - are considered to be promising simple tools for cardiovascular research and clinical studies. HRV analysis has been commercially available and widely used in clinical studies based on a guideline published in 1996 (Malik, 1996).

Beat-to-beat variations of RR-interval and systolic/diastolic blood pressure can be shown in a time-series analysis. It is noted that the time domain analysis was initially based on simple statistics, for example the standard deviation of RR-interval

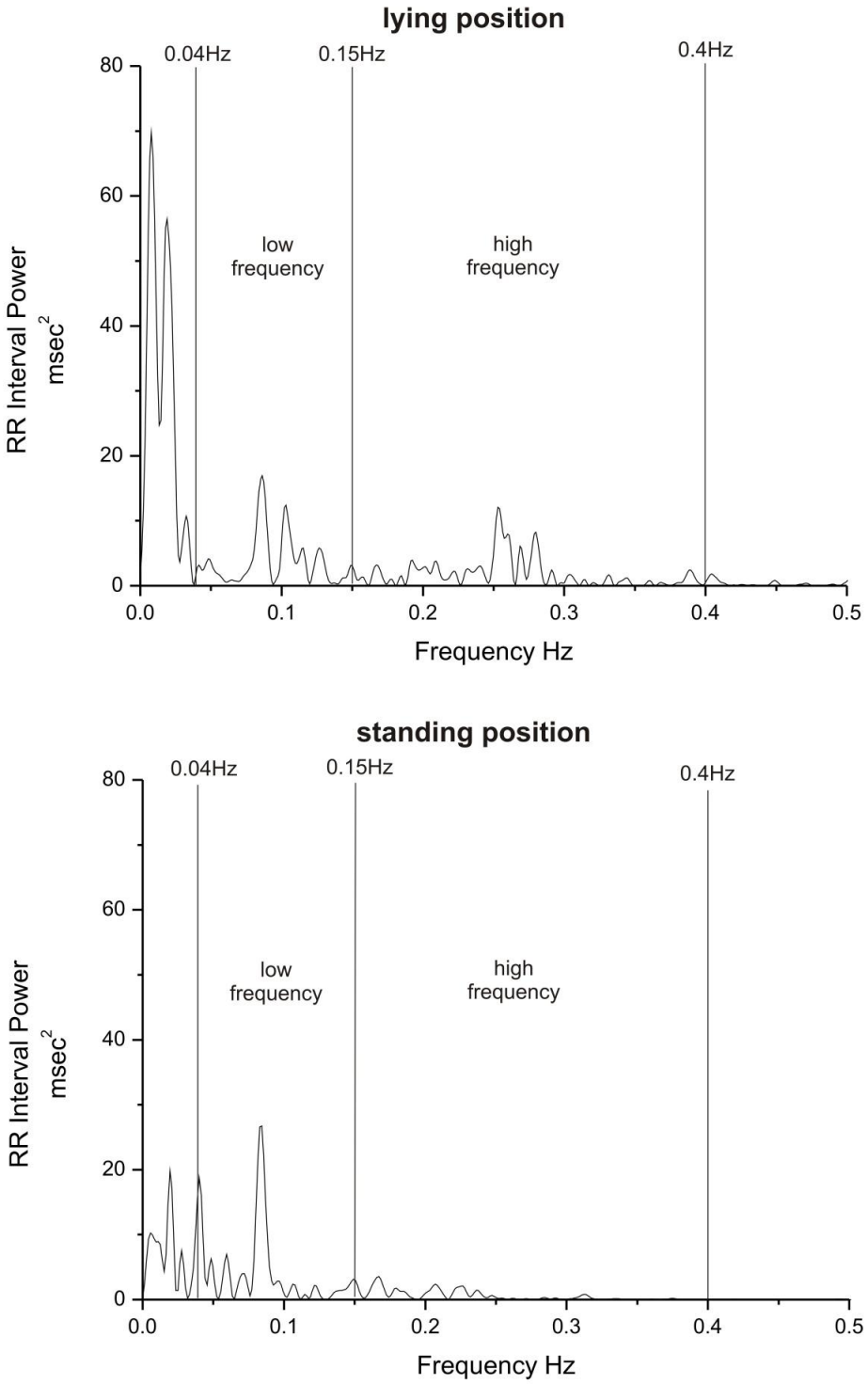

Figure 5. Using Fast Fourier Transform, the RR-interval fluctuations are decomposed into two components (powers) in low frequency (LF, 0.04-0.15 Hz) and high frequency (HF, 0.15-0.40 Hz) domains. The absolute unit of the power is expressed as squared milliseconds. In a representative subject, from lying to standing (top panel compared to bottom panel), LF power tends to increase or remains unchanged, whilst HF power significantly reduced; therefore, the ratio of LF/HF increased, suggesting a sympathovagal balance shifting.

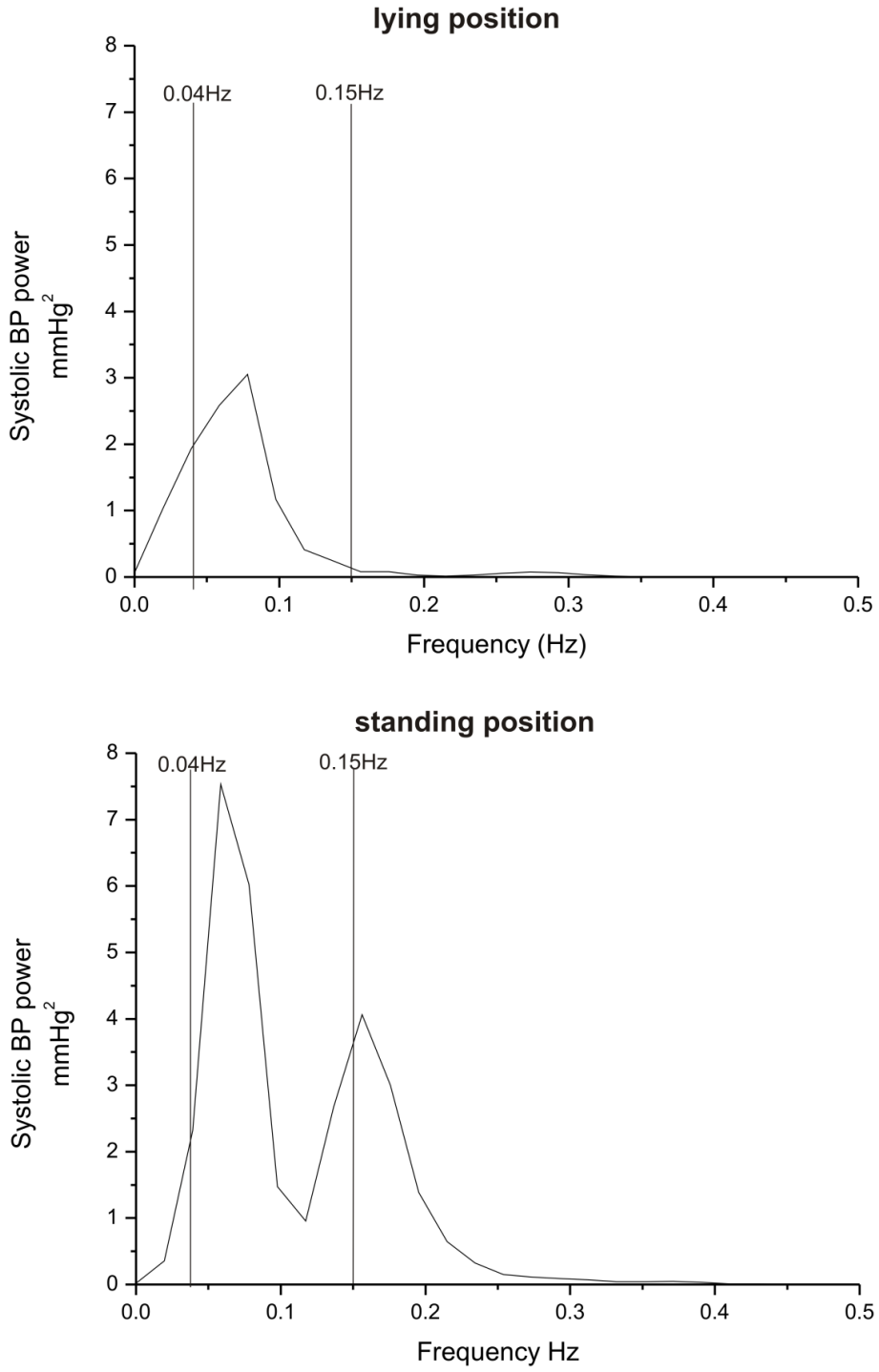

Figure 6. The beat-to-beat blood pressure fluctuations (amplitude changes) can also be decomposed into components in frequency domains; in the above subject in Figure 5, the diagrams show that, from lying to standing, low frequency (0.04-0.15 Hz) spectral power of systolic blood pressure fluctuations significantly increased (the same result also shown in diastolic blood pressure power).

variation, which does not provide any information about sympathetic and parasympathetic modulation (Malik, 1996).

The various phenomena, such as heart period and arterial pressure variations can also be understood by decomposing their oscillatory components using a fast Fourier transformation, defined by the amount of frequency present in different parts of the spectrogram (See Chapter One, Section 3.3, Figure 3.). LF spectral power represents mainly cardiac sympathetic modulation, and HF spectral power represents vagal control to the cardiac pacemaker tissue (Akselrod *et al.*, 1981; Pagani *et al.*, 1986) (See Chapter One, Section 3.3, Figure 4.).

In humans, both in the resting state and under circumstances of daily stimuli, the peaks (amplitudes) within the two frequency bands, i.e. LF (0.04-0.15 Hz) and HF (0.15-0.40 Hz) are evident (Pomeranz *et al.*, 1985). Under resting conditions, vagal tone prevails, and variations in heart period are largely dependent on vagal modulation. The vagal and sympathetic activities constantly interact (Malliani *et al.*, 1991). In response to stressors, cardiac sympathovagal balance is likely to shift towards sympathetic predominance in most circumstances (Pagani *et al.*, 1986).

For any commercial equipment designed to analyse short-term HRV should incorporate non-parametric and preferably also parametric spectral analysis. The analysis based on regular sampling of the tachograms (RR-intervals in time series) should be offered in all cases. Non-parametric spectral analysis (FFT) should employ at least 512 or preferably 1024 points for 5 min recordings. The commonly used spectral windows are Hanning, Hamming, and triangular. Hanning window was chosen here (Malik, 1996).

Measurements of LF and HF power components is initially made in absolute values of power (ms^2); but LF and HF can be measured in normalised units (nu), which represent the relative value of each power component in proportion to the total power minus the very low frequency (VLF) component (see Chapter One, Section 4.2.). The representation of LF and HF components in n.u. emphasises the controlled and balanced behaviour of the two branches of the autonomic nervous system (Malik, 1996).

HRV provides unique information on the parasympathetic arc of autonomic nervous system. Vagal activity is the major contributor to the HF component of HRV (Baumert *et al.*, 2009; Eckberg *et al.*, 1985). Disagreement exists in respect to the LF component. Some studies suggest that when expressed in normalised units, LF spectral power is a quantitative marker for sympathetic modulations (Montano *et al.*, 1994; Pagani *et al.*, 1986), other studies support LF component reflecting contributions of both sympathetic and vagal activity (Sleight *et al.*, 1995). Consequently, the LF/HF ratio is considered by some investigations (Montano *et al.*, 1994; Pagani *et al.*, 1986) to mirror sympathovagal balance (Malliani *et al.*, 1991; Montano *et al.*, 2009). Indeed, power spectral analysis of HRV provides correct functional information on autonomic nervous modulation to stressors (Furlan *et al.*, 2000; Pagani *et al.*, 1997).

6.2 LF component of BPV

The continuous finger arterial pressure provided an accurate estimate for the absolute value of radial intra-arterial pressure in healthy subjects and patients with essential hypertension, during both resting state and various laboratory stimuli, such as hand-grip, cold pressor test, valsalva mauver, diving test, IV phenylephrine and tri-nitroglycerine, as well as leg raising and LBNP; the results of blood pressure variability and cardiovagalbaroreflex function are also identical between the two methods of blood pressure recordings (Parati *et al.*, 1989). In response to orthostatic stress, the non-invasive finger blood pressure waveform may also replace the role of invasive arterial pressure recording (Imholz *et al.*, 1990). A review collected from 43 papers regarding the accuracy of finger blood pressure in comparison with intra-arterial pressure, suggests for the assessment of beat-to-beat changes in blood pressure and BPV, finger diastolic and mean blood pressure are reliable alternatives to values derived from invasive arterial pressure. Differences in finger systolic blood pressure may be of greater deviation from the intra-arterial pressure, but are of no clinical relevance. The authors conclude that finger blood pressure recording is recommended for clinical investigation for autonomic dysfunction and syncope, although the absolute blood pressure values should be collaborated (Imholz *et al.*, 1998a).

6.3 In comparison with muscle sympathetic nerve activity and norepinephrine spillover methods

The most direct measurement of efferent sympathetic nerve activity in human is the tip of the fine needle (recording electrode) inserted into a muscle fascicle of the peroneal nerve around the knee level, so as to record the signals of peripheral sympathetic nerve firing by microneurography technique. This is termed as muscle sympathetic nerve activity (MSNA)(Burke *et al.*, 1977; Sundlof *et al.*, 1977). MSNA has been used in many physiological studies (Fu *et al.*, 2012; Fu *et al.*, 2004b). Similar to RR-interval and blood pressure fluctuations with low and high frequency components, the sympathetic neural activity also exhibits components in two low-and high- frequency domains (Eckberg *et al.*, 1985).

Pagani et al. (Pagani *et al.*, 1997) examined 8 healthy subjects with vasoactive drugs induced blood pressure changes to characterise the correlations between the frequency components of cardiovascular variability (HRV and BPV) and sympathetic neural variability (MSNA variability). Nitroprusside induced decrease in blood pressure is associated with LF components of RR-interval, systolic BP and MSNA variabilities predominate relative to HF; whereas phenylephrine induced hypertensive effect causes opposite changes in frequency components of these variabilities. Blood pressure changes are closely related to absolute values of MSNA (both bursts and amplitudes), as well as the normalised units of LF and HF components of MSNA. Furthermore, LF components of cardiovascular variabilities (HRV and BPV) are tightly correlated to LF components of MSNA variability. The study demonstrated that during acute stress (changes in blood pressure), spectral power of cardiovascular variability provides functional evidence in characterising the modulations of autonomic neural activity in healthy humans (Pagani *et al.*, 1997). Furlan et al. (Furlan *et al.*, 2000) investigated the coupling of cardiovascular and neural variabilities in 10 healthy subjects during orthostatic stress (without blood pressure change). Head up tilt 75 degree induced a decrease in central venous pressure, but blood pressure remained unchanged. This orthostatic stimulus is associated with clear signs of sympathetic activation characterised by increases in heart rate, MSNA and plasma norepinephrine. The major findings are that during tilt,

LF components of HRV, BPV and MSNA variabilities all increased significantly; the LF spectral power of MSNA is in proportion to the increase of overall MSNA (bursts/min); LF components of MSNA variability have significantly greater coherence with that of cardiovascular variability during tilt than at rest, suggesting cardiovascular variability is particularly valuable marker in characterising autonomic modulation during sympathetic excitation (Furlan *et al.*, 2000).

Breathing activity may affect the cardiovascular variability as a marker in reflecting functional information on autonomic modulation during laboratory challenge. A recent study found in response to hand-grip exercise, cold pressor and hypoxia, MSNA increased during both controlled breathing (20 cycles per min) or spontaneous breathing in 12 health subjects; however, HRV LF spectral power (n.u.) achieve parallel increases only in selected conditions compared with MSNA (DeBeck *et al.*, 2010). Here it is found that spontaneous breathing with normal rate (approximate 10-12 times per minute) and tidal volume is more physiological and practical in correctly analysing cardiovascular variability. We recommend good instruction and practice for subjects regarding normal spontaneous breathing prior to experiment.

It is reported that in response to meal ingestion, whole-body and forearm plasma noradrenaline spillover, and LF component of BPV increased significantly, while LF component of HRV remained unaltered postprandially in healthy young men (Vaz *et al.*, 1995b). To our knowledge, meal ingestion as a subtle stressor does not necessarily evoke changes in HRV spectral power when sympathetic-mediated vasoconstrictor effect is sufficient to compensate the haemodynamic perturbation in healthy young men (see result chapter). It is also possible that central nervous system may prioritise the peripheral regional sympathetic activation according to different types of stressors (Cox *et al.*, 1995; Wallin *et al.*, 1992) and individual's physiological conditions (Cozzolino *et al.*, 2010; Lembo *et al.*, 1992; Lipsitz *et al.*, 1993; Tentolouris *et al.*, 2003).

Kingwell et al. compared the three techniques: HRV, MSNA, and norepinephrine spillover in subjects during resting state (Kingwell *et al.*, 1994). Their subjects were grouped under two sympathetic conditions: 1) sympathetic activation: normal ageing

and cardiac failure; 2) cardiac sympathetic denervation: pure autonomic failure (PAF), post cardiac transplantation, dopamine-β-hydroxylase (DβH) deficiency. In ageing and cardiac failure groups, MSNA and cardiac norepinephrine spillover are markedly higher than counterpart control groups, whereas HRV LF power is significantly low. This indicates the mechanisms of decreased cardiac adrenergic receptor sensitivity and postsynaptic signal transduction may result in the functional deficit in ageing population and patients with cardiac failure. In cardiac sympathetic denervation groups, all three markers were consistently in low levels in patients with PAF or early after cardiac transplantation. Two years after cardiac transplantation, cardiac norepinephrine spillover level returned to normal, but HRV LF power remained markedly reduced. The authors suggest that nerve re-innervation may be patchy particularly in the sinus node region, the lack of HRV LF power indicates a functional deficit rather than the concept of “near-normal” based on neurochemical evidence. DβH deficiency refers to the failure of neurochemical synthesis for norepinephrine. The patient with DβH exhibited exaggerated high level of MSNA, but cardiac norepinephrine spillover level and HRV LF power were very low, suggesting an electrochemical uncoupling associated with functional deficit. Finally, in resting state, cardiac norepinephrine spillover is correlated to MSNA in healthy subjects, but not to HRV LF power, suggesting power spectral analysis of HRV provides overall functional evidence and is not direct measurement as either cardiac norepinephrine spillover level, or sympathetic neural firing rate (Kingwell *et al.*, 1994). Furthermore, HRV LF/HF ratio is a useful marker in evaluating the sympathovagal balance during stressors (Malliani *et al.*, 1991). A study shows the non-invasive HRV LF/HF ratio during sympathetic stimulation induced by the standing position correlated significantly with cardiac norepinephrine spillover, as an index of cardiac sympathetic nerve activity in patients with mild to moderate chronic heart failure (Tygesen *et al.*, 2001).

7. Sequence method baroreflex sensitivity (sBRS)

7.1 Time domain analysis of baroreflex sensitivity

In response to beat-to-beat blood pressure changes, baroreceptors provide instantaneous information to the brainstem, and blood pressure changes are reflexly modulated by the two efferent arcs of autonomic nervous system. An activation of baroreceptor by a rise in blood pressure results in an increase in cardioinhibitory parasympathetic neuron discharge, and a reduction in sympathetic neural firing to the heart and vasculature; and vice versa. Baroreceptor denervation is related to exaggerated blood pressure excursions, but not the absolute value (setpoint) of blood pressure (Cowley Jr *et al.*, 1973). Cardiovascular disease is associated with impairment of the inhibitory mechanism of baroreflex (negative feedback), leading to imbalanced sympathovagal outflows to the heart, and thus adrenergic activation (La Rovere *et al.*, 1998).

Baroreceptor-mediated heart rate reflex to arterial pressure can be estimated by a simple *time domain* analysis, termed the sequence method of spontaneous BRS. The sequence method was first described by Parati et al. (Parati *et al.*, 1988), based on the identification of three or more consecutive heart beats in which progressive increases/decreases in systolic blood pressure are followed by progressive lengthening/shortening in RR-interval. The threshold values for beat-to-beat changes in systolic blood pressure and RR-interval are 1mmHg and heart period 6ms respectively. The BRS is computed as the slope between changes in systolic blood pressure and RR-interval. All computed slopes are finally averaged to obtain the BRS (La Rovere *et al.*, 2008).

7.2 The use of Hemolab – Analyzer for estimation of sequence method Baroreflex Sensitivity

Analyzer is one of widely used software provides estimates of sequence method baroreflex sensitivity. The software is available online and free to be downloaded: http://www.haraldstauss.com/HemoLab/HemoLab.php.

Arterial pressure data can be loaded directly from LabChart 7.2 into the Analyzer (Figure 7.). A manual for Hemolab software is also available as a guide for calculation of baroreflex sensitivity.

During physiological condition, baroreceptors mediated neural-reflex buffers the changes in beat-to-beat blood pressure by altering cardiac cycles (RR intervals); blood pressure rise may induce a sequence of increases in cardiac cycle (decreases in heart rate), and vice versa. To estimate this sequence changes, a slope may reflect this physiological regulation. The software is to identify all the slopes that contain 4 or more of this consecutive baroreflex-mediated blood pressure and RR-interval sequence changes (slopes) within a time period (Figure 7.). Each slope is calculated by linear regression to ensure a cut-off r value of the correlation that is greater than 0.8. Only those sequences that is highly correlated ($r>0.8$) are used in the final estimation for baroreflex sensitivity, i.e. average value of the slopes.

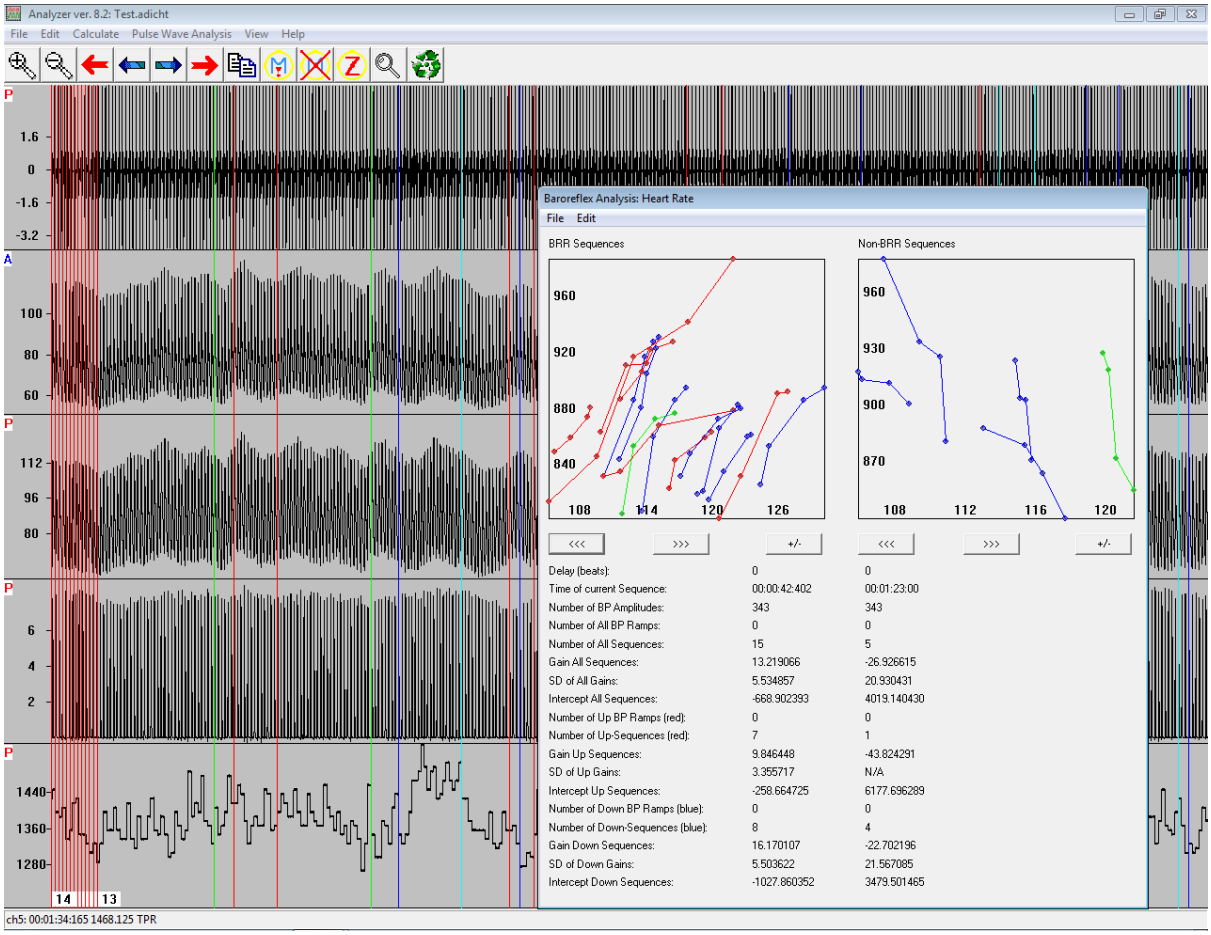

Figure 7. In physiological conditions, beat-to-beat blood pressure changes are likely to be buffered by baroreflex-mediated heart rate responses, i.e. the higher the blood pressure, the longer the heart-beat interval, and vice versa. The diagram shows the software “Analyzer” can detect and calculate those with 4 or more of this sequence changes (defined as baroreflex response – BRR) in blood pressure and heart rate in a period of time, reflecting baroreflex sensitivity. More information is available at: http://www.haraldstauss.com/HemoLab/HemoLab.php.

Chapter Three: Result One

This study has been submitted to the journal "Autonomic Neuroscience: Basic and Clinical" for publication and is currently under second review, tilted as "Quiet standing after carbohydrate ingestion induces sympathoexcitatory and pressor responses in young healthy males"

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Quiet standing after carbohydrate ingestion induces sympathoexcitatory and pressor responses in young healthy males

Abstract

Objective - To investigate the role of the sympathetic nervous system in the cardiovascular response to quiet standing in the postprandial state.

Method - Following a 30 min pre-ingestion phase, 14 healthy young male subjects consumed a 600kcal carbohydrate-rich meal. Arterial blood pressure (BP) and heart rate (HR) were recorded for a further 120 min. Measurements were obtained (Finometer) in both the supine (5 min) and standing (5min) condition every 30 mins. Power spectral analysis of RR-interval and BP variability was calculated, and heart rate responses to the baroreceptor reflex were calculated to estimate spontaneous baroreflex sensitivity (sBRS). Derived stroke volume was measured to track changes to postural stress in the postprandial state.

Results - Quiet standing increased RR LF nu power, ratio of RR LF/HF, and low frequency systolic /diastolic BP power (SBP / DBP LF power), and decreased RR HF nu power and sBRS before, and after eating. Postprandially SBP / DBP LF power increased and sBRS decreased in both lying and standing conditions. During standing postprandially DBP and mean arterial pressure increased (P<0.01). Increased BP is associated with increased stroke volume (P< 0.05) in the early phase, and increased SBP/ DBP LF power (P<0.01, P<0.05) in the later phase. SBP LF power is inversely correlated with SV in the postprandial state (P<0.001, R^2=0.96).

Conclusion: The findings suggest a sympathetic activation mediated by baroreflex resetting. Quiet standing in the postprandial state enhances sympathetic activity, increasing BP. Stroke volume may be a compensatory factor stabilising BP during standing early in the postprandial state.

Keywords: orthostatic stress, meal ingestion, blood pressure, sympathetic nervous activity, stroke volume.

Abbreviations:

LF: low frequency
HF: high frequency
n.u.: normalised unit
sBRS: spontaneous baroreceptor reflex sensitivity
MAP: mean arterial pressure
BP: blood pressure
HR: heart rate
SV: stroke volume
CO: cardiac output
TPR: total peripheral resistance

Introduction

Obesity, which is frequently associated with disorders such as hypertension (Landsberg, 2001), dyslipidaemia, diabetes (metabolic syndrome) (Grassi *et al.*, 1995; Mancia *et al.*, 2007) and sleep apnoea (Grassi *et al.*, 2005) amongst others, is a major cause of morbidity throughout the world. Physical inactivity is related to low energy expenditure and prolonged sitting is an independent risk factor to all causes mortality (Hamilton *et al.*, 2007; Patel *et al.*, 2010; Van Der Ploeg *et al.*, 2012). A recent study shows that uninterrupted sitting following a carbohydrate ingestion may acutely elevate blood glucose and insulin levels in overweight /obese subjects (Dunstan *et al.*, 2012). Reduce workplace sitting time and/or interrupt the time of sitting can improve cardiovascular health (Van Uffelen *et al.*, 2010). Standing increases energy expenditure in contrast to either supine or sitting position (Levine *et al.*, 2000), this may be attributed to the involvement of isometric quadriceps and lower leg muscle contraction (Chow *et al.*, 2011; Masani *et al.*, 2008). Short periods of standing may be a useful approach to reduce sitting time and break up time spent sitting (Alkhajah *et al.*, 2012), and a potential solution to combat excessively sedentary lifestyle (Hamilton *et al.*, 2007; Patel *et al.*, 2010; Van Der Ploeg *et al.*, 2012).

Responses to assuming an upright posture in the initial stage, i.e. short term quiet standing, is mainly controlled by autonomic nervous system (Smit *et al.*, 1999). Short term standing unloads cardiopulmonary receptors and baroreceptors (Abboud *et al.*, 1979; Taylor *et al.*, 1995), and is associated with a reflex sympathetic activation (Cooke *et al.*, 1999; Furlan *et al.*, 2000) and regional vasoconstriction to counteract gravitation-induced venous pooling (Abboud *et al.*, 1979); and also a reduction in parasympathetic activity with an increase in heart rate (Cooke *et al.*, 1999; Taylor *et al.*, 1995). Maintenance of normal arterial blood pressure is critical in order to ensure appropriate circulation of blood to different parts of the body as required. A failure to maintain normal arterial blood pressure can lead to postural hypotension (Freeman, 2008; Smit *et al.*, 1999), or hypertension (Fessel *et al.*, 2006; Streeten *et al.*, 1985), if counter-regulatory systems such as the baroreceptor reflex fail to operate correctly.

Individuals spend approximately 50% of daytime in the postprandial state. Feeding behaviour is centrally controlled to maintain a balance of energy intake to energy expenditure (Schwartz *et al.*, 2000). There is growing evidence that food intake and insulin secretion increase sympathetic nerve activity and increase sympathetic-baroreflex gain as estimated from recordings of muscle sympathetic nerve activity(Fagius *et al.*, 1994; Young *et al.*, 2010a). A reduction in parasympathetic activity is also found after meal ingestion (Cozzolino *et al.*, 2010; Hayano *et al.*, 1990).

In the modern world, meals rich in carbohydrates are common. However, the autonomic control and haemodynamic effects of a brief loading with carbohydrate rich food and its interaction with brief periods of short term of standing are less clearly understood.

Here the objective was to determine if there is an interaction between food intake and posture on arterial blood pressure and changes in sympathetic nerve activity and baroreflex function. To investigate interactions between food intake and posture, the effect of a defined breakfast meal on changes in cardiovascular function during lying and standing was determined.

Heart rate was measured continuously using the electrocardiogram, and arterial blood pressure was recorded continuously using finger photoplethysmography. Changes in efferent sympathetic nerve activity were estimated by spectral analysis (fast Fourier transformation) of systolic and diastolic arterial blood pressure in the low frequency range (Pagani *et al.*, 1986). The sequence method was used to estimate the sensitivity of the heart-rate baroreflex (Steptoe *et al.*, 1990).

Research Design and Methods

Study participants

Fourteen males were recruited from a population of post-graduates students in our Department. Subjects were non-smokers on no medication (age31.4 ± 2.9 years; height 179.6 ± 7.9cm; weight 75.9 ± 7.2kg; Body Mass Index (BMI) 23.6 ± 2.6kg/m^2(healthy weight range 18.50 - 24.99kg/m^2)). Subjects were instructed not to drink water 1.5 hours prior to experiments, and to abstain from caffeinated beverages and food for 12 h, alcohol for 24 h, and moderate or strenuous physical

activity for 48 h prior to the experimental sessions. After receiving a detailed verbal and written explanation of the intended experimental protocol and measurements, each subject provided informed consent. All experimental procedures and protocols were approved by the Human Ethics Committee of Macquarie University, Sydney, NSW, Australia.

Recording

Electrocardiogram (ECG) and BP (finger photoplethysmography; Finometer Pro, Ohmeda, Amsterdam, Holland) were measured continuously. ECG was sampled at 1000 Hz and stored for off-line analysis (LabChart 7.2 and Powerlab8/30, ADInstruments, Bella Vista, Sydney, NSW, Australia). BP files were recorded at a sampling rate of 200 Hz for further power spectral analysis. The subject's left arm was placed with the left hand (testing hand) at the level of the heart at all times (Imholz *et al.*, 1998b). Brachial arterial blood pressure was recorded from the right arm with an automated sphygmomanometer (Microlife A100 PLUS, AG,9443Widnau, Switzerland) to confirm the accuracy of the Finometer measurements of absolute blood pressure. All other hemodynamic variables were downloaded with Finolink and derived from Beatscope software (FMS, Finapres Medical Systems BV, Amsterdam, The Netherlands). Details of the methodology used by the Finometer software ("beatscope") to calculate different haemodynamic parameters are available from http://www.finapres.com/site/index.php(Wesseling *et al.*, 1993). With this approach it is also possible to track changes of cardiac output (CO), stroke volume (SV)and total peripheral resistance (TPR) (Bogert *et al.*, 2005). This has been widely used in various clinical studies (Gisolf *et al.*, 2004; Hu *et al.*, 2011; Krediet *et al.*, 2005). A recent study reveals that cardiac output measured using Modelflow analysis agreed well with that measured by the acetylene rebreathing technique during tilting in a recent study ($R = 0.74$, $P < 0.01$) (Fu et al., 2012).

Before starting the experiment, subjects were asked to maintain breathing frequency at 0.2Hz by following a metronome until the subjects were confident and comfortable with this breathing pattern. To avoid mental stress, subjects were then asked to approximate this frequency and depth spontaneously during the experiment. Breathing was also carefully supervised by investigators, and adjusted if the

breathing deviated excessively during recording in both supine and upright positions throughout the study (Bernardi *et al.*, 2000; Bloomfield *et al.*, 2001; Taylor *et al.*, 1995).

Experimental protocol

All subjects were fasted overnight and studies were conducted from 0900 to 1230. The laboratory temperature is central air-conditioned and maintained at 23°C throughout the experiment. In order to minimise the light and other external stimulation, all subjects were kept in a semi-dark laboratory room (light-off and curtain on) and instructed not to read book / newspapers, listen to music or chat with the investigator throughout the experiment. Subjects were encouraged and supervised to be awake, and report any discomfort. The investigator was also responsible to ask any discomfort from time to time.

ECG and Finometer were connected and the validity of signals was ensured prior to the first 20 minutes resting supine period in fasting state. Baseline measurements of all parameters (brachial BP, 5 minutes of ECG and Finger blood pressure recording) were obtained in the supine state after the resting period. Subjects then stood and after 2 minutes of hemodynamic equilibration data was recorded again. Subjects were then fed a standard breakfast. The breakfast was an ordinary meal style, including 30g Weet-Bix Bites (wild berry) (Sanitarium Health and Wellbeing Australia), 100ml Original Milk (Dairy Farmer Pty Ltd Australia), 170ml Low Fat Fruit Yogurt (Dairy Farmer Pty Ltd Australia), 200ml Orange Juice (The Daily Juice Company, Australia), one medium-sized banana (all sourced from Woolworth Ltd, Australia). The food formula is a 600kcal carbohydrate rich mixed meal (semi-liquid): 118g carbohydrate (including 85g sugar) (78%), 20g protein (13%), 6g fat (8%), sodium 300mg. All subjects were instructed to consume the meal within 10-12minutes, and then came back to the laboratory and lying supine with ECG and Finometer reconnected. The first 30minute time point postprandially was defined as starting from the first mouth of food intake (10-12 minutes of eating time) until 18-20minutes after the meal with supine resting state. Then recordings were repeated for 30 minute time intervals for a further 2 hours after the meal. Each time-interval

incorporates 12 minutes recording time (5 minutes lying and 7 minutes standing) followed by 18 minutes of supine resting state. (Fig 1)

Data analysis

Power spectral analysis of RR interval was calculated with the HRV module in the commercial software of LabChart. (LabChart 7.2, ADInstruments, Bella Vista, Sydney, NSW, Australia). Power spectrum analysis of systolic and diastolic blood pressure (SBP and DBP) was performed using custom written scripts with Spike2 software (Cambridge Electronic Design Limited, Cambridge, England). All analyses were performed from stable haemodynamic regions with a duration of 5 minutes that was free of ectopic beats and any technical artefacts.

Fast Fourier Transform (Hanningwindow; 512 block size) was applied to the R-R interval, SBP and DBP in time series to obtain power spectral estimates of HRV and BPV (Malik, 1996; Pagani *et al.*, 1986). The low-frequency refers to 0.04–0.15 Hz and high-frequency refers to 0.15–0.40 Hz. Signal powers of each band were calculated as integrals under the respective power spectral density functions and expressed in normalised unit for HRV and absolute units for BPV ($mmHg^2$). Spontaneous baroreceptor reflex sensitivity (sBRS) (RR interval to systolic BP) was evaluated with HemoLab software (http://haraldstauss.com/HemoLab/HemoLab.php), using the sequence method that identifies sequences of four or more heart beats, where BP and pulse interval change in the same direction (Bertinieri *et al.*, 1985). A delay of 0-2 physiological beat cycles between systolic blood pressure and pulse interval was used to provide the most representative estimates of BRS (Steptoe *et al.*, 1990).

Statistical analysis

All statistical analyses were calculated using GraphPad Prism software (version 6). Statistical significance was evaluated using two-way ANOVA and Bonferroni post-hoc tests for group analysis (lying posture versus standing posture, to the meal ingestion effects); within group analysis (either lying or standing posture), i.e. meal ingestion effects over time were evaluated using one-way ANOVA with repeated measures followed by Bonferroni post-hoc tests. Group averaged values of SBP LF power, SV and CO at each postprandial time point of both supine and upright

positions were used, and univariate correlations of SBP LF power to SV and CO were calculated (Pearson's R_2). Data are presented as means ± standard error mean (SEM). All statistical differences were considered significant at $P < 0.05$.

Results

Hemodynamic response to orthostatic stress and meal ingestion

A fall in SBP (-12mmHg, $P<0.01$) during quiet standing from the supine position was noted, but only in the fasting state. DBP and MAP were unaffected by the orthostatic challenge both before and after meals ($P=0.51$, $P=0.30$, respectively). SBP, DBP and MAP in both supine and upright postures increased and reached a maximum 60 min after meal ingestion ($P< 0.0001$) (Fig 2A-C). Orthostatic stress increased HR during quiet standing compared to the supine position ($P< 0.0001$, Fig 2D). In the postprandial state, HR started to increase from 30min in both supine and upright positions and these increases reached the maximum level at 90 min ($P<0.0001$). Stroke volume (SV) was significantly lower during quiet standing compared to that seen in the supine state at all time-points ($P<0.0001$, Fig. 2E). In response to meal ingestion, SV increased significantly in the early postprandial stage (30min) during quiet standing but not in the supine position ($P<0.0001$), and returned to baseline after 60-120min. Notably, SV was significantly lower during quiet standing at baseline, 60min, 90min and 120min (change%=(SVsupine-SVupright)/SVsupine: 26.8%, 29.0%, 31.3% and 31.3%, $P< 0.0001$), with the exception of SV 30min (21.6%) in the postprandial state. In other words, the postprandial rise in SV during quiet standing at 30min attenuated the postural decrease in SV ($P<0.05$) (Fig 2E). Cardiac output (CO) was unchanged in response to orthostatic challenge at baseline and in the postprandial state ($P=0.56$); However, there was a significant elevation in CO in response to meal ingestion that was sustained throughout the 120min period in the postprandial state compared with the baseline recordings ($P< 0.0001$, Fig 2F). Orthostatic stress did not affect total peripheral resistance (TPR) ($P=0.94$, Fig. 2G). TPR decreased by 10% and reached a minimum at 30min after meal ingestion ($P< 0.0001$) remaining stable for the remainder of the recordings (Fig. 2G).

Autonomic control to orthostatic stress and meal ingestion

During quiet standing, RR LF (nu) variance was significantly increased (P<0.0001), while the RR HF (nu) variance was decreased significantly (P<0.0001) at all time_points. This contributed to significant increases in RR LF/HF ratios (P<0.0001) (Fig 3A-C). Similarly, orthostatic stress led to significantly greater SBP LF and DBP LF powers (P<0.0001) (Fig 3D, G). There was no difference in the LF/HF ratios of SBP and DBP powers (P=0.26, P=0.74). sBRS was significantly lower in upright positions than that of supine positions at all time-points (P<0.0001) (Fig 2H).

Meal ingestion did not change RR LF (nu), RR HF (nu) variances and LF/HF ratios (P=0.36, P=0.74, P=0.33, respectively) (Fig 3A-C). In contrast, there were increases in SBP LF power in the supine state (P<0.01), and graded increases in both SBP LF and DBP LF power during quiet standing in the postprandial state (P<0.01) (Fig 3D, G). During standing, SBP LF power increased from 27.8mmHg^2 (at baseline and 30min) to 39.1mmHg^2 at 60min (+41%), 48.7mmHg^2 at 90min (+75%), and 50.1mmHg^2 at 120min (+80%) (P<0.0001). Similarly, DBP LF power increased from 16.7mmHg^2 at baseline to 23.0mmHg^2 at 90min (+38%), and 23.6mmHg^2 at 120min (+41%) (P<0.0001).

sBRS fell after meal ingestion in both supine and upright positions (P<0.0001). In the supine state, sBRS decreased from 25.4 ms/mmHg at baseline to 16.4ms/mmHg at 60min after meal ingestion (-35.4 %); during quiet standing sBRS decreased from 10.4ms/mmHg at baseline to 6.8ms/mmHg at 60min (-34.6 %). After 60min, sBRS remained at these levels until the end of 120min recordings. (Fig 2H)

Interactions of autonomic and haemodynamic variables to position × time effect

DBP, MAP and HR increased during standing over time in the postprandial state (p<0.01, p<0.05, p<0.01 Fig 2B-D). SV rose during standing early in the postprandial state (at 30min) (P<0.05) (Fig 2E). An enhancement in SBP LF power and DBP LF power was observed during standing later in the postprandial state (60min onwards) (P<0.01, P<0.05) (Fig 3D,G). There was no position× time effect on sBRS (p=0.11) (Fig 2H).

Correlation of SV, CO and SBP LF power after meal ingestion

Correlations between the averaged values of haemodynamic and autonomic parameters are found. There is strong inverse correlation between the SV and SBP LF power (P<0.001, R^2=0.96), and between CO and SBP LF power (P=0.001, R^2=0.83) at each time point in the postprandial state (Fig 4).

Discussion

The current study investigated the cardiovascular autonomic control and hemodynamic changes to orthostatic stress in the postprandial state in healthy males.

The primary new findings are:

1) Standing after carbohydrate rich meal ingestion causes a transient enhancement in SBP and DBP LF power; associated with orthostatic hypertension in the postprandial state. The data suggest a central sympathetic activation to maintain BP and SV during standing in the postprandial state, but not in the supine state.
2) A strong inverse correlation between SV/CO and SBP LF power in the postprandial state was observed; and in the early stage (30min) after meal ingestion a surge in SV may be important in stabilising BP during orthostatic stress.

Cardiovascular autonomic control in the postprandial state: lying vs standing. (Fig 5)

Our data are consistent with previous studies that report meal-induced increases in muscle sympathetic nerve activity (MSNA) in the resting supine state (Fagius *et al.*, 1994; Young *et al.*, 2010a), and demonstrate the efficacy of the non-invasive finger-cuff approach in making this assessment. Meal ingestion induces a physiological hyperinsulinaemia, that is associated with an enhanced gain of the sympathetic-baroreflex (Young *et al.*, 2010a). This enhancement of the sympathetic-baroreceptor reflex during physiological hyperinsulinaemia is comparable to the effect of insulin infusion achieved with hyperinsulinaemic euglycaemic clamp in normal young subjects (Young *et al.*, 2010a). The current study showed that short periods (7min) of standing (recording in the last 5min), causes increases in SBP and DBP LF power

over time following meal ingestion in the standing, but not the lying state. An effect that is independent of the gravitation-induced sympathetic activation. It was recently reported that insulin can act at the arcuate nucleus in the hypothalamus to initiate and enhance arterial sympathetic baroreflex function and lumbar sympathetic nerve activity (SNA) in rat (Cassaglia *et al.*, 2011). Thus, we speculate that the additional hyperinsulinaemia provoked by standing (Jamerson *et al.*, 1993; Lembo *et al.*, 1993) causes a sympathoactivation mediated by direct actions of insulin at central autonomic sites, possibly including the arcuate nucleus. Insulin is transported across the blood brain barrier, and may gain access to neurons within the arcuate nucleus via receptor-mediated endocytosis (Banks, 2004). The highly permeable microvasculature of the ventromedial arcuate nucleus is believed to enable circulating substances, such as insulin to be delivered directly to neurons at this site (Ciofi, 2011). This process accelerates an insulin-induced sympathetic activation. Thus, it is possible that a brief period (7min out of each 30min) of quiet standing that elicits an acute hyperinsulinaemia in the postprandial state induces a transient postural sympathetic activation, as observed in the current study.

As noted above meal ingestion combined with standing is known to cause a marked increase in plasma insulin levels that leads to sympathetic activation (Mancia *et al.*, 2007). The hyperinsulinaemia that follows meal ingestion and standing is then associated with marked reductions in glucose uptake (Laakso *et al.*, 1990). Previous studies in human forearm, suggest that sympathoactivation can cause insulin resistance since there is an association between reduced forearm glucose utilisation and vasoconstriction mediated by thigh cuff inflation and lower body negative pressure (Jamerson *et al.*, 1993; Lembo *et al.*, 1993). Evidence for adrenergically mediated insulin resistance was subsequently obtained in experiments where intra-arterial infusion of noradrenaline was shown to directly reduce glucose uptake (Jamerson *et al.*, 1994).

It is observed that, DBP rose over time following meal ingestion in the standing position; an effect that was not seen in the lying condition. This difference was also reflected in the MAP. It is known that both low and high doses of insulin within the physiological range do not elevate arterial pressure (Anderson *et al.*, 1991), because of the local insulin-mediated vasodilation in the limb muscles (Baron, 1994;

Muniyappa *et al.*, 2007). However, we cannot exclude the possibility that a transient hyperinsulinaemia, provoked by standing, in addition to that caused by carbohydrate ingestion, may be a causal pathway to stimulate a hypertensive response(Landsberg, 2001; Mancia *et al.*, 2007). Growing evidence shows that pro-opiomelanocortin (POMC) neurons in the arcuate and paraventricular nuclei (Kishi *et al.*, 2003) are crucial for sympathetic response (Ward *et al.*, 2011) and hypertensive effects independent of changes in body weight or body mass index (Purkayastha *et al.*, 2011). In addition, changes in sympathetic function may also be due to actions of insulin on vagal afferent neurons that project to the nucleus tractus solitarius (Dallman *et al.*, 2007; Warne *et al.*, 2008) resulting in complex effects on blood pressure.

On the other hand, the increase in MAP observed here appears to be due to an increase in sympathetic nervous activity (Abboud *et al.*, 1979) and DBP (Streeten *et al.*, 1985) that is not balanced by excessive venous pooling (insulin-induced vasodilation), and decreased venous return (Streeten *et al.*, 1985). This is consistent with the current data that graded decreases in stroke volume are correlated with graded increases in SNA during standing after meal ingestion.

Responses to postural change are widely quoted as providing validation for the methodology of power spectral analysis of heart rate and blood pressure variability(Pagani *et al.*, 1986). The approach of estimating changes in low-frequency spectral power is known to correspond closely to findings obtained using simultaneous direct measurement of SNA (Pagani *et al.*, 1997). A critical advantage of the power spectral approach is that it is simple to perform and entirely non-invasive(Grassi *et al.*, 1999). Furthermore, recordings can be obtained from the same individual over prolonged periods and for prolonged time periods with no discomfort. The current study shows that, in response to quiet standing, there are a decrease in RR HF power and an increase in RR LF power and ration of LF/HF, suggesting that autonomic control shifts towards sympathoexcitation. Similarly, the increases in SBP LF power and DBP LF power in response to postural stress are of indicative of an increase in sympathetic outflow to the vasculature.

Previously, a modest, but significant increase in RR LF power at 80min after a 400kcal mixed liquid meal ingestion (Lipsitz *et al.*, 1993) was reported in young

healthy subjects, although plasma norepinephrine was not increased. However, the low frequency band defined in that study was 0.01-0.15Hz, which is slightly different from that used in the current study (0.04-0.15Hz) (Malik, 1996). Tentolouris et al. found that the ratio of RR LF/RR HF power is increased, but there was no change in RR LF power, following a carbohydrate rich meal in lean healthy females (Tentolouris *et al.*, 2003); this may not be comparable to our data since our subjects are all males (Fu *et al.*, 2005; Hart *et al.*, 2009a). Power spectral analysis is a tool used to describe the sympathovagal balance, but it may not be invariably consistent with the sympathetic nerve traffic (Eckberg, 1997); in addition, the fast Fourier transform (FFT) method is relatively focusing more on broadband powers (0.04-0.15Hz), whereas the autoregressive method is more suitable for examining rhythmic fluctuations of BP and RR interval driven by a fixed rate oscillator (0.1Hz) (Cozzolino *et al.*, 2010; Hayano *et al.*, 1990). Interestingly, regional distributions of noradrenaline levels can be different after meal ingestion – using the spill-over method. For example, cardiac noradrenaline spill-over remains unchanged, although MSNA and whole-body noradrenaline spill-over levels increase (Cox *et al.*, 1995; Vaz *et al.*, 1995b).

The unchanged RR power after meal ingestion in the current study may be explained as follows: 1) A carbohydrate rich meal rather than a mixed meal was used in the current study. We observed an attenuation of sBRS in response to this meal a finding that is in keeping with previous studies showing that an oral glucose tolerance test and hyperinsulinaemic euglycaemic clamp reduce baroreflex sensitivity (Madden *et al.*, 2008; Straznicky *et al.*, 2012). 2) Many elements of food consumption may influence gut mechanoreceptors and chemoreceptors, thereby affecting neural responses and changes in efferent autonomic activity. These elements may include: caloric value, food composition, pattern of intake and hormonal state (Jansen *et al.*, 1995; Schwartz *et al.*, 2000; van Baak, 2008). 3) Sympathetic effects on the vasculature can be cardiac cycle dependent(Charkoudian *et al.*, 2005), and we postulate that a shorter cardiac cycle (faster heart rates) after carbohydrate rich meal ingestion may reduce cardiac noradrenaline release thereby decreasing efferent sympathetic outflow to the sinus node. 4) It is also possible that

some normal healthy subjects, with relatively less insulin sensitivity, may show a blunted sympathetic cardiac autonomic response to insulin (Bergholm *et al.*, 2001).

An increase in stroke volume during standing in early phase of postprandial state

In the postprandial state, a reduction in systemic vascular resistance is important in enhancing the perfusion to skeletal muscle and increasing glucose uptake in normal subjects (Baron *et al.*, 1995). The time to reach half-maximal rates of skeletal muscle blood flow was 40-60 min (Baron, 1994). This is consistent with our finding that the reduction in peripheral vascular resistance (TPR) peaked soon after eating (at the 30min time point) in both the supine and upright postures, indicating a maximal postprandial vasodilatation effect at this time (Someya *et al.*, 2008). In compensating for the vasodilatatory effects of meal ingestion, a sympathetically-mediated vasoconstriction occurs that is due in part to the central actions of insulin (Brooks, 2010; Scherrer *et al.*, 1997). Time is required for insulin to redistribute and cross the blood brain barrier and trigger an increase in nerve activity (Banks, 2004). As observed in the current study, the sympathoexcitation after meal ingestion was only apparent after 60mins, a time period that is consistent with the time course of action of insulin acting centrally (Anderson *et al.*, 1991; Vollenweider *et al.*, 1993). A key finding of this study is that a significant surge in stroke volume (SV) in the early period in the postprandial state (~30min) during quiet standing, at least in male subjects, may be important in compensating for a lack of sympathetic vasoconstriction.

The current study also reveals a strong inverse correlation between SV and CO, and the SBP LF power in the postprandial state. This finding suggests an important physiological role for the surge in SV during quiet standing in compensating for a lack of sympathetic vasoconstriction early after eating (Charkoudian *et al.*, 2005). It is noteworthy that all of the subjects in this study are healthy young males since this correlation is particular clear in males (Charkoudian *et al.*, 2005), but not in females (Hart *et al.*, 2009a). Evidence is accumulating that a smaller and less distensible left ventricle leads to a steeper Frank-Starling relationship in deconditioned hearts (Levine *et al.*, 1997), in female subjects (Fu *et al.*, 2004a; Fu *et al.*, 2005), and in

patients with orthostatic tachycardia syndrome (Fu *et al.*, 2010) where an excessive reduction in SV occurs during orthostasis. Thus, the current study indicates that a surge in SV during a gravitational challenge in the early phase after eating in healthy males can be of physiologically importance in maintaining a stable BP during this period.

Perspectives

Carbohydrate ingestion is associated with hyperinsulinaemia and sympathetic activation (Berne *et al.*, 1989; Young *et al.*, 2010a). Reflex-mediated sympathetic activation may also induce acute hyperinsulinaemia (Jamerson *et al.*, 1993; Lembo *et al.*, 1993), a phenomenon supported by the findings here in the post-prandial state. The main findings of the current study (Fig. 5) are that carbohydrate ingestion combined with standing causes transient regional insulin resistance, with associated sympathoexcitation and pressor response as a physiological adaptation for nutrient delivery (Guyenet, 2006). Long term and/or repetitive exposure to this excessive sympathetic activity may cause insulin resistance and impaired glucose metabolism (Mahfoud *et al.*, 2011; Mancia *et al.*, 2007), leading to hypertension (Landsberg, 2001).

The current study aimed to examine cardiovascular regulation to the interplay of nutrition and orthostasis as a normal daily activity, but not simply targeting on the relationship between hyperinsulinaemia and sympathetic activation that has been well demonstrated (Anderson *et al.*, 1991; Berne *et al.*, 1989; Young *et al.*, 2010a).Carbohydrate ingestion induces increase in blood glucose level and hyperinsulinaemia (Berne *et al.*, 1989; Fagius *et al.*, 1994; Young *et al.*, 2010a), and a carbohydrate rich meal is used in the current study. Meal ingestion is a subtle and useful stressor in examining autonomic response (Cozzolino *et al.*, 2010; Lipsitz *et al.*, 1993), minimal external interference and mental stress during the experiment are appreciated, such as avoidance of repetitive blood sample collection and prolonged needle insertion to peroneal nerve(Berne *et al.*, 1989). Clinically, the straightforward non-invasive approach described here may be useful and of prognostic value in longitudinal studies that require an estimate of sympathetic activity or autonomic modulation(La Rovere *et al.*, 1998). This straightforward

approach may also find a place in monitoring treatment of hypertension in individual patients.

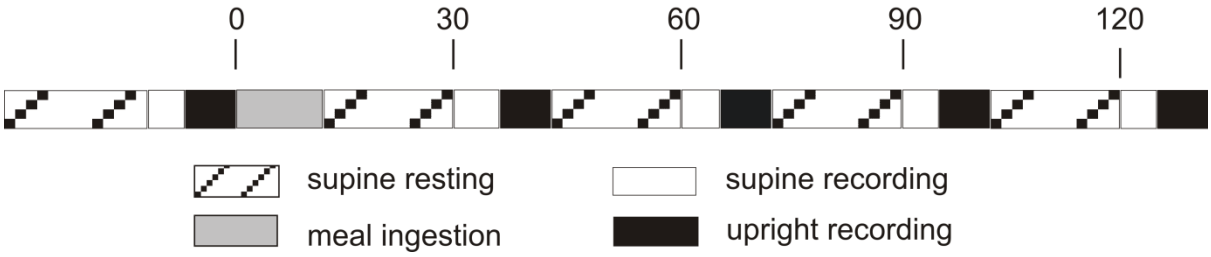
Conclusion

The current study demonstrates that orthostatic stress may evoke an increase in efferent sympathetic outflow to the vasculature after carbohydrate rich meal ingestion, with associated increases in orthostatic BP, suggesting a central resetting of sympathetic baroreflex control of BP. Furthermore, the current study provides new evidence for an inverse correlation between SV and sympathetic nerve activity in the postprandial state. The rise in SV soon after meal ingestion may be crucial in stabilising BP during orthostatic stress.

In Chapter Four and Five, the work described here is extended to determine the ageing and gender effect on autonomic modulation, and autonomic dysfunction in glaucoma patients.

Figures and legends

Fig 1. Experimental Protocol



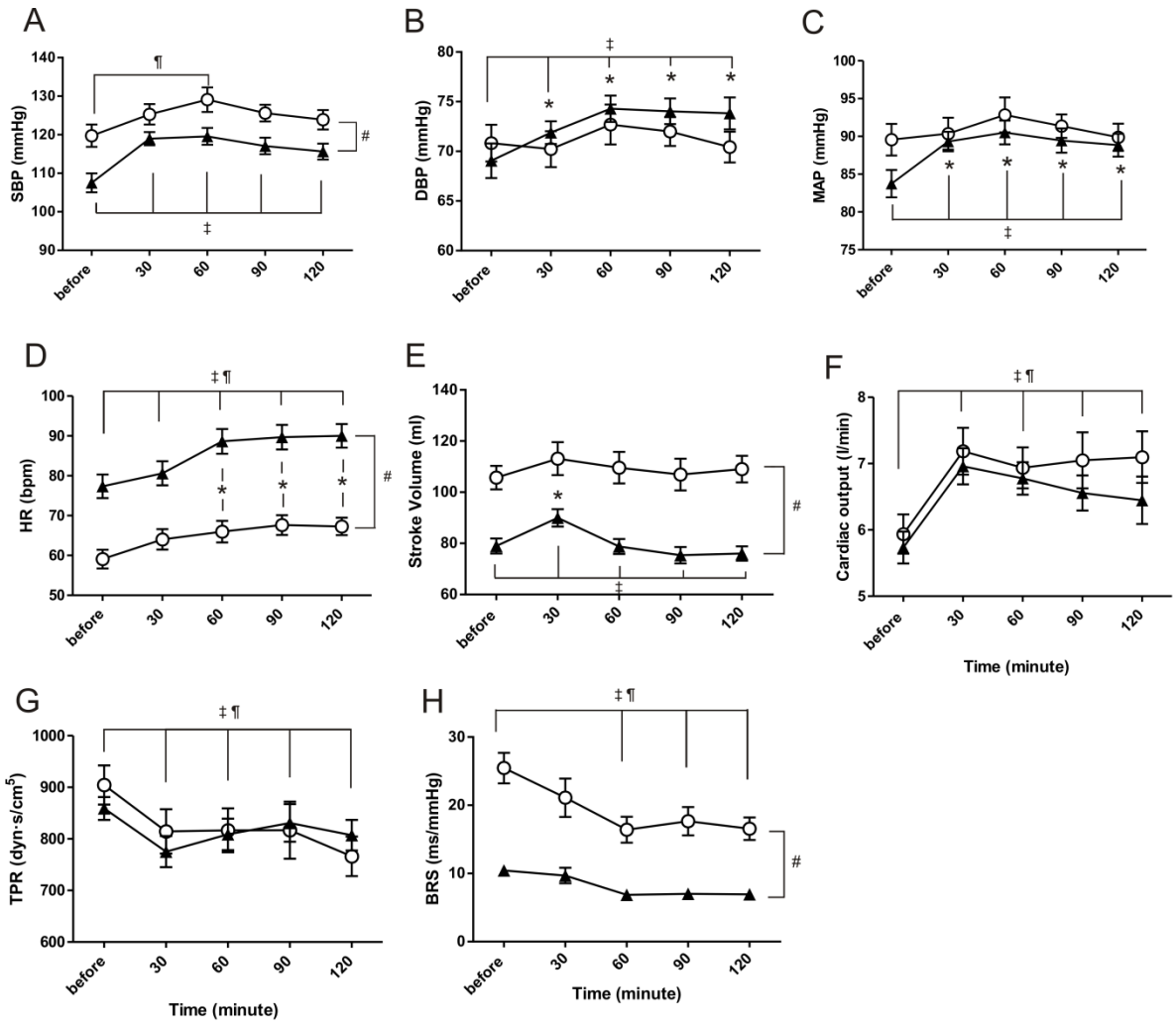


Fig 2, Haemodynamic responses to orthostatic stress and meal ingestion

# p<0.01, Orthostatic stress

† p<0.05, ‡ p<0.01, Meal ingestion during quiet standing;

§ p<0.05,¶ p<0.01, Meal ingestion in the supine state;

* p<0.05, interaction between orthostatic stress and meal ingestion.

▲ during quiet standing ○ in the supine state

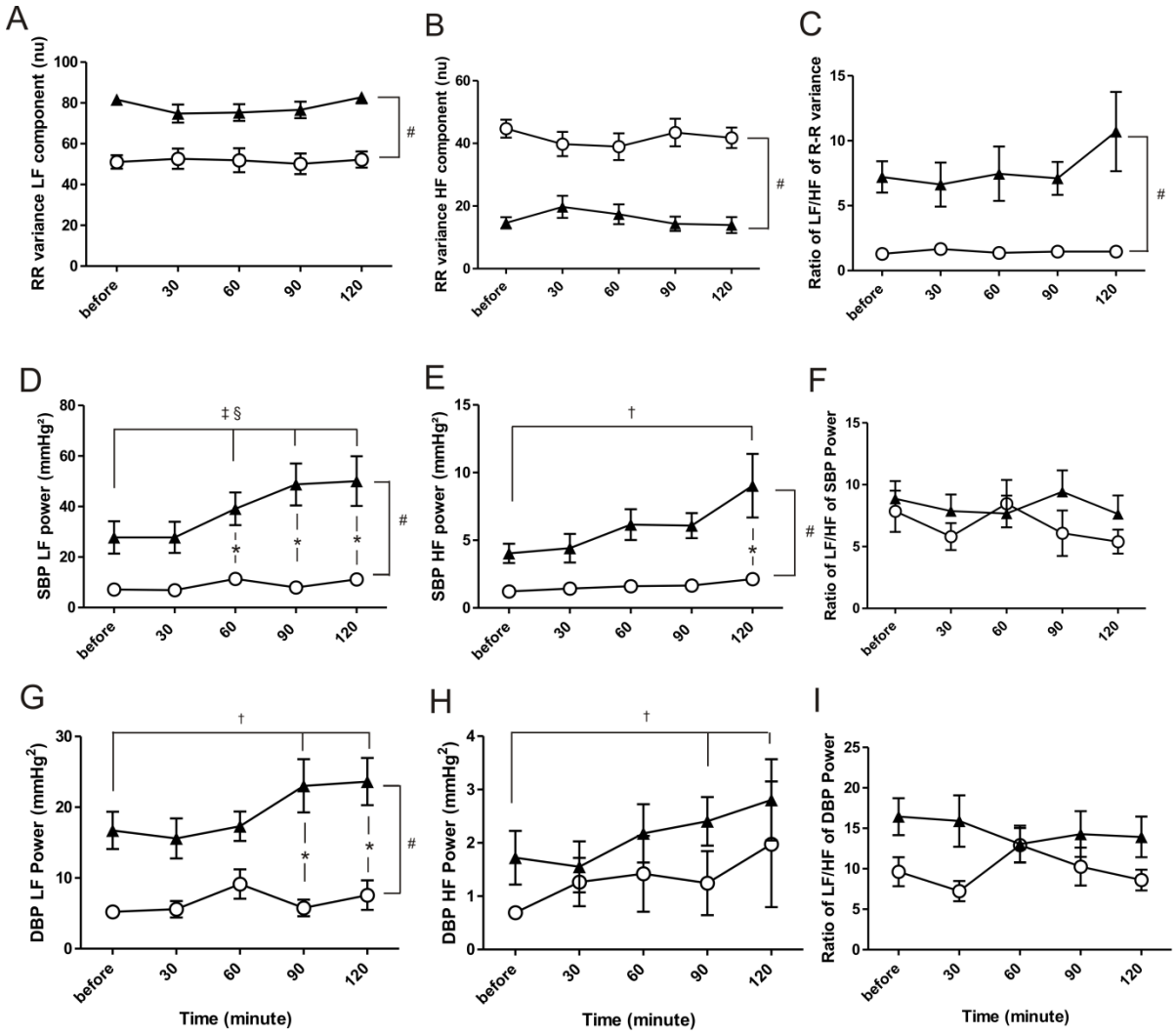


Fig 3, Autonomic control to orthostatic stress and meal ingestion
# p<0.01, Orthostatic stress
† p<0.05, ‡ p<0.01, Meal ingestion during quiet standing;
§ p<0.05,¶ p<0.01, Meal ingestion in the supine state;
* p<0.05, interaction between orthostatic stress and meal ingestion.
▲ during quiet standing ○ in the supine state

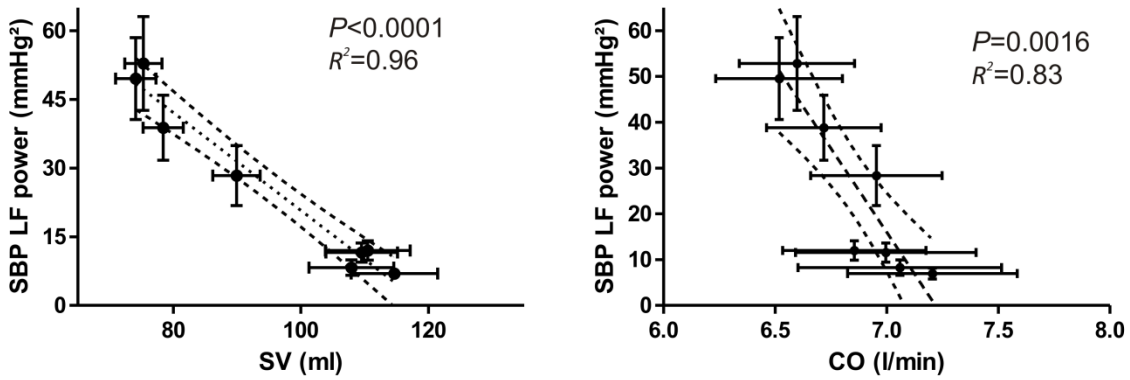
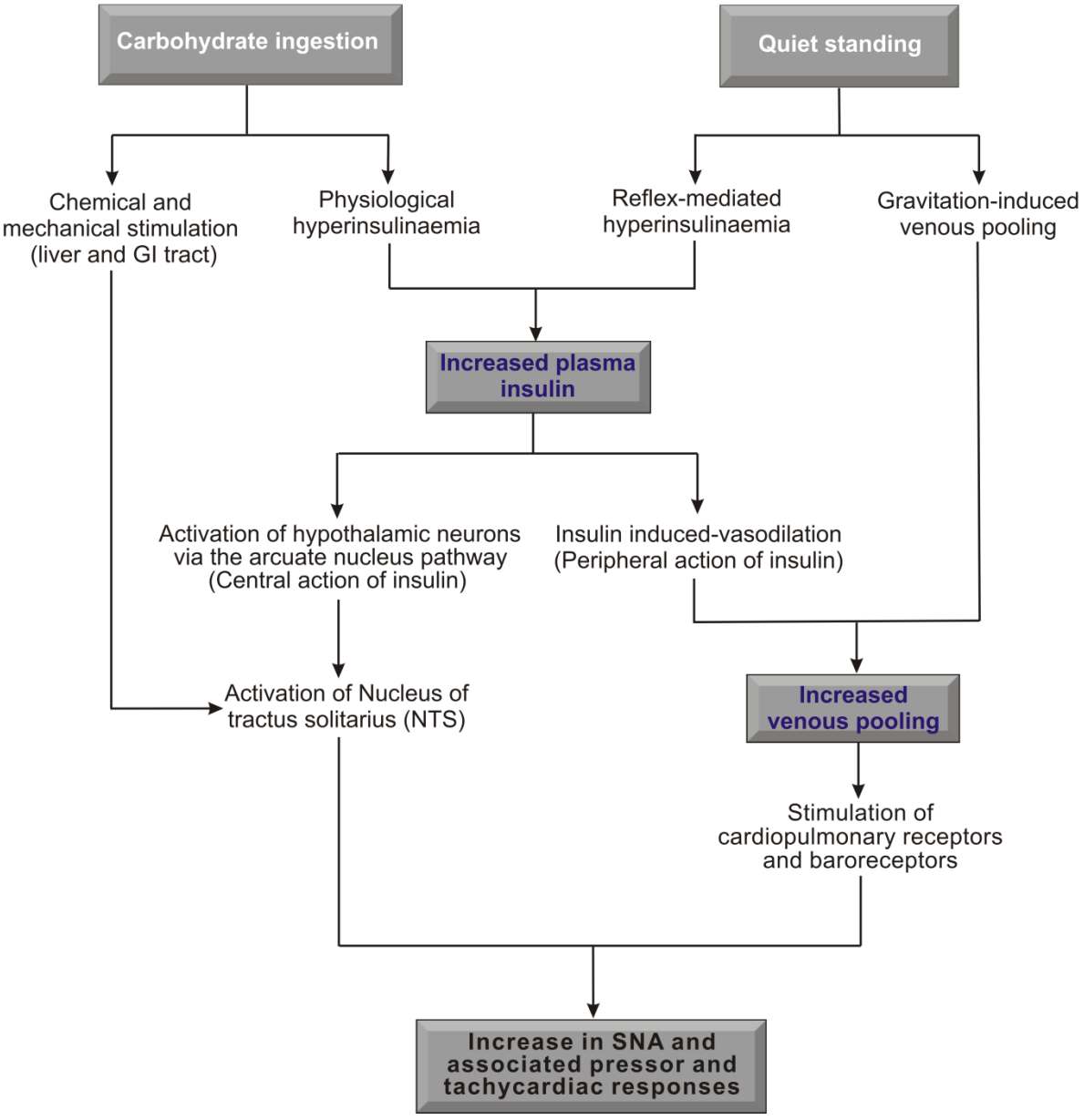


Fig 4, Grouped data of SBP LF power correlates to stroke volume (SV) and cardiac output (CO) at each postprandial time point of both supine and upright positions

Fig 5. Quiet standing after carbohydrate ingestion induced sympathoexcitatory and pressor response



Chapter Four: Result Two

This study is prepared to be submitted to the journal "American Journal of Physiology - Regulatory Integrative and Comparative Physiology" for publication, titled as "Ageing and Sex Effects on Cardiovascular Autonomic Regulation to Orthostatic Stress and Meal Ingestion"

Lei Cao, Stuart L Graham, Paul M Pilowsky

Ageing and Sex Effects on Cardiovascular Autonomic Regulation to Orthostatic Stress and Meal Ingestion

Abstract

Background: ageing and gender effects on autonomic regulation are associated with cardiovascular health.

Methods: Autonomic regulation to meal ingestion and orthostatic stress was examined in 14 young males, 14 older males and 21 older females. Continuous ECG and finger arterial pressure was recorded in the lying and standing position before and after eating a standard meal. Biochemical markers were screened. Power spectral analysis for heart rate and blood pressure variability and the sequence method for baroreflex sensitivity were used to quantify autonomic regulation.

Results: Basal SBP LF power is higher in older men than either young men or older women, and is positively correlated to MAP only in older men (P=0.035, r=0.56). In response to orthostatic stress, all three groups exhibited increases in SBP LF power, HRV LF nu and HRV LF/HF, and decreases in HRV HF nu and sBRS. In response to meal ingestion, SBP LF power increased in young and older men, but not in older women; in contrast, HRV LF nu and HRV LF/HF increased, HRV HF nu and sBRS reduced in older women, but not in young and older men. In response to the interplay of orthostatic stress and meal ingestion, young men showed enhanced SBP LF power with pressor response, but not in older groups.

Conclusion: ageing is associated with higher basal sympathetic tone, augmented sympathetic mediated vasoconstrictor effect, and attenuated autonomic and haemodynamic responses. Meal ingestion induced sympathetic activation to the vasculature is evident in men, but women favours cardiac autonomic modulation.

Key Words: autonomic, sympathetic, age, gender, orthostatic stress, meal ingestion, heart rate variability

Abbreviations:

LF: low frequency

HF: high frequency

MAP: mean arterial pressure

SBP: systolic blood pressure

DBP: diastolic blood pressure

HR: heart rate

HRV LF nu: RR interval LF power (component) in normalised unit

HRV HF nu: RR interval HF power (component) in normalised unit

HRV LF/HF ratio: RR interval LF/HF power (component) ratio

SBP LF power: Systolic blood pressure low frequency power

sBRS: sequence method for baroreflex sensitivity evaluation

Introduction

Ageing effect on autonomic regulation has been found to be associated with cardiovascular disease (Ferrari *et al.*, 2003; Jansen *et al.*, 1995; Lipsitz *et al.*, 1993). As humans age, basal sympathetic tone increases and the attenuation of autonomic regulation to daily stressors commonly occurs(Laitinen *et al.*, 2004). A study found that, with comparable normal level of oestrogen in a group of younger and postmenopausal women, parasympathetic control is the main regulator of cardiovascular system in young women, but sympathetic dominates in postmenopausal women (Lavi *et al.*, 2007). This may underlie the increased prevalence of cardiovascular disease in postmenopausal women.

Gender effects on autonomic regulation have recently been recognised (Hart *et al.*, 2009a; Hart *et al.*, 2012b; Huikuri *et al.*, 1996). Young women exhibited lower tonic autonomic support of arterial blood pressure and less effective baroreflex buffering than men (Christou *et al.*, 2005). Charkoudian et al. demonstrated that cardiac output (CO) inversely correlated to muscle sympathetic nerve activity (MSNA) at rest in young men, suggesting a balance between cardiac output and MSNA plays important role in regulating blood pressure (Charkoudian *et al.*, 2005). Interestingly, this correlation is absent in young women (Hart *et al.*, 2009a). Menstrual cycle affects sympathetic responses during passive tilting in young women (Fu *et al.*, 2009). Recent studies show that in young women, MSNA mediated vasoconstrictor effects and blood pressure changes, not only balanced by CO (Fu *et al.*, 2012), but also offset by the oestradiol induced β-adrenergic vasodilation effect in young women(Hart *et al.*, 2011; Wenner *et al.*, 2013). In postmenopausal women, oestrogen replacement therapy improved arterial baroreflex gain of MSNA, but did not changes the resting arterial pressure itself (Hunt *et al.*, 2001). The characteristics of autonomic regulation to daily stressors in older men and women (postmenopausal) remain unclear.

In response to orthostatic stress middle-aged women haveless effective cardiovagal baroreflex control than men of a similar age (Huikuri *et al.*, 1996). Head up tilting induces markedly increased autonomic responses in health subjects, with higher plasma noradrenaline and renin levels in older men than in older women (Geelen *et*

al., 2002). However, cardiovascular autonomic regulation markers, such as heart rate and blood pressure variabilities, and baroreflex sensitivity may not differ between genders (Laitinen *et al.*, 2004). It is recently demonstrated meal ingestion is a subtle laboratory stimulus than orthostatic stress, and may elicit distinguishing features of autonomic regulation (Cozzolino *et al.*, 2010). Gender effects on autonomic regulation to meal ingestion remain to be studied.

Power spectral analysis of heart rate and blood pressure variabilities is well established method in estimating autonomic modulation and has been widely used in clinical studies. We therefore aimed to investigate 1) ageing effect on autonomic regulation in basal state and in response to orthostatic stress and meal ingestion, 2) sex effect on autonomic regulation in basal state and in response to orthostatic stress and meal ingestion, 3) ageing and sex effects on autonomic and haemodynamic responses to the interplay of the orthostatic stress and meal ingestion. We hypothesised that, 1) ageing may be associated with attenuation in sympathetic regulation to orthostatic stress and meal ingestion (young males vs older males); 2) in comparison to older men with an increase invascular sympathetic outflow to meal ingestion, postmenopausal women may exhibit an increase in cardiac sympathetic response.

Research Design and Methods

Study participants

The study was approved by the Ethics Committee at Macquarie University, NSW, Australia. All subjects provided written informed consent.

In a preliminary study (see Chapter Three, Result one), 14 healthy male subjects were recruited from our medical school. Age 31.4 ± 2.9 years; height 179.6 ± 7.9cm; weight 75.9 ± 7.2kg; Body Mass Index (BMI) 23.6 ± 2.6 kg/m^2. All subjects were normotensive and were not taking any medications.

All older participants were recruited from communities through local newspaper advertisements, and fulfilled inclusion criteria: age of 40-80 years; no history of

significant cardiovascular diseases other than hypertension, i.e. stroke, heart attack, unstable angina, cardiac arrhythmia, heart failure; no previously diagnosed diabetes and not on anti-diabetic medications; no history of major cardiac surgery, any recent surgical operation and hospitalisation. Exclusion criteria were: cardiac arrhythmia identified during the experiment, i.e. atrial fibrillation and/or frequent ectopic beats. Liver function, renal function, lipid profile and fasting blood glucose and insulin level were screened prior to the experiment. (See Chapter Two, Methodology for detailed information)

Experimental protocol

As described in Chapter Three, Result One.

Recordings

As described in Chapter Two, Methodology and Chapter Three, Result One.

Assessment of autonomic regulation

As described in Chapter Two, Methodology and Chapter Three, Result One.

Spontaneous cardiac baroreflex function

As described in Chapter Two, Methodology. In summary, spontaneous baroreceptor reflex sensitivity (RR interval to systolic BP) was evaluated with HemoLab software (http://haraldstauss.com/HemoLab/HemoLab.php), using the sequence method that identifies sequences of four or more heart beats, where BP and pulse interval change in the same direction (Bertinieri *et al.*, 1985). A delay of 0-2 (young adults) and 0-5 (older subjects) physiological beat cycle(s) between systolic blood pressure and pulse interval was used to provide the most representative estimates of BRS.

Hemodynamic responses

As described in Chapter Three, Result One.

Statistical analysis

All statistical analyses were calculated using GraphPad Prism software (version 6). Subject profiles, clinical characteristics and the hemodynamic and autonomic data were evaluated by using unpaired *t* tests between study groups. Statistical significance for hemodynamic and autonomic responses was evaluated using two-

way ANOVA (repeated measures) (i.e. meal ingestion effects over time between lying and standing posture). Univariate correlations were calculated (Pearson R^2). A two-tailed P value <0.05 was regarded as statistically significant. Data are presented as means ± SEM in figures and means ± SD in tables.

Results

Baseline comparison of autonomic markers between groups: young males (N=14) vs older males (N=14), older males (N=14) vs older females (N=21)

In the fasting resting state (baseline), older males had higher HRV LF/HF ratio and lower sBRS than young males. There was no difference in SBP LF power between young and older males. Older males had higher SBP LF power and HRV LF/HF ratio than the older females, but similar sBRS (Table 1.) (Figure 1.).

Furthermore, there is a positive correlation between SBP LF power and MAP in older males (P=0.035, r=0.56), but not in young males (P=0.86, r=-0.05) and older females (P=0.88, r=0.04) (Figure 2.).

Autonomic and hemodynamic responses to orthostatic stress and meal ingestion in young healthy men (N=14)

In response to orthostatic stress before and after meal ingestion, HRV LF nu increased, HRV HF nu decreased and HRV LF/HF ratio increased, as well as SBP LF power increased and sBRS decreased (all P<0.01) (Figure 3.). HR increased (P<0.01) but MAP remained stable (Figure 4.). SV decreased (P<0.01), while CO and TPR remained unaltered.

In response to meal ingestion, both in lying and standing positions, SBP LF power increased and sBRS decreased (both P<0.01). HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged (Figure 3). Both MAP and HR increased (both P<0.01), i.e. from fasting to postprandial $_{max}$, lying MAP increased from 89.5 to 92.9mmHg (by 3.4mmHg), standing MAP increased from 83.7 to 90.5mmHg (by 6.8mmHg); lying HR increased from 59.1 to 67.6 bpm (by 8.5 bpm), standing HR increased from 77.3 to 90.0 bpm (by 12.7 bpm) (Figure 4.). Early after meal ingestion, SV and CO increased and TPR decreased (all P<0.01); SV and CO

returned to baseline level over time after meal ingestion, TPR remained in a lower level in the postprandial state (Figure 4.).

There are an enhancement of SBP LF power (P=0.0029) (Figure 3.) and an associated pressor response (P=0.024) during quiet standing in the postprandial state in young male subjects. Early after eating (30min), SV in standing augmented (P=0.026), compared to supine state other postprandial time points (Figure 4.).

Autonomic and hemodynamic responses to orthostatic stress and meal ingestion in older men (N=14) and older women (N=21)

In response to orthostatic stress, HRV LF nu increased, HRV HF nu decreased and HRV LF/HF ratio increased in both older males and females. SBP LF power markedly increased and sBRS decreased in both males and females (Figure 3.). MAP significantly increased in males, but not in females (P=0.055); HR increased in both male and females (Figure 4.). SV decreased (P<0.01, P<0.05), and CO remained unchanged in both males and females; TPR increased in older men (P<0.01), but not in older women (P=0.07) (Figure 4.).

In response to meal ingestion, HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged in males. In contrast, in females, HRV LF nu appeared no change (P=0.051), HRV HF nu decreased (P<0.01) and HRV LF/HF ration increased (P<0.05). SBP LF power markedly increased in males (P<0.01), but there was no such response in females (P=0.99). sBRS had no response in males (P=0.18), but markedly decreased in females (P<0.01) (Figure 3.). MAP had no change in males (P=0.96) and in females (P=0.057). HR increased in both males and females (both P<0.01). SV decreased in older males (P=0.01), but not in older females. CO and TPR remained unchanged in both genders. (Figure 4.)

Changes of autonomic markers in response to orthostatic stress in fasting state, and postprandial state: young males (N=14) vs older males (N=14), older males (N=14) vs older females (N=21)

In fasting state, orthostatic stress induced changes in SBP LF power are identical between young men and older men, and between older men and older women. The changes in HRV LF/HF ratio (sympathovagal shifting reserve) from lying to standing

are similar between young men and older men; whereas older women exhibited markedly smaller changes in HRV LF/HF ratio than men of a similar age ($P<0.05$). The magnitude of reduction in sBRS is significantly greater in young men compared to older men ($P<0.01$), older men and older women did not show difference. (Figure 5.)

In postprandial state, as mentioned above, SBP LF power were markedly increased in young and older males, whereas older females exhibited significantly less changes in SBP LF power than older males ($P<0.05$). The orthostatic stress induced changes in HRV LF/HF ratio become significantly different in young men and older men, young men has greater sympathovagal shifting reverse than older men ($P<0.01$). The changes in HRV LF/HF ratio become similar in older males and females. The changes in sBRS still greater in young men than older men ($P<0.05$), and there is no difference between genders. (Figure 5.)

Discussion

The primary new findings are that:

Ageing effects:

1) basal cardiac and vascular sympathetic tone is higher, and cardiac vagal baroreflex function is lower, in older men than in young men, and that SBP LF power is correlated to MAP in older men, but not in young men;

2) quiet standing in the postprandial state induces vascular sympathetic activation coupled with a pressor response in normotensive young, but not in older men.

Gender effects:

1) basal cardiac and vascular sympathetic tone is higher in older men than older women; SBP LF power is not correlated to MAP in older women;

2) in response to meal ingestion older men and women exhibit distinctive features of autonomic regulation, i.e. there is increased vascular sympathetic outflow in older men, but in older women cardiac sympathetic activation and

vagal inhibition with an associated reduction in baroreflex cardiac vagal function were observed.

Ageing effects on autonomic regulation to orthostatic stress and meal ingestion

We find that, in the resting condition, SBP LF power in older men is markedly higher than in young men, suggesting an enhancement of sympathetic outflow to the vasculature with aging. This is consistent with previous evidence that resting muscle sympathetic nerve activity (MSNA) chronically increases with age in human (Sundlof *et al.*, 1978). As reported here, cardiac vagal spectral power (heart period; 0.15-0.40 Hz) is lower in older men at rest, shifting the sympathovagal balance towards sympathetic predominance. This change may be associated with an attenuated cardiovagal baroreflex function (Jones *et al.*, 2001). In older adults, the higher level of vascular sympathetic tone is related to a lower reserve of sympathetic vasoconstrictor responses to laboratory stimuli (standing, eating), and the lower level of cardiac vagal baroreflex function may indicate a poorer ability to increase heart rate as required, to compensate for daily stressors, such as eating or standing (Fu *et al.*, 2004b; Jones *et al.*, 2001; Sundlof *et al.*, 1978). As a consequence, autonomic dysfunction may be associated with an increased incidence of cardiovascular diseases (Lucini *et al.*, 2002a).

Another major finding is that there is a positive relationship of mean arterial pressure with sympathetic outflow (SBP-LF power; 0.04 – 0.15 Hz) to the vasculature in the resting condition in older men (>60 years old; r=0.56; 31% of variance). A recent study examining the relationship between mean arterial pressure and MSNA in males > 40 years of age reported an r value of 0.37 (14% of variance) (Hart *et al.*, 2012a), studies in men younger than 40 report no relationship between mean arterial pressure and sympathetic activity using frequency analysis (this study) or microneurography (Hart *et al.*, 2012a; Sundlof *et al.*, 1978). To our knowledge this is the first study to demonstrate this relationship using photoplethysmography, rather than microneurography (Sundlof *et al.*, 1978) supporting the utility of this non-invasive approach in longitudinal measures of sympathetic function with aging in the clinic. The significant variability of resting MSNA observed in young men (Sundlof *et*

al., 1977) may be due to other factors that adapts the short-term blood pressure regulation, such as cardiac output (Charkoudian *et al.*, 2005) and peripheral vascular adrenergic responsiveness (Charkoudian *et al.*, 2006). As humans age, sympathetic neural control of the circulation becomes predominant, and is characterised by a positive relation of MAP with sympathetic nerve activity contributing to the pathogenesis of hypertension (Hart *et al.*, 2009b; Narkiewicz *et al.*, 2005).

Our data shows that, in response to quiet standing, HRV LF power nu in older men (and older women, see below) does not increase when compared to young men, suggesting a blunted cardiac sympathetic activation (Pagani *et al.*, 1986). This is in keeping with previous studies showing that aging is associated with attenuated sympathetic response (Jones *et al.*, 2001).

Our study also demonstrates for the first time that quiet standing in the postprandial state enhances sympathetic outflows to the vasculature and is associated with pressor and tachycardiac responses in young men, but not in older men. The sympathetic activation and blood pressure elevation may be of physiologically importance in adapting the combined stressors (Guyenet, 2006). (A diagram for proposed mechanism: See Chapter Three - Result One, Figure 5.)

In the early postprandial state, splanchnic hyperaemia and orthostasis-mediated lower body venous pooling are buffered by the baroreflex mechanism. Lack of this adaptation may influence circulatory haemostasis and impair nutrient delivery during various stimuli in the postprandial state (Chaudhuri *et al.*, 1992; Puvi-Rajasingham *et al.*, 1997). In the present study, we find the upright posture- induced reduction in stroke volume is significantly less prominent 30minutes after eating in young men, but not in older men. We cannot exclude the possibility that a less distensible ventricle (stiffer heart) in aging may influence circulatory homeostasis (Fu *et al.*, 2012; Gisolf *et al.*, 2004; Levine *et al.*, 1997).

On the other hand, in the later postprandial state, carbohydrate ingestion is associated with sympathetic activation (Berne *et al.*, 1989; Young *et al.*, 2010a), due primarily to hyperinsulinemia (Anderson *et al.*, 1991). Therefore, sympathetic activation and pressor response during standing in the postprandial state may

represent a better physiological reserve of insulin-mediated sympathoexcitatory effect in young adults (Young *et al.*, 2010a; Young *et al.*, 2010b).

Gender effects on autonomic regulation to orthostatic stress and meal ingestion

The current study investigated autonomic regulation in older postmenopausal women and age-matched older men at resting state and in response to orthostatic stress and meal ingestion.

Our data shows that basal SBP LF power is higher in older men than older women, and basal SBP LF power is positively correlated with MAP in older men, but not in older women. β-adrenergic mediated vasodilation (Mallem *et al.*, 2005) may offset α-adrenergic vasoconstriction. Lack of β-adrenergic mediated vasodilator effect may explain the increased cardiovascular risk in postmenopausal women (Hart *et al.*, 2011). Our data suggest that, in basal state, the sympathetic mediated vasoconstrictor effect is higher in older men than in postmenopausal women of a similar age. β-adrenergic receptor function on blood vessels may not be enough to explain the high cardiovascular risk in postmenopausal women and other mechanisms should be sought (Wenner *et al.*, 2013).

In the current study, older men exhibited higher cardiac autonomic modulation to quiet standing (orthostatic stress) in the fasting state, and a higher vascular sympathetic outflow than older women during orthostatic stress in the postprandial state (Fig 5.). This is in keeping with previous studies showing that men have greater baroreflex-mediated sympathetic control for maintenance of blood pressure (Barnett *et al.*, 1999; Geelen *et al.*, 2002; Shoemaker *et al.*, 2001). Notably, we report here for the first time that older men and women exhibited distinctive features of autonomic regulation in response to meal ingestion, i.e. there is marked enhancement of sympathetic outflow to the vasculature and unaltered cardiac autonomic response in older men; whereas in older women, vascular sympathetic outflows remained unchanged with cardiac sympathovagal balance shifting towards sympathetic predominance.

The possible mechanisms may be:

1) it is well documented that women have a lower sympathoadrenal activity-related autonomic support of blood pressure and less effective baroreflex buffering to the blood pressure changes (Christou *et al.*, 2005);

2) it is also reported, in response to handgrip exercise, that the increase in sympathetic outflow to the peripheral vasculature was lower in women than men, suggesting a blunted metaboreflex in women (Jarvis *et al.*, 2011);

3) postmenopausal women may be more sensitive to 17β-oestradiol exposure than men of the same age with respect to autonomic cardiovascular regulation, leading to lower peripheral vasoconstrictor tone. A recent study demonstrates that compared to women with normal orthostatic tolerance, women susceptible to orthostatic intolerance are more sensitive to 17β-oestradiol exposure; showing a decrease in forearm vascular resistance accompanied with compensatory increases in baroreflex-mediated cardiovagal heart rate responses (Wenner *et al.*, 2013).

4) the cardiac autonomic responses may reflect a compensatory modulation to arterial pressure fluctuation (DeBoer *et al.*, 1987; Sleight *et al.*, 1995), which seems less effectively buffered by the sympathetic outflow to the vasculature in females (Christou *et al.*, 2005).

Some subjects with mild hypertension (but without cardiovascular diseases) were included in the current study. Hypertension may be associated with abnormal sympathetic response to stressors (Lembo *et al.*, 1992).We noted that the baseline diastolic blood pressure in older men (78.29±8.84mmHg, mean±SD) was slightly higher than that in older women (70.67±8.00mmHg, mean±SD). Indeed, the blood pressure values in both group are in normal range (<90mmHg), and the small difference in blood pressure between groups might not be biologically significant.

In the current study we developed a novel protocol to investigate autonomic responses to orthostatic stress and meal ingestion and their interaction over time. This study therefore replicates one of the most common activities of daily life and uses time and frequency domain techniques to assess the effects on cardiovascular,

parasympathetic and sympathetic function in old, young, male, female subjects. Importantly, this was achieved non-invasively, yet the findings correlate well with invasive studies such as microneurography, noradrenaline spillover and glucose clamp. The approach is therefore useful for studies on large numbers of subjects and can be easily achieved at any location.

Limitations

The current study did not examine changes in insulin levels over time in the postprandial state, mainly because our study aimed to: 1) develop a non-invasive approach to evaluate autonomic responses to orthostatic stress before and after meal ingestion, without targeting the relationship between postprandial plasma insulin and sympathetic nerve activity (Anderson *et al.*, 1991; Berne *et al.*, 1989; Young *et al.*, 2010a); and 2) compare autonomic responses in normal population with age, gender differences and in pathological conditions. Meal ingestion seems to be a relatively subtle stressor (Cozzolino *et al.*, 2010) in our hands, requiring around two hours to observe the full autonomic response. For these reasons, we attempted to minimise the interference and associated mental stress that would occur with repetitive blood collection and needle insertion (Berne *et al.*, 1989). To mitigate this limitation, carbohydrate rich meal ingestion was used to ensure hyperinsulinaemia, which is known to increase sympathetic outflow to the vasculature (MSNA) in the postprandial state (Berne *et al.*, 1989; Young *et al.*, 2010a).

Conclusion

The present study provides new evidence that men and women rely on different integrated physiological mechanisms to maintain a stable blood pressure in response to daily stressors, and that the mechanisms change with age and gender can be altered in disorders that affect autonomic function adversely.

Legends

Fig 1. Baseline autonomic markers comparison: age and gender

Fig 2.Relationships of SBP LF power with MAP in young men (N=14), older men (N=14) and older women (N=19).

SBP LF power is positively correlated to MAP in older men (Pearson's r), but the relationship exists neither in young men nor in older women.

Fig 3.Autonomic responses to orthostatic stress and meal ingestion amongst young men (N=14), older men (N=14) and older women (N=21)

In response to orthostatic stress, in all groups, SBP LF power increased, HRV LF nu increased, HRV HF nu decreased, HRV LF/HF ratio increased, and sBRS decreased. In response to meal ingestion, SBP LF power increased in young and older men ($P<0.01$), but not in older women. Conversely, in older women, HRV LF nu increased, HRV HF nu decreased, and HRV LF/HF ratio increased ($P<0.05$, $P<0.01$, $P<0.05$), with a reduction in sBRS ($P<0.01$), but the HRV responses remained unchanged in young and older men.

White circle: lying position; filled triangle: standing position.

Fig 4.Hemodynamic responses to orthostatic stress and meal ingestion amongst young men (N=14), older men (N=14) and older women (N=21)

In response to orthostatic stress, MAP increased only in older men ($P<0.01$), but not in young men and older women. HR increased in all three groups ($P<0.01$).

In response to meal ingestion, HR increased in all groups (all $P<0.01$). MAP increased only in young men ($P<0.01$), but not in older subjects.

White circle: lying position; filled triangle: standing position.

Fig 5. Changes of autonomic modulation to orthostatic stress in fasting and postprandial state

As described in the Text.

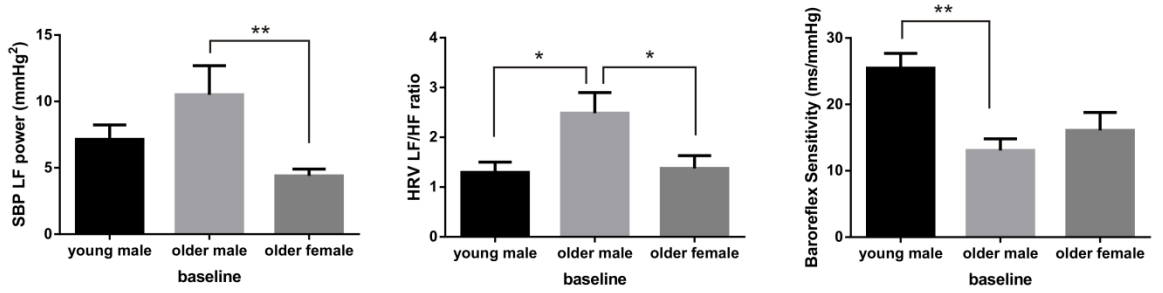
Figures

Table 1. Baseline comparison of autonomic and hemodynamic markers between young males, older males and older females

	Young Male(N=14)	Old Male (N=14)	Old Female (N=21)	P value
Age, years	31.4 ± 2.9	65.00 ± 7.87	64.05 ± 1.65	**
BMI, Kg/m2	23.6 ± 2.6	24.73 ± 2.8	25.34 ± 1.29	
Hemodynamic				
Systolic BP, mmHg	122.4 ± 10.14	127.40 ± 13.21	120.80 ± 14.59	
Diastolic BP, mmHg	74.36 ± 6.09	78.29 ± 8.84	70.67 ± 8.00	^
HR, bpm	56.57 ± 10.41	58.57 ± 7.1	61.48 ± 6.68	
Autonomic				
SBP LF power, mmHg2	7.14 ± 4.07	10.51 ± 8.15	4.40 ± 2.21	
HRV LF power, ms2	1083 ± 944.0	454.6 ± 322.7	452.3 ± 453.9	*
HRV HF power, ms2	812.7 ± 704.6	391.6 ± 539.3	450.6 ± 544.5	
HRV LF/HF ratio	1.29 ± 0.75	2.49 ± 1.54	1.38 ± 1.17	* ^
BRS (ms/mmHg)	25.45 ± 8.39	13.06 ± 6.54	16.08 ± 11.78	**

Data shown as Mean±SD; P value is calculated by unpaired t-test between groups.
young male vs old male (age difference): * P<0.05, ** P<0.01
older male vs older female (gender difference): ^ P<0.05, ^^ P<0.01

Fig 1. Baseline autonomic markers comparison: age and gender



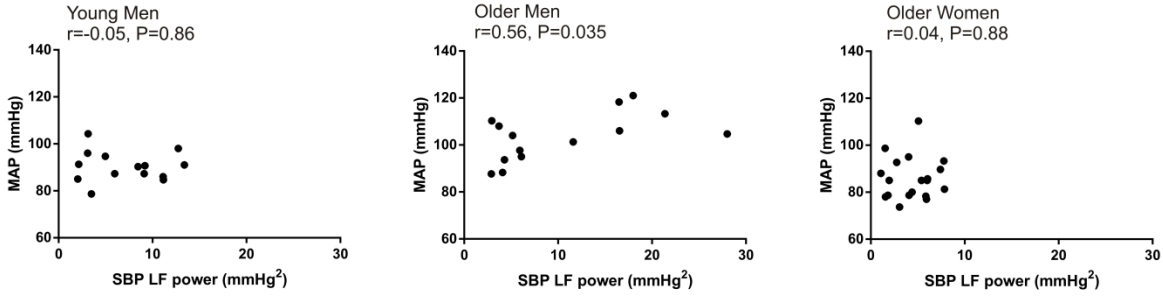


Fig 2. Relationships of SBP LF power with MAP in young men (N=14), older men (N=14) and older women (N=19). SBP LF power is positively correlated to MAP in older men, but the relationship of MAP with SBP LF power exists neither in young men nor in older women.

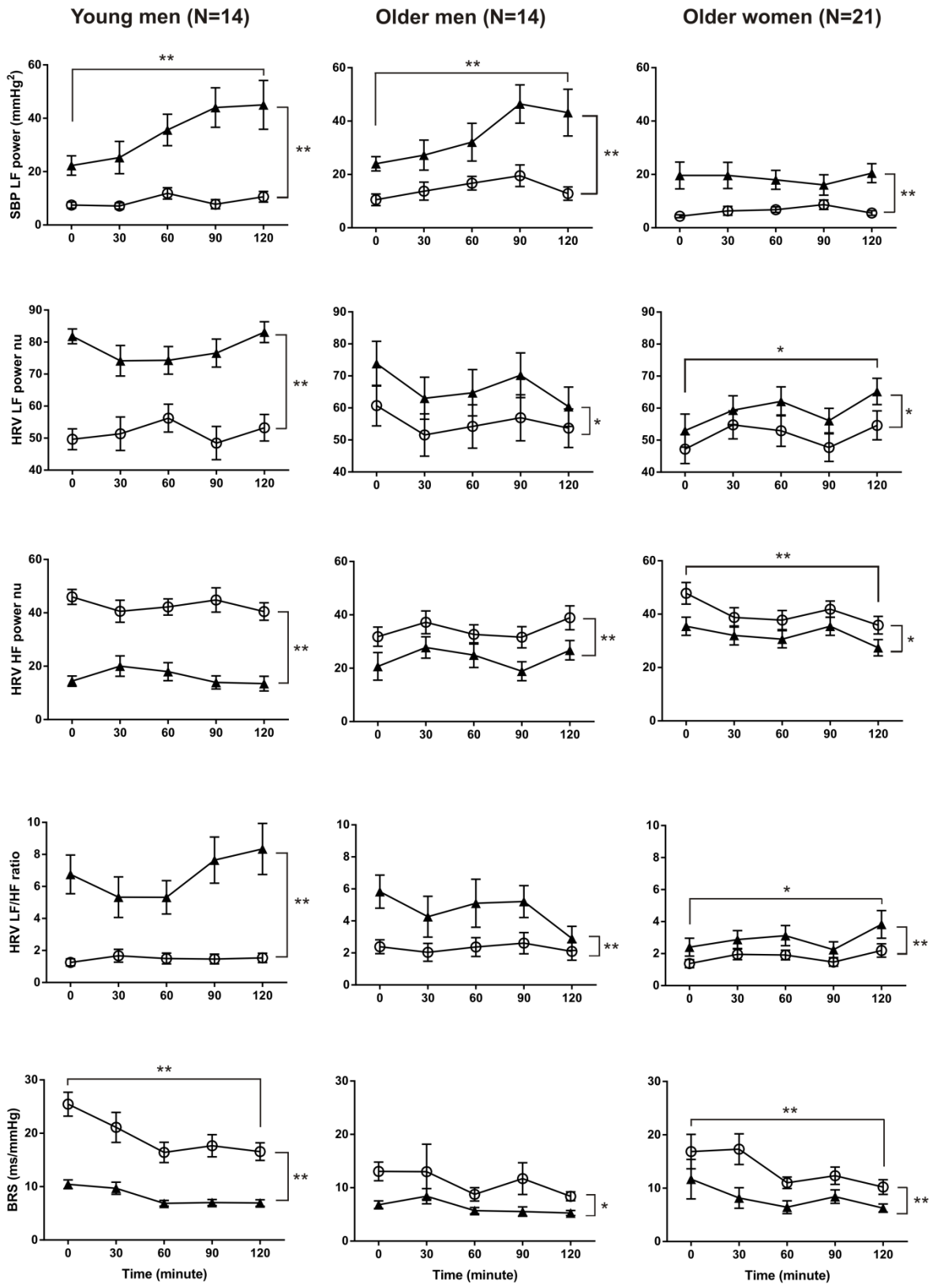
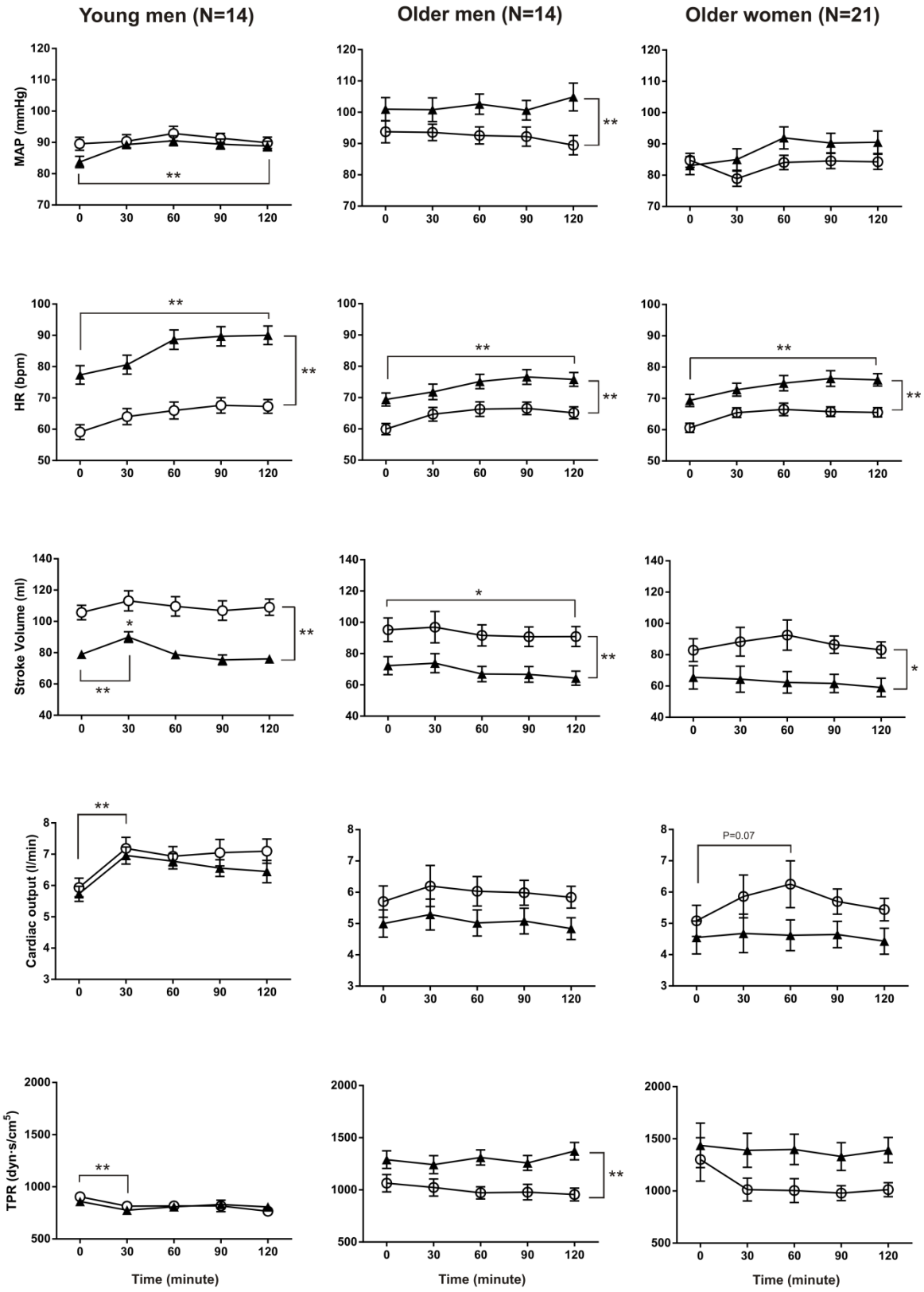


Fig 3. Autonomic responses to orthostatic stress and meal ingestion amongst young men, older men and older women

Fig 4. Hemodynamic responses to orthostatic stress and meal ingestion amongst young men, older men and older women



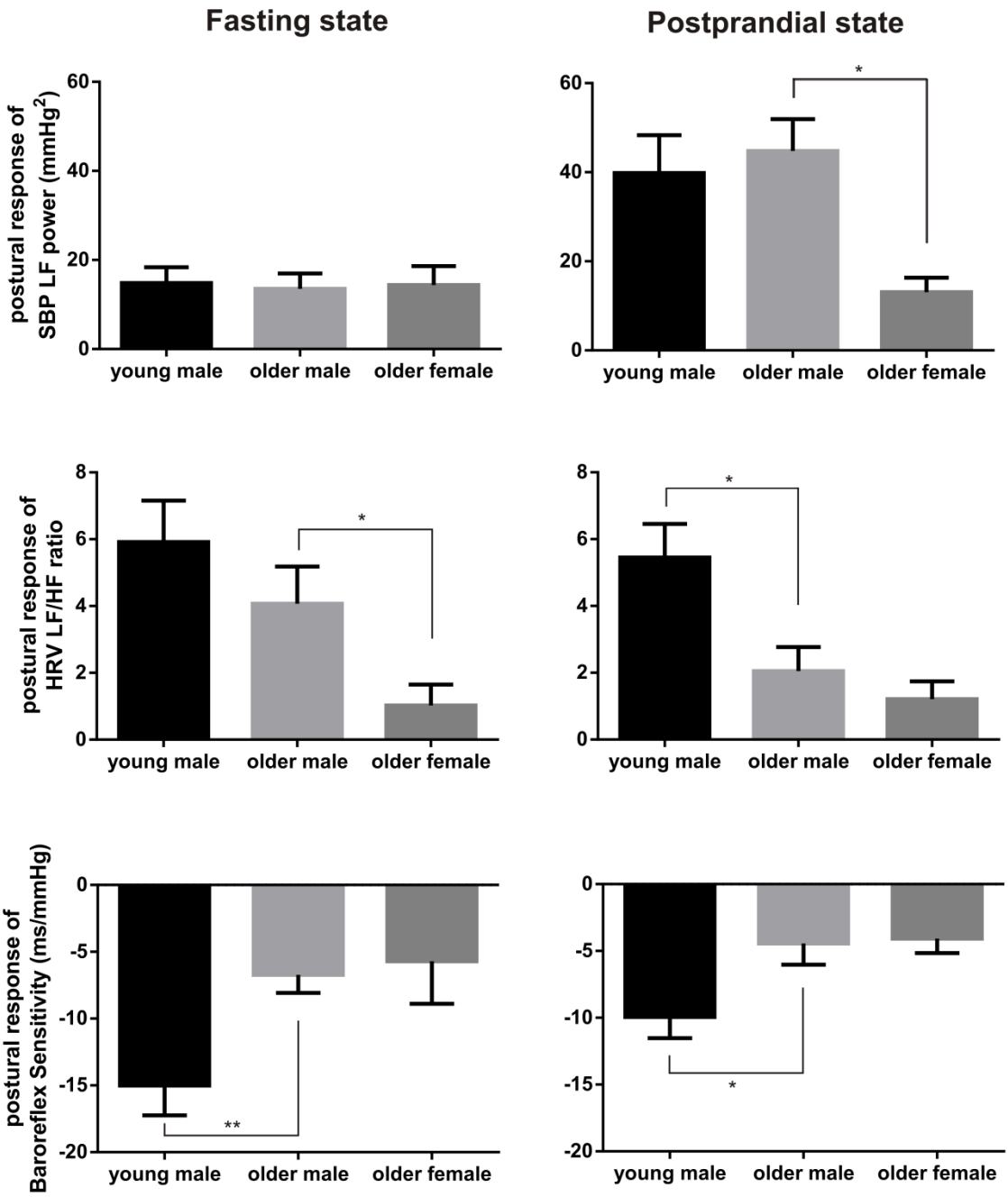


Fig 5. Changes of autonomic modulation to orthostatic stress in fasting and postprandial state

Chapter Five: Result Three

This study is prepared to be submitted to the journal "Investigative Ophthalmology and Visual Science" for publication, titled as "Autonomic Dysfunction in Glaucoma: autonomic responses to meal ingestion and orthostatic stress differ in normal tension and high tension glaucoma"

Lei Cao, Stuart L Graham, Paul M Pilowsky

Autonomic Dysfunction in Glaucoma: autonomic responses to meal ingestion and orthostatic stress differ in normal tension and high tension glaucoma

Abstract

Background– Systemic autonomic dysfunction may underlie the pathogenesis of the spectrum of glaucoma, from normal tension glaucoma (NTG) to primary open angle glaucoma (POAG). Whether or not NTG and POAG manifest features of autonomic dysfunction and the existence of gender differences in vascular risk in NTG remains unknown.

Methods and Results - We examined autonomic regulation in 21 NTG, 32 POAG, and 43 age-gender-matched controls. Continuous ECG and finger arterial pressure was recorded in the lying and standing position before and after eating a standard meal. Biochemical markers were screened. Power spectral analysis for heart rate and blood pressure variability and the sequence method for baroreflex sensitivity were used to quantify autonomic regulation. Compared to controls, cardiac autonomic responses were attenuated in both NTG and POAG to orthostatic stress. Notably, in response to meal ingestion, there exist cardiac autonomic failure with hypotension in POAG and cardiac sympathetic hyper-responsiveness in NTG. NTG females exhibited exaggerated postprandial cardiac sympathetic activation

compared to control females. Hypertensive POAG showed postprandial sympathetic over-activation. Topical β-blockers inhibited the postprandial sympathetic hyper-responsiveness, but decreased mean arterial pressure early postprandially. Fasting glucose is positively correlated to fasting insulin in control and POAG; and inversely correlated to postprandial standing SBP LF power in control, but not in POAG.

Conclusions – The distinctive features of systemic autonomic dysfunction underlie the vascular risk, and the degree of chronic elevation of intraocular pressure in NTG and POAG. Older women are at a higher risk of developing NTG. Hypertension is an additional risk factor in POAG regardless of gender. Taken together, the findings suggest that both NTG and POAG are part of a systemic autonomic cardiovascular disorder.

Key Words: autonomic, sympathetic, gender, hypertension, orthostatic stress, meal ingestion, heart rate variability, intraocular pressure (IOP)

Abbreviations:

LF: low frequency

HF: high frequency

MAP: mean arterial pressure

SBP: systolic blood pressure

DBP: diastolic blood pressure

HR: heart rate

HRV LF nu: RR interval LF power (component) in normalised unit

HRV HF nu: RR interval HF power (component) in normalised unit

HRV LF/HF ratio: RR interval LF/HF power (component) ratio

SBP LF power: Systolic blood pressure low frequency power

sBRS: sequence method for baroreflex sensitivity evaluation

Glaucoma Study – Main Text

Introduction

Glaucoma is one of the most common causes of blindness, with a loss of retinal ganglion cells, leading to visual field defects (Weinreb *et al.*, 2004). Growing evidence indicates that vascular risk underlies the pathogenesis of glaucoma in normal tension glaucoma (NTG), and in those with elevated intraocular pressure (IOP), regardless of the pathological role of elevated intraocular pressure (IOP) (Flammer *et al.*, 2007). By convention, primary open angle glaucoma (POAG) refers to all cases of glaucoma regardless of IOP level (despite the fact that many clinicians refer to POAG as only those with elevated IOP levels). High tension glaucoma (HTG) refers to those with a documented IOP >21mmHg (although the term HTG is rarely used clinically). The subtype of POAG with an IOP of less than 21mmHg is termed NTG. More recently, it was suggested that the two forms of glaucoma (NTG and HTG) share a similar pathogenic pathway from a vascular perspective (Mroczkowska *et al.*, 2013), and it is proposed that the term NTG is of little value (Sommer, 2011). For the purposes of this study we have used the term POAG to represent the HTG subgroup.

A study suggests that patients with both NTG and POAG exhibit similar autonomic dysfunction (Brown *et al.*, 2002). Autonomic dysfunction is found as an early marker of pathogenesis that contributes to the incidence of cardiovascular disease (Lucini *et al.*, 2005; Lucini *et al.*, 2002a; Lucini *et al.*, 2002b), and may be a harbinger of hypertension and diabetes (Lucini *et al.*, 2002a; Straznicky *et al.*, 2012). We therefore hypothesise that distinguishing features of autonomic dysfunction may also exist in the two forms of glaucoma and along the entire glaucomatous optic neuropathy process. In other words, the distinctive features of autonomic dysfunction may underlie the pathogenesis of glaucoma and/or correlate with the different degrees of chronic IOP elevation in both NTG and HTG. Given the known gender differences in cardiovascular regulation (Christou *et al.*, 2005; Hart *et al.*, 2011) and prevalence of cardiovascular disease in older women (Bugiardini *et al.*, 2005; Mosca *et al.*, 1997), we also investigated whether or not older women (postmenopausal) may be at additional vascular risk in NTG (vulnerability to IOP elevation). Since hypertension may be an independent risk factor for the development of POAG

(Newman-Casey *et al.*, 2011), we then examined whether or not sympathetic overexcitation may underlie the pathogenesis of POAG patients with hypertension.

Quiet standing induces orthostatic stress and is a well-established approach in the examination of baroreflex-mediated autonomic function (Montano *et al.*, 1994; Pagani *et al.*, 1986). Carbohydrate meal ingestion is associated with splanchnic hyperaemia (Someya *et al.*, 2008) and sympathetic activation (Berne *et al.*, 1989; Young *et al.*, 2010a). Compared with orthostatic stress, meal ingestion provides a unique window to examine autonomic function and may further differentiate autonomic dysfunction in normal aging and pathological conditions (Cozzolino *et al.*, 2010; Lipsitz *et al.*, 1993). Power spectral analysis of heart rate and blood pressure variabilities is a widely used method to evaluate sympathetic and parasympathetic responses to daily stressors (Malik, 1996; Malliani *et al.*, 1991; Pagani *et al.*, 1986; Pagani *et al.*, 1997).

The current study initiated a prospective cross-sectional study to examine autonomic responses in age and gender-matched subjects and in glaucoma patients, including both NTG and POAG. It was aimed to investigate 1) whether or not there are similarities and differences in the autonomic responses between the two forms of glaucoma; 2) whether or not older women have an additional vascular risk in NTG; 3) the pathological role of hypertension in POAG; 5) topical β-blockers effects on autonomic and hemodynamic responses in glaucoma patients; and 6) relationships between metabolic and autonomic markers.

Research design and Methods

Study participants

The study was approved by the Ethics Committee at Macquarie University, NSW, Australia. All subjects provided written informed consent.

32 POAG, 21 NTG patients and 43 volunteer subjects fulfilled inclusion and exclusion criteria, and served for the clinical study (Figure 1. Recruitment Flowchart). Previously diagnosed glaucoma patients were recruited by a consultant ophthalmologist (S.L.G.) and included both POAG and NTG patients (n=36). In

addition, patients (n=20) and control volunteers (n=52) were recruited from the general population via an advertisement in the local newspaper and from Glaucoma Australia. Accuracy of diagnosis was confirmed on medical records and a retinal nerve fibre layer (RNFL) scan (Spectralis OCT, Heidelberg Eng, Germany) after the experimental study. Control volunteers were defined as normal based on the medical history screening, results of ophthalmic examination, and a RNFL scan. For any control subjects who were suspect in RNFL scan results, further full clinical investigations were conducted (S.L.G.) to confirm, or exclude the diagnosis of glaucoma or other pathology. All participants fulfilled inclusion criteria: age of 40-80 years; no history of significant cardiovascular diseases other than hypertension, i.e. stroke, heart attack, unstable angina, cardiac arrhythmia, heart failure; no previously diagnosed diabetes and not on anti-diabetic medications; no history of major cardiac surgery, any recent surgical operation and hospitalisation. Exclusion criteria were: cardiac arrhythmia identified during the experiment, i.e. atrial fibrillation and/or frequent ectopic beats; community-sourced patients with other types of glaucoma, i.e. pigment dispersion glaucoma. Two volunteers could not be differentiated as either early glaucoma or normal based on clinical investigations and were excluded. In total, 108 subjects were recruited for the study (European derived population accounts for 95% of study subjects); eligible subjects were used for data analysis (n=96), including POAG (n=32), NTG (n=21), and control (n=43) (Figure 1. Recruitment Flowchart).

Clinical investigations

A medical history was taken in each subject paying particular attention to eye and cardiovascular disease, and medication use. Anthropometric measurements comprised body weight, BMI, waist circumference, and waist/hip ratio. Fasting blood tests for renal function, liver function, lipid profile, and Blood Sugar Level (BSL) and plasma insulin levels were taken in all subjects.

Experimental Protocol

As described in Chapter Three, Result One.

Assessment of autonomic regulation

As described in Chapter Two, Methodology.

Spontaneous cardiac baroreflex function

As described in Chapter Two, Methodology.

Hemodynamic responses

As described in Chapter Three, Result One and Chapter Four, Result Two.

Statistical analysis

All statistical analyses were calculated using GraphPad Prism software (version 6). Subject profiles, clinical characteristics and the hemodynamic and autonomic data were evaluated by using unpaired *t* tests or chi-square tests (Fisher's exact tests) between study groups. Statistical significance for hemodynamic and autonomic responses were evaluated using two-way ANOVA (repeated measures) (i.e. meal ingestion effects over time between lying and standing posture). Univariate correlations were calculated (Pearson R^2). A two-tailed P value <0.05 was regarded as statistically significant. Data are presented as means ± SEM in figures and means ± SD in tables.

Results

Clinical characteristics of study groups

General profile, metabolic parameters, and hemodynamic data were comparable in POAG (n=32) and NTG (n=21) patients to control subjects (n=43). POAG patients showed higher fasting BSL and blood pressure in both lying and standing positions than control subjects, as well as more history of hypertension and prescribed vasoactive drugs (Table 1. Subjects Profile).

Data from subjects prescribed oral vasoactive drugs was considered separately. Thus, 36 control subjects (14 males and 22 females) and 37 glaucoma patients (14 males and 23 females) (χ^2 test, *P*=1), including 19 NTG and 18 POAG, were studied to evaluate autonomic and hemodynamic responses to orthostatic stress and meal ingestion. All female subjects were postmenopausal and without hormone

replacement therapy (one woman in the control group was excluded due to her younger age and peri-menopausal status).

Hemodynamic and autonomic responses in control (N=36) and glaucoma groups (N=37)

In response to orthostatic stress, HRV LF nu increased, HRV HF nu decreased, and therefore, HRV LF/HF ratio increased; SBP LF power increased and sBRS decreased in both control subjects and glaucoma patients (Figure 2.). MAP increased and HR increased in both control subjects and glaucoma patients (Figure 3.).

In response to meal ingestion, HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged in controls subjects. In contrast, in glaucoma patients, HRV LF nu increased, HRV HF nu reduced, and HRV LF/HF ratio increased. In both control, and glaucoma subjects, SBP LF power increased, and sBRS decreased (Figure 2.). In controls MAP remained unchanged, but was unstable in glaucoma subjects, i.e. decreased 30min after meal ingestion and elevated 60min after meal ingestion. HR increased in both control and glaucoma subjects (Figure 3.).

Hemodynamic and autonomic responses in glaucoma patients with (N=17) or without (N=20) topical β-blocker eye drop

In response to orthostatic stress, HRV LF nu increased slightly, but significantly, in β-blocker users, but not in non- β-blocker patients. HRV HF nu decreased in both β-blocker users and non- β-blocker patients. The HRV LF/HF ratio did not change in β-blocker users, but increased in non- β-blocker patients. SBP LF power increased and sBRS decreased in both the β-blocker and non- β-blocker groups. MAP did not change in both non- β-blocker group and β-blocker users; HR increased in both groups (Figure 4.).

In response to meal ingestion, HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged in β-blocker users. In non-β-blocker patients, HRV LF nu increased, HRV HF nu reduced, and HRV LF/HF ratio increased. SBP LF power increased and sBRS decreased in both groups. MAP elevated in the postprandial

state in non-β-blocker patients (P<0.01), but decreased in β-blocker users early after meal ingestion (at 30min) (P=0.01); HR increased in both groups (Figure 4.).

Baseline hemodynamic and autonomic data in the fasting state does not differ between the three groups

BP, HR, sBRS, HRV and BPV were comparable (Table 2.).

Autonomic and hemodynamic responses to postural stress in fasting and in the early postprandial state (30min time point)

In response to orthostatic stress, HRV LF nu increased, HRV HF nu decreased and the HRV LF/HF ratio increased in control subjects. HRV HF nu decreased in NTG and POAG, however, HRV LF nu and HRV LF/HF ratio did not increase in NTG or POAG subjects. sBRS decreased and SBP LF power increased in all groups in response to orthostatic stress before and after a meal (Figure 5). MAP increased in control, but there was no change in NTG and POAG patients. HR increased in control, NTG and POAG groups. In addition, total peripheral resistance (TPR) increased in control, but there was no change in NTG and POAG patients. Cardiac output decreased in control and POAG, but not in NTG (Figure 6.).

Early after meal ingestion (30 min), in POAG patients, HRV LF nu decreased, HRV HF nu increased and HRV LF/HF ratio decreased. In contrast, HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged in control subjects and NTG patients. SBP LF power and sBRS remained unchanged in control, NTG and POAG groups (Figure 5.).

Early after meal ingestion, MAP fell (P<0.01) in POAG patients from 97.3mmHg to 86.7mmHg in the supine state and from 100.5 mmHg to 93.4 mmHg during quiet standing. TPR also decreased (P<0.01) in POAG patients, i.e. from 1231 dyn·s/cm^5 to 1032 dyn·s/cm^5 in the supine state and from 1334 dyn·s/cm^5 to 1161 dyn·s/cm^5 during quiet standing. MAP remained stable in control and NTG subjects, as well as TPR. HR all increased in three groups. Cardiac output increased in control, but did not change in NTG and POAG patients (Figure 6.).

Within the 18 POAG patients, those not prescribed topical β-blockers (N=8) also exhibited decreased HRV LF nu and HRV LF/HF ratio, although MAP remained unchanged. On the other hand, a postprandial depressor response was found in POAG patients prescribed topical β-blockers (96±4mmHg vs 84±7 mmHg – lying; 102±5 mmHg vs 92±6 mmHg - standing; P<0.02).

Autonomic and hemodynamic responses to postural stress in fasting and in the later phases after eating (60, 90, 120min time points).

In control subjects, orthostatic stress increased HRV LF nu, a decrease in HRV HF nu and an increase in HRV LF/HF ratio in both fasting and postprandial states. In NTG patients, from lying to standing, HRV LF nu did not increase, but HRV HF nu decreased and HRV LF/HF ratio increased. In POAG patients, although HRV HF nu significantly reduced, the response of HRV LF nu was absent and HRV LF/HF ratio did not increase. SBP LF power increased and sBRS inhibited in control, NTG and POAG subjects (Figure 7.). MAP increased when assuming the upright position in control subjects and POAG, but remained unchanged in NTG (Figure 7.). HR increased in all three groups.

Meal ingestion increased HRV LF nu, reduced HRV HF nu and increased the HRV LF/HF ratio during lying or standing in NTG patients (P<0.01, P<0.05, P<0.01). The HRV LF/HF ratio increased by 44.7% (from1.41 to 2.04) in the lying position and 91.7% (from 2.29 to 4.39) in the standing position. In contrast, HRV LF nu, HRV HF nu and HRV LF/HF ratio remained unchanged in control subjects and in POAG patients (Figure 7.). Within these 19 NTG patients, those who were not prescribed topical β-blockers (N=12) also showed HRV HF nu decreased and HRV LF/HF ratio increased. Meal ingestion induced increases in SBP LF power in the later phase of the postprandial state in control, NTG and POAG subjects. In addition, quiet standing in the later phase of the postprandial state induced a further enhancement in SBP LF power in POAG patients, but this effect was not observed in either control or NTG groups. There were significant decreases in sBRS in the later phase after meal ingestion in control, NTG and POAG groups. There is no change in MAP from the fasting state to the later phase of the postprandial state in control, NTG and POAG groups (Figure 7.). HR increased in all three groups.

Autonomic responses to meal ingestion and orthostatic stress between control females (N=21) and NTG females (N=13)

We also compared NTG females with control females. In NTG female patients (N=13), HRV LF nu increased, HRV HF nu reduced, and HRV LF/HF ratio increased in response to orthostatic stress. In response to meal ingestion, although HRV LF nu did not change, HRV HF nu significantly decreased and the HRV LF/HF ratio increased. During quiet standing in the postprandial state, the HRV LF/HF ratio increased significantly from 1.33 in fasting state to 5.2 at 90 min (3 fold), and to 3.53 by 120min (2 fold) in NTG females (Figure 8. A,B). We also compared the change in postprandial autonomic responses (i.e. postprandial $_{\text{mean of90+120min}}$ - fasting) during quieting standing between control females and NTG females. The changes of HRV LF nu $_{\text{postprandial-fasting}}$ did not differ, but the reduction of HRV HF nu $_{\text{postprandial-fasting}}$ was greater in NTG females than control females, and the increase of HRV LF/HF ratio $_{\text{postprandial-fasting}}$ was markedly higher in NTG females than control females (3.04±1.14 vs 0.05±0.59) (P=0.016) (Figure 8. C,D).

Responses of SBP LF power between normotensive and hypertensive subgroups of POAG patients

Since POAG patients (N=32) had higher DBP and MAP than age-gender matched control subjects (N=43) in the fasting state (Table 1), the POAG patients were divided into normotensive (DBP below 90 mmHg; n=15) , or hypertensive (DBP above 90 mmHg; n=17) in the lying and/or standing position, regardless of history of hypertension and vasoactive drug treatment (normotensive subgroup (5/10), hypertensive subgroup (11/6); χ^2 test (fisher's), P=0.16).

In response to orthostatic stress, SBP LF power was elevated in both normotensive and hypertensive subgroups in both fasting and postprandial states (Figure 9. C,D). HRV LF nu did not change, HRV HF nu was markedly reduced and the HRV LF/HF ratio increased in both subgroups (Figure 9. A,B).

In response to meal ingestion, HRV LF nu increased, HRV HF nu reduced and the HRV LF/HF ratio increased in the hypertensive subgroup, but remained unchanged in the normotensive subjects (Figure 9. A, B). SBP LF power increased in

hypertensive subjects, while SBP LF power remained unchanged in normotensive subjects (Figure 9. C, D). After meal ingestion, both SBP LF power and HRV responses were markedly enhanced from 60min and remained at a high level until 120min in both the lying and standing positions

In addition, in the fasting state, SBP LF powers were comparable in both lying and standing positions between normotensive and hypertensive subgroups. In the postprandial state the hypertensive subgroup exhibited higher SBP LF powers (i.e. mean value of time points of 60, 90, 120mins) in both lying and standing positions compared with the normotensive subgroup (Figure 9. E, F, G, H).

Correlation of BSL to insulin and SBP LF power in POAG and control groups

To determine the relationship of fasting BSL to fasting plasma insulin, and postprandial standing SBP LF power between POAG patients (N=32) and control subjects (N=43).

Fasting BSL is positively correlated to the fasting insulin level in POAG (r=0.39, P=0.03) and control (r=0.35, P=0.02) groups (Figure 10. A, B). Fasting BSL is also negatively correlated to the mean (time points of 30, 60, 90, 120mins) or maximal value (time point of 120mins) of postprandial standing SBP LF power in the control group (Figure 10. D, F). However, these correlations between metabolic and sympathetic markers are entirely absent in the POAG group (r=-0.09, P=0.65; r=-0.19, P=0.32) (Figure 10. C, E).

Discussion

The primary new finding is that during orthostatic stress, both NTG and POAG exhibited an attenuated cardiac sympathetic response with a loss of the normal shift in sympathovagal balance towards a sympathetic predominance, particularly in the early postprandial state. More importantly the two forms of glaucoma manifested distinct features of autonomic dysfunction in response to meal ingestion. Meal ingestion in NTG subjects caused cardiac sympathetic over-activation, while in POAG subjects cardiac autonomic failure with hypotension was apparent in the postprandial state. Therefore, the data support the idea that glaucoma is part of a spectrum of disorders associated with autonomic dysfunction. Thus global autonomic

dysregulation may underlie, and correlate with, vascular disorders of the eye, and different degrees of chronic IOP elevation in both NTG and POAG. The current experimental data suggest ocular vascular dysregulation, as part of a global cardiovascular dysregulation may be additional risks in older female adults with NTG and in hypertensive subjects with POAG.

Autonomic dysfunction during orthostatic stress in both NTG and POAG

A previous study reported that both POAG and NTG patients manifest an impaired baroreflex-mediated autonomic dysfunction to neck suction (Brown *et al.*, 2002). Assuming an upright posture is a normal daily activity and orthostatic stress is a well-established approach to examine autonomic function in humans (Montano *et al.*, 1994; Pagani *et al.*, 1986). The current study investigated, for the first time, baroreflex-mediated autonomic regulation in response to orthostatic stress before and after eating in both POAG and NTG subjects. The current study finds that during orthostatic stress cardiac sympathetic activation and vagal inhibition are intact with the sympathovagal balance shifting towards sympathetic predominance in age-matched control subjects. In contrast, the cardiac sympathetic response and sympathovagal balance remained unchanged in both NTG and POAG, suggesting a blunted cardiac sympathetic response to orthostatic stress.

A blunted cardiac sympathetic response to orthostatic stress occurs in aging populations (Laitinen *et al.*, 2004), essential hypertensive patients (Radaelli *et al.*, 1994), pre-hypertensive humans (Lucini *et al.*, 2002a) and in chronic psychosocial stress (Lucini *et al.*, 2005). Assuming an upright posture is a normal daily activity and induces a redistribution of the cardiac output. Although cerebral autoregulation compensates for changes in posture, perfusion in the brain may still be challenged in susceptible individuals (Van Lieshout *et al.*, 2003). Impaired cardiac output in human (MAP<80mmHg) may also jeopardise ocular perfusion, as seen in patients with autonomic failure exhibiting a significant decrease in ocular perfusion while standing up (Singleton *et al.*, 2003). It is therefore conceivable that cardiac autonomic dysfunction may chronically disturb cerebral blood flow and cause unstable ocular blood flow. It is well documented that vascular dysregulation, i.e. the instability of ocular blood flow underlies the pathogenesis in both NTG and HTG (Flammer *et al.*,

2007; Flammer *et al.*, 2002). Systemic autonomic dysfunction may be an initial pathological factor. Certainly, it is recognised that autonomic dysfunction during orthostatic stress can be a harbinger of cardiovascular ischemic events (Lucini *et al.*, 2005; Lucini *et al.*, 2002a; Lucini *et al.*, 2002b; Straznicky *et al.*, 2012).

As shown above, NTG and POAG manifest common features of autonomic dysfunction to orthostatic stress, we therefore also hypothesise that there may exist distinguishing features of autonomic dysfunction along a continuum of the two forms of glaucoma. Compared to orthostatic stress, meal ingestion is a rather subtle metabolic stress that may further highlight the disparity of autonomic responses in diseases (Cozzolino *et al.*, 2010; Lipsitz *et al.*, 1993; Straznicky *et al.*, 2012). Orthostatic stress will lead to vasoconstrictionin hindlimb, while meal ingestion will cause vasodilation in the mesenteric bed. Furthermore, carbohydrate ingestion is associated with sympathetic activation (Berne *et al.*, 1989; Young *et al.*, 2010a).

Postprandial autonomic failure and depressor response in POAG

A major new finding of the current study is that soon after eating (30min time point), there is a significant depressor response (a reduction in MAP of 7 mmHg in lying and 11mmHg in standing) and a decrease in peripheral vascular resistance, accompanied with cardiac paradoxical sympathovagal response in POAG patients, i.e. sympathetic inhibition and vagal enhancement (Furlan *et al.*, 1998). In contrast, in both NTG and age-matched control subjects, mean arterial pressure was stable with unchanged vascular resistance and a functional cardiac autonomic response. Indeed, the postprandial hypotensive effect in POAG patients may directly cause unstable ocular perfusion as seen in patients with autonomic failure (Singleton *et al.*, 2003). A previous study reported that cold provocation that induced a normal pressor response in control subjects was accompanied by stable ocular blood flow, whereas in POAG patients, the blood pressure failed to increase accompanied with a significant decrease in ocular blood flow, suggesting a concurrent systemic autonomic failure and instability of ocular blood flow in POAG (Gherghel *et al.*, 2004a). The current study supports this idea with experimental demonstration of autonomic dysfunction and provides new evidence that POAG patients may undergo

repetitive systemic autonomic failure in daily life, such as in the postprandial state; a situation that is perhaps more physiologically relevant than the cold pressor test.

The cardiac autonomic response early after eating is unlikely to be attributable to sympathoexcitation evoked by hyperinsulinemia(Anderson *et al.*, 1991). After meal ingestion, splanchnic vascular dilatation peaks in the early, but not the later phase, of the postprandial state (Someya *et al.*, 2008). The autonomic nervous system regulates postprandial splanchnic vasodilation, and failure of autonomic regulation may lead to postprandial hypotension: most commonly in the elderly and patients with dysautonomia(Lipsitz *et al.*, 1993). Similar to orthostasis induced venous pooling, postprandial splanchnic vasodilation may also induce cardiopulmonary mechanoreceptor overstimulation and an excessive sympathetic outflow in patients with syncope experience (Mosqueda-Garcia *et al.*, 2000). Following head-up tilt, cardiac sympathetic inhibition and vagal predominance were observed as a sign preceding vasovagal syncope (Furlan *et al.*, 1998). Notably, we observed a similar coupling of cardiac efferent sympathetic inhibition and vagal enhancement early after eating in POAG subjects, suggesting a paradoxical sympathovagal response to meal ingestion. This observed autonomic failure may be subclinical since all our patients did not exhibit clinical symptoms and signs during the experiments. The data also show that in young healthy men and in older subjects in the control group there are normal cardiac autonomic responses along with an increased stroke volume and increased cardiac output. Therefore, it can be postulated that, in POAG patients, the cardiac autonomic failure with associated insufficient elevation in cardiac output may contribute to the postprandial depressor response (Mosqueda-Garcia *et al.*, 2000). In addition, in POAG patients, there is a marked reduction in peripheral vascular resistance but without attenuated sympathetic outflow to the vasculature. We speculate that this dissociation is consistent with impaired β_2-adrenergic receptor function in the vasculature and/or increased afferent responsiveness of cardiopulmonary receptor resulting in autonomic failure in POAG patients. Based on our sub-analysis, it is also not simply an effect of β-blocker therapy. Further studies are needed to determine this.

Postprandial sympathetic hyper-responsiveness in NTG

Notably, compared to age- and gender- matched control subjects, there is cardiac sympathetic activation in NTG patients in the later phase of postprandial state. The current study is the first investigation of postprandial autonomic responses in glaucoma patients. We find a cardiac sympathetic hyper-responsiveness to meal ingestion in NTG patients. Food intake is controlled by the central nervous system (Schwartz *et al.*, 2000). Within the hypothalamus, the arcuate nucleus is the key site (Cassaglia *et al.*, 2011) that mediates the central sympathoexcitatory actions of insulin (Scherrer *et al.*, 1997). Intravenous insulin infusion induces central sympathetic over-reactivity in patients with essential hypertension (Lembo *et al.*, 1992). The current finding of postprandial sympathetic hyper-responsiveness may therefore help to explain the underlying causes of higher sympathetic nerve activity observed during 24 hours ECG recordings in NTG patients (Wierzbowska *et al.*, 2012).

Since meal ingestion is an essential normal daily behaviour, abnormal sympathetic activation may trigger endothelial dysfunction (Hijmering *et al.*, 2002). The extent to which different dietary compositions may affect these changes remains to be determined. Evidence of endothelial dysfunction was observed in NTG patients, including higher basal plasma endothelin-1 and abnormal endothelin-1 response to laboratory stimuli (Kaiser *et al.*, 1995; Nicolela *et al.*, 2003). In addition, chronic ocular administration of endothelin-1 can induce ocular ischemia glaucomatous optic neuropathy in primates (Cioffi *et al.*, 2004).

The current study compared the sympathetic response in age-matched female control subjects (N=21) with female NTG patients (N=13). Female NTG patients manifested an exaggerated cardiac sympathovagal balance and a shift towards sympathetic predominance postprandially. This result confirms the abnormal cardiac sympathovagal response in NTG patients and suggests that women may be at additional risk for vascular events.

The heart and the eye as ischemia-sensitive organs may share similar common features (Flammer *et al.*, 2013). Variant angina with normal or non-obstructive

angiography occurs more frequently in women, and is likely due to endothelial dysfunction (Bugiardini *et al.*, 2004; Bugiardini *et al.*, 2005). Recently, a new approach to the examination of the retinal microcirculation (as index of endothelial function) may predict the risk of coronary artery disease (CAD) – particularly in women (Liew *et al.*, 2008; McGeechan *et al.*, 2009). Thus, the current study indicates that sympathetic-mediated endothelial dysfunction in women may be additional vascular risk factors in NTG, and may share a similar pathological process to variant angina (Bugiardini *et al.*, 2005). We also postulate that the coexistence of abnormal heart rate variability and retinal vascular response in CAD patients (Heitmar *et al.*, 2011) may precede frank atherosclerosis on coronary angiography (Bugiardini *et al.*, 2005; Liew *et al.*, 2008).

Postprandial sympathetic hyper-responsiveness in POAG with DBP>90mmHg

Our data show that POAG patients have higher DBP and MAP (both lying and standing) than age-gender-matched control subjects in the fasting state. In comparison to normotensive POAG subjects (DBP < 90mmHg) , the current study finds that POAG patients with DBP ≥ 90mmHg, manifested hyper-responsive sympathetic outflows to the heart and vasculature in the later phase of the postprandial state (Lembo *et al.*, 1992). The current study suggests that sympathetic hyper-responsiveness in response to daily stressors in hypertension, over a prolonged period, triggers end-organ damage, including glaucoma (Newman-Casey *et al.*, 2011).

Relationship between fasting blood sugar level (BSL) and postprandial sympathetic outflow

Fasting insulin levels, HOMA (the homeostasis model assessment) and QUICKI (quantitative insulin sensitivity check index) are accurate surrogate markers for evaluating insulin sensitivity in the absence of a euglycaemic clamp (Katz *et al.*, 2000; Matthews *et al.*, 1985). These markers are matched between POAG patients and control subjects in our study. In addition, we also found strong positive correlations between fasting BSL and fasting insulin in both POAG and control subjects. Thus, it is likely that insulin sensitivity is comparable between study groups.

Another major finding of the current study is that fasting BSL is inversely correlated to postprandial standing sympathetic outflow to the vasculature in control subjects, but this correlation is absent in POAG patients. This suggests that, in the normal population, fasting BSL may be a useful marker for the evaluation of postprandial sympathetic responsiveness. Carbohydrate loading induces a physiological hyperinsulinemia (Young *et al.*, 2010a). Quiet standing may also provokes a transient increase in plasma insulin due to acute forearm insulin resistance (Jamerson *et al.*, 1993). A lower fasting BSL may indicate a better insulin reserve, i.e. a greater production and secretion of insulin from pancreatic β cells, associated with a greater centrally mediated sympathetic response (Berne *et al.*, 1989; Paton, 1998; Scherrer *et al.*, 1997).

Given the comparable insulin sensitivity in POAG and control subjects, the absence of an inverse relationship in POAG patients (both normal DBP and high DBP subgroups) may be due not to the different plasma insulin level, but to hypertension-associated sympathetic hyper-responsiveness to hyperinsulinemia as mentioned above (Lembo *et al.*, 1992).

Limitations

The current study did not examine changes in insulin levels over time in the postprandial state, mainly because our study aimed to: 1) develop a non-invasive approach to evaluate autonomic responses to orthostatic stress before and after meal ingestion, without targeting the relationship between postprandial plasma insulin and sympathetic nerve activity (Anderson *et al.*, 1991; Berne *et al.*, 1989; Young *et al.*, 2010a); and 2) compare autonomic responses between normal control subjects and glaucoma patients. Meal ingestion seems to be a relatively subtle stressor (Cozzolino *et al.*, 2010) in our hands, requiring around two hours to observe the full autonomic response. For these reasons, we attempted to minimise the interference and associated mental stress that would occur with repetitive blood collection and needle insertion (Berne *et al.*, 1989). To mitigate this limitation, carbohydrate rich meal ingestion was used to ensure hyperinsulinemia, which is known to increase sympathetic outflow to the vasculature (MSNA) in the postprandial state (Berne *et al.*, 1989; Young *et al.*, 2010a).

Conclusion

In conclusion, compared to normal controls, both POAG and NTG exhibit similar cardiac autonomic dysfunction in response to orthostatic stress. Notably, the current study demonstrates that in response to meal ingestion POAG manifested a postprandial cardiac autonomic failure associated with a significant depressor response in the early postprandial phase. NTG subjects manifested a cardiac sympathetic hyper-responsiveness in the late postprandial phase. Female gender in NTG, and hypertension (DBP≥90mmHg) in POAG, may be an additional vascular risk. Fasting blood sugar level seems to be a new and simple predictor of the sympathetic response in the postprandial state.

Thus, the current study provides an important mechanistic insight into the distinctive pathological features of autonomic dysfunction that may underlie both the vascular pathogenesis and the different degrees of chronic IOP elevation seen in glaucoma.

Legends

Fig 1. Recruitment Flowchart

Fig 2. Autonomic responses to orthostatic stress and meal ingestion in control (N=36) and glaucoma (N=37) subjects

In response to orthostatic stress, in control and glaucoma subjects, SBP LF power, HRV LF nu increased, and HRV LF/HF ratio increased; HRV HF nu decreased with associated reduction in sBRS (all P<0.01).

Notably, in response to meal ingestion, in control subjects, HRV responses maintained unchanged. However, in glaucoma patients, HRV LF nu and HRV LF/HF ratio increased (P<0.05, P<0.01), and HRV HF nu decreased with associated reduction in sBRS (both P<0.01).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 3. Hemodynamic responses to orthostatic stress and meal ingestion in control (N=36) and glaucoma (N=37) subjects

In response to orthostatic stress, in control and glaucoma subjects, MAP and HR increased (both P<0.01).

In response to meal ingestion, HR increased in both control and glaucoma groups (both P<0.01). MAP maintained stable in control subjects, but altered in glaucoma patients (P<0.05).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 4. Autonomic responses to orthostatic stress and meal ingestion between β-blocker eye drop user (N=17) and non β-blocker eye drop patients (N=20)

In response to orthostatic stress, HRV HF nu reduced (P<0.01) in both β-blocker user and non β-blocker patients. HRV LF/HF ratio increased only in non β-blocker patients (P<0.01), but not in β-blocker user.

In response to meal ingestion, HRV responses remained unchanged β-blocker user. However, HRV LF nu increased, HRV HF nu decreased, and HRV LF/HF ratio increased in non β-blocker patients (all P<0.05).

In response to orthostatic stress, MAP remained unaltered irrespective of topical β-blocker, while HR increased in both groups. After meal ingestion, MAP decreased early postprandially in patients with beta-blocker, whereas gradually increased until reaching peak at 90min in non-beta-blocker group.HR increased in the postprandial state in both groups.

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 5. Autonomic responses to orthostatic stress and meal ingestion in fasting and early postprandial phase (30min after eating)

In response to orthostatic stress, in control subjects, HRV LF nu increased, HRV HF nu decreased, and HRV LF/HF ratio increased (all P<0.01). However, in NTG and POAG patients, although HRV HF nu reduced (P<0.05), HRV LF nu and HRV LF/HF ratio was not increased.

From fasting to early postprandial phase, in both lying and standing positions, HRV responses remained unchanged in control subjects and NTG patients. In contrast, in POAG patients, HRV LF nu decreased (P<0.01), HRV HF nu increased (P<0.05), and HRV LF/HF ratio decreased (P<0.05).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 6. Hemodynamic responses to orthostatic stress and meal ingestion in fasting and early postprandial phase (30min after eating)

In response to orthostatic stress, in control subjects, although CO reduced (P<0.01), MAP elevated (P<0.01) accompanied with a significant increase in TPR (P<0.01). However, the responses of MAP, TPR and CO were absent in both NTG and POAG patients.

From fasting to early postprandial phase, in control subjects, with a significant elevation in CO (P<0.01), MAP maintained stable and TPR remained unchanged. Conversely, in POAG subjects, with an insufficient response in CO (P<0.06), MAP significantly fell (P<0.01), accompanied with a reduction in TPR (p<0.01).

Total peripheral resistance (TPR), cardiac output (CO)

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 7. Autonomic and hemodynamic responses to orthostatic stress and meal ingestion in fasting and later postprandial phase (60, 90, 120 mins)

In response to orthostatic stress, in control subjects, HRV LF nu increased, HRV HF nu decreased, and HRV LF/HF ratio increased with an elevation in MAP (all $P<0.01$). However, in NTG and POAG patients, the increases in HRV LF nu were absent, although there were reductions in HRV HF nu in both of them, and an increase in HRV LF/HF ratio ($P<0.05$) in NTG patients.

From fasting to later phase of postprandial state, in both lying and standing positions, HRV responses remained unchanged in control and POAG subjects. In contrast, in NTG patients, HRV LF nu increased, HRV HF nu decreased, and HRV LF/HF ratio increased (all $P<0.01$).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 8. Postprandial cardiac responses in NTG females and control females

In the later phase of postprandial state (90 and 120 minute time points), during standing position, HRV LF/HF ratio markedly increased in NTG females (Fig 8 A, B). Compared to control females, there are greater reduction in HRV HF nu and increase in HRV LF/HF ratio in NTG females (both $P<0.05$) (Fig 8 C, D).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 9 A,B,C,D. Responses of HRV LF/HF ratio and SBP LF power to orthostatic stress and meal ingestion in Normotensive (Normal- DBP) and hypertensive (High-DBP) subgroups of POAG patients

In response to orthostatic stress, both HRV LF/HF ratio and SBP LF power increased in normotensive and hypertensive subgroups (both $P<0.05$).

In response to meal ingestion, hypertensive subgroup exhibited marked increases in HRV LF/HF ratio and SBP LF power from 60min to 120min ($P=0.01$, $P<0.01$). In contrast, HRV LF/HF ratio and SBP LF power remained unchanged in normotensive subgroup ($P=0.46$, $P=0.15$).

*P<0.05, **P<0.01 Data shown as mean±SEM

White Circle: Lying; Filled Triangle: Standing

Fig 9 E,F,G,H. SBP LF powers in the fasting and postprandial states between Normotensive (Normal-DBP) and Hypertensive (High-DBP) subgroups

In fasting state, SBP LF powers ($mmHg^2$) were comparable between normotensive and hypertensive subjects, in both lying (5.19 ± 0.70 vs 10.12 ± 2.43, P=0.08) and standing positions (14.72 ± 1.94 vs 17.63 ± 2.90, P=0.42). In the postprandial state, the mean values (in time points of 60,90 and120min) of SBP LF powers were significantly higher in hypertensive subjects than that of normotensive subjects, in both lying (10.79 ± 1.81vs 5.20 ± 0.55, P<0.01) and standing positions (26.84 ± 3.42 vs 14.72 ± 1.94, P<0.01).

**P<0.01 Data shown as mean±SEM

Fig 10. Correlations of fasting Blood Sugar Level (BSL) to insulin and SBP LF power in POAG and control subjects

Fig 10 A, B. Fasting BSL is positively correlated to fasting insulin in both POAG and control subjects.

Fig 10 C, D. Fasting BSL is inversely correlated to the mean values of postprandial standing SBP LF power (at time points of 60, 90, 120min) in control subjects, but the correlation does not exist in POAG subjects.

Fig 10 E, F. Fasting BSL is inversely correlated to the maximal value of postprandial standing SBP LF power (at 120min time point) in control subjects, but the correlation does not exist in POAG subjects.

Figures and Tables

Table 1. Subject profile and baseline characteristics (N=96)

Profile	Control (N=43)	NTG (N=21)	P value	POAG (N=32)	P value
Age, years	64.72 ± 8.09	63.24 ± 8.81	0.51	66.72 ± 6.35	0.25
Sex (female), N (%)	26, 60.5%	14, 66.7%	0.78	17, 53.1%	0.64
Height, m	1.67 ± 0.09	1.67 ± 0.08	0.81	1.70 ± 0.10	0.20
Weight, kg	71.31 ± 16.17	67.19 ± 10.24	0.29	74.97 ± 13.61	0.30
BMI, kg/m2	25.43 ± 5.20	24.19 ± 3.20	0.32	25.90 ± 3.99	0.67
Waist Circumference, cm	91.11 ± 13.50	87.69 ± 10.43	0.31	92.44 ± 11.33	0.65
Waist/hip ratio	0.90 ± 0.07	0.87 ± 0.09	0.13	0.89 ± 0.08	0.66
Creatinine clearance, mL/min	84.35 ± 23.07	83.28 ± 18.90	0.85	92.72 ± 24.97	0.14
Medical history					
History of hypertension, N (%)	5, 11.6%	1, 5%	0.65	16, 50.0%	0.0005*
Vasoactive medications, N (%)	8, 18.6%	2, 9.5%	0.48	14, 43.8%	0.02*
Family Hx of cardiovascular diseases, N (%)	21, 48.9%	9, 42.9%	0.79	19, 59.4%	0.48
Eyedrop (β-blocker), N (%)	0, 0%	8, 38.1%		21, 65.6%	
Family history of glaucoma, N (%)	11, 25.6%	9, 42.9%	0.25	11, 34.4%	0.45
Metabolic (fasting state)					
Plasma glucose, mmol/L	5.34 ± 0.53	5.29 ± 0.39	0.66	5.73 ± 0.90	0.02*
Serum insulin, mU/L	6.61 ± 3.19	7.38 ± 3.75	0.39	8.16 ± 6.27	0.17
IS-QUICKI	0.36 ± 0.03	0.36 ± 0.03	0.55	0.35 ± 0.03	0.12
HOMA-IR	1.59 ± 0.86	1.75± 0.91	0.52	2.19 ± 2.09	0.10
Triglycerides, mmol/L	1.08 ± 0.54	1.26 ± 0.88	0.33	1.24 ± 0.64	0.25
Cholesterol, mmol/L	5.38 ± 0.85	5.51 ± 0.67	0.53	5.45 ± 1.18	0.74
Hemodynamic (fasting state)					
SBP, mmHg (lying)	123.10 ± 14.54	124.70 ± 13.20	0.68	129.20 ± 15.15	0.08
DBP, mmHg (lying)	73.47 ± 9.12	76.62 ± 8.52	0.19	79.34 ± 11.94	0.02*
MAP, mmHg (lying)	90.01 ± 10.22	92.63 ± 8.86	0.32	95.95 ± 12.48	0.03*
HR, beats/min (lying)	60.21 ± 7.38	57.38 ± 7.07	0.15	59.84 ± 9.35	0.85
SBP, mmHg (standing)	130.90 ± 17.82	130.90 ± 12.04	0.10	138.10 ± 14.43	0.06
DBP, mmHg (standing)	83.23 ± 8.85	84.76 ± 7.75	0.50	88.31 ± 11.35	0.03*
MAP, mmHg (standing)	99.12 ± 10.92	100.10 ± 8.57	0.71	104.90 ± 11.52	0.03*
HR, beats/min (standing)	69.21 ± 9.35	65.90 ± 9.58	0.19	69.06 ± 10.92	0.95

Data expressed as mean±SD. P value is calculated by unpaired t test between groups: NTG vs Control, POAG vs Control.

*P<0.05.

Table 2. Hemodynamic and autonomic data in fasting state in study subjects without taking vasoactive drugs

	Control (N=36)	NTG (N=19)	P value	POAG (N=18)	P value
Lying position					
SBP, mmHg	122.50 ± 15.10	124.70 ± 11.14	0.57	126.50 ± 16.03	0.37
DBP, mmHg	73.50 ± 9.01	76.89 ± 8.70	0.18	77.44 ± 11.84	0.18
MAP, mmHg	89.83 ± 10.37	92.84 ± 8.36	0.28	93.80 ± 12.89	0.23
HR, beats/min	60.72 ± 7.23	57.68 ± 7.33	0.15	58.78 ± 7.87	0.37
HRV LF power, ms^2	445.2 ± 402.2	337.5 ± 194.1	0.29	397.8 ± 287.9	0.68
HRV LF power, nu	52.80 ± 22.28	47.77 ± 21.54	0.42	59.26 ± 22.52	0.32
HRV HF power, ms^2	419.0 ± 529.8	457.8 ± 412.8	0.79	273.8 ± 319.0	0.32
HRV HF power, nu	41.27 ± 18.09	45.24 ± 16.64	0.43	35.49 ± 18.22	0.27
HRV LF/HF ratio	1.82 ± 1.40	1.44 ± 1.21	0.33	2.30 ± 1.96	0.09
SBP LF power, $mmHg^2$	7.04 ± 6.17	11.13 ± 9.77	0.08	9.30 ± 8.65	0.28
BRS, ms/mmHg	14.61 ± 9.80	16.45 ± 13.14	0.58	11.88 ± 6.58	0.29
Standing position					
SBP, mmHg	130.60 ± 18.22	130.90 ± 11.51	0.95	139.10 ± 14.39	0.09
DBP, mmHg	83.47 ± 9.11	84.79 ± 8.13	0.59	87.83 ± 10.14	0.12
MAP, mmHg	99.18 ± 11.26	100.20 ± 8.73	0.74	104.90 ± 10.56	0.08
HR, beats/min	70.14 ± 8.77	66.16 ± 10.03	0.13	68.56 ± 9.92	0.55
HRV LF power, ms^2	383.5 ± 334.3	448.3 ± 339.6	0.5	208.9 ± 164.7	0.048*
HRV LF power, nu	61.52 ± 26.10	49.76 ± 25.47	0.12	62.85 ± 20.52	0.85
HRV HF power, ms^2	200.2 ± 261.3	298.7 ± 242.4	0.2	131.5 ± 157.4	0.31
HRV HF power, nu	29.50 ± 18.12	38.06 ± 17.95	0.11	26.43 ± 12.52	0.52
HRV LF/HF ratio	4.02 ± 3.92	2.28 ± 2.86	0.09	3.30 ± 2.27	0.48
SBP LF power, $mmHg^2$	21.18 ± 16.08	19.57 ± 10.96	0.72	16.65 ± 9.21	0.28
BRS, ms/mmHg	8.79 ± 9.88	7.29 ± 3.81	0.56	7.68 ± 8.23	0.69

Data expressed as Mean ± SD; P value is calculated by unpaired t-test between groups, i.e. Control vs NTG, Control vs POAG

Fig 1 Subjects recruitment flowchart

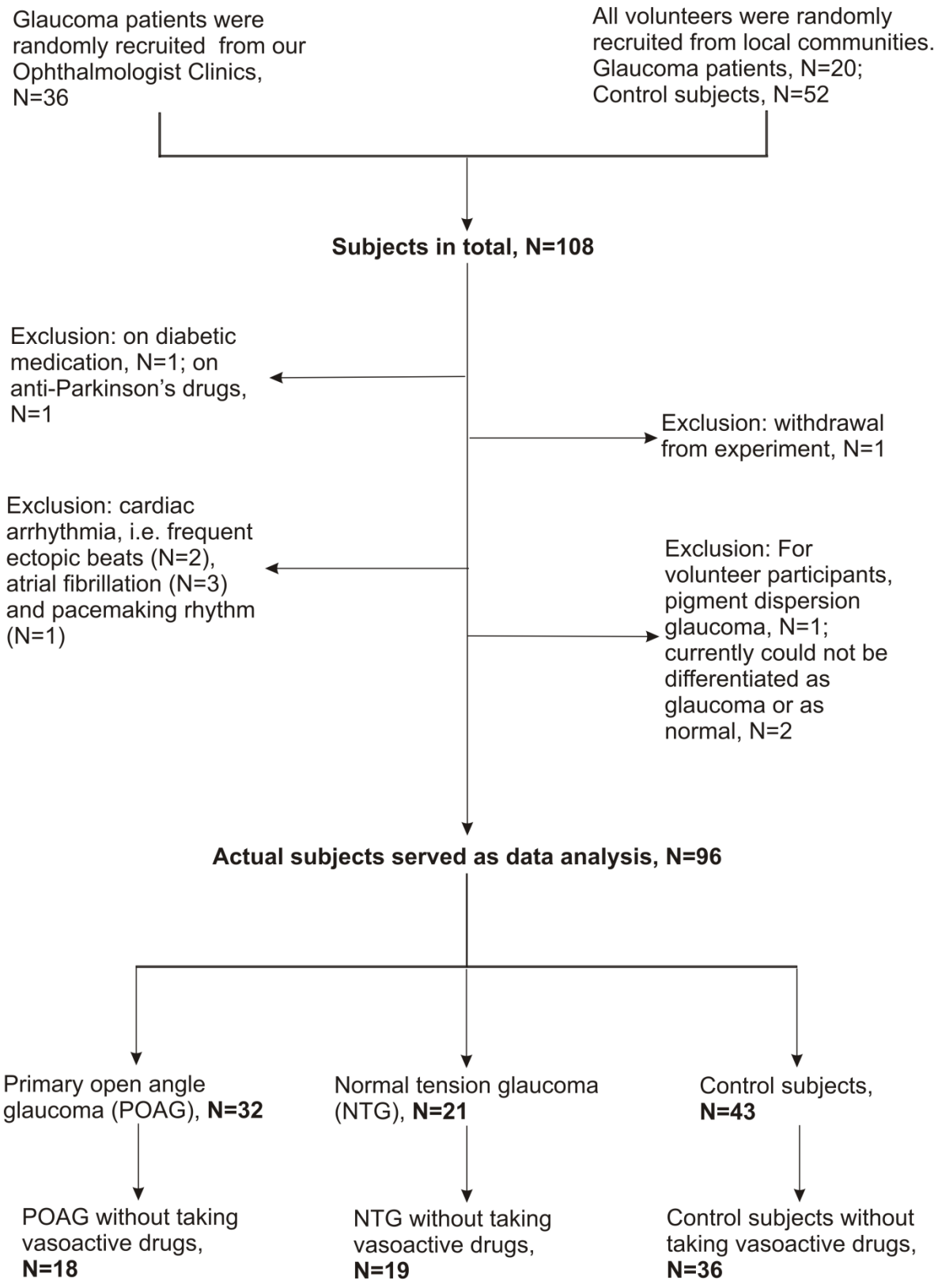


Fig 2. Autonomic responses to orthostatic stress and meal ingestion

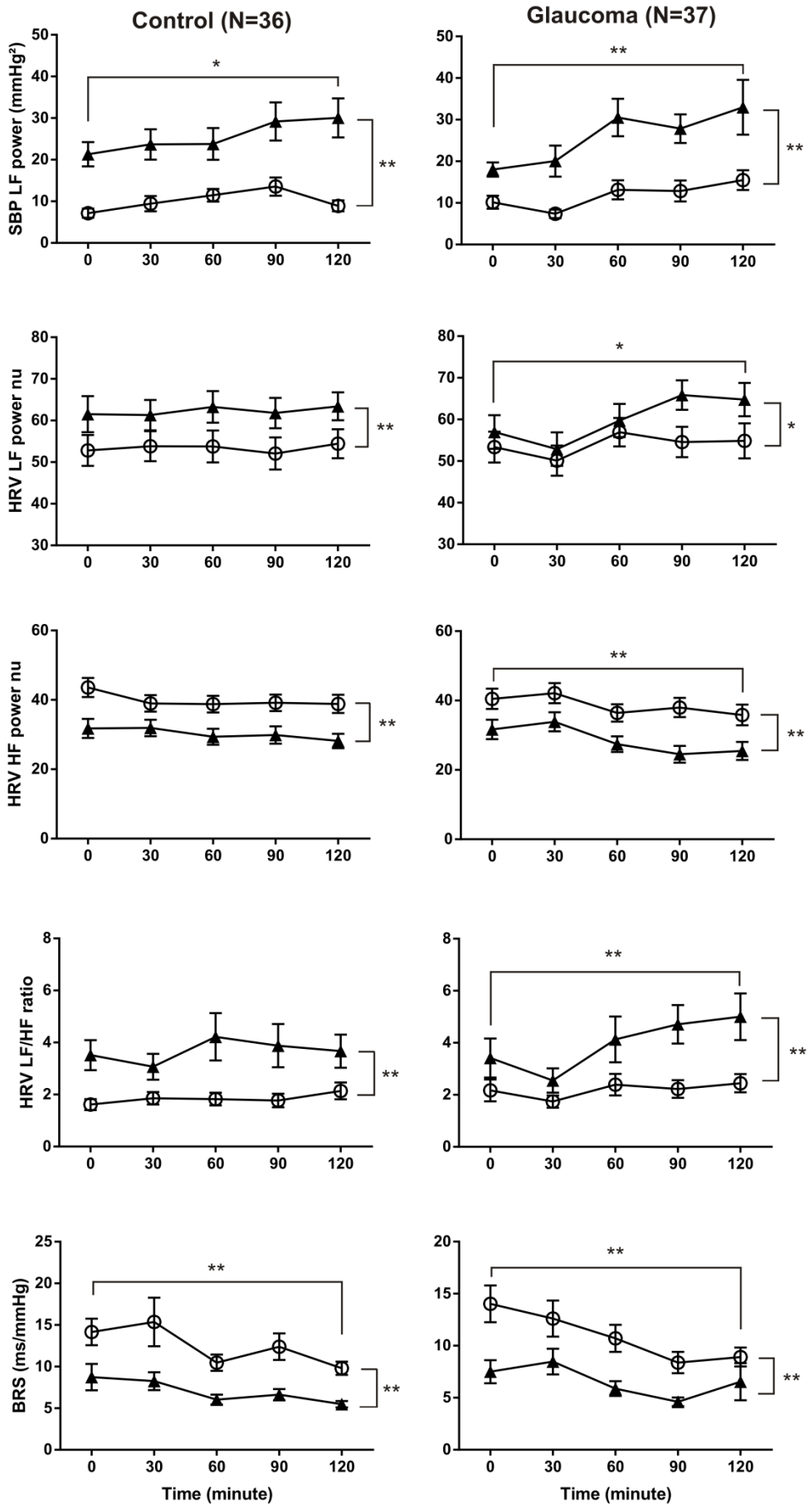


Fig 3. Hemodynamic responses to orthostatic stress and meal ingestion

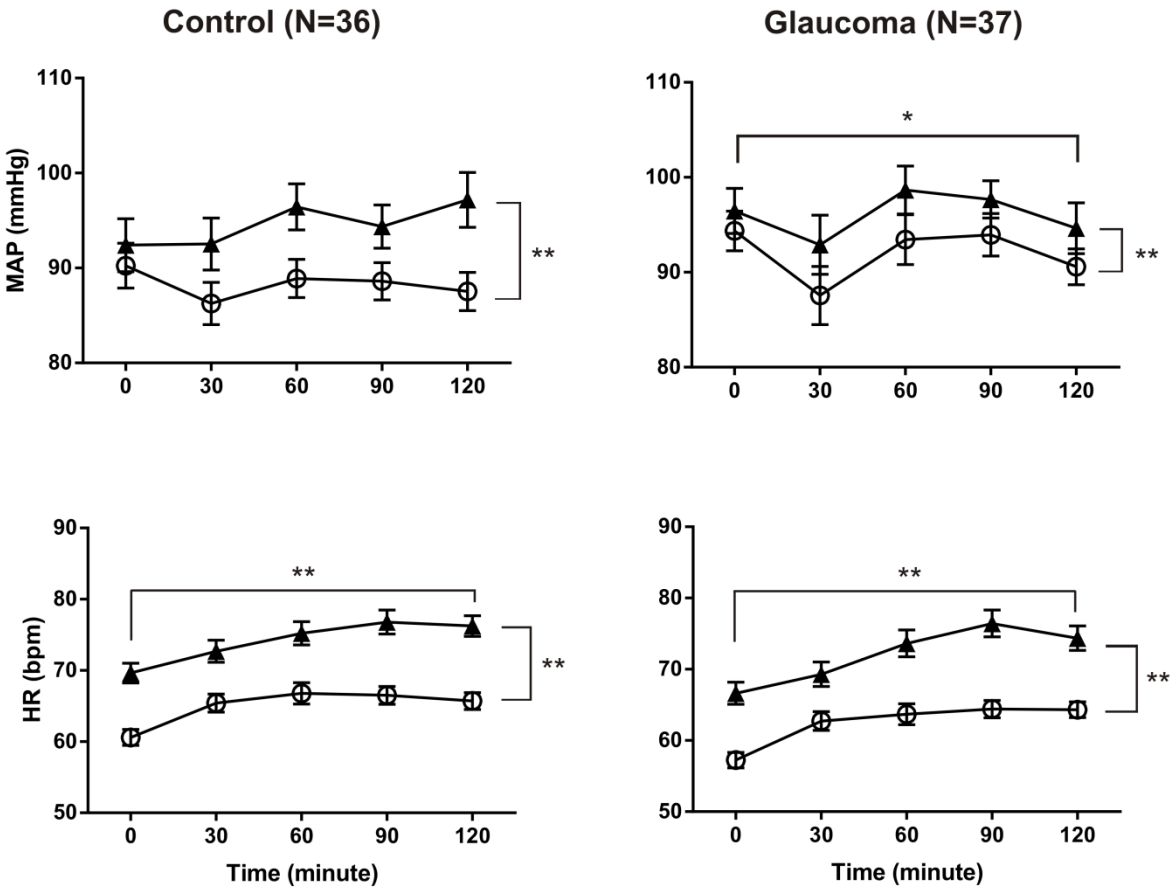


Fig 4. Autonomic responses between β-blocker users and Non- β-blocker patients

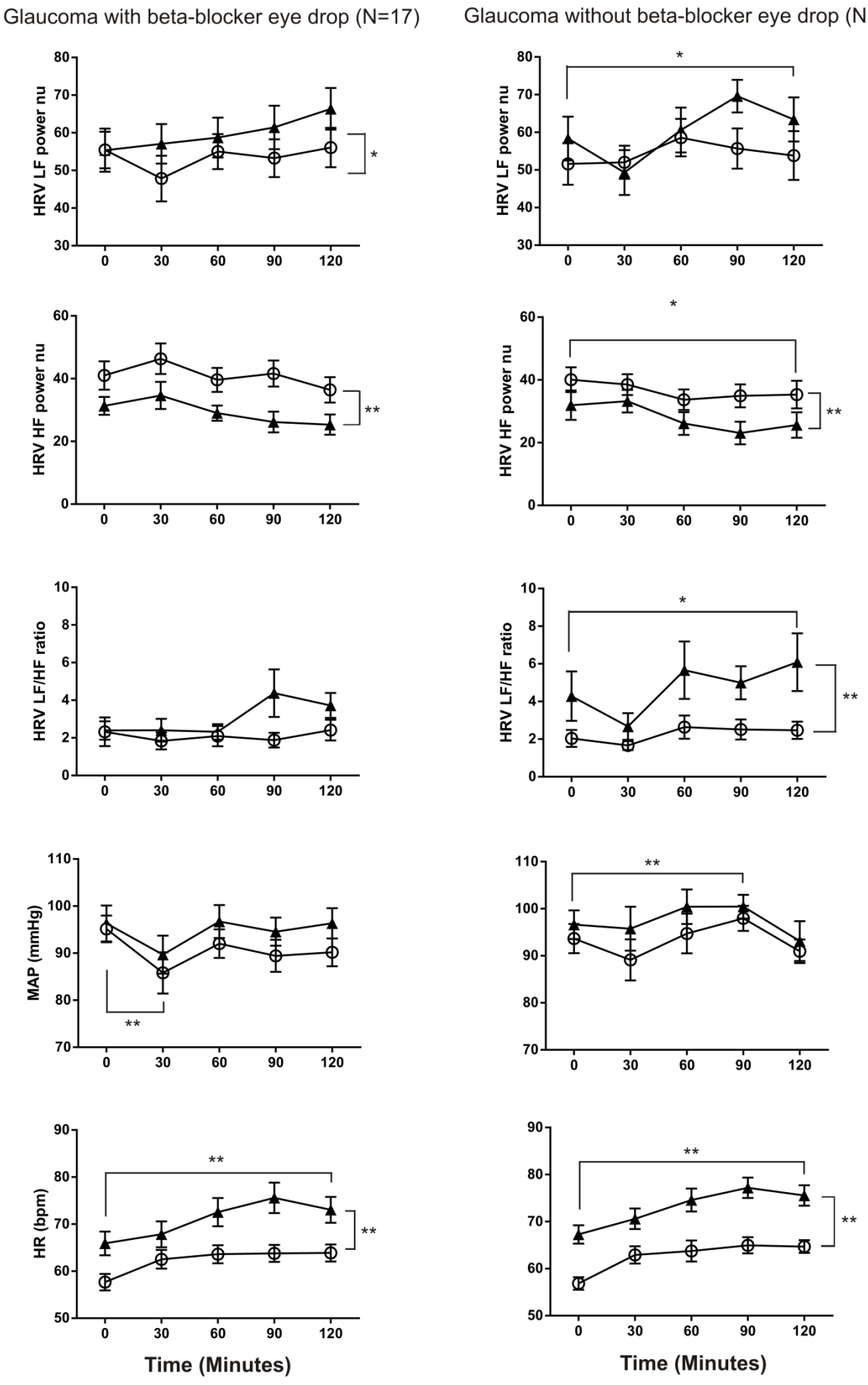


Fig 5. Autonomic responses to orthostatic stress and meal ingestion in fasting and early postprandial phase (30min after eating)

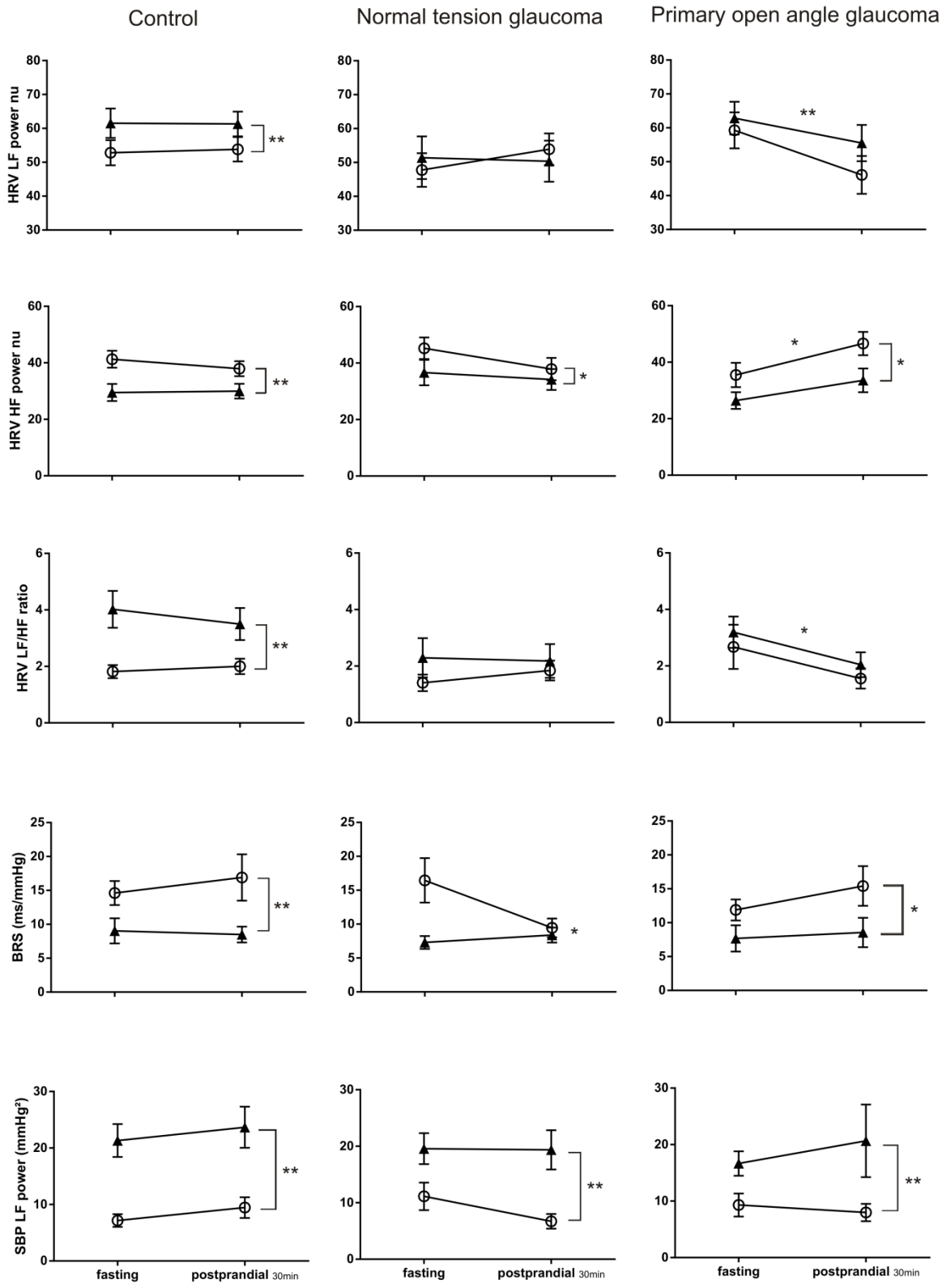


Fig 6. Hemodynamic responses to orthostatic stress and meal ingestion in fasting and early postprandial phase (30min after eating)

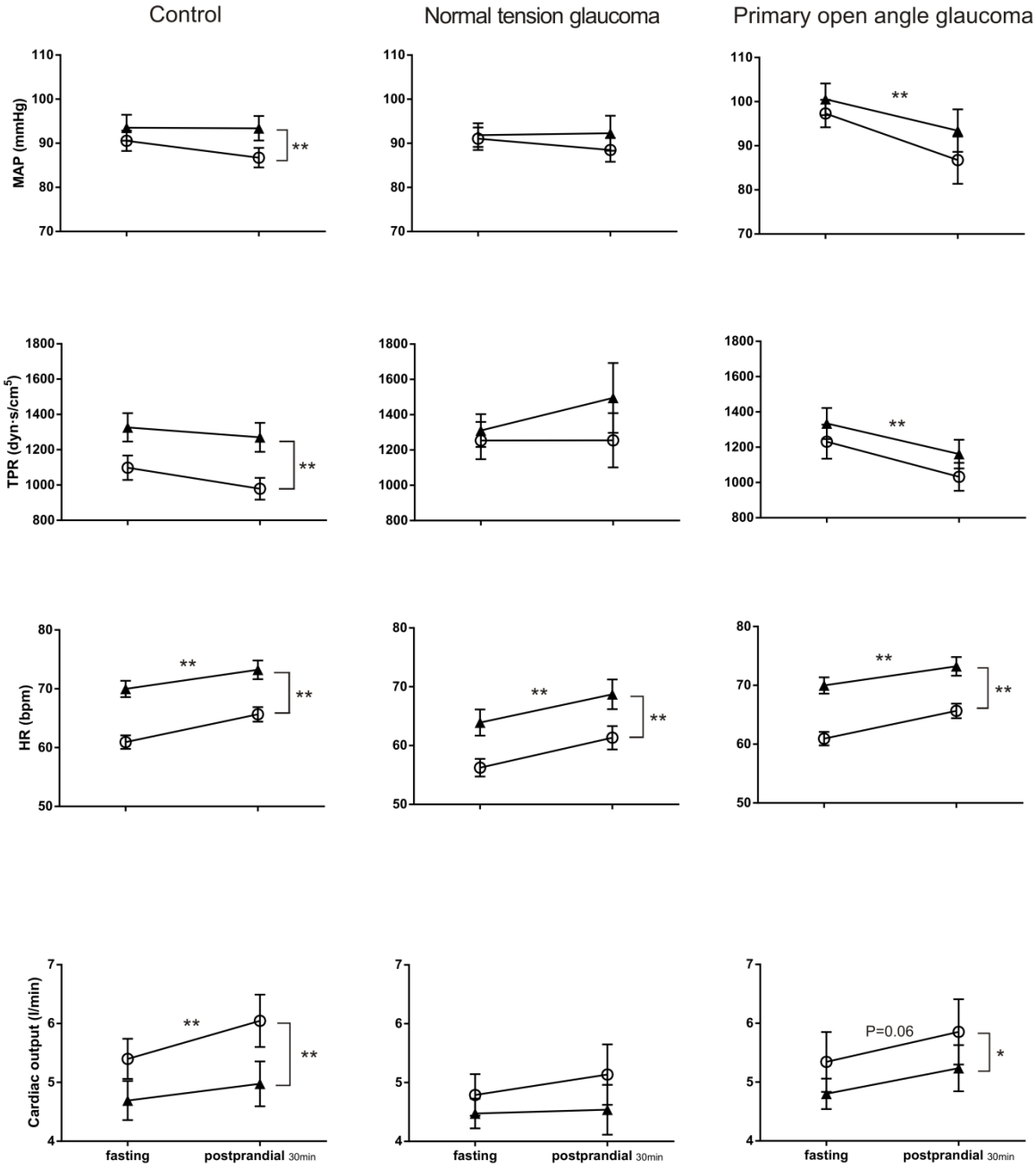


Fig 7. Autonomic and hemodynamic responses to orthostatic stress and meal ingestion in fasting and later postprandial phase (60, 90, 120 mins)

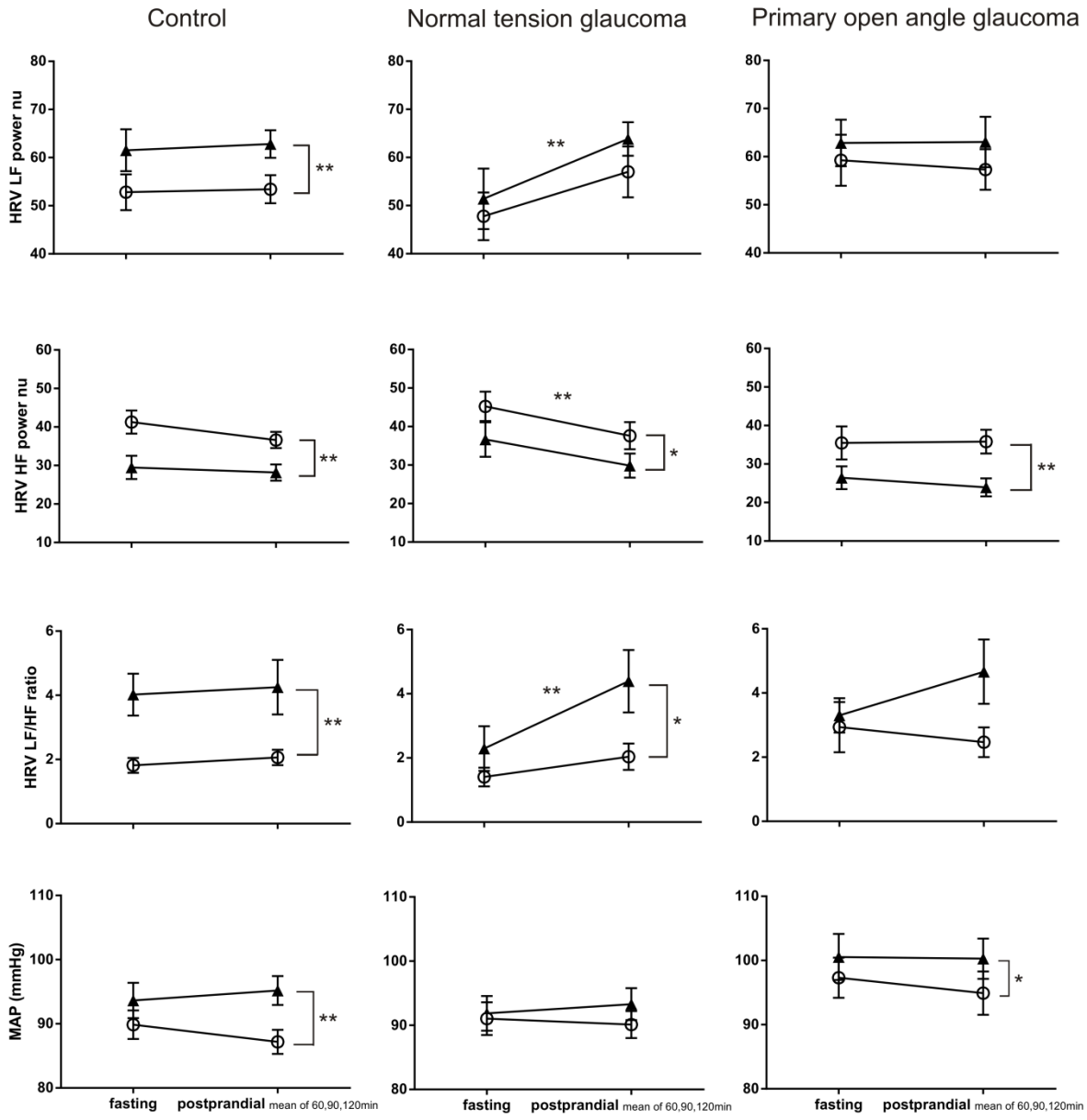


Fig 8. Postprandial cardiac responses in NTG females and control females

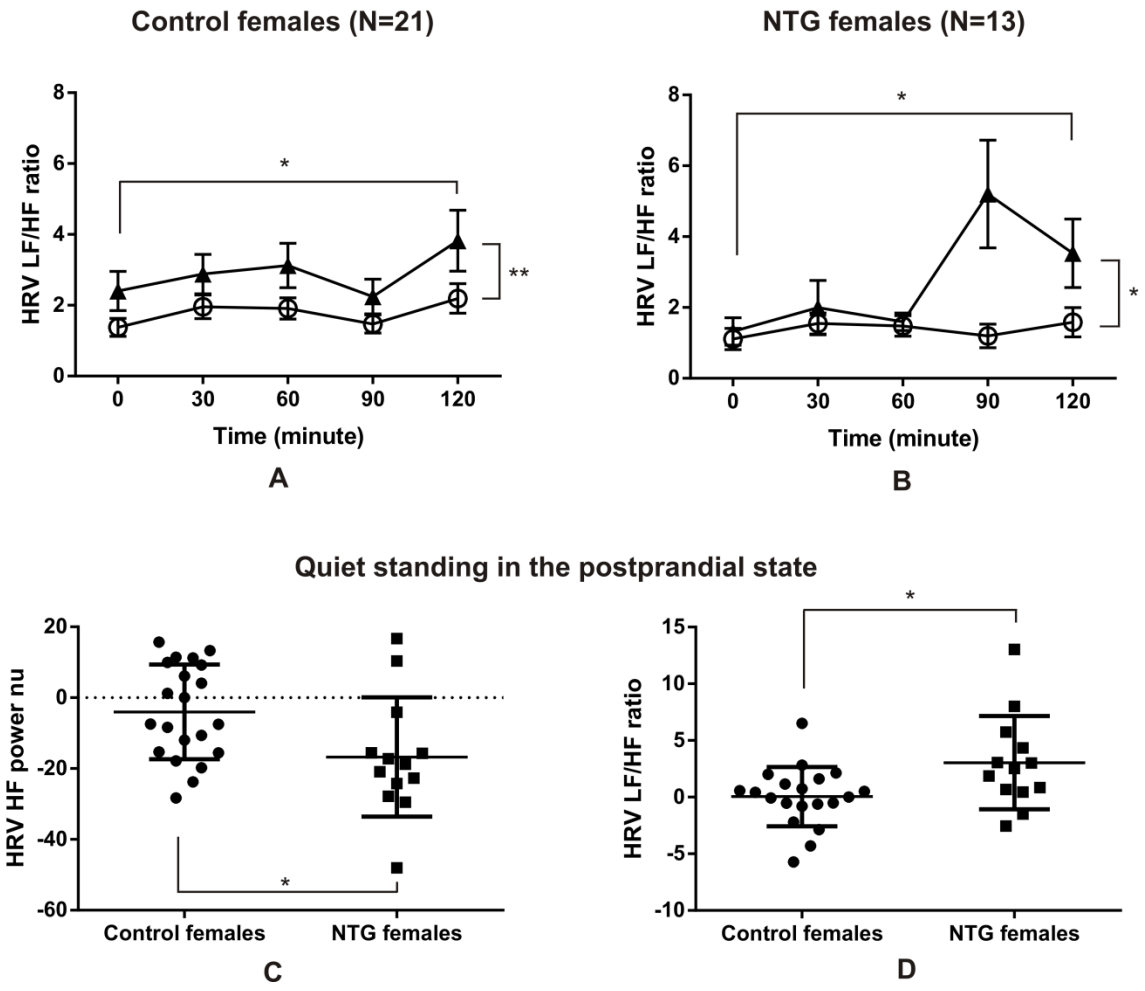


Fig 9 A,B,C,D. Responses of HRV LF/HF ratio and SBP LF power to orthostatic stress and meal ingestion in Normotensive (Normal- DBP) and hypertensive (High-DBP) subgroups of POAG patients

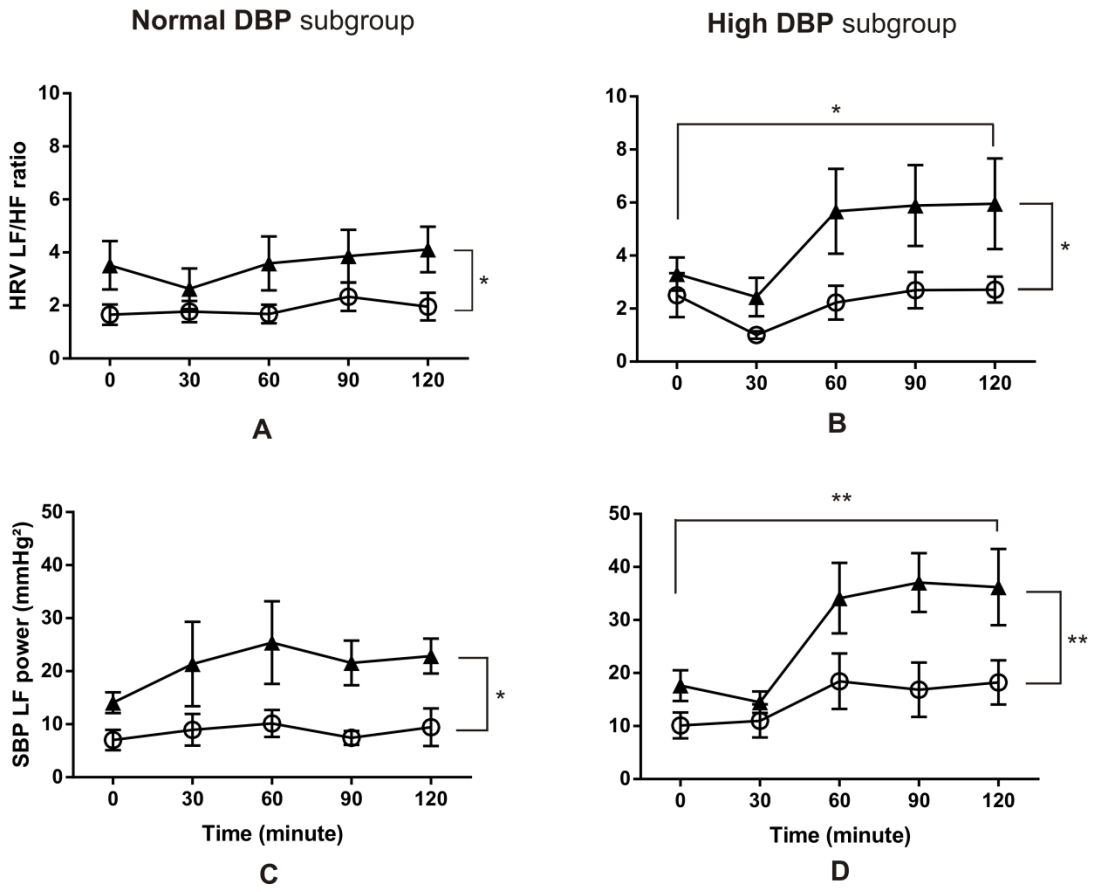


Fig 9 E,F,G,H. SBP LF powers in the fasting and postprandial states between Normotensive (Normal-DBP) and Hypertensive (High-DBP) subgroups

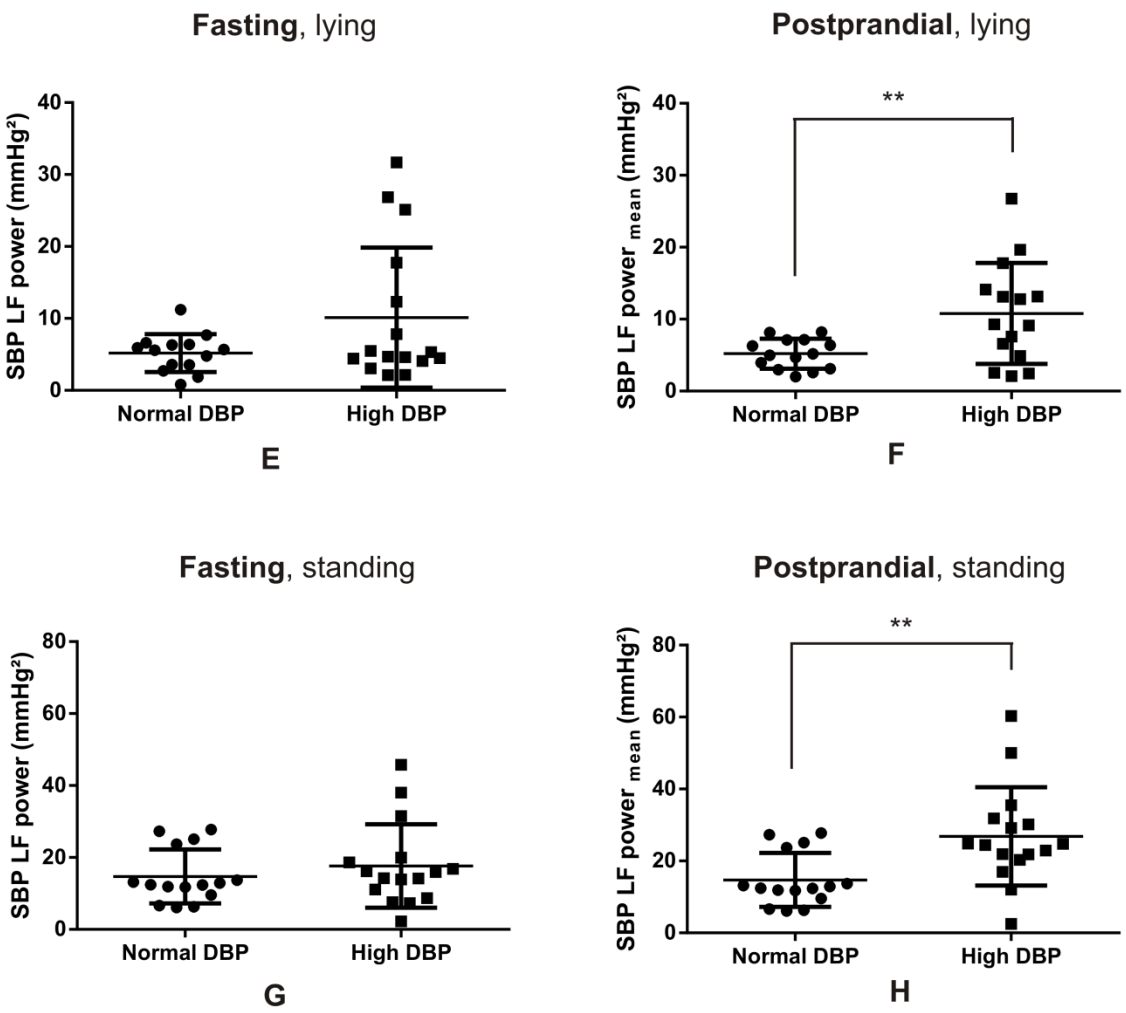
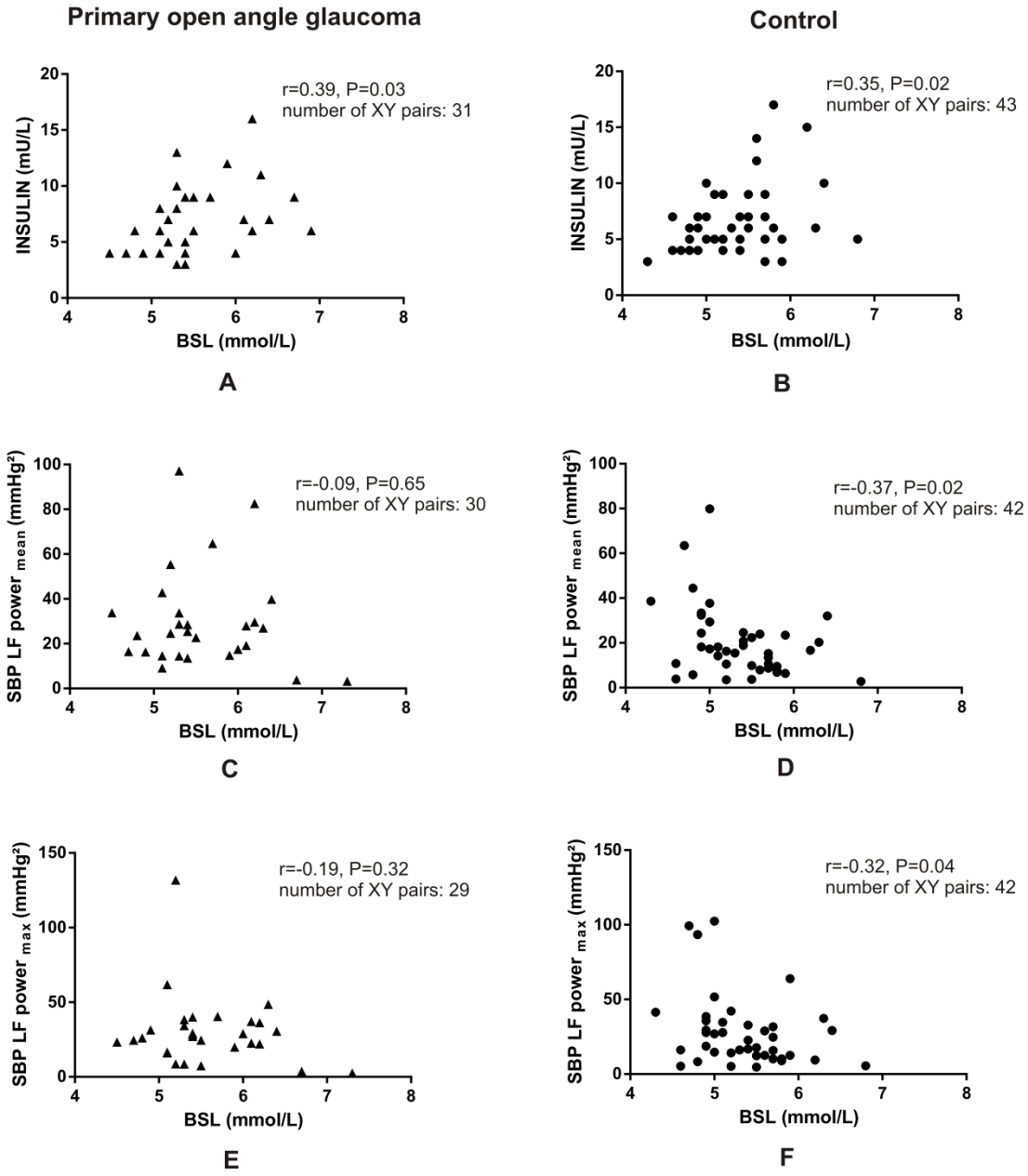


Fig 10. Correlations of fasting Blood Sugar Level (BSL) to insulin and SBP LF power in POAG and control subjects



End of Result Chapters

Chapter Six: Conclusion

Autonomic regulation plays an important role in cardiovascular haemostasis. Autonomic dysfunction is associated with the pathogenesis of various cardiovascular diseases, such as hypertension, diabetes, sleep apnoea, ischaemic heart disease, and obesity.

Orthostatic stress as a reliable laboratory stimulus has been favourable to provide valid information on sympathetic and parasympathetic modulation of autonomic nervous system via power spectral analysis of HRV and BPV. The non-invasive approach of spectral power of HRV and BPV together with head up tilt test (or standing position) is widely used in clinical and research studies in predicting cardiovascular diseases.

Meal ingestion is also a normal daily activity and has recently been recognised as a useful laboratory stimulus, not only due to its close relation to obesity, the most common health issue in our modern society, but also as a subtle laboratory stressor, meal ingestion elicits useful information in reflecting the functional autonomic responses in different groups of population.

The current studies demonstrated the following principal new findings:

In healthy young male subjects, quiet standing enhances vascular sympathetic outflow with associated pressor response after carbohydrate ingestion. This acute physiological effect to the interplay of nutrition and orthostasis exhibited in young men is not present in older subjects, suggesting an attenuated cardiovascular and autonomic regulation in ageing population.

Meal ingestion, but not orthostatic stress, elicited distinguishing features of autonomic modulation between older males and females, i.e. in response to meal ingestion, there is an increase in sympathetic outflow to the vasculature in older men, whereas older women favour a reduction in parasympathetic control to the heart, associated with cardiac sympathovagal balance shifting towards sympathetic predominance, and a decrease in heart rate baroreflex sensitivity. The mechanisms for the increased prevalence of cardiovascular disease in postmenopausal women remain unclear. The study provides new functional information and attracts attention for this discussion.

The main section of the study investigated the cardiovascular and autonomic function in a large cohort of glaucoma patients. The above well-established non-invasive approaches are used, i.e. HRV and BPV as markers of autonomic modulation; and the newly-developed laboratory stimuli to mimic daily stressors, i.e. quiet standing, meal ingestion, and the interplay of nutrition and orthostasis. It is demonstrated that there is autonomic dysfunction to orthostatic stress in both primary open angle glaucoma (POAG) and normal tension glaucoma (NTG) patients. More importantly, the study provides new evidence that POAG patients manifested autonomic failure early after meal ingestion, with associated depressor response; NTG patients exhibited sympathetic hyper-responsiveness later after meal ingestion. Again, similar to the above gender effects on autonomic regulation, meal ingestion elicited distinguishing features of autonomic modulation between POAG and NTG. The current study shed light on the important pathogenesis of glaucoma, i.e. glaucoma may be a systemic cardiovascular autonomic disorder, and the distinct features of autonomic dysfunction may underlie the development and progression of the two forms of glaucoma.

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APPENDIX

Ethics application reference-5201001044- Final approval

Dear Prof Pilowsky

Re: "Analysis of heart rate and blood pressure variability in response to postural challenges, mental stress and other stressors of the autonomic nervous system" (Ethics Ref: 5201001044)

Thank you for your recent correspondence. Your response has addressed the issues raised by the Human Research Ethics Committee and you may now commence your research.

The following personnel are authorised to conduct this research:

Prof Paul Pilowsky- Chief Investigator/Supervisor
Ms Lei Cao Mmed- Co-Investigator

Please note the following standard requirements of approval:

1. The approval of this project is conditional upon your continuing compliance with the National Statement on Ethical Conduct in Human Research (2007).

2. Approval will be for a period of five (5) years subject to the provision of annual reports. Your first progress report is due on 27 October 2011.

If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. If the project has been discontinued or not commenced for any reason, you are also required to submit a Final Report for the project.

Progress reports and Final Reports are available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

3. If the project has run for more than five (5) years you cannot renew approval for the project. You will need to complete and submit a Final Report and submit a new application for the project. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).

4. All amendments to the project must be reviewed and approved by the Committee before implementation. Please complete and submit a Request for Amendment Form available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

5. Please notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that affect the continued ethical acceptability of the project.

6. At all times you are responsible for the ethical conduct of your

research in accordance with the guidelines established by the University. This information is available at the following websites:

http://www.mq.edu.au/policy/

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/policy

If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of Final Approval to an external organisation as evidence that you have Final Approval, please do not hesitate to contact the Ethics Secretariat at the address below.

Please retain a copy of this email as this is your official notification of final ethics approval.

Yours sincerely
Dr Karolyn White
Director of Research Ethics
Chair, Human Research Ethics Committee

Final Approval- Ethics application reference-5201100552 (D)

Dear Prof Pilowsky

Re: "Analysis of heart rate and blood pressure variability in response to postural challenges, mental stress and other stressors of the autonomic nervous system" (Ethics Ref: 5201100552)

Thank you for your recent correspondence. Your response has addressed the issues raised by the Human Research Ethics Committee and you may now commence your research.

The following personnel are authorised to conduct this research:

Prof Paul Pilowsky- Chief Investigator/Supervisor
Dr Stuart Graham & Mr Lei Cao- Co-Investigators

NB. STUDENTS: IT IS YOUR RESPONSIBILITY TO KEEP A COPY OF THIS APPROVAL EMAIL TO SUBMIT WITH YOUR THESIS.

Please note the following standard requirements of approval:

1. The approval of this project is conditional upon your continuing compliance with the National Statement on Ethical Conduct in Human Research (2007).

2. Approval will be for a period of five (5) years subject to the provision of annual reports. Your first progress report is due on 31 August 2012.

If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. If the project has been discontinued or not commenced for any reason, you are also required to submit a Final Report for the project.

Progress reports and Final Reports are available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

3. If the project has run for more than five (5) years you cannot renew approval for the project. You will need to complete and submit a Final Report and submit a new application for the project. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).

4. All amendments to the project must be reviewed and approved by the Committee before implementation. Please complete and submit a Request for Amendment Form available at the following website:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics/forms

5. Please notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that affect the

continued ethical acceptability of the project.

6. At all times you are responsible for the ethical conduct of your research in accordance with the guidelines established by the University. This information is available at the following websites:

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If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of Final Approval to an external organisation as evidence that you have Final Approval, please do not hesitate to contact the Ethics Secretariat at the address below.

Please retain a copy of this email as this is your official notification of final ethics approval.

Yours sincerely
Dr Karolyn White
Director of Research Ethics
Chair, Human Research Ethics Committee

Ethics amendment ref: 5201100552- Amendment approved

Dear Lei,

Thank you for your email and response regarding your amendment request. The following amendment has been approved:

1. To expand the number of independent variables in regression analysis models to understand the association between glaucoma and markers of metabolic syndrome. To assess this, the following components will be measured:
    - blood sugar level
    - serum insulin level
    - lipids (triglyceride, low-density-lipoprotein cholesterol, high-density-lipoprotein cholesterol)
    - renal function tests (urea, creatinine, uric acid, sodium, and potassium)
    - liver function tests (albumin, alanine transaminase, asparatate transaminase, total bilirubin, conjugated bilirubin)

2. Addition of an information sheet for the participants to act as a guide for them on the day of the study. This also includes a statement about informing the researchers about any food requirements they may have.

3. The Information and Consent form has been amended to reflect these changes.

Please note that in the 6th paragraph of your Information and Consent form after the section "hand grip test (grasping a dynamometer and sustaining a fixed, isometric contraction for 3 minutes at 30% maximum effort whilst recording continues for another 5 minutes" is missing a bracket at the end of that sentence. That sentence is also not clear about what changes occur between the "3 minutes at 30% maximum effort" and the "recording continues for another 5 minutes". Please reword this sentence to be more explicit and correct the typo.

Please forward the amended Information and Consent form for our records.

Please do not hesitate to contact the Ethics Secretariat if you have any questions or concerns.

Regards,

Kate
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Office of the Deputy Vice Chancellor (Research)

Ethics Secretariat

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